

**The Role of ATP and Adenosine
in Nociception and Inflammatory Pain**

By

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Do mo mhuintir ar fad idir Mhuintir Uí Dhubhda agus Muintir Mhic Oireachtaigh,
pé ar bith áit ina bhfuil siad.

Declaration

I declare that this thesis was composed entirely by myself, and that the work on which it is based is my own with the following exceptions:

The electrophysiological studies of cat corneal nociceptors were carried out in collaboration with Prof. Carlos Belmonte, Dr. Juana Gallar and Ms. Marie Carmen Acosta from the Instituto de Neurociencias, Universidad de Alicante, Spain.

Dr. A. Ramón Gutiérrez (Departamento de Oftalmología, Universidad de Murcia) performed the photorefractive keratectomy of the cat cornea.

Dr. Carmen De Felipe (Instituto de Neurociencias, Universidad de Alicante) retrogradely labelled the murine trigeminal ganglia.

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Abstract

The development of novel analgesics would be facilitated if the mechanisms underlying nociception and inflammatory pain were fully understood. Adenosine 5'triphosphate (ATP) and adenosine can cause pain in humans when applied to a blister base, but the algogenic mechanism of action is still unclear. Cells contain millimolar concentrations of ATP, which is released into the extracellular space when the cells are damaged, and is subsequently metabolised to adenosine. Consequently, levels of the purines are increased in damaged, inflamed or ischemic tissues and this makes them ideal candidates to signal the presence of tissue injury. It is thought that ATP and adenosine might be involved in the initiation of pain by directly or indirectly activating distinct subtypes of P2 or P1 receptors respectively. In this thesis, behavioural, electrophysiological, and immunohistochemical techniques were used to test the hypothesis that ATP and adenosine are involved in the initiation of pain by directly and/or indirectly activating nociceptors innervating the cornea and the knee joint *in vivo*.

ATP and ATP analogues were administered to the normal cat cornea and the normal rat knee joint under pentobarbitone anaesthesia and their effects on the discharge of nociceptors innervating these tissues were recorded. The effects of inflammation caused by photorefractive keratectomy of the cornea or Freund's adjuvant induced monoarthritis of the knee joint on the sensitivity to the purines was also determined. In behavioural studies, ATP analogues were instilled into the eyes of conscious rats and any changes in behaviour indicative of pain were assessed. To establish whether the P2X₃ receptor subtype for ATP was expressed in the cell bodies of mouse corneal and rat knee joint neurones in the trigeminal and dorsal root

ganglia respectively, these cells were retrogradely labelled using fluorogold and subsequently examined for co-localisation of fluorogold fluorescence with P2X₃ immunoreactivity. Adenosine and adenosine analogues were also administered to the normal and arthritic rat knee joint and, in behavioural studies, the effect of adenosine agonists, adenosine antagonists and increasing the levels of endogenous adenosine on the pain and inflammation associated with experimental arthritis were determined.

Immunoreactivity to P2X₃ receptors was found in cell bodies of mouse corneal nociceptors, but none of the ATP analogues tested excited cat corneal nociceptors or caused pain when instilled into the eyes of conscious rats. The P2X₃ subtype was also expressed in knee joint neurones in the dorsal root ganglia. ATP, the stable P2X₁ and P2X₃ selective agonist, $\alpha\beta$ -methylene ATP and the P2 agonists, ATP γ S and benzoylbenzoyl ATP (BzATP), caused a rapid-onset, short-lasting increase in action potential discharge from nociceptors innervating the rat knee joint. These responses were antagonised by the P2 antagonist PPADS. ATP and ATP γ S also caused a delayed-onset, long-lasting increase in firing which was probably mediated by adenosine A₁ receptors since adenosine, and the A₁ selective agonists GR79236 and CPA evoked a similar response. These slow-onset responses were antagonised by the A₁ selective antagonist DPCPX. Paradoxically, systemic injections of DPCPX were not analgesic in behavioural studies, while the adenosine uptake inhibitor, dipyridamole, which increases the extracellular levels of endogenous adenosine, was. GR79236 had no effect on the pain of arthritis but did possess anti-inflammatory properties. The ability of ATP to indirectly activate rat knee joint nociceptors via P2X₇ receptors expressed on inflammatory cells was assessed by injecting high concentrations of BzATP, ATP γ S and ATP intra-

particularly to the knee joint and monitoring their effects on spontaneous and bradykinin-evoked neural discharge. BzATP did not cause any increase the basal action potential discharge rate nor did it sensitise the nociceptors to bradykinin. The data from the other agonists was complicated by their metabolism to adenosine but, like BzATP, no evidence was found for a sensitising effect.

This data supports the hypothesis that ATP and its metabolite, adenosine can directly excite nociceptors innervating the rat knee joint via P2X and A₁ receptor subtype(s), respectively. However, it does not support a role for P2X mediated initiation of pain from the cat or rat cornea nor does it indicate that ATP could cause pain via an indirect action on inflammatory cells. These findings have implications for the development of novel therapies for the treatment of pain.

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List of commonly used abbreviations

AC	adenylate cyclase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
Ca ²⁺	calcium cation
°C	degrees Celcius
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CO ₂	carbon dioxide
DAG	diacylglycerol
DRG	dorsal root ganglion/ganglia
ED ₅₀	dose causing 50% of maximum response
FCA	Freund's complete adjuvant
g	gram
G-protein	guanyl nucleotide binding protein
H ⁺	hydrogen ion
i.a.	intra-arterial
i.art.	intra-articular
IP ₃	inositol triphosphate
i.p.	intra-peritoneal
K ⁺	potassium ion
kg	kilogram
log	logarithm
LPS	lipopolysaccharide
M	molar concentration
m	metre
m (prefix)	milli (10 ⁻³)
MAN	medial articular nerve
mg	milligram
mg kg ⁻¹	milligrams per kilogram

ms ⁻¹	metres per second
µg	microgram
ml	millilitre
mm	millimetre
n	number of observations
n (prefix)	nanogram
Na ⁺	sodium ion
NaCl	sodium chloride
NSAID	non-steroidal anti-inflammatory drug
P	statistical probability
p (prefix)	picogram
PBS	phosphate buffered saline
PLC	phospholipase C
PLD	phospholipase D
PKC	protein kinase C
s	second
sem	standard error of the mean
TG	trigeminal ganglion/ganglia
UTP	uridine triphosphate
w/v	weight per volume

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

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Chapter 1: General introduction

Severe chronic pain is a major symptom of rheumatoid arthritis (RA) and one of the main aims of treating this disease is to improve the quality of life of patients by providing pain relief. The non-steroidal anti-inflammatory drugs (NSAIDs) are the most effective analgesic compounds used clinically to treat RA pain but their prolonged use is associated with severe side effects including renal dysfunction (Lifschitz, 1983) and gastrointestinal bleeding (Goodwin, 1987). These drugs inhibit the enzymes responsible for the production of a group of peripherally-acting inflammatory mediators, the prostanoids, which are largely thought to contribute to inflammatory pain by sensitising the primary afferent nociceptor (Levine *et al.*, 1993; Vane, 1971). This indicates that the interaction between the nociceptors innervating inflamed tissues and the mediators produced during inflammation have a crucial role to play in generating and maintaining inflammatory pain. For this reason, considerable efforts have been made to establish the effects of endogenous inflammatory mediators on nociceptors and nociceptive behaviours in order to facilitate the development of novel analgesics to treat inflammatory pain.

The purines adenosine 5'triphosphate (ATP) and adenosine are prime candidates as endogenous mediators of inflammatory pain because extracellular levels of both are increased during inflammation (Gordon, 1986). That these purines might be involved in producing pain was first alluded to by Stoner and Green (1945) after they found that intra-arterial injections of ATP produced "tingling pains" in the forearms of humans. Since then, ATP and adenosine have been implicated in many of the peripheral and central mechanisms of pain (Cronstein *et al.*, 1996; Keil & Salter, 1996). In order to ascertain whether manipulation of purinoceptor

pharmacology is a viable therapeutic target for the treatment of pain, it is crucial to determine the effects of these purines on nociceptive afferents innervating both normal and inflamed tissues, and on nociceptive behaviours in conscious animals.

In this introductory chapter, the peripheral sensors through which painful stimuli are perceived will be described, followed by a description of the mechanisms through which inflammatory mediators can modulate nociceptor function. The nociceptors innervating the cornea and the knee joint will then be described as these tissues were used in the present study. Finally, the literature suggesting that ATP and adenosine play a role nociception and inflammatory pain will be reviewed.

1.1 NOCICEPTION

The ability to sense pain has two main functions: it allows the organism to detect potentially injurious external stimuli resulting in the initiation of the appropriate protective response, and it provides the means to sense if tissue has been injured. Nociception is the detection of such events by specific pain-sensing receptors called nociceptors. The terms “nociception” and “nociceptor” are derived from Sherrington’s (1906) proposal at the start of this century that “noci-receptors” exist that are able to detect stimuli “capable of compromising the integrity of the organism”. Although Sherrington proposed the presence of nociceptors almost a century ago, definitive evidence of their existence was only presented 30 years ago (see below).

1.1.1 DISCOVERY OF NOCICEPTORS

The meticulous investigations of the early psychophysicists, in particular von Frey (1894; 1922) yielded the first solid scientific evidence that pain was a sense distinct

from touch and was mediated by specific sense organs. Von Frey described separate sensitivities to pain, touch, warm and cold at different punctate regions of the body. Furthermore, the pain spots were always associated with areas that were solely innervated by unspecialised nerve endings, the so-called “free” nerve endings. Following the introduction of electrophysiological techniques for recording from sensory nerves innervating peripheral tissues, it was shown that thinly myelinated (A δ -fibres) and unmyelinated axons (C-fibres) were activated by noxious stimuli, whereas the largest myelinated fibres (A β -fibres) responded to innocuous stimuli (Clarke *et al.*, 1935; Gasser & Erlanger, 1927; Heinbecker *et al.*, 1932; Zotterman, 1933). These studies suggested that the thin fibres were responsible for the detection of potentially injurious stimuli.

The suggestion that the small diameter fibres were the specific detectors of pain was soon disputed. In 1960, Iggo reported that he had recorded from a number of individual cat cutaneous C-fibres that responded to mechanical stimuli ranging from the innocuous to the noxious and stated that the unmyelinated fibres “can no longer be regarded as exclusively nociceptive in function” (Iggo, 1960). Other studies at the time also demonstrated that the thin fibres were not exclusively nociceptive (Hunt & McIntyre, 1960a-c). The equivocal data from electrophysiological experiments led Malzack and Wall (1962) to state that “there can no longer be any doubt that the temporal and spatial patterns of nerve impulses provide the basis of our sensory perceptions”. Thus even a few decades ago, the very existence of receptors for noxious stimuli (Sherrington’s “noci-receptors”) was still in dispute.

The controversy was finally resolved by Perl and colleagues in the late sixties. Burgess and Perl (1967) described the response characteristics of over 500 thinly myelinated cat cutaneous afferents to mechanical and thermal stimulation. They reported that 74 of these A δ -fibres had mechanical thresholds many times higher than the other A δ -fibres and they responded to noxious thermal stimulation and pinching of the skin. They named these afferents “nociceptors” for the first time using a term derived from Sherrington. Shortly thereafter, Bessou and Perl (1969) reported the response characteristics of a population of unmyelinated C-fibres innervating the skin of the cat’s hindlimb. About 50% of the afferents they recorded were activated by innocuous mechanical stimulation, but two groups were defined as nociceptors: 40% were activated by intense mechanical stimulation, high temperatures and chemical stimulation, and 10% responded to intense mechanical stimulation only. The former group was called the polymodal nociceptors and the second were called mechanonociceptors.

1.1.2 Mechanical nociceptors

1.1.2 CLASSIFICATION OF NOCICEPTORS

To date, nociceptors innervating a wide variety of tissues including the skin (Campbell & Meyer, 1996), cornea (Belmonte & Gallar, 1996), joints (Schaible & Schmidt, 1996), muscle (Mense, 1996) and viscera (Cervero, 1996) have been described. Unfortunately, no one system is used for the classification of nociceptors (see Lynn, 1996) and this can lead to difficulties in comparing the work of different groups.

The main criteria used to classify nociceptors are the stimulus required to excite them, the conduction velocity of action potentials along their axes and the target tissue they innervate. For example, sensory fibres innervating the cat’s cornea

that are activated by mechanical, chemical and thermal stimulation and which conduct in the C-fibre range ($<2\text{ms}^{-1}$) are termed corneal C-fibre polymodal nociceptors (Gallar *et al.*, 1993). Nociceptors innervating the cat cornea and the rat knee joint were investigated in the present study and these will be described in more detail later in this chapter.

1.1.3 EXCITATION OF NOCICEPTORS

Noxious mechanical, thermal and chemical stimuli are detected when the peripheral terminals of nociceptors are depolarised to the threshold for action potential initiation. Depolarisation occurs either as a result of an increased permeability of the membrane to ions such as sodium (Na^+) and calcium (Ca^{2+}), or a reduced permeability to potassium ions (K^+) (Bevan, 1996). The action potential(s) are then transmitted to the central nervous system (CNS) and, depending of the state of sensitisation of central sites, pain is sensed (Millan, 1999).

1.1.3.1 Mechanical excitation

Very little is known about the mechanisms through which high-intensity mechanical stimulation causes action potential generation in nociceptors. Most of the current knowledge of mechanosensitivity has come from studies of the mechanosensitive channel of large conductance (MscL) from *Eschericia coli* (reviewed by Sukharev *et al.*, 1997) and from genetic studies of the nematode, *Caenorhabditis elegans* (reviewed by Tavernarakis & Driscoll, 1997).

Purified MscL protein forms functional mechanosensitive channels when reconstituted into artificial lipid bilayers (Häse *et al.*, 1995) indicating that stretching of the lipid bilayer directly opens the channel. In *C. elegans*, mutations of various

genes render the nematode insensitive to touch (Tavernarakis & Driscoll, 1997). One of the genes required by *C. elegans* for mechanosensitivity is thought to encode a mechanosensitive ion-channel because it is homologous to the amiloride-sensitive sodium channel and amiloride is well known to inhibit mechanosensitive ion-channels (Hamill *et al.*, 1992). Similar ion-channels may account for mechanosensitivity in mammalian nociceptors, but this is still unknown. Mutations of other *C. elegans* genes, including those encoding extracellular matrix and cytoskeletal components, also confer mechanical insensitivity on the nematode. It is still unclear what role extracellular matrix and cytoskeletal components play in mechanical transduction but it has been suggested that mechanical stimuli produce changes in the overall structural architecture of cells which results in intracellular biochemical signals (see Ingber, 1997).

Further functional investigations of the MscL protein from *E. coli* and studies of mutated genes from *C. elegans* will undoubtedly extend our knowledge of mechanosensitivity but for now, the mechanisms underlying selective sensitivity to high threshold mechanical stimulation which is characteristic of nociceptors remains unknown.

1.1.3.2 Thermal excitation

Although relatively little is known regarding the transduction of noxious cold stimuli, the mechanism through which noxious heat is detected has largely been revealed over the past two years. The first vanilloid receptor (VR-1) was cloned and found to be gated by noxious heat and by capsaicin (8-methyl-N-vanillyl-6-noneamide), the compound that makes peppers taste hot (Caterina *et al.*, 1997). Capsaicin and noxious heat gate a non-selective cation-channel (the VR-1 receptor)

which is highly permeable to divalent cations. Capsaicin has long been recognised as a peripheral nociceptive excitatory substance and therefore, the detection of tissue-damaging heat stimuli probably follows the activation of vanilloid receptors expressed on the peripheral terminals of nociceptive afferents (Tominaga *et al.*, 1998).

1.1.3.3 Chemical excitation

In general, acute nociceptor activation, for example during a mechanical insult, leads to a transient sensation of pain of little clinical relevance. However, in inflamed tissues, a variety of chemical mediators are released which activate and/or sensitise the primary afferent nociceptor leading to heightened, and sometimes chronic, pain. The mechanisms through which inflammatory mediators excite or sensitise nociceptors will be considered in Section 1.2.2 below.

1.2 INFLAMMATORY PAIN

When an inflammatory response is triggered by tissue injury or the presence of an antigenic foreign body one of the main symptoms is pain. Inflammatory pain is characterised by ongoing spontaneous pain, a painful sensation following normally innocuous stimuli and enhanced pain to noxious stimuli. Although the nomenclature is not clearly defined (Meyer *et al.*, 1996; Woolf *et al.*, 1998), pain arising from a normally innocuous stimulus is commonly called allodynia, while an increased response to a noxious stimulus is termed hyperalgesia, and this is the context in which these terms will be used in this thesis. Sensitisation of both peripheral nociceptors and central pathways are involved in causing these enhanced pain states.

The present investigation is concerned with changes in the excitability of the peripheral terminals of nociceptors and how this contributes to inflammatory pain. Thus, in this section, the discovery of peripherally-acting inflammatory pain mediators will be outlined, followed an account of the mechanisms through which these mediators cause peripheral sensitisation.

1.2.1 HISTORICAL PERSPECTIVE

The four main symptoms of inflammation - redness, heat, swelling and pain were first stated two millennia ago by Celsus (30 BC – 30 AD). In the late 18th century, Hunter (1894) published one of the most detailed descriptions of the signs of inflammation based on his observations in humans and experiments with animals. On inflammatory pain, Hunter stated:

“Thus a tendon has very little sensation when injured in a natural state; but let that tendon become inflamed, or otherwise diseased, and the sensation shall be very acute.”

Over a century later, Sir Thomas Lewis (1942) was the first to consider the processes underlying the hypersensitivity associated with inflamed tissue:

“The hyperalgesic skin, according to my theory, is one which has been brought to this state by the action of certain tissue substances upon the nerve endings, the latter being rendered hyperexcitable.”

Lewis had come to his perceptive theory regarding the actions of chemical tissue factors after a consideration of his and others' experiments with histamine. Histamine (decarboxylated amino acid histidine) caused an inflammation-like response when injected into the skin (Eppinger, 1913). Furthermore, a histamine-like substance could be extracted from skin which caused itch in addition to the redness, wheal and flare of the “triple response” when re-injected into normal skin (Harris, 1927). The itching caused by histamine and the histamine-like substance, as well as by minor

tissue injuries producing the triple response, was prolonged when the circulation to the affected skin was stopped. These observations led Lewis (1927) to conclude that stopping the circulation caused the retention in the tissues of an H-substance released from the damaged cells. He also suggested that more severe injury caused the release of a pain-producing substance from cells because he noted that intra-dermal injection of skin extracts could also cause pain (Lewis, 1935). At this time few endogenous chemical mediators had been identified but neither of those that were known, namely histamine or acetylcholine, nor changes in pH, tonicity or potassium, could account for the pain-producing substance in the skin extract (Lewis, 1942).

Rather surprisingly, the pain aspect of the inflammatory response was largely ignored after Lewis until the work of Keele and colleagues (reviewed in Keele & Armstrong, 1964). These authors had developed a novel method of testing the algogenic properties of chemical substances by applying them to the base of experimentally-induced blisters in human volunteers (Armstrong *et al.*, 1951). The blisters were raised by placing a plaster containing cantharidin (the active principle from *Cantharis vesicatoria*, the blistering beetle) on the skin of the forearm. Subsequently, the base was exposed by aspirating the blister fluid and removing the separated epidermis. A large number of chemicals, endogenous mediators, body fluids and insect venoms were tested for algogenic properties on the blister base. Among the substances found to cause pain were bradykinin, 5-hydroxytryptamine (5-HT) and substance P, as well as knee joint fluids from patients with RA. Since then, the effects of these and other substances on the primary afferent nociceptor and the contribution of inflammatory mediators to the pain associated with inflammation have been studied in more detail.

1.2.2 PERIPHERAL MECHANISMS

Combined evidence from various lines of investigation can indicate the mechanism through which inflammatory mediators modulate nociceptor function. For example, immunohistochemistry can be used to identify the cells in which the receptors for inflammatory mediators are expressed. If they are localised to the cell bodies of afferents in sensory ganglia, this suggests a direct effect of the mediator on the nociceptor. However, if the receptors are expressed on inflammatory cells, this could imply an indirect mechanism of action. Functional studies of sensory cell bodies or inflammatory cells *in vivo* or *in vitro* reinforce these basic investigations. Another method of investigating the effect of algogenic mediators on nociceptor function is to record from the axon of the afferents *in vivo* while applying the test substance locally to the tissue they innervate. The latency to onset of the expected response usually gives a good indication of whether the effect is direct or indirect. A preferable method would be to perform intracellular recordings of electrical activity in the nociceptive terminal while applying the test substance but this is not possible because of the size of the terminals and their close association with the tissues they innervate.

Evidence from these types of studies have indicated that some inflammatory mediators act *directly* on the sensory nerve terminal to excite it and/or to sensitise it to other chemical, mechanical or thermal stimuli, whereas others affect nociceptor function *indirectly* by causing the release of algogenic or sensitising mediators from other cells (Levine & Tawio, 1994). A schematic diagram of the mechanisms of inflammatory pain is shown in Figure 1.1.

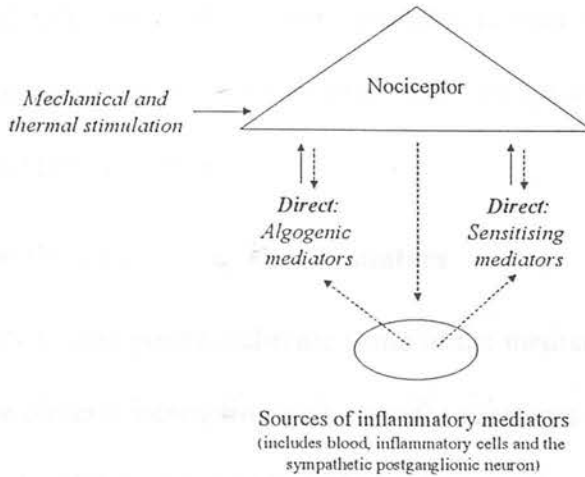


Figure 1.1 Schematic diagram of the peripheral mechanisms of inflammatory pain. Substances released from inflammatory cells, the sympathetic postganglionic neurone, the nociceptor itself, and derived from other sources can directly or indirectly activate and/or sensitise the primary afferent nociceptive afferent. Adapted from (Levine & Tawio, 1994). See text for details.

1.2.2.1 Directly acting algogenic mediators

Algogenic chemicals and inflammatory mediators that are thought to cause pain by directly activating nociceptive afferents include capsaicin, bradykinin, 5-HT, acetylcholine and protons (Kress & Reeh, 1996). Some algogens (for example, 5-HT via the 5-HT₃ receptor subtype) can depolarise the cell membrane rapidly because they bind to specific ligand-gated ion-channel linked receptors. Other algogens (for example, bradykinin via the B₂ receptor subtype) bind to receptors that are coupled to guanyl-nucleotide-binding (G) protein and cause depolarisation via second messenger signalling cascades. Although the exact means through which G-protein-linked receptors cause membrane depolarisation is still unclear, the pathway through which bradykinin B₂ receptor subtypes do so is relatively well characterised. Binding of bradykinin to B₂ receptors expressed on sensory neurones causes the activation of

phospholipase C (PLC) which cleaves membrane lipids to form inositol triphosphate (IP₃) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) which can phosphorylate membrane ion-channels and receptors leading to membrane depolarisation (Bevan, 1996).

1.2.2.2 Directly acting sensitising mediators

Bradykinin, PGE₂ and prostacyclin are some of the mediators thought to sensitise the nociceptor by directly interacting with specific receptors on the nociceptor terminal (Kress & Reeh, 1996). Adenylate cyclase (AC) mediated increases in intracellular cyclic adenosine monophosphate (cAMP) have been implicated in nociceptor sensitisation (Levine & Tawio, 1994) although the mechanism through which they do so is not clear. There is some evidence that sensitising substances which increase cAMP levels (for example, prostaglandin E₂ (PGE₂) via the EP₂ receptor subtype) reduce the membrane permeability to K⁺, thus depolarising the terminal and increasing the likelihood that action potentials will be initiated (Bevan, 1996).

1.2.2.3 Indirectly acting mediators

Interleukin-1 β (IL-1 β) is a potent, indirectly acting pro-nociceptive inflammatory mediator which is produced by a variety of non-neuronal cells (including macrophages, mast cells, fibroblasts and synoviocytes) during inflammation (Bianchi *et al.*, 1998). This cytokine causes the release of a variety of pro-nociceptive inflammatory mediators; for example it 1) stimulates PGE₂ release from cultured human synovial cells (Dayer *et al.*, 1986), 2) induces nerve growth factor production *in vitro* (Yoshida & Gage, 1992) and *in vivo* (Safieh-Garabedian *et al.*, 1995), and 3) up-regulates levels of substance P in sympathetic neurones *in vitro* (Hart *et al.*, 1991; Jonakait *et al.*, 1990) and increases transport of tachykinin receptors to the peripheral

terminals of sensory neurones *in vivo* (Jeanjean *et al.*, 1995). PGE₂, substance P and nerve growth factor can then cause pain directly and/or indirectly.

Due to the excitatory and/or sensitising effects of inflammatory mediators, nociceptors innervating inflamed tissues have an increased basal rate of discharge, a reduced threshold for activation and an augmented response to stimuli. Furthermore, during inflammation more nociceptors are excited by any given stimulus and nociceptors that were previously inactive are now activated (Coggeshall *et al.*, 1983; Guilbaud *et al.*, 1985; Schaible & Schmidt, 1985; Schaible & Schmidt, 1988). The elevated C-fibre activity during inflammation increases the excitability of spinal neurones by releasing glutamate and substance P so that the response to all sensory input, including that to low threshold stimuli, is augmented (Ma & Woolf, 1995; Woolf, 1983; Woolf & Thompson, 1991; Woolf & Wall, 1986; Xu *et al.*, 1992). Central sensitisation also occurs due to increased activity of A β -fibres which express substance P during inflammation (Neumann *et al.*, 1996).

Both peripheral and central sensitisation contribute to inflammatory pain but their relative contribution is not known. This study focused on the peripheral roles of ATP and adenosine in nociception and inflammatory pain and this was largely investigated in the cornea and the knee joint.

1.3 NOCICEPTORS INNERVATING THE NORMAL AND INFLAMED CORNEA AND KNEE JOINT

1.3.1 THE CORNEA

The cornea is an ideal tissue in which to study nociception because it is an accessible, avascular, very simple structure with a high density of nociceptive nerve

terminals. Apart from the merits of investigating corneal nociceptors in basic research, knowledge of the mechanisms of corneal pain would aid the development of novel analgesics for corneal inflammatory pain. Although the cornea is an avascular tissue, blood-derived inflammatory cells can be detected at the edges of a corneal wound within 2-3 hours of injury, and the tissue has a population of mast cells (Bazan, 1990). Intense and prolonged corneal pain can arise as a result of accidental corneal erosions, bulbous keratopathy and following photorefractive surgery for the correction of myopic conditions. As well as these severe pain states, discomfort and irritation are frequent complaints of contact lens wearers.

1.3.1.1 Corneal innervation

A vast number of publications dealing with the innervation of the cornea in many species, including members of the mammalian, avian, amphibian, reptilian and fish classes, have been published (see Zander & Wedell, 1951). The cornea is innervated (primarily) by afferents originating in the trigeminal ganglion (TG). The afferents travel to the eye via the ophthalmic nerve, which innervates the tissue either directly, via long ciliary nerves, or indirectly after first passing to the ciliary body, which in turn sends short ciliary nerves to the eye. The long and short ciliary nerves join to form the mixed ciliary nerve, which pierces the sclera at the posterior pole of the eyeball around the optic nerve (Attias, 1912). From here, they travel radially around the eyeball towards the cornea, and at the interface between the conjunctiva and the cornea, the limbus, the nerve axons form an “episcleral pericorneal plexus” (Zander & Wedell, 1951). It is from this “ring-like scaffold” of nerve bundles that nerves penetrate the cornea from the limbus. The nerve axons extend into the cornea parallel to the surface where they branch repeatedly resulting in a plexiform arrangement of

nerve fibres distributed in many layers of the corneal stroma with the greatest density towards the superficial layers. From the plexiform arrangement, many axons ascend vertically and penetrate the epithelium between cells of the basal layer. Here they divide into as many as 15 daughter axons that continue to branch as they travel vertically between cells towards the corneal surface (Zander & Wedell, 1951).

The resulting enormous number of axon terminals can terminate at any level within the epithelium with many ending a few microns from the corneal surface. This continuous branching of corneal sensory fibres makes the cornea the most densely innervated structure in the body; the cornea is thought to have approximately 300 – 600 times more sensory endings than the skin and 20 – 40 times more than the tooth pulp (Rozsa & Beuerman, 1982).

1.3.1.2 Corneal nociceptors

Tower (1940) was the first to record action potentials from corneal afferents. He reported that the receptive fields of mechanosensitive corneal afferents were large (50-200mm²) and frequently extended into the adjacent limbus and conjunctiva, an observation later confirmed by Lele and Waddell (1959) and Belmonte's group (Giraldez *et al.*, 1979). In more recent years, Belmonte and his colleagues (Belmonte & Gallar, 1996; Belmonte *et al.*, 1991; Belmonte & Giraldez, 1981; Gallar *et al.*, 1993; Giraldez *et al.*, 1979) have identified three main functional classes of corneal nociceptors; mechanosensitive, polymodal and cold (reviewed by Belmonte & Gallar, 1996).

Mechanonociceptors respond exclusively to high threshold mechanical stimulation and always conduct in the A δ -fibre range (Belmonte *et al.*, 1991). Polymodal nociceptors are activated by mechanical, thermal and chemical

stimulation. These units conduct in either the A δ or C-fibre range and are the most abundant type of unit found in the cornea (Belmonte *et al.*, 1991; Gallar *et al.*, 1993). Cold sensitive afferents are always C-fibres and they respond selectively to cooling of the cornea (Gallar *et al.*, 1993).

The functional properties of nociceptors innervating the cat cornea are modified following experimental photorefractive surgery (Gallar *et al.*, 1997). In this initial report, a small area (6mm diameter, 70 μ m depth) of the cornea was removed and the properties of nociceptors innervating the area surrounding or within wound were examined (12-48 hours later). Interestingly, only small proportion of nociceptors (A δ -polymodal; J. Gallar and M.C. Acosta, personal communication) with receptive fields in the wound retained sensitivity to mechanical or chemical stimulation, whereas fibres bordering the ablation, were sensitised to these stimuli. Furthermore, nociceptors with receptive fields in both areas had an increased rate of spontaneous discharge. Thus, these spontaneous and stimulus-evoked discharges might contribute to inflammatory pain arising from the cornea following laser surgery for myopia.

1.3.2 THE KNEE JOINT

Knee joint pain is common in RA (Sculco, 1998) and detailed knowledge of the peripheral mechanisms that cause articular pain would facilitate research into mechanisms of preventing it. The innervation and functional properties of the cat knee joint has been studied in detail and will be described in this section.

1.3.2.1 Knee joint innervation

Early comprehensive studies of the anatomy of the cat's knee joint were made by Gardner (1944), Skoglund (1956) and Freeman and Wyke (1967). More recently, electron microscope studies by Heppelmann *et al.* (1990) have provided a detailed morphological analysis of the nociceptive afferent endings innervating the joint.

According to Freeman and Wyke (1967), the cat knee joint is innervated by two groups of articular nerves which they classified as primary and accessory. The former comprises the posterior, medial and lateral articular nerves whereas the latter arise from nerves innervating the muscles around the knee joint. Since the medial articular nerve (MAN) was recorded from in the present study, it will be the only one discussed further. In the cat, the MAN usually arises from the saphenous nerve and traverses the antero-medial aspect of the thigh, alongside the descending genicular artery and vein, to the medial aspect of the knee joint. At this point it splits into two branches which spread out and supply the medial and antero-medial aspects of the fibrous capsule of the knee joint, the medial collateral ligament, the medial part of the annular ligament attached to the medial meniscus, the ligamentum patellae and the infrapatellar fat pad, and the medial part of the patellar periosteum (Freemans & Wyke, 1967).

The MAN of the cat contains approximately 630 afferent fibres and 500 unmyelinated sympathetic efferent fibres (Langford & Schmidt, 1983). Of the afferents, the vast majority are thinly myelinated ($A\delta$ -fibres, termed Group III by Langford and Schmidt) or unmyelinated (C-fibres, Group IV) fibres (21% and 70% respectively). The remainder (9%), are fast conducting, large diameter afferents ($A\beta$ -fibres, Group II). The terminals of the $A\delta$ -fibres consist of 3 main areas; 1) the

proximal area which is myelinated and runs within a perineural sheath, 2) more distally, the nerve is unmyelinated but still runs within the perineural sheath and 3) the distal area begins after the perineural sheath ends (Heppelmann *et al.*, 1990). The distal endings of the A δ -fibres run into the tissues they innervate and split into 2-4 branches. The endings of the C-fibres have only two main areas: the proximal area runs within the perineural sheath and the distal area, running into the tissues, is outside it. Approximately two thirds of the distal terminal of both fibre types are covered by Schwann cells, with the remaining one third left bare. These “bare areas” were presumed by Heppelmann and his co-workers (1990) to be the receptive sites of the afferents.

1.3.2.2 Knee joint nociceptors

Detailed studies of the functional properties of the thinly myelinated A δ -fibres and unmyelinated C-fibres innervating the cat knee joint have been carried out by Schaible, Schmidt and colleagues (Coggeshall *et al.*, 1983; Schaible & Schmidt, 1983a,b). These authors have comprehensively investigated the responses of afferents innervating the knee to joint movements and mechanical stimulation and have concluded that the majority of A δ and C-fibre afferents of the MAN are probably associated with signalling joint pain. Thus, 55% of A δ -fibres and 70% of C-fibres are strongly activated by noxious joint movements and high threshold mechanical stimulation or are only excited during inflammation.

The authors have also investigated the properties of thinly myelinated or unmyelinated afferents of the MAN following the induction of an acute inflammation (induced by intra-articular injection of kaolin and carrageenan, Schaible & Schmidt, 1985; Schaible & Schmidt, 1988). In the inflamed knee joint, the vast majority of A δ

and C-fibres are spontaneously active (75% and 83% respectively) and fire at a rate more than double that of normal joints. In addition, most have low thresholds and enhanced responses to movements and mechanical probing. The increased spontaneous discharge of nociceptors innervating the inflamed knee joint is thought to contribute to the spontaneous pain associated with inflammation, whereas the reduced threshold and augmented discharge of these nociceptors probably contributes allodynia and hyperalgesia respectively (Schaible & Schmidt, 1996).

1.4 EXTRACELLULAR ATP AND ADENOSINE

1.4.1 SOURCES

Purines are ubiquitous and essential to man, being involved in the production of cellular energy and the synthesis of the nucleic acids, DNA and RNA. The main sources of intracellular purines are *de novo* synthesis and recycling of used nucleosides and bases within the cell (reviewed by Stone & Simmonds, 1991). The intracellular concentration of ATP is very high, between 5-10mM, and high local concentrations can result when it is released from cells (see DiVirgilio *et al.*, 1996). One of the main mechanisms by which this purine is released into the extracellular space is through non-specific membrane perturbations. However, it is also released from cells during hypoxic stress, as well as from activated inflammatory cells, sensory nerves and platelets (Gordon, 1986).

1.4.2 METABOLISM

Once released, ATP is rapidly metabolised by enzymes located at the cell surface as outlined in Figure 1.2 (reviewed by Zimmermann *et al.*, 1998). The main enzymes

responsible for the hydrolysis of extracellular ATP are ectoATP diphosphohydrolase and ectoATPase. The former has an equal preference for ATP and ADP as substrates and can therefore hydrolyse ATP to AMP via ADP. EctoATPase specifically metabolises ATP to ADP, which is subsequently metabolised to AMP by specific ADPases. 5'nucleotidase catalyses the formation of adenosine from AMP and adenosine deaminase is responsible for adenosine breakdown.

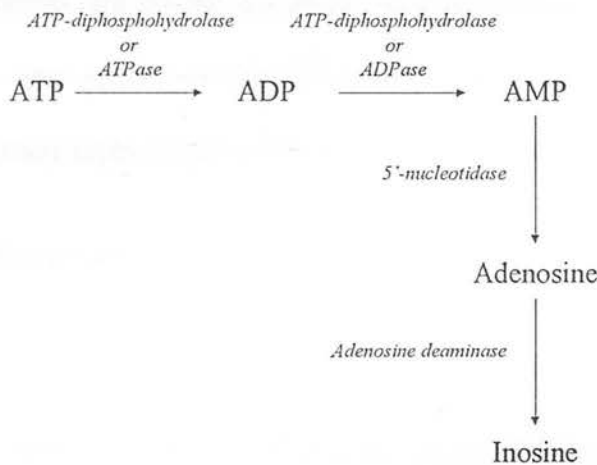


Figure 1.2 Main pathways and enzymes (indicated by italic font) involved in the extracellular degradation of purines. See text for details. Modified from Zimmermann (1998).

A number of compounds that inhibit the activity of extracellular ATP metabolising enzymes have been reported of which 6-N,N-diethyl-beta, gamma-dibromomethylene-D-ATP (ARL67156, formally FPL 67156) is the most effective (Zimmermann & Braun, 1996). ARL67156 potentiates the contractile effect of ATP in the rabbit ear artery preparation (Crack *et al.*, 1995), sympathetic neurotransmission in the guinea pig vas deferens (Westfall *et al.*, 1996) and parasympathetic neurotransmission in the guinea pig bladder (Westfall *et al.*, 1997).

Interestingly, P₂ receptor antagonists, including PPADS and suramin, can also inhibit ecto-nucleotidases (Beukers *et al.*, 1995; Bültmann *et al.*, 1996; Chen *et al.*, 1996; Crack *et al.*, 1994; Stout & Kirley, 1995; van der Ven & Hinds, 1996; Ziganshin *et al.*, 1995a,b; Ziganshin *et al.*, 1996). In the study by Crack *et al.* (1994), the data obtained was analysed using a theoretical model which indicated that simultaneous receptor antagonism and ecto-nucleotidase inhibition could result in the “self-cancellation” of the antagonistic and enzyme-inhibitory effects in certain situations. Compounds that inhibit adenosine deaminase have been recognised for a number of years and some, such as deoxycoformycin and pentostatin are used clinically to treat some types of cancer (Kane *et al.*, 1992; O'Dwyer *et al.*, 1988).

1.4.3 PURINE RECEPTORS

1.4.3.1 Historical perspective

Drury and Szent-Györgi (1929) presented the first evidence of the extracellular actions of purines when they reported that adenosine and AMP had various biological effects including arterial vasodilation. Five years later, Gillespie (1934) showed differential effects of ATP compared to adenosine and AMP on cat and rabbit blood pressure and contraction of guinea-pig ileum implying that there were different extracellular receptors for ATP compared to adenosine and AMP. The first real evidence for the existence of an adenosine receptor was provided by De Gubareff & Sleator (1965) who showed that the cardiovascular effects of adenosine were blocked by caffeine.

Interest in the extracellular actions of endogenous purines grew after Holton (1959) demonstrated that ATP was released from sensory nerves and Berne (1963) proposed that adenosine was the physiological mediator responsible for the coronary vasodilation during occurring during myocardial hypoxia. A few years later, Burnstock *et al.* (1970) suggested that the nonadrenergic noncholinergic substance released from autonomic nerves supplying the intestinal smooth muscle was ATP and its metabolite adenosine. Thus, the existence of “purinergic nerves” and “purinergic receptors” in target tissues were postulated. The first classification system for these “purinergic receptors” was proposed by Burnstock in 1978 where the putative ATP receptors were termed P₂-purinergic and the adenosine receptors were termed P₁-purinergic (Burnstock, 1978).

The ability of adenosine to both stimulate and inhibit the activity of adenylate cyclase soon led to the subclassification of P₁ purinoceptors into adenosine A₁ and A₂ subtypes (Londos *et al.*, 1980; van Calker *et al.*, 1979). The A₂ receptors were further subdivided into A_{2A} and A_{2B} subtypes based on the presence of high and low affinity binding sites for adenosine in the rat brain (Bruns *et al.*, 1986; Daly *et al.*, 1983). A third adenosine receptor was proposed to exist based on pharmacological studies in atrial tissues (Ribeiro & Sebastião, 1986), but this was later disputed (Kennedy *et al.*, 1992). However, an adenosine receptor with a novel pharmacological profile, different to that proposed by Ribeiro and Sebastião, has been cloned and termed the A₃ subtype (Zhou *et al.*, 1992).

The P₂ purinoceptors were subclassified into P_{2X} and P_{2Y} subtypes based on a comprehensive review of the effects of ATP and its various analogues in a wide variety of biological systems (Burnstock & Kennedy, 1985). In the following year,

Gordon (1986) described actions of ATP in platelets and mast cells that did not conform to the P_{2X}/P_{2Y} classification and he named the receptors responsible P_{2T} and P_{2Z} respectively. A fifth P_2 receptor subtype, the P_{2U} receptor, at which the pyrimidine nucleotide uridine 5'-triphosphate (UTP) and ATP were equipotent was soon identified (O'Connor *et al.*, 1991). Finally, a sixth subtype at which adenine dinucleotide polyphosphates were effective was termed P_{2D} (Castro *et al.*, 1992; Hilderman *et al.*, 1991). These six subtypes of P_2 purinoceptor (P_{2X} , P_{2Y} , P_{2T} , P_{2Z} , P_{2U} and P_{2D}) were recognised by the first Subcommittee of the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) for Purinoceptor Classification (Fredholm *et al.*, 1994). Abbracchio and Burnstock (1994) soon proposed that all P_2 purinoceptors could be classified as subtypes of P_{2X} (ion-channels) and P_{2Y} (G protein-coupled) families. This was based on the evidence (summarised by Dubyak (1991)) that all P_2 purinoceptors are either ion-channel gated or G protein-coupled, and on new data following the cloning of a number of receptors in the mid-1990's. Later Fredholm *et al.* (1997) suggested that the term purinoceptor was no longer valid as there was clear evidence for the existence of receptors that were structurally similar to ATP receptors but were preferentially activated by pyrimidines (Communi & Boeynaems, 1997). This nomenclature system has now been adopted and for the rest of this thesis the term "receptor" will be used instead of "purinoceptor".

1.4.3.2 Current classification

The recent developments in ATP and adenosine receptor classification, structure, distribution and function have been comprehensively reviewed by Ralevic and

Burnstock (1998). This section will briefly outline the classification and pharmacology of the receptors relevant to the present investigation.

1.4.3.2.1 P1 receptors

The three adenosine receptor subtypes (A_1 , A_{2A} and A_{2B}) proposed by pharmacological studies, and the fourth subtype (A_3) have now been cloned (Ralevic & Burnstock, 1998). All are members of the G-protein coupled receptor superfamily with seven transmembrane domains. Adenosine receptors mediate their effects by initiating a variety of second messenger signalling cascades and can be distinguished pharmacologically using selective agonists and antagonists (Table 1.1).

Table 1.1 Functional characteristics of cloned P1 receptor subtypes expressed in *Xenopus* oocytes or mammalian cells.

Subtype	Signal transduction system	Selective agonist	Selective antagonist	Reference
A ₁	↓AC ↑PLC ↑PLD	CPA GR79236	DPCPX	(Mahan <i>et al.</i> , 1991; Reppert <i>et al.</i> , 1991)
A _{2A}	↑AC	CGS21680	ZM241385	(Maenhaut <i>et al.</i> , 1990).
A _{2B}	↑AC ↑PLC	None available	None available	(Pierce <i>et al.</i> , 1992; Rivkees & Reppert, 1992; Stehle <i>et al.</i> , 1992)
A ₃	↓AC ↑PLC	2Cl-IB-MECA	I-ABOX	(Zhou <i>et al.</i> , 1992)

Abbreviations: 2Cl-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-5'-N-methylcarbamoyl)adenosine; CPA, N⁶-cyclopentyladenosine; DPCPX, 1,3,-dipropyl-8-cyclopentylxanthine; CGS21680, 2-[p-(2-carbonyl-ethyl(-phenylethylamino)-5'-N-ethylcarboxamido)adenosine; GR79236, N-[1S, trans, 2-hydroxycyclopentyl] adenosine; I-ABOX, 3-(3-iodo-4-aminobenzyl)-8-(4-oxycetata)phenyl-1-propylxanthine; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol.

1.4.3.2.2 P2 receptors

1.4.3.2.2.1 P2X receptors

Seven subtypes of P2X (P2X₁₋₇) receptors have been cloned from mammalian tissue and pharmacologically characterised (see Ralevic & Burnstock, 1998). All of the subtypes are ATP-gated ion-channels permeable to Na⁺, K⁺ and Ca²⁺. There are currently no selective agonists or antagonists available to discriminate responses mediated by the various P2X subtypes *in vivo*. However, recombinant receptors expressed in *Xenopus* oocytes or mammalian cells can be pharmacologically distinguished based their sensitivity to a stable ATP analogue, αβ-methylene ATP (αβmeATP), the P2 antagonists pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic

acid (PPADS) and suramin, their rate of desensitisation and the agonist potency order at the receptors (Table 1.2).

Table 1.2 Functional characteristics of cloned P2X receptor subtypes expressed in *Xenopus* oocytes or mammalian cells. Modified from (Collo *et al.*, 1996).

Subtype	$\alpha\beta$ meATP sensitivity	Antagonist sensitivity	*Desensitisation	Agonist potency ratio	Reference
P2X ₁	Yes	Yes	Rapid	2meSATP \geq ATP $>$ $\alpha\beta$ meATP $>>$ ADP	(Valera <i>et al.</i> , 1994).
P2X ₂	No	Yes	Slow	ATP=ATP γ S= 2meSATP	(Brake <i>et al.</i> , 1994).
P2X ₃	Yes	Yes	Rapid	2meSATP $>>$ ATP $>\alpha\beta$ meATP	(Chen <i>et al.</i> , 1995; Lewis <i>et al.</i> , 1995)
P2X ₄	No	No	Slow	ATP $>$ ATP γ S $>$ 2meSATP $>$ ADP= $\alpha\beta$ meATP.	(Bo <i>et al.</i> , 1995)
P2X ₅	No	Yes	Slow	ATP $>$ 2meSATP $>$ ADP	(Collo <i>et al.</i> , 1996)
P2X ₆	No	No	Slow	ATP $>$ 2meSATP $>$ ADP	(Collo <i>et al.</i> , 1996)
P2X ₇	No	Partial	Slow	BzATP $>$ ATP $>>$ 2meSATP $>$ ATP γ S $>$ ADP	(Surprenant <i>et al.</i> , 1996).

* Rate at which the inward current evoked by agonist application wanes. Rapid desensitisation occurs within hundreds of milliseconds in the continued presence of agonist.

Abbreviations: 2meSATP, 2-methylthio ATP; ATP γ S, adenosine 5'-O-(3-thiotriphosphate; BzATP, 3'-O-(4-benzoyl)benzoyl ATP.

1.4.3.2.2.2 P2Y receptors

P2Y receptors are receptors for purine and pyrimidine nucleotides that couple to second messenger systems through G-proteins causing increases in PLC levels, inhibition of AC and N-type Ca²⁺ channels and activation of K⁺ channels (for a comprehensive review see Ralevic & Burnstock, 1998). To date, a number of mammalian P2Y receptor subtypes (including P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁)

have been cloned, and a number of other sequences have been cloned from non-mammalian sources. The focus of this thesis is on the rapid effects of ATP mediated by P2X receptors and further discussion of P2Y receptors is not relevant to the present investigation.

1.4.4 THE ROLE OF ATP IN NOCICEPTION

A number of early studies in humans revealed that intra-arterial or intra-dermal injections of ATP caused pain. The first report was that of Stoner and Green (1945) who were investigating the cardiovascular reactions to tourniquet application and the possible role of ATP in mediating the observed cardiovascular effects. A minor part of their study involved injecting ATP into brachial artery and they noted it caused “tingling pains” in the forearms. This effect was simply noted and not discussed further by the authors. A number of years later, Keele and Armstrong (Armstrong *et al.*, 1957) reported that ATP caused pain in humans when applied to the base of a cantharidin-induced blister, an observation that was only examined in detail 20 years later (Bleehan *et al.*, 1976; Bleehen & Keele, 1977). These studies demonstrated that exogenous ATP had the capacity to cause pain but did not reveal anything about the mechanism through which it was having its effect.

The first indication that ATP might cause pain by *directly* activating the nociceptive afferents was provided by Jahr and Jessell (1983) who reported that ATP depolarised rat dorsal root ganglia (DRG) neurones in dissociated cell culture, a finding later confirmed by many authors (summarised by Bean & Friel, 1990). Interest in the potential role of ATP as an endogenous algogen intensified following the cloning of a number of P2X receptor subtypes and the discovery that one of these (P2X₃) was solely expressed in sensory ganglia (Chen *et al.*, 1995). Even more

significantly, the P2X₃ subtype was selectively expressed in small diameter cells of the DRG and TG; the cell bodies of the thinly myelinated or unmyelinated primary afferents. Thus, indirect evidence was accumulating that ATP, acting at P2X receptor subtype(s), had a specific role to play in the direct activation of nociceptors.

Despite all the evidence from cell culture experiments, there was still very little evidence that ATP could activate the peripheral terminals of these sensory afferents. Research at the Glaxo Institute of Applied Pharmacology (GIAP) at this time (Trezise & Humphrey, 1996) showed that P2X receptor agonists excited cutaneous afferents of the tail in the neonatal rat tail-spinal cord preparation *in vitro*, thus suggesting that functional P2X receptors were expressed on the peripheral terminals of sensory afferents. However, unless it could be shown that these receptors are functionally expressed on peripheral *nociceptive* terminals *in vivo*, then they could not be implicated in the algogenic effects of ATP.

The study of Chen *et al.* (1995 see above) stimulated widespread interest in the role of ATP in nociception because P2X receptors represented a previously unexplored target for analgesic drug development. Their report was soon followed by a number of reviews pointing to the potential therapeutic role of P2X receptor antagonists in treating many types of pain, including that of inflammation (Burnstock, 1996; Burnstock & Wood, 1996).

1.4.5 THE ROLE OF ATP IN INFLAMMATORY PAIN

ATP is an excellent candidate for signalling tissue damage because it is a ubiquitous cytoplasmic cell constituent and is released from injured, ischaemic and inflamed tissues. Levels of ATP are increased in inflamed tissues (Gordon, 1986) and it is found in the synovial fluid of patients with rheumatoid arthritis (Park *et al.*, 1996;

Ryan *et al.*, 1991). A variety of cells are thought to release ATP during the inflammatory process including activated macrophages and neutrophils (see DiVirgilio *et al.*, 1996), activated platelets (Born, 1962) and injured cells. It is also possible that ATP is released from the activated sensory nerve terminal itself (Holton, 1959) thereby contributing to neurogenic inflammation. Furthermore, it has been shown that the activity of ATP metabolising enzymes is attenuated in the synovial fluid of patients with rheumatoid arthritis thereby increasing the half-life of the purine (Park *et al.*, 1996). Thus, both enhanced release and reduced metabolism of ATP probably contributes to its accumulation during inflammation.

If functional P2X receptors are expressed on the peripheral terminals of nociceptive afferents, there is evidence from *in vitro* studies that they might be particularly suited to signalling pain under inflammatory conditions. In dissociated cell culture, acidification (Li *et al.*, 1997; Li *et al.*, 1996) and substance P (Hu & Li, 1996) augment the ATP-induced depolarisation of sensory neurones. If this augmentation occurs in nociceptors *in vivo*, the excitatory effects of ATP could be significantly increased during inflammation, because tissue inflammation is associated with a decrease in pH (Farr *et al.*, 1985) and an increase in the levels of substance P (Marshall *et al.*, 1990). In addition to its potential direct actions on the nociceptive afferent, ATP has a multitude of indirect actions through which it may contribute to inflammatory pain.

It has been known for 30 years that ATP causes the release of histamine from rat peritoneal mast cells *in vitro* (Sugiyama & Yamasaki, 1969). The receptor mediating this effect has several unusual properties: ATP⁴⁻ is the most potent agonist and low concentrations evoke apparently normal histamine release, whereas high

concentrations cause leakage of cytoplasmic cell constituents (Cockcroft & Gomperts, 1979a,b; Cockcroft & Gomperts, 1980). Responses mediated by the same receptor were subsequently found on a range of inflammatory and immune cells and the receptor was termed P2Z (Gordon, 1986), later identified as the seventh member of the P2X receptor family (P2X₇ Surprenant *et al.*, 1996). The effects of endogenous ATP on P2X₇ receptors is unknown but evidence from *in vitro* and *in vivo* studies (see below) suggests that it may contribute significantly to inflammatory pain.

Mast cells contain a multitude of inflammatory mediators including histamine, prostaglandin D₂ (PGD₂), 5-HT, leukotriene B₄ (LTB₄), platelet activating factor (PAF), tumour necrosis factor (TNF), some interleukins and an IL-1 converting enzyme (Arnason & Malone, 1995). It is well known that ATP induces histamine and PGD₂ release from mast cells (Jaffar & Pearce, 1990), but release of other mediators has not been investigated. However, because high concentrations of ATP cause the formation of a large diameter non-specific pore in mast cells, it is probable that ATP releases many of the other mediators too.

In addition to the effects of ATP on mast cells, it was shown early in this decade that the pro-nociceptive and pro-inflammatory cytokine IL-1 β was released from mouse peritoneal macrophages primed with bacterial cell wall lipopolysaccharide (LPS) during apoptosis induced by ATP (Hogquist *et al.*, 1991a). IL-1 β is produced as an intracellular biologically inactive 35 kDa precursor, pro-IL-1 β , in response to inflammatory stimuli such as LPS (Hogquist *et al.*, 1991b). However, the production of pro-IL-1 β , and the processing and release of active IL-1 β are distinct events (Chin & Kostura, 1993), and a separate stimulus is required to

cause an increase in extracellular cytokine levels. The early findings of Hogquist *et al.* (1991) have been confirmed over the past few years and ATP, via the P2X₇ subtype, is now known to be a potent stimulator of pro-Il-1 β processing and release from LPS-primed macrophages and microglia *in vitro* (Ferrari *et al.*, 1997a,b; Ferrari *et al.*, 1996; Perregaux & Gabel, 1994). This process also occurs *in vivo* as intra-peritoneal injection of ATP in mice also stimulates Il-1 β release from LPS-primed peritoneal macrophages (Griffiths *et al.*, 1995). It is still not known whether endogenous ATP stimulates Il-1 β release and contributes to pathophysiological conditions. If so, blockade of the P2X₇ receptor could inhibit the release of the cytokine and prevent its pro-nociceptive and pro-inflammatory effects.

ATP could also contribute to inflammatory pain via actions at receptors other than P2X subtypes. For example, small quantities of PGE₂ are released from cultured human rheumatoid synovial cells stimulated with ATP and UTP, and the release is markedly increased when the cells are pre-treated with Il-1 (Loredo & Benton, 1998). This effect is mediated by a G-protein coupled P2Y subtype (P2Y₂, formerly P2U). NSAIDs are currently the most effective analgesic treatment for RA and these compounds inhibit the enzymes responsible for prostaglandin production (Schiff, 1997). Thus, inhibition of PGE₂ release from rheumatoid synovial cells through pharmacological manipulation of the P2Y₂ receptor for ATP may have potential for the treatment of arthritic pain. The P2Y₂ subtype also mediates the release of arachidonic acid from cultured astrocytes (Chen & Chen, 1998; Stella *et al.*, 1997) which may contribute to central inflammatory processes, such as following stroke or head trauma.

Thus, the *in vitro* evidence that has accumulated to date suggests that extracellular ATP may be a direct chemical excitant of nociceptors, which could be of particular importance during inflammation. It also indicates that ATP might indirectly affect nociceptor function by causing the release of pro-nociceptive inflammatory mediators. However, in order to extend these proposals, the effects of ATP agonists on the discharge of nociceptive afferents *in vivo* must be determined. ATP release might be doubly important in pain because the nucleotide is rapidly metabolised to adenosine, which in-itself is thought to be involved in nociception.

1.4.6 THE ROLE OF ADENOSINE IN NOCICEPTION

Evidence from human and animal studies indicates that adenosine plays a complex role in the generation of pain (Keil & Salter, 1996; Sawynok, 1998). Like many of the potential endogenous inflammatory mediators, adenosine was first shown to cause pain in humans following application to the blister base (Bleehen & Keele, 1977). During the following decade, interest in the algescic properties of adenosine grew after it was shown to cause angina pectoris-like pain following intravenous infusion in man (Sylvén *et al.*, 1986). Because adenosine is released during myocardial ischemia (Haneda *et al.*, 1989), it was proposed to be the mediator of anginal pain. Another study showed that exercise-induced angina pain was reduced by aminophylline, a non-specific adenosine receptor antagonist (Crea *et al.*, 1990), thus indicating that ischemia-induced cardiac pain was partly caused by endogenous adenosine release.

Cardiac pain caused by adenosine is thought to result from the activation of adenosine A₁ receptors (Crea & Gaspardone, 1997) because the adenosine A₁ receptor selective antagonists, bamiphylline and N-6-endonorboran-1-yl-9-

methyladenine reduce chest pain induced by the nucleoside (Bertolet *et al.*, 1996; Gaspardone *et al.*, 1995). Bamiphylline also reduces exercise-induced anginal pain (Gaspardone *et al.*, 1993) further supporting the hypothesis that anginal-pain is caused by the release of endogenous adenosine. Adenosine is thought to be a directly-acting algescic-agent because it excites canine cardiac sympathetic afferent nerves *in vivo* (Uchida *et al.*, 1969) and this effect appears to be mediated by the A₁ subtype (Dibner-Dunlap *et al.*, 1993).

In behavioural studies of rodents, local adenosine injections are also pronociceptive. Following intra-dermal injection into the rat hind paw, adenosine lowers the mechanical threshold required to initiate a paw-withdrawal reflex (Taiwo & Levine, 1990). The nucleoside also enhances the nociceptive responses to subcutaneous formalin in the rat (Doak & Sawynok, 1995) and mouse (Karlsten *et al.*, 1992). In these studies, the nucleoside was not inherently algescic, that is, it did not elicit nociceptive responses when administered alone. Unlike the human situation where A₁ receptor activation is thought to mediate the algescic effects of adenosine, in rodents local A₂ receptor activation is thought to be responsible for the pronociceptive effects of the purine, whereas A₁ receptor activation prevents the adenosine-induced hyperalgesia. Although it is difficult to establish the mechanism of action of adenosine from whole animal behavioural studies, Taiwo and Levine (1990) suggested that adenosine-induced hyperalgesia was due to a direct action of the purine on A₂ receptors expressed on the sensory afferent. Thus, the effect had an onset-latency similar to that of the directly acting hyperalgesic agent, PGE₂, and remained after the depletion of neutrophils, sympathectomy and the inhibition of cyclooxygenase enzymes (Taiwo & Levine, 1990).

Thus it seems that activation of the A₂ subtype can excite or sensitise nociceptors, whereas activation of the A₁ subtype can cause excitation or, somewhat paradoxically, inhibition of sensitisation. Both subtypes are expressed on sensory neurones (Huang *et al.*, 1995) in the DRG supporting a direct effect of the nucleoside. Furthermore, as mentioned above, the nucleoside activates canine cardiac sympathetic afferents (Uchida *et al.*, 1969); it also excites cat carotid chemosensory afferents (McQueen & Ribeiro, 1981) and has recently been shown to activate pulmonary C-fibres (Hong *et al.*, 1998). Activation of carotid body chemosensory nerves is apparently mediated by the A₂ subtype (Monteiro & Ribeiro, 1987), whereas pulmonary C-fibre activation occurs via the A₁ receptor subtype (Hong *et al.*, 1998). The apparent contradictory effects of adenosine on the nociceptive afferent nociception are probably effected by various second messenger systems. Adenosine induced hyperalgesia is attributed to the increased formation of cAMP, a mechanism common to PGE₂-induced hyperalgesia, whereas inhibition of hyperalgesia by A₁ receptor activation is thought to be caused by a decrease in cAMP levels (Levine & Tawio, 1994). However, A₁ receptors are coupled to other second messenger systems (Fredholm *et al.*, 1996) and it is feasible that sensory neurone excitation may be a result of PKC activation, such as occurs following bradykinin activation of B₂ receptor.

Adenosine A₁ receptors have a higher affinity for the nucleoside compared to A₂ subtypes. Thus, at low concentrations of adenosine, A₁ effects probably predominate with A₂ effects becoming apparent at higher concentrations. The overall effect of adenosine's actions on the nociceptive afferent terminal probably depends

on the local concentration of the purine and could lead to pro- or anti-nociceptive effects.

In addition to the local effects of adenosine on the primary afferent nociceptor, the nucleoside is well known to have anti-nociceptive effects in the spinal cord. In animals, intrathecal administration of the nucleoside is analgesic in a variety of acute and chronic inflammatory pain states, and this effect is primarily attributed to the activation of the A₁ receptor (Sawynok, 1998). Activation of spinal A₁ receptors in humans also abolished tactile allodynia in one patient (Karlsten & Gordh T, 1995) and intrathecal adenosine was analgesic in a variety of experimental pain tests in humans (Rane *et al.*, 1998).

1.4.7 THE ROLE OF ADENOSINE IN INFLAMMATORY PAIN

The direct excitatory effects of adenosine on the nociceptive afferent nerve terminal may be augmented during inflammation. *In vivo*, substance P enhances the response of canine ischaemia-sensitive ventricular afferents to adenosine (Huang *et al.*, 1995). Thus, it is possible that substance P released from activated sensory nerves innervating inflamed tissues may increase the local algescic properties of endogenous adenosine thereby contributing to inflammatory pain. The nucleoside may also contribute to the pain of inflammation by releasing pro-nociceptive mediators from inflammatory cells. Stimulation of mast cells with adenosine A₃ receptor agonists causes 5-HT and histamine release and produces pain in rats (Sawynok *et al.*, 1997).

Adenosine is well known to have a variety of effects that can promote or inhibit the inflammatory process depending on the receptor activated (see Cronstein *et al.*, 1996) and these could lead to downstream pro-nociceptive or anti-nociceptive effects respectively. For example, activation of A₁ receptors on human neutrophil

enhances phagocytosis and superoxide anion generation by the cells *in vitro*, whereas stimulation of the neutrophil A₂ subtype inhibits these processes (Salmon & Cronstein, 1990). Similarly, treatment of cultured human neutrophils with adenosine A₁ receptor agonists promotes adherence to endothelial cells and chemotaxis, whereas A₂ agonists inhibit neutrophil adherence (Cronstein *et al.*, 1990; Cronstein *et al.*, 1992). Adenosine, acting on the A₂ subtype, also potently inhibits the release of certain pro-inflammatory cytokines including tumour necrosis factor α (TNF α) from inflammatory cells (for example see Eigler *et al.*, 1997; Parmely *et al.*, 1993; Sajjadi *et al.*, 1996; Thiel & Chouker, 1995; Wagner *et al.*, 1998). It also enhances the release of the anti-inflammatory cytokine, Il-10 from monocytes (Le Moine *et al.*, 1996), probably via the A₂ receptor subtype (Haskó *et al.*, 1996). Thus, in general, activation of the A₁ receptor subtype is pro-inflammatory, whereas activation of the A₂ subtype is anti-inflammatory. It is thought that the overall outcome of these opposing effects is dependent on the local adenosine concentration: at low concentrations the pro-inflammatory effects (A₁ receptor mediated) prevail, whereas at high concentrations the anti-inflammatory effects (A₂ receptor mediated) predominate (Cronstein *et al.*, 1996).

The antifolates are a group of compounds used as chemotherapeutic agents in the treatment of some cancers (Fleisher, 1993). Interestingly, antifolates (including methotrexate and sulphasalazine) are used in low doses as disease modifying anti-rheumatic drugs used clinically to treat rheumatoid arthritis and they are thought to act by increasing endogenous adenosine levels (Cronstein *et al.*, 1996; Cronstein *et al.*, 1993; Gadangi *et al.*, 1996).

1.5 AIMS OF THIS STUDY

In the present study, electrophysiological, behavioural and immunohistochemical approaches were used to investigate the potential role of ATP and adenosine in the initiation and/or maintenance of pain and the mechanism(s) through which they act.

The hypothesis that ATP activates nociceptors either directly, by acting on functional P2X receptor subtype(s) expressed on nociceptive afferent terminals, or indirectly, by activating the P2X₇ receptor subtype on inflammatory cells was tested, and the hypothesis that the major metabolite of ATP, adenosine, directly excites nociceptors was also investigated. The effect of inflammation caused by injection of Freund's Complete Adjuvant (FCA) into the rat knee joint on the direct excitatory effect of the purines was investigated, and in behavioural studies, the role of adenosine in maintaining inflammatory allodynia was assessed. Adjuvant arthritis is a commonly used model of human RA and will be described in more detail in the next chapter (Section 2.1.2.1).

The overall aim of this thesis was to contribute to the understanding of how extracellular purines modulate nociceptor activity and in doing so, to evaluate the validity of peripheral purine receptors as potential targets for therapeutic intervention in inflammatory pain.

Chapter 2: Methods

A total of 179 male Wistar rats, 8 adult cats and 4 adult mice were used in the present study. The studies using rats were carried out at The University of Edinburgh under UK Home Office regulations (Project licence, PPL 60/1860; Personal license, PIL 60/5794), and the experiments using cats or mice were carried out at the Universidad de Alicante, Spain.

2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

2.1.1 THE CAT CORNEA

2.1.1.1 Anesthesia and surgical preparation

Adult cats of either sex were anaesthetised with pentobarbitone (40mg kg^{-1} i.p.) and maintained in an areflexic state throughout experiments by slow infusion of anaesthetic (3mg kg hour^{-1} , via a cannula in the saphenous vein). The trachea was cannulated and the cats breathed room air spontaneously. End tidal CO_2 and rectal temperature were continuously monitored and maintained at around 4% and $36\text{-}38^\circ\text{C}$ respectively. After finishing an experiment in one eye, cat were kept overnight under pentobarbitone anaesthesia (infusion at 3mg kg hour^{-1} intravenously, i.v.) and the other eye was used on the following day.

2.1.1.2 Cilliary nerve dissection

The superior and lateral walls of the orbital cavity were removed and the extrinsic muscles of the eye were cut to expose the cilliary nerves. The cavity produced was filled with warm mineral oil and a mixed cilliary nerve was dissected from the underlying connective tissue and cut centrally to eliminate efferent activity. The

nerve was split using fine forceps until small filaments containing 1-3 single afferent fibres were isolated.

2.1.1.3 Recording mixed ciliary nerve activity

Afferent activity in filaments of mixed ciliary nerve was recorded using bipolar silver-silver chloride wire electrodes connected to a computerised recording system. The raw nerve signal was amplified (Neurolog NL103 and NL105), filtered (Neurolog NL115), and displayed on an oscilloscope (Tektronix 5113) connected to a loudspeaker. The amplified nerve signal was also stored on an FM magnetic tape for analysis off-line. For data analysis, output from the tape was digitised, filtered and passed via a 1401 interface (Cambridge Electronic Design (CED)) to a personal computer operating Spike2 software (CED). The arrangement used for storing and analysing afferent nerve activity is summarised in Figure 2.1.

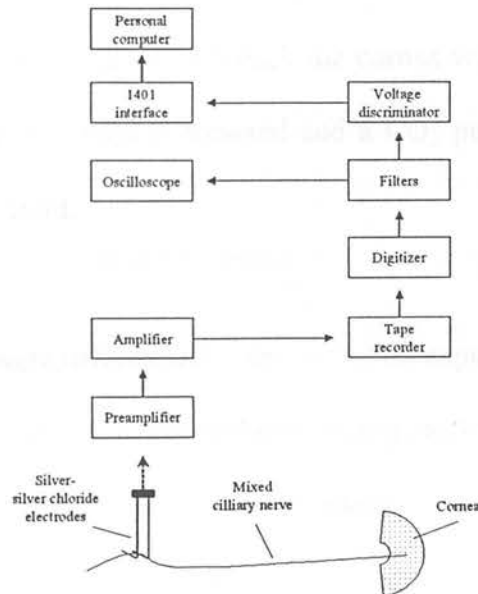


Figure 2.1 Schematic diagram of the arrangement used to record, store and analyse the afferent activity in mixed ciliary nerves innervating the cat cornea.

2.1.1.4 Identification of units

Afferent units recorded from were classified according to their mechanosensitivity, chemosensitivity and conduction velocity.

Mechanosensitivity

The receptive field of each the afferent was identified by lightly brushing the cornea with a fine wet paint brush.

Chemosensitivity

Chemosensitive afferents were identified because they were activated by low pH which was achieved by directing a jet of carbon dioxide to the corneal receptive field. CO₂ (98.5%) from a gas cylinder was humidified and passed through a flow regulator to a glass pipette (inner diameter: 1mm). The pipette was lowered to 5mm from the cornea by a micromanipulator and the gas flow was adjusted so that air alone did not stimulate the afferents. The pipette was raised from the cornea and flushed with CO₂ so that the gas would reach the cornea with negligible delay once the flow was started. It was then re-lowered and a CO₂ pulse of 30s duration was applied to the receptive field.

Conduction Velocity

Conduction velocities were determined at the end of an experiment by measuring the time taken for action potentials to reach the recording electrodes following electrical stimulation of the receptive field (conduction velocity = conduction distance/action potential delay). A pair of silver wire electrodes were used to apply suprathreshold electric shocks (0.1-0.5ms, 0.5-3mA) to the afferent receptive field. Conduction distance was established by placing a short piece of 8.0 gauge thread on the corneal

surface between the recording and stimulating electrodes and measuring its length with a ruler.

2.1.1.5 Drug administration

Drugs were applied to the cornea for 30s using a piece of solution-soaked tissue paper (approximate area: 6mm^2). Careful application of vehicle (phosphate buffered saline, PBS) by this method did not increase afferent discharge, but in some cases placing the tissue on the cornea caused a transient mechanically-evoked stimulation easily distinguishable from the drug-induced discharges. In some cases, drugs were applied to the receptive field as a $50\mu\text{l}$ drop from a pipette; control drops of PBS had no effect on afferent discharge. The area was repeatedly washed with saline from a pipette after drug application. To reduce the risk of tachyphylaxis, at least 5 - 20 minutes were allowed to pass between successive drug applications.

2.1.1.6 Spike sorting

Nerve activity from each filament (containing 1-3 separate afferent fibres) was recorded on tape for sorting off-line. Spike2 software was used to generate templates for each individual action potential it identified. The experiment on tape was then replayed and each separate action potential was sorted, classified as an event and displayed on a separate channel. Markers were used to indicate the point at which a drug injection was made.

2.1.1.7 Data analysis

Event data on each channel was expressed as discharge frequency (the number of action potentials per unit time) and the effect of CO_2 , drug or vehicle application was determined by comparing the discharge frequency following application with that

before application. The 15s pre-injection period was taken as the control. The response was expressed as the change in action potential discharge frequency according to the calculation below. When no clear response was obtained, the activity in a fixed time of 15s post-injection was measured.

Definitions:

$\Sigma\chi$: The total number of action potentials counted in the control and test time periods, t_{control} and t_{test} respectively (expressed as impulses).

$\bar{\chi}$: The frequency of action potential discharge in the control and test time periods (expressed as impulses s^{-1}).

$$\begin{aligned} \text{Thus,} \quad & \bar{\chi}_{\text{control}} = \Sigma\chi_{\text{control}} / t_{\text{control}} \\ \text{and,} \quad & \bar{\chi}_{\text{test}} = \Sigma\chi_{\text{test}} / t_{\text{test}} \end{aligned}$$

Calculations:

Delta $\bar{\chi}$: The change in action potential discharge frequency evoked by the drug.

$$\text{Thus,} \quad \text{delta } \bar{\chi} = \bar{\chi}_{\text{test}} - \bar{\chi}_{\text{control}}$$

2.1.2 THE KNEE JOINT

2.1.2.1 Freund's adjuvant induced arthritis

Polyarthritis induced by injection of FCA (heat-killed *Mycobacterium spp.* in mineral oil) into the rat tailbase or footpad is a widely used model of human rheumatoid arthritis (RA, see Billingham, 1983; Rainsford, 1982). Injection of FCA results in a severe polyarthritis after about 10 days which is most severe in the joints of the hindlimbs, where there is bone destruction, damage to tendons and loss of cartilage. There are also lesions in the ears, eyes, nose, skin, tail and genitals as well as severe weight loss (Pearson, 1956; Pearson, 1963; Pearson & Wood, 1963). FCA causes

inflammatory allodynia and/or hyperalgesia as measured using the Randall-Selitto test (Calvino *et al.*, 1987), tail flick test (Colpaert, 1987), hotplate test (Hara *et al.*, 1984), rotarod grip strength (Perrine & Takesue, 1968), squeezing with forceps (Hirose & Jyoyama, 1971), foot bend procedure (Calvino *et al.*, 1987) and transcutaneous electrical stimulation (Okuyama & Aihara, 1984). These tests measure the increased sensitivity to an acute stimulus (Colpaert, 1987), but spontaneous pain has also been assessed by measuring several parameters that are indicative of pain. These include decreases in body weight (Calvino *et al.*, 1987; Colpaert, 1987), reduced locomotor activity (Calvino *et al.*, 1987), scratching behaviour (De Castro Costa *et al.*, 1981), irritability (Colpaert *et al.*, 1982) and hyperventilation (Colpaert & van den Hoogen, 1983). Polyarthritic rats also preferentially drink water that contains analgesic drugs indicating that the arthritis is associated with chronic pain (see Colpaert, 1987).

Scientific and ethical questions have been raised regarding the use of severely affected polyarthritic animals (see Casey & Dubner, 1989). This has led to the development of less severe models of RA where a monoarthritic lesion confined to one joint is induced (Butler *et al.*, 1992; Donaldson *et al.*, 1993; Grubb *et al.*, 1991; Grubb *et al.*, 1988). In the present investigation, a modified version of the method described by these authors was used to induce monoarthritis in the rat knee joint.

2.1.2.1.1 Induction of monoarthritis

Localised experimental arthritis was induced in the left knee joint of male Wistar rats by intra-articular (i.art.) injection of FCA under transient halothane anaesthesia (5% in oxygen). The left knee joint was swabbed with alcohol and FCA was injected i.art. (0.15ml of 1mg ml⁻¹ *Mycobacterium tuberculosis* in paraffin oil) using a 26-gauge

needle inserted through the infrapatellar ligament just beneath the patella. A number of rats were injected with sterile paraffin oil (0.15 ml, i.art.) as controls. Following recovery from anaesthesia, the rats were housed (up to four animals per cage) on a 12-hour light/dark cycle with free access to water and standard laboratory food until they were used.

2.1.2.2 Assessment of arthritis for afferent nerve recordings

The rats were used for electrophysiological recordings 14-21 days following induction of arthritis. During this time the general condition and mobility of the animals was monitored daily by observing their behaviour in the cages. Knee joint diameter was measured using a sliding calipers on the day the rats were used for neural experiments.

2.1.2.3 Anesthesia and surgical preparation

Rats were anaesthetised with pentobarbitone (60mg kg^{-1} i.p., supplemented hourly with 6mg i.v., see below). Body temperature was maintained at 38°C by an automated heating blanket (Harvard Apparatus Limited) connected to a thermistor probe inserted into the rectum. A midline incision was made in the ventral aspect of the neck and the trachea was cannulated (catheter OD: 2.10mm) to facilitate spontaneous breathing of room air. The right carotid artery was cannulated (catheter OD: 0.75mm) so that arterial blood pressure could be continuously recorded via a pressure transducer (Bell and Howell, 4-442-0002) linked to a computerised recording system (MacLab/8 and Macintosh LC475). The right femoral vein was cannulated (catheter OD: 0.63mm) for hourly administration of pentobarbitone. A cannula (catheter OD: 0.63mm) was inserted into the abdominal aorta (at the level of

the iliac bifurcation) via the right femoral artery for close-arterial injection of drugs to the left knee joint.

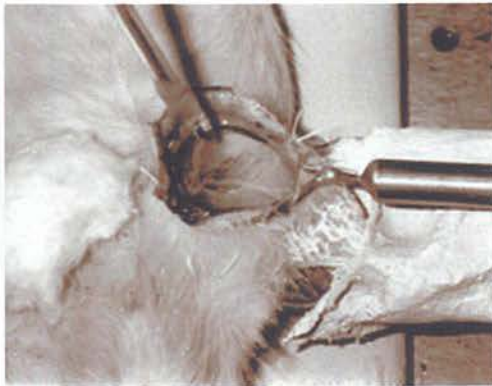
2.1.2.4 Medial articular nerve dissection

The left leg was fixed to a support and the skin on the medial aspect of the limb was cut to expose three branches of the MAN where they leave the saphenous nerve. The skin flaps were used to form a trough, which was filled with heavy paraffin oil. The saphenous nerve was cut centrally to eliminate efferent neural activity. One of the branches of the MAN was dissected from the underlying connective tissue and split using fine forceps until a small filament, usually containing 1-4 individual afferent fibres was isolated.

2.1.2.5 Recording medial articular nerve activity

Afferent activity of small filaments of the MAN was recorded using bipolar platinum-iridium wire electrodes (Figure 2.2).

a)



b)

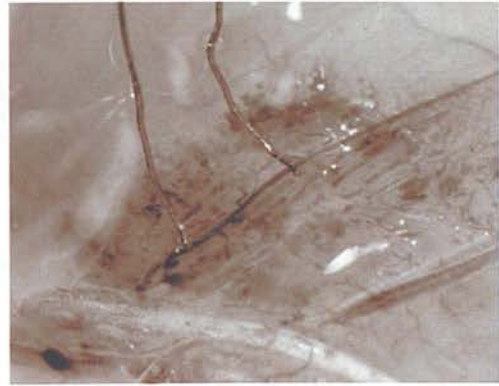


Figure 2.2 Photographs of the preparation used to record neural activity in the MAN innervating the rat left knee joint. Part a) shows the position of the limb and the paraffin-filled pool. Part b) shows an expanded view of the MAN on the electrodes.

The raw nerve signal was amplified (Neurolog NL103 & NL105), displayed on an oscilloscope (Gould 1604), digitised (Sony Digital Audio Processor PCM-701ES) and stored on videotape (Sony VideoCassette Recorder EV-C2000E PAL). The digitised signal was also filtered (Neurolog NL115) and displayed on the oscilloscope after passing through a voltage discriminator (Digitimer D.130) connected to a loud speaker. For data analysis, the signal was passed via a Micro1401 interface (Cambridge Electronic Design, CED) to a personal computer (RM PC-5200) operating Spike2 software (Spike2 version 2.24, CED). All electrical activity above positive and negative trigger levels was captured on-line and data files were saved for analysis off-line. Figure 2.3 shows the arrangement used for recording and storing afferent nerve activity.

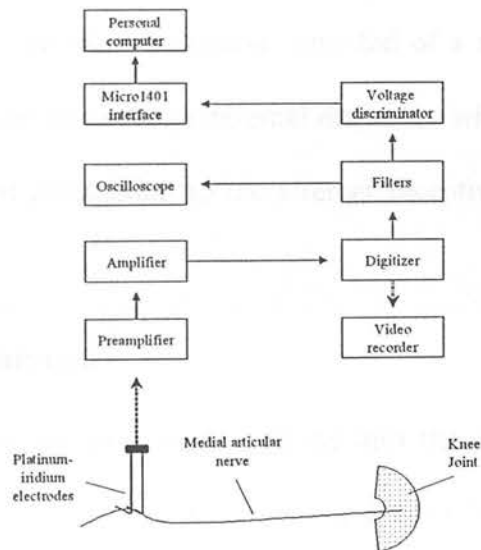


Figure 2.3 Schematic diagram of the arrangement used to record, store and analyse the afferent activity in the MAN innervating the rat knee joint.

2.1.2.6 Identification of units

Afferent units recorded from were classified according to their mechanosensitivity, chemosensitivity and conduction velocity.

Mechanosensitivity

The receptive field of each afferent was identified by probing the knee joint capsule with a hand held plastic probe of approximately 1mm tip diameter.

Chemosensitivity

Single low doses of the algogens capsaicin (10nmol) and bradykinin (9nmol) were administered by close intra-arterial (i.a.) injection (see Section 2.1.2.7 below) at some point during each experiment to determine the chemosensitivity of the afferents.

Conduction Velocity

Conduction velocities were determined as described for the cornea (Section 2.1.1.4). In the knee joint, the stimulating electrode consisted of a silver wire core isolated from a metal cylinder casing of 1mm external diameter, which was used to deliver suprathreshold electrical stimulation to the afferent receptive field (0.3 – 5V, 1ms pulse duration at 1Hz).

2.1.2.7 Drug administration

For close i.a. injections, agonists were injected into the abdominal aorta via the femoral cannula in a volume of 0.1ml, and washed in with 0.2 ml of saline (NaCl 0.9% w/v in distilled water). The injection was completed within 2s. To reduce the risk of tachyphylaxis, at least 5 - 20 minutes were allowed to pass between successive injections. Antagonists were injected over 10s (0.1ml 100g⁻¹ body

weight). Injection of drug vehicle, in most cases PBS, did not evoke any afferent excitation.

In a small number of animals, drugs were administered by i.art. injection into the knee joint in a volume of 0.05 – 0.1ml using a 26-gauge needle inserted beneath the patella through the infrapatellar ligament. To determine whether the presence of the needle in the knee joint influenced neural activity, spontaneous afferent discharge and that evoked by agonists was recorded prior to insertion of the needle. The needle, fitted to a syringe containing drug solution, was then inserted into the joint space and secured with a clamp. Drawing 20-30 μ l of air into the syringe after the drug solution prevented leakage of drug into the joint during the subsequent recording. Spontaneous activity and responses to agonists were then recorded. Because the rat knee joint volume is 0.15-0.20 ml, only one or two injections were performed per knee.

2.1.2.8 Estimation of local drug concentration

It is not possible to determine the precise local concentration of drugs at the knee joint terminals following i.a. or i.art. injection. However, if it is assumed that the drugs are distributed in a blood volume of 1ml, or a synovial volume of 0.1ml, respectively, it can be estimated from the following equation:

$$\text{Molar concentration} = \text{number of moles/number of litres}$$

The estimated local concentration of all drugs used in the rat knee joint neural recordings is given in Appendix II.

2.1.2.9 Data sorting

Nerve activity from each filament was sorted off-line using Spike2. This program was used to scan the waveform channel and generate templates for each individual

action potential it identified. The channel was then reclassified and each separate action potential was extracted and displayed on a separate channel as individual wavemark data. Markers were used to indicate the point at which a drug injection was made.

2.1.2.10 Data analysis

The knee joint data was analysed in a similar manner as described in Section 2.1.1.7 above for the corneal data. Briefly, the effect of a drug injection was determined by comparing the discharge after drug injection with that before injection, and the response was expressed as the change in the action potential discharge frequency. The effect of a drug on MAN discharge was also expressed as the change in the total number of action potentials firing as given by the calculation below. When no clear drug response was obtained, the activity in a fixed time of 15s post-injection was measured.

Delta $\Sigma\chi$: The change in the total number of action potentials evoked by the drug.

$$\text{Thus, } \Delta \Sigma\chi = \Sigma\chi_{\text{test}} - (\bar{\chi}_{\text{control}} * t_{\text{test}})$$

This calculation assumes that the basal discharge would remain unchanged in the absence of a stimulus.

Where,

$\Sigma\chi_{\text{test}}$: The total number of action potentials counted in the test time period, t_{test} (expressed as impulses).

$\bar{\chi}_{\text{control}}$: The frequency of action potential discharge in the control time period.

t_{test} : The test time period.

2.2 BEHAVIOURAL STUDIES

2.2.1 THE RAT EYE

In conscious male Wistar rats, the effects of topical application of drugs to the eye on eye blinking and wiping was assessed.

2.2.1.1 Drug administration

The rats were held while 5 μ l drops of drugs were instilled into the eyes using a pipette. The effect of vehicle instillation in one eye was determined initially and 10 minutes was allowed before drug was applied to the contralateral eye. Twenty minutes were allowed between successive instillations to the same eye.

2.2.1.2 Assessment of nociceptive responses

Rats were placed in an empty cage and the number of blinks and forepaw wipes of the treated eye were counted in the first minute post instillation of drug.

2.2.2 THE RAT KNEE JOINT

2.2.2.1 Induction of monoarthritis

Localised experimental arthritis was induced in the left knee joint of male Wistar rats by i.art injection of FCA as described in Section 2.1.2.1.1. For controls, some rats were injected with sterile paraffin as described.

2.2.2.2 Assessment of arthritis in behavioral studies

Throughout the development and maintenance of monoarthritis the general health of the rats was monitored by measuring body weight. The allodynia and swelling associated with adjuvant-induced arthritis was assessed by measuring hindlimb weight bearing and knee joint swelling as described below.

Weight-bearing allodynia

The distribution of body weight between the arthritic (left) and normal (right) hindlimbs of monoarthritic rats was measured quantitatively using a the dual channel weight averager (GlaxoWellcome/Linton Instruments, see Clayton *et al.* (1997)). This instrument consisted of a pair of force transducers connected to a digital read-out that displayed the mean weight on each transducer over a fixed time (Figure 2.4). The rats were placed in a clear perspex box so that they were standing with each hindlimb on a separate transducer and the mean weight on each foot was measured over a period of 7s. The allodynia induced by walking was assessed qualitatively by visual observation of the rats mobility using the following scores:

- 0 = normal
- 1 = slight limp, paw touched cage floor most of the time
- 2 = moderate limp, paw briefly touched cage floor
- 3 = severe limp, paw never touched cage floor



Figure 2.4 Photograph of the weight averager used to assess weight-bearing allodynia in monoarthritic rats.

Swelling:

The inflammation in the knee joint was measured quantitatively using a sliding calipers (Figure 2.5) and qualitatively by visual examination of the arthritic knee joint using the following scores:

- 0 = normal
- 1 = mild swelling
- 2 = moderate swelling
- 3 = severe swelling

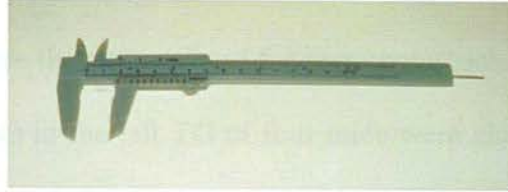


Figure 2.5 Photograph of the calipers used to measure knee joint diameter in monoarthritic rats.

2.2.2.3 Data analysis

The cumulative change in measurements over the duration of the trial from a selected control value was determined for each rat, and the change for each rat was expressed as a mean for the group.

2.3 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.3.1 ARTHRITIS INDUCTION

FCA or sterile paraffin-oil was injected into the left knee joint of male Wistar rats as described previously. The rats were used for retrograde labelling one week later and/or tissue was removed for histological and immunohistochemical studies two weeks later.

2.3.2 RETROGRADE LABELING OF DORSAL ROOT GANGLIA

One week after induction of arthritis, both knee joints of one male Wistar rat were injected with the retrograde tracer, fluorogold (Schmued & Fallon, 1986), to label knee joint afferent nerves in the DRG as described by Salo and Theriault (1997). The

rat was transiently anaesthetised with halothane and 5 μ l of 2% fluorogold in distilled water was injected into the knee joint just beneath the patella using a Hamilton syringe and a 26G needle. This was followed after a few minutes by 15 μ l of 0.1M phosphate buffer to precipitate the dye in the synovial space. A survival time of one week was allowed before the rat was fixed for immunohistochemistry.

Corneal afferents in the left TG of four mice were also retrogradely labelled using fluorogold, and the right TG were used as controls (performed by Dr. Carmen De Felipe at the Universidad de Alicante). The TG were removed five days later, post-fixed in paraformaldehyde (4% in 0.01M sodium phosphate buffer) and sent to Edinburgh for immunohistochemical studies.

2.3.3 FIXATION

Normal, control (paraffin-injected) and arthritic rats were anaesthetised with pentobarbitone (60mg kg⁻¹ i.p.). A midline incision was made at the abdomen, the abdominal aorta was anterogradely cannulated (catheter OD: 1.34mm) and the vena cava was cut to allow bleeding of the animal. The animal was perfused with 10ml of heparinised saline (500units kg⁻¹) followed by 50-100ml of 10% formal saline via the aortic cannula. The left and right knee joints, and DRGs (levels L3, L4, L5 and L6) were removed and post-fixed in 10% formal saline. All tissue was then processed (post-fixed, decalcified and paraffin-embedded), sectioned (3 μ M sections) and mounted on glass slides by Mr. Steven MacKell and Ms. Helen Caldwell (Department of Pathology, University of Edinburgh).

2.3.4 HISTOLOGY

Paraffin-embedded sections of normal, in addition to ipsilateral and contralateral knee joints of arthritic and control rats were stained using haematoxylin and eosin, and covered with glass coverslips using Pertex mountant. The sections were subsequently examined using light microscopy by Dr. Donald Salter (Department of Pathology, University of Edinburgh).

2.3.5 IMMUNOHISTOCHEMISTRY

In the present study, polyclonal antibodies raised in the rabbit against rat P2X₃ receptors (kindly donated by Dr. E.J. Kidd) were used to confirm the presence of P2X₃ receptors in rat and mouse sensory neurones in the DRG and TG respectively. Antibody labelling was indirectly viewed using the Avidin-Biotin Complex method (ABC) which is shown schematically in Figure 2.6. In the ABC method used, complexes of streptavidin and biotinylated horseradish peroxidase (HRP-ABC), or biotinylated alkaline phosphatase (AP-ABC) were visualised using the chromogens 3,3'-diaminobenzidine tetrahydrochloride (DAB) or Vector Red respectively.

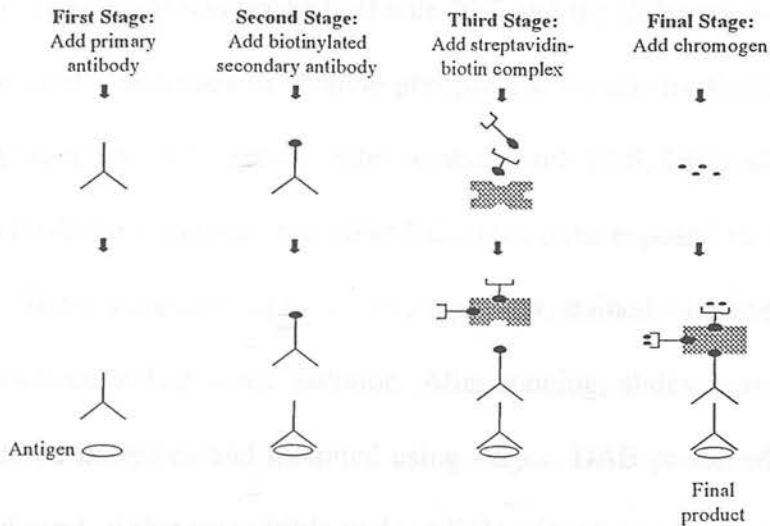


Figure 2.6 Schematic diagram showing the principles of the Avidin-Biotin method used to locate $P2X_3$ receptor in rat and mouse tissues.

2.3.6 $P2X_3$ IMMUNOHISTOCHEMISTRY METHOD

Sections were dewaxed in xylene for 10 minutes and placed in 95% alcohol. If horseradish peroxidase was used as the tracer, the sections were put in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. Sections were then dehydrated in alcohol and washed with running tap water. The slides were subsequently mounted on individual perspex slide holders, placed in a staining trough (Sequenza) and washed with PBS (all washes were 3-5 minutes). Non-specific staining was blocked by incubating the slides with normal swine serum diluted 1:5 with 0.1% triton in PBS (NSS-tPBS) for 10 minutes (all incubations were at room temperature except where stated). Sections were then incubated with either i) $P2X_3$ antibody or ii) pre-immune serum (PI, the serum from the host animal in which the primary antibody was raised before exposure to the immunogen) diluted 1:2000 in NSS-tPBS for 72 hours at 4°C. After the primary incubation, sections were washed with PBS, blocked with NSS-tPBS (10 minutes) and incubated in biotinylated secondary swine-anti-rabbit antibody (1:500 in NSS-tPBS) for 2 hours.

The secondary antibody was washed off with PBS and the slides were incubated with either horseradish peroxidase or alkaline phosphatase Avidin-Biotin Complex (HRP-ABC or AP-ABC) for 30 minutes. After washing with PBS, HRP-ABC slides were exposed to DAB for 5 minutes and AP-ABC slides were exposed to Vector Red for 30 minutes. Slides were washed in running tap water, stained with haematoxylin and “blued” with Scott’s Tap Water Solution. After staining, slides were dehydrated in alcohol, cleared in xylene and mounted using Pertex. DAB produced a dark-brown reaction end product that was visible under a light microscope. Vector Red produced a red end product that was also visible under the light microscope, but since it is a red fluorescent dye (maximally absorbent under green light), it was primarily examined under a fluorescence microscope.

2.4 DETERMINATION OF ATP CONCENTRATION IN SYNOVIAL PERFUSATE

2.4.1 ARTHRITIS INDUCTION

Arthritic and control rats were induced as described and the rats were used two weeks post-induction.

2.4.2 WITHDRAWAL OF SYNOVIAL FLUID

Normal and arthritic male Wistar rats were anaesthetised with pentobarbitone (60mg kg^{-1} i.p.). The rats were placed on their backs and one of the hindlimbs was held firmly. Two 26-gauge needles were inserted through the infrapatellar ligament beneath the patella as described by Malone (1991). One needle was used as an inflow needle and the other as an outflow needle. 1ml of 1mM EDTA in PBS was slowly

injected into the knee joint via the inflow needle and the perfusate was aspirated from the hub of the outflow needle and collected into ice-cold Epindorf tubes. EDTA was used to try to limit ATP degradation (ecto-nucleotidases are dependant on divalent cations (Banerjee, 1981)). Samples were immediately centrifuged (200g for 10 minutes at 4°C) in a refrigerated centrifuge (Micromax RF, International Equipment Company) to remove cells. After centrifugation, the supernatant was weighed by transferring it into epindorfs on ice that had been “zeroed” on a balance and samples were placed in dry-ice and sent to GIAP for analysis of ATP concentration. Although centrifugation was probably traumatic and caused ATP release from the cells, it was necessary because freezing and thawing would rupture the cell membrane and cause considerable ATP release. This was confirmed in an earlier investigation, which showed that the perfusate ATP concentration correlated with the level of blood in the samples (data not shown in this thesis).

2.5 STATISTICAL ANALYSIS

Unpaired t-tests were used to analyse differences between the means of two normally distributed groups of data. When the sample size was too small to determine whether or not it followed a Gaussian distribution, the Mann-Whitney test was used. Student’s paired t-test analysis was used when experiments were performed to produce normally distributed data with in-experiment controls. One way analysis of variance, followed by Tukey’s multiple comparison (post hoc) test, was used to compare the means of more than two normally distributed groups.

Chapter 3: The direct role of ATP in corneal nociception

3.1 INTRODUCTION

Photorefractive keratectomy for myopia is associated with severe post-operative ocular pain (Stein *et al.*, 1994). Topical instillation of local anaesthetic is widely used to control the pain, but prolonged use causes poor wound healing and other complications (Verma & Marshall, 1996). An improved method of treating this type of post-operative pain is urgently needed, as reflected by recent clinical trials (for example see Assouline *et al.*, 1998; Frangouli *et al.*, 1998; Lim-Bon-Siong *et al.*, 1998). Research strategies aimed at finding effective treatments for corneal pain could be devised if the mechanisms underlying it were fully understood. The cornea is innervated by trigeminal afferents and it is known that six subtypes of ATP-gated ion-channel receptors (P2X₁₋₆) are expressed in the cell bodies of neurones within the trigeminal ganglion (Collo *et al.*, 1996). If it can be shown that P2X receptors are involved in nociceptive transmission from the cornea, this would have implications for the treatment of corneal pain.

To establish whether P2X receptors play a role in corneal nociception, it is crucial to determine whether functional receptors are expressed on the peripheral terminals of trigeminal afferents innervating the cornea. In cell culture, neurites of rat tooth pulp trigeminal neurones are depolarised by ATP and also by the P2X₁ and P2X₃ receptor selective agonist $\alpha\beta\text{meATP}$ (Cook *et al.*, 1997). These neurones were assumed to be nociceptive because the predominant sensation evoked from tooth pulp by various stimuli is pain (Ahlquist *et al.*, 1984; Anderson & Matthews, 1967; Edwall & Olgart, 1977). The Cook *et al.* (1997) investigation indicates that

functional $\alpha\beta\text{meATP}$ -sensitive P2X receptors are expressed away from the cell bodies of assumed nociceptors *in vitro*, but whether they are on the peripheral terminals of defined nociceptors *in vivo* is still unknown.

The present investigation used electrophysiological and behavioural methods to test the hypothesis that functional P2X receptors are expressed on the peripheral terminals of corneal nociceptors *in vivo*. The neural discharge of nociceptive afferents innervating the corneas of anaesthetised cats was recorded and the effects of applying P2 agonists to their receptive fields were investigated. The cornea is an ideal tissue for electrophysiological studies of nociception because it has a high density of nociceptors (20-40 times more than tooth pulp (Rozsa & Beuerman, 1982)) with easily explored receptive fields. The effect of photorefractive corneal keratectomy on the sensitivity of corneal nociceptors to the purines was also briefly investigated as this has previously been shown to alter the sensitivity of corneal polymodal nociceptors to chemical or mechanical stimulation (Gallar *et al.*, 1997). The cornea is also ideal for behavioural studies since, like the tooth pulp, pain is the main sensation aroused from it (see Belmonte & Gallar, 1996). Thus, to determine the behavioural correlates of the electrophysiological studies, P2 receptor agonists were instilled into the eyes of conscious rats and any resulting behaviours indicative of painful stimulation were noted. In the final section of this study, cell bodies of corneal afferents in the mouse TG were labelled using a retrograde fluorescent tracer and immunohistochemical methods were used to investigate whether P2X receptor immunoreactivity co-localised with the tracer.

3.2 METHODS

3.2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

Electrophysiological recordings were carried out as described in Section 2.1.1. Briefly, seven normal adult cats (3.2 ± 0.2 kg) were anaesthetised with pentobarbitone (40 mg kg^{-1} i.p., supplemented hourly i.v.) and the trachea and right saphenous vein cannulated. One cat (3.5 kg) following photorefractive keratectomy (performed by Dr. A.R. Gutierrez, Departamento de Oftalmologia, Universidad de Murcia, Spain) was also used. Extracellular recordings from filaments of mixed ciliary nerves with receptive fields in the cornea were performed as described previously (Belmonte *et al.*, 1991). Drugs were applied to the cornea for 30s via a piece of solution-soaked tissue or in a $50 \mu\text{l}$ drop. Data are expressed as mean change in action potential frequency \pm sem.

3.2.2 BEHAVIOURAL STUDIES

Behavioural studies were performed as described in Section 2.2.1. In summary, drug solutions ($5 \mu\text{l}$ drop) were instilled into the eyes of four conscious male Wistar rats (283 ± 7 g) and the number of blinks and forepaw wipes of the eye, behaviours associated with nociception (see Gonzalez *et al.*, 1993), were counted in the first minute post-instillation.

3.2.3 IMMUNOHISTOCHEMICAL STUDIES

The left TG of four mice were retrogradely labelled from the left cornea using fluorogold and the right TG were used as controls (performed by Dr. C. De Felipe,

Instituto de Neurociencias, Universidad de Alicante, Spain). The processing and detection of P2X₃ immunoreactivity was carried-out as described in Section 2.3.

3.3 RESULTS

3.3.1 AFFERENT NERVE RECORDINGS *IN VIVO*

Neural discharge was recorded from 56 afferent fibres innervating 13 eyes from 7 normal cats. Afferent activity was also investigated in 6 fibres innervating 1 eye after central corneal keratectomy.

3.3.1.1 Characterisation of afferent nerve fibres

3.3.1.1.1 Conduction velocities, mechanosensitivity and chemosensitivity

The afferents were classified as A δ -fibre polymodal nociceptors, C-fibre polymodal nociceptors or A δ -fibre mechanonociceptors based on their conduction velocities, mechanosensitivity and sensitivity to CO₂ stimulation. A δ -fibre polymodal nociceptors were fast conducting ($5.02 \pm 0.09 \text{ms}^{-1}$), activated by stroking the receptive field with a fine wet paintbrush, and by a 30s pulse of CO₂ directed at the receptive field (Figure 3.1). C-fibre polymodal nociceptors were slowly conducting ($0.77 \pm 0.08 \text{ms}^{-1}$) and responded to mechanical and CO₂ stimulation (Figure 3.1). A δ -fibre mechanonociceptors (conduction velocity: $9.58 \pm 3.06 \text{ms}^{-1}$) responded to mechanical stimulation of the receptive field but were not excited by CO₂.

In normal cats, 64% (36/56 afferents) of the afferents recorded from were A δ -fibre polymodal nociceptors, 20% (11/56 afferents) were C-fibre polymodal nociceptors and 16% (9/56 afferents) were mechanonociceptors. The 6 fibres innervating the laser-treated cornea were A δ -fibre polymodal nociceptors.

3.3.1.1.2 Location of afferent receptive fields

Nociceptors with corneal receptive fields were selected for this study. However, a small number of the afferents had receptive fields that extended into the surrounding sclera, that is, they were cornea/scleral afferents. The effect of application of P2X receptor agonists to the scleral receptive field of these fibres will be presented in Section 3.3.1.8.

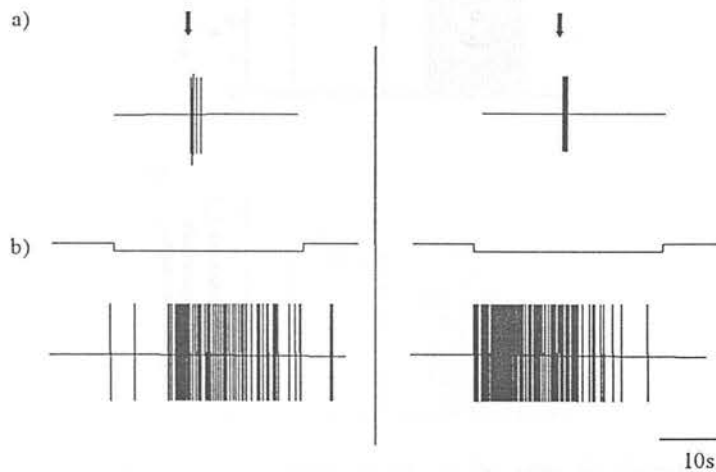


Figure 3.1 Neural discharges evoked by a) mechanical (at arrow) and b) CO₂ stimulation (for 30s marker) of the corneal receptive field of single A δ - (first panel) and C-fibre (second panel) polymodal nociceptors.

3.3.1.2 CO₂-evoked excitation of corneal polymodal nociceptors

The response latency, response duration and change in action potential discharge frequency from the basal rate (A δ -polymodal: 0.2 ± 0.4 impulses s⁻¹; C-polymodal: 0.2 ± 0.5 impulses s⁻¹) caused by CO₂ in 19 A δ -fibre and 9 C-fibre polymodal nociceptors innervating the cornea were compared. The onset latency of the excitation in C-fibres was significantly shorter ($P < 0.05$, unpaired t-test) than that in A δ -fibres, which may have reflected deeper terminal endings of the latter, but no other differences between the responses were apparent (Figure 3.2).

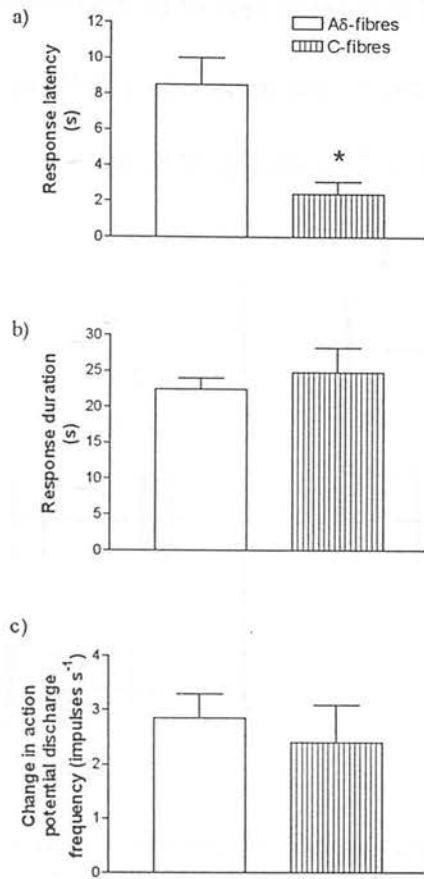


Figure 3.2 Pooled data showing the a) onset latency, b) duration and c) change in discharge frequency of the response evoked by a 30s pulse of CO₂ in corneal Aδ-fibre and C-fibre polymodal nociceptors. Columns represent mean ± sem. Aδ-fibres n=19, C-fibres n=9. *P<0.05 unpaired t-test; faster onset in C-fibres compared to Aδ-fibres.

3.3.1.3 Lack of P2X-mediated activation of corneal polymodal nociceptors

The P2X₁ and P2X₃ subtype selective agonist, αβmeATP (100μM), the non-selective P2 agonist, ATPγS (100μM) and the endogenous ligand at P2 receptors, ATP (100μM) did not evoke a response from Aδ-fibre or C-fibre polymodal nociceptors when the drugs were applied to the cornea using a piece of tissue paper (Figure 3.3 and Figure 3.4). A wider range of doses of αβmeATP was studied these also failed to stimulate the nociceptors (30μM: 0.07±0.10 impulses s⁻¹; 1000μM: 0.65±0.14 impulses s⁻¹, n=3 Aδ-polymodal nociceptors per concentration). αβmeATP (100μM)

and ATP (100 μ M) were also applied to the cornea as a 50 μ l drop, but application by this method also failed to elicit a change in action potential discharge ($\alpha\beta$ meATP: 0.06 \pm 0.22 impulses s⁻¹; ATP: 0.07 \pm 0.23 impulses s⁻¹, n=3 A δ -fibres each).

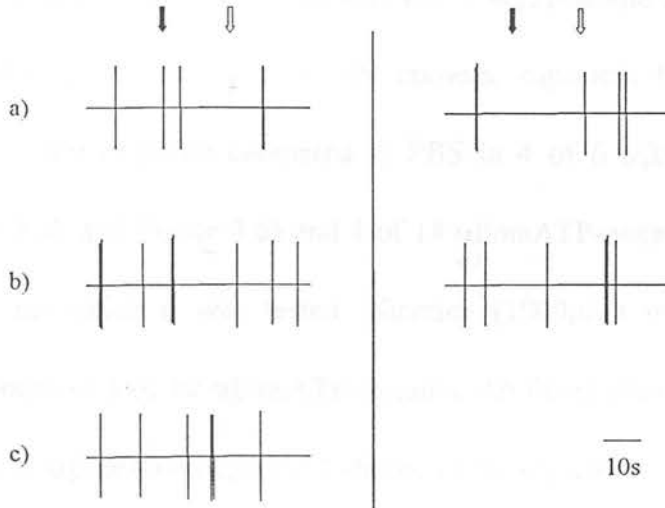


Figure 3.3 Neural discharge showing the lack of response evoked by application (at first arrow) of a) $\alpha\beta$ meATP 100 μ M, b) ATP γ S 100 μ M and c) ATP 100 μ M to the receptive field of corneal A δ -fibre (first panel) and C-fibre (second panel) polymodal nociceptors. The second arrow indicates when the cornea was washed.

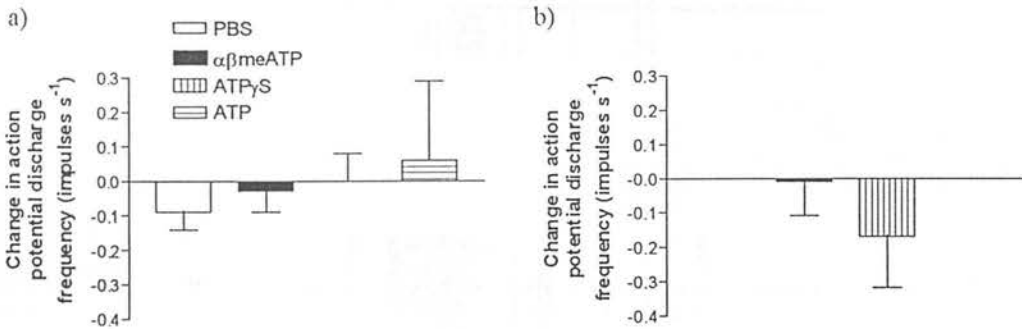


Figure 3.4 Pooled data showing the absence of effect of PBS, $\alpha\beta$ meATP 100 μ M, ATP γ S 100 μ M and ATP 100 μ M on the action potential discharge frequency in a) A δ -fibre and b) C-fibre corneal polymodal nociceptors. Data is from the first 10s after application of the drugs via a piece of tissue paper. Columns represent mean \pm sem. PBS: A δ -fibres n=5, C-fibres not done; $\alpha\beta$ meATP: A δ -fibres n=25, C-fibres n=9; ATP γ S: A δ -fibres n=7, C-fibres n=4; ATP: A δ -fibres n=5, C-fibres not done.

3.3.1.4 Capsaicin & nicotine-evoked excitation of corneal polymodal nociceptors

Although polymodal nociceptors were not excited by the purines, the algogens capsaicin and nicotine were used to confirm that it was possible to excite them using other pharmacological agents. In normal corneas, capsaicin ($0.1 \mu\text{M}$) evoked a significant ($P < 0.05$) response compared to PBS in 4 of 6 $\alpha\beta\text{meATP}$ -negative C-fibres (Figure 3.5b and Figure 3.6) and 1 of 14 $\alpha\beta\text{meATP}$ -negative $A\delta$ -fibres ($2.03 \text{ impulses s}^{-1}$) on which it was tested. Nicotine ($1000 \mu\text{M}$) evoked a significant ($P < 0.05$) response in 3 of 10 $\alpha\beta\text{meATP}$ -negative $A\delta$ -fibres (Figure 3.5b and Figure 3.6) and 1 of 2 $\alpha\beta\text{meATP}$ -negative C-fibres ($4.98 \text{ impulses s}^{-1}$) on which it was tested.

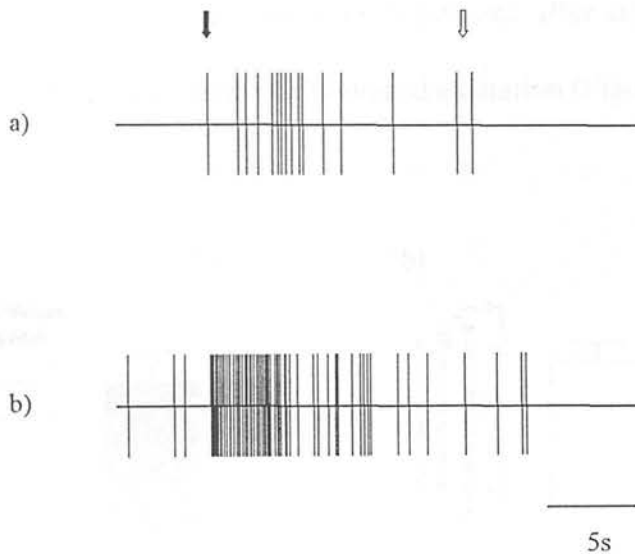


Figure 3.5 Neural discharge showing typical responses evoked by application (at first arrow) of a) capsaicin $0.1 \mu\text{M}$ to the corneal receptive field of a C-fibre polymodal nociceptor and b) nicotine $1000 \mu\text{M}$ to the corneal receptive field of an $A\delta$ -fibre polymodal nociceptor. Second arrow indicates when the cornea was washed.

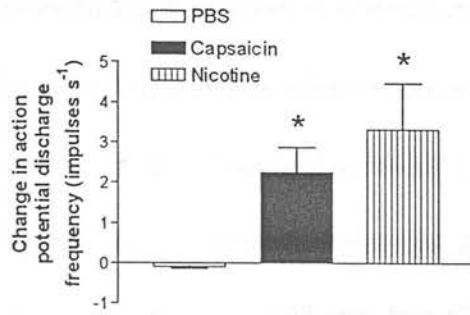


Figure 3.6 Pooled data showing the change in action potential discharge frequency evoked by capsaicin $0.1 \mu\text{M}$ and nicotine $1000 \mu\text{M}$ from polymodal nociceptors. Columns represent mean \pm sem. Capsaicin: C-fibres $n=4$; nicotine: $A\delta$ -fibres $n=3$; PBS: $A\delta$ -fibres $n=5$. * $P < 0.05$ unpaired t -test versus PBS.

3.3.1.5 Lack of P2X-mediated modulation of the CO_2 response

Although $\alpha\beta\text{meATP}$ did not excite corneal nociceptors directly, any potential augmentation or attenuation of the CO_2 response in polymodal nociceptors was determined. The magnitude of the CO_2 response in polymodal nociceptors was measured 1 or 5 minutes before and 1 or 5 minutes after $\alpha\beta\text{meATP}$. The P2X receptor agonist had no effect on the CO_2 -evoked excitation (Figure 3.7).

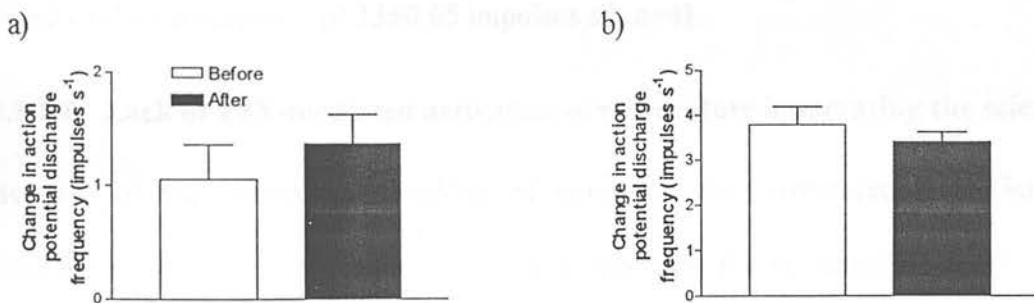


Figure 3.7 Pooled data showing the response evoked by a 30s pulse of CO_2 a) 1 minute before & 1 minute after, and b) 5 minutes before & 5 minutes after $\alpha\beta\text{meATP}$ ($100 \mu\text{M}$). Columns represent mean \pm sem. Pooled data from one $A\delta$ -fibre and two C-fibre polymodal nociceptors.

3.3.1.6 Lack of P2X-mediated excitation of corneal mechanonociceptors

In normal cornea, 16% (9/56 afferents) of afferents recorded from were classified as A δ -fibre mechanonociceptors. The effect of $\alpha\beta$ meATP (100 μ M) on action potential discharge was investigated in 2 of these afferents but it failed to elicit a response (change in discharge frequency, 1st unit: 0.03 impulses s⁻¹; 2nd unit: -0.12 impulses s⁻¹).

3.3.1.7 Lack of P2X-mediated activation of nociceptors innervating the keratectomised cornea

The location of the afferent receptive field relative to the excised section of the cornea, that is, whether they are in, near, or far from the ablation, affects the response characteristics of the nociceptors (Gallar *et al.*, 1997). In the present study, the polymodal A δ -fibres had corneal receptive fields near to the ablation. The afferent discharge evoked by CO₂ in these nociceptors was not significantly different to that in normal corneas (normal A δ -fibres: 2.85 \pm 0.44 impulses s⁻¹, n=19; keratectomy A δ -fibres: 2.27 \pm 0.19 impulses s⁻¹, n=4). As in the normal corneas, $\alpha\beta$ meATP (100 μ M) failed to elicit a response (0.13 \pm 0.05 impulses s⁻¹, n=4).

3.3.1.8 Lack of P2X-mediated activation of nociceptors innervating the sclera

Because of the extensive branching of corneal nerve axons (see the General Introduction), some of the receptive fields extend into the surrounding sclera. This unusual property allows the testing of the same afferent in avascular (cornea) and vascularised (sclera) regions.

Two of the A δ -polymodal nociceptors innervating normal eyes and one innervating the keratectomised eye had receptive fields that extended into the sclera.

No response to $\alpha\beta\text{meATP}$ ($100\mu\text{M}$) was observed when it was applied to either the corneal or the scleral receptive field of these afferents (Table 3.1).

Table 3.1 Absence of excitation following application of $\alpha\beta\text{meATP}$ ($100\mu\text{M}$) to the corneal or scleral receptive fields of A δ -fibre polymodal nociceptors.

	Change in discharge frequency (impulses s ⁻¹)	
	Corneal	Scleral
A δ -fibre from normal eye	0.1	0
A δ -fibre from normal eye	-0.5	0
A δ from keratectomised eye	0	-0.2

3.3.2 BEHAVIORAL STUDIES

3.3.2.1 Lack of nociceptive behaviour following instillation of P2X receptor agonists into the eye

In a pilot study in one conscious rat, single instillations of a number of P2 receptor agonists into the eyes did not induce any behavioural changes indicative of pain (Table 3.2). In a more detailed investigation in four rats, the number of blinks and wipes of the eye evoked following instillation of $\alpha\beta\text{meATP}$ (10 - $1000\mu\text{M}$) did not differ significantly from that following vehicle instillation or from the basal rate (Figure 3.8).

Table 3.2 Blinks and wipes in the first minute post-instillation of ATP and ATP γ S into the eye. Data are from single instillations. Basal data from Figure 3.8 are included for comparison.

	Blinks (number min ⁻¹)	Wipes (number min ⁻¹)
Basal rate	2.3±0.9	0±0
ATP 100 μ M	3	0
ATP 1000 μ M	2	0
ATP γ S 100 μ M	2	0
ATP γ S 1000 μ M	2	0

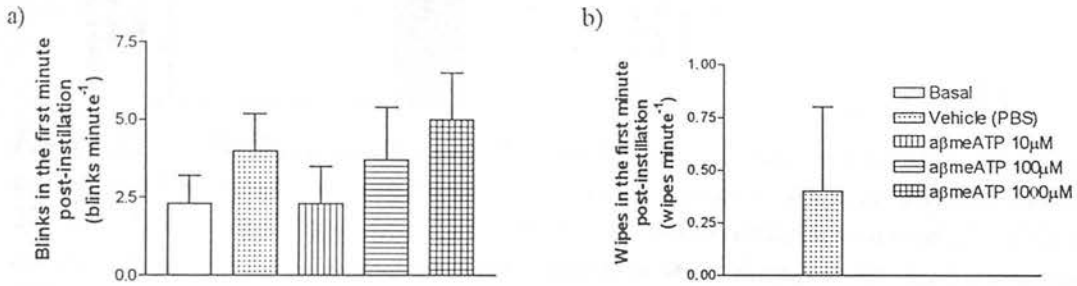


Figure 3.8 Pooled data showing the number of a) blinks and b) wipes in the first minute post instillation of $\alpha\beta$ meATP (10-1000 μ M) and vehicle into the eye. The basal rates are shown for comparison. Columns represent mean \pm sem.

3.3.2.2 Nociceptive behaviour following instillation of capsaicin and nicotine into the eye

Threshold concentrations of capsaicin (10 μ M) and nicotine (1000 μ M) both caused a significantly higher ($P < 0.01$) number of eye blinks compared to their respective vehicles (Figure 3.9a). However, the mean number of wipes evoked by capsaicin and nicotine was not statistically different to that caused by vehicles (Figure 3.9b). Higher doses of the algogens would have caused more pain and would presumably

have induced significant wiping of the eye but this was not investigated to avoid undue suffering.

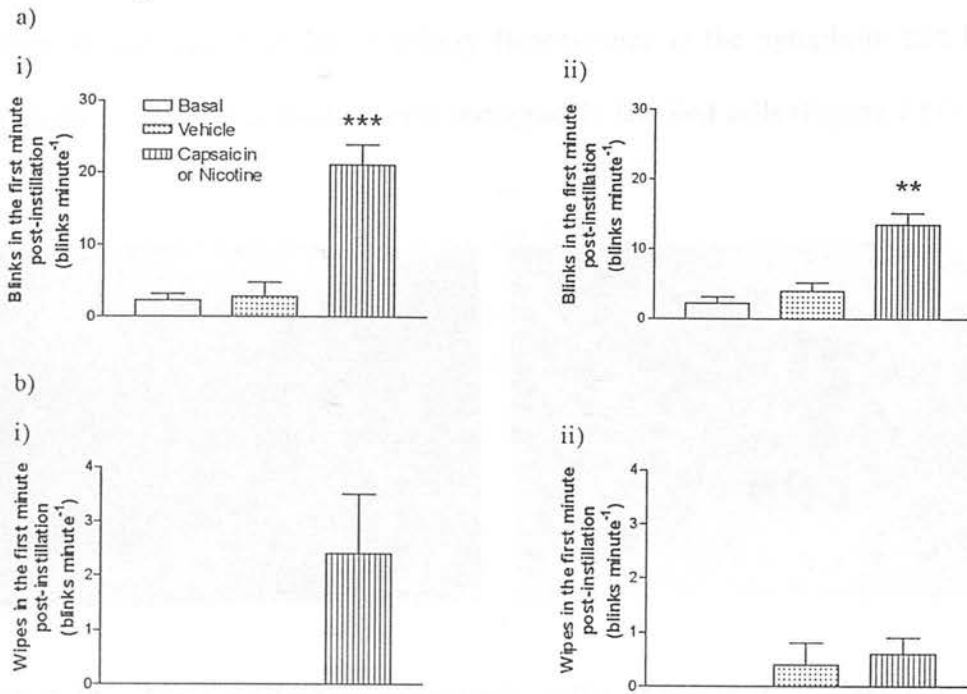


Figure 3.9 Pooled data showing the number of a) blinks and b) wipes in the first minute post instillation of capsaicin $10\mu\text{M}$ (i) or nicotine $1000\mu\text{M}$ (ii) into the eye. The basal rates and the number evoked by capsaicin vehicle (ethanol & PBS) and nicotine vehicle (PBS) are shown for comparison. Columns represent mean \pm sem. *** $P < 0.0001$ unpaired *t*-test capsaicin versus vehicle. ** $P < 0.01$ unpaired *t*-test nicotine versus vehicle.

3.3.3 IMMUNOHISTOCHEMICAL STUDIES

3.3.3.1 P2X₃ immunoreactivity in corneal trigeminal ganglion cell bodies

Two sections from the left and right TG, fluorogold-labelled and control respectively, of four mice were examined for P2X₃-immunoreactivity and/or co-localisation with fluorogold fluorescence. Each section clearly showed P2X₃-immunoreactivity throughout the cytoplasm in discrete cells using DAB (not shown) or Vector Red visualisation (Figure 3.10). Although no attempt was made to quantify the proportion of cells labelled, approximately half of the cells in each section were

labelled with the P2X₃ antibody. Retrogradely labelled cells were identified by the presence of scattered particles of silvery fluorescence in the cytoplasm and P2X₃-immunoreactivity co-localised in some retrogradely labelled cells (Figure 3.11)

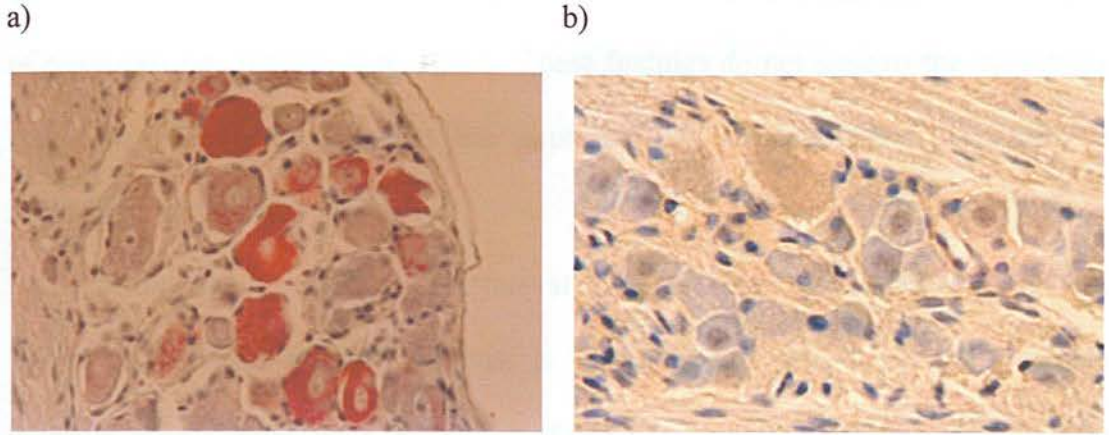


Figure 3.10 Light field photomicrographs (x40) of two sections from one mouse trigeminal ganglion showing a) discrete P2X₃-immunoreactivity visualised with Vector Red in a number of cells and b) the absence of staining when the sections were incubated with pre-immune serum.

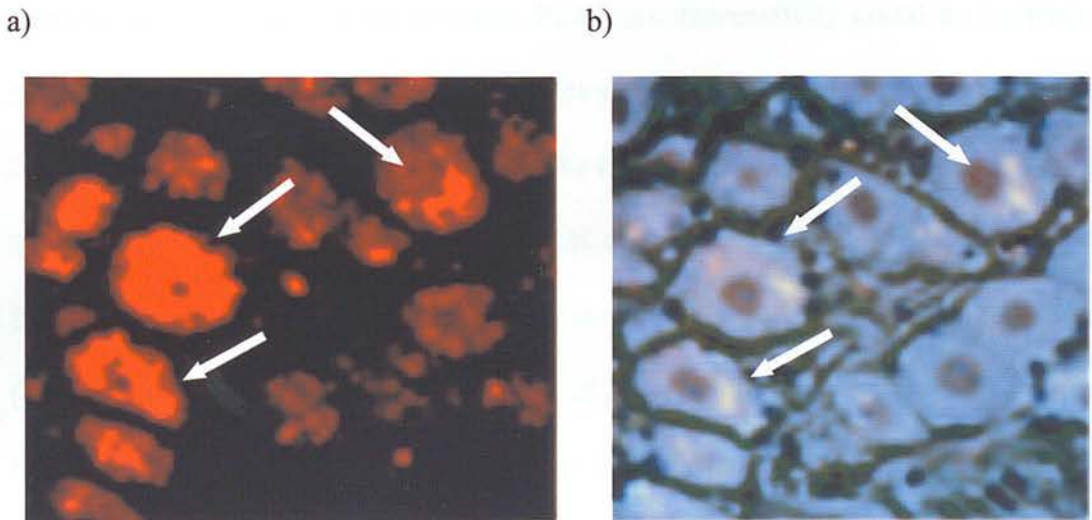


Figure 3.11 Fluorescence micrographs (x40) of a single mouse trigeminal ganglion section illustrating a) P2X₃-immunoreactivity visualised with Vector Red and b) fluorogold fluorescence. Fluorogold and Vector Red fluorescence co-localised in the cells indicated by the arrows.

3.3.4 DISCUSSION

Although P2X₃ receptor immunoreactivity was found in mouse corneal trigeminal neurones, P2 receptor agonists did not excite or sensitise nociceptors innervating the cat cornea nor did they evoke nociceptive behaviours after instillation into the eyes of conscious rats (Dowd *et al.*, 1997). These findings do not support the hypothesis that functional P2X receptors are expressed on the peripheral terminals of nociceptive afferents *in vivo*.

Antibodies directed against the rat P2X₃ receptor were used (Kidd *et al.*, 1998) because this receptor subtype is selectively expressed in, and probably mediates depolarisation of a population of trigeminal tooth pulp nociceptors *in vitro* (Cook *et al.*, 1997). It was surprising that $\alpha\beta$ meATP (a stable P2X₁ and P2X₃ subtype selective agonist), ATP γ S (a non-selective P2 receptor agonist) and ATP (the endogenous ligand at P2 receptors) did not excite cat corneal nociceptors or evoke nociceptive behaviours in the rat since P2X₃-immunoreactivity could be located in corneal neurones in the mouse TG. Species differences are unlikely to account for the negative behavioural data because P2X₃ mRNA transcripts or immunoreactivity has previously been detected in rat TG (Collo *et al.*, 1996; Llewellyn-Smith & Burnstock, 1998; Xiang *et al.*, 1998), and nerve fibres of the rat corneal epithelium (Vulchanova, 1998). However, expression of P2X₃ receptors in the cat TG or cornea have not been investigated and therefore, it is possible that the lack of excitation of cat corneal nociceptors reflects an absence of the receptor protein. Species differences in expression of P2X₃ receptors is known to exist: in rats, it is selectively expressed in sensory ganglia (Collo *et al.*, 1996) whereas in humans, it is only expressed in the spinal cord and heart (GarciaGuzman *et al.*, 1997). Because the

distribution of P2X receptors in the cat TG is unknown, it is not possible to exclude that species differences accounted for our electrophysiological results. However, the possibility that the P2 agonists did not penetrate to the nociceptive terminals because of biological, physical or electrochemical barriers was also considered.

ATP and ATP γ S are rapidly metabolised to adenosine *in vivo* (see Chapters 5 and 6) and corneal ecto-nucleotidase activity could conceivably have prevented them from reaching the nociceptors. However, $\alpha\beta$ meATP is not degraded by the enzyme and therefore the presence of enzymatic barriers cannot be invoked to explain our results. The other pharmacological agents tested, capsaicin and nicotine, excited polymodal nociceptors, as did CO₂, which mediates its effects by lowering interstitial pH (Chen *et al.*, 1995). Capsaicin and nicotine have lower molecular weights (162 and 305 respectively) than ATP, ATP γ S or $\alpha\beta$ meATP (551, 547 and 505 respectively) and could potentially have traversed the outer corneal epithelial layers more easily than the purines. However, high molecular weight algogenic peptides such as bradykinin (MW 1060) can activate corneal nociceptors (Belmonte *et al.*, 1994) and thus the size of the P2 agonists should not have influenced their ability to gain access to the nerve endings. The possibility that the electrically charged form of ATP that activates P2X receptors (ATP⁴⁻, see Fedan *et al.*, 1990) may have been prevented from penetrating to the nerve terminals was also considered. This is unlikely because nicotine is also a charged compound ((-)-nicotine) and it activated cat corneal nociceptors and caused behaviours related to pain after instillation into the rat eye.

One of the peculiar aspects of recording from nociceptors innervating the cornea is that it is possible to investigate the responses of the same afferent to drugs

in avascular and vascular regions. If excitation is observed following application of drugs to the sclera but not the cornea, this indicates that vascular effects, such as vasoconstriction resulting in hypoxia or production of a blood-derived inflammatory mediator, probably account for the response. In the present investigation, no change in discharge was observed when $\alpha\beta$ meATP was applied to the corneal or scleral receptive fields of nociceptors. This confirmed that the absence of excitation observed in the cornea could not be accounted for by the lack of a vascular effect.

Another explanation for the lack of a P2X mediated response in this study is the very slow penetration of the agonists to the nerve endings. Although nerves in the cornea terminate within a few microns of the surface (Zander & Wedell, 1951), the capsaicin-evoked response in the electrophysiological studies was delayed in onset (approximately 5s, see Figure 3.5, pooled data not shown). In contrast, capsaicin, when injected into rat lower abdominal artery, activates nociceptors in the knee joint with a latency of approximately 1s even though it has a far greater distance to travel (Dowd *et al.*, 1998c, and see Chapter 4). Slow penetration is likely to lead to gradual increases in the local concentration of drugs, which may have caused receptor desensitisation. Recombinant P2X₃ receptors mediate a transient current when expressed in HEK 293 cells (Lewis *et al.*, 1995) and therefore this subtype would be expected to desensitise rapidly. Interestingly, in small diameter sensory neurones (associated with nociception) dissociated from the rat DRG, capsaicin activates a slowly-desensitising current (Li *et al.*, 1999). If similar properties are associated with capsaicin-induced responses in trigeminal neurones, this could explain why capsaicin evoked a response from corneal nociceptors in the present study despite the slowly increasing concentration of agonist.

Aside from possibility of species differences or receptor desensitisation, the obvious implication of this work is that functional P2X receptors are not expressed in corneal nociceptive endings *in vivo* despite the presence of functional P2X receptors and P2X₃ receptor immunoreactivity in TG (the present immunohistochemical data and Cook *et al.*, 1997). This is supported by recent findings in the tooth pulp because ATP and analogues failed to activate tooth pulp afferents in anaesthetised cats (Li *et al.*, 1998; Matthews *et al.*, 1997). If these receptors are not expressed functionally on the peripheral terminals of sensory afferents, then why are they expressed functionally at the cell bodies of sensory neurones?

Interestingly, P2X₃ immunohistochemical staining is very intense in the endoplasmic reticulum and Golgi apparatus of trigeminal sensory neurones (Llewellyn-Smith & Burnstock, 1998). It is conceivable that under certain conditions these receptors might be transported to, and expressed on the sensory cell body membrane. Thus, the artificial environment found in dissociated cell culture might stimulate the functional expression of the receptors, thereby explaining why P2X mediated responses are obtained in trigeminal neurones *in vitro*. Consistent with this is the demonstration that few P2X mediated responses can be obtained from rat DRG neurones when they are maintained *in vitro* with the peripheral roots intact, whereas when these cells are dissociated, most of them exhibit ATP and $\alpha\beta$ meATP-evoked inward currents (Stebbing *et al.*, 1998). If some *in vitro* conditions stimulate the functional expression of P2X receptors, does an equivalent physiological trigger exist that would cause the receptors to be functionally expressed on the peripheral terminals of nociceptive afferents?

One possibility is that the receptors might only be functionally expressed under inflammatory conditions or following injury. This is known to happen with other receptors. For example, bradykinin B₁ receptors are not expressed in normal tissue but *de novo* synthesis and expression occurs after exposure to some inflammatory mediators (Bouthillier *et al.*, 1987; deBlois *et al.*, 1991). The proportion of rat trigeminal neurones that are immunoreactive to the P2X₃ antibody increases 4-10 days following injury to the mandibular inferior alveolar nerve (Eriksson *et al.*, 1998). These authors suggested that up-regulation of P2X₃ receptors following nerve injury could mediate the abnormal firing of injured sensory nerves. Consistent with this, the P2X₃ subtype accumulates at sites proximal to trigeminal nerve injury (Cook *et al.*, 1997; Eriksson *et al.*, 1998). Since photorefractive corneal keratectomy undoubtedly injures the nociceptive afferents, it is possible that the P2X₃ receptor might be up-regulated and functionally expressed at these sites. If so, this could represent a novel mechanism underlying the heightened pain in humans following laser treatment for myopia. The present pilot investigation does not agree with this proposal because $\alpha\beta$ meATP did not elicit an increase in action potential discharge from polymodal nociceptors innervating the keratectomised cat cornea. However, we only performed electrophysiological studies in one cat at 18 hours post-surgery and further studies at different time points would better answer the question of whether functional P2X expression occurs following corneal nerve injury. Furthermore, it is possible that the normal mechanisms underlying post-operative corneal sensitisation were unusual in this cat because the responses of polymodal nociceptors to CO₂ were not augmented in comparison to those normal corneas and this is a feature of nociceptors innervating keratectomised cat corneas (Gallar *et al.*,

1997). It would be necessary to repeat the experiments in more severely sensitised keratectomised corneas before the possibility that P2X receptors mediate post-operative pain is excluded.

Overall, the results do not support the hypothesis that functional P2X receptors are expressed on the peripheral terminals of nociceptors innervating the cat or rat cornea *in vivo* despite the finding that the P2X₃ subtype is expressed in mouse corneal neurones in the TG. However, our data does not preclude a role for the functional expression of P2X receptors in certain pain states. It is also possible that the negative data may be due to species differences or the slow penetration of P2X agonists and the resulting desensitisation of the receptors. Nevertheless, our data indicates that pharmacological manipulation of P2X receptor function is unlikely to provide a useful therapeutic target for treating corneal pain.

Chapter 4: Adjuvant-induced monoarthritis of the rat knee joint

4.1 INTRODUCTION

Adjuvant-induced polyarthritis, caused by injection of FCA into the rat tailbase or footpad, is the best characterised animal model for human RA (see Billingham, 1983; Rainsford, 1982). However, the arthritic lesions of polyarthritis are widespread and the animal suffers considerable distress and incurs dramatic weight loss (Owen, 1980). In order to reduce the severity of the arthritis, investigators have developed monoarthritic models of arthritis where the inflammation is confined to a single joint, frequently the knee or ankle. One of the earliest monoarthritic models was developed in this laboratory by administration of a low dose of FCA subdermally around the ankle joint of the rat (Grubb *et al.*, 1988). Over the last decade, this model has been characterised and used as a tool in pharmacological studies of nociception and inflammation (Asghar, 1995; Birrell *et al.*, 1990; Donaldson *et al.*, 1993; Grubb *et al.*, 1991; Grubb *et al.*, 1988; McQueen *et al.*, 1991). Although the model has been used successfully in these studies, using the ankle joint for arthritis induction and electrophysiological studies has a number of disadvantages.

Some investigators have suggested that injection of FCA into the synovial space is the preferable method of arthritis induction and have argued that subdermal injections into the tissue overlying the joint causes a superficial inflammation rather than a true articular arthritis (Butler *et al.*, 1992). However, i.art. injections into the rat ankle joint are very difficult to carry out without causing injury because of the complex anatomy and number of small bones in this joint. It is easier to induce

arthritis in the rat knee joint because this joint has a relatively large infrapatellar ligament through which needles can easily be inserted.

Electrophysiological recordings of the neural activity in nerves innervating the rat ankle joint are made from the primary articulo-cutaneous ramus (PACR), a branch of the tibial nerve (Guilbaud *et al.*, 1985). One of main limitations of using this preparation is that considerable surgery is required to sever the cutaneous branches of the PACR to ensure that the recorded nerve activity is from the articular divisions only. This can cause bleeding around the electrodes that can interfere with the electrical recording. Furthermore, invasive surgery and the associated tissue damage has the potential to sensitise the ankle tissues. There are fewer difficulties associated with electrophysiological recordings from the MAN innervating the rat knee joint because it is readily accessible and less surgery is required to expose it. To date, most of the electrophysiological studies of afferents innervating the knee joint have been carried out in the cat by Schaible, Schmidt and colleagues (Coggeshall *et al.*, 1983; Schaible & Schmidt, 1983a & b; Kanaka *et al.*, 1985; Schaible & Schmidt, 1985; Grigg *et al.*, 1986; Schaible & Schmidt, 1988; Dorn *et al.*, 1991). Since it is desirable to limit the use of cats in medical research, a preparation for electrophysiological recording from the rat knee joint was developed in this laboratory (Kelly, 1998 and see Dowd *et al.*, 1998c).

FCA-induced monoarthritis of the rat knee joint has been used previously in pharmacological studies by a number of authors (Bileviciute *et al.*, 1994; Bileviciute *et al.*, 1995; Davis & Perkins, 1993; Laird *et al.*, 1997; McDougall *et al.*, 1995). However, to date, none of these arthritic rats have been fully characterised electrophysiologically, behaviourally and histologically. The aim of this study was to

confirm that i.art. injection of FCA into the rat knee joint induces a monoarthritic lesion and that the electrophysiological preparation is a viable method of recording from MAN afferents innervating normal and arthritic knee joints. Neural recordings were made from filaments of the MAN and the discharge characteristics of afferents innervating normal and arthritic joints were compared. In behavioural studies, the allodynia and swelling quantified with chronic inflammation were assessed using a recently developed weight-bearing device (Clayton *et al.*, 1997) and by measuring knee joint diameter respectively. Histological studies were also carried-out to confirm that adjuvant injection induced an inflammatory response in the knee joint. The overall aim of this study was to evaluate the electrophysiological preparation, and the neural and behavioural features of the monoarthritic model for use in subsequent investigations of the role of ATP and adenosine in nociception and inflammatory pain.

4.2 METHODS

4.2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

Arthritis induction and neural recordings were carried out as described in Section 2.1.2. Briefly, arthritis was induced by i.art. injection of FCA (0.10-0.15ml) into the left knee joint of rats under halothane anaesthesia 14-21 days prior to the neural experiments. 80 normal and 24 arthritic rats (body weight range 240-380g; mean \pm sem 318 \pm 10g) were used for the electrophysiological studies. Animals were anaesthetised with pentobarbitone (60mg.kg⁻¹ i.p., supplemented hourly i.v.) and the trachea, and right carotid and femoral arteries were cannulated. Neural activity in small filaments of the MAN was recorded using bipolar platinum-iridium wire

electrodes and data was analysed off-line using Spike 2 software. Drugs were administered by close-arterial injection via the cannula in the right femoral artery.

4.2.2 BEHAVIOURAL STUDIES

Behavioural assessment of the rats was carried out as described in Section 2.2.2. In summary, body weight, hindlimb weight bearing and knee diameters were measured once daily from three days pre-injection (Day -3) to 15 days post-injection (Day 15) of FCA (n=5 rats) or sterile paraffin (n=4 rats) into the knee joint (injections were made on Day 0).

4.2.2.1 Statistical analysis

To determine whether there were any differences between FCA and paraffin injected groups, the cumulative change from a selected control period in body weight, hindlimb weight bearing and joint swelling was determined. The control period was the mean of the measurements on Days -2 and 0, and this was subtracted from the mean of the measurements on Days 1,2,4 and 8. Data was analysed using an unpaired t-test.

4.3 RESULTS

4.3.1 AFFERENT NERVE RECORDINGS *IN VIVO*

4.3.1.1 Characterisation of afferent nerve fibres

Neural discharge was recorded from 165 afferent fibres innervating 80 normal knee joints and 82 afferent fibres innervating 24 arthritic knee joints. The afferents were classified as C-fibre polymodal nociceptors or A δ -mechanoreceptors based on their

conduction velocities, mechanosensitivity and whether or not they were activated by the algogen capsaicin.

C-fibre polymodal nociceptors had slow conduction velocities ($1.07 \pm 1.03 \text{ms}^{-1}$), were excited by capsaicin (3-30nmol i.a.), and could be activated by probing the knee joint capsule with a hand held plastic probe (Figure 4.1 and see Section 4.3.1.3 below). $A\delta$ -mechanoreceptors had faster action potential conduction velocities ($4.70 \pm 0.77 \text{ms}^{-1}$) and did not respond to capsaicin. 63% (104/165) of afferents innervating normal knee joints and 54% (44/82) innervating arthritic knee joints were C-fibre polymodal nociceptors and 37% (61/165) of afferents innervating normal knee joints and 46% (38/82) innervating arthritic knee joints were $A\delta$ -mechanoreceptors. A small number of $A\beta$ -mechanoreceptors were also identified (low threshold mechanosensitive units with conduction velocities greater than 10ms^{-1} that were not activated by capsaicin), but these were not studied. The rats used in these experiments were used in subsequent investigations of the role of ATP and adenosine in nociception and inflammatory pain. Because ATP is released from cells by non-specific membrane perturbations (see General Introduction), mechanical stimulation of the joints was kept to a minimum and mechanical thresholds were not systematically determined to avoid release of the purine and possible tissue desensitisation.

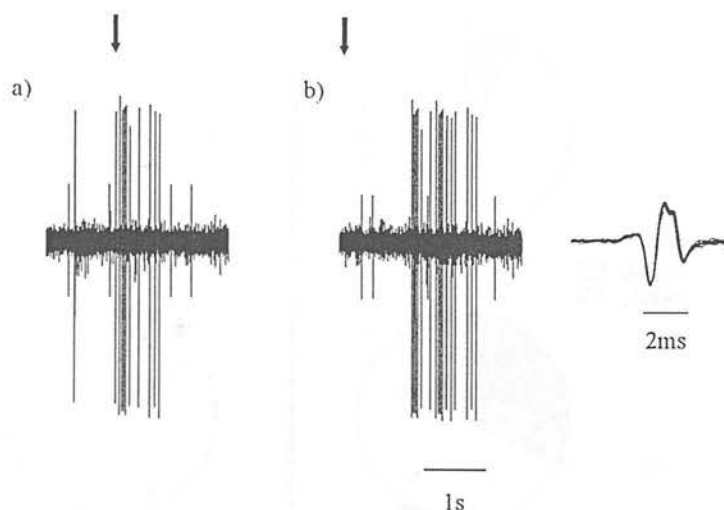


Figure 4.1 Response evoked from a single C-fibre polymodal nociceptor (far right) by a) by mechanical stimulation of the knee joint with a hand-held plastic probe (at arrow) and b) close-arterial injection of capsaicin (10nmol, at arrow).

4.3.1.2 Basal discharge of nociceptors innervating normal and arthritic joints

Previous electrophysiological studies have shown that there are two characteristic features of the basal discharge of afferents innervating adjuvant arthritic joints in comparison with normal joints. Firstly, nerves innervating arthritic joints have a higher proportion of afferents with basal discharge and secondly, the rate of discharge of those afferents which are spontaneously active is higher in arthritic joints (Guilbaud *et al.*, 1985). In the present investigation, A δ -fibres innervating normal knee joints (n=61) were always silent, and in arthritic joints only 3 of 38 (8%) A δ afferents were spontaneously active (Figure 4.2 and Figure 4.3). In contrast, 16% (17/104) of C-fibre polymodal nociceptors innervating normal knee joints had on-going discharge, whereas 32% (14/44) of those innervating arthritic joints were spontaneously active and discharged at a significantly ($P < 0.05$) higher rate (Figure 4.2 and Figure 4.3).

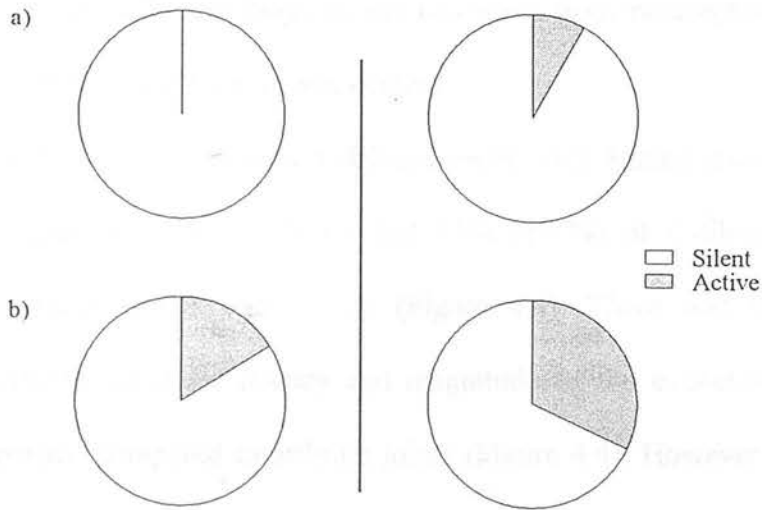


Figure 4.2 Proportion of a) $A\delta$ -mechanoreceptors and b) C-fibre polymodal nociceptors with spontaneous discharge innervating normal (first panel) and arthritic (second panel) knee joints.

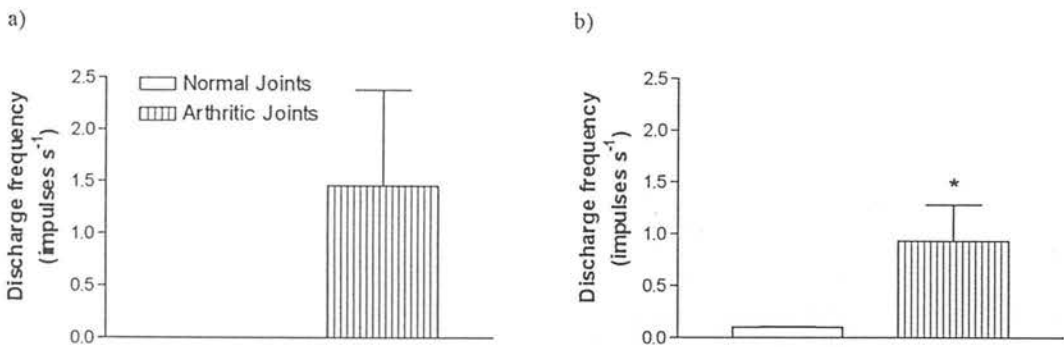


Figure 4.3 Spontaneous discharge rate of a) $A\delta$ -mechanoreceptors and b) C-fibre polymodal nociceptors innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints: $A\delta$ -fibres none with basal discharge, C-fibres $n=17$; arthritic joints: $A\delta$ -fibres $n=3$, C-fibres $n=14$. * $P<0.05$, unpaired t -test; higher frequency in C-fibres innervating arthritic compared to normal joints.

4.3.1.3 Capsaicin and bradykinin-evoked excitation of C-fibre nociceptors innervating normal and arthritic joints

Capsaicin (3-30nmol, i.a.) evoked a fast onset, high frequency, transient response from all C-fibres innervating normal and arthritic knee joints (Figure 4.1 and Figure

4.4). No significant difference between the responses from nociceptors innervating normal compared to arthritic joints was evident.

Bradykinin (2-30nmol, i.a.) evoked a delayed onset, long lasting increase in action potential discharge from 88% (48/54) and 71% (17/24) of C-fibres innervating normal and arthritic joints respectively (Figure 4.4). There was no significant difference between the onset latency and magnitude of the excitation in C-fibres innervating normal compared to arthritic joints (Figure 4.4). However, the duration of the bradykinin response in arthritic joints was significantly ($P<0.05$) prolonged compared to normal joints (Figure 4.4) resulting in an increase (although not statistically significant) in the total number of action potentials firing in arthritic joints (Figure 4.4). None of the $A\delta$ -fibres recorded from were activated by the kinin.

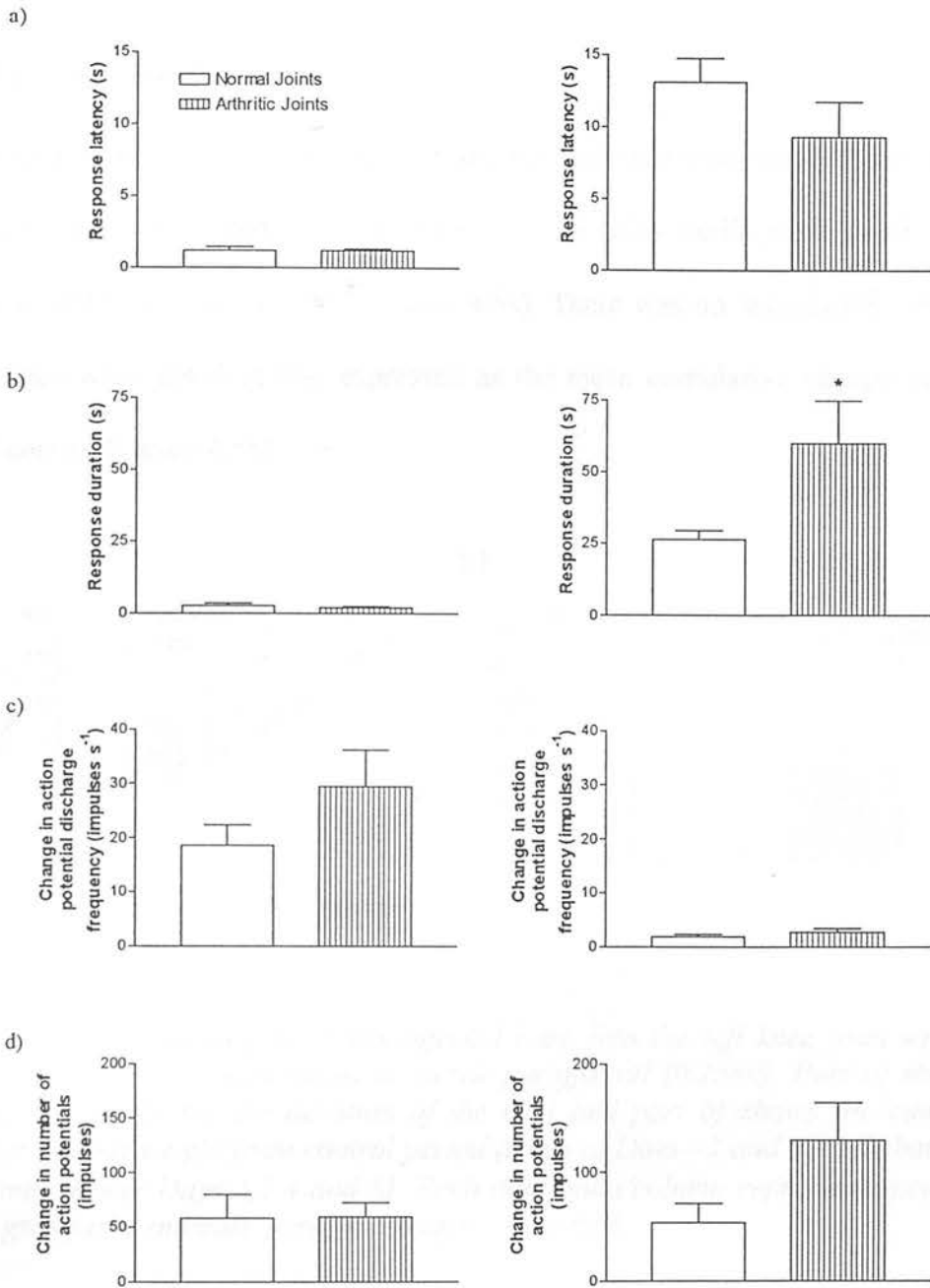


Figure 4.4 Capsaicin (3nmol, figures on the left) and bradykinin (9nmol, figures on the right) evoked excitation of C-fibre polymodal nociceptors innervating the normal and arthritic knee joints. Latency to onset (a), duration (b), changes in action potential frequency (c) and number (d) are shown. Data are from experiments in which capsaicin and bradykinin were alternately the first and second drug injected. Columns represent mean \pm sem. Capsaicin: normal joints $n=6$, arthritic joints $n=15$; bradykinin: normal joints $n=10$, arthritic joints $n=9$. * $P < 0.05$ unpaired t -test; longer duration of bradykinin response in arthritic versus normal joints.

4.3.2 BEHAVIOURAL STUDIES

4.3.2.1 Body weight

The mean body weight of rats injected into the left knee joint with FCA (0.15ml of 1mg ml^{-1} , i.art) was similar to that of rats injected with sterile paraffin oil (0.15ml, i.art) up to Day 8 post-injection (Figure 4.5a). There was no statistically significant difference when the data was expressed as the mean cumulative change in weight from control (Figure 4.5b).

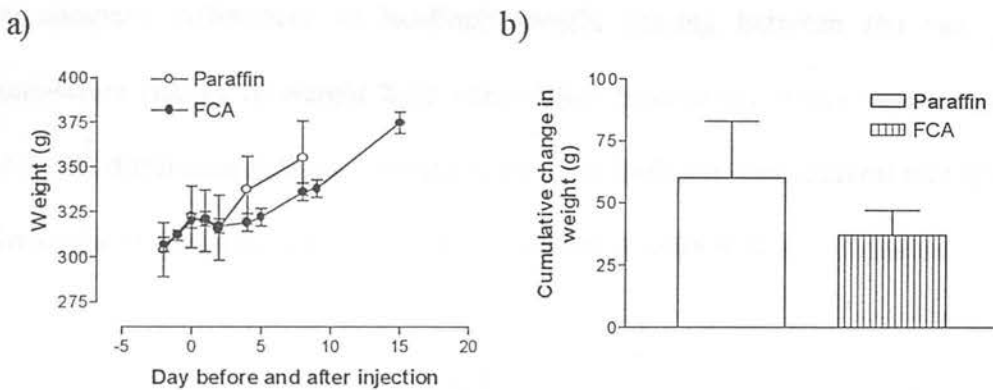


Figure 4.5 Body weight of rats injected i.art. into the left knee joint with FCA (0.15ml of 1mg ml^{-1} suspension) or sterile paraffin oil (0.15ml). Part a) shows the change in weight for the duration of the trial and part b) shows the cumulative change in body weight from control period (mean of Days -2 and 0, and change was determined over Days 1, 2, 4 and 8). Each data point/column represents mean \pm sem. FCA group $n=5$ animals, paraffin group $n=4$ animals.

4.3.2.2 Allodynia

Following injection of paraffin oil into the left knee joint, there was a small shift in hindlimb weight bearing from the left to the right hindlimb (Figure 4.6a). In the FCA group, this shift was pronounced and significant (Figure 4.6b), and was manifest as a three-legged gait or a limp when the animals were observed walking in their cages.

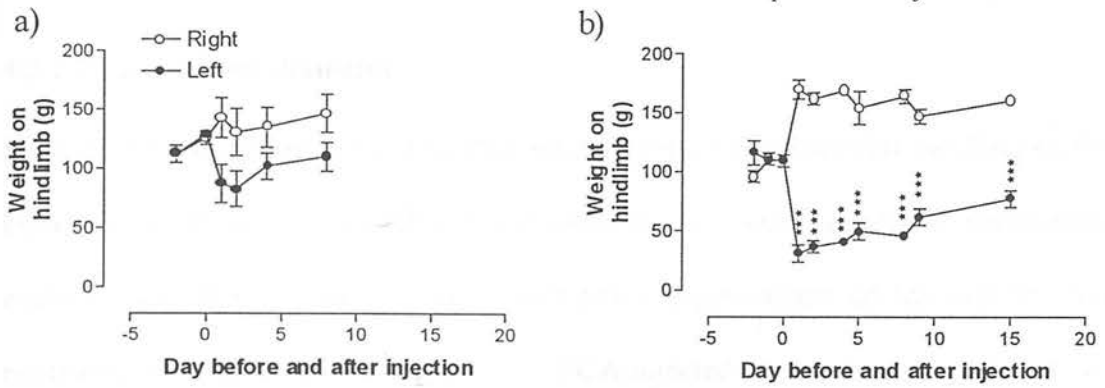


Figure 4.6 Hindlimb weight bearing on the left and right hindlimbs of the a) paraffin-injected and b) FCA-injected groups. Each data point represents mean \pm sem. *** $P < 0.001$ unpaired t -test. Paraffin group $n = 4$ animals, FCA group $n = 5$ animals.

To compare differences in hindlimb weight bearing between the two groups, cumulative change in weight from control was determined. There was a significant ($P < 0.01$) difference between weight bearing on both the contralateral and ipsilateral hindlimbs between the FCA and paraffin groups (Figure 4.7).

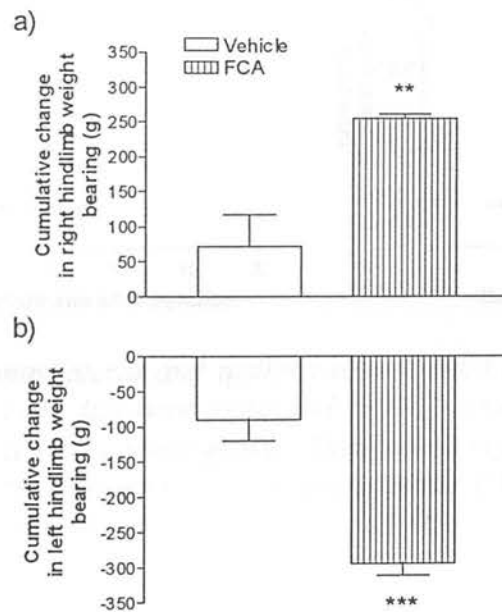


Figure 4.7 Cumulative change from control period in a) contralateral and b) ipsilateral hindlimb weight bearing: effect of paraffin (0.15ml, i.art. left knee joint) and FCA (0.15ml of 0.15mg ml^{-1} suspension, i.art. left knee joint). Columns represent mean \pm sem. Control was mean of Days -2 and 0 , and change was determined over Days $1, 2, 4,$ and 8 . *** $P < 0.001$, ** $P < 0.01$ FCA vs vehicle, unpaired t -test.

4.3.2.3 Knee joint diameter

Subjective examination of the rats showed that there was substantial swelling of the FCA-injected, but not the paraffin or contralateral knee joints, which was particularly evident up to Day 2 post-injection. Quantitative measurement of the arthritic rats confirmed that there was swelling of the FCA-injected knee joint compared to the contralateral knee joint, whereas in the control rats, the diameter of the paraffin-injected knee was similar to that of the contralateral knee (Figure 4.8). Cumulative data is shown in Figure 4.9 and confirms that there was significant ($P < 0.0001$) swelling in the ipsilateral joint of the FCA group compared with that of the paraffin group, whereas there was no significant difference between the injected and non-injected knees of the paraffin group.

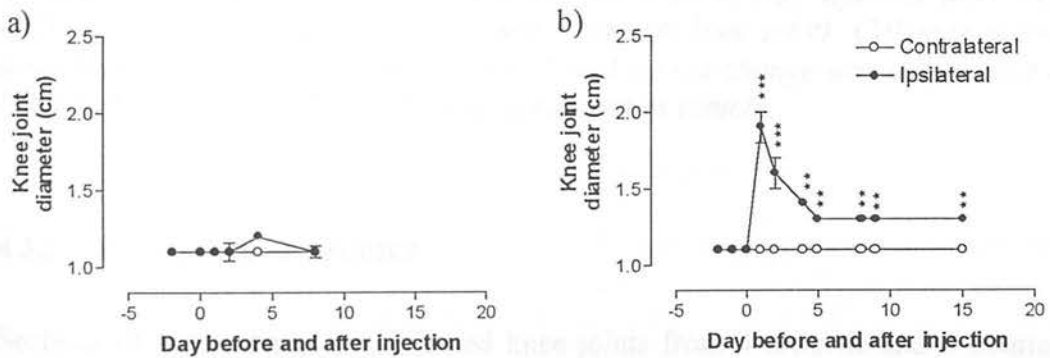


Figure 4.8 Contralateral and ipsilateral knee joint diameter of the a) paraffin-injected (0.15ml, i.art. left knee joint) and b) FCA-injected (0.15ml of 0.15mg ml⁻¹ suspension, i.art. left knee joint) groups. Data points represents mean \pm sem. Paraffin group n=4 animals, FCA group n=5 animals. ***P>0.001, **P<0.01 ipsilateral vs contralateral, unpaired t-test.

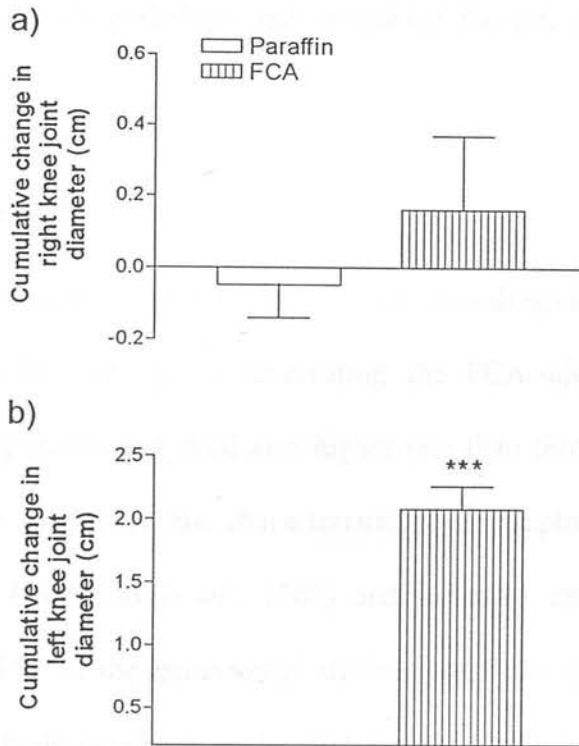


Figure 4.9 Cumulative change from control period in a) contralateral and b) ipsilateral knee joint diameter: effect of paraffin (0.15ml, i.art. left knee joint) and b) FCA (0.15ml of 0.15mg ml⁻¹ suspension, i.art. left knee joint). Columns represent mean \pm sem. Control was mean of Days -2 and 0, and change was determined over Days 1, 2, 4 and 8. *** $P < 0.0001$ unpaired *t*-test vs vehicle.

4.3.3 HISTOLOGICAL STUDIES

Sections of injected and non-injected knee joints from 4 arthritic and 4 control (2 paraffin-injected and 2 normal) rats (14 days post-injection) were stained using haematoxylin and eosin by Ms. H. Caldwell and examined using light microscopy by Dr. D.S. Salter (both from The Department of Pathology, University of Edinburgh). The histological studies confirmed the presence of an inflammatory cell infiltrate, synovial proliferation, fibroplasia and new bone formation in the arthritic knee joints (photographs not shown in this thesis). The contralateral knee joints showed no signs

of an inflammatory pathology and resembled the left and right knee joint of the control rats.

4.4 DISCUSSION

The main finding of the electrophysiological recordings in this study is that more C-fibre polymodal nociceptors innervating the FCA-injected rat knee joint were spontaneously active and fired at a higher rate than those innervating normal knee joints. These properties are characteristic of nociceptors innervating arthritic rat ankle joints (Guilbaud *et al.*, 1985) and inflamed cat knee joints (Schaible & Schmidt, 1985). In the behavioural studies, the FCA-injected animals consistently shifted their body weight onto the contralateral hindlimbs indicating that they were experiencing discomfort in the injected joint, and the FCA-injected knee joints were significantly swollen compared to paraffin-injected or non-injected knees. In addition, histological studies revealed signs of an inflammatory reaction including the presence of inflammatory cell infiltrates and synovial hypertrophy. These findings confirm that i.art. injection of FCA into the rat knee joint induces an arthritic lesion confined to the ipsilateral knee joint.

Histological studies have shown that the MAN innervating the cat's knee joint contains approximately equal numbers of afferent and sympathetic efferent fibres (Langford & Schmidt, 1983). Approximately 70% of the afferents axons are unmyelinated (C-fibre) and 21% are thinly myelinated ($A\delta$ -fibre), whereas only 9% have large myelinated ($A\beta$ -fibre) axons. In the present study, 63% of all rat MAN afferents recorded from were C-fibre polymodal nociceptors and the remainder (37%) were $A\delta$ -fibre mechanoreceptors. A small number of fibres conducting in the

A β -fibre range were also identified but these were not studied. These proportions reflect the histological findings of Langford and Schmidt (1983). No efferent neural activity was recorded because the saphenous nerve was cut centrally to ensure all discharge was from afferent fibres only. These findings validate this knee joint electrophysiological preparation as a viable method of recording from the rat MAN. By definition, nociceptors detect potentially tissue damaging stimuli or existing tissue damage (see General Introduction). The C-fibre afferents recorded from in the present study were undoubtedly nociceptive because they were activated by the pain-producing substance, capsaicin, a property that is frequently used to identify nociceptors. Furthermore, the majority (70%, see Schaible & Schmidt, 1996) of C-fibres innervating the cat knee joint are thought to serve nociceptive functions. However, it is not possible to say definitively whether the A δ -mechanoreceptors recorded from were nociceptors because no stimuli known to activate knee joint A δ -fibre nociceptors were applied (for example, high threshold mechanical stimulation or extreme knee joint movements, see Coggeshall *et al.*, 1983; Schaible & Schmidt, 1983a,b). Mechanical thresholds were not investigated in the present study to avoid tissue desensitisation, and responses to joint movements were not determined because the recording electrodes are located very near to knee joint in the rat preparation (approximately 8-10mm) and manipulations of the knee joint would have dislodged them. Despite the limitations of the present study with regard to classification of A δ -fibre function, studies of the cat knee joint have shown that 55% of MAN A δ -fibres are nociceptive while the remainder serve proprioceptive functions (see Schaible & Schmidt, 1996). Thus, it is not unreasonable to assume that

approximately half of the MAN A δ -fibres innervating the rat knee joint are also nociceptors.

In FCA-injected rat knee joints (14-21 days post-injection), twice as many C-fibre polymodal nociceptors were spontaneously active compared to normal joints, and they had significantly higher (although still low) resting discharges. Previous electrophysiological studies of afferents innervating inflamed knee joints have been carried-out following kaolin and carrageenan-induced acute inflammation in the cat (Coggeshall *et al.*, 1983; Schaible & Schmidt, 1985). In this model, more than twice as many C-fibres and A δ -fibres are spontaneously active and these have basal discharge frequencies more than twice as high as afferents innervating normal knee joints. This contrasts with the finding of the present study where very few A δ -fibres (8%) innervating FCA-injected joints were spontaneously active. Interestingly, Schaible and Schmidt (1988), have reported that some A β and A δ -fibres innervating the cat knee joint become spontaneously active within the first hour after inducing an acute inflammation, whereas there is a delay of a few hours before C-fibres and other populations of A δ -fibres display resting discharge. It is possible that the presence of spontaneously active C-fibres but not A δ -fibres innervating the rat knee joint 2-3 weeks following the induction of arthritis reflects differences in the sensitisation or recruitment of different sub-classes of nociceptors at different times during the development of arthritis. It is not feasible to verify this because it would require continuous recording MAN activity in anaesthetised rats over a 2-3 week period. Another feature of nociceptors innervating arthritic or inflamed joints is that they have reduced thresholds and increased responsiveness to stimuli such as mechanical probing or joint movements (Coggeshall *et al.*, 1983; Guilbaud *et al.*, 1985; Schaible

& Schmidt, 1985) but this was not studied in the present investigation for reasons given above.

It has also been reported that C-fibre polymodal nociceptors innervating adjuvant-arthritic rat ankle joints have an increased sensitivity of the algogen capsaicin, and a reduced sensitivity to bradykinin (Asghar, 1995). The former was attributed to the sensitising actions of inflammatory mediators, whereas desensitisation or down-regulation of bradykinin receptors due to elevated levels of endogenous bradykinin during inflammation was implicated in the latter. However, in the present study, the excitation of rat knee joint C-fibre nociceptors by capsaicin was similar in normal and arthritic joints, and the discharge elicited by bradykinin had a longer duration in arthritic joints. Interestingly, the enzymes responsible for bradykinin degradation are inhibited by acidic pH, such as would be expected to occur during inflammation (Edery & Lewis, 1962). This could explain why the bradykinin response duration was increased in arthritic joints. However, if high endogenous bradykinin levels cause articular receptor desensitisation or down regulation, a reduced response to the kinin would have been expected in arthritic joints. Because this was not observed, this may indicate that the arthritic lesion in the knee joint was not as severe as that in the ankle joint. This would also explain why no sensitisation to capsaicin was detected. Consistent with this, Asghar (1995) reported higher spontaneous discharge rates of C-fibres innervating arthritic ankle joints (3.4 ± 0.46 impulses s^{-1} , $n=198$ C-fibres), compared to that found in the present study in C-fibres innervating arthritic rat knee joints (0.93 ± 0.35 impulses s^{-1}).

In the present behavioural studies, rats that had received an i.art. injection of FCA consistently limped or had a three-legged gait suggesting that walking, which is

normally painless, was painful after induction of arthritis. The FCA-induced allodynia was quantified using a weight bearing device which was recently validated as a method of assessing allodynia in rats (Clayton *et al.*, 1997). While the subjective observation of the rats gave an indication of “walking allodynia”, the quantitative measurement assessed “standing allodynia”, that is, the pain caused by placing weight on the injected limb. The arthritic rats redistributed their body weight such that most of it was placed on the non-injected hindlimb. Control rats that were injected i.art. with sterile paraffin oil also shifted their weight slightly (but not significantly) from the ipsilateral hindlimb which probably reflects some tissue damage following the insertion of a needle through the infrapatellar ligament. The allodynia experienced by the arthritic rats was significantly greater than that experienced by the control rats and remained consistent throughout the duration of the study.

FCA also induced swelling in the ipsilateral knee joints that was very pronounced in the first two days and subsided to a plateau by the fourth day post-injection. Early swelling that drops to a constant but significantly swollen level is a common feature of FCA-induced arthritis in the rat ankle joint (Asghar, 1995). Both the early and late swelling is reduced by known anti-inflammatory agents and the ability of novel agents to reduce it is a method of investigating their anti-inflammatory properties. In the present study, the injected knee joint was significantly swollen relative to the contralateral knee joint or the paraffin-injected knee joints for the duration of the study. However, the difference between the inflamed and non-inflamed joints was very small after the initial swelling had subsided. Thus, the potential anti-

inflammatory properties of agents would be more easily determined in the early phase of adjuvant-induced arthritis in the knee joint.

The data obtained from the electrophysiological and behavioural studies, in addition to the results of the histological studies, confirms that FCA induces a mild monoarthritis in the rat knee joint. The overall aim of this investigation was to assess the value of the electrophysiological preparation and the monoarthritic model for further study into the role of ATP and adenosine in nociception and inflammatory pain.

One of the main disadvantages of this study is that different aspects of inflammatory pain were quantified in the neural and behavioural studies: in the former, spontaneous discharge, the neural correlate of spontaneous pain was recorded, whereas in the latter, standing allodynia was assessed. Thus, it is not possible to state which afferents are involved in signalling pain produced by weight bearing on the inflamed knee joint. However, during acute inflammation of the cat knee joint, almost all of the C- and A δ - fibre nociceptors respond to innocuous movements and low threshold mechanical stimulation, indicating that they play a role in causing articular allodynia (see Schaible & Schmidt, 1996). Therefore it is reasonable to assume that the thinly myelinated afferents innervating the rat knee joint also contribute to allodynia. The behavioural correlate of elevated spontaneous discharge, spontaneous pain was not assessed in this investigation because it is difficult to quantify and has been the subject of some debate (Colpaert, 1987). It would have been relatively easy to assess the neural and behavioural components of mechanical hyperalgesia but this was not done because the clinical relevance of mechanically probing the knee joint is questionable.

The data indicates that the electrophysiological preparation is a viable method of recording from nociceptive C-fibre afferents innervating the rat knee joint. However, without further characterisation of the A δ -fibres, it is not possible to state unequivocally whether they are nociceptive or proprioceptive. Functional classification of the afferents would be facilitated if the electrophysiological preparation was developed to allow the joint position to be manipulated during the experiments (perhaps using a modified version of the model described for the cat (Schaible & Schmidt, 1983a,b)). This type of stimulation is more clinically relevant in comparison to mechanically probing the joint because the afferents signalling the normal movements of the joint can be identified.

The monoarthritis model is limited as a means to investigate the potential therapeutic properties of purine compounds for a number of reasons. The spontaneous discharge of nociceptors innervating arthritic joints was very low (0.93 ± 0.35 impulses s^{-1}), which would make any reductions caused by peripherally-acting compounds with putative analgesic effects difficult to assess. For a similar reason, the effect of potential anti-inflammatory compounds on the swelling induced by FCA would be difficult to determine. Interestingly, the degree of ankle joint monoarthritis induced by FCA in the rat is determined by the dose of adjuvant used (Donaldson *et al.*, 1993). Thus, it would be useful to determine whether higher doses injected into the knee would cause a more severe inflammation with higher basal discharge and more swelling. Finally, there is currently no means of determining the effects of potential analgesic compounds on discharge evoked by quantified innocuous or noxious stimulation (the correlates of allodynia or hyperalgesia respectively), which could be determined if the neural preparation was developed to allow the joint to be moved.

In conclusion, this study confirmed that i.art. injection of FCA into the rat knee caused a mild monoarthritis confined to the joint. However, in order to fully exploit the potential of the knee joint in pain studies it is recommended that different doses of FCA are used to try to induce a more severe arthritic response, and that electrophysiological preparation be developed to allow the joint to be moved during the recording.

Chapter 5: The direct role of ATP in knee joint nociception

5.1 INTRODUCTION

One of the primary aims of treating RA is to improve the quality of life of patients by providing relief from the chronic pain associated with the condition. The most effective analgesic compounds used to treat RA pain are the NSAIDs, but their prolonged use is associated with severe side effects including renal dysfunction (Lifschitz, 1983) and gastrointestinal bleeding (Goodwin, 1987) which may give rise to anaemia (Davies *et al.*, 1996). The other agents used to treat RA (including corticosteroids, methotrexate, sulfasalazine, gold, D-penicillamine and hydroxychloroquine) are primarily aimed at reducing inflammation and preventing joint destruction, and they have little or no inherent analgesic properties (Schiff, 1997). The inadequacies of the current treatments provide the motivation to search for novel analgesics to provide relief from the pain of RA.

Knee joint pain and tissue damage are common in RA (Sculco, 1998). The development of novel analgesics for articular pain would be facilitated if the mechanisms responsible for its production were fully understood. The knee is innervated by sensory nerves arising in the DRG where mRNA for six of the ATP-gated ion-channel receptor subtypes (P2X₁₋₆) are expressed (Collo *et al.*, 1996). ATP has long been known to cause cutaneous pain (Bleehen & Keele, 1977; Keele & Armstrong, 1964) and, if ATP acting on P2X receptors is involved in mediating joint pain, then this would have implications for the treatment of RA pain.

There is compelling indirect evidence that P2X receptors in the DRG might be involved in sensory signalling. In the rat, mRNA for the P2X₃ subtype is

selectively expressed in sensory ganglia (Collo *et al.*, 1996) and within the DRG the message is localised to small diameter afferent neurones commonly associated with nociception (Chen *et al.*, 1995). These findings have led investigators to believe that P2X receptor subtypes might be involved in the initiation of pain (Burnstock, 1996; Burnstock & Wood, 1996). It is known from studies on dissociated cells in culture that ATP gates an inward current in DRG neurones (Bean, 1990; Bean *et al.*, 1990; Bouvier *et al.*, 1991; Jahr & Jessell, 1983) which has recently been shown to be mediated by P2X subtypes (Lewis *et al.*, 1995). However, there is some concern that this functional expression of P2X receptors might be an experimental artefact induced by the dissociation of neurones and subsequent maintenance in the *in vitro* artificial environment (Stebbing *et al.*, 1998). These authors reported that very few DRG neurones were depolarised by P2X receptor agonists when dorsal ganglia, dorsal roots and sciatic nerve are maintained together *in vitro*. In contrast, most of the cells displayed a P2X-mediated response when the DRG neurones were dissociated. In order to establish whether P2X receptors might be involved in the initiation of joint pain, it is crucial to determine whether functional receptors are expressed on the peripheral terminals of articular nociceptive afferents *in vivo*.

The two electrophysiological studies focusing on P2X mediated excitation of nociceptive afferents *in vivo* have provided no evidence that functional P2X receptors are expressed on nociceptors (Dowd *et al.*, 1997; Matthews *et al.*, 1997). This is supported by the behavioural data presented in Chapter 3 of this thesis where $\alpha\beta\text{meATP}$ did not evoke nociceptive responses when instilled into the eye (Dowd *et al.*, 1997). However, these reports conflict with reports that $\alpha\beta\text{meATP}$ elicits painful behaviours when it is injected into the rat footpad (Bland-Ward & Humphrey, 1997).

If it can be shown that functional P2X receptors are present on nociceptive terminals *in vivo*, it follows that ATP might be a directly-acting endogenous mediator of inflammatory pain. Levels of the nucleotide are increased in inflamed or damaged tissues (Gordon, 1986) and it is present in the synovial fluid of patients with arthritis (Park *et al.*, 1996; Ryan *et al.*, 1991). Furthermore, during RA, synovial pH falls (Farr *et al.*, 1985) and levels of substance P are increased (Marshall *et al.*, 1990); factors that enhance the actions of ATP on dissociated sensory neurones *in vitro* (Hu & Li, 1996; Li *et al.*, 1996). Thus, locally released ATP may contribute to pain by acting on P2X receptors associated with nociceptive afferents in the joint, and this action could be enhanced in inflammatory conditions such as arthritis.

The aim of this study was to test the hypothesis that functional P2X receptors are expressed on rat knee joint nociceptors, and to investigate whether any excitation is modified in afferents innervating monoarthritic joints. Neural discharge was recorded from nociceptors innervating normal and arthritic rat knee joints and the response to locally injected ATP and ATP analogues was measured. $\alpha\beta\text{meATP}$ was the main ATP analogue used in this study for two reasons: it is resistant to metabolism by extracellular ATP metabolising enzymes and it is a relatively selective agonist for the P2X₁ and P2X₃ receptor subtypes. In addition, cell bodies of knee joint afferents in the DRG were labelled with the retrograde fluorescent tracer, fluorogold, and sections of labelled DRGs were subsequently stained with P2X₃ receptor antibodies to determine whether P2X₃ receptors are expressed in knee joint neurones.

5.2 METHODS

5.2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

Arthritis induction and neural recordings were carried out as described in Section 2.1.2. A total of 58 normal and 24 arthritic rats (weight range 240-380g; mean \pm sem 323 \pm 9g; arthritis induced 14-21 days before the experiments) were used in the present investigation. Animals were anaesthetised with pentobarbitone (60mg.kg⁻¹ i.p., supplemented hourly i.v.) and they were prepared surgically as described. Neural activity in small filaments of the MAN was recorded and data was analysed off-line. In most experiments, drugs were administered by close-arterial injection via a cannula in the right femoral artery, but in a small number, they were injected i.art. into the knee joint in a volume of 0.1 ml.

5.2.2 IMMUNOHISTOCHEMICAL STUDIES

Retrograde labelling and immunohistochemical studies were carried out as described in Section 2.4. Briefly, sections of the left and right DRGs (at levels L3, L4 and L5) of 4 control (2 normal and 2 paraffin-injected) and 4 arthritic (2 weeks arthritic) rats were immunohistochemically stained for P2X₃ immunoreactivity using Vector Red or DAB as chromogens. In one arthritic rat, the cell bodies of knee joint afferents in the DRG were retrogradely labelled by injection of fluorogold into both knee joints of one rat one week post-induction of arthritis and the DRG were removed one week later.

5.2.3 DETERMINATION OF ATP CONCENTRATION IN SYNOVIAL PERFUSATE

Withdrawal of synovial fluid for determination of ATP concentration was performed as described in Section 2.4. Briefly, normal and arthritic male Wistar rats were anaesthetised with pentobarbitone (60mg kg⁻¹ i.p.) and two 26-gauge needles were inserted into the joint. 1ml of 1mM EDTA was injected into the knee joint via one needle and the perfusate was aspirated from the hub of the other. Samples were immediately centrifuged and the supernatant was stored at -70°C.

5.3 RESULTS

5.3.1 AFFERENT NERVE RECORDINGS *IN VIVO*

5.3.1.1 Characterisation of afferent nerve fibres

Neural discharge was recorded from a total of 114 afferent fibres innervating 58 normal knee joints and 82 afferent fibres innervating 24 arthritic knee joints. The afferents were classified as C-fibre polymodal nociceptors or A δ -fibre mechanoreceptors based on their conduction velocities, mechanosensitivity and sensitivity to capsaicin (see previous chapter, Section 4.3.1.1.). Of the afferents recorded, 66% (75/113) from normal joints and 50% (41/82) from arthritic joints were classified as C-fibre polymodal nociceptors, and 34% (38/113) from normal joints and 50% (41/82) from arthritic joints were classified as A δ -fibre mechanoreceptors.

5.3.1.2 $\alpha\beta$ meATP-evoked excitation of articular afferents

5.3.1.2.1 Proportion of afferents activated by $\alpha\beta$ meATP

The P2X₁ and P2X₃ receptor agonist, $\alpha\beta$ meATP (1-600 nmol, injected i.a., evoked a rapid onset, short duration increase in action potential discharge in 43% (32/75) of C-fibres and 84% (16/19) of A δ -fibres on which it was tested in normal joints, and 44% (11/25) of C-fibres and 63% (24/38) of A δ -fibres in arthritic joints (Figure 5.1 and Figure 5.2). The nucleotide vehicle, PBS (0.1ml i.a.) did not stimulate any of the afferents on which it was tested (change in discharge frequency 0.02 ± 0.1 impulses s⁻¹ n=12 afferents).

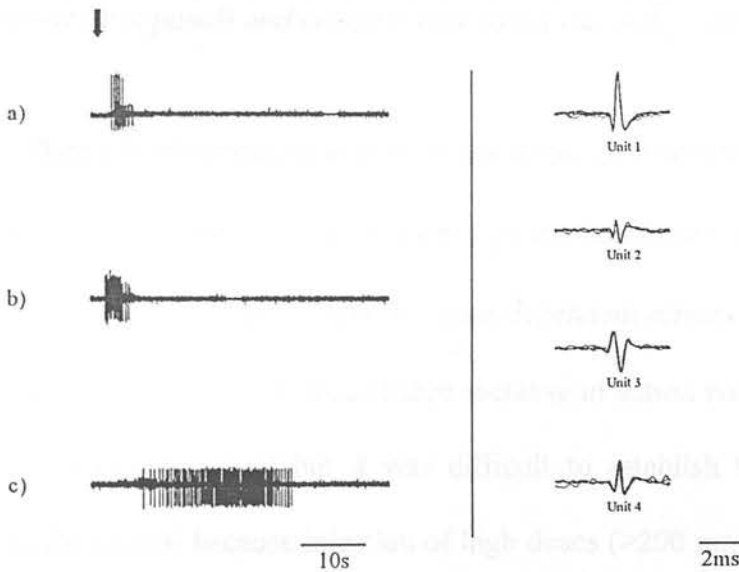


Figure 5.1 Neural discharge (first panel) showing the response evoked from a filament of the MAN innervating a normal knee joint by close-arterial injection of a) $\alpha\beta$ meATP (60nmol, activated units 1 & 2). The excitation evoked by b) capsaicin (10nmol, activated units 3 & 4) and c) bradykinin (9 nmol, activated unit 3) are shown for comparison. The individual units that responded are shown (second panel).

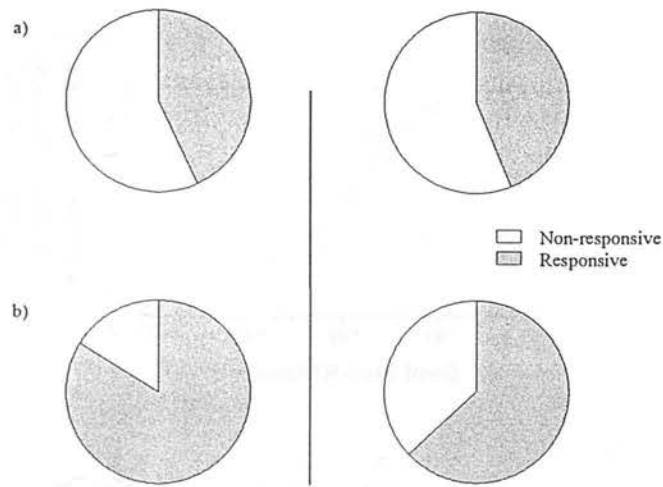


Figure 5.2 Pie charts showing the proportion of a) C-fibre polymodal nociceptors and b) A δ -fibre mechanoreceptors activated by $\alpha\beta$ meATP (1-600nmol i.a.) in normal (first panel) and arthritic knee joints (second panel).

5.3.1.2.2 Dose-related increase in action potential discharge evoked by $\alpha\beta$ meATP

Four C-fibres and four A δ -fibres from normal joints, and three C-fibres and two A δ -fibres from arthritic joints were tested for dose-dependent effects of $\alpha\beta$ meATP. The P2X receptor agonist evoked a dose-related increase in action potential discharge in both fibre types (Figure 5.3) but it was difficult to establish the true maximum response to the agonist because injection of high doses (>200 nmol) caused receptor desensitisation and marked cardiovascular and ventilatory effects (McQueen *et al.*, 1998). Consequently, *apparent* ED₅₀ values for activation of C-fibres and A δ -fibres were determined from individual experiments.

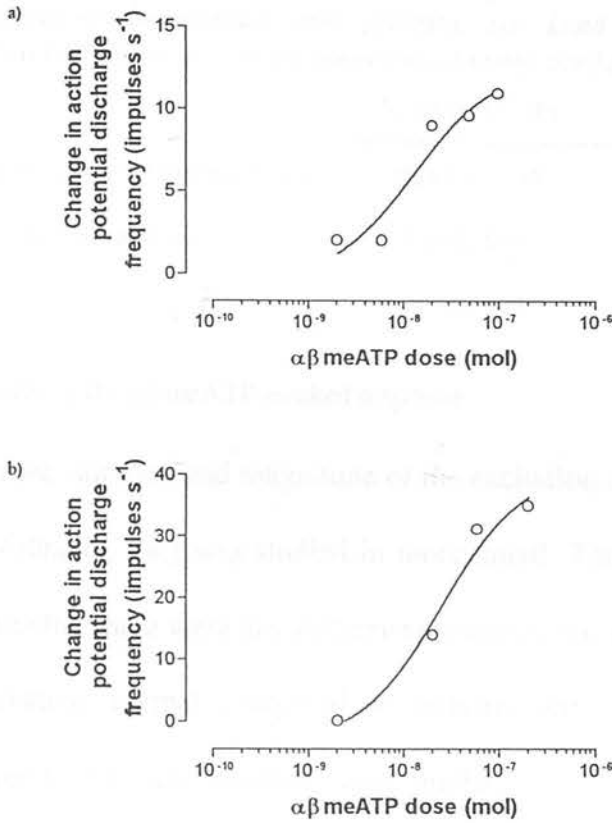


Figure 5.3 Typical log dose-response curves showing the dose related increase in action potential discharge frequency evoked by close-arterial injection of $\alpha\beta$ meATP in a) a single C-fibre polymodal nociceptor innervating a normal knee joint and b) a single $A\delta$ -fibre mechanoreceptor innervating an arthritic joint.

The mean apparent ED_{50} for $\alpha\beta$ meATP-evoked excitation of C-fibres was not significantly different to that of $A\delta$ -fibres, nor was there any significant difference between the mean apparent ED_{50} values of the nucleotide in normal and arthritic joints Table 5.1.

Table 5.1 Mean apparent ED_{50} (nmol) for $\alpha\beta$ meATP mediated excitation of afferents innervating the normal and arthritic rat knee joint. Mean \pm sem. No significant differences between means were found using the Mann-Whitney test.

	Normal joints	Arthritic joints
C-fibre polymodal nociceptors	41 \pm 34, n=4	96 \pm 17, n=3
A δ -mechanoreceptors	26 \pm 8, n=4	47 \pm 22, n=2

5.3.1.2.3 Features of the $\alpha\beta$ meATP-evoked response

The delay to onset, duration and magnitude of the excitation caused by a single dose of $\alpha\beta$ meATP (60nmol, i.a.) was studied in more detail. The data was analysed to determine 1) whether there were any differences between the responses evoked from afferents innervating normal compared to arthritic joints and 2) whether the responses evoked from C- and A δ -fibres were similar.

5.3.1.2.3.1 Onset-latency

$\alpha\beta$ meATP rapidly activated the knee joint neurones following injection into the lower abdominal artery (Figure 5.1). Figure 5.4 shows the pooled data from C-fibre polymodal nociceptors and A δ -fibre mechanoreceptors innervating normal and arthritic joints. The latencies were similar in C-fibres and A δ -fibres, and were not significantly changed in arthritic joints.

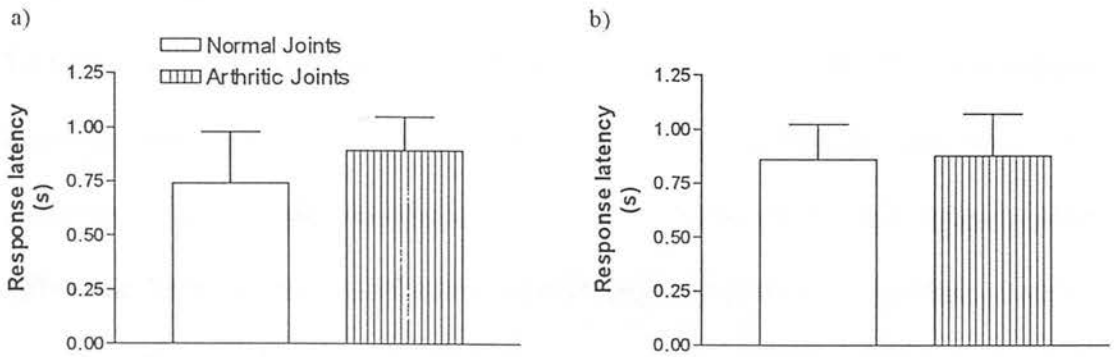


Figure 5.4 Pooled data showing the response onset latency in a) C-fibre polymodal nociceptors and b) A δ -fibre mechanoreceptors following injection of $\alpha\beta$ meATP (60nmol i.a.) in normal and arthritic joints. Columns represent mean \pm sem. C-fibres: normal joints n=5, arthritic joints n=9; A δ fibres: normal joints n=11, arthritic joints n=9.

5.3.1.2.3.2 Duration

The nucleotide transiently increased action potential discharge in knee joint articular fibres (Figure 5.5). As shown in Figure 5.5, there was no significant difference between the response duration in each fibre type between normal or arthritic joints.

Responses were also similar in C-fibres and A δ -fibres.

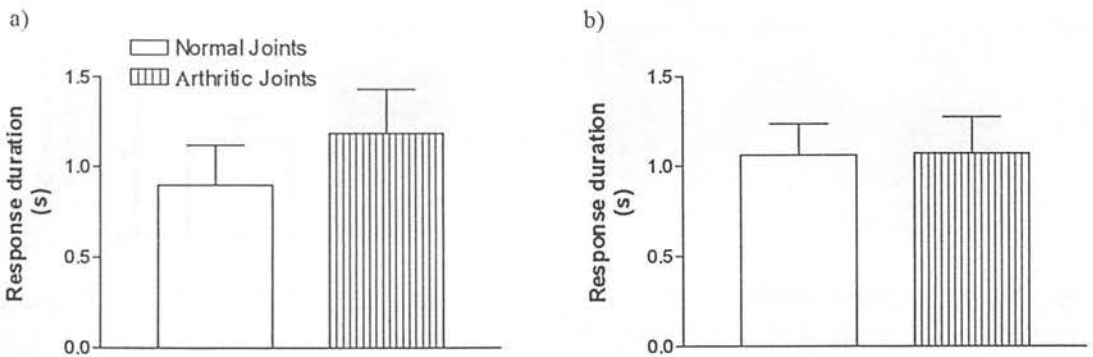


Figure 5.5 Pooled data showing the response duration in a) C-fibre polymodal nociceptors and b) A δ -fibre mechanoreceptors following injection of $\alpha\beta$ meATP (60nmol i.a.) in normal and arthritic joints. Columns represent mean \pm sem. C-fibres: normal joints n=5, arthritic joints n=9; A δ fibres: normal joints n=11, arthritic joints n=9.

5.3.1.2.3.3 Magnitude

To take into account the transience of the response to $\alpha\beta\text{meATP}$, the instantaneous discharge frequency *and* the total number of action potentials that fired were quantified. As with the response latency and duration, there was no significant difference between the action potential discharge frequency or number of action potentials that fired following close-arterial injection of $\alpha\beta\text{meATP}$ in normal or arthritic joints, and responses in C- and A δ -fibres were also similar (Figure 5.6).

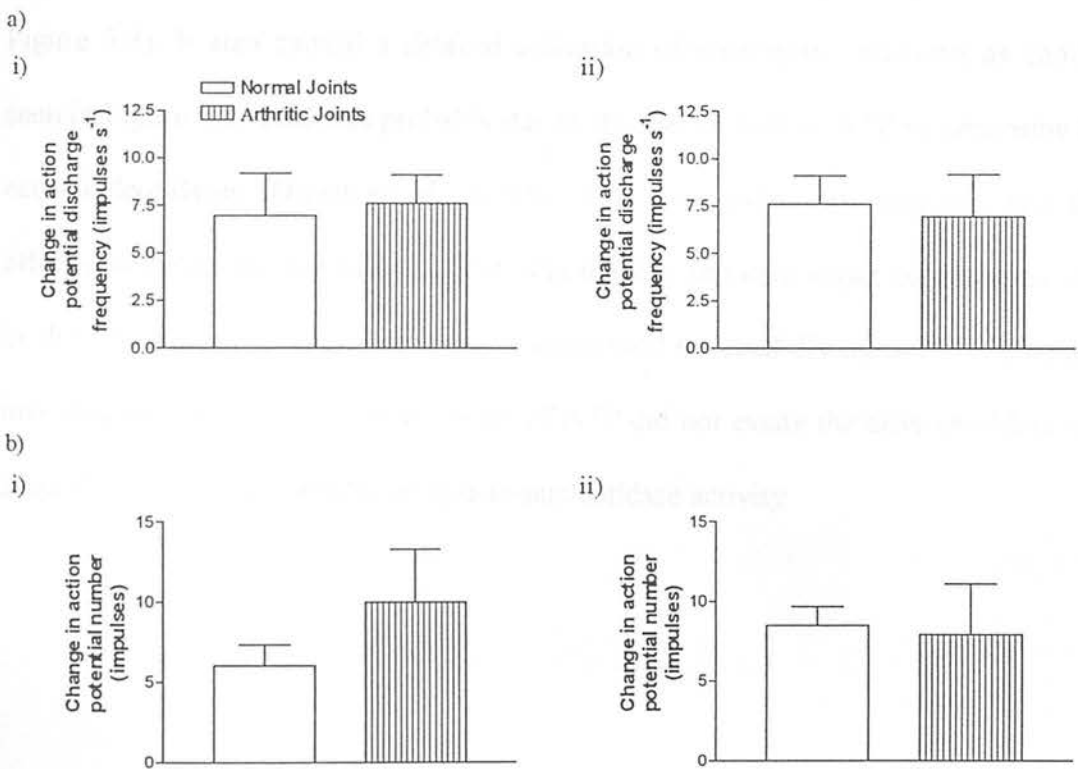


Figure 5.6 Pooled data showing the change in a) the action potential discharge frequency and b) the total number of action potentials that fired from C-fibre polymodal nociceptors (i) and A δ -fibre mechanoreceptors (ii) following injection of $\alpha\beta\text{meATP}$ (60nmol i.a.) in normal and arthritic joints. Columns represent mean \pm sem. C-fibres: normal joints n=5, arthritic joints n=9; A δ fibres: normal joints n=11, arthritic joints n=9.

5.3.1.3 ATP-evoked excitation of articular afferents

5.3.1.3.1 Proportion of afferents activated by ATP

The endogenous ligand for P2X receptors, ATP (2000nmol, i.a.), evoked a rapid-onset, short-lasting increase in action potential discharge in 40% (6/15) of C-fibres and 69% (9/13) of A δ -fibres on which it was tested in normal joints, and 38% (14/37) of C-fibres and 39% (15/38) of A δ -fibres in arthritic joints (Figure 5.7 and Figure 5.8). It also caused a delayed activation of nociceptive afferents as can be seen in Figure 5.7. This was probably due to the metabolism of ATP to adenosine by ecto-nucleotidases (Dowd *et al.*, 1998a). The slow excitatory response and the afferent populations excited by ATP during the fast and slow-onset components will be discussed in more detail in the next chapter (and see final discussion). Preliminary investigations revealed that lower doses of ATP did not excite the afferents (data not shown). This was also attributed to ecto-nucleotidase activity.

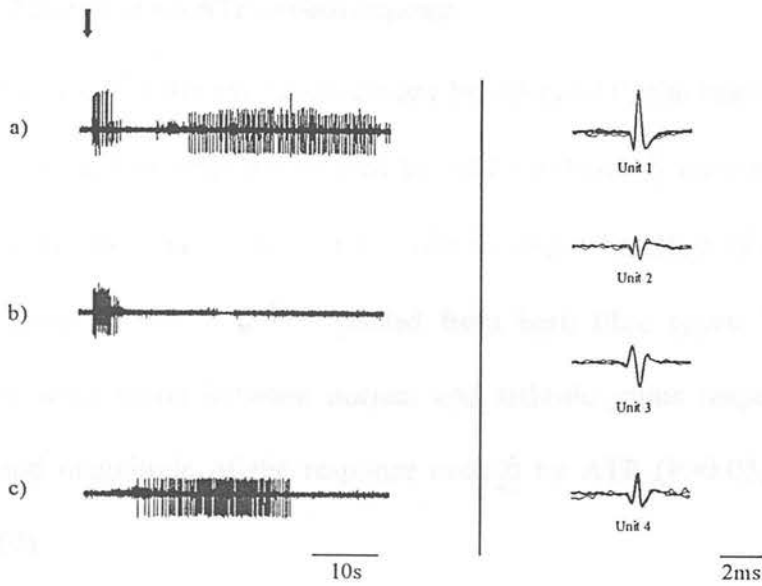


Figure 5.7 Neural discharge (first panel) showing the response evoked from a filament of the MAN by close-arterial injection of a) ATP (2000nmol, activated units 1 & 2 during the fast response and units 3 & 4 during the slow response), b) capsaicin (10nmol, activated unit 3 & 4) and c) bradykinin (9 nmol, activated unit 3). The afferent fibres that responded are shown (second panel). Note that different units were excited during the fast and slow components.

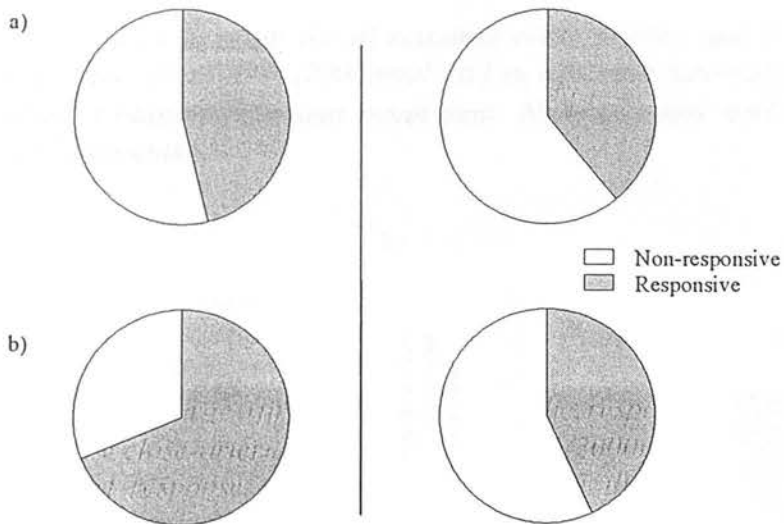


Figure 5.8 Pie charts showing the proportion of a) C-fibre polymodal nociceptors and b) A δ -fibre mechanoreceptors that were rapidly activated by ATP (2000nmol i.a.) in normal (first panel) and arthritic knee joints (second panel).

5.3.1.3.2 Features of the ATP-evoked response

As was observed for the excitation caused by $\alpha\beta\text{meATP}$, the latency, duration and magnitude of the fast response evoked by ATP (2000nmol) were similar in C- and A δ -fibres (separate data not shown). In order to assess the effect of adjuvant arthritis on this excitation, the data was pooled from both fibre types. No significant differences were found between normal and arthritic joints respect to the delay, duration and magnitude of the response evoked by ATP ($P>0.05$, Figure 5.9 and Figure 5.10).

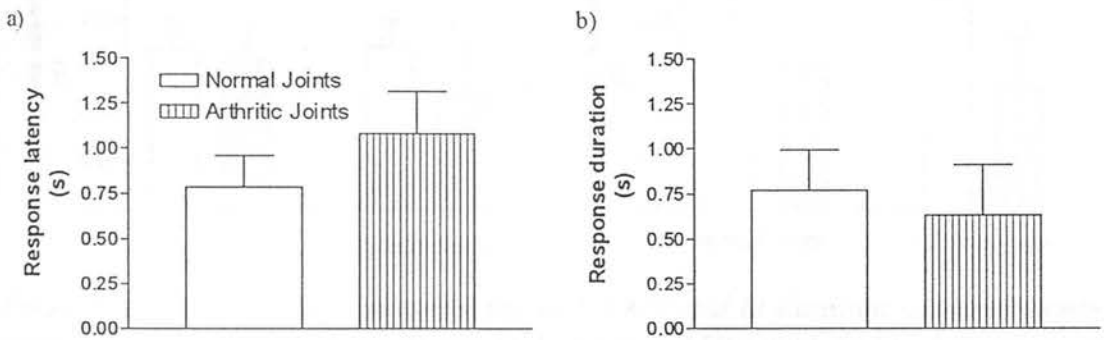


Figure 5.9 Pooled data showing the a) response onset latency and b) response duration following injection of ATP (2000nmol i.a.) in afferents innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints n=7 afferents; arthritic joints n=7 afferents.

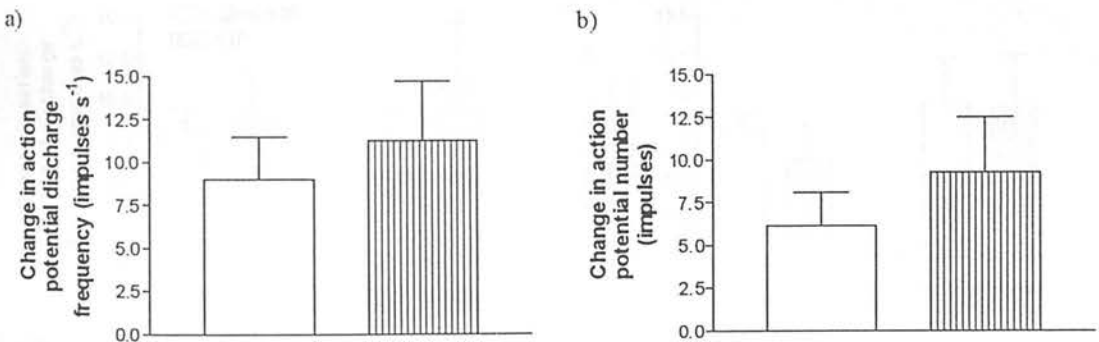


Figure 5.10 Pooled data showing the change in a) the action potential discharge frequency and b) the number of action potentials firing in afferents innervating normal and arthritic joints following injection of ATP (2000nmol i.a.). Columns represent mean \pm sem. Normal joints n=7 afferents; arthritic joints n=7 afferents.

5.3.1.4 Comparison between the $\alpha\beta$ meATP and ATP-evoked excitations

During experiments in which $\alpha\beta$ meATP (60nmol i.a.) and ATP (2000nmol i.a.) were both tested, fibres which were excited by $\alpha\beta$ meATP also invariably responded to ATP (normal joints n=26 afferents; arthritic joints n=37 afferents) and there was no significant difference between the responses evoked by the two nucleotides with respect to response onset latency, duration or size (Figure 5.11 and Figure 5.12).

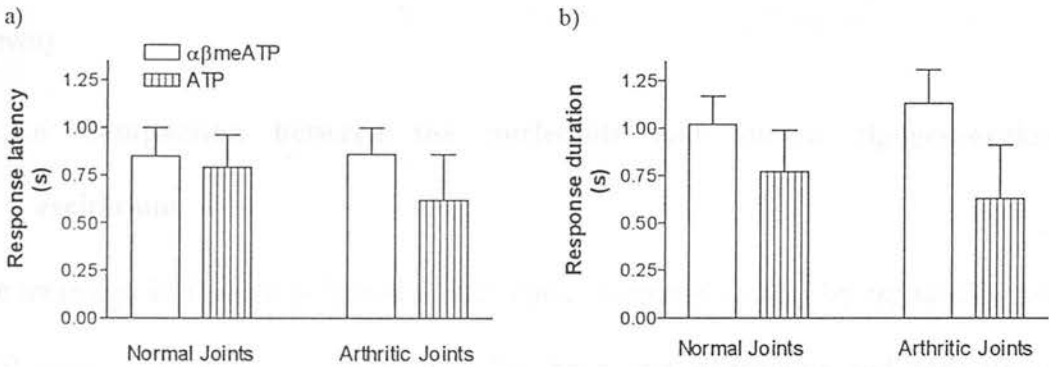


Figure 5.11 Pooled data showing the a) latency and b) duration of the response following injection of $\alpha\beta$ meATP (60nmol i.a.) and ATP (2000nmol i.a.) in afferents innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints: $\alpha\beta$ meATP n=14 afferents; ATP n=7 afferents; arthritic joints: $\alpha\beta$ meATP n=15 afferents; ATP n=7 afferents.

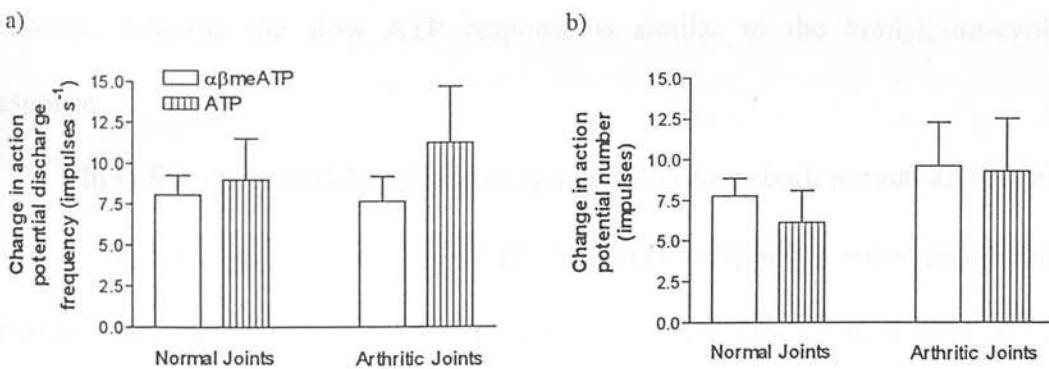


Figure 5.12 Pooled data showing the change in a) the action potential discharge frequency and b) the number of action potentials firing in afferents innervating normal and arthritic joints following injection of $\alpha\beta$ meATP (60nmol) and ATP (2000nmol i.a.). Columns represent mean \pm sem. Normal joints: $\alpha\beta$ meATP n=14 afferents; ATP n=7 afferents; arthritic joints: $\alpha\beta$ meATP n=15 afferents; ATP n=7 afferents.

5.3.1.5 Factors affecting the ATP-evoked excitation

In three experiments, normal saline (NaCl 0.9% (w/v) in distilled water) was acidified (pH 4) by perfusing with CO₂ and the resulting carbonic acid was injected (0.2ml i.a., n=3 afferents) 10s before ATP. In two separate experiments, the effect of injecting substance P (20nmol i.a., n=3 afferents) 10s before ATP was investigated. Neither pre-injection significantly affected the ATP-evoked excitation (data not shown).

5.3.1.6 Comparison between the nucleotide and known algogen-evoked excitations

The increases in C-fibre polymodal nociceptor discharge caused by $\alpha\beta$ meATP and ATP were compared to those evoked by the potent exogenous and endogenous algogens, capsaicin (10nmol) and bradykinin (9nmol) respectively. Figure 5.1 and Figure 5.7 clearly show that the response evoked by $\alpha\beta$ meATP and the fast response evoked by ATP resemble (in terms of latency and duration) the capsaicin-evoked response, whereas the slow ATP response is similar to the bradykinin-evoked response.

In C-fibre polymodal nociceptors (pooled data from both normal and arthritic joints), the latencies of the $\alpha\beta$ meATP and ATP responses were significantly ($P<0.05$) shorter than that of the bradykinin-evoked excitation (Figure 5.13a), whereas they were similar to that evoked by capsaicin (Figure 5.14a). The increased action potential discharge following injection of the nucleotides also had a significantly ($P<0.05$) shorter duration than that evoked by bradykinin (Figure

5.13b), but it was also significantly ($P < 0.05$) more transient than that evoked by capsaicin (Figure 5.14b).

Although equipotent doses were not used, the size of the responses evoked by capsaicin, bradykinin and the purines are compared in Figure 5.15. The change in action potential discharge frequency elicited by the purines was smaller than that evoked by capsaicin (though this did not reach statistical significance for ATP) but was significantly higher than that evoked by bradykinin (Figure 5.15a). The change in the total number of action potentials that fired following injection of $\alpha\beta\text{meATP}$ or ATP was significantly ($P < 0.05$) smaller than the change evoked by capsaicin or bradykinin (Figure 5.15b).

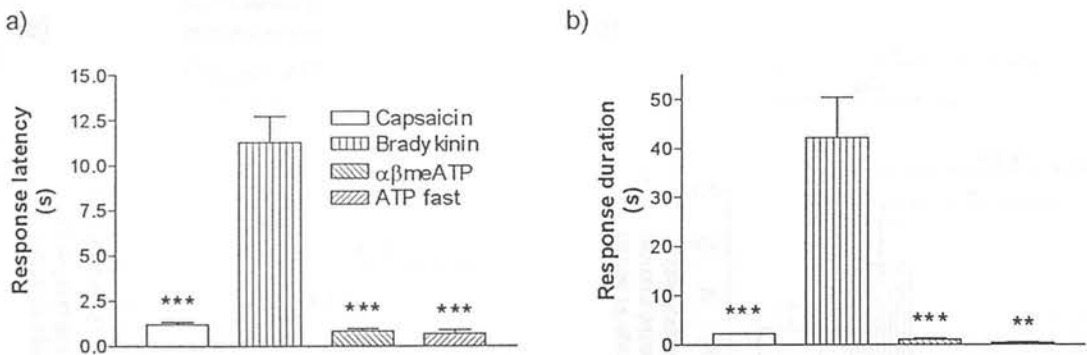


Figure 5.13 Pooled data from C-fibre polymodal nociceptors innervating normal and arthritic joints showing the a) onset latency and b) duration of the response evoked by capsaicin (10nmol), bradykinin (9nmol), $\alpha\beta\text{meATP}$ (60nmol) and ATP (2000nmol). Columns represent mean \pm sem. Capsaicin $n=21$, bradykinin $n=19$, $\alpha\beta\text{meATP}$ $n=14$, ATP $n=7$. *** $P < 0.0001$, ** $P < 0.01$ unpaired t -tests compared to bradykinin.

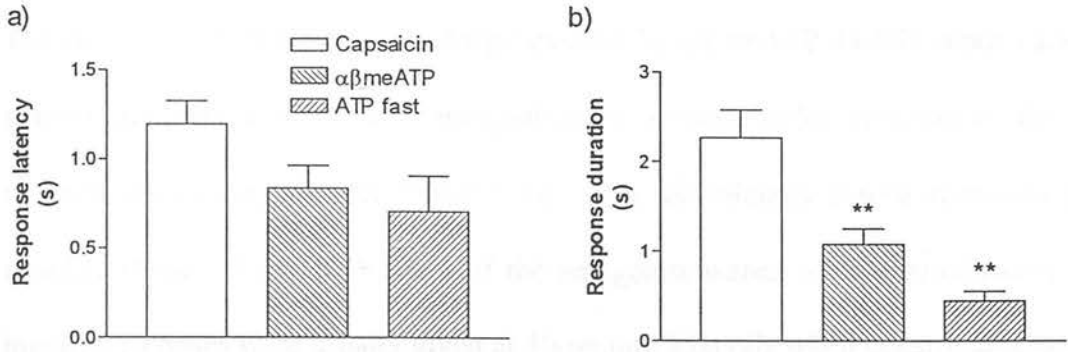


Figure 5.14 Data from the figure above for capsaicin (10nmol), $\alpha\beta\text{meATP}$ (60nmol) and ATP (2000nmol) with an expanded y-axis scale showing the a) onset latency and b) duration of the responses evoked. Columns represent mean $\pm\text{sem}$. Capsaicin n=21, $\alpha\beta\text{meATP}$ n=14, ATP n=7. ** $P<0.01$ unpaired t-tests compared to capsaicin.

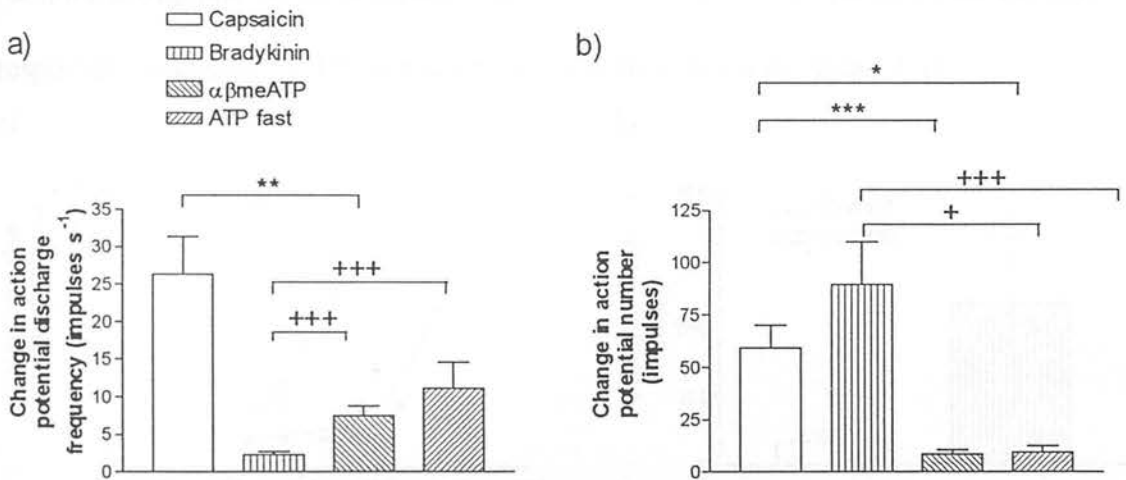


Figure 5.15 Pooled data from C-fibre polymodal nociceptors innervating normal and arthritic joints showing the change in a) the action potential discharge frequency and b) the total number of action potentials that fired in response to capsaicin (10nmol), bradykinin (9nmol), $\alpha\beta\text{meATP}$ (60nmol) and ATP (2000nmol). Columns represent mean $\pm\text{sem}$. Capsaicin n=21, bradykinin n=19, $\alpha\beta\text{meATP}$ n=14, ATP n=7. Part a) ** $P<0.01$ unpaired t-test versus capsaicin. +++ $P<0.0001$ unpaired t-test versus bradykinin. Part b) *** $P<0.0001$ and * $P<0.05$ unpaired t-test compared to capsaicin. +++ $P<0.0001$ and + $P<0.05$ unpaired t-test versus bradykinin.

5.3.1.7 Effect of PPADS on the $\alpha\beta$ meATP and ATP-evoked excitation

The dose-related increase in discharge evoked by $\alpha\beta$ meATP (1-600 nmol i.a.) in normal and arthritic joints was antagonised by a single bolus injection of the P2 receptor antagonist, PPADS ($16\mu\text{mol kg}^{-1}$ i.a.), 10 minutes before re-testing the agonists (Figure 5.16). The effect of the antagonist waned after approximately 10 minutes, so doses were usually given at 10-minute intervals when constructing dose-response curves. PPADS also antagonised the fast response evoked by a single dose of $\alpha\beta$ meATP (60nmol) or ATP (2000nmol) (Figure 5.17) without affecting the delayed response to ATP (see Chapter 6). The lack of effect of the adenosine A_1 receptor antagonist DPCPX ($3\mu\text{mol kg}^{-1}$ i.a., 10 minutes pre-treatment) on the fast responses evoked by the P2 receptor agonists is also shown in Figure 5.17.

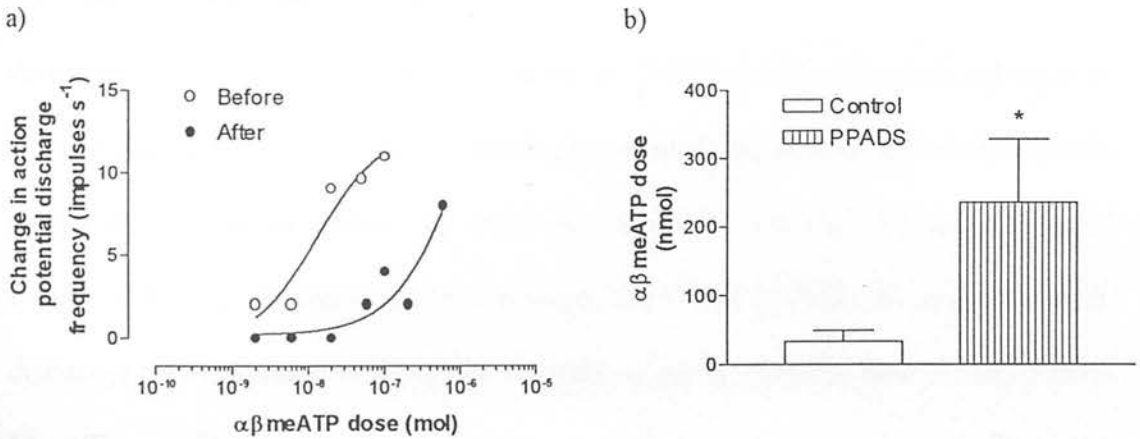


Figure 5.16 Effect of PPADS ($16\mu\text{mol kg}^{-1}$ i.a.) on a) the dose related increase in afferent discharge evoked by $\alpha\beta$ meATP in a single C-fibre polymodal nociceptor and b) on the mean apparent ED_{50} for $\alpha\beta$ meATP. Columns represent mean \pm sem. * $P < 0.05$ unpaired t -test compared to control responses. Pooled data from normal and arthritic joints. Control $n=8$ afferents; PPADS $n=4$ afferents.

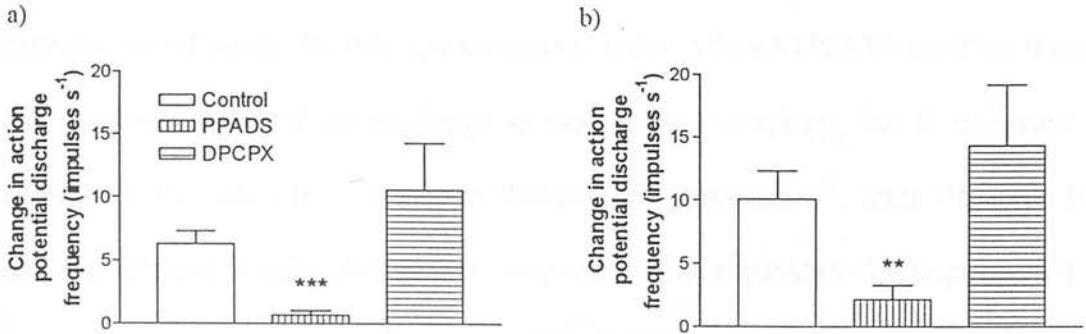


Figure 5.17 Pooled data from C-fibres and A δ -fibres innervating normal and arthritic joints showing the effect of PPADS ($16\mu\text{mol kg}^{-1}$ i.a.) and DPCPX ($3\mu\text{mol kg}^{-1}$ i.a.) on the fast increase in afferent discharge evoked by a) $\alpha\beta\text{meATP}$ (60nmol) and b) ATP (2000nmol). Columns represent mean \pm sem. *** $P < 0.0001$ and ** $P < 0.01$ Student's paired *t*-test compared to control responses. $\alpha\beta\text{meATP}$: PPADS $n=10$, DPCPX $n=4$; ATP: PPADS $n=5$, DPCPX $n=4$.

5.3.1.8 Effect of PPADS on the spontaneous discharge of nociceptors innervating arthritic joints

As discussed in the previous chapter, one of the features of adjuvant-induced arthritis is an increased rate of spontaneous discharge in afferents innervating arthritic joints compared to normal joints. In order to establish whether endogenous ATP contributed to the increased basal discharge, the effect of PPADS on spontaneous discharge was examined in 15 of the 24 arthritic joints. Activity was recorded from 53 afferent fibres; 26 were C-fibre polymodal nociceptors and 27 were A δ -mechanoreceptors. Ten (38%) of the C-fibres were spontaneously active but only two were excited by exogenous $\alpha\beta\text{meATP}$ (60nmol , i.a.) or ATP (2000nmol , i.a.). Two (7%) of the A δ -fibres were spontaneously active but neither were excited by $\alpha\beta\text{meATP}$. Administration of PPADS ($16\mu\text{mol kg}^{-1}$ i.a.) did not reduce the firing rate of the 10 C-fibre afferents nor did it reduce the firing rate of the two spontaneously active A δ -fibres (Figure 5.18). However, PPADS would only be

expected to influence the two spontaneously active $\alpha\beta$ meATP/ATP-positive fibres: the antagonist reduced the discharge in one of the recordings, but it increased it slightly in the other (unit 1 before PPADS: 0.5 impulses s^{-1} , after PPADS: 0.3 impulses s^{-1} ; unit 2 before PPADS: 0.3 impulses s^{-1} , after PPADS: 0.4 impulses s^{-1}).

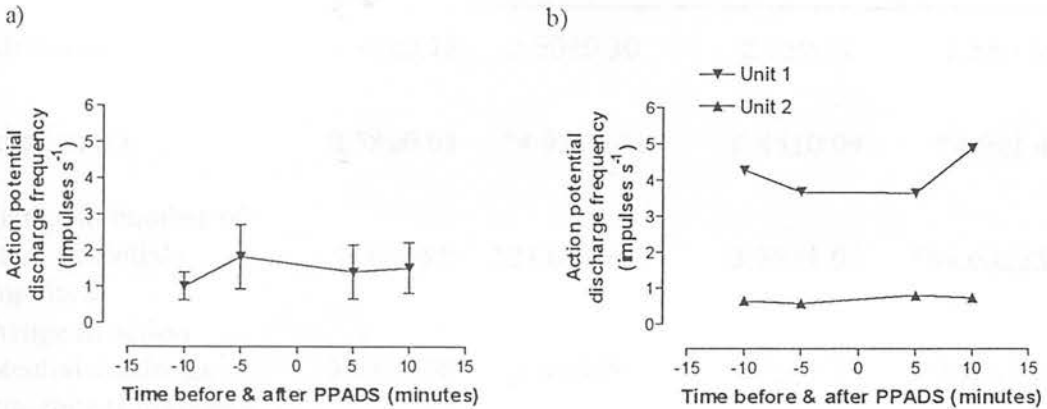


Figure 5.18 Data from a) 10 C-fibres and b) 2 A δ -fibres showing the lack of any significant effect of PPADS (16 μ mol kg^{-1} i.a.) on the elevated discharge frequency of afferents innervating arthritic joints.

5.3.1.9 Excitation following intra-articular injection of $\alpha\beta$ meATP and ATP

In order to exclude vascular mediated effects and to confirm that close-arterial injections of the P2 receptor agonists were exciting joint afferents, single doses of $\alpha\beta$ meATP (60nmol) and ATP (2000nmol) were injected directly into the normal knee joint synovial space in a three experiments. When these nucleotides were injected i.art., the response lasted for a significantly longer time ($P < 0.05$) than that evoked from the same afferents when injected i.a., and thus the total number of action potentials that fired was also significantly greater (Table 5.2).

Table 5.2 Comparison of the response evoked from the same afferents by i.a. and i.art. injection of $\alpha\beta$ meATP (60nmol) and ATP (2000nmol). Mean \pm sem, n=4 afferents per injection, either C or A δ fibres. 20 minutes were allowed between successive injection in the same animals. *P<0.05 unpaired t-test, duration and number of impulses greater following i.art. injection.

	$\alpha\beta$ meATP		ATP	
	i.a.	i.art.	i.a.	i.art.
Latency (s)	2.46 \pm 0.15	2.50 \pm 0.30	2.5 \pm 0.02	1.5 \pm 0.05
Duration (s)	0.58 \pm 0.08	*4.92 \pm 1.72	0.45 \pm 0.04	*4.9 \pm 1.46
Change in number of action potentials (impulses)	2.5 \pm 0.62	*21.66 \pm 9.57	3.39 \pm 1.06	*54.66 \pm 23.99
Change in action potential discharge frequency (impulses s ⁻¹)	3.93 \pm 0.98	4.4 \pm 0.99	7.56 \pm 1.23	9.88 \pm 2.00

Vehicle injection (PBS 0.1 ml) evoked no response (0.01 \pm 0.02 impulses s⁻¹, n=3 afferents).

The presence of the needle in the knee joint for the duration of the recording had no significant effect on spontaneous discharge (control: 0.01 \pm 0.02 impulses s⁻¹; after insertion of needle: 0.01 \pm 0.03 impulses s⁻¹).

5.3.1.10 ATP γ S and BzATP-evoked excitation

The P2 agonist ATP γ S (900nmol i.a.) was tested on 19 afferents from three normal knee joints (12 C-fibres and 7 A δ -fibres). Like ATP, it evoked a biphasic response consisting of a fast component followed by a delayed increase in action potential discharge (see next chapter) in three of the A δ -fibres on which it was tested (latency 0.9 \pm 0.4s, duration 0.7 \pm 0.2s, change in action potential frequency 9.78 \pm 3.6 impulses s⁻¹). In one afferent on which they were tested, PPADS but not DPCPX, antagonised the ATP γ S evoked fast-onset response (Table 5.3).

Table 5.3 Characteristics of the fast-onset response evoked by close-arterial injection of ATP γ S (900nmol) in one A δ -fibre mechanoreceptor. Mean \pm sem, n=injections. 15 minutes were allowed between successive injections.

	Control n=3	After DPCPX n=1	After PPADS n=1
Latency (s)	0.15 \pm 0.06	0	-
Duration (s)	1.10 \pm 0.24	0.6	-
Change in number of action potentials (impulses)	6.50 \pm 0.60	6.3	0
Change in action potential discharge frequency (impulses s ⁻¹)	6.38 \pm 0.90	4	0

ATP γ S (900nmol i.art) was also tested on 16 afferents (9 C-fibres and 7 A δ -fibres) by i.art. injection. Injection by this route also evoked a fast-onset, short duration, increase in action potential discharge (latency 5.1 \pm 2.5s, duration 5.8 \pm 1.9s, change in discharge frequency 7.3 \pm 2.3 impulses s⁻¹) in 1 C-fibre and 3 A δ -fibres. A delayed increase in action potential discharge was also evoked (see next chapter).

The P2X agonist, BzATP (700nmol i.a.) was tested on two C-fibres and 5 A δ -fibres; it also evoked a fast and transient excitation from two of the A δ afferents (latency 1.30 \pm 0.10s, duration 0.65 \pm 0.15s, change in discharge frequency 9.8 \pm 6 impulses s⁻¹). No delayed onset response was evident. Intra-articular injection of this agonist in two normal knee joints did not evoke any increase in action potential discharge.

5.3.2 CARDIORESPIRATORY EFFECTS OF P2X RECEPTOR AGONISTS

During the course of these experiments, it was noticed that intra-arterial injection of $\alpha\beta$ meATP (1-600nmol) and ATP (2000nmol) evoked a Bezold-Jarisch

cardiorespiratory reflex (B-J; Bezold, 1867) comprising of bradycardia, hypotension and apnoea (which was preceded by a transient hyperventilation). This reflex was studied in more detail in separate experiments following i.v. injection of the agonist (via the right external jugular vein) because administration by this route was approximately 10 times more potent at eliciting it (McQueen *et al.*, 1998; McQueen *et al.*, 1997). The nucleotide-evoked B-J reflex was largely investigated by Dr. D.S. McQueen and the results will only be summarised here (for more details see McQueen *et al.*, 1998).

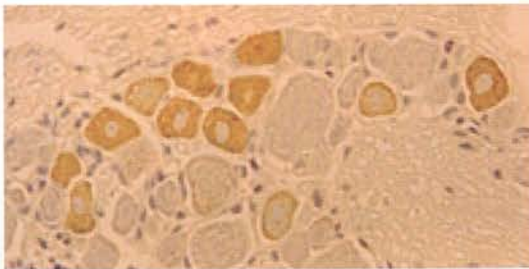
The reflex bradycardia (and resulting hypotension) evoked by $\alpha\beta$ meATP (0.6-600nmol i.v.; ED_{50} for $\alpha\beta$ meATP-induced bradycardia 14.6 ± 3.8 nmol) was significantly ($P<0.05$) reduced by bilateral vagotomy, atropine ($2.8\mu\text{mol kg}^{-1}$, i.v.) or PPADS ($17\mu\text{mol kg}^{-1}$, i.v.), and was unaffected by cutting the carotid sinus nerves. The apnoeic component of the reflex (ED_{50} for $\alpha\beta$ meATP-induced apnoea: 47.1 ± 8.5 nmol) was significantly ($P<0.05$) reduced by vagotomy or PPADS, and was unaffected by atropine or by cutting the carotid sinus nerves. The hyperventilation that preceded the apnoea (ED_{50} for $\alpha\beta$ meATP-induced hyperventilation: 23.3 ± 6.0 nmol) was significantly reduced ($P<0.05$) by cutting the sinus nerves, potentiated by vagotomy or PPADS, and was unaffected by atropine.

Interestingly, neural recordings from vagal afferents showed that 62% of units recorded from (primarily pulmonary inflation receptors but also some unidentified units) were rapidly activated by $\alpha\beta$ meATP (ED_{50} 22 ± 5 nmol) and this was blocked by PPADS. The nucleotide also excited carotid chemoreceptor afferents (ED_{50} 23 ± 9 nmol), an action that was unaffected by PPADS.

5.3.3 IMMUNOHISTOCHEMICAL STUDIES

Two sections from the left and right L3, L4 and L5 DRG of 4 arthritic, 2 paraffin-injected and 2 normal rats were examined for P2X₃-immunoreactivity using DAB or VR visualisation. P2X₃-immunoreactivity was clearly seen throughout the cytoplasm in discrete cells (Figure 5.19 and Figure 5.20) in all sections examined. Although, no attempt was made to quantify the proportion or distribution of cells labelled, approximately half of the small cells, and only a small number of large cells were labelled. The DRG of one monoarthritic rat were retrogradely labelled from both knee joints using fluorogold. Retrogradely labelled cells were identified by the presence of scattered particles of silvery fluorescence in the cytoplasm and P2X₃-immunoreactivity co-localised in some retrogradely labelled cells (Figure 5.20).

a)



b)

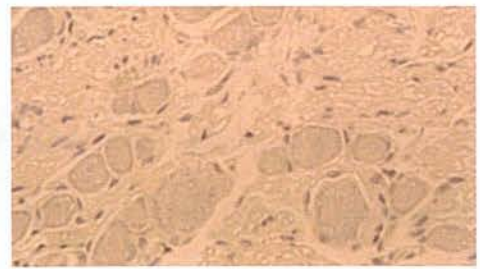


Figure 5.19 Light field photomicrographs (x40) of two sections from one normal rat DRG (L3) showing a) discrete P2X₃-immunoreactivity visualised with DAB in a number of small cells and b) the absence of staining when the sections were incubated with pre-immune serum.

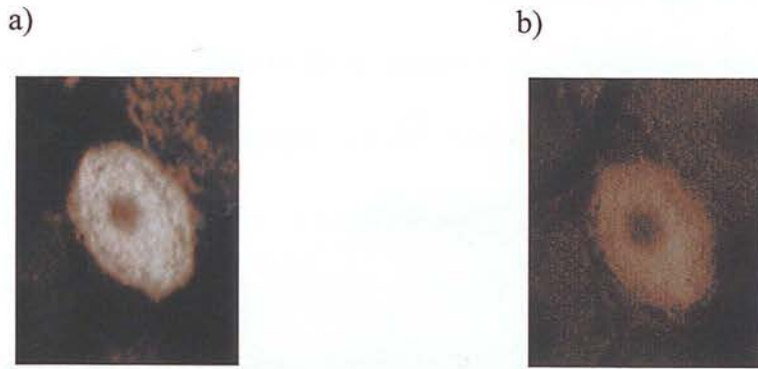


Figure 5.20 Fluorescence micrographs ($\times 1000$) of a single section from an arthritic rat DRG (L3) showing a) P2X₃-immunoreactivity visualised with Vector Red and b) fluorogold fluorescence. Fluorogold and Vector Red fluorescence colocalised in this cell.

5.3.4 DETERMINATION OF ATP CONCENTRATION IN SYNOVIAL PERFUSATE

The results of the firefly-luminescence assay for ATP concentration in the synovial perfusate are pending.

5.4 DISCUSSION

The main finding of this study is that P2 and P2X receptor agonists evoke a rapid, short-lasting excitation from approximately half of the C-fibre polymodal nociceptors innervating the rat knee joint. This evidence supports the hypothesis that functional P2X receptors are present on the peripheral terminals of a sub-population of nociceptive afferents innervating the rat knee joint *in vivo*. However, the purine analogues also excited most of A δ -fibre mechanoreceptors recorded from, of which half are probably nociceptors (see previous chapter). This precludes a purely nociceptive function for rat articular afferent P2X receptors.

Afferents that were activated by the P2X₁ and P2X₃ receptor agonist, $\alpha\beta$ meATP, also invariably responded to ATP, the endogenous ligand at P2 receptors. No significant difference between the responses evoked by the agonists at the doses used were evident, and the response to both was antagonised by the P2 antagonist PPADS. This data suggests that $\alpha\beta$ meATP and ATP were acting on the same P2 receptor subtype(s).

The evidence supports the hypothesis that the receptor(s) mediating the excitation is a ligand-gated ion-channel expressed on the peripheral terminals of the articular afferents. Following close-arterial injections, fast-onset excitation is typically observed with algogens known to mediate their effects through specific ion-channel receptors expressed on sensory nerve terminals, whereas slow onset responses are observed with algogens acting through G-protein coupled receptors expressed on the nerve terminal (Birrell *et al.*, 1990). Capsaicin rapidly increased C-fibre nociceptor discharge with a delay to onset that was similar to that observed following injections of $\alpha\beta$ meATP or ATP in the same recordings, whereas the bradykinin evoked response was significantly longer in onset. Since capsaicin acts directly on the sensory nerve terminal via a specific vanilloid ion-channel receptor (Caterina *et al.*, 1997), it is probable that the fast responses to $\alpha\beta$ meATP and ATP are also mediated via direct actions on the afferent nerve terminals. Furthermore, the nucleotides also evoked a response when injected into the knee joint, which indicates that the excitation was not secondary to vascular effects.

The cell bodies of knee joint sensory fibres are in the DRG where mRNA for six of the ATP-gated cation-channel receptor subtypes (P2X₁₋₆) are expressed (Collo *et al.*, 1996). There are currently no selective pharmacological tools available for use

in vivo that would enable us to discriminate definitively between responses mediated by these subtypes. To complicate the matters even further, $\alpha\beta$ meATP can evoke a variety of responses from sensory neurones *in vitro*, which suggests that different $\alpha\beta$ meATP-sensitive P2X receptors are functionally expressed in the cells. Thus, $\alpha\beta$ meATP evokes transient and persistent currents from dissociated rat DRG neurones (Li *et al.*, 1999) and from cultured trigeminal nociceptors (Cook *et al.*, 1997). Since the P2X₁ and P2X₃ subtypes are the only P2X receptors sensitive to $\alpha\beta$ meATP, either or both might underlie the nucleotide evoked excitation of sensory neurones. However, the P2X₃ subtype, but not to the P2X₁ subtype, is present in trigeminal nociceptors (Cook *et al.*, 1997) indicating that the latter cannot be responsible for the $\alpha\beta$ meATP-evoked currents in these cells. $\alpha\beta$ meATP and ATP evoke a rapidly-desensitising inward current from HEK293 cells expressing recombinant P2X₃ receptors (Lewis *et al.*, 1995) which is analogous with the transient phenotype of sensory neurones. Persistent current in trigeminal nociceptors is explained by heteropolymerisation of P2X₃ subunits with P2X₂ subunits because nucleotides stimulate a slowly-desensitising current from HEK293 cells cotransfected with P2X₃ and P2X₂ cDNA (Lewis *et al.*, 1995). Thus, functional P2X₃ homomeric and P2X_{2/3} heteropolymeric channels, associated with rapidly and slowly-desensitising inward current phenotypes respectively, seem to be expressed in sensory neurones. Which, if any of these receptors was responsible for the excitation observed in the present study?

Selective agonists or antagonists for the various P2X receptor subtypes, as well as analogues which would discriminate between heteropolymeric and homopolymeric receptors, would enable the receptor(s) underlying the excitation of

the rat knee joint afferents to be characterised. Unfortunately, these are not available yet. The other P2 agonists used in the present investigation, ATP γ S and BzATP, are not selective at P2X subtypes. Another possible method of identifying the receptors would be to perform neural experiments in P2X₂ and/or P2X₃ knockout rats, but again, these have not yet been generated. A mouse P2X₃ receptor knock-out line has been generated at GIAP, but a method of recording from the mouse MAN would have to be developed in order to investigate the responses to nucleotides in these animals. One potential means of determining whether rapidly or slowly-desensitising P2X receptor(s) mediated the excitation observed in the present study is to closely examine the duration of the action potential discharge elicited by the nucleotides. When they were injected i.a., $\alpha\beta$ meATP and ATP evoked a transient (0.5-1s) excitation but when they were injected i.art. the nucleotides evoked a significantly longer discharge (~5s). If rapidly-desensitising P2X₃ homomeric channels mediated the discharge then concentration-dependant effects (considerably higher local concentration following i.art. injection) would not account for the longer duration because these would still have desensitised rapidly. This data suggests that rapid clearance of the nucleotides by the blood largely accounted for the transience of the excitation following i.a. injection. These findings largely implicate the slowly-desensitising P2X_{2/3} heteropolymer, at which inward current fades within seconds (Cook & McCleskey, 1997), in the nucleotide-evoked afferent excitation. However, it is not possible to exclude a role for homomeric P2X₃ receptors for a number of reasons. Firstly, following i.art. injection of ATP, the duration of the action potential firing evoked was quite varied (range 1.9s-8.9s, data not shown), possibly indicating the involvement of more than one type of receptor. Secondly, Cook *et al.* (1997)

found that transient (milliseconds) inward current evoked by P2X receptor agonists from trigeminal nociceptors was associated with considerably longer action potential discharge (~0.5s). Thus, rapidly-desensitising P2X₃ homomers may underlie the shorter duration excitation evoked by the nucleotides. It would be interesting to perform more experiments where the purines are injected i.art. to determine if there are populations of afferents with distinct action potential discharge kinetics.

The functional expression of P2X receptor subtype(s) on the peripheral terminals of sensory afferents represents a novel mechanism through which extracellular ATP may contribute to sensory processing. One of the *in vivo* situations where local levels of endogenous ATP are increased is during inflammation. Since the present study has confirmed that functional P2X receptors are expressed on the peripheral terminals of a subpopulation of knee joint nociceptors, the ATP released during articular inflammation might contribute to pain. As mentioned in the General Introduction, inflammatory pain is characterised by ongoing pain, in addition to stimulus-induced allodynia and hyperalgesia. For reasons discussed in the previous chapter, the latter two components were not investigated using electrophysiological techniques in this thesis. The spontaneous pain associated with knee joint inflammation is manifest as an increase in the number and rate of discharge of spontaneously active nociceptive afferents (Schaible & Schmidt, 1996), a feature of the monoarthritic model used in the present investigation. One of the main incentives for investigating P2X receptors is the possibility that antagonists at these receptors may have therapeutic potential in treating inflammatory pain. However, it is unlikely that endogenous ATP acting on the P2X subtype(s) expressed on nociceptive afferents contributed to the increased basal discharge of C-fibre afferents

innervating arthritic joints because very few of the spontaneously active afferents were ATP sensitive. Because exogenous ATP (i.a. injection) excited these afferents, this suggests that the endogenous ATP concentration was not high enough to activate the P2X receptor(s). Perfusate from normal and arthritic knee joints was collected for determination of ATP concentration, but as mentioned, the results of the assay are pending. It could be argued that the dose of exogenous ATP (2000nmol, i.a.) was higher than endogenous levels expected during chronic monoarthritis. The dose of ATP injected in this study was estimated to have yielded a local concentration of 2mM at the afferent terminals (see Appendix III). This concentration is lower than that that could potentially accumulate under conditions of continuous ATP release during inflammation (the intracellular concentration of ATP is between 5-10mM, leading to high local levels due to release from damaged, necrotic or apoptotic cells (see DiVirgilio *et al.*, 1996)). It is probable that the chronic arthritic lesion was not severe enough to cause adequate ATP release, and that any ATP released was rapidly metabolised. Perhaps a more severe inflammation would cause more ATP release, or possibly ATP release varies during different stages of adjuvant-arthritis development. It would be interesting to measure the ATP concentration in severely arthritic joints or at different stages of the arthritic process, and to correlate these with spontaneous nociceptor discharge.

Based on recent evidence gained from *in vitro* and *in vivo* studies, it is rather surprising that the afferents innervating the arthritic joints were not sensitised to the exogenous nucleotides. Experimental arthropathies are associated with lowered pH and increased substance P concentrations (Ahmed *et al.*, 1995; Tulamo *et al.*, 1989) and both of these factors augment the ATP-evoked current in rat DRG neurones *in*

in vitro (Hu & Li, 1996; Li *et al.*, 1996). *In vivo*, ATP and $\alpha\beta$ meATP evoke nociceptive behaviour following intra-plantar injection (see below) and these are enhanced during carrageenan or ultraviolet irradiation-induced inflammation, or following injection of PGE₂ (Hamilton *et al.*, 1999). Although the levels of hydrogen ions, substance P or PGE₂ in arthritic rat knee joints was not determined in the present investigation, the absence of any sensitisation could indicate that the monoarthritic lesion was not severe enough to induce changes that would alter the sensitivity of the afferents to P2X receptor agonists. In order to resolve this, it would be desirable to investigate the responses evoked from afferents innervating more severely inflamed joints. An alternative explanation is that, for example, pH and Substance P-sensitive P2X receptors were not responsible for the nucleotide responses in the present experiments. Consistent with this, bolus i.a. injections (10s, pre-injection) of acidic saline or substance P did not augment the ATP evoked excitation of knee joint afferents. However, it is unlikely that transient exposure of the terminals to these “sensitising-agents” following i.a. injection would have given time for sensitisation to occur. Other methods of exposing the afferents to carbonic acid or substance P, perhaps by infusing them or even injecting them i.art., would probably reveal whether they can sensitise rat knee joint afferents to P2X receptor agonists.

This study has revealed that P2X receptors are present on approximately half of the C-fibre polymodal nociceptors and most of the A δ -fibres innervating the rat knee joint. Given that (in the cat), 1) the C-fibres and half of the A δ -fibres of the MAN innervating the knee joint are nociceptive (see previous chapter), whereas the remainder of the A δ -fibres innervating the joint are proprioceptive, and 2) that the role of knee joint afferents largely seems to relate to signalling joint movements

(Schaible & Schmidt, 1996), the most important question to ask is what is the functional significance of these receptors? At this point it is worth considering the pathophysiological or physiological events that might cause ATP release. One is during inflammation, but it is also known that membrane distortions release ATP and that the nucleotide is released when cells are mechanically stimulated (Nakamura & Strittmatter, 1996). It is conceivable that tissue-deforming but non-injurious stimuli, such as low intra-articular pressure, low-intensity mechanical pressure or stretch, might cause low levels of ATP release *in vivo*, whereas noxious events, such as high intra-articular pressure, acute tissue-damaging mechanical insults or stretch (or inflammation), might cause high levels of ATP release. Interestingly, in dissociated rat DRG neurones, lower concentrations of $\alpha\beta$ meATP are required to activate *transient* inward current, whereas higher concentrations are needed to evoke *persistent* current (Li *et al.*, 1999). Therefore, it is tempting to speculate that transient current might be associated with non-nociceptive afferents, and persistent current with nociceptive afferents.

In this scheme, proprioceptive A δ -afferents innervating the joint would be transiently activated by ATP release during innocuous joint movements, thereby rapidly signalling to the CNS that the joint has moved. During an acute stretching of the joint beyond its normal working range, the P2X receptor on nociceptive A δ and C-fibre afferents would be activated, because higher levels of the nucleotide would be released (see Figure 5.21). In the absence of a continued signal (motor reflexes would return the joint to its normal position and ATP would be hydrolysed) the noxious stimulus would only be transient. Although there is little evidence to support this proposal at present, the proposed structure for P2X receptors resembles that of

the channel thought to be responsible for mechanosensitivity in *Caenorhabditis elegans* (Surprenant *et al.*, 1995 and see General Introduction), and it has been suggested that these receptors might be involved in mechanosensation (Cook *et al.*, 1997). Furthermore, one of the metabotropic ATP receptor subtypes (P2Y₁) has been implicated in proprioceptive mechanosensation because mRNA for this subtype is present in large diameter DRG neurones and functional expression of the protein in *Xenopus* oocytes renders the cells sensitive to touch (Nakamura & Strittmatter, 1996). However, excitation of metabotropic receptors on sensory neurones (such as that excited by bradykinin in this thesis) usually has a relatively long onset latency, whereas mechanical stimuli are sensed rapidly. It is possible that P2X receptors might be responsible for the initial detection of mechanical stimuli, but other, perhaps metabotropic receptors, may be involved in signalling sustained mechanical stimuli.

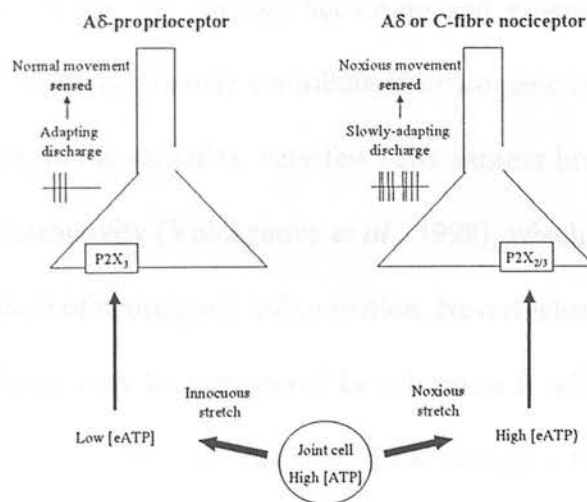


Figure 5.21 A proposed mechanism for P2X receptor mediated detection of innocuous and noxious joint movements. Innocuous movement causing low extracellular ATP (eATP) concentrations would activate the rapidly-desensitising P2X subtype (P2X₃ homomer?), whereas high concentrations would activate the slowly-desensitising subtype (P2X_{2/3} heteromultimer?). The former would not evoke painful sensations, whereas the latter would.

This proposal could also be accommodated to provide a role for P2X receptors in inflammatory pain. During inflammation local release of high concentrations of ATP would cause fluctuating activation and desensitisation of the population of afferents with slowly-desensitising P2X receptors. P2X receptors on nociceptors are highly permeable to calcium (Cook *et al.*, 1997) and increases in intracellular calcium concentration, particularly if mediated via persistent inward current, may produce longer-term changes in excitability. This is thought to occur following capsaicin activation of sensory neurones *in vitro*: vanilloid receptor(s) activation causes calcium influx which triggers nitric oxide synthase activity, leading to cyclic guanosine monophosphate (cGMP) production (Wood *et al.*, 1989). cGMP may then activate protein kinases that sensitise the afferent (for example see Qian *et al.*, 1996). Furthermore, there has been speculation the ATP itself may be released from activated sensory nerve terminals (Holton, 1959), if so, this could create a positive feedback mechanism for sensory activation and sensitisation. It has also been suggested that P2X receptors may contribute to neurogenic inflammation (Cook *et al.*, 1997). However, in the rat DRG, very few cells express both substance P and P2X₃ subtype immunoreactivity (Vulchanova *et al.*, 1998), which largely excludes a P2X₃ mediated *initiation* of neurogenic inflammation. Nevertheless, the ATP-evoked action potential discharge may be augmented by substance P release from other C-fibre terminals (Hu & Li, 1996). In this way, P2X subtypes may play a role in *sustaining* the neurogenic inflammation caused by substance P (see Figure 5.22). If more ATP is released from cells that are that are compromised during inflammation by membrane distortions – thus P2X receptors may also play a role in allodynia or hyperalgesia during inflammation.

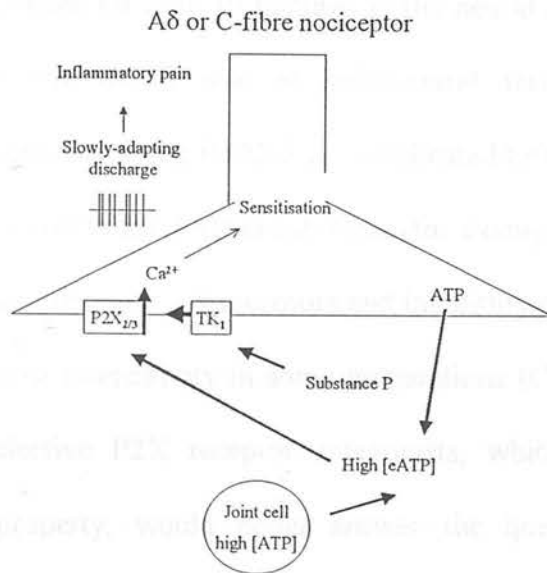


Figure 5.22 A proposed mechanism for initiation of inflammatory pain by P2X receptors. The high concentrations of extracellular ATP (eATP) during inflammation would stimulate the slowly-desensitising P2X subtype (P2X_{2/3} heteromultimer?) expressed on C- or Aδ-fibre nociceptors. In the continued presence of agonist, the intracellular Ca²⁺ concentration would be increased which could lower the threshold of the nociceptors and contribute to allodynia and hyperalgesia. Excitation of the afferents would induce the release of ATP, which could feedback on the terminals. The substance P released from neuropeptide containing C-fibre nociceptors would enhance the excitation of afferents by ATP and promote neurogenic inflammation.

However, the data provided by the current study can be interpreted in many other ways, and the proposal outlined in Figure 5.21 and Figure 5.22 is highly speculative and it cannot be tested at present because of the lack of pharmacological tools. Nevertheless, it could probably be determined whether i.art. injection of ATP or $\alpha\beta$ meATP evokes rapidly-adapting action potential discharge from proprioceptive afferents, and slowly-adapting discharge from nociceptive afferents by performing i.art. injections as described above. The hypothesis that P2X receptors are involved in signalling joint movements, or afferent sensitisation to movement during inflammation could also be determined by injecting PPADS i.a. while flexing or extending the normal or arthritic joint respectively. The effect of the antagonist on “weight-bearing” allodynia in the monoarthritis model was not investigated in this

study because its effects lasted for only 10 minutes in the neural experiments and it was unlikely to serve any useful role in behavioural testing. In addition, interpretation of experiments involving PPADS is complicated by the fact that it also inhibits the enzymes responsible for ATP metabolism (for example see Chen *et al.*, 1996). Simultaneous antagonism of P2X receptors and inhibition of ATP breakdown leads to apparent antagonist insensitivity in some preparations (Crack *et al.*, 1994). The development of selective P2X receptor antagonists, which lack this ecto-nucleotidase blocking property, would better answer the question of whether endogenous ATP contributes to mechanosensation in normal joints, and/or spontaneous pain, allodynia or hyperalgesia in chronically inflamed joints.

Although there is no evidence that the suggested proposal (in Figure 5.21 and Figure 5.22) has any functional relevance, there are a number of observations that support it. In the present study, fewer A δ -mechanoreceptors innervating arthritic joints were excited by $\alpha\beta$ meATP (normal 84%, arthritic 63%) or ATP (normal 69%, arthritic 39%) which would be consistent with desensitisation of rapidly-adapting P2X receptors on proprioceptors by higher ATP levels in inflamed tissues. Furthermore, sub-plantar (Bland-Ward & Humphrey, 1997), or intra-plantar (Hamilton *et al.*, 1999) injections of stable and unstable P2X receptor agonists into the rat hindpaw stimulate nociceptive behaviours lasting for up to 5 minutes that are abolished by neonatal capsaicin treatment. It is very unlikely that slow clearance of the analogues from the tissue accounted for this since 1) ATP is rapidly metabolised by nucleotidases and therefore would not be in the tissue for this length of time, 2) even the slow-desensitising P2X receptor desensitises within seconds (Cook & McCleskey, 1997), and 3) i.art. injection of nucleotides evokes an excitation lasting

Chapter 6: The direct role of adenosine in knee joint nociception

6.1 INTRODUCTION

Antifolate drugs, including methotrexate and sulfasalazine, are some of the main disease-modifying treatments for RA (vanEde *et al.*, 1998). These compounds increase the levels of endogenous adenosine and it has been proposed that this effect accounts for their anti-inflammatory properties (Baggott *et al.*, 1993; Cronstein, 1994; Cronstein *et al.*, 1996; Cronstein *et al.*, 1993). However, antifolate therapy is limited by adverse side effects (Sandoval *et al.*, 1995; Schnabel & Gross, 1994; vanEde *et al.*, 1998) and investigators have started to investigate alternative, more selective methods of increasing endogenous adenosine as potential anti-inflammatory treatments (Cronstein *et al.*, 1996; Firestein, 1996).

One of the main rationales for developing anti-rheumatic drugs is to provide relief from the pain of the disease. Novel anti-inflammatory treatments that increase peripheral adenosine levels may have severe limitations as analgesic agents and may even exacerbate inflammatory pain because the nucleoside is known to cause pain in humans when it is applied to a blister base (Bleehen & Keele, 1977) and when it is injected intradermally (Pappagallo *et al.*, 1993) or intravenously (Gaspardone *et al.*, 1995). Research strategies aimed at developing adenosine compounds for the treatment of RA would be facilitated if the mechanisms underlying the pain-producing effects of adenosine were fully understood.

The peripheral pro-nociceptive effects of adenosine could be mediated by direct and/or indirect actions on the primary afferent nociceptor either to excite it or

sensitise it to other stimuli. Adenosine can depolarise the cell bodies of primary afferent nociceptors *in vitro* (Huang *et al.*, 1995) and it excites primary sensory nerves *in vivo* (McQueen & Ribeiro, 1981). The nucleoside also induces mechanical hyperalgesia in conscious rats following intradermal injection, apparently through direct actions on the nociceptive afferent (Taiwo & Levine, 1990). In addition to these direct actions, sub-cutaneous administration of the purine causes nociceptive behaviours in rats by releasing histamine and 5-hydroxytryptamine from mast cells (Sawynok *et al.*, 1997).

A major source of extracellular adenosine is the metabolism of ATP by ecto-nucleotidases (Gordon, 1986). As reported in the previous chapter (and see Dowd *et al.*, 1998a), close-arterial injection of ATP to the rat knee joint, evokes a biphasic excitation of joint afferents consisting of a rapid response, followed by a delayed increase in discharge. The stable ATP analogue, $\alpha\beta$ -methylene ATP, which is not metabolised to adenosine, did not elicit the slow response. These findings suggest that adenosine might be responsible for the delayed increase in afferent discharge following i.a. injection of ATP, and indicates that the nucleoside might be involved in nociception in the knee joint by directly activating articular nociceptors.

The primary aim of the present electrophysiological study was to test the hypothesis that adenosine directly activates nociceptive afferents innervating the rat knee joint. Because substance P is released during adjuvant-induced arthritis (Ahmed *et al.*, 1995) and this tachykinin has previously been shown to augment the response of canine ventricular sensory afferents to adenosine (Huang *et al.*, 1995), experiments were also performed to determine whether the afferent responses to the nucleoside were affected in the arthritic rat knee joint. In order to determine the role

of endogenous adenosine in maintaining inflammatory pain associated with monoarthritis, the effects of adenosine receptor agonists, antagonists and the nucleoside uptake inhibitor, dipyridamole, on weight-bearing allodynia was investigated in behavioural studies.

6.2 METHODS

6.2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

These experiments were carried out as described in Section 2.1.2. Briefly, arthritis was induced in the left knee joint and rats were used for neural recordings 14-21 days later. A total of 17 normal and 19 arthritic rats (weight range 235-350g; mean \pm sem 313 \pm 10g) were used in the present investigation. Animals were anaesthetised with pentobarbitone (60mg.kg⁻¹ i.p., supplemented hourly i.v.) and surgically prepared. Bipolar platinum-iridium wire electrodes were used to record extracellular neural activity in MAN afferents and data was analysed off-line using Spike2. In most experiments, drugs were administered by close-arterial injection via a cannula in the right femoral artery, but in a small number, they were injected i.art. into the knee joint.

6.2.2 BEHAVIOURAL STUDIES

Body weight, knee joint swelling and weight-bearing allodynia were assessed as described in Sections 2.2.2.

6.2.2.1 Dipyridamole and DPCPX trial

In this study, four treatment groups (dipyridamole, DPCPX, DPCPX & dipyridamole, and vehicle) each with six male Wistar rats were used. Body weight,

weight-bearing and knee diameters were measured from Day -3 to Day 14 post-induction of arthritis, where arthritis was induced on Day 0. From Day -1 to Day 5, the rats were injected as follows: the dipyridamole group received DPCPX vehicle (5% DMSO, 2% 1M NaOH, 93% PBS; 0.1ml kg⁻¹ i.p.) followed 10 minutes later by dipyridamole (40mg kg⁻¹ i.p.); the DPCPX group received DPCPX (1mg kg⁻¹ i.p.) followed 10 minutes later by dipyridamole vehicle (PBS; 0.1ml kg⁻¹ i.p.); the combined group received DPCPX (1mg kg⁻¹ i.p.) followed 10 minutes later by dipyridamole (40mg kg⁻¹ i.p.) and the vehicle group received DPCPX vehicle (0.1ml kg⁻¹ i.p.) followed 10 minutes later by dipyridamole vehicle (0.1ml kg⁻¹ i.p.). All measurements were made blind to drug treatment one hour after the injections.

6.2.2.2 GR79236 and DPCPX trial

Four treatment groups with six rats each were used in this study (GR79236, DPCPX, DPCPX & GR79236, and vehicle). As in the dipyridamole study, arthritis was induced after the measurements on Day 0 and the rats were measured from Day -3 to Day 14 post-induction of arthritis. From Day -1 to Day 5, the rats were injected as follows: the GR79236 group received DPCPX vehicle (0.1ml kg⁻¹ i.p.) and GR79236 (0.3mg kg⁻¹ s.c.); the DPCPX group received DPCPX (1mg kg⁻¹ i.p.) and GR79236 vehicle (PBS; 0.05ml kg⁻¹ s.c.); the combined group received DPCPX (1mg kg⁻¹ i.p.) and GR79236 (0.3mg kg⁻¹ s.c.) and the vehicles group received DPCPX vehicle (0.1ml kg⁻¹ i.p.) and dipyridamole vehicle (0.05ml kg⁻¹ s.c.). The DPCPX injections (including vehicle) were administered three times daily (7am, 1pm and 6:30pm) and the GR injections (including vehicle) were given 30 minutes after the first and last DPCPX injection (7:30am and 7pm). The rats were measured blind to drug treatment 1 hour after the morning injections (8:30am).

6.2.2.3 Statistical analysis

To determine whether there were any differences between drug treatment groups, the cumulative change from a selected control period in body weight, hindlimb weight bearing and joint swelling was determined. The control period was the mean of the measurements on Days -1 and 0, and this was subtracted from the mean of the measurements on Days 1-5. The data was analysed using one way ANOVA followed by Tukey's multiple comparison test.

6.3 RESULTS

6.3.1 AFFERENT NERVE RECORDINGS *IN VIVO*

6.3.1.1 Characterisation of afferent nerve fibres

Neural discharge was recorded from a total of 40 single afferent fibres innervating 17 normal knee joints and 77 single afferent fibres innervating 19 arthritic knee joints. The afferents were classified as C-fibre polymodal nociceptors or A δ -fibre mechanoreceptors based on their conduction velocities, mechanosensitivity and sensitivity to capsaicin as described in Section 4.3.1.1. Of the afferents recorded, 58% (23/40 afferents) from normal joints and 51% (39/77 afferents) from arthritic joints were classified as C-fibre polymodal nociceptors and 43% (17/40 afferents) from normal joints and 49% (38/77) from arthritic joints were classified as A δ -fibre mechanoreceptors.

6.3.1.2 Adenosine-evoked excitation of articular afferents

6.3.1.2.1 Proportion of afferents activated by adenosine

A single bolus injection of adenosine (370nmol i.a.) evoked an increase in action potential discharge, that was delayed in onset and long lasting from 82% (18/22 afferents) of C-fibres and 19% (3/16 afferents) of A δ -fibres on which it was tested in normal joints and 71% (15/21 afferents) of C-fibres and 17% (2/12 afferents) of A δ -fibres in arthritic joints (Figure 6.1 and Figure 6.2). The representative nerve recordings in Figure 6.1 show the adenosine-evoked excitatory response in comparison with the discharge elicited by the algogens capsaicin and bradykinin. Because so few A δ -fibres were activated by the purine, the data presented in this chapter will be from C-fibre nociceptors only. Adenosine was dissolved in PBS, and as reported in the previous chapter, vehicle did not excite any of the afferents on which it was tested (0.1ml i.a., change in discharge frequency 0.02 ± 0.1 impulses s^{-1} n=12 afferents).

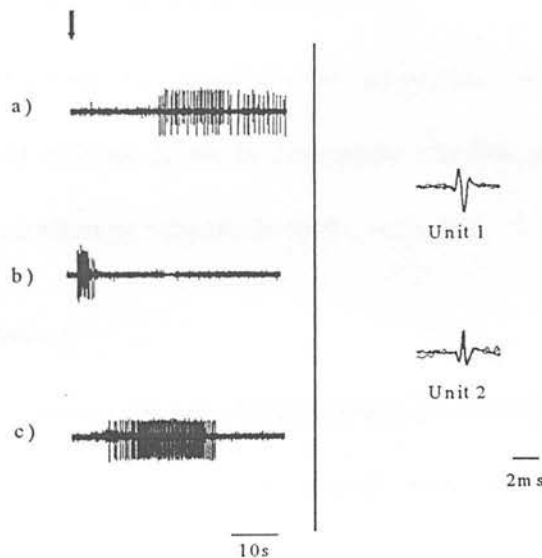


Figure 6.1 Neural discharge (first panel) showing the response evoked from a filament of the MAN innervating a normal knee joint by close-arterial injection of a) adenosine (370nmol, activated units 1 & 2), b) capsaicin (10nmol, activated units 1 & 2) and c) bradykinin (9 nmol, activated unit 1). The afferent fibres that responded are shown (second panel).

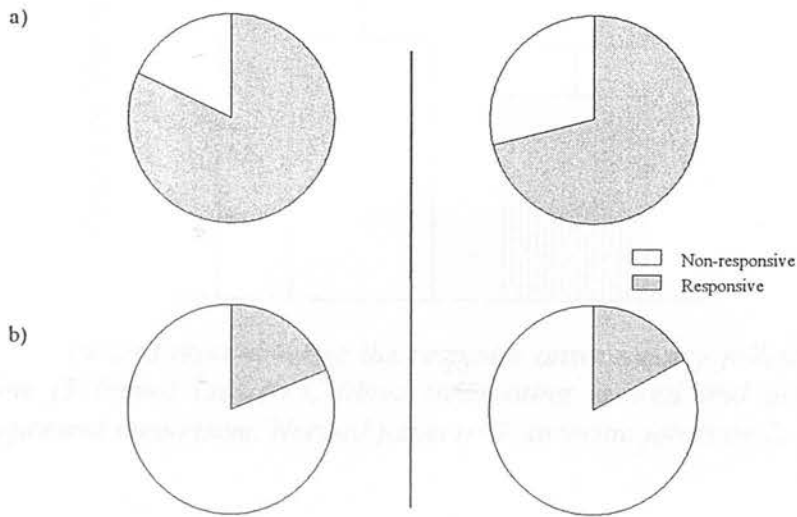


Figure 6.2 Pie charts showing the proportion of a) C-fibre polymodal nociceptors and b) A δ -fibre mechanoreceptors that were activated by adenosine (370nmol i.a.) in normal (first panel) and arthritic knee joints (second panel).

6.3.1.2.2 Features of the adenosine-evoked response

The excitation following i.a. injection of adenosine (370nmol) was compared between normal and arthritic joints to determine whether adjuvant-arthritis affected the onset latency, duration or magnitude of the response.

6.3.1.2.2.1 Onset-latency

The increase in action potential discharge evoked by adenosine was delayed in onset in normal joints (Figure 6.3) and the latency was not significantly different in arthritic joints.

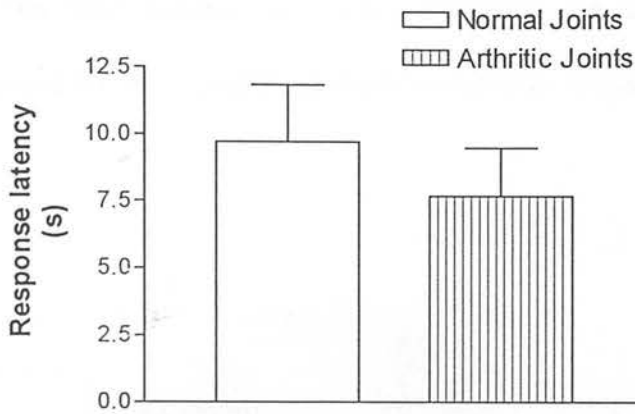


Figure 6.3 Pooled data showing the response onset latency following injection of adenosine (370nmol i.a.) in C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints n=9; arthritic joints n=7.

6.3.1.2.2.2 Duration

As illustrated in Figure 6.4, adenosine caused a long-lasting increase in C-fibre discharge that was similar in normal and arthritic joints.

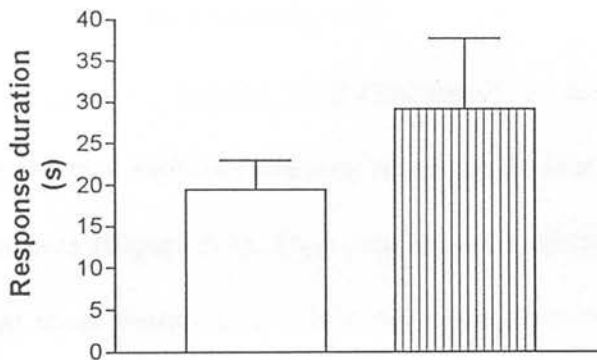


Figure 6.4 Pooled data showing the duration of the response evoked by adenosine (370nmol i.a.) from C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints n=9; arthritic joints n=7.

6.3.1.2.2.3 Magnitude

The change in action potential discharge frequency evoked by adenosine in C-fibres innervating normal joints was not significantly different from that in arthritic joints

(Figure 6.5a). The total number of action potentials that fired was also not significantly affected by the presence of adjuvant-arthritis in the knee joint (Figure 6.5b).

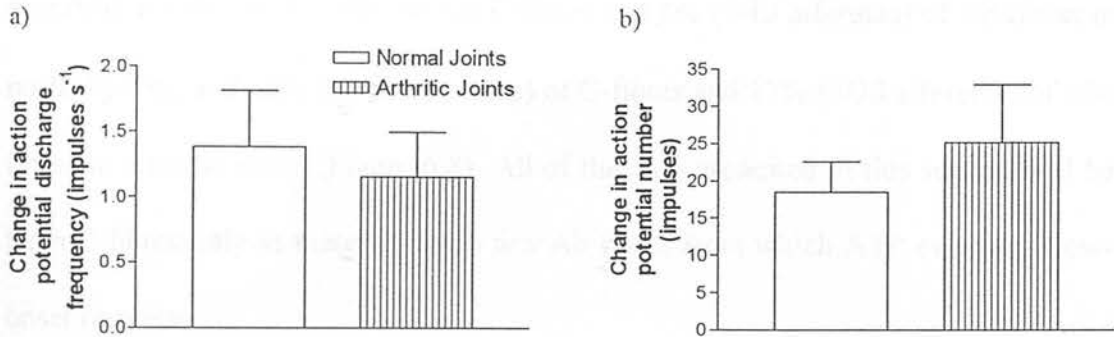


Figure 6.5 Pooled data showing the change in a) the C-fibre discharge frequency and b) the total number of action potentials that fired following close-arterial injection of adenosine (370nmol) to normal and arthritic knee joints. Columns represent mean \pm sem. Normal joints n=9; arthritic joints n=7.

6.3.1.3 ATP-evoked excitation of articular afferents

6.3.1.3.1 Proportion of afferents activated by ATP

As mentioned in the previous chapter, ATP (2000nmol) evoked a biphasic increase in neural discharge from a multiunit filament of the MAN that consisted of fast and slow-onset components (Figure 6.6). Data analysis of responses in single afferent fibres revealed that some fibres were rapidly *and* slowly activated by the nucleotide whereas others exhibited one component only. There was also a small population of knee joint afferents that were not activated following injection of ATP. The populations of C-fibre nociceptors and A δ -fibre mechanoreceptors innervating normal and arthritic joints which were (or not) excited following i.a. injection of ATP are shown in Figure 6.7.

The fast onset response was apparently mediated by the action of ATP on P2X receptors (Dowd *et al.*, 1998c and see previous chapter) and will not be discussed further in this chapter. The slow component of the ATP excitation was observed in 73% (11/15 afferents) of C-fibres and 8% (1/13 afferents) of A δ -fibres in normal joints, and 62% (23/37 afferents) of C-fibres and 13% (5/38 afferents) of A δ -fibres in arthritic joints (Figure 6.8). All of the data presented in this section will be from C-fibres only as there were too few A δ -fibres from which ATP evoked a slow-onset response.

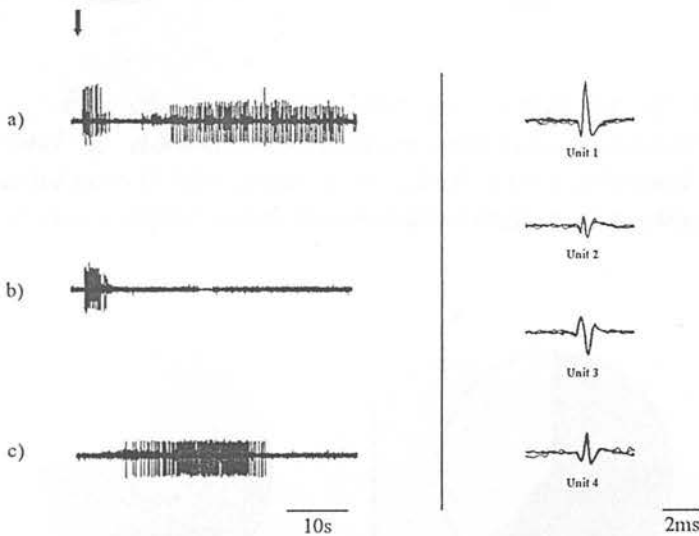


Figure 6.6 Neural discharge (first panel) showing the response evoked from a filament of the MAN by close-arterial injection (at arrow) of a) ATP (2000nmol, activated units 1 & 2 during the fast response and units 3 & 4 during the slow response), b) capsaicin (10nmol, activated unit 3 & 4) and c) bradykinin (9 nmol, activated unit 3). The individual units that responded are shown (second panel). Note that different units were excited during the fast and slow components of the ATP-evoked excitation.

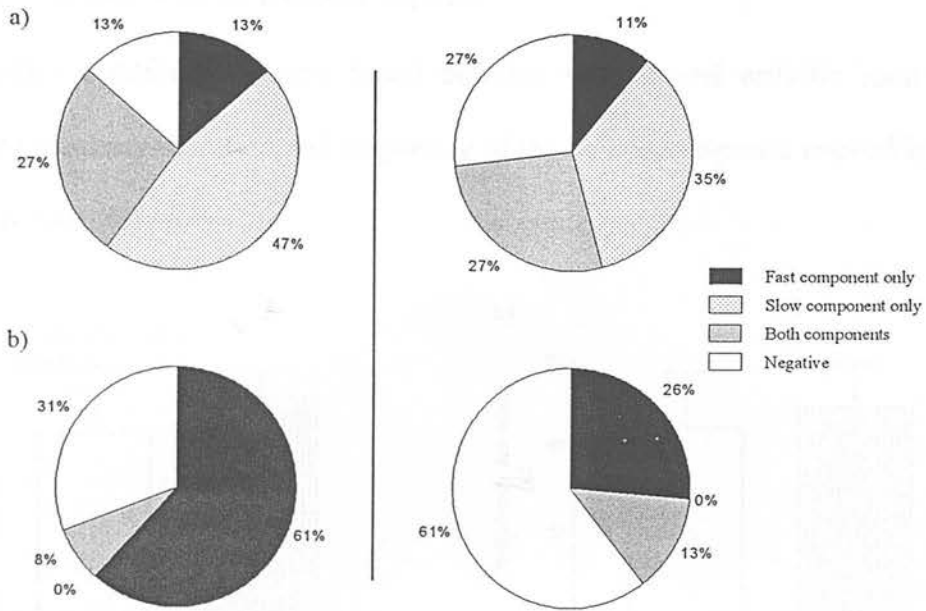


Figure 6.7 Pie charts showing the proportion of a) C-fibre polymodal nociceptors and b) Aδ-mechanoreceptors innervating normal (first panel) and arthritic (second panel) knee joints from which ATP (2000nmol i.a.) evoked rapid-onset, delayed-onset, rapid and delayed-onset excitation, or neither components.

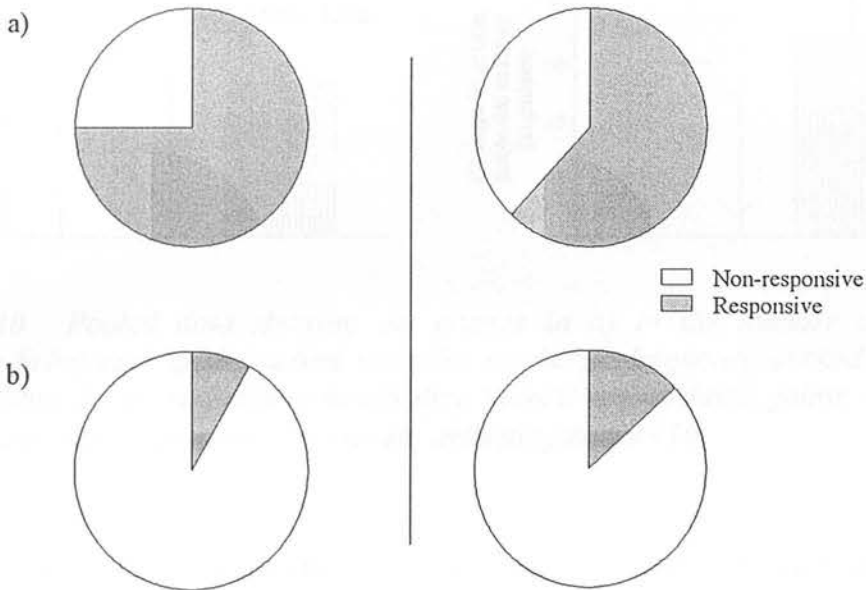


Figure 6.8 Pie charts showing the proportion of a) C-fibre polymodal nociceptors and b) Aδ-fibre mechanoreceptors innervating normal (first panel) and arthritic (second panel) knee joints from which ATP (2000nmol i.a.) evoked a slow-onset response.

6.3.1.3.2 Features of the ATP-evoked response

No significant differences were found between normal and arthritic joints with respect to the delay, duration and magnitude of the delayed response evoked by ATP (Figure 6.9 and Figure 6.10).

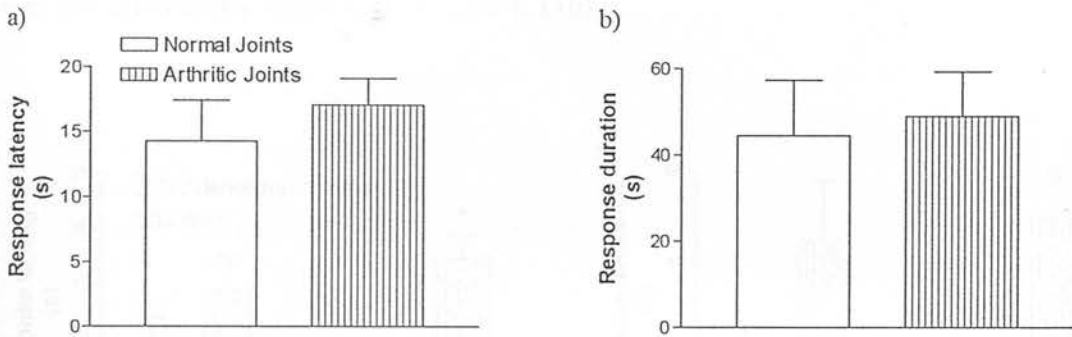


Figure 6.9 Pooled data showing the a) onset latency and b) duration of the slow-onset response evoked by ATP (2000nmol i.a.) in C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints n=10; arthritic joints n=19.

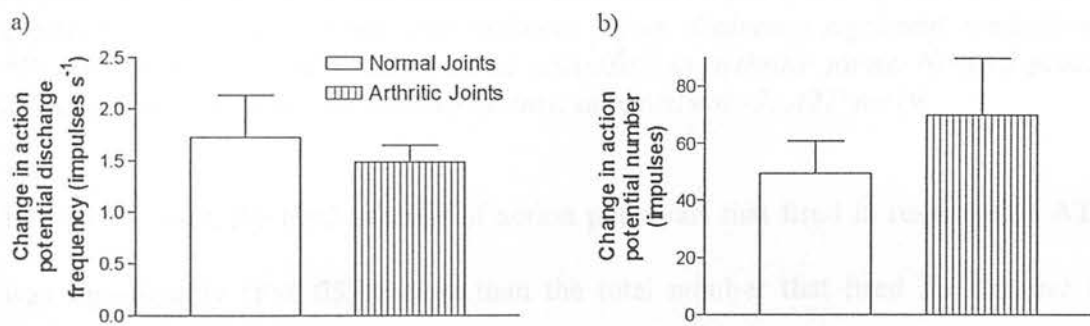


Figure 6.10 Pooled data showing the change in a) in the number of action potentials firing and b) the action potential discharge frequency evoked by ATP (slow, 2000nmol i.a.) in C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. Normal joints n=10; arthritic joints n=19.

6.3.1.4 Comparison between the excitation evoked by adenosine and ATP

In experiments where adenosine and ATP were both injected, all of the C-fibres that were excited by adenosine also responded with a delayed excitation to ATP (normal n=8; arthritic n=14). In normal joints the delay to onset of the responses caused by

the purines were similar but in arthritic joints, the latency of the ATP evoked excitation was significantly ($P < 0.05$) longer compared to that of adenosine (Figure 6.11a). The duration of the response evoked ATP tended to be longer than the response evoked by adenosine in both normal and arthritic joints but this difference was not statistically significant (Figure 6.11b).

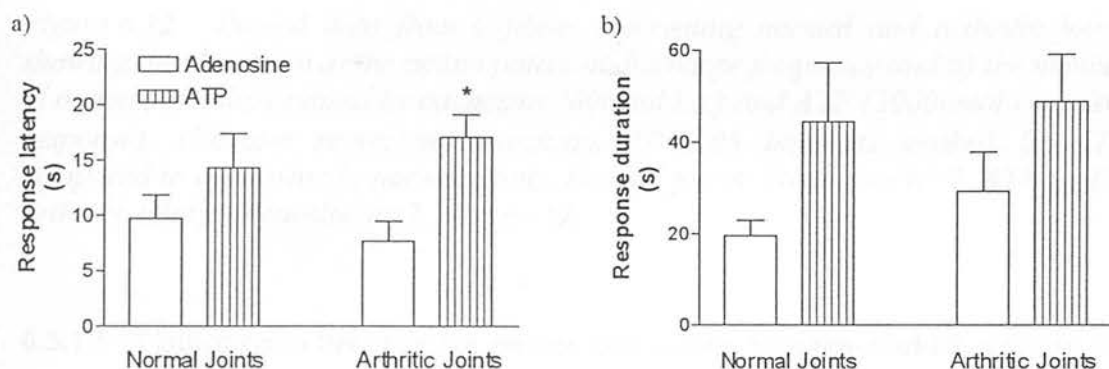


Figure 6.11 Pooled data showing the a) latency and b) duration of the slow response following injection of adenosine (370nmol i.a.) and ATP (2000nmol i.a.) in C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. * $P < 0.05$ latency for ATP compared to adenosine in arthritic joints. Normal joints: adenosine $n=9$; ATP $n=10$; arthritic joints: adenosine $n=7$; ATP $n=19$.

In normal joints, the total number of action potentials that fired in response to ATP was significantly ($P < 0.05$) greater than the total number that fired in response to adenosine although the frequencies of the responses were similar (Figure 6.12). The same pattern was seen in arthritic joints but the total number of action potentials that fired following injection of ATP was not significantly greater than the total number evoked (Figure 6.12).

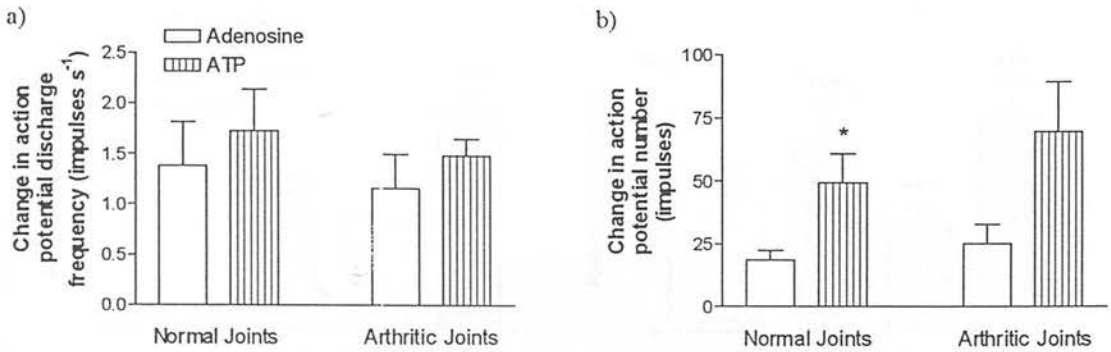


Figure 6.12 Pooled data from C-fibres innervating normal and arthritic joints showing the change in a) the action potential discharge frequency and b) the number of action potentials evoked by adenosine (60nmol i.a.) and ATP (2000nmol i.a., slow response). Columns represent mean \pm sem. * $P < 0.05$ impulses evoked by ATP compared to adenosine in normal joints. Normal joints: adenosine $n=9$, ATP $n=10$; arthritic joints: adenosine $n=7$, ATP $n=19$.

6.3.1.5 Comparison between the purine and known algogen-evoked excitations

The increases in C-fibre nociceptor discharge caused by adenosine and ATP were compared to those evoked by the potent exogenous and endogenous algogens, capsaicin and bradykinin respectively. Figure 6.1 and Figure 6.6 clearly show that the temporal pattern of the response evoked by adenosine and the slow response evoked by ATP resemble the bradykinin-evoked response, whereas the fast ATP response is similar to the capsaicin-evoked response.

In order to compare response caused by the purines to those following capsaicin and bradykinin, data from normal and arthritic joints was pooled. In C-fibres, there was no significant difference between the latency or duration of the slow-onset response evoked by adenosine and ATP in comparison with bradykinin, but they did differ significantly from capsaicin (Figure 6.13).

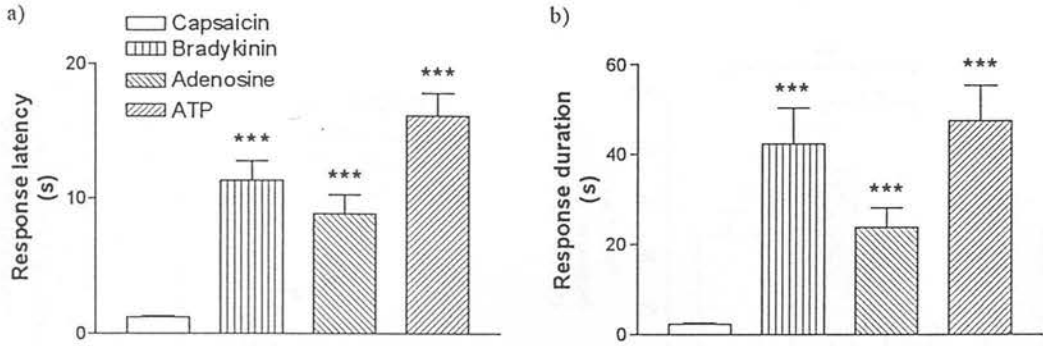


Figure 6.13 Pooled data from C-fibre polymodal nociceptors innervating normal and arthritic joints showing the a) onset latency and b) duration of the responses evoked by i.a. injection of capsaicin (10nmol), bradykinin (9nmol), adenosine (370nmol) and ATP (2000nmol, slow response). Columns represent mean \pm sem. Capsaicin $n=21$, bradykinin $n=19$, adenosine $n=16$, ATP $n=29$. *** $P < 0.0001$ unpaired t -test compared to capsaicin.

The size of the response evoked by capsaicin, bradykinin and the purines are compared in Figure 6.14. The change in the action potential discharge frequency following the injection of the purines was significantly smaller ($P < 0.001$) than that evoked by capsaicin, but was similar to that evoked by bradykinin. The change in the total number of action potentials that fired following injection of adenosine, but not ATP, was significantly ($P < 0.05$) smaller than the change evoked by capsaicin or bradykinin (Figure 6.14).

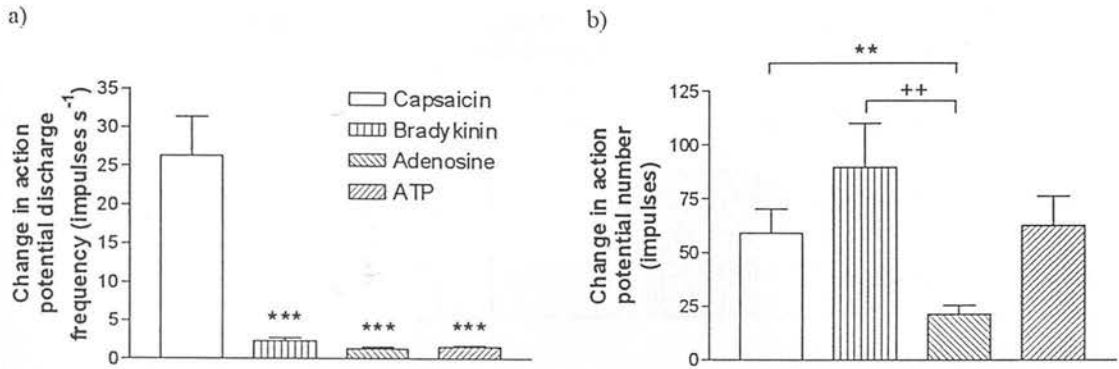


Figure 6.14 Pooled data from C-fibre polymodal nociceptors innervating normal and arthritic joints showing the change in a) the action potential discharge frequency and b) the total number of action potentials that fired in response to i.a. injection of capsaicin (10nmol), bradykinin (9nmol), adenosine (370nmol) and ATP (2000nmol, slow response). Columns represent mean \pm sem. Capsaicin $n=21$, bradykinin $n=19$, adenosine $n=16$, ATP $n=29$. Part a) *** $P<0.001$ unpaired t -test compared to capsaicin. Part b) ** $P<0.01$ unpaired t -test compared to capsaicin, ++ $P<0.01$ unpaired t -test compared to bradykinin.

6.3.1.6 Characterisation of the receptor mediating the slow response to adenosine and ATP

6.3.1.6.1 Responses evoked by selective adenosine receptor agonists

The adenosine A₁ receptor selective agonists, CPA (30nmol i.a.) and GR79236 (85 nmol i.a.) also evoked a slow-onset, long duration increase in the discharge of adenosine-positive C-fibres (Figure 6.15). In contrast, the adenosine A₂ receptor agonist CGS21680 (190nmol i.a.) had no effect on adenosine-positive C-fibre activity (normal joint: 0.01 ± 0.2 impulses s⁻¹, $n=2$; arthritic: 0.02 ± 0.1 impulses s⁻¹, $n=2$).

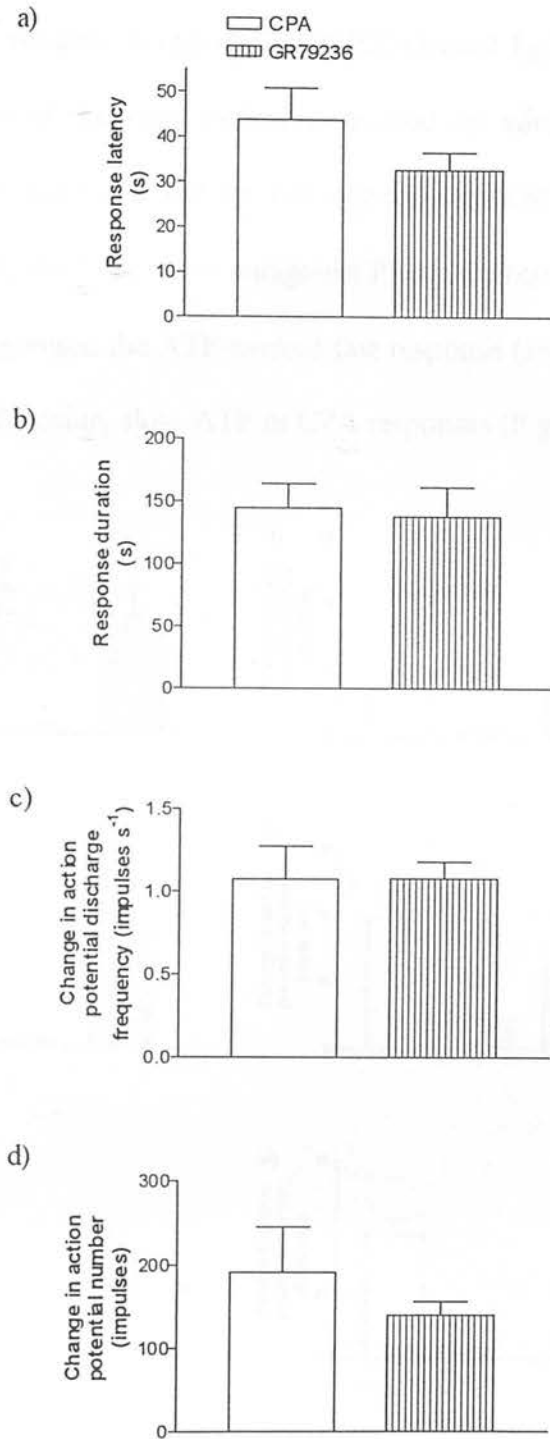


Figure 6.15 Summary of a) response onset latency b) response duration c) change in discharge frequency and d) change in action potential number following injection of CPA (30nmol i.a.) or GR79236 (85nmol i.a.). Pooled data from C-fibres innervating normal and arthritic joints. Columns represent mean \pm sem. CPA: normal joints $n=11$, arthritic joints $n=5$; GR79236: normal joints $n=4$.

6.3.1.6.2 Effect of purinoceptor antagonists on the purine-evoked responses.

The adenosine A_1 receptor antagonist, DPCPX ($3\mu\text{mol kg}^{-1}$ i.a., 10 minutes pre-treatment) antagonised the slow excitation evoked by adenosine, ATP and CPA (Figure 6.16) but it did not affect the fast response evoked by ATP (see previous chapter). In contrast, the P2 receptor antagonist PPADS ($16\mu\text{mol kg}^{-1}$ i.a., 10 minutes pre-treatment) antagonised the ATP-evoked fast response (see previous chapter) but did not block the adenosine, slow-ATP or CPA responses (Figure 6.16).

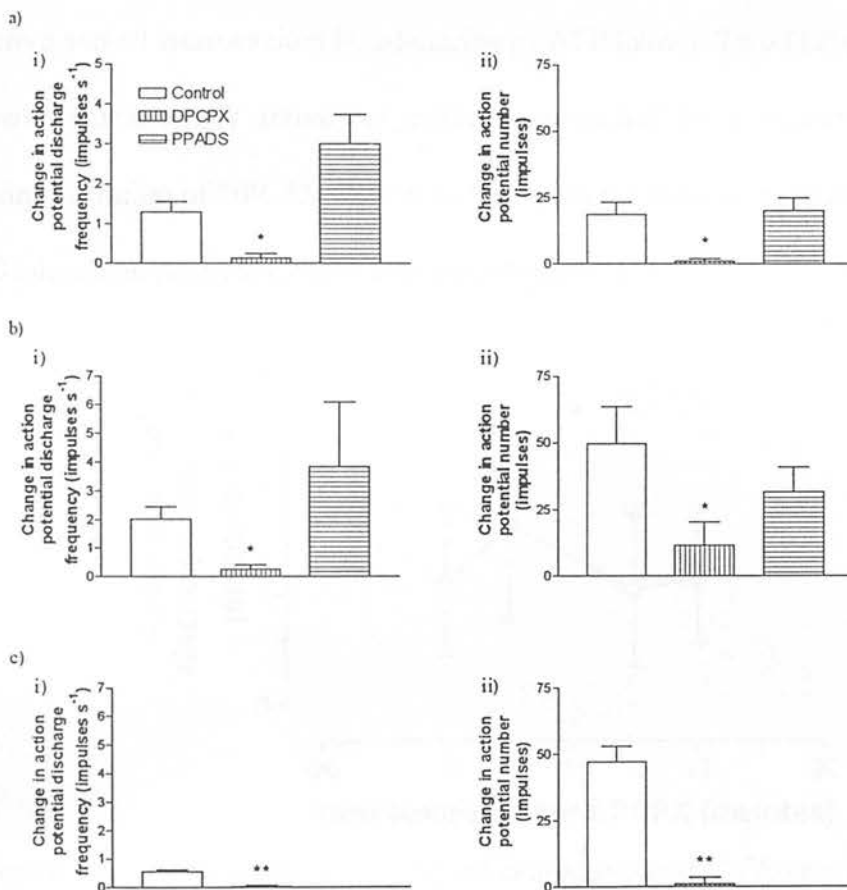


Figure 6.16 Pooled data from C-fibres innervating normal and arthritic joints showing the effect of DPCPX ($3\mu\text{mol kg}^{-1}$ i.a.) and PPADS ($16\mu\text{mol kg}^{-1}$ i.a.) on the magnitude of the responses evoked by a) adenosine (370nmol), b) ATP (2000nmol) and c) CPA (30nmol). The changes in action potential discharge frequency (i) and number of action potentials (ii) are shown. Columns represent mean \pm sem. * $P < 0.05$ and ** $P < 0.01$ Student's paired *t*-test compared to control responses. Adenosine: DPCPX $n=5$, PPADS $n=4$; ATP: DPCPX $n=6$, PPADS $n=3$; CPA: DPCPX $n=5$, PPADS not done.

6.3.1.7 Effect of DPCPX on the spontaneous discharge of nociceptors innervating arthritic joints

In order to establish whether endogenous adenosine contributed to the increased basal discharge seen in arthritic joints, the effect of DPCPX on spontaneous discharge was examined in 9 arthritic joints. The neural discharges of 21 C-fibres and 15 A δ -fibres were recorded. Ten (48%) of the C-fibres were spontaneously active and all were excited by adenosine or ATP (slow). Two (13%) of the A δ -fibres were spontaneously active but neither was excited by adenosine or ATP (slow). Administration of DPCPX ($3\mu\text{mol kg}^{-1}$ i.a.) did not reduce the basal firing rate of the 10 adenosine-positive C-fibre afferents (Figure 6.17).

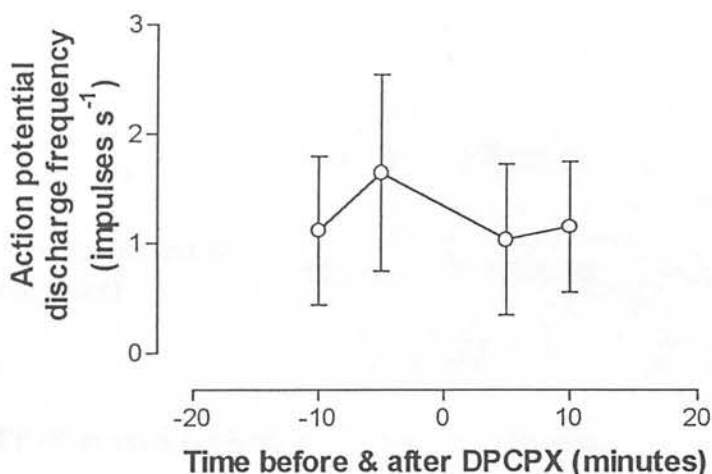


Figure 6.17 Pooled data from 10 adenosine-positive C-fibres showing the lack of effect of DPCPX ($3\mu\text{mol kg}^{-1}$ i.a.) on the elevated rate of spontaneous discharge of nociceptors innervating arthritic joints.

6.3.1.8 Excitation following intra-articular injection of adenosine and ATP

Adenosine (370 nmol) evoked a delayed increase in C-fibre discharge when injected i.art, and ATP (2000 nmol) caused a biphasic increase (Table 6.1). The slow

excitation following i.art. injection had a similar onset-latency and discharge frequency to those following i.a. injection in the same afferents. However, the increase in action potential discharge lasted for a significantly ($P < 0.001$) longer time following i.art. injection and thus, significantly ($P < 0.001$) more action potentials fired in total.

*Table 6.1 Comparison of the responses evoked by i.art. and i.a. injection of adenosine (370nmol) or ATP (2000nmol). *** $P < 0.001$ Mann-Whitney test vs i.a. injections, $n=3$ C-fibres per injection.*

	Adenosine		ATP	
	i.a.	i.art.	i.a.	i.art.
Latency (s)	10±2	14±3	15±2	12±3
Duration (s)	27±6	***202±37	33±6	***179±23
Change in action potential frequency (impulses s ⁻¹)	1.70±0.47	1.37±0.34	1.26±0.78	1.37±0.23
Change in number of action potentials (impulses)	46±13	***276±46	42±26	***245±41

6.3.1.9 ATP γ S-evoked excitation of articular afferents

The P2 agonist, ATP γ S (900nmol i.a.) was tested on 19 afferents from four normal knee joints (12 C-fibres and 7 A δ -fibres). Like ATP, it evoked a biphasic increase in action potential discharge consisting of a fast-onset (see previous chapter), followed by a delayed-onset increase in action potential discharge. The slow-onset response was evoked in 6 of 19 afferents tested (delay 15±4s, duration 22±4, change in number of action potentials 50±15 impulses, $n=6$ (3 C-fibres and 3 A δ -fibres)). The

adenosine A₁ receptor antagonist DPCPX was tested on the response evoked from three afferents and it was found to antagonise the slow component (see Table 6.2).

Table 6.2 Characteristics of the slow-onset response evoked from C-fibre nociceptors by close-arterial injection of ATP γ S (900nmol). *** $P < 0.0001$ Mann-Whitney versus control, $n = 3$ afferents per injection.

	Control	DPCPX
Latency (s)	20 \pm 14	
Duration (s)	34 \pm 18	
Change in action potential frequency (impulses s ⁻¹)	3.5 \pm 0.3	***0.05 \pm 0.01
Change in number of action potentials (impulses)	86 \pm 51	***5 \pm 3

ATP γ S (900nmol) was also tested on 16 afferents (9 C-fibres and 7 A δ -fibres) by i.art. injection. Injection by this route also evoked a fast-onset (see Chapter 5) and a slow onset response, the latter was evoked from 20% (4/16 afferents, 2 C-fibres and 2 A δ -fibres) of afferents. As with ATP and adenosine, the duration and total number of impulses evoked by i.art. injection of this agonist were significantly greater than those following i.a. injection, whereas the latency and change of discharge frequency were similar (Table 6.3).

Table 6.3 Comparison of the responses evoked by i.art. and i.a. injection of ATP γ S (700mmol). *** $P < 0.001$ Mann-Whitney test versus i.a. injections, $n=3$ C-fibres per injection.

	i.a.	i.art.
Latency (s)	20 \pm 14	15 \pm 7
Duration (s)	34 \pm 18	***265 \pm 41
Change in action potential frequency (impulses s ⁻¹)	3.5 \pm 0.3	2.7 \pm 0.9
Change in number of action potentials (impulses)	86 \pm 51	***715 \pm 238

6.3.2 BEHAVIOURAL STUDIES

6.3.2.1 Dipyridamole and DPCPX trial

6.3.2.1.1 General observations

In this study, four treatment groups each with six male Wistar rats were used (dipyridamole, DPCPX, DPCPX & dipyridamole, and vehicle). The groups injected with the nucleoside transport inhibitor, dipyridamole, either alone or after DPCPX, appeared sluggish and lethargic during the drug treatment period. This was probably due to the known sedative and motor inhibitory effects of adenosine (see Sawynok & Sweeney, 1989). The effects were not apparent in rats injected with DPCPX alone or vehicles.

6.3.2.1.2 Body weight

The weight of the rats was measured throughout the trial to monitor their general health (Figure 6.18). All rats, including the vehicle group, lost weight during the drug treatment period. In order to assess differences in weight loss, the mean cumulative change in weight from a selected control (mean of Days -1 and 0) over Days 1-5 was determined (Figure 6.19). The group injected with DPCPX (1mg kg^{-1} i.p., once daily) 10 minutes before dipyridamole, lost significantly ($P < 0.01$) more weight over Days 1-5 in comparison with vehicle. Rats injected with either compound alone also lost more weight than the rats injected with vehicles, but this did not reach statistical significance.

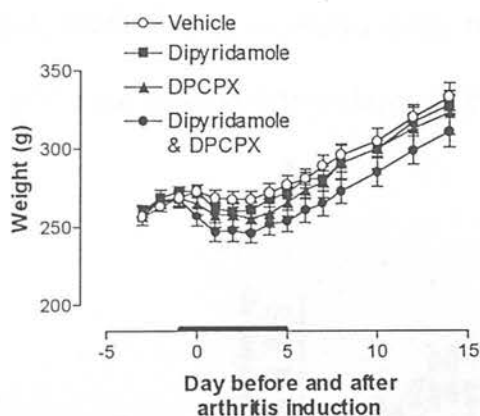


Figure 6.18 Effect of injection of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.) on the body weight of monoarthritic rats. Rats were injected with DPCPX 10 minutes before dipyridamole. The thick line above the x-axis indicates the 7-day treatment period.

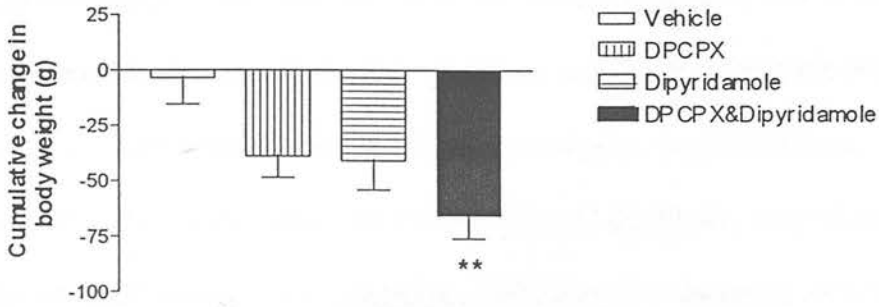


Figure 6.19 Cumulative change in body weight from control period: effect of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 10 minutes before dipyridamole. Columns represent mean \pm sem. Control was mean of Days -1 and 0, and change was determined over Days 1-5. ** $P < 0.01$ vs vehicle, one way AVOVA followed by Tukey's multiple comparison test.

6.3.2.1.3 Allodynia

The weight placed on each hindlimb was measured using the dual channel weight averager (Clayton *et al.*, 1997) and original data is shown in Figure 6.20.

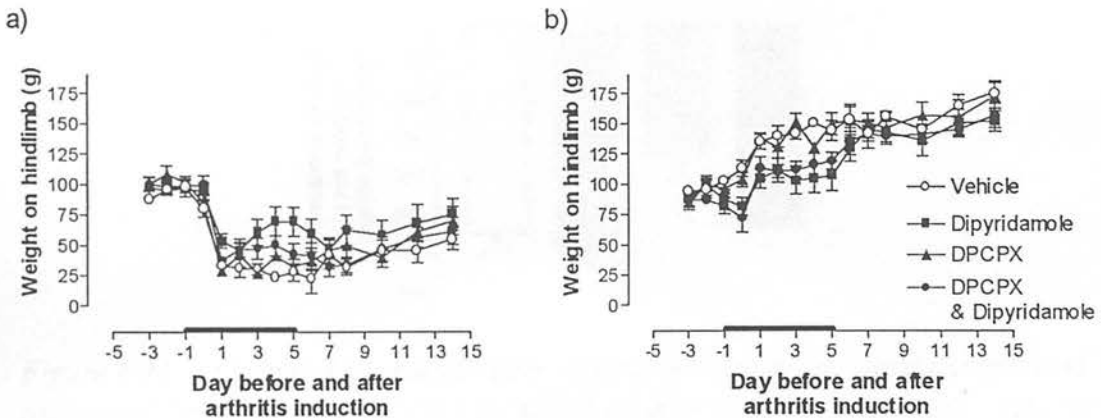


Figure 6.20 Effect of injection of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.) on a) ipsilateral and b) contralateral hindlimb weight bearing in monoarthritic rats. Rats were injected with DPCPX 10 minutes before dipyridamole. The thick line above the x-axis indicates the 7-day treatment period.

The cumulative change in left and right hindlimb weight bearing is shown in Figure 6.21. When compared to the vehicle-treated group, rats injected with dipyridamole, which increases endogenous extracellular adenosine concentrations, placed significantly ($P < 0.05$) more weight on their ipsilateral hindlimbs, and significantly less weight on their contralateral hindlimbs, indicating an analgesic effect of the compound. When DPCPX was injected before dipyridamole, this effect was not observed. DPCPX alone did not have any significant effect on the hindlimb weight-bearing.

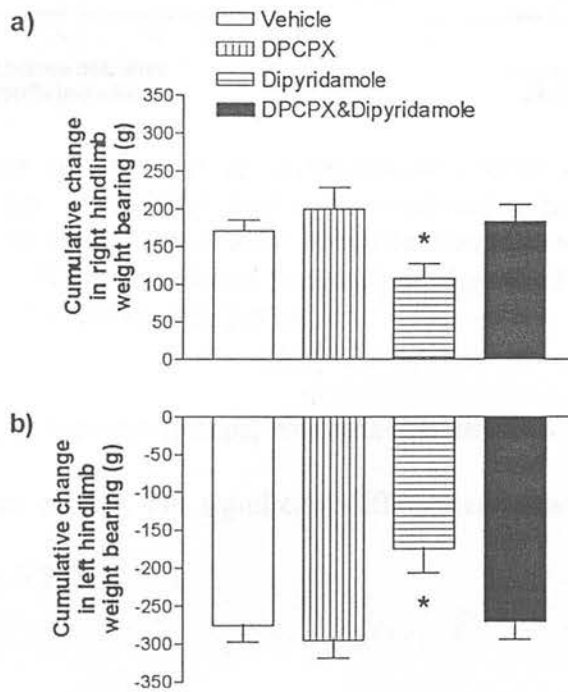


Figure 6.21 Cumulative change from control period in a) contralateral and b) ipsilateral hindlimb weight-bearing: effect of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 10 minutes before dipyridamole. Columns represent mean \pm sem. Control was mean of Days -1 and 0 , and change was determined over Days $1-5$. * $P < 0.05$ dipyridamole vs vehicle one way AVOVA followed by Tukey's multiple comparison test.

6.3.2.1.4 Knee diameter

The knee joint diameter was measured in order to give an indication of the potential anti-inflammatory properties of the adenosine-related compounds. The original joint diameter data is shown in Figure 6.22.

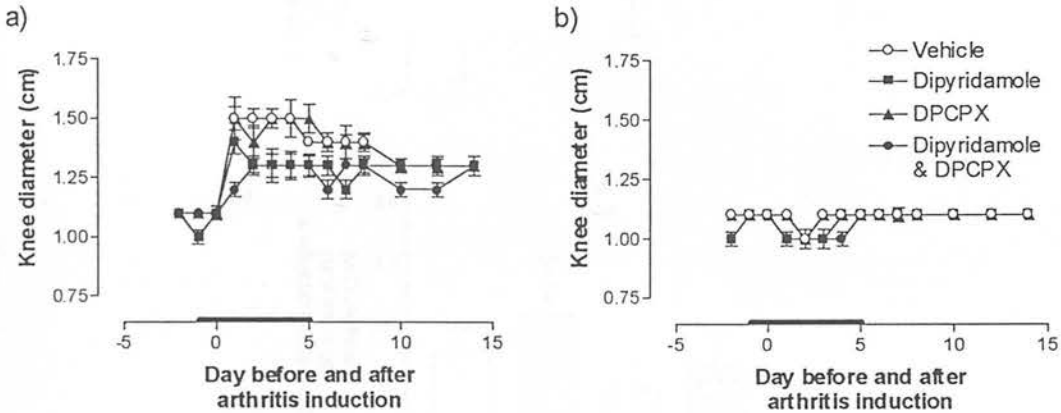


Figure 6.22 Effect of injection of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.) on a) ipsilateral and b) contralateral knee joint diameter in monoarthritic rats. Rats were injected with DPCPX 10 minutes before dipyridamole. The thick line above the x-axis indicates the 7-day treatment period.

To assess differences between groups, data was expressed as the cumulative change over Days 1-5 from control. No significant differences between any of the groups were found (Figure 6.23).

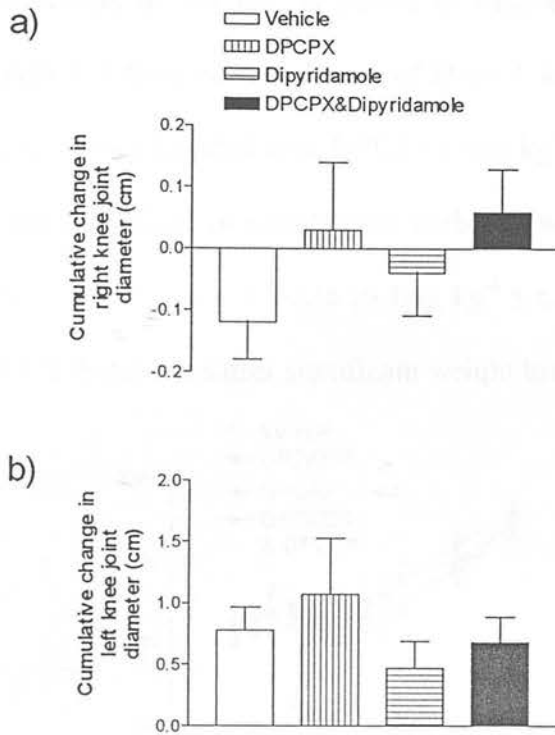


Figure 6.23 Cumulative change from control period in a) contralateral and b) ipsilateral knee joint diameter: effect of dipyridamole (40mg kg^{-1} i.p., once daily), DPCPX (1mg kg^{-1} i.p., once daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 10 minutes before dipyridamole. Columns represent mean \pm sem. Control was mean of Days -1 and 0 , and change was determined over Days $1-5$. No significant differences between groups were detected using one way ANOVA followed by Tukey's multiple comparison test.

6.3.2.2 GR79236 and DPCPX trial

6.3.2.2.1 General observations

Four treatment groups with six rats each were used in this study (GR79236, DPCPX, DPCPX & GR79236, and vehicle). Sedative and locomotor inhibitory effects were apparent in the rats injected with the adenosine A_1 receptor selective agonist GR79236. No apparent motor or depressant effects were seen in the other groups.

6.3.2.2.2 Body weight

The mean body weight of the rats is shown in Figure 6.24 and the cumulative changes over Days 1-5 from control (mean of Days 1 and 2) are shown in Figure 6.25. The group that were injected with DPCPX (1mg kg^{-1} i.p., 3 times daily), lost a significant amount of weight in comparison with the vehicle injected group. The groups that were injected with GR79236 (0.3mg kg^{-1} s.c., 2 times daily) or DPCPX followed by GR79236 did not suffer significant weight loss.

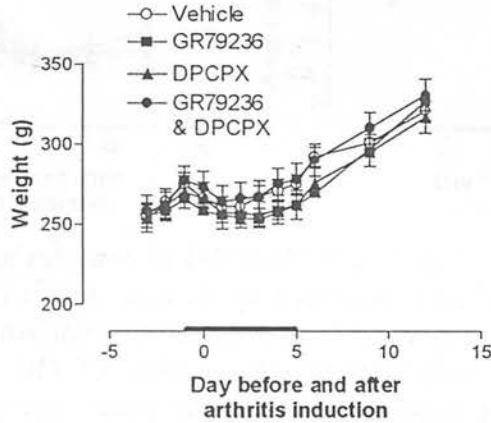


Figure 6.24 Effect of injection of GR79236 (0.3mg kg^{-1} s.c., 2 times daily), DPCPX (1mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1ml kg^{-1} i.p.) on the body weight of monoarthritis rats. Rats were injected with DPCPX 30 minutes before GR79236. The thick line above the x-axis indicates the 7-day treatment period.

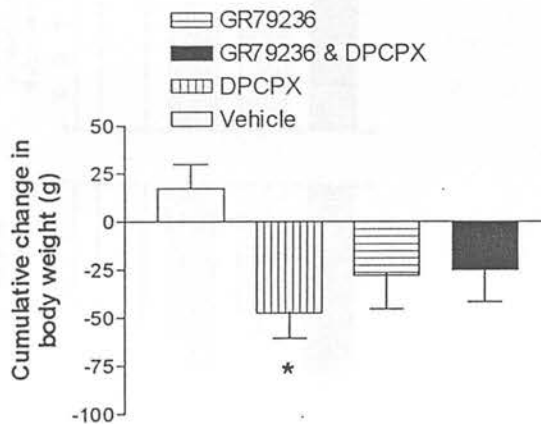


Figure 6.25 Cumulative change in body weight from control period: effect of GR79236 (0.3mg kg^{-1} s.c., 2 times daily), DPCPX (1mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 30 minutes before GR79236. Columns represent mean \pm sem. Control was mean of Days -1 and 0, and change was determined over Days 1-5. * $P < 0.05$ DPCPX vs vehicle, one way AVOVA followed by Tukey's multiple comparison test.

6.3.2.2.3 Allodynia

Original data is shown in Figure 6.26 and cumulative results are shown in Figure 6.27. No significant differences between groups were found.

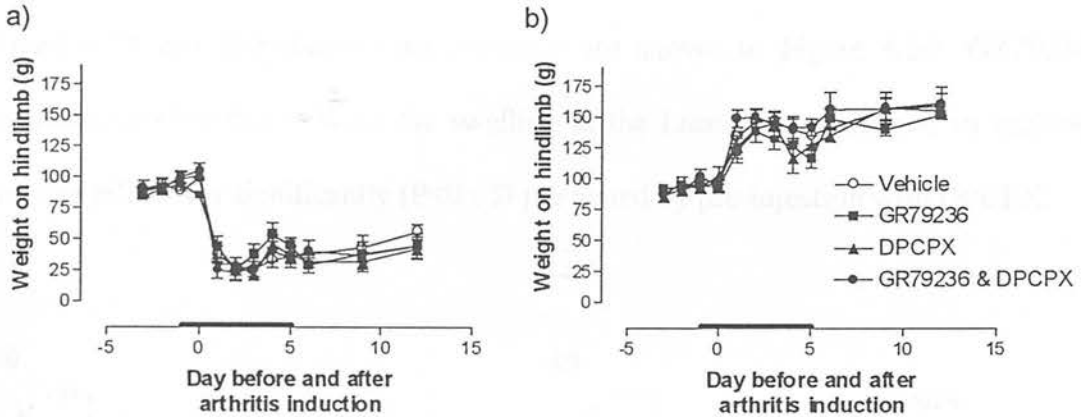


Figure 6.26 Effect of injection of GR79236 (0.3mg kg^{-1} s.c., twice daily), DPCPX (1mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1ml kg^{-1} i.p.) on a) ipsilateral and b) contralateral hindlimb weight bearing in monoarthritic rats. Rats were injected with the DPCPX solutions 30 minutes before GR79236 solutions. The thick line above the x-axis indicates the 7-day treatment period. This figure is for information only (see Figure 6.23 for statistical analysis).

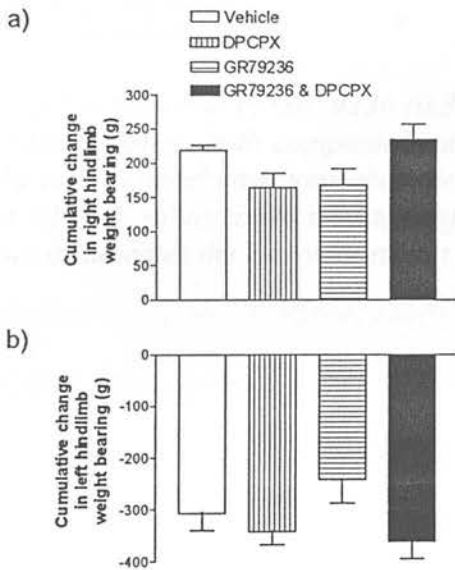


Figure 6.27 Cumulative change in a) contralateral and b) ipsilateral hindlimb weight-bearing from control period: effect of GR79236 (0.3mg kg^{-1} s.c., 2 times daily), DPCPX (1mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 30 minutes before GR79236. Columns represent mean \pm sem. Control was mean of Days -1 and 0 , and change was determined over Days $1-5$. No significant differences between groups were observed using one way AVOVA followed by Tukey's multiple comparison test.

6.3.2.2.4 Knee diameter

The original knee joint diameter measurements are illustrated for information only in Figure 6.28 and cumulative measurements are shown in Figure 6.29. GR79236 significantly ($P < 0.05$) reduced the swelling in the knee joint compared to vehicle, and this effect was significantly ($P < 0.05$) prevented by pre-injection with DPCPX.

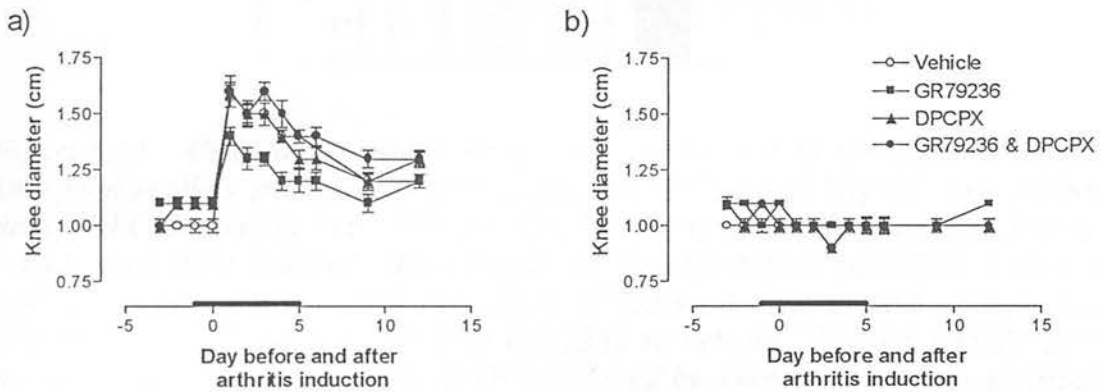


Figure 6.28 Effect of injection of GR79236 (0.3 mg kg^{-1} s.c., twice daily), DPCPX (1 mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1 ml kg^{-1} i.p.) on a) ipsilateral and b) contralateral knee joint diameter in monoarthritic rats. Rats were injected with the DPCPX solutions 30 minutes before GR79236 solutions. The thick line above the x-axis indicates the 7-day treatment period.

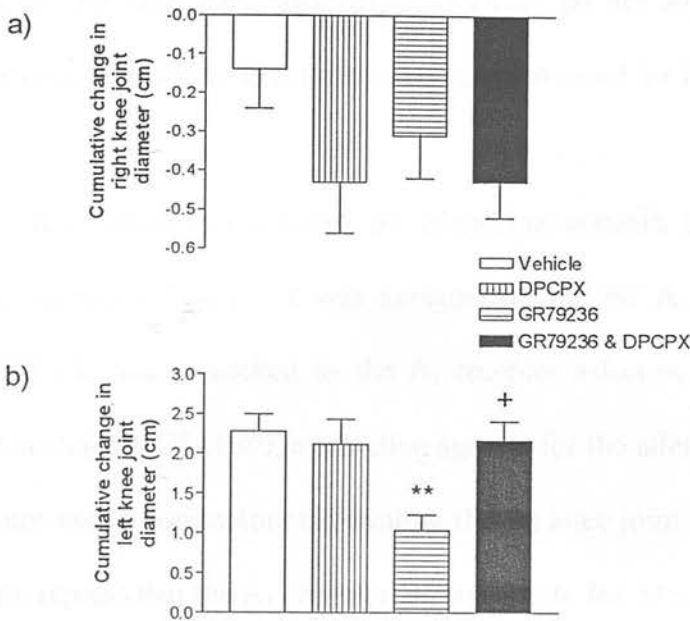


Figure 6.29 Cumulative change in a) contralateral and b) ipsilateral hindlimb knee joint swelling from control period: effect of GR79236 (0.3mg kg^{-1} s.c., 2 times daily), DPCPX (1mg kg^{-1} i.p., 3 times daily), both compounds and vehicles (0.1ml kg^{-1} i.p.). Rats were injected with DPCPX 30 minutes before GR79236. Columns represent mean \pm sem. Control was mean of Days -1 and 0, and change was determined over Days 1-5. * $P < 0.05$ GR79236 vs vehicle, + $P < 0.05$ DPCPX pre-treatment on GR79236 one way ANOVA followed by Tukey's multiple comparison test.

6.4 DISCUSSION

The main finding from the work in this section is that adenosine excites the majority of C-fibre polymodal nociceptors innervating the rat knee joint (Dowd *et al.*, 1998a; Dowd *et al.*, 1998b). However, the adenosine A_1 receptor antagonist, DPCPX, which antagonised the adenosine-evoked response, does not reduce the elevated basal discharge of adenosine-positive C-fibres innervating arthritic joints, nor does it reduce the weight-bearing allodynia associated with adjuvant arthritis. Paradoxically, dipyridamole, which increases extracellular adenosine levels, is anti- rather than pro-nociceptive in adjuvant arthritic rats. These findings support the hypothesis that

adenosine excites rat knee joint nociceptors, but they do not support an algogenic role for endogenous adenosine in inflammatory pain induced by FCA in the rat knee joint.

The C-fibre excitation induced by adenosine appears to be mediated by adenosine A₁ receptors because it was antagonised by the A₁ receptor selective antagonist, DPCPX, and mimicked by the A₁ receptor selective agonists CPA and GR79236. In contrast, CGS21680, a selective agonist for the adenosine A_{2A} receptor subtype, did not excite nociceptors innervating the rat knee joint. These findings are consistent with reports that the A₁ receptor is responsible for adenosine-induced pain in humans (Ahlgren & Levine, 1993; Gaspardone *et al.*, 1995; Lee & Yaksh, 1996; Pappagallo *et al.*, 1993) and for activating canine cardiac and rat pulmonary afferents *in vivo* (Dibner-Dunlap *et al.*, 1993; Hong *et al.*, 1998; Huang *et al.*, 1995). However, the results of the present investigation contrast with findings of Taiwo and Levine (1990) who reported that intra-dermal injection of A₂, but not A₁ adenosine receptor agonists caused mechanical hyperalgesia in the rat hind-paw. These authors also found that adenosine A₁ receptor activation prevented the sensitisation induced by A₂ receptor agonists or by PGE₂, and these effects were attributed to direct effects on the primary afferent nociceptor. However, the effects of adenosine receptor agonists on noxious stimulus-evoked discharge (neural correlate of hyperalgesia) were not investigated in the present study. Thus, the present data is not directly comparable with the Taiwo and Levine investigation. It also cannot be excluded that the second messenger systems activated by adenosine receptors expressed on the knee joint nociceptors differs from those stimulated in the rat hindpaw. In the rat hindpaw, increases in intracellular cAMP levels were implicated in adenosine A₂

receptor mediated hyperalgesia in the hindpaw, whereas, decreases in cAMP accounted for A₁ receptor mediated inhibition of sensitisation (Taiwo & Levine, 1990). Adenosine A₁ receptors, like bradykinin B₂ receptors, are also coupled to PKC (Fredholm *et al.*, 1996), which may have accounted for the excitation seen in the present investigation. This could be tested by pre-treating rats with a PKC inhibitor and determining whether afferent excitation by adenosine can still be elicited.

The nucleotide, ATP, evoked a biphasic increase in afferent discharge. In normal knee joints, the slow component was similar in size, latency and duration to the adenosine-evoked response, and was antagonised by DPCPX. Thus, the delayed increase was likely to be mediated by adenosine, produced by metabolism of ATP, also acting at metabotropic adenosine A₁ receptors. Interestingly, different populations of afferents were excited during the fast and slow components of the ATP-evoked excitation. As this chapter is largely concerned with adenosine's effects, the potential significance of this will be considered in the General Discussion.

The latency to onset of the slow responses evoked by adenosine and ATP did not differ significantly from that of the directly acting algogen, bradykinin, a finding compatible with adenosine having a direct mode of action on the primary afferent terminals. Excitation of joint nociceptors by adenosine and ATP could be evoked by i.art. injection of the purines, suggesting that the excitatory responses obtained were not secondary to effects on the vasculature. The evidence obtained makes it probable that the excitation of nociceptive afferents evoked by adenosine, either injected or

produced by the metabolism of ATP, resulted from a direct action on nociceptive nerve terminals.

The latency of the ATP-evoked slow response in arthritic knees was increased relative to its latency in normal joints, and with respect to the latency of the response to adenosine in arthritic joints. This might be due to changes in the activity of ATP-metabolising enzymes in the arthritic knee joints because it is known that the half-life of ATP in the synovial fluid of patients with arthritis is increased (Park *et al.*, 1996), that is, it is metabolised more slowly. This would explain why the latency to onset of the ATP response is increased in arthritic joints, but the latency to onset of the adenosine response is not.

The cellular concentration of ATP is in the millimolar range and release of the nucleotide into the extracellular space during inflammation is expected to lead to high local concentrations of both ATP and adenosine (Gordon, 1986). The dose of exogenous adenosine used in the present study (370nmol i.a.) was estimated to produce a local concentration of 0.37mM, considerably less than that which is estimated to occur *in vivo* during inflammation. Since exogenous adenosine activated most knee joint C-fibre polymodal nociceptors, it was expected that endogenous adenosine might have contributed to the elevated discharge of nociceptors innervating arthritic joints. However, DPCPX, which antagonised the excitatory effects of close-arterially injected nucleoside, did not reduce the elevated spontaneous activity of adenosine-sensitive nociceptors, suggesting that these afferents were not tonically activated by endogenous adenosine. However, as discussed in the previous chapter, the mild chronic arthritis induced by FCA may not have been sufficient to cause adequate ATP release, and hence sufficiently high

extracellular adenosine production. Consistent with the suggestion that the arthritis was too mild, it was expected that afferents innervating arthritic joints might be sensitised to the effects of the purines because substance P levels are increased in adjuvant arthritis (Ahmed *et al.*, 1995) and this tachykinin augments the adenosine-evoked excitation of canine cardiac neurones (partly mediated by the A₁ subtype, Huang *et al.*, 1995). However, the afferent responses to adenosine and ATP were unaffected by arthritis in the knee joint in the present study, possibly indicating that levels of the tachykinin were not increased in the knee joints (or that substance P does not enhance the response of rat knee joint nociceptors to adenosine). It would be valuable to assess the effect of DPCPX on spontaneous discharge during electrophysiological studies of more severely inflamed rat knee joints.

Although the electrophysiological studies do not support a causative role for endogenous adenosine (acting via the A₁ subtype) in spontaneous nociceptor discharge in the monoarthritic rat, the present study does not preclude a role for adenosine in stimulus-evoked afferent discharge. Furthermore, the nucleoside may be released in a phasic manner, be enhancing the actions of another endogenous algogen, or influencing nociceptors over a longer time scale than the 3-4 hours investigated in the present experiments. In order to study the longer term effects of endogenous adenosine, the effects of the nucleoside uptake inhibitor, dipyridamole, the adenosine A₁ receptor selective antagonist, DPCPX, and the A₁ receptor selective agonist, GR79236, were investigated during the early phase of adjuvant arthritis.

Despite the finding that adenosine excites nociceptors via A₁ receptors, DPCPX, injected either once or three times daily, did not have any analgesic effects in the hindlimb weight bearing test during the initial phase of monoarthritis. This

finding suggests that insufficient levels of the adenosine are released during the early phase, or that adenosine is not involved in causing this type of inflammatory pain. However, interpretation of the behavioural studies is complicated because the drugs were injected systemically and could have mediated their effects through peripheral, spinal and/or supraspinal sites. Adenosine is well known to cause *analgesia* following spinal administration, an effect primarily mediated by A₁ receptors and thought to contribute to opioid analgesia (see Keil, 1996; Sawynok, 1998). If spinal adenosine is tonically released during this phase of experimental arthritis, antagonism of spinal adenosine A₁ receptors could have disguised any analgesic effects of antagonising A₁ receptors on the nociceptive afferents. In order to separate the spinal and peripheral effects of A₁ receptor antagonism, it would be interesting to investigate the effect of administration of DPCPX into the joint on the development of arthritic pain. However, this would not be feasible because the effects of DPCPX last for only 4 hours (N. Clayton, personal communication) and it would be injurious to repeatedly inject the rat knee joint. Furthermore, DPCPX is usually dissolved in a solution containing the noxious agent, dimethylsulphoxide, which would have a local analgesic effect following i.art. injection. Thus, unless 1) a method of infusing the rat joint (perhaps by implanting a cannula) is developed and 2) a “non-noxious solution soluble” selective A₁ receptor antagonist is used, then it will not be possible to determine the effects of antagonising articular A₁ receptors on inflammatory pain.

In contrast to the results of the neural recordings, dipyridamole, which raises endogenous extracellular adenosine levels, was analgesic in the weight-bearing test. This effect was not observed when the rats were pre-treated with DPCPX indicating an A₁ receptor mediated analgesic effect. This analgesia could have been due the

actions of adenosine at spinal A₁ receptors. In order to examine further the effects of A₁ receptor activation, the adenosine A₁ receptor agonist, GR79236, was administered. This agonist had no effect on inflammatory pain as assessed using the weight-bearing device. Considering that A₁ receptors have consistently been shown to mediate analgesia at spinal sites in many acute and chronic pain models (Sawynok, 1998), and that i.p. dipyridamole was analgesic in the present study, it is surprising that no analgesia was observed following i.p. injection of GR79236. This is a relatively new adenosine agonist and its use *in vivo* has been limited to date. Therefore, little is known about the penetration of this agonist to spinal sites following subcutaneous injection. Thus, analgesic levels of the compound may not have been reached in the spinal cord.

The knee joint diameter was measured in order to assess the anti-inflammatory potential of the adenosine compounds. Agents that raise endogenous adenosine levels have repeatedly been shown to have anti-inflammatory effects largely thought to be due to adenosine A₂ receptor activation (Cronstein, 1994; Cronstein, 1995; Cronstein *et al.*, 1996; Firestein, 1996; Firestein *et al.*, 1994). In the present investigation, rats injected with dipyridamole, either alone or following DPCPX, tended to have less inflamed joints than the other groups although this did not reach statistical significance. Nevertheless, this suggests that adenosine accumulation may also be anti-inflammatory in this monoarthritis model, and that the A₁ subtype does not mediate this effect. It would be valuable to explore this possibility further by trying different dipyridamole injection protocols in order to generate high endogenous adenosine levels. Interestingly, GR79236 significantly reduced swelling of arthritic knee joints during the drug treatment period indicating

that it has anti-inflammatory properties in the monoarthritis model. Although the anti-inflammatory effects of adenosine are largely attributed to A₂ receptor activation (Cronstein, 1994; Cronstein *et al.*, 1996), activation of A₁ receptors is also known to have anti-inflammatory properties (it prevents neutrophil accumulation) *in vivo* (Lesch *et al.*, 1991; Schrier *et al.*, 1990). The mechanism through which GR79236 mediated its anti-inflammatory effect is not known, but it is possible that the agonist prevented neutrophil accumulation at the site of the arthritic lesion. Detailed histological studies of GR79236-treated monoarthritic rat knee joints would confirm this. Interestingly, A₁ receptors are normally associated with pro-inflammatory effects *in vitro* (Cronstein, 1994; Cronstein *et al.*, 1996). Since the present study has demonstrated an anti-inflammatory effect of GR79236, the physiological relevance of the pro-inflammatory effects of A₁ receptors is questionable.

In summary, the data presented in this chapter have shown that half of the C-fibre nociceptors innervating the rat knee joint express functional adenosine A₁ receptors. Thus, extracellular adenosine, which increases following ATP release during inflammation, could act directly on the nociceptors to exacerbate inflammatory pain. The present investigation has not explored the contribution of adenosine to stimulus-evoked afferent discharge, but as discussed in the previous chapter, ATP, and therefore adenosine, is released from cells during mechanical stimulation indicating a possible role in allodynia and/or hyperalgesia. The effects of endogenous adenosine on weight-bearing allodynia were investigated in behavioural studies, and despite the peripheral analgesic effects of adenosine, dipyridamole, which increases endogenous extracellular adenosine levels, was anti-allodynic in this test, possibly via a central mechanism of action. This suggests that the potential peripheral

algescic effects of adenosine accumulation by agents such as nucleoside transport, adenosine kinase and adenosine deaminase inhibitors is overcome probably through opposing central effects. Thus, if these agents are developed as disease-modifying anti-rheumatic drugs, they are unlikely to exacerbate the pain of RA, and may even alleviate it.

Chapter 7: The indirect role of ATP in knee joint nociception

7.1 INTRODUCTION

Interleukin-1 β (Il-1 β) is a potent pro-inflammatory cytokine that is released from a variety of cells including macrophages, mast cells, synoviocytes and fibroblasts during inflammation and which plays a key role in the pathogenesis of RA (Feldmann *et al.*, 1996). The significance of this cytokine as a pro-inflammatory mediator in RA is highlighted by the fact that agents that inhibit the effects of Il-1 β are currently in clinical trials for the treatment of the disease (see Arend & Dayer, 1995). Il-1 β is also known to have potent pro-nociceptive properties (Bianchi *et al.*, 1998) and probably contributes to the pain associated with RA. This is supported by the preliminary results of an early clinical trial showing that treatment of RA patients with interleukin receptor antagonist alleviates joint pain (Campion *et al.*, 1996). If the mechanisms underlying the release of Il-1 β during disease states were fully understood, novel therapeutic strategies for the treatment of RA could be developed.

Il-1 β is synthesised in the cytoplasm from an inactive precursor, pro-Il-1 β , by Il-1 β -converting enzyme (ICE, Cerretti *et al.*, 1992; Molineaux *et al.*, 1993; Thornberry *et al.*, 1992). One of the best characterised triggers of intracellular pro-Il-1 β accumulation *in vitro* is LPS (Giri *et al.*, 1985). In LPS treated cells, the interleukin precursor and ICE coexist in the cell and a secondary stimulus is needed to activate ICE and release active Il-1 β (Hogquist *et al.*, 1991b; Perregaux & Gabel, 1994). ATP is one of the known triggers of Il-1 β release from LPS-primed macrophages both *in vitro* (Ferrari *et al.*, 1997a; Perregaux & Gabel, 1994) and *in vivo* (Griffiths *et al.*, 1995). This effect is mediated by the P2X₇ subtype of ATP-

gated cation-channel receptors (Ferrari *et al.*, 1997a) which are expressed on a variety of inflammatory and immune cells (Collo *et al.*, 1997; Divirgilio, 1995). If this action is physiologically relevant *in vivo*, then the actions of ATP at the P2X₇ receptor could contribute significantly to inflammatory pain by releasing Il-1 β . Thus, antagonists of P2X₇ receptors could have potential for the treatment of RA.

This study tested the hypothesis that ATP indirectly excites and/or sensitises articular nociceptors by causing Il-1 β release from LPS pre-treated cells in the rat knee joint *in vivo* via P2X₇ receptors. Intra-articular injection of Il-1 β into the knee joint has previously been shown to increase the spontaneous and bradykinin-evoked discharge of nociceptors innervating the joint (Kelly, 1998; Kelly *et al.*, 1996; Kelly *et al.*, 1997). Thus, in the present study, two P2 receptor agonists at the P2X₇ subtype, ATP γ S and BzATP were injected i.art. into the knee joint, either alone or following pre-treatment with LPS, and their effects on spontaneous and bradykinin-evoked discharge of nociceptors innervating the knee joint were recorded.

7.2 METHODS

7.2.1 AFFERENT NERVE RECORDINGS *IN VIVO*

Electrophysiological studies were carried out as described in Section 2.1.2. In brief, extracellular neural activity in the MAN innervating the left knee joint was recorded under pentobarbitone anaesthesia and data was analysed off-line.

7.2.1.1 Experimental protocols

These experiments were carried-out as a number of pilot studies to determine the appropriate dose and pre-treatment time of LPS. In most experiments, drugs were injected i.art. to the knee joint, but in a small number, they were injected i.p.

7.2.1.1.1 Intra-articular injections

Six normal male Wistar rats, and eight rats following pre-treatment with LPS (see below) were used in this section (weight range 286-342g; mean±sem 317±24g)

Normal joints:

Dose-response data for bradykinin-evoked excitation of the knee joint nociceptors following close-arterial injection (i.a.) was obtained. The P2 agonists ATPγS (900nmol, i.art.) and BzATP (700nmol, i.art.) were then injected i.art. and the spontaneous discharge over the following 30 minutes was recorded. 30-45 minutes post-injection of the nucleotides, the bradykinin injections were repeated.

LPS pre-treated joints:

In four joints LPS (0.01-1μg, i.art.) was injected 2 hours prior to injection of the P2 receptor agonists during the neural recordings. The rest of the experiment was performed as described for normal joints. In four other rats, LPS (1-5μg, i.art.) was injected the day before the neural recording, 24 hours prior to the nucleotide injections.

7.2.1.1.2 Intra-peritoneal injections

In a seven male Wistar rats (weight range 298-310g; mean±sem 306±4g), LPS (10mg kg⁻¹) was injected i.p. 2 hours before ATP (9000nmol, i.p.). Spontaneous discharge was recorded throughout the experiment.

7.3 RESULTS

7.3.1 AFFERENT NERVE RECORDINGS *IN VIVO*

7.3.1.1 Intra-articular injections

Neural discharge was recorded from a total of 23 single afferent fibres innervating six normal knee joints, 11 afferents innervating four knee joints before and/or 2 hours after i.art injection of lipopolysaccharide (LPS, 0.01 – 1µg) and 16 afferents innervating four knee joints 24 hours after pre-treatment with LPS (1 – 5µg i.art.). The fibres were classified as C-fibre polymodal nociceptors or Aδ-fibre mechanoreceptors as described in Section 4.3.1.1. Of the afferents recorded from, 42% (28/66 afferents) were classified as C-fibre polymodal nociceptors and 58% (38/66) were classified as Aδ-fibre mechanoreceptors.

7.3.1.1.1 Effect of LPS pre-treatment on neural discharge

The effect of LPS pre-treatment alone was analysed to determine what effect the endotoxin had on the discharge of knee joint afferents. No difference was detected between the results obtained with the different doses of LPS and therefore, data were pooled for each dose.

7.3.1.1.1.1 Spontaneous discharge

The effect of LPS (0.01 – 1µg i.art., 2 hours pre-treatment) on the spontaneous discharge of 11 afferents was investigated in 4 experiments. Of the afferents recorded, only 2 of 9 C-fibres (22%) and 1 out of 2 Aδ-fibres (50%) were spontaneously active before LPS. After injection of LPS (2 hours post-injection), no additional nociceptors were recruited. The two C-fibres, but not the Aδ-fibre,

continued to display basal activity (Figure 7.1a). It was not possible to determine whether LPS had any significant effect on the neural discharge because so few nociceptors were spontaneously active (Figure 7.1b).

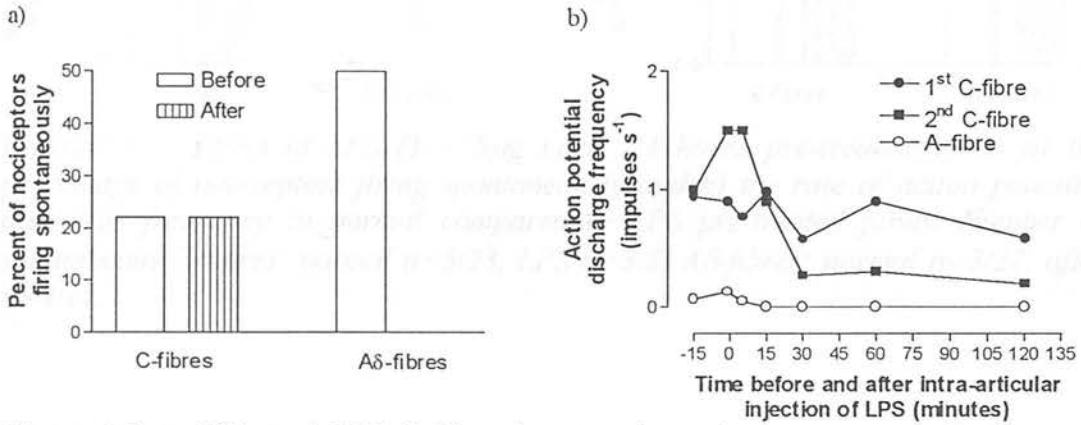


Figure 7.1 Effect of LPS (0.01 – 1µg i.art.) on the spontaneous discharge of nociceptors. Part a) shows the percentage of nociceptors firing spontaneously before and 2 hours after LPS (C-fibres: before and after n=2/9; Aδ-fibres: before n=1/2, after n=0/2). Part b) shows the rate of action potential discharge of the two C-fibres and the Aδ-fibre before and over the 2 hours following injection of LPS.

In four animals LPS (1 – 5µg) was injected i.art. into the left knee joint under transient halothane anaesthesia 24 hours before the neural experiment. To determine whether the LPS pre-treatment significantly affected the basal discharge of afferents innervating these joints, the results obtained were compared with those from afferents innervating the 10 normal knee joints investigated in this section. A greater proportion of afferents discharged spontaneously after LPS (3/5 C-fibres and 4/11 Aδ-fibres) compared to normal knee joints (5/23 C-fibres and 3/27 Aδ-fibres) but there was no significant difference in the rate of afferent firing (Figure 7.2).

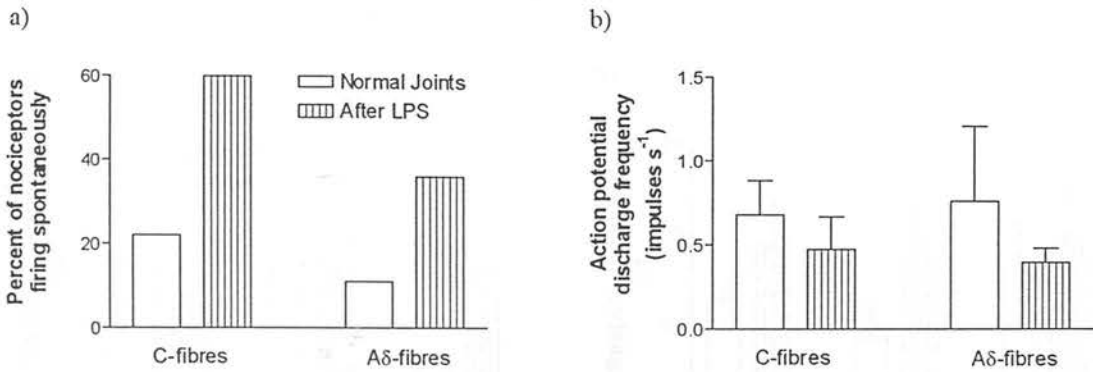


Figure 7.2 Effect of LPS ($1 - 5\mu\text{g}$ i.art., 24 hours pre-treatment) on a) the percentage of nociceptors firing spontaneously and b) the rate of action potential discharge frequency in normal compared to LPS pre-treated joints. Number of spontaneous C-fibres: normal $n=5/23$, LPS $n=3/5$; A δ -fibres: normal $n=3/27$, after $n=4/11$.

7.3.1.1.1.2 Bradykinin-evoked neural discharge

Five of the 11 C-fibres recorded before and after LPS ($0.01 - 1\mu\text{g}$ i.art., 2 hours pre-treatment) were activated by bradykinin (3, 10 and 30nmol i.a.). The effect of LPS on the response evoked by *each individual dose* of bradykinin was investigated in these afferents. Ideally, it would have been more appropriate to investigate the effect of LPS on the bradykinin ED_{50} for activation of the afferents, but this was not feasible because only a three-point dose-response curve was constructed.

LPS had no significant effect on the latency or duration of the response evoked by bradykinin (Figure 7.3), but both the discharge frequency and the total number of action potentials evoked by the algogen tended to be increased following LPS pre-treatment (Figure 7.4). However, only the number of action potentials evoked by the highest dose (30nmol) of bradykinin was significantly ($P < 0.05$) increased after LPS (Figure 7.4b).

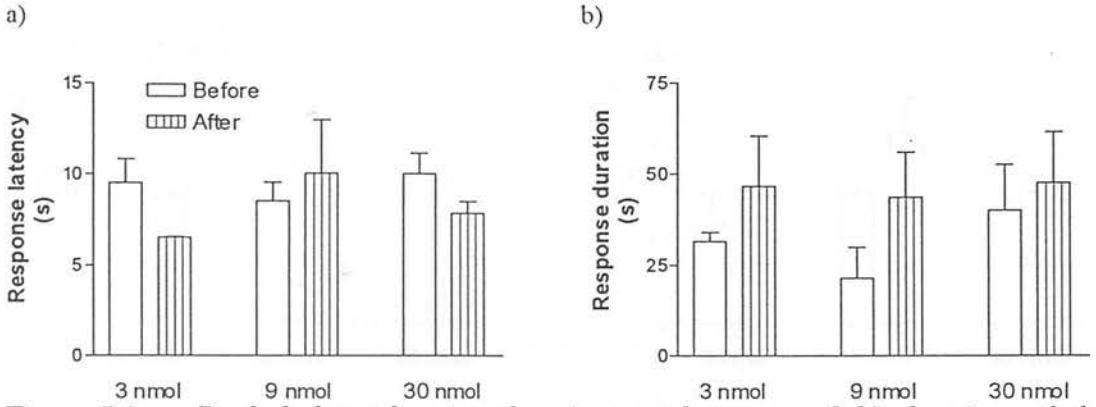


Figure 7.3 Pooled data showing the a) onset latency and b) duration of the response evoked by bradykinin (3, 9 and 30nmol, i.a.) from 5 C-fibres innervating the knee joint before and after LPS (0.01 – 1 μ g, i.art., 2 hours pre-treatment). Columns represent mean \pm sem.

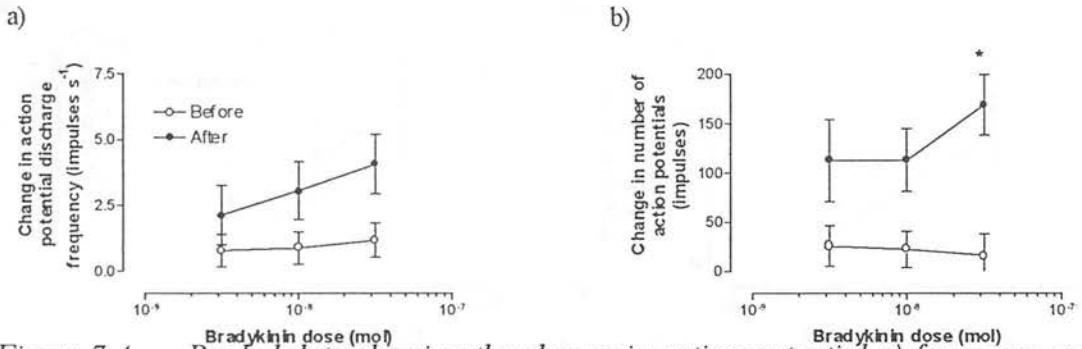


Figure 7.4 Pooled data showing the change in action potential a) frequency and b) number evoked by bradykinin (3, 9 and 30nmol, i.a.) in 5 C-fibres innervating the knee joint before and after LPS (0.01 – 1 μ g, i.art., 2 hours pre-treatment). Data points represent mean \pm sem. * $P < 0.05$ Student's paired *t*-test after compared with before.

Six (3 C-fibres and 3 A δ -fibres) of the afferents recorded from after LPS (1–5 μ g i.art., 24 hours pre-treatment) were excited by bradykinin. When compared to normal joints, the response evoked by bradykinin in LPS pre-treated joints was not significantly different with respect to latency, duration or size (Figure 7.5 and Figure 7.6).

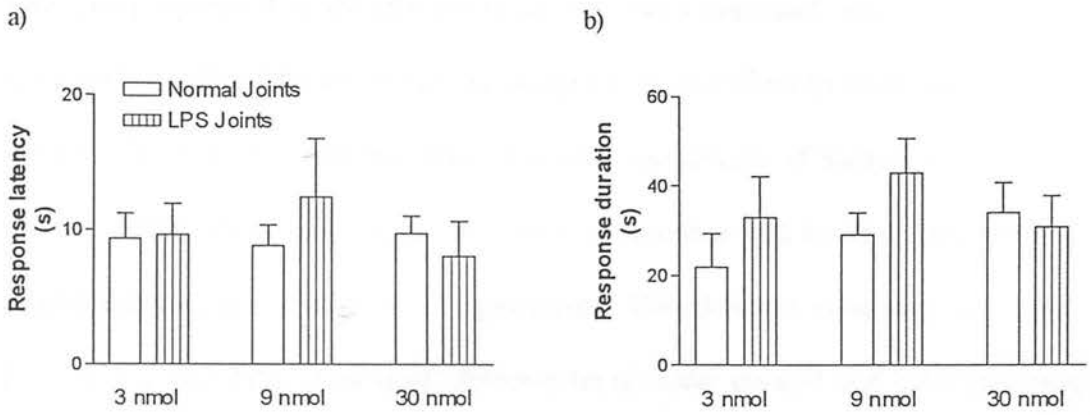


Figure 7.5 Pooled data from 3 C-fibres and 3 A δ -fibres innervating the knee joint showing the a) onset latency and b) duration of the response evoked by bradykinin (3, 9 and 30nmol, i.a.) in normal and LPS pre-treated (1 – 5 μ g, i.art., 24 hours pre-treated) joints. Columns represent mean \pm sem.

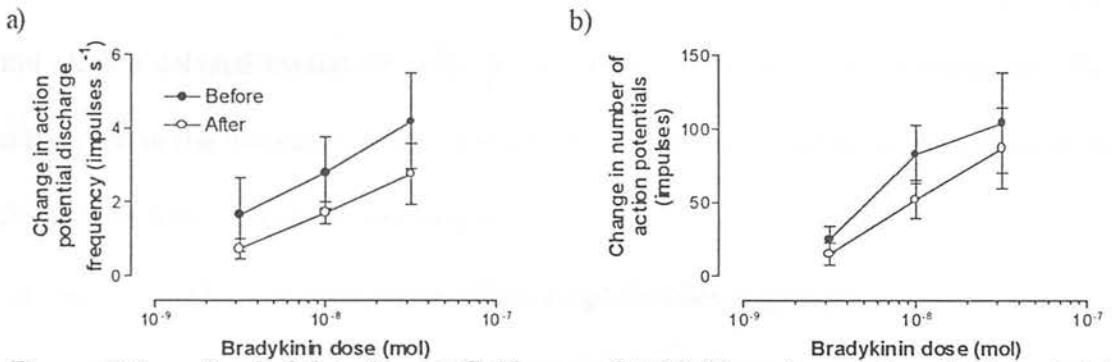


Figure 7.6 Pooled data from 3 C-fibres and 3 A δ -fibres innervating the knee joint showing the change in action potential a) frequency and b) number evoked by bradykinin (3, 10 and 30nmol, i.a.) in normal and LPS pre-treated (1 – 5 μ g, i.art., 24 hours pre-treatment) joints. Columns represent mean \pm sem.

7.3.1.1.2 Effect of ATP γ S on neural discharge

7.3.1.1.2.1 Spontaneous neural discharge

ATP γ S (900nmol, i.art.) was tested on 15 afferents (8 C-fibres and 7 A δ -fibres) innervating 4 normal knee joints and 24 afferents (11 C-fibres and 13 A δ -fibres) innervating 4 knee joints 2 hours after pre-treatment with LPS (0.01 – 1 μ g i.art.). Intra-articular injection of the agonist evoked a biphasic increase in action potential discharge consisting of a fast-onset response, apparently mediated by the P2X

subtype(s) expressed on the afferent terminals, and a sustained, slow-onset response, apparently mediated by adenosine A₁ receptors, as described in Sections 5.3.1.8 and 6.3.1.8. The fast response has been discussed previously (Chapter 5) and not be considered further in this chapter, but the slow response will be described because it caused difficulties in the present experiments. This delayed excitation was seen in 33% (5/15) and 71% (17/24) of afferents innervating normal and LPS pre-treated joints respectively. Fibres that were slowly activated following i.art. injection of the nucleotide were also slowly activated by i.a. administration of adenosine (370nmol) or ATP (2000nmol, data not shown). Similarly, fibres from which i.art. ATP γ S did not elicit a delayed excitation, were not activated by i.a. injection of adenosine. No difference in the discharge of C-fibres and A δ -fibres was evident, so data from both fibre types were pooled for this section.

Discharge of afferents from which ATP γ S evoked a slow response:

In normal joints, the increase in action potential discharge in afferents from which ATP γ S evoked a slow response (2 C-fibres and 3 A δ -fibres) was greatest at 1 minute post-injection and declined over the following 20 minutes but did not return to pre-injection levels (Figure 7.7a). After pre-treatment with LPS, a similar pattern was observed in 10 C-fibres and 7 A δ -fibres, but the increase in frequency remained elevated for longer (Figure 7.7a). However, this augmentation did not reach statistical significance when the data was expressed as a cumulative change from the pre-injection discharge frequency (Figure 7.7b). Because evidence from earlier experiments suggested that the slow response evoked by ATP γ S was mediated by adenosine acting at adenosine A₁ receptors, the A₁ receptor selective antagonist DPCPX (3 – 6 μ mol, i.a., 10 minutes pre-treatment) was used to try to block the

sustained increase. However, the antagonist had no significant effect on the elevated rate of action potential discharge following ATP γ S injection in LPS pre-treated knees (Figure 7.7). DPCPX was not studied in normal joints.

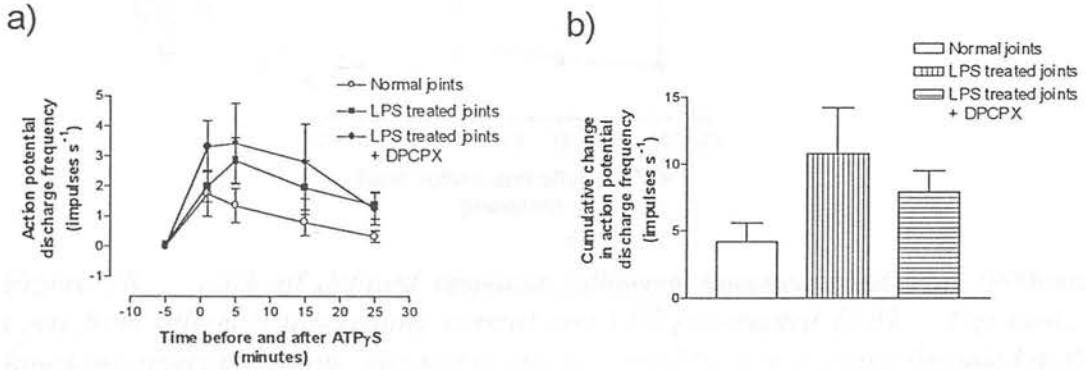


Figure 7.7 The delayed response evoked by ATP γ S (900nmol, i.art) from afferents innervating normal and LPS pre-treated (0.01 – 1 μ g i.art., 2 hours pre-injection) joints. Part a) shows the discharge frequency up to 25 minutes post-injection, and part b) shows the cumulative change from the pre-injection frequency. The lack of effect of the adenosine A₁ receptor antagonist DPCPX (3 – 6 μ mol, i.a. 10 minutes pre-treatment) on the delayed increase in afferent discharge is shown. The fast response evoked by ATP γ S is not included in this graph. Data points/columns represent mean \pm sem. Normal joints: n=5 afferents; LPS treated joints: without DPXPX n=7afferents, with DPCPX n=10 afferents.

Discharge of afferents from which ATP γ S did not evoke a slow response:

ATP γ S did not evoke a slow response from 10 afferents (6 C-fibres and 4 A δ -fibres) innervating normal joints and 7 afferents (2 C-fibres and 5 A δ -fibres) innervating LPS pre-treated joints. In these afferents, no effect on basal discharge was observed following injection of ATP γ S when measured over 25 minutes (Figure 7.8).

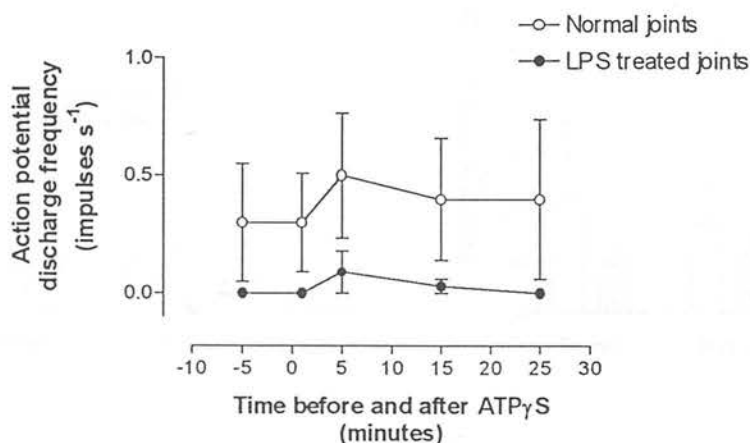


Figure 7.8 Lack of delayed response following injection of ATP γ S (900nmol, i.art) from afferents innervating normal and LPS pre-treated (0.01 – 1 μ g i.art., 2 hours pre-injection) joints. The fast response evoked by ATP γ S is not included in this graph. Data points represent mean \pm sem. Normal joints: n=10 afferents; LPS treated joints: without n=7 afferents.

7.3.1.1.2.2 Bradykinin-evoked neural discharge

The effect of a single bolus i.art. injection of ATP γ S (900nmol, 30 minutes pre-treatment) on the response evoked by bradykinin (3, 9 and 30nmol, i.a.) was investigated in 7 afferents (6 C-fibres and 1 A δ -fibre) innervating 4 normal knee joints and 8 afferents (7 C-fibres and 1 A δ -fibre) innervating 4 knee joints 2 hours after i.art. injection of LPS (0.01 – 1 μ g). ATP γ S had no significant effect on the latency, duration, or size of the response evoked by bradykinin either in normal or LPS pre-treated joints (Figure 7.9 and Figure 7.10).

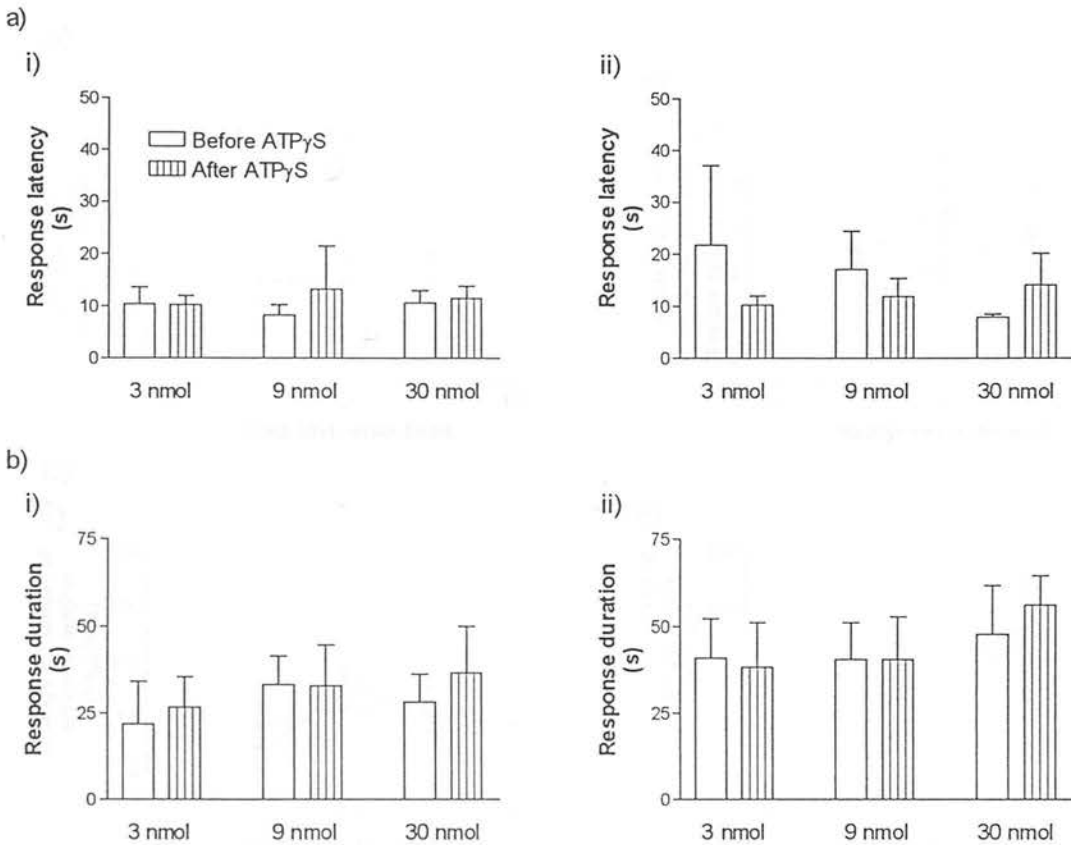


Figure 7.9 Pooled data showing the a) onset latency and b) duration of the response evoked by bradykinin (3, 9 and 30nmol, i.a.) before and after injection of ATP γ S (900nmol i.art, 30 minutes pre-treatment) from nociceptors innervating normal (i) and LPS injected (0.01 – 1 μ g, i.art., 2 hours pre-treatment) knee joints (ii). Columns represent mean \pm sem. Normal joints n=7 afferents; LPS pre-treated joints n=8.

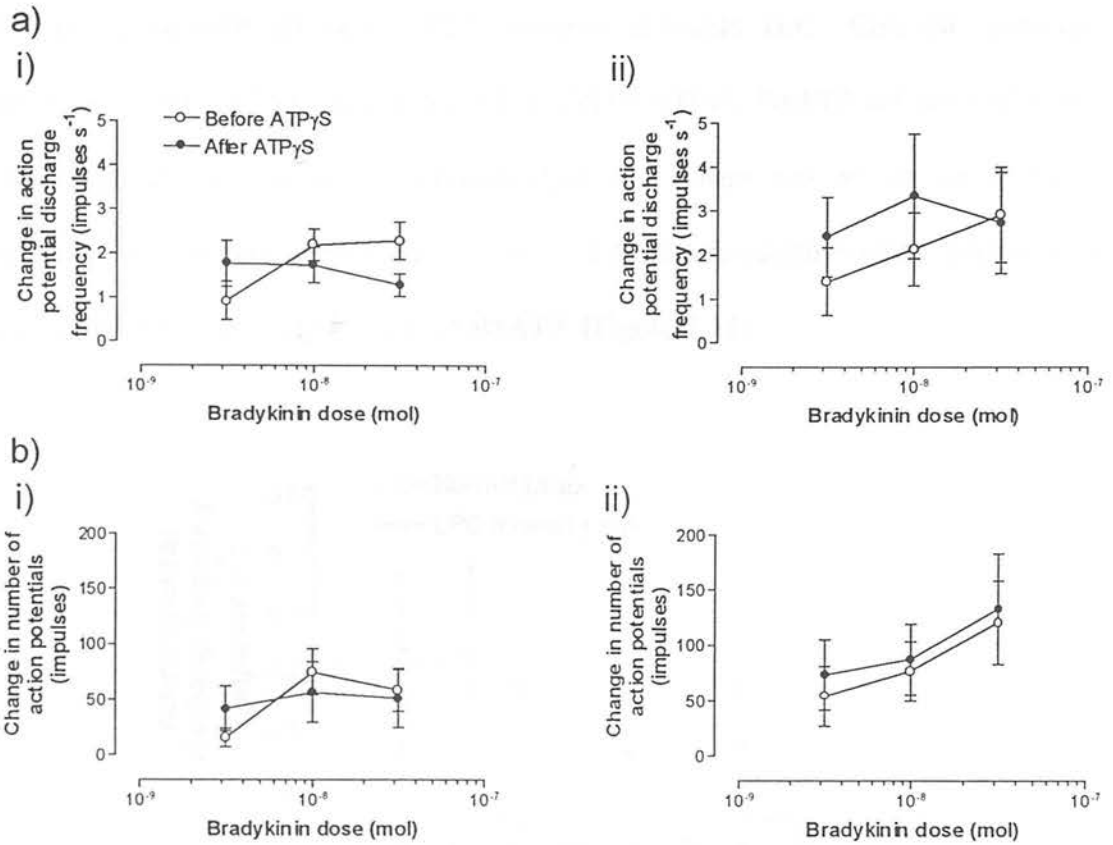


Figure 7.10 Pooled data showing the change in action potential a) discharge frequency and b) number evoked by bradykinin (3, 9 and 30nmol, i.a.) before and after injection of ATP γ S (900nmol i.art, 30 minutes pre-treatment) from nociceptors innervating normal (i) and LPS injected (0.01 – 1 μ g, i.art., 2 hours pre-treatment) knee joints (ii). Data points represent mean \pm sem. Normal joints n=7 afferents; LPS pre-treated joints n=8.

7.3.1.1.3 Effect of BzATP on neural discharge

7.3.1.1.3.1 Spontaneous neural discharge

BzATP (700nmol, i.art.) was tested on 7 afferents (2 C-fibres and 5 A δ -fibres) innervating two normal knee joints and 10 afferents (3 C-fibres and 7 A δ -fibres) innervating three knee joints 24 hours after pre-treatment with LPS (1 – 5 μ g i.art.). Intra-articular injection of the agonist evoked a fast-onset increase in action potential discharge, which was probably mediated by the afferent P2X receptor(s) because it is

known to activate all seven P2X receptor channels (I.C. Chessell, personal communication, and see Section 6.3.1.10). Unlike ATP γ S, BzATP did not evoke any slow-onset increase in action potential discharge. There was no change in basal discharge of afferents innervating normal or LPS pre-treated joints when studied over a 25-minute period after injection of BzATP (Figure 7.11).

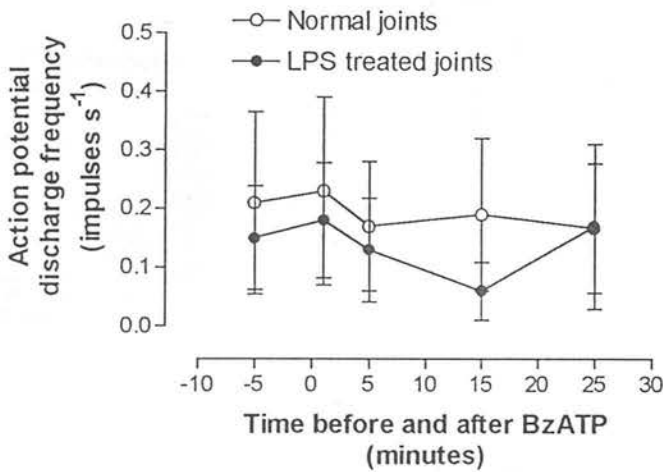


Figure 7.11 Lack of effect of BzATP (700nmol, i.art.) on the spontaneous discharge of afferents innervating normal and LPS pre-treated joints (1 – 5 μ g i.art., 24 hours pre-injection). The fast response evoked by BzATP is not included in this graph. Data points represent mean \pm sem. Normal joints n=7 afferents, LPS joints n=10 afferents.

7.3.1.1.3.2 Bradykinin-evoked neural discharge

The effect of BzATP (700nmol i.art., 30 minutes pre-treatment) on the response evoked by bradykinin (3, 9 and 30nmol, i.a.) was investigated in 2 afferents (C-fibres) innervating two normal knee joints and 6 afferents (3 C-fibres and 3 A δ -fibres) innervating three knee joints 24 hours after an i.art injection of LPS (1 – 5nmol). BzATP had no significant effect on the latency or duration (Figure 7.12) of the response evoked by bradykinin either in normal or LPS pre-treated joints.

However, there was a tendency for bradykinin to evoke a smaller response both in terms of the change in action potential discharge frequency and number after BzATP in LPS pre-treated joints (Figure 7.13). But only the number of action potentials evoked by the middle dose (9nmol) of bradykinin in LPS joints was significantly ($P<0.05$) reduced after BzATP (Figure 7.13).

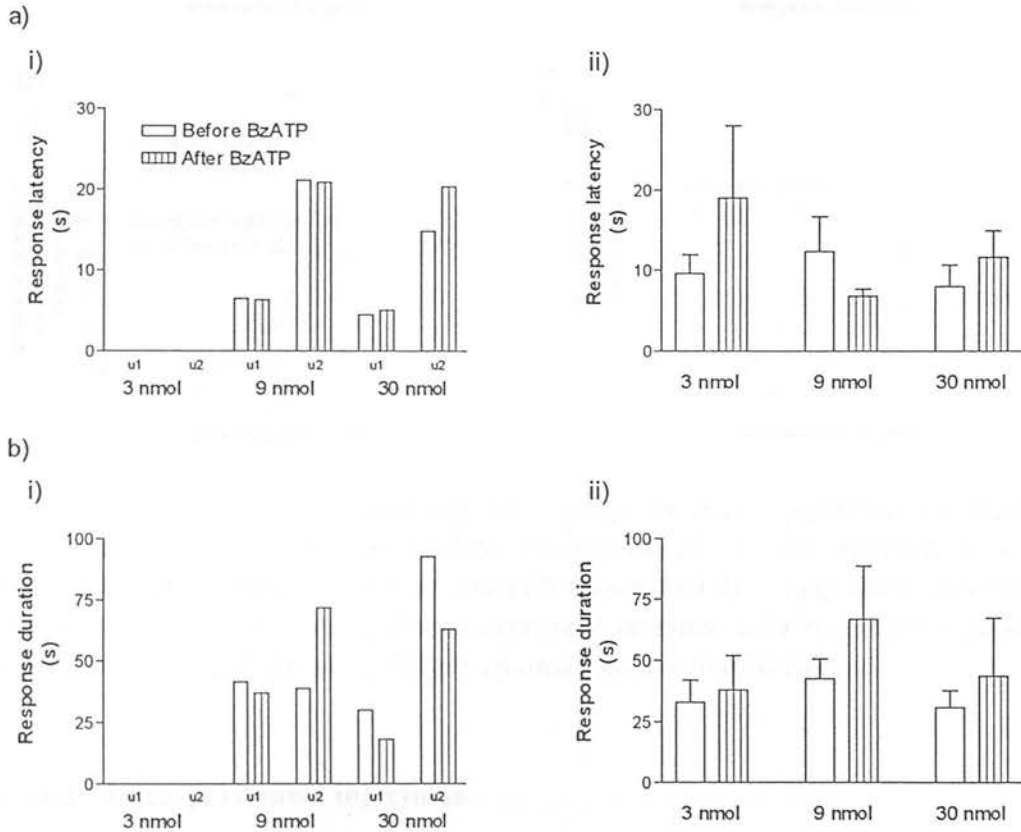


Figure 7.12 Bar charts showing the a) onset latency and b) duration of the response evoked by bradykinin (3, 9 and 30nmol, i.a.) from nociceptors before and after injection of BzATP (700nmol i.art., 30 minutes pre-treatment) innervating normal (i) and LPS injected (0.01 – 1 μ g, i.art., 24 hours pre-treatment) knee joints (ii). Columns represent original data (i) or mean \pm sem (ii). Normal joints n=2 afferents; LPS pre-treated joints n=6 afferents.

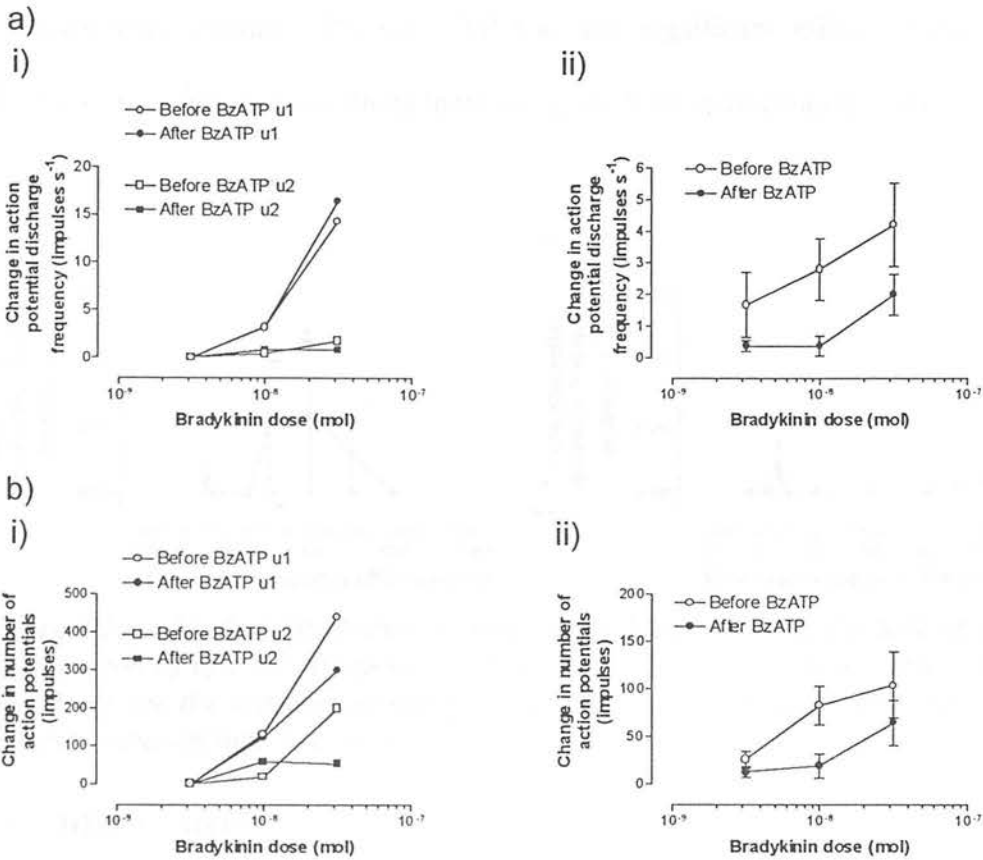


Figure 7.13 Pooled data showing the change in action potential a) discharge frequency and b) number evoked by bradykinin (3, 9 and 30nmol, i.a.) from nociceptors innervating normal (i) and LPS injected (0.01 – 1 μ g, i.art., 2 hours pre-treatment) knee joints (ii). Columns represent original data (i) or mean \pm sem (ii). Normal joints n=2 afferents; LPS pre-treated joints n=6 afferents.

7.3.1.2 Intra-peritoneal injections

Neural discharge was recorded from a total of 32 single afferent fibres innervating 7 knee joints: 75% (24/32) were classified as C-fibre polymodal nociceptors and 25% (8/32) were classified as A δ -fibre mechanoreceptors.

7.3.1.2.1 LPS on spontaneous discharge

The effect of i.p. injection of ATP (9000nmol) on the spontaneous discharge of 11 C-fibres and 5 A δ -fibres 2 hours after injection of LPS (10mg kg⁻¹) was investigated in

3 experiments. Neither LPS nor ATP had any significant effect on the rate of discharge of C-fibres or A δ -fibres innervating the knee joint (Figure 7.14).

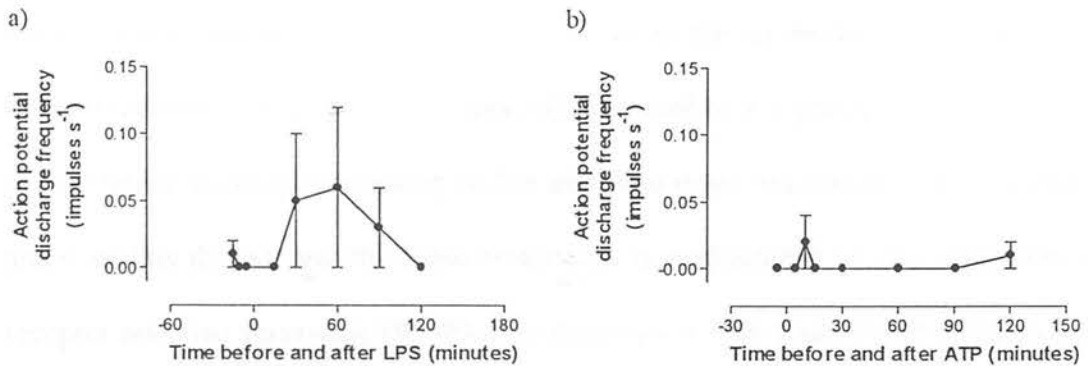


Figure 7.14 Pooled data from C-fibres and A δ -fibres showing the lack of effect of i.p. injection of a) LPS (10mg kg⁻¹) and b) ATP 9000nmol (2 hours after LPS pre-treatment) on the rate on spontaneous discharge of afferents. Data is from 11 afferents innervating 3 knee joints.

7.4 DISCUSSION

Intra-articular injections of ATP γ S and BzATP, compounds that are agonists at P2X₇ receptors, did not significantly increase either the basal or the bradykinin-evoked discharge of nociceptors innervating the normal or LPS pre-treated joint that did not respond to adenosine. However, injection of ATP γ S, but not BzATP led to a prolonged increase in discharge of afferents that were excited by adenosine. The response peaked within minutes of the injection and slowly declined over the next 30 minutes. This pattern of activity does not correspond to that previously observed following injection of Il-1 β into the knee (Kelly, 1998) and so it is unlikely to involve release of this cytokine. These findings do not support the hypothesis that activation of P2X₇ receptors in the knee joint leads to the release of Il-1 β *in vivo*.

The main problem experienced during this investigation was the lack of stable and selective P2X₇ receptor agonists. ATP γ S is an agonist at many of the P2X

and P2Y receptor subtypes and although it was originally thought to be resistant to ecto-nucleotidase hydrolysis, it is now known to be metabolised in certain tissues (Casalheira & Sebastião, 1992). Furthermore, the nucleotide seems to be rapidly metabolised to adenosine following i.a. injection in the rat because, analogous with the endogenous ligand at P2 receptors, ATP, it evokes a biphasic increase in knee joint afferent discharge consisting of fast and slow-onset excitations (see Chapters 5 and 6 of this thesis), and the latter component is antagonised by the adenosine A₁ receptor selective antagonist DPCPX (see Section 6.3.1.9). Thus, ATP_γS can also act at P1 receptor subtypes following its degradation to adenosine. The other P2 agonist used in this study, BzATP, is also unstable *in vivo* and is not selective for the P2X₇ receptor subtype (I.C. Chessell, personal communication). Interestingly, BzATP does not evoke a slow-onset increase in afferent discharge, indicating that its metabolite is not an agonist at the adenosine A₁ receptors expressed on the knee joint afferent terminals. Despite these obstacles, the fact that both of these agonists evoked a rapid-onset increase in action potential discharge, probably mediated by the P2X subtype(s) expressed on the afferent terminals (see Section 5.1.1.9), indicates that they were active as parent compounds before metabolism and therefore would have had time to act at P2X₇ receptors.

ATP_γS has previously been shown to evoke the release of Il-1 β from LPS-primed but not normal murine peritoneal macrophages *in vivo* (Griffiths *et al.*, 1995). Release of sufficient Il-1 β would be expected to increase the activity of nociceptors (Fukuoka *et al.*, 1994; Kelly, 1998; Kelly *et al.*, 1996; Kelly *et al.*, 1997). In the present study, injection of ATP_γS led to a sustained slow-onset increase in action potential discharge in 33% of nociceptors innervating normal joints and 71% of

nociceptors innervating LPS pre-treated joints. Initially these results suggested that the nucleotide might be releasing some algogenic mediator such as Il-1 β , but further studies indicated that this increase in discharge was probably mediated by adenosine because 1) adenosine itself causes a sustained increase in discharge when injected into the knee joint (see Section 6.3.1.8) and 2) ATP γ S causes a biphasic increase in action potential discharge following close-arterial injection, the slow component of which is antagonised by DPCPX (see Section 6.3.1.9). In this thesis, the duration of the knee joint afferent discharge evoked by purines has consistently been longer following i.art. injection in comparison with i.a. injection, probably because of different clearance rates from the terminals. The other result indicating that the sustained increase in discharge following i.art. injection of ATP γ S did not result from Il-1 β release was the unexpected pattern of the elevated activity. In the work by Kelly (1998), the basal discharge increases within minutes after injection of Il-1 β into the knee joint and continues to rise for up to one hour post-injection. In the present study, the elevated discharge peaked within the first minute or so following injection and thereafter, declined for the remainder of the recording time. The pattern of discharge observed following injection of ATP γ S is consistent with the slow removal of an excitatory substance rather than the release of a pro-inflammatory and pro-nociceptive mediator.

The delayed excitation observed following injection of ATP γ S was a considerable complication in the present studies because it would have masked any increase in discharge due to Il-1 β release. DPCPX was used to try to block this excitation but it failed to do so. This is probably because of the relatively high local concentration of adenosine in the knee joint compared to the antagonist. DPCPX is a

competitive antagonist at adenosine A₁ receptors (von der Leyen *et al.*, 1989) and high concentrations of agonist would displace the antagonist from the receptors.

Because the delayed increase in discharge evoked by ATP γ S could not be antagonised by DPCPX, the only way to reveal whether the P2 agonist could have caused Il-1 β release was to examine the discharge of afferents from which the nucleotide did not evoke a slow response. However, no increase in basal discharge or enhancement of bradykinin-induced excitation was observed in these fibres. BzATP would not have masked any increase secondary to the release of inflammatory mediators because this agonist did not cause any sustained increase in action potential discharge. However, there was no indication that this ATP analogue released Il-1 β or any other algogenic mediator.

Overall, the data obtained do not support the hypothesis that activation of P2X₇ receptors in the knee joint leads to Il-1 β release. Since ATP and ATP γ S have previously been shown to release Il-1 β from LPS-primed macrophages *in vivo* (Griffiths *et al.*, 1995), it was rather surprising that an Il-1 β induced increase in afferent discharge was not seen in the present study. A range of LPS doses (0.01-1 μ g) were injected 2 hours before injection of the P2 agonists. This correlates with the doses and pre-treatment times used by Griffiths *et al.* (1995) where 1 μ g of the endotoxin was administered i.p. two hours before injection of the purine nucleotides. The doses of P2 agonists used in the present study were also based on those previously reported to release Il-1 β from LPS-primed macrophages *in vivo*. Thus, in the mouse peritoneal macrophage study, ATP γ S was injected at concentrations up to 10mM (Griffiths *et al.*, 1995). In the present investigation, the nucleotide probably achieved a synovial fluid concentration of 9mM following i.art. injection (see

APPENDIX II). Interestingly, the dose-response relationship for ATP-evoked release of Il-1 β from human macrophages *in vitro* is bell shaped (Ferrari *et al.*, 1997a). Thus, higher ATP concentrations (5mM) release less Il-1 β , whereas lower concentrations (1mM) release more of the cytokine. Although it is not possible to extrapolate from *in vitro* studies of isolated human macrophages to *in vivo* studies of the rat knee joint, this finding indicates that a wider dose range of the purine nucleotides should be investigated in future studies.

The probable explanation for the lack of enhanced spontaneous and bradykinin-evoked discharge seen in our studies is that insufficient Il-1 β was released to alter the responsiveness of nociceptors innervating the joint. In the study by Griffiths *et al.* (1995), nanogram quantities of the cytokine were released from LPS-primed peritoneal macrophages. This level of Il-1 β is adequate to increase the spontaneous discharge of cutaneous afferents in the rat hindpaw (100pg-1 μ g used by Fukuoka *et al.*, 1994), and to induce spontaneous discharge and bradykinin sensitisation of nociceptors innervating the rat knee joint *in vivo* (1-100ng used by Kelly, 1998). However, it is unlikely that the levels of cytokine release measured by Griffiths *et al.* (1995) were achieved in the present study because of the different resident macrophage populations in the peritoneal cavity and the synovial space. The peritoneal cavity has high numbers of resident macrophages which can easily be detected in lavage fluid (De Halleux *et al.*, 1973), whereas normal synovial fluid is acellular. In contrast, the synovial fluid of RA joints is enriched with cells, including neutrophils and macrophages and furthermore, the RA synovium has high numbers of macrophages in the cellular infiltrate known as pannus (Feldmann *et al.*, 1996). Thus, the lack of Il-1 β release in our experiments may be because of low numbers of

resident articular macrophages. It would be valuable to repeat the present experimental protocols using the monoarthritic rat knee joint as pronounced cellular infiltration occurs in the synovial layer of the knee joint in this model (see Section 4.3.3).

Despite the findings of the present study, ATP (probably via the P2X₇ subtype) is a potent trigger of Il-1 β processing and release from inflammatory cells *in vivo* (Griffiths *et al.*, 1995) and *in vitro* (Ferrari *et al.*, 1997a; Ferrari *et al.*, 1997b; Perregaux & Gabel, 1994). The methods used in the present study may not be appropriate for determining the role of macrophage P2X₇ receptors in nociception because of the low number of resident macrophages in the normal knee joint. It is also difficult to selectively investigate P2X₇ receptor activation *in vivo* because of the lack of stable and selective agonists for this subtype. The only known antagonist with selectivity for P2X₇ receptors is oxidised ATP, but this antagonist is unsuitable for use *in vivo* because it requires long (2 hour) incubation times in the presence of the receptors (Murgia *et al.*, 1993). Whether or not P2X₇ receptors play a role in inflammation and inflammatory pain will only be revealed if a potent, selective P2X₇ receptor antagonist suitable for use *in vivo* is developed and tested in animal models. Alternatively, the development of inflammation and arthritic states could be investigated in genetically modified rodents that do not express the P2X₇ receptor. In addition, the levels of Il-1 β could be measured in the synovial fluid of normal and arthritic P2X₇ knockouts to investigate the contribution of this receptor subtype to Il-1 β release *in vivo*. Future studies should reveal whether release of Il-1 β by ATP's actions at P2X₇ receptors contributes significantly to the pain of RA or other inflammatory diseases. If so, antagonists at the P2X₇ receptor would have potential,

not only for the treatment of arthritic pain, but also as disease modifying anti-rheumatic drugs by preventing the pro-inflammatory effects of IL-1 β .

Chapter 8: General Discussion

The results obtained in this thesis have shown that distinct populations of nociceptive and probable proprioceptive afferents innervating the rat knee joint express functional P2X receptor subtype(s) and/or A₁ receptors, for ATP and its metabolite adenosine, respectively. In contrast, no indication that functional P2X receptors are expressed on nociceptors innervating the cat or rat cornea was found. These findings support the hypothesis that nociceptors innervating the rat knee joint can be directly excited by ATP and adenosine, but they do not support a direct role for these purines in activating corneal nociceptors. The indirect excitatory effects of ATP were also investigated in the rat knee joint, but no evidence that the purine releases pro-nociceptive mediators from synovial cells was obtained. The findings of this thesis contribute to the understanding of how extracellular purines modulate nociceptor activity.

The direct-excitatory role of ATP and adenosine in nociception was largely investigated through electrophysiological recordings of the rat knee joint. Neural recordings from MAN afferents innervating this joint showed that most of them express P2X and/or A₁ receptor subtypes. Close-arterial injection of ATP, which was rapidly metabolised to adenosine, revealed that there were substantial differences in purine receptor expression between C-fibre nociceptors and A δ -fibres, which immediately suggested different sensory roles for the purines (see Figure 6.6).

Because the majority (approximately 70%) of C-fibre nociceptors were slowly excited following ATP injection (attributed to the action of adenosine), this suggested a direct analgesic role for adenosine, a proposal which is supported by human studies (Bertolet *et al.*, 1996; Bleehen & Keele, 1977; Gaspardone *et al.*,

1995; Sylven *et al.*, 1986). Interestingly, previous authors have reported a peripheral anti-nociceptive role for adenosine acting at the A_1 subtype in the rat hind-paw (Karlsten *et al.*, 1992; Sawynok *et al.*, 1998; Taiwo & Levine, 1990), and this has led to the suggestion that i.art. injections of adenosine might be analgesic (Keil & Salter, 1996). However, it is well known that nociceptors innervating different tissues have different functional properties (Walters, 1996), and there is no reason to assume that identical sensory mechanisms exist in the rodent hindpaw and knee joint. Remarkably, very few (approximately 10%) $A\delta$ -fibres were slowly excited by ATP, which largely indicates a largely C-fibre nociceptor-specific role for the nucleoside. The excitation of C-fibre nociceptors by adenosine was not prone to desensitisation because when it, ATP γ S or ATP (both degraded to adenosine) were injected into the rat knee, the elevation in discharge lasted for up to 25 minutes (for example, see Figure 7.7). This indicates that the adenosine receptors on the peripheral terminals of knee joint nociceptors are slowly-adapting in the continued presence of agonist, a property that would be important in signalling the pain associated with chronically inflamed tissues. Moreover, prolonged activation of neuropeptide-containing C-fibre terminals could contribute to neurogenic inflammation. Thus, based on the evidence obtained in this thesis, it is reasonable to speculate that extracellular adenosine, resulting from the metabolism of ATP, plays a significant role in the initiation and maintenance of inflammatory pain.

In contrast to the population of afferents excited by adenosine, ATP activated the majority of $A\delta$ -fibres (approximately 70% in normal joints), and almost half of the C-fibres (approximately 40%) innervating the knee joint (mediated by P2X receptor subtype(s)), indicating that the nucleotide may play nociceptive and

proprioceptive roles. Whereas the A₁ receptor-mediated excitation lasted for over 20 minutes following i.art. injection, that mediated by P2X receptor(s) only lasted for seconds. This adapting response would not be very advantageous in terms of signalling chronic inflammatory pain. However, this does not preclude a role for P2X receptors in sensitising the nociceptive afferent or contributing to neurogenic inflammation. As discussed in Chapter 5, P2X receptors might be involved in signalling noxious and/or innocuous joint movements. This was not investigated in the present thesis because the neural preparation was not set-up to allow the joint to be moved. Therefore, this proposal is largely based on circumstantial evidence: the proposed structure for P2X receptors resembles that of mechanosensitive ion-channels (Surprenant *et al.*, 1995); ATP is released from cells that are mechanically stimulated (Nakamura & Strittmatter, 1996), and the thinly myelinated afferents innervating the cat knee joint are activated by joint movements (Coggeshall *et al.*, 1983).

The role of ATP and adenosine in inflammatory pain was investigated using electrophysiological and behavioural techniques. The antagonists, PPADS and DPCPX, blocked the effects of exogenous ATP and adenosine respectively, but did not lower the spontaneous discharge afferents innervating arthritic joints. This indicates that endogenous extracellular purine levels were too low to excite the afferents and argues against a role for the purines in contributing to the spontaneous pain associated with chronic adjuvant arthritis. However, as described in Chapter 4, the arthritis induced was mild, and sufficient ATP might not have been released from synovial cells during the chronic phase. Furthermore, the effects of antagonists on stimulus-induced afferent discharge, the neural correlate of allodynia and/or

hyperalgesia, were not examined. In the behavioural studies, the effects of pharmacological agents on the development of joint pain was examined. It was not possible to investigate the role of ATP however, because of the lack of suitable antagonists for use *in vivo*. Interestingly, there was no indication that systemic injection of DPCPX had any anti-allodynic effect, but paradoxically, dipyridamole, which prevents adenosine uptake, had. Interpretation of the behavioural studies is complicated by concurrent peripheral, spinal and central effects, which can be opposing. Nevertheless, the findings indicate that increasing the levels of endogenous extracellular adenosine causes analgesia, perhaps through activation of spinal adenosine A₁ receptors (Sawynok, 1998) because the effect was not seen when DPCPX was injected prior to dipyridamole.

The direct excitatory effects of ATP were also investigated using electrophysiological techniques in the cat cornea, and behavioural techniques in the rat eye. In contrast to the knee joint, there was no evidence that functional P2X receptors are expressed on nociceptors innervating the cat or rat cornea, despite finding that P2X₃ immunoreactivity was present in trigeminal ganglion cells retrogradely labelled from the mouse cornea. This may reflect differences in nociceptor characteristics between the knee joint and the cornea, perhaps indicating that whatever functional role P2X receptors play in the knee joint is not required in the highly-specialised corneal tissue. Alternatively, the receptors that are constitutively expressed in the rat knee joint may only be expressed in the cornea under certain situations, perhaps during inflammation or following injury. However, there is the possibility that slow penetration of the nucleotides to the afferent

terminals caused receptor desensitisation and thus, the cornea may not be a suitable preparation for studying P2X receptors.

In addition to the direct excitatory effects of ATP, the indirect excitatory effects of this nucleotide were investigated by monitoring spontaneous and bradykinin-evoked discharge following administration of P2X₇ receptor agonists into the normal and LPS pre-treated knee joint. The experiments were complicated by the lack of a stable, selective P2X₇ agonist: ATP γ S and BzATP were both rapidly degraded, the former to adenosine and the latter to an unknown compound. Despite these complications, no evidence was obtained that P2X₇ receptor activation caused Il-1 β release. Previous experiments *in vivo* have shown that a single bolus i.p. injection of ATP is sufficient to release Il-1 β from LPS-primed mouse peritoneal macrophages (Griffiths *et al.*, 1995). This makes it unlikely that rapid nucleotide metabolism and insufficient exposure of the P2X₇ receptors to agonist accounted for the lack of sensitisation following ATP γ S and BzATP. The probable explanation is that the resident macrophage population in the normal rat knee joint was too low thereby not producing adequate levels of Il-1 β .

8.1 FUTURE WORK AND CONCLUSIONS

The experiments described in this thesis have answered certain questions and raised several. One of the main unanswered questions is the subtype of P2X receptor mediating the rat knee joint afferent excitation. In order to determine this, P2X subtype selective agonists or antagonists, or specific P2X subtype knock-out rats would have to be developed. The main question that will then remain is the

functional significance of the P2X receptor subtype(s) for ATP and the A₁ receptors for adenosine that are expressed on rat knee joint afferents.

It is crucial to determine whether distinct P2X subtypes are expressed on different populations of afferents. With the currently available pharmacological and genetic tools, it would only be possible to determine whether rapidly-adapting and/or slowly-adapting P2X subtypes are involved in mediating the afferent excitation. As discussed in Chapter 5, this could be achieved by careful quantification of the duration of action potential discharge following i.art. injection of nucleotides into the joint. In order to reveal whether these are expressed on, for example, nociceptive vs proprioceptive afferents, the rat knee joint preparation would have to be developed to allow the different sensory classes to be distinguished. A mechanical indentation generator, which delivers quantitative mechanical stimuli to the knee joint, has been developed in-house (Department of Preclinical Veterinary Sciences, University of Edinburgh) for use in the laboratory. This will enable future researchers to distinguish afferents responding to innocuous and noxious mechanical stimuli. However, delivering acute mechanical stimuli to the knee joint capsule has little clinical relevance and damages the tissue. Therefore, it would be preferable to use a modified version of the technique described by Schaible and Schmidt (1983a,b) to enable the joint to be moved during the neural recordings. If this was done, the hypothesis that P2X receptors are involved in signalling joint stretch could be determined by measuring the afferent discharge to innocuous and noxious movements before and after PPADS.

One of the surprising aspects of the electrophysiological investigations was that there was no indication that purines were released during chronic inflammation

or that afferents innervating arthritic joints were sensitised to exogenous ATP and adenosine. As this may have resulted from the mildness of the arthritic lesion, it is recommended that higher doses of adjuvant be used to induce arthritis in future studies. It could then be determined whether PPADS and/or DPCPX could reduce the elevated basal discharge in afferents innervating these joints. Furthermore, the role of P2X and/or A₁ receptors in causing the enhanced response of articular afferents to movement during inflammation (Schaible & Schmidt, 1985) could also be determined. Once P2X subtype selective antagonists are developed that are suitable for use *in vivo*, the effect of P2X subtypes on spontaneous pain, allodynia and hyperalgesia could be determined in behavioural studies of monoarthritic rats.

Until these studies are completed, it will not be possible to conclude that the direct actions of ATP and/or adenosine on the nociceptive afferent terminal play a role in causing the inflammatory pain associated with chronic FCA-induced arthritis in the rat knee joint. Regarding the indirect effects of ATP that were preliminarily investigated in this thesis, P2X₇ receptor knock-out mice have become available at GIAP and it will be interesting to monitor the development of arthritis in these rodents. Meanwhile, it would be valuable to repeat the experiments described in Chapter 7 using arthritic rat knee joints in order to test the hypothesis that ATP would induce sensitisation in joints with a higher synovial macrophage population.

One of the aims of this thesis was to evaluate the potential of purine receptors as targets for treating inflammatory pain. Given the widespread expression of excitatory A₁ receptors for adenosine on rat knee joint nociceptors, it is probable that endogenous nucleoside has a role to play in modulating articular nociceptor function. However, adenosine has a variety of pro-nociceptive and pro-inflammatory effects,

in addition to opposing anti-nociceptive and anti-inflammatory actions and any therapy aimed at modulating adenosine receptor function will have to take this into account. P2X receptors for ATP are also widely expressed on rat knee joint afferents, and it is probable that endogenous nucleotide has an important role in modulating afferent input from the joint to the CNS. However, because of the lack of pharmacological and genetic tools for investigating these receptors, this can not be determined yet, and it is too early to tell whether manipulation of P2X receptor pharmacology will have any clinical relevance for the treatment of pathological pain.

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Drugs and solutions used in this investigation

Drug Name	Manufacturer	Strength	Quantity
Aspirin	Parke-Davis	5 grains	100
Codeine	Parke-Davis	1/2 grain	100
Phenacetin	Parke-Davis	2 grains	100
Salicylic acid	Parke-Davis	5 grains	100
Urea	Parke-Davis	10 grains	100
Urea solution	Parke-Davis	10%	100

APPENDIX I

Drugs and solutions used in this investigation

Drug Name	Manufacturer	Strength	Quantity
Aspirin	Parke-Davis	5 grains	100
Codeine	Parke-Davis	1/2 grain	100
Phenacetin	Parke-Davis	2 grains	100
Salicylic acid	Parke-Davis	5 grains	100
Urea	Parke-Davis	10 grains	100
Urea solution	Parke-Davis	10%	100

Sources and solvents of drugs used

Abbreviation	Chemical name of drug	Supplier	Solvent for stock solution
Adenosine		Sigma	PBS
ATP	Adenosine 5' triphosphate disodium salt	Sigma	PBS
ATP γ S	adenosine 5'-O-(3-thiotriphosphate)	Sigma	PBS
$\alpha\beta$ meATP	$\alpha\beta$ -methylene ATP lithium salt	Sigma	PBS
Bradykinin		Sigma	PBS
BzATP	benzoylbenzoyl ATP	Sigma	PBS
CGS21680	2- <i>p</i> -(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride	RBI	PBS
Capsaicin	8-methyl-N-vanillyl-6-nonenamide	Sigma	Tween 80 10%, ethanol 10%, PBS
CPA	N ⁶ -cyclopentyladenosine	Sigma	PBS
DPCPX	8-cyclopentyl-1,3-dipropylxanthine	Sigma	DMSO 5%, 1M NaOH 2%, PBS
GR79236	N-[(1 <i>S</i> , <i>trans</i>)-2-hydroxypentyl] adenosine	Glaxo Wellcome	PBS
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium	Tocris	PBS
Substance P		RBI	Dissolved in distilled H ₂ O, with NaCl to 0.9% added

Saline:

100ml contained 0.9g NaCl in distilled water

PBS:

100ml contained 40.5ml 0.2M Na₂HPO₄.12H₂O in saline, 9.5ml 0.2M NaH₂PO₄.2H₂O in saline

Sources of reagents used in immunohistochemical procedures

Reagent	Supplier
P2X ₃ antiserum	Gift from GIAP
S-100 antiserum	DAKO
Normal swine serum	Harlan Serolabs
Biotinylated swine-anti-rabbit antiserum	DAKO
P2X ₃ Pre-immune antiserum	Gift from GIAP
Alkaline phosphatase ABC kit	DAKO
Horseradish peroxidase ABC kit	DAKO
Vector red	Vector
DAB	Sigma

10% Formalin:

100ml had 10ml 40% formaldehyde, 0.9g NaCl and 90ml distilled water.

Solutions used in immunohistochemical procedures

Solution	Method of preparation
Avidin Biotin Complex Buffer: <i>pH</i> 7.6	To 24ml 0.2M Tris and 38ml 0.1N HCl added distH ₂ O to 80ml; checked pH, added distH ₂ O to 100ml
DAB solution:	To 100μl frozen DAB aliquot added 100μl H ₂ O ₂ and 4.8ml DAB buffer
<i>Frozen DAB aliquot:</i>	125mg DAB in 5ml distH ₂ O. Aliquoted into 100μl volumes.
<i>DAB buffer: pH</i> 7.6	To 24ml 0.2M Tris, 38ml 0.1N HCl and 38ml distH ₂ O added 68mg imiadazole; checked pH.
<i>0.2M Tris:</i>	24.23g Trisma base in 1000ml distH ₂ O
<i>0.1N HCl:</i>	8.5ml conc. HCl in 1000ml distH ₂ O
Phosphate buffered saline: <i>pH</i> 7.3	Made from commercial tablets (OXOID). Dissolved 1 tablet in 100ml distH ₂ O. Autoclaved at 115°C for 10 minutes
Scott's Tap Water:	2g K(CO ₃) ₂ and 20g MgSO ₄ in distH ₂ O
Tris buffered saline: <i>pH</i> 7.6	Diluted Tris 10* 1/10 in normal saline; checked pH
<i>Normal saline:</i>	42.5g NaCl in 5000ml distilled water (distH ₂ O)
<i>Tris 10*:</i>	To 500ml of 0.5M Tris added N HCL to pH 7.6. Made up to 2000ml with distH ₂ O; checked pH
<i>0.5M Tris:</i>	30.25g Trisma base in 500ml distH ₂ O
<i>Normal HCl:</i>	85ml conc. HCl in 1000ml distH ₂ O
Vector Red Buffer: <i>pH</i> 8.2	25ml 0.2M Tris, 22.9ml 0.1N HCl and 32.1ml distH ₂ O; checked pH; made to 100ml with distH ₂ O.
Trypsin Digestion Solution: <i>pH</i> 7.6-7.8	100mg trypsin and 100mg CaCl in distH ₂ O

APPENDIX II

Estimated concentrations of drugs used

Estimated local concentrations of drugs
used in rat knee joint neural experiments

	Drug	Dose (nmol)	Volume distributed in (ml)	Estimated local concentration (mM)
Close-arterial injections				
	Adenosine	370	1.0	0.370
	ATP	2000	1.0	2.000
	ATP γ S	900	1.0	0.900
	$\alpha\beta$ meATP	1-600	1.0	0.001-0.600
		60	1.0	0.060
	Bradykinin	2-30	1.0	0.002-0.030
		9	1.0	0.009
	BzATP	700	1.0	0.700
	CGS21680	190	1.0	0.190
	Capsaicin	3-30	1.0	0.003-0.030
		10	1.0	0.010
	CPA	30	1.0	0.030
	GR79236	85	1.0	0.085
	Substance P	20	1.0	0.020
	Drug	Dose (nmol)	Volume distributed in (ml)	Estimated local concentration (mM)
Intra-articular injections				
	Adenosine	370	0.1	3.7
	ATP	2000	0.1	20.0
	ATP γ S	900	0.1	9.0
	$\alpha\beta$ meATP	60	0.1	0.6
	BzATP	700	0.1	7.0

APPENDIX III

Publications

286P ACTIVATION BY P2X PURINOCEPTOR AGONISTS OF SENSORY NERVES INNERVATING THE RAT KNEE JOINT

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Six different types of ATP-gated ion-channel receptors (P2X₁₋₆) are expressed in dorsal root ganglia (DRG) and ATP evokes a depolarising current when applied to isolated DRG neurones (Jahr & Jessell, 1983). These findings have led investigators to believe that P2X purinoceptors, in particular the P2X₃ subtype, which is found only in sensory ganglia, may be involved in peripheral nociception (see Burnstock & Wood, 1996). The aim of this study was to determine whether $\alpha\beta$ methylene ATP ($\alpha\beta$ meATP), a P2X agonist, can activate nociceptive DRG afferents innervating the rat knee joint *in vivo*.

Male Wistar rats (mean weight 335g, range 250-420g) were anaesthetised with pentobarbitone (60mg.kg⁻¹ i.p., supplemented hourly i.v.) and the trachea, right carotid artery, right femoral artery and vein were cannulated. Extracellular recordings were made from filaments of the medial articular nerve (MAN) innervating the left knee joint using techniques similar to those described previously for the rat ankle joint (Birrell *et al.* 1991). Drugs were applied by close intra-arterial injection into the lower abdominal aorta via the femoral cannula. Data are expressed as the mean change in action potential frequency \pm s.e.mean.

Recordings were made from 60 mechanosensitive fibres with receptive fields in the knee joint. Conduction velocities were mainly in the C-fibre range (<2 m.s⁻¹) but some Ad fibres (2-5 m.s⁻¹) were also recorded. 49% of these afferents were excited by $\alpha\beta$ meATP (6-600nmol, Table 1). The responses had a very short delay to onset (200nmol: 0.68 \pm 0.2s) and were of short duration (200nmol: 1.6 \pm 0.3s). Half of the $\alpha\beta$ meATP-sensitive afferents also responded to capsaicin (3-20nmol) with a

similar duration of response (10nmol: 1.6 \pm 0.4s) but with a longer onset latency (10nmol: 1.9 \pm 0.3s).

Table 1. Change in action potential frequency evoked by saline, capsaicin and $\alpha\beta$ meATP injected i.a. Mean \pm s.e.mean, n = 6-12.

Basal discharge (impulses.s ⁻¹)	saline (0.3ml)	capsaicin (10nmol)	$\alpha\beta$ meATP (200nmol)
0.07 \pm 0.02	0.06 \pm 0.15	28.7 \pm 8.8	7.3 \pm 2.3

Preliminary data suggests that P2X receptor antagonists may inhibit the $\alpha\beta$ meATP-evoked discharge (suramin 60mg.kg⁻¹ i.a., apparent mean agonist ED₅₀ before: 24 \pm 8 nmol; after: 267 \pm 168 nmol n=3; PPADS 10mg.kg⁻¹ i.a., apparent agonist ED₅₀ before: 13 \pm 3 nmol; after: 81 \pm 14 nmol n=4).

Our results shows that nociceptors in the rat knee joint can be excited by a P2X purinoceptor agonist. This suggests that $\alpha\beta$ meATP-sensitive P2X receptors are involved in the initiation of nociceptive input to the central nervous system which is consistent with recent finding of algescic responses to $\alpha\beta$ meATP in the conscious rat (Bland-Ward & Humphrey, 1997).

Birrell, G.J., McQueen, D.S. *et al.* (1991) *Br. J. Pharmacol.*, 101, 715-721.

Bland-Ward, P. & Humphrey, P.P.A. (1997) *Br. J. Pharmacol.* (in press).

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Jahr, C.E. & Jessell, T.M. (1983) *Nature*, 304, 730-733.

348P NOCICEPTORS OF THE CAT AND RAT CORNEA ARE NOT EXCITED BY P2X PURINOCEPTOR AGONISTS

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ATP causes pain when applied to a blister base in humans (Bleehan and Keele, 1977) and the recent finding that six of the seven known ATP-gated ion-channel receptors (P2X₁₋₆) are expressed in the trigeminal and dorsal root ganglia (Collo *et al.*, 1996) suggests that ATP may play a role in peripheral nociception. The aim of our studies was to establish electrophysiologically whether P2X purinoceptor agonists activate polymodal nociceptors in an avascular preparation, the cat cornea, and to determine if there is a behavioural correlate when the drugs are applied to the rat eye.

Five adult cats were anaesthetised with pentobarbitone (40mg.kg⁻¹ i.p., supplemented hourly i.v.) and the trachea and right saphenous vein cannulated. Extracellular recordings from filaments of mixed ciliary nerves with receptive fields in the cornea were performed as described previously (Belmonte *et al.*, 1991). Drugs were applied to the cornea for 30s via solution-soaked tissue. Data are expressed as mean change in action potential frequency \pm s.e.mean. For behavioural studies, drug solutions (5 μ l drop) were instilled into the eyes of conscious male Wistar rats (283 \pm 7g; n=4) and the number of blinks and forepaw wipes of the eye, behaviours associated with nociception, were counted in the first minute post-instillation.

Recordings were made from C-fibre polymodal nociceptors (conduction velocity 0.77 \pm 0.08ms⁻¹, basal discharge 0.20 \pm 0.50 impulses.s⁻¹, n=9) all of which were excited by a 30s jet of CO₂ (2.86 \pm 0.40 impulses.s⁻¹) and 4 of which were also excited by

capsaicin 0.1 μ M (2.48 \pm 0.89 impulses.s⁻¹). Application of α β meATP (30-100 μ M), ATP γ S (100-1000 μ M) or ATP (100 μ M) did not cause any significant change in action potential frequency (α β meATP 100 μ M; 0.20 \pm 0.04 impulses.s⁻¹). Similar results were obtained with 29 A δ fibres (conduction velocity 5.80 \pm 0.70ms⁻¹). In conscious rats, threshold concentrations of capsaicin and nicotine caused a significant blink response whereas α β meATP did not (Table 1).

Table 1. Number of blinks and wipes in the first minute post-instillation of vehicles, capsaicin, nicotine and α β meATP into the rat eye. Mean \pm s.e.mean. *P<0.01 Mann-Whitney versus vehicles. n = instillations.

	Blinks	Wipes	n
Basal (events.min ⁻¹)	2.3 \pm 0.9	0 \pm 0	4
Capsaicin vehicle (EtOH/PBS)	2.8 \pm 2.0	0 \pm 0	5
Capsaicin 10 μ M	21.1 \pm 2.8*	2.4 \pm 1.1	10
Phosphate buffered saline (PBS)	4.0 \pm 1.2	0.4 \pm 0.4	5
Nicotine 1000 μ M in PBS	13.5 \pm 1.7*	0.6 \pm 0.3	12
α β meATP 10 μ M in PBS	2.3 \pm 1.2	0 \pm 0	6
α β meATP 100 μ M in PBS	3.7 \pm 1.7	0 \pm 0	6
α β meATP 1000 μ M in PBS	5.0 \pm 1.5	0 \pm 0	6

In conclusion, nociceptors of the cornea were not excited by P2X purinoceptor agonists in our experiments. Our data suggests that functional P2X receptors are not expressed on peripheral terminals of trigeminal afferents in the cat cornea and α β meATP sensitive P2X receptors, if present, do not stimulate nociceptive responses in the rat cornea. Thus it is unlikely that P2X receptors are involved in pain sensation from the cornea of the cat or rat.

Belmonte, C., Gallar, J., Pozo, M.A. *et al.* (1991) *J. Physiol.*, 437, 709-725.
 Bleehan, T. & Keele, C.A. (1977) *Pain*, 3, 367-377.
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P2X receptor-mediated excitation of nociceptive afferents in the normal and arthritic rat knee joint

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1 We tested the hypothesis that functional P2X receptors are present on peripheral terminals of primary afferent articular nociceptors in the rat knee joint. Neural activity was recorded extracellularly from the medial articular nerve innervating the knee joint in rats anaesthetized with pentobarbitone.

2 The selective P2X receptor agonist, $\alpha\beta$ methylene ATP ($\alpha\beta$ meATP), and the endogenous ligand, ATP, caused a rapid short-lasting excitation of a sub-population of C and A δ nociceptive afferent nerves innervating normal knee joints when injected intra-arterially or intra-articularly, and this effect was antagonized by the non-selective P2 receptor antagonist PPADS.

3 Induction of a chronic (14–21 days) unilateral inflammatory arthritis of the knee joint using locally injected Freund's adjuvant neither increased or decreased responsiveness of joint nociceptors to $\alpha\beta$ meATP or ATP.

4 Our results support the hypothesis that $\alpha\beta$ meATP-sensitive P2X receptors are expressed on peripheral nociceptive afferents in the rat knee joint suggesting that they may be involved in the initiation of nociception and pain.

Keywords: ATP; P2X receptors; pain; nociception; sensory neurones; arthritis

Introduction

Adenosine 5'-triphosphate (ATP) causes pain when applied to a blister base in humans (Bleehan & Keele, 1977) and the nucleotide also depolarises rat dorsal root ganglion neurones *in vitro* (Jahr & Jessell, 1983), suggesting that it may play a role in nociception. The effects of extracellular ATP are mediated through ionotropic (P2X) and metabotropic (P2Y) receptors (Fredholm *et al.*, 1994), and seven of each have been cloned to date (for review see North & Barnard, 1997). Indirect evidence is accumulating which supports a role for ATP in the initiation of pain by acting on putative P2X receptors expressed on nociceptive afferent nerve terminals (see Burnstock & Wood, 1996).

It has been established that mRNA for six of the seven known subtypes of P2X receptor are expressed in sensory ganglia (Collo *et al.*, 1996) and the P2X₃ subtype is selectively expressed in these ganglia (Chen *et al.*, 1995; Lewis *et al.*, 1995). P2X₃ mRNA in rat dorsal root ganglia is localized to small diameter afferent neurones (Chen *et al.*, 1995) which are commonly associated with nociception. In rat trigeminal ganglia, cell bodies of nociceptors innervating the tooth pulp are immunoreactive to antibodies for this receptor, whereas non-nociceptive neurones are not (Cook *et al.*, 1997). However, the results from behavioural studies that have been performed to investigate the role of P2X receptors in nociception are conflicting. For example, the stable P2X₁ and P2X₃ receptor agonist, $\alpha\beta$ methylene ATP ($\alpha\beta$ meATP), evokes nociceptive responses when injected into the rat food pad (Bland-Ward & Humphrey, 1997), but not when it is instilled into the rat eye (Dowd *et al.*, 1997a).

Functional P2X receptors are thought to be expressed on some sensory afferent nerves in the rat because ATP and $\alpha\beta$ meATP excite vagal and chemosensory primary afferents in this species (Trezise *et al.*, 1993; Khakh *et al.*, 1995; McQueen

et al., 1997, 1998). Although there is indirect evidence that ATP excites peripheral nociceptors (Bland-Ward & Humphrey, 1997), there is little direct evidence concerning the actions of ATP on nociceptive afferents, and that which exists does not appear to support a role for $\alpha\beta$ meATP-sensitive P2X receptors in nociception. For example, in the cat, polymodal nociceptors innervating the cornea (Dowd *et al.*, 1997a), and sensory afferents innervating tooth pulp (Matthews *et al.*, 1997), were not excited by $\alpha\beta$ meATP. There is currently no direct evidence linking functional P2X receptors and peripheral nociceptive neurones in rat.

Levels of ATP are increased in inflamed or damaged tissues (Gordon, 1986) and the nucleotide is found in the synovial fluid of patients with arthritis (Ryan *et al.*, 1991; Park *et al.*, 1996). The actions of ATP on sensory neurons *in vitro* are enhanced by acidification (Li *et al.*, 1996, 1997), and a fall in synovial pH occurs in human rheumatoid arthritis (Farr *et al.*, 1985) and in experimentally-induced inflammatory arthritis (Tulamo *et al.*, 1989). Thus, locally released ATP may contribute to pain by acting on P2X receptors associated with nociceptive afferents in the joint, and this action could be enhanced in inflammatory conditions such as arthritis. Alternatively, desensitization or down-regulation of P2X receptors might result from the continued presence of ATP within the inflamed joint capsule. What effect exogenous ATP actually has on nociceptive afferents innervating chronically inflamed joints needs to be established.

The aim of our study was to test the hypothesis that functional P2X receptors are expressed on primary afferent articular nociceptors. We recorded the neural discharge of nociceptors innervating the rat knee joint and measured the response to locally injected P2 receptor agonists. We also determined whether these responses were modified in joints with adjuvant-induced arthritis, a commonly used model for human arthritis (Rainsford, 1982). A preliminary account of some of the work has been presented (Dowd *et al.*, 1997b).

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Methods

Experiments, licensed under U.K. Home Office regulations, were performed on 30 normal and 18 arthritic male Wistar rats (body weight range 240–380 g; mean \pm s.e.mean 325 ± 11 g).

Induction of arthritis

Freund's Complete Adjuvant (FCA, 0.10–0.15 ml of 1 mg ml⁻¹ *Mycobacterium tuberculosis* in paraffin oil, Sigma) was injected intra-articularly into the left knee joint of rats under halothane anaesthesia (5% in oxygen). Animals were used for electrophysiological recordings 14–21 days post-induction at which time a mild persistent unilateral arthritis was present and associated with swelling of the knee (mean increase in diameter of 30% from 1.0 ± 0.01 cm to 1.3 ± 0.03 cm, $n = 18$).

Surgical procedures

Animals were anaesthetized with pentobarbitone (60 mg kg⁻¹ i.p., supplemented hourly with 6 mg i.v. via a cannula inserted into the right femoral vein). The trachea and right carotid artery were cannulated, and respiration and arterial blood pressure were continuously monitored. Body temperature was maintained at 38°C by an automated heating blanket connected to a thermistor probe inserted into the rectum. A cannula was inserted into the lower abdominal artery through the right femoral artery for close arterial injection of drugs to the left knee joint.

Electrophysiological recordings

The left leg was fixed to a support and the skin on the medial aspect of the limb was cut to expose three branches of the medial articular nerve (MAN) where they leave the saphenous nerve. The skin flaps were used to form a trough, which was filled with heavy paraffin oil.

The saphenous nerve was cut centrally to eliminate efferent neural activity. One of the branches of the MAN was dissected from the underlying connective tissue and neural discharge from small filaments containing 1–4 afferent fibres was recorded using bipolar platinum-iridium wire electrodes as described previously for the ankle joint (Grubb *et al.*, 1991). Briefly, neural activity was recorded digitally on videotape and analysed off-line using a pulse-height voltage discriminator linked to a personal computer operating Spike 2 (CED, Cambridge) software. Single action potentials, identified by the size and shape of the spike, were counted separately. The receptive fields of the afferents were identified by probing the knee joint's capsule with a hand held plastic probe to activate fibres with mid-high mechanical thresholds. However, because ATP is released from damaged tissues, the mechanical thresholds were not systematically measured to avoid damaging or desensitizing the capsular tissue. Conduction velocities (conduction distance/action potential delay) were determined at the end of an experiment from the time taken for the action potential to reach the recording electrodes following a depolarizing stimulus applied to the receptive field using a stimulating electrode.

Drug administration

For intra-arterial (i.a.) injections, agonists were injected in a volume of 0.1 ml, washed in with 0.2 ml of saline (0.9% w/v sodium chloride), the injection being completed within 2 s.

Repeatable responses to agonists were obtained before using antagonists. The minimum interval between successive agonist doses was 20 min. Antagonist was injected i.a. over 10 s (0.1 ml 100 g⁻¹ body weight) at least 10 min before agonists were re-tested. In some cases it was necessary to administer an additional dose of antagonist if its effects were observed to be waning.

In six normal animals, drugs were administered by intra-articular (i.art.) injection to the knee joint using a 26-gauge needle inserted through the infrapatellar ligament just beneath the patella. To determine whether the presence of the needle in the knee joint influenced neural activity, spontaneous afferent discharge and that evoked by agonists was recorded prior to insertion of the needle. The needle, fitted to a syringe containing drug solution, was then inserted into the joint space and secured with a clamp. Drawing 20–30 μ l of air into the syringe after the drug solution prevented leakage of drug into the joint during the subsequent recording. Spontaneous activity and responses to agonists were then recorded. Because the rat knee joint volume is 0.15–0.20 ml, we performed only one or two injections (0.1 ml i.art.) per knee.

Data analysis

The effect of a drug or vehicle injection was determined by comparing the action potential discharge frequency following drug injection with that in the 15 s pre-injection period. Data are expressed as the mean change in action potential frequency \pm s.e.mean. Marked receptor desensitization and cardiovascular effects can occur following injection of $\alpha\beta$ meATP (McQueen *et al.*, 1997), so high doses (>200 nmol) were not routinely used. Consequently, it was difficult to establish the true maximum response to this agonist, and data from individual experiments were expressed as *apparent* ED₅₀ values. Differences between means were analysed statistically using the Mann-Whitney test and the null hypothesis rejected at the $P \geq 0.05$ level.

Drugs

Adenosine 5' triphosphate disodium salt (ATP), $\alpha\beta$ -methylene ATP lithium salt and 8-methyl-N-vanillyl-6-nonenamide (capsaicin) were purchased from Sigma and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium (PPADS) was from Tocris. All drugs were dissolved in phosphate buffered saline (PBS) except for capsaicin, which was dissolved in Tween 80 (10% v/v), ethanol (10% v/v) and PBS.

Results

Characterization of afferent fibres

Neural discharge was recorded from a total of 104 single afferent fibres from 42 knee joints. The afferents were classified as C-fibre polymodal nociceptors (61%) or A δ -mechanonociceptors (39%) based on their conduction velocities, mechanosensitivity and whether or not they were activated by the algogen, capsaicin. C-fibre polymodal nociceptors were slowly conducting (1.08 ± 0.17 ms⁻¹), excited by capsaicin (10 nmol: 24.0 ± 3.9 impulses s⁻¹), and were activated by mechanical stimulation of the joint capsule. A δ -mechanonociceptors were faster conducting (3.42 ± 0.54 ms⁻¹) and did not respond to capsaicin. Some A β -mechanoreceptors were also identified (low threshold mechanosensitive units with conduc-

tion velocities greater than 10 ms^{-1}), but these were not studied.

Comparison of basal discharge in nociceptive afferents from normal and arthritic joints

We recorded from 57 afferent fibres (57% C, 43% A δ) innervating normal knee joints and 47 (53% C, 47% A δ) innervating arthritic knee joints. Previous electrophysiological studies in the rat ankle joint have shown two characteristic features in adjuvant-induced arthritis. These are: (1) that nerves innervating arthritic joints have a higher proportion of spontaneously active afferents than those innervating normal joints (2) the rate of discharge of those afferents which are spontaneously active is higher in arthritic joints (Guilbaud *et al.*, 1985). In our experiments A δ -mechanonociceptors innervating normal knee joints ($n=23$) were always silent, and in arthritic joints only 1 of 17 (6%) A δ afferents was spontaneously active ($0.1 \text{ impulses s}^{-1}$). In contrast, 16% (5/34 afferents) of C-fibre polymodal nociceptors innervating normal knee joints had low basal levels of spontaneous activity ($0.10 \pm 0.01 \text{ impulses s}^{-1}$), whereas 32% (10/31 afferents) of those innervating arthritic joints were active and discharged at significantly higher rate ($0.39 \pm 0.01 \text{ impulses s}^{-1}$, $P < 0.05$ versus normal joints).

Histological assessment of the injected and contralateral knee joints of six rats (one normal, one vehicle-treated, and four injected with adjuvant) confirmed the presence of an inflammatory lesion manifest as an inflammatory cell infiltrate, synovial proliferation, fibroplasia, oedema and new bone formation in the adjuvant-injected knees (data not shown).

Responses to $\alpha\beta\text{meATP}$ in normal and arthritic knee joints

The P2X receptor agonist, $\alpha\beta\text{meATP}$ (1-600 nmol i.a.), evoked an increase in action potential discharge frequency in 55% of C-fibre polymodal nociceptors and 65% of A δ -mechanonociceptors recorded from normal joints. Pooled data are shown because the responses evoked from both types of afferent fibre were similar. The increase in discharge following $\alpha\beta\text{meATP}$ was rapid in onset, of short duration (Figure 1) and was dose-related (Figure 2); injection of vehicle evoked no response

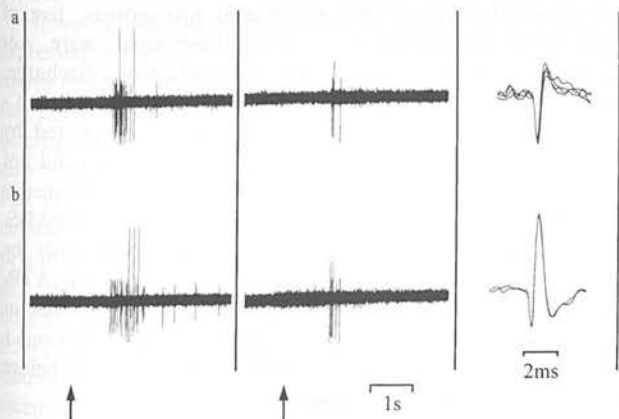


Figure 1 Oscilloscope traces showing the discharge evoked from a nerve filament innervating a normal knee joint by close-arterial injection (at arrow) of (a) $\alpha\beta\text{meATP}$ (60 nmol) and (b) ATP (2000 nmol). The first panel shows the control responses and the second panel shows the responses 10–30 min after injection of the P2 purinoceptor antagonist, PPADS ($16 \mu\text{mol kg}^{-1}$ i.a.). The third panel shows the two action potentials that were counted.

(PBS 0.1 ml: $0.01 \pm 0.01 \text{ impulses s}^{-1}$). Injection of higher doses of $\alpha\beta\text{meATP}$, or repeating lower doses at short (<15 min) intervals, caused tachyphylaxis, i.e. a loss of responsiveness to previously effective doses (but not to mechanically-induced excitation) which lasted for about 20–30 min.

$\alpha\beta\text{meATP}$ also evoked an increase in action potential discharge in 46% of C-fibres and 89% of A δ -fibres recorded from arthritic joints. Adjuvant-induced arthritis did not affect the mean apparent ED_{50} for excitation of the afferents caused by $\alpha\beta\text{meATP}$ (normal: $31 \pm 12 \text{ nmol}$, $n=9$; arthritic: $57 \pm 24 \text{ nmol}$, $n=5$). Similarly, as shown in Figure 3, the magnitude, latency to onset, and duration of the response evoked by a single mid-range dose of $\alpha\beta\text{ATP}$ (60 nmol i.a.) did not differ significantly between normal and arthritic joints.

Responses to ATP in normal and arthritic knee joints

ATP, the endogenous ligand for P2X receptors, also evoked a rapid short-lasting increase in action potential discharge in 51% of C-fibre polymodal nociceptors and 81% of A δ -mechanonociceptors on which it was tested in normal joints, and in 43% of C-fibres and 88% of A δ -fibres in arthritic joints. Adjuvant-induced arthritis of the knee joint did not affect the magnitude, latency to onset, or duration of the response evoked by a single high dose of ATP (2000 nmol; see Figure 3). During experiments in which ATP and $\alpha\beta\text{meATP}$ were both tested ($n=58$ afferents), fibres which were excited by ATP also invariably responded to $\alpha\beta\text{meATP}$ and there was no significant difference between the responses evoked by the two nucleotides with respect to response amplitude, onset latency and duration (Figure 3). Seventeen of the fibres that responded to both $\alpha\beta\text{meATP}$ and ATP were C-fibre polymodal nociceptors that also responded to capsaicin. The latency to onset of responses to $\alpha\beta\text{meATP}$ and ATP did not differ significantly from that to capsaicin in these fibres ($\alpha\beta\text{meATP}$ 60 nmol: $0.8 \pm 0.1 \text{ s}$; ATP 2000 nmol: $0.6 \pm 0.3 \text{ s}$; capsaicin 10 nmol: $1.1 \pm 0.2 \text{ s}$). ATP also evoked a delayed increase in discharge which may be due to activation of adenosine receptors following metabolism of ATP by ectonucleotidase to adenosine (data not shown).

Effect of $\alpha\beta\text{meATP}$ and ATP injected i.art. into the knee joint

To confirm that we were investigating responses in joint afferents, we injected $\alpha\beta\text{meATP}$ and ATP directly into the knee. Injection of either substance evoked a transient increase in action potential discharge, whereas i.art. injection of the same volume of vehicle had no effect ($\alpha\beta\text{meATP}$ 60 nmol: $4.4 \pm 1.0 \text{ impulses s}^{-1}$; ATP 2000 nmol: $9.9 \pm 2 \text{ impulses s}^{-1}$;

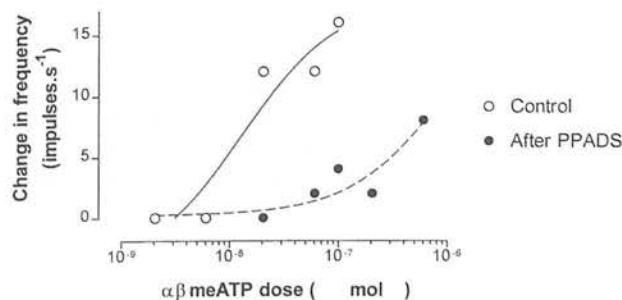


Figure 2 Dose-related increase in afferent discharge evoked by close-arterial injection of $\alpha\beta\text{meATP}$ in a single C-fibre innervating a normal knee joint before and after PPADS ($16 \mu\text{mol kg}^{-1}$ i.a.).

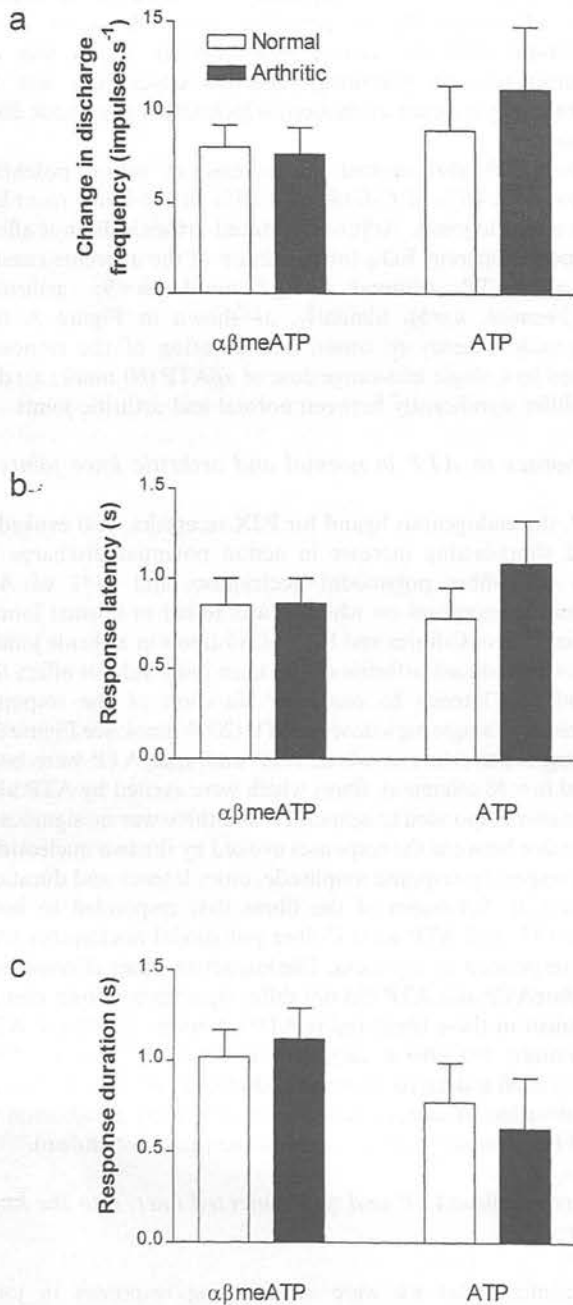


Figure 3 Summary of (a) increase in nociceptive discharge (b) latency to onset of the excitation and (c) duration of the response following i.a. injection of either $\alpha\beta$ meATP (60 nmol) or ATP (2000 nmol). Data is shown for normal ($\alpha\beta$ meATP: $n=14$; ATP $n=7$) or chronically arthritic joints ($\alpha\beta$ meATP: $n=15$; ATP $n=7$). There was no significant difference with either agonist between the responses obtained from normal and arthritic joints.

saline (PBS) 0.1 ml: 0.01 ± 0.02 impulses s^{-1} ; $n=3$ for each). The presence of the needle in the knee joint for the duration of the recording had no significant effect on the spontaneous discharge of the afferents (control: 0.01 ± 0.02 impulses s^{-1} ; after insertion of needle: 0.01 ± 0.03 impulses s^{-1} , $n=9$ units).

Effects of PPADS on the nociceptive responses to $\alpha\beta$ meATP and ATP

The increase in discharge evoked by $\alpha\beta$ meATP (1-600 nmol i.a.) in normal and arthritic joints was antagonized by the P2X receptor antagonist, PPADS (16 μ mol kg^{-1} , i.a.) as illustrated

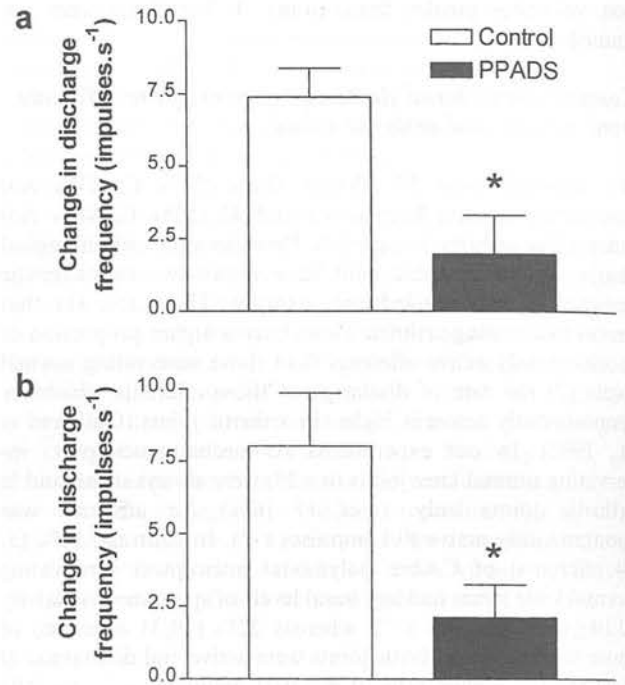


Figure 4 Response evoked by close-arterial injection of (a) $\alpha\beta$ meATP (60 nmol) and (b) ATP (2000 nmol) before and after PPADS (16 μ mol kg^{-1} i.a., 10 min pre-treatment). Pooled data from afferents innervating normal and arthritic knee joints ($\alpha\beta$ meATP: $n=27$ afferents before, $n=11$ afferents after; ATP: $n=10$ afferents before, $n=6$ afferents after). Columns represent mean \pm s.e.mean. * $P < 0.05$ compared to control responses, Mann-Whitney test.

in Figure 1, Figure 2 and Figure 4. The mean apparent ED_{50} for $\alpha\beta$ meATP was 31 ± 12 nmol ($n=9$) before and 258 ± 84 nmol ($n=4$) after PPADS ($P < 0.01$). PPADS also antagonized the initial response to ATP (Figure 4), without affecting the delayed ATP response (data not shown).

Effect of PPADS on spontaneous discharge from nociceptive afferents innervating arthritic joints

In order to establish whether endogenous ATP contributed to the increased basal discharge, the effect of PPADS on spontaneous discharge was examined in nine of the eighteen arthritic joints. Activity was recorded from 17 afferent fibres; nine of which were C-fibre polymodal nociceptors, five of which were excited by ATP. The other eight were A δ -mechanonociceptors which lacked any spontaneous discharge, and six of these were responsive to ATP. Four (44%) of the C-fibres were spontaneously active, and two were excited by ATP. Administration of PPADS (16 μ mol kg^{-1} , i.a.) did not reduce the firing rate of the four C-fibre afferents (frequency before PPADS: 0.5 ± 0.5 impulses s^{-1} ; 10 min after PPADS: 0.4 ± 0.4 impulses s^{-1}). However, PPADS would only be expected to influence the two spontaneously active ATP-positive fibres: the antagonist reduced the discharge in one of the recordings, but it increased it slightly in the other (unit 1 before: 0.50 impulses s^{-1} ; after 0.3 impulses s^{-1} , unit 2: before 0.3 impulses s^{-1} ; after 0.4 impulses s^{-1}).

Discussion

The main finding from this *in vivo* pharmacological study is that ATP and $\alpha\beta$ meATP both cause a rapid short-lasting excitation of a sub-population of nociceptive afferent nerve

fibres innervating the rat knee joint. Chronic adjuvant-induced arthritis did not significantly affect the responsiveness of these joint sensors to the purinoceptor agonists studied. This evidence supports the hypothesis we were testing, namely that functional P2X receptors are present on the peripheral terminals of primary afferent articular nociceptors. Activation of these purinoceptors excites nociceptive afferents in normal as well as in chronically inflamed arthritic joints.

The delay to onset of joint afferent responses to intra-arterially injected algogens gives some indication of the transduction mechanism through which these substances mediate their effects. Thus, excitation with fast onset is typically observed with algogens known to mediate their effects through specific ion channels receptors expressed on the sensory nerve terminals, whereas slow onset responses are observed with algogens acting through G-protein coupled receptors expressed on the nerve terminal (Birrell *et al.*, 1990). We found that capsaicin rapidly increased afferent discharge with a delay to onset which was similar to that observed following injections of $\alpha\beta$ meATP or ATP in the same recordings. Since capsaicin acts directly on the sensory nerve terminal via a specific vanilloid ion-channel receptor (Caterina *et al.*, 1997), it is probable that the fast responses to $\alpha\beta$ meATP and ATP are also mediated via direct actions on the afferent nerve terminals, rather than through an intermediary. We also established that injection of $\alpha\beta$ meATP or ATP directly into the knee joint evoked rapid excitation, which provides additional evidence that responses observed following i.a. injection of the agonists were not secondary to effects on the vasculature.

In the dorsal root ganglia, where the cell bodies for knee joint nociceptors are located, mRNA for six of the ATP-gated cation channel receptors (P2X₁₋₆) are expressed (Collo *et al.*, 1996). Only two of these receptors, namely the P2X₁ and P2X₃ subtypes, are sensitive to the P2X agonist, $\alpha\beta$ meATP, and the non-selective P2 antagonist, PPADS. There are currently no selective pharmacological tools available for use *in vivo* that would enable us to discriminate between responses mediated by these subtypes. However, previous studies have shown P2X₃ mRNA to be selectively expressed in sensory ganglia (Lewis *et al.*, 1995) and nociceptive afferents in the rat tooth pulp have recently been shown to possess immunoreactivity for the P2X₃, but not the P2X₁, receptor subtype (Cook *et al.*, 1997). This restricted localization of the P2X₃ receptor suggests that it may play an important role in the initiation of primary afferent depolarization.

However, the properties of homomeric P2X₃ receptors expressed in HEK293 cells are very different from those of the native receptor in isolated sensory (nodose ganglion) neurones (see Lewis *et al.*, 1995), and this had led to the suggestion that heteropolymerization of P2X receptors may occur *in vivo*. The only heteropolymer which displays the phenotype of the native P2X receptor of sensory neurones ($\alpha\beta$ meATP sensitive, slowly desensitizing and increases in affinity for ATP with decreasing pH) is the P2X_{2/3} heteropolymer (Lewis *et al.*, 1995; Stoop *et al.*, 1997). In our experiments, the excitation of joint nociceptors by $\alpha\beta$ meATP and ATP lasted for approximately 0.5–1.0 s which is longer than expected if the responses were mediated by homomeric P2X₃ since responses to ATP at these receptors desensitize within milliseconds. Thus it is possible that the P2X_{2/3} heteropolymer mediated the fast excitation evoked by $\alpha\beta$ meATP and ATP in our experiments.

One of the features of the ATP-evoked response in cultured sensory neurones is an augmentation of the response with decreasing pH (Li *et al.*, 1996, 1997). This may mean that the fall in tissue pH during inflammation (see Introduction) could sensitise nociceptive terminals to ATP. However, we found the

overall response of joint nociceptors to injected $\alpha\beta$ meATP and ATP was not significantly affected by the presence of adjuvant arthritis in the knee joint. Although the pH of synovial fluid was not measured in our experiments, we consider it reasonable to assume that it fell because pH is known to decrease in rheumatoid arthritis (Farr 1985) and during synovitis associated with infectious arthritis in horses (Tulamo *et al.*, 1989). Our results suggest that the augmentation of ATP-evoked responses by the decrease in pH observed in sensory neurones *in vitro* does not occur during adjuvant-induced arthritis *in vivo*. Alternatively, pH-sensitive P2X receptors are not responsible for the excitation mediated by ATP in our experiments.

Inflammation is also associated with an increase in the level of extracellular ATP which can be released from damaged cells (Gordon, 1986), platelets (Born & Kratzer, 1984), some inflammatory cells (Di Virgilio *et al.*, 1996; Ferrari *et al.*, 1997) and perhaps the sensory nerve terminal itself (Holton, 1959). The continuous exposure of nociceptors to endogenous ATP could cause chronic desensitization, equivalent to that observed in some inflammatory cells where the P2X-mediated response is only unmasked following pre-treatment with the ATP-metabolising enzyme apyrase which metabolizes the ATP released from the cells themselves (see Di Virgilio *et al.*, 1996). However, in the chronically inflamed arthritic knee joints, the nociceptive afferents remained sensitive to the P2X receptor agonists, suggesting that either high levels of extracellular ATP are not found in the inflamed joint in this model of arthritis, or that endogenous ATP does not desensitize the afferent P2X receptor involved. Since nociceptive afferents retain their sensitivity to ATP during chronic inflammation, endogenously released ATP could contribute to the associated pain and hyperalgesia by exciting them.

The pain and hyperalgesia of adjuvant-induced arthritis is manifest as an increase in the number and rate of discharge of spontaneously active nociceptive afferents (Guilbaud *et al.*, 1985; McQueen *et al.*, 1991). In our experiments, basal discharge was higher in afferents innervating arthritic joints, but it was not possible to establish the extent to which endogenous ATP contributed to this sensitization because only a small proportion of the spontaneously active afferents were ATP-sensitive. Further studies, perhaps involving a more severe arthritis to cause greater ATP release from more severely inflamed tissues, are required. In addition, interpretation of experiments involving PPADS is complicated by the fact that, in common with other P2 receptor antagonists, it also inhibits the enzymes responsible for ATP metabolism (Khakh *et al.*, 1995). Simultaneous antagonism of P2X receptors and inhibition of ATP breakdown leads to apparent antagonist insensitivity in some preparations (Crack *et al.*, 1994). The development of selective P2X receptor antagonists, which lack this ectonucleotidase-blocking property, would better answer the question of whether endogenous ATP contributes to the sensitization of nociceptors seen in inflammatory models.

It is worth speculating as to why activation of P2X receptors excites nociceptors in joints, whereas this did not occur when $\alpha\beta$ meATP was tested electrophysiologically on cat corneal nociceptors (Dowd *et al.*, 1997a) or cat tooth pulp afferents (Matthews, 1997). Species differences could be invoked to explain the discrepancies, or fundamental differences may exist in the populations of P2X receptor subtypes associated with particular afferent nerve terminals at different sites in the body. Further studies would be needed to explore these possibilities. However, since the response elicited is dependent on the concentration of drug, the speed with which the agonists reach the receptor is important; slow penetration

may be associated with receptor desensitization, which is one possible explanation for the differences observed. We may have detected P2X-mediated excitation of joint afferents by our use of close-arterial bolus injections to deliver optimal concentrations of agonists to the receptors.

Our results support the hypothesis that functional $\alpha\beta\text{meATP}$ -sensitive P2X receptors are present on a sub-

population of peripheral nociceptive afferent nerves in the rat knee joint. ATP can excite these receptors in normal and in chronically inflamed joints. This data supports the suggestion that P2X receptors are involved in the initiation of nociception and pain and that they may represent a potential target for the development of new analgesics.

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Adenosine excites nociceptive afferents of the rat knee joint via adenosine A₁ receptors

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Adenosine causes pain in humans when injected intradermally (Gaspardone *et al.* 1995), but it is not clear whether it has a direct action on nociceptive nerve terminals. The aim of this study was to determine whether adenosine excites nociceptive afferents innervating the rat knee joint and to characterize the receptor involved.

Male Wistar rats (mean weight 305 g, range 250–355 g, $n = 10$) were anaesthetized with pentobarbitone (60 mg kg⁻¹ i.p., supplemented hourly i.v.) and the trachea, right carotid artery, right femoral artery and vein were cannulated. Extracellular recordings were made from filaments of the medial articular nerve innervating the left knee joint as described previously for the rat ankle joint (Birrell *et al.* 1990). Drugs were injected into the abdominal aorta via the femoral cannula.

Recordings were made from twenty-two mechanosensitive afferent fibres with receptive fields in the knee joint (basal activity 0.06 ± 0.02 impulses s⁻¹ (i.p.s.); mean \pm s.e.m.). Adenosine evoked an increase in increased afferent discharge in ten (46%) of the fibres (370 nmol: 2.8 ± 0.7 i.p.s.). All the adenosine-sensitive afferents were slowly conducting C-fibres (1.1 ± 0.2 m s⁻¹), which were also excited by capsaicin (10 nmol: 18.7 ± 3.4 i.p.s.) and bradykinin (9 nmol: 2.2 ± 0.8 i.p.s.). The latency of the adenosine-evoked response was not significantly different from that of bradykinin (adenosine: 9.3 ± 2.6 s; bradykinin: 10.2 ± 2.5 s; $P > 0.05$, Mann-Whitney test), and this delay is consistent with nociceptor activation via G protein coupling. The adenosine A₁ receptor agonist CPA also excited the adenosine-positive afferents (30 nmol: 1.3 ± 0.2 i.p.s., $n = 3$) and the A₁ receptor-selective antagonist DPCPX (3 μ mol kg⁻¹) antagonized adenosine-mediated excitation (370 nmol: before, 2.8 ± 0.7 i.p.s; after, 0.1 ± 0.1 i.p.s.; $P < 0.05$, Mann-Whitney).

Our results show that adenosine acts via adenosine A₁ receptors to activate a population of C-fibre nociceptors innervating the rat knee joint. Since adenosine levels are elevated in inflamed tissues, the nucleoside may contribute to the development and maintenance of pain associated with arthritis.

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Adenosine A₁ receptor-mediated excitation of nociceptive afferents innervating the normal and arthritic rat knee joint

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1 We tested the hypothesis that adenosine excites nociceptive primary afferents innervating the knee joint.

2 Neuronal recordings were made from fine nerve filaments innervating the knee joint in rats anaesthetized with pentobarbitone. Drugs were injected close-arterially (i.a.) or into the articular space (i.art.). We studied normal and chronically inflamed arthritic joints, the latter 14–21 days after a single intra-articular injection of Freund's Complete Adjuvant, performed under halothane anaesthesia.

3 Adenosine injected i.a. caused delayed (approximately 10 s) excitation of the majority of polymodal C-fibre afferents, and had similar effects when injected directly into the joint.

4 Adenosine triphosphate (ATP) had biphasic effects on discharge, a fast (<1 s) excitation was followed by a delayed increase similar to that seen with adenosine.

5 The adenosine A₁ receptor agonists N⁶-cyclopentyladenosine (CPA) and N-[(1*S*,*trans*)-2-hydroxypropyl] adenosine (GR79236) also excited the C-fibre afferents. The A₁ antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) antagonized the responses evoked by adenosine, CPA, and the delayed increase seen after ATP, indicating that excitation of the nociceptive afferents was mediated *via* adenosine A₁ receptors.

6 Adenosine and ATP evoked delayed excitatory effects of similar magnitude, regardless of whether or not the knee joint was chronically inflamed. The increased basal discharge observed in arthritic joints was unaffected by DPCPX, which implies that the increase in spontaneous activity associated with arthritis is unlikely to involve tonically released adenosine.

7 The results support the hypothesis that adenosine excites primary afferent nociceptive nerve terminals in the rat knee joint, an effect mediated by adenosine A₁ receptors. ATP, adenosine, and A₁ receptors may play a role in generating the peripheral nociceptive (pain) signal.

Keywords: Adenosine; A₁ receptors; pain; nociception; sensory neurones; arthritis

Introduction

Evidence from human and animal studies suggests that adenosine plays a complex role in the generation and modulation of peripheral nociception (Keil & Salter, 1996; Sawynok, 1998). Adenosine has been reported to cause pain in humans when it is applied to a blister base (Bleehan & Keele, 1977), injected intradermally (Pappagallo *et al.*, 1993), or injected intravenously (Gaspardone *et al.*, 1995); it is also associated with the pain of angina pectoris (Sylvén, 1993). However, others report that adenosine is anti-nociceptive in, for example, neuropathic pain (Belfrage *et al.*, 1995). Similarly, in animal studies adenosine is reported to cause both hyperalgesia and analgesia (Taiwo & Levine, 1990).

The complexity of adenosine's actions in nociception may result from activation of different adenosine receptors, four of which have been cloned (A₁, A_{2A}, A_{2B} and A₃; Collis & Hourani, 1993), and a combination of indirect actions mediated *via* inflammatory cells, and direct actions on primary afferent nociceptors. For example, adenosine acts on A₃ receptors to cause pain indirectly *via* the release of histamine and 5-hydroxytryptamine from mast cells (Sawynok *et al.*, 1997). It might also cause pain by directly exciting primary afferent nociceptors; adenosine-induced cardiac pain is considered to be mediated by a direct action on adenosine A₁ receptors located on cardiac afferents (Sylvén, 1993). This

suggestion is supported by the observation that neurones in dorsal root (sensory) ganglia, which are excited by ischaemia and thought to mediate the pain of angina, are excited by adenosine in the dog (Huang *et al.*, 1995).

A major source of extracellular adenosine is the metabolism of ATP by ectonucleotidases (Gordon, 1986). We have recently shown that ATP excites nociceptive afferents innervating the rat knee joint (Dowd *et al.*, 1998a): it evoked a biphasic excitation consisting of a rapid increase in discharge (mediated by P2X receptors) followed by a delayed excitation. The delayed increase did not occur when we used the stable ATP analogue, $\alpha\beta$ -methylene-ATP, which is resistant to ectoATPase and therefore not rapidly metabolized to adenosine. This evidence suggested that adenosine might be responsible for the delayed increase in afferent discharge that we obtained with ATP. If so, this would support the view that adenosine causes pain by exciting primary afferent nociceptors.

The present study was undertaken to test the hypothesis that adenosine excites nociceptive primary afferents innervating the rat knee joint. We used selective agonists and antagonists to characterize the type of adenosine receptor(s) involved, and also investigated whether chronic adjuvant-induced inflammatory arthritis affected the responsiveness of knee joint afferents to adenosine in anaesthetized rats. A preliminary report on this work has been published (Dowd *et al.*, 1998b).

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Methods

Experiments were licensed under U.K. Home Office regulations and performed on 11 normal and 13 arthritic male Wistar rats (mean body weight 360 g, range 270–450 g).

Induction of arthritis

Freund's Complete Adjuvant (0.10–0.15 ml of *Mycobacterium tuberculosis* 1 mg ml⁻¹ in paraffin oil, Sigma) was injected intra-articularly into the left knee joint of rats under halothane anaesthesia (5% in oxygen). Animals were used for electrophysiological recordings 14–21 days post-induction, at which time a mild persistent unilateral arthritis was present and associated with swelling (30% increase in diameter) and hyperalgesia of the knee.

Surgical procedure

Animals were anaesthetized with pentobarbitone (60 mg kg⁻¹ i.p., supplemented hourly with 6 mg i.v. via a cannula inserted into the right femoral vein). The trachea and right carotid artery were cannulated and arterial blood pressure was continuously monitored. Body temperature was maintained at 38°C by an automated heating blanket connected to a thermistor probe inserted into the rectum. A cannula was inserted into the lower abdominal artery through the right femoral artery for close arterial injection of drugs to the left knee joint.

Electrophysiological recordings

Afferent neural discharge was recorded from a branch of the medial articular nerve which was cut centrally to eliminate efferent neural activity. The methods used have been described previously (Grubb *et al.*, 1991; Dowd *et al.*, 1998a). Briefly, neural activity was recorded via bipolar extracellular wire electrodes, and recorded digitally on videotape. The neural signal was analysed off-line using a pulse-height voltage discriminator linked to a personal computer operating Spike2 software (CED, Cambridge, U.K.). Single action potentials, identified by the size and shape of the spike, were counted separately. The receptive fields of the afferents were identified by probing the knee joint capsule with a hand held plastic probe (tip diameter 1 mm; Von Frey threshold for receptor activation >40 g mm⁻²). Conduction velocities were determined at the end of experiments.

Drug administration

Agonists were injected in a volume of 0.1 ml, washed in with 0.2 ml of saline (0.9% w/v sodium chloride), the i.a. injection being completed within 2 s. Repeatable responses to agonists were obtained before using antagonists. Antagonists were injected i.a. over 10 s (in a volume of 0.1 ml 100 g⁻¹ body weight) at least 10 min before agonists were re-tested. In three normal animals, drugs were administered by intra-articular injection into the knee joint using a 26-gauge needle inserted through the infrapatellar ligament just beneath the patella.

Data analysis

The effect of a drug, or vehicle, injection was determined by comparing the action potential discharge frequency after drug injection with that in the 15 s pre-injection period. Data are expressed as the mean change in action potential frequency

± s.e.mean. Differences between means were analysed statistically using the Mann-Whitney test and the null hypothesis rejected at the 0.05 level of probability.

Drugs

Adenosine, adenosine 5'-triphosphate disodium salt (ATP), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N⁶-cyclopentyladenosine (CPA), bradykinin and 8-methyl-N-vanillyl-6-nonenamide (capsaicin) were purchased from Sigma; 2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680) from RBI, and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium (PPADS) from Tocris. N-[(1*S*,*trans*)-2-hydroxypropyl] adenosine (GR79236) was a gift from GlaxoWellcome, Stevenage, U.K. All drugs were dissolved in phosphate buffered saline (PBS) except for capsaicin, which was dissolved in Tween 80 (10% v/v), ethanol (10% v/v) and PBS, and DPCPX which was dissolved in DMSO (8% v/v), 1 M NaOH (2% v/v), and PBS.

Results

Action potentials were recorded from a total of 61 afferent nerve fibres, 25 innervating normal knee joints in 11 rats, and 41 innervating chronically inflamed joints in 13 rats; 48% of afferents innervating normal joints and 61% of those innervating arthritic joints were C-fibre polymodal nociceptors (mean conduction velocity: 1.08 ± 0.17 m s⁻¹) which were excited by capsaicin (10 nmol i.a.) and mechanical stimulation of the joint capsule. The remaining 52% of afferents innervating normal joints and 39% innervating arthritic joints were Aδ-afferents (conduction velocity: 3.43 ± 0.54 m s⁻¹) which were excited by mechanical stimulation of the joint, but not by capsaicin.

Adenosine (370 nmol) evoked an increase in action potential discharge in 80% (8/10 afferents) of C-fibres and 25% (3/12 afferents) of Aδ-fibres innervating normal knee joints. It also increased activity in 77% (17/22 afferents) of C-fibres and 27% (3/11 afferents) of Aδ-fibres innervating arthritic knee joints (Figure 1). Although the responses were similar in both fibre types, all the results presented in this paper are from C-fibres as there were too few adenosine-positive Aδ-fibres to study systematically. The magnitude, onset latency and duration of the adenosine-evoked response did not differ significantly between normal and arthritic joints (Figure 2). The drug vehicle (PBS, 0.3 ml i.a.) had no effect on discharge in adenosine-sensitive afferents (mean increase 0.01 ± 0.01 impulses s⁻¹, *n* = 6). There was no evidence of desensitization when successive doses of adenosine were administered at intervals of 15 min.

ATP (2000 nmol i.a.) evoked a biphasic increase in neural discharge consisting of a fast-onset response followed by a delayed or 'slow' excitation (see Figure 1). We have previously shown that the fast desensitizing component is mediated by P2X receptors (Dowd *et al.*, 1998a), and it will not be discussed further in this paper. The slow component was evoked in 78% (7/9 afferents) of C-fibres and 10% (1/10 afferents) of Aδ-fibres innervating normal knee joints, and 83% (19/23 afferents) of C-fibres and 19% (3/16 afferents) of Aδ-fibres innervating arthritic joints. The magnitude and duration of the slow ATP-evoked response in C-fibres was not significantly different for arthritic joints, in comparison with normal joints, but the latency to onset was significantly longer in the arthritic joints (Figure 2). There was no evidence for desensitization of the slow response to ATP.

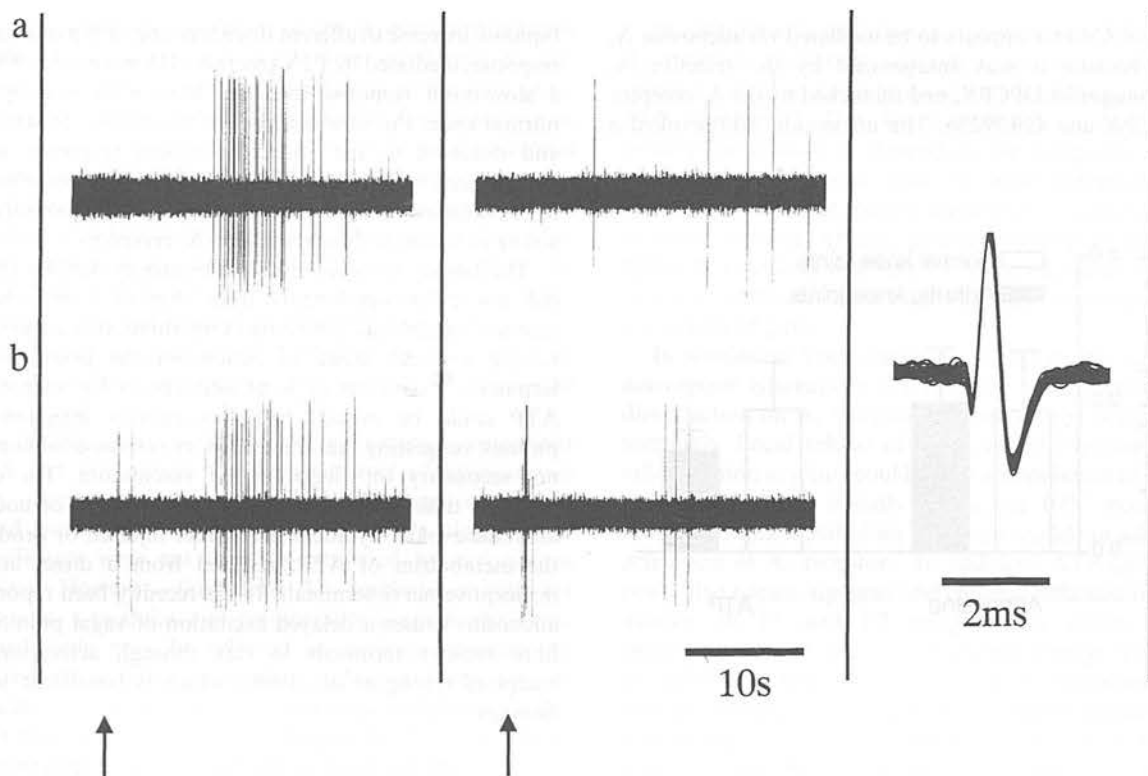


Figure 1 Oscilloscope traces showing the discharge evoked from a nerve filament innervating a normal knee joint by i.a. injection (at arrow) of (a) adenosine (370 nmol) and (b) ATP (2000 nmol). The first panel shows control responses, the second shows the responses 10–30 min after injection of DPCPX ($3 \mu\text{mol kg}^{-1}$). Note that the slow responses to adenosine and ATP are antagonized by DPCPX while the fast-onset response to ATP is not. The third panel shows the C-fibre that responded to adenosine and ATP.

In experiments during which adenosine and ATP were both tested, all the fibres that were excited by adenosine also responded with a delayed excitation to ATP (normal $n=8$ afferents; arthritic $n=22$ afferents). In normal joints there was no significant difference with respect to response magnitude, latency and duration between the responses evoked by the purines (Figure 2). However, in arthritic joints, although there was no significant difference with respect to response magnitude and duration, the latency to onset of the slow ATP response was significantly longer than that of the adenosine response (Figure 2: ATP 2000 nmol 17.8 ± 2.4 s; adenosine 370 nmol 9.1 ± 2.0 s, $P < 0.05$ Mann-Whitney).

In order to confirm that we were recording from afferents innervating the knee joint, we injected adenosine and ATP intra-articularly in three animals. Adenosine evoked a delayed increase in C-fibre afferent discharge when injected i.art., and ATP caused a biphasic increase (adenosine 370 nmol: 2.05 ± 0.59 impulses s^{-1} ; ATP (slow component) 2000 nmol: 2.2 ± 0.54 impulses s^{-1} ; PBS 0.1 ml: 0.01 ± 0.02 impulses s^{-1} ; $n=2$ for each).

We compared the latencies to onset of the slow excitation evoked by the purines with those of the directly acting algogens, bradykinin and capsaicin. In C-fibres innervating normal knee joints, the onset latencies for the purines did not differ significantly from that for bradykinin. However, they were significantly ($P < 0.05$) longer in comparison with the capsaicin response (adenosine 370 nmol: 8.8 ± 1.9 s $n=4$; ATP 2000 nmol: 9.3 ± 2.6 s $n=8$; bradykinin 9 nmol: 10.2 ± 2.5 s $n=12$; capsaicin 10 nmol: 1.1 ± 0.2 s $n=12$). The latency of the fast ATP response was similar to that of the capsaicin response (Dowd *et al.*, 1998a).

We used selective purinoceptor agonists and antagonists to characterize the purinoceptor(s) mediating the delayed

excitatory responses to adenosine and ATP. The adenosine A_1 receptor agonists, CPA (30 nmol i.a.) and GR79236 (85–285 nmol i.a.) also evoked a delayed increase in afferent discharge (CPA: see Figure 3; GR79236: 1.02 ± 0.17 impulses s^{-1} , $n=6$ afferents innervating normal joints). In contrast, the adenosine A_2 receptor agonist CGS21680 (190 nmol i.a.) had no effect on afferent discharge (normal joint: 0.01 ± 0.2 impulses s^{-1} , $n=2$; arthritic: 0.02 ± 0.1 impulses s^{-1} , $n=2$). The excitation evoked by adenosine and ATP was unaffected by the P2 receptor antagonist PPADS ($16 \mu\text{mol kg}^{-1}$ i.a.), whereas, the adenosine A_1 receptor antagonist, DPCPX ($3 \mu\text{mol kg}^{-1}$ i.a.) blocked the slow excitation associated with adenosine, ATP and CPA (Figure 3).

A significantly higher rate of spontaneous discharge was observed in C-fibre afferents innervating arthritic knee joints, in comparison with those recorded from normal joints (normal joints: 0.1 ± 0.01 impulses s^{-1} ; arthritic: 0.39 ± 0.01 impulses s^{-1} , $P < 0.05$). To determine whether endogenous adenosine contributed to this increase in basal activity, we studied the effect of DPCPX on the spontaneous discharge recorded from afferents innervating arthritic joints. DPCPX ($3 \mu\text{mol kg}^{-1}$ i.a.) did not reduce the spontaneous discharge of adenosine-positive C-fibre afferents (spontaneous discharge before: 0.4 ± 0.2 impulses s^{-1} ; after DPCPX: 0.3 ± 0.2 impulses s^{-1} , $n=6$).

Discussion

Our main finding is that adenosine excites the majority of C-fibre polymodal nociceptors innervating the rat knee joint. It also activated some $A\delta$ -fibre afferents, but detailed study of these was not feasible because of their small number. The

excitation of C-fibres appears to be mediated *via* adenosine A_1 receptors because it was antagonized by the selective A_1 receptor antagonist DPCPX, and mimicked by the A_1 receptor agonists CPA and GR79236. The nucleotide ATP evoked a

biphasic increase in afferent discharge consisting of a fast-onset response, mediated by P2X receptors (Dowd *et al.*, 1998b), and a slow-onset response from rat knee joint nociceptors. In normal knees the slow component was similar in size, latency and duration to the adenosine-evoked response, and was antagonized by DPCPX. Thus, the delayed increase was likely to be mediated by adenosine, produced by metabolism of ATP, acting at metabotropic adenosine A_1 receptors.

The latency to onset of the responses evoked by adenosine did not differ significantly from that of a directly acting algenon, bradykinin, a finding compatible with adenosine also having a direct mode of action on the primary afferent terminals. Excitation of joint nociceptors by adenosine and ATP could be evoked by intra-articular injection of the purines, suggesting that the excitatory responses obtained were not secondary to effects on the vasculature. The evidence obtained makes it probable that the excitation of nociceptive afferents evoked by adenosine, either injected or produced by the metabolism of ATP, resulted from a direct action on nociceptive nerve terminals. It has recently been reported that adenosine causes a delayed excitation of vagal pulmonary C-fibre sensory terminals in rats through activation of A_1 receptors (Hong *et al.*, 1998), which is consistent with our findings.

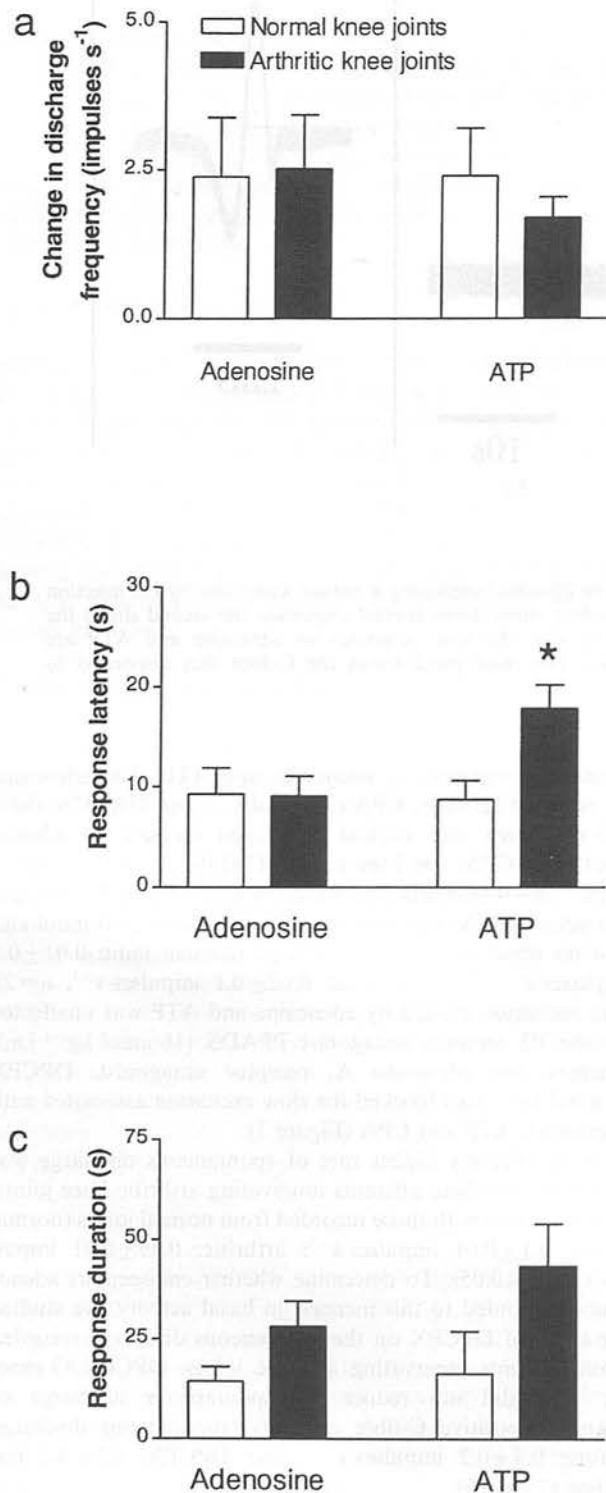


Figure 2 Summary of (a) amplitude (b) latency to onset and (c) duration of the response evoked in C-fibre afferents following i.a. injection of either adenosine (370 nmol) or ATP (2000 nmol). Data shown is from C-fibres innervating normal (adenosine: $n=8$; ATP $n=4$) or chronically arthritic (adenosine: $n=10$; ATP $n=13$) joints. Columns represent mean \pm s.e.mean. * $P < 0.05$ ATP response latency in arthritic joints compared to (i) ATP response latency in normal joints and (ii) compared to adenosine response latency in arthritic joints.

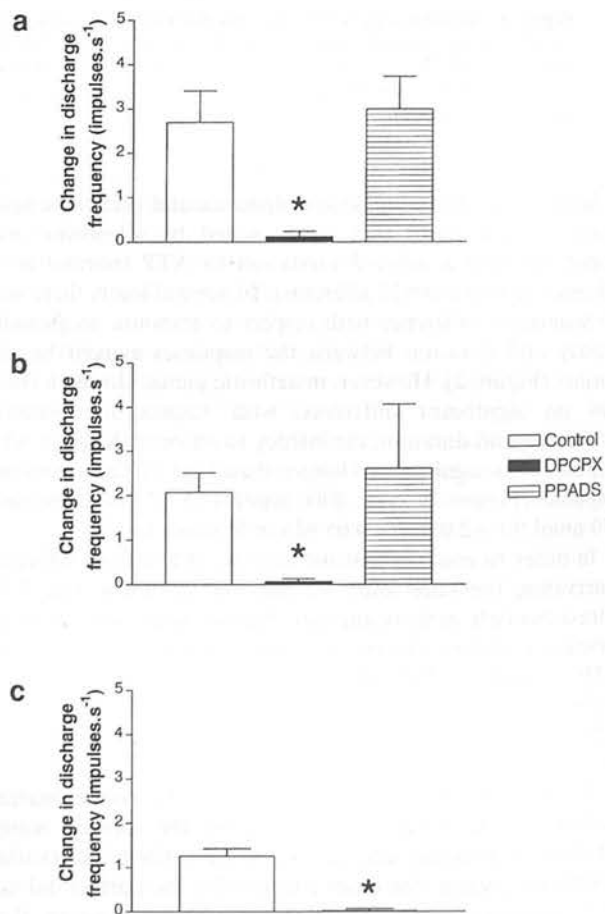


Figure 3 Slow-onset excitation evoked by intra-articular injection of (a) adenosine (370 nmol), (b) ATP (2000 nmol) and (c) CPA (30 nmol) before and after either DPCPX ($3 \mu\text{mol kg}^{-1}$) or PPADS ($16 \mu\text{mol kg}^{-1}$). Pooled data from C-fibres innervating normal and arthritic knee joints (adenosine: control $n=16$, after DPCPX $n=4$, after PPADS $n=4$; ATP: control $n=12$, after DPCPX $n=3$, after PPADS $n=5$; CPA: control $n=8$, after DPCPX $n=5$). Columns represent mean \pm s.e.mean. * $P < 0.05$ compared to control responses, Mann-Whitney test.

The magnitude and duration of the A₁-mediated afferent response to adenosine and ATP were unaffected by the presence of mild adjuvant-induced arthritis in the knee joint. However, the latency of the ATP-evoked slow response in arthritic knees was increased relative to its latency in normal joints, and also with respect to the latency for the response to adenosine in arthritic joints. This might be due to changes in the activity of ATP-metabolising enzymes in the arthritic knee joints because it is known that the half-life of ATP in the synovial fluid of patients with arthritis is increased (Park *et al.*, 1996), that is, it is metabolized more slowly. This could explain why the latency to onset of the ATP response is increased in arthritic joints but the latency to onset of the adenosine response is not.

The finding that the adenosine A₁ receptor antagonist DPCPX did not reduce the elevated level of spontaneous activity observed in adenosine-sensitive nociceptive afferents recorded from chronically arthritic knee joints suggests that these afferents were not tonically activated by endogenous adenosine. However, this does not preclude a role for adenosine as a modulator of peripheral nociceptor sensitivity. The nucleoside may be released in a phasic manner, be enhancing the actions of another endogenous algogen, or influencing nociceptors over a longer time scale than the 3–4 hours that we were able to investigate in our experiments. Adenosine might also be important in the development of joint hyperalgesia, something our experiments on chronically inflamed joints did not address.

In contrast with this algogenic effect of peripheral adenosine A₁ receptor activation, activation of spinal adenosine A₁ receptors is associated with antinociception (see Sawynok, 1998 for review). Thus the overall role of A₁ receptors in nociception is likely to depend on the comparative release of adenosine in the spinal cord, the local concentration in the periphery and on the relative number of A₁ receptors. Because of these opposing effects, careful targeting of peripheral or spinal A₁ receptors will have to be considered if adenosine A₁ receptor antagonists or agonists are developed for the alleviation of pain.

In conclusion, both adenosine and ATP can excite the same nociceptive afferents in the rat knee joint, apparently by a direct action on A₁ receptors located on the peripheral nerve terminals. Local release of ATP during hypoxaemia and/or inflammation in joints could excite a population of nociceptive primary afferents, initially acting *via* P2X receptors then, following rapid metabolism of the nucleotide to adenosine, *via* activation of A₁ receptors. In addition, ATP and adenosine could also release algogens and enhance inflammation through actions on P1 and P2 receptors on nearby cells (e.g. synoviocytes, mast cells, blood vessels; Panayi, 1992; Scott *et al.*, 1994). The role of adenosine and A₁ receptors associated with the peripheral terminals of nociceptive primary afferents innervating articular joints merits further study with a view to understanding their contribution to the development and maintenance of chronic pain associated with inflammatory joint disease.

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246P P2X RECEPTOR ACTIVATION EVOKES A BEZOLD-JARISCH-LIKE REFLEX IN ANAESTHETISED RATS

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During studies on rat articular nociceptors (Dowd et al, 1997) we observed that the P2X purinoceptor agonist $\alpha\beta$ -methylene-ATP ($\alpha\beta$ Me) caused bradycardia, hypotension and apnoea, which are characteristics of a Bezold-Jarisch-like (BJ) vagal reflex (see Kraye, 1961). It has been shown that activation of P2X receptors depolarises rat isolated vagal afferents and nodose neurones (see Khakh et al, 1995). We tested the hypothesis that a B-J reflex can be evoked in anaesthetised rats by activation of P2X receptors associated with vagal afferents.

Male Wistar rats (376±19g, n=18) were anaesthetised with pentobarbitone (60 mg.kg⁻¹ i.p., supplemented hourly i.v.), and the trachea, femoral arteries and right jugular vein cannulated. Ventilation was monitored using an electrospirometer, arterial blood pressure via a pressure transducer, and the output signals recorded on a MacLab-8; bradycardia (Δ beats.min⁻¹), apnoea (duration, s), and hyperventilation (Δ ml.min⁻¹) were measured. Action potentials were recorded from sensory nerves using bipolar wire electrodes. Drugs were dissolved in 0.9% w/v NaCl and injected i.v. as a bolus over 2s at intervals of 5-10 min.

ATP (0.2-5 μ mol i.v.) or $\alpha\beta$ Me (0.6-594 nmol i.v.) evoked short-lasting (1-10s) dose-related bradycardia with associated hypotension, and apnoea; high doses caused desensitisation. Transient hyperventilation (1-5s) preceded the apnoea. Bilateral mid-cervical vagotomy significantly reduced the bradycardia and apnoea; cutting the carotid sinus nerves (CSN) abolished the

hyperventilation (Table 1). The P2 purinoceptor antagonist PPADS antagonised the reflex apnoea and bradycardia, but not the hyperventilation. Neural recordings showed that $\alpha\beta$ Me excited vagal inflation (e.g. 40 nmol: basal 11.8 increased to 25.6 impulses.s⁻¹, 5s duration) and arterial chemoreceptor (40 nmol: basal 3.4 increased to 14.2 impulses.s⁻¹, 2.4s duration) afferents.

Table 1. Apparent ED₅₀ values for $\alpha\beta$ Me (moles) before and after denervation or PPADS (10 mg.kg⁻¹). Mean \pm s.e.mean *P<0.05, Mann-Whitney test versus control in *n* experiments.

	Bradycardia	Apnoea	Hyperventilation
Control	1.5±0.4x10 ⁻⁸ 12	4.9±0.9x10 ⁻⁸ 13	2.3±0.6x10 ⁻⁸ 15
Vagi cut	>1x10 ⁻⁶ *	3.4±1.4x10 ⁻⁷ * 5	3.5±1.6x10 ⁻⁹ * 5
CSN cut	4.4±2.8x10 ⁻⁸ 3	4.2±1.0x10 ⁻⁸ 4	>1x10 ⁻⁶ * 4
PPADS	4.4±2.8x10 ⁻⁷ * 3	>1x10 ⁻⁶ * 3	2.4±1.6x10 ⁻⁹ * 3

We conclude that activation of P2X purinoceptors on vagal afferents elicits a BJ reflex in rats; activation of P2X receptors in the carotid bodies causes hyperventilation. PPADS antagonises vagal but not chemoreceptor responses to $\alpha\beta$ Me. The BJ reflex could be useful for the functional study and characterisation of drugs acting at P2X receptors, and may be of patho-physiological importance if evoked by endogenous ATP.

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Activation of P2X receptors for adenosine triphosphate evokes cardiorespiratory reflexes in anaesthetized rats

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1. We tested the hypothesis that activation of P2X receptors associated with vagal afferent nerves can evoke a Bezold–Jarisch (B–J) depressor reflex in anaesthetized rats.
2. Injection of $\alpha\beta$ -methylene ATP ($\alpha\beta$ -MeATP; 0.6–600 nmol i.v.) evoked a dose-dependent B–J reflex comprising bradycardia, hypotension and apnoea in rats anaesthetized with pentobarbitone. Apnoea was commonly preceded by hyperventilation. Bilateral vagotomy significantly reduced the bradycardia and most of the apnoeic response without affecting hyperventilation, and unmasked a vasopressor response. Hypotension and apnoea were subject to desensitization, and ATP was about 100 times less potent than $\alpha\beta$ -MeATP in evoking the B–J reflex.
3. ED₅₀ values for responses to $\alpha\beta$ -MeATP were: bradycardia 14.6 ± 3.8 nmol; apnoea 47.1 ± 8.5 nmol; hyperventilation 23.3 ± 6.0 nmol, $n = 14$. The ED₅₀ for apnoea was significantly greater than that for bradycardia or hyperventilation ($P < 0.05$). Atropine ($2.8 \mu\text{mol (kg body wt)}^{-1}$ i.v.) antagonized the reflex bradycardia and hypotension.
3. The P2 antagonists suramin ($14 \mu\text{mol (kg body wt)}^{-1}$ i.v.) and PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.) antagonized the bradycardic and apnoeic components of the reflex response to $\alpha\beta$ -MeATP, without reducing the vasopressor or hyperventilatory responses to the agonist.
4. Recordings from vagal afferents showed that pulmonary inflation receptors were activated by $\alpha\beta$ -MeATP in 62% of units recorded (ED₅₀ 22 ± 5 nmol) and this was blocked by PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.); unidentified vagal afferents were also activated.
5. $\alpha\beta$ -MeATP activated carotid chemoreceptor afferents (ED₅₀ 23 ± 9 nmol), an action that was unaffected by PPADS or suramin.
6. The results support the hypothesis that P2X receptor subtypes for ATP are associated with specific sensory nerves that form part of the homeostatic mechanism for cardiovascular and respiratory regulation and these receptors therefore have physiological, pathological and therapeutic significance.

P2X purinoceptors are multimeric ATP-gated cation channels (see recent reviews by Humphrey *et al.* 1995; North, 1996; Buell, Collo & Rassendren, 1996) and seven separate P2X receptor subunits have been cloned (Valera *et al.* 1994; Suprenant, Rassendren, Kawashima, North & Buell, 1996). The distribution of these receptors has been studied by *in situ* hybridization, and in rat they are found in the brain and spinal cord, particularly within areas such as the substantia gelatinosa where primary afferent nerve fibres synapse (Collo *et al.* 1996). Autoradiographic studies have confirmed the presence of abundant P2X receptor binding sites in rat brain and spinal cord (Tuyau, Hansen, Dampney,

Balcar & Bennett, 1997), and P2X receptors are also associated with sensory neurones in the periphery (Evans & Suprenant, 1996). For example, the nodose (vagal) ganglion expresses RNAs for six of the seven known P2X receptors, including the P2X₃ variant that appears only to be present in sensory ganglia (Kidd, Grahames, Simon, Michel, Barnard & Humphrey, 1995; Collo *et al.* 1996). P2X₃ subunits have been detected immunohistochemically in small trigeminal nerve sensory fibres and endings in rat tooth pulp, and neurites of dissociated cultured neurones from the trigeminal ganglion were depolarized by pressure-ejected ATP (Cook, Vulchanova, Hargreaves, Elde & McCleskey, 1997).

Functional studies involving the vagus nerve have shown that intravenously administered ATP slows the cat heart via reflex actions involving vagal afferent and efferent nerve fibres (Emmelin & Feldberg, 1948), and ATP is known to activate a vagal reflex in dog and human (see Pelleg, Hurt & Michelson, 1990). More recently, Trezise, Kennedy & Humphrey (1993) and Trezise, Bell, Kennedy & Humphrey, (1994) demonstrated that activation of P2X receptors depolarizes nerve fibres in the rat isolated vagus. Whole-cell patch-clamp recordings from rat nodose neurones in culture confirmed that ATP depolarized neurones, as did the non-degradable agonist $\alpha\beta$ -methylene ATP ($\alpha\beta$ -MeATP; Khakh, Humphrey & Suprenant, 1995) which activates a subset of P2X receptors, including P2X₁, P2X₃ and P2X₂-P2X₃ heteromers (Buell, Collo & Rassendren, 1996). It has also been shown that ATP excites vagal C fibre nerve terminals in the dog lung, an effect that appeared to be mediated by P2X receptors because $\alpha\beta$ -MeATP caused the same effect, which was antagonized by the P2 antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; Pelleg & Hurt, 1996).

The Bezold-Jarisch (B-J) reflex comprises a triad of apnoea, bradycardia and hypotension that was originally described as a cardiodepressor-vagal chemoreflex evoked via the stimulation of cardiac receptors by intravenously administered veratrine (Bezold & Hirt, 1867; Dawes & Comroe, 1954). ATP is reported to be one of the 'detector substances' that can also activate this complex reflex (Jarisch, 1941; Dawes & Comroe, 1954). The B-J reflex involves various cardiopulmonary sensory receptors: most of the cardiodepression and vasodepression results from activation of vagal afferents in the heart, particularly the left ventricle (coronary chemoreflex, Dawes & Comroe, 1954), whereas apnoea and about 10% of the cardiovascular depression arise from stimulation of vagal sensory receptors in the lungs, in cats and dogs anaesthetized with pentobarbitone (Dawes, 1947).

While studying the effects of purinoceptor agonists on nociceptor afferents in anaesthetized rats (Dowd, McQueen, Chessell & Humphrey, 1997), we observed that reflex bradycardia, hypotension and apnoea occurred in response to intra-arterial administration of $\alpha\beta$ -MeATP. The reflex was obtained during some, but not all, of the experiments and it seemed to be influenced by the anaesthetic agent used. We have not found any reports concerning activation of the B-J reflex by ATP in rat, so the present investigation was undertaken to test the hypothesis that activation of P2X receptors associated with vagal afferents can evoke a B-J cardiorespiratory reflex in this species. We evoked cardiorespiratory reflexes by i.v. injection of $\alpha\beta$ -MeATP and other less stable P2 purinoceptor agonists, including ATP. However, since ATP is rapidly degraded by nucleotide enzymes *in vivo* (Holton, 1959), it is of limited use in experiments because the cardiovascular and respiratory effects seen following its injection will result from mixed actions on P1 and P2 purinoceptors for adenosine and ATP

respectively; adenosine is a major metabolite of ATP and it causes bradycardia, hypotension and hyperventilation (Drury & Szent-Györgi, 1929; Reid, Watt, Penny, Newby, Smith & Routledge, 1991). We determined which sensory nerves contribute to the reflex in animals anaesthetized with pentobarbitone by using a combination of selective denervation and electrophysiological recordings from afferent nerves. The antagonists suramin and PPADS were used in conjunction with the purinoceptor agonists to characterize the type of purinoceptor associated with the sensory nerves involved in evoking the B-J reflex. A preliminary account of the work has been published (McQueen, Moores, Dowd, Bond, Chessell & Humphrey, 1997).

METHODS

Animals and anaesthesia

Experiments were licensed under UK Home Office regulations. Male Wistar rats ranging between 250 and 550 g in body weight were anaesthetized with either pentobarbitone (60 mg (kg body wt)⁻¹ i.p., mean weight 361 ± 13 g, $n = 26$, followed at hourly intervals with 6 mg i.v., if necessary - dependent on response to applying pressure to a limb joint and the basal heart rate and blood pressure (BP) or urethane (single dose 0.6 ml (100 g body wt)⁻¹ i.p. of 25% w/v aqueous ethyl carbamate; mean weight 374 ± 37 g, $n = 6$). In three experiments the rat (mean weight 374 ± 25 g) was anaesthetized with trichloroethylene and the brain and spinal cord destroyed by pithing, as described by Gillespie & Muir (1967). A servo-controlled heating blanket (Harvard) maintained rectal temperature at 38 °C.

Ventilation and blood pressure

The trachea was cannulated and connected to a pneumotachograph head linked to an electrospirometer (Mercury Electronics CS5) and a computerized recording system (MacLab-8 and Macintosh LC475 computer) for measuring and recording tracheal air flow, respiratory frequency, tidal volume and respiratory minute volume (RMV). The animals breathed room air spontaneously, apart from the pithed rats and the majority of those used for neural recordings, which were artificially ventilated and neuromuscularly blocked with gallamine (8 mg i.v. at hourly intervals, together with 6 mg pentobarbitone in the neural experiments). Both femoral arteries were cannulated, one catheter being used to record arterial blood pressure via a BP transducer (Bell & Howell) linked to the MacLab, the other being used for taking samples (0.2 ml) of arterial blood for measurement of arterial oxygen pressure (P_{a,O_2}), and arterial carbon dioxide pressure (P_{a,CO_2}) and pH (Ciba Corning 238 analyser) during the experiment. Heart rate was measured from the BP recording, with the time scale expanded to allow the number of pulses in a fixed period to be counted pre- and post-injection. The right external jugular vein was cannulated for intravenous administration of drugs.

Surgical procedures

In five experiments the vagi were sectioned at mid-cervical level, and in two cases the nodose ganglia were subsequently excised. The carotid sinus nerves (left and right side) were identified at their junction with the glossopharyngeal (IX cranial) trunk and both the left and right nerves were sectioned in four experiments. Denervation was confirmed by the abolition of the reflex hyperventilation that was evoked before denervation by the peripheral chemoreceptor stimulant sodium cyanide (2 μ mol i.v.).

Neural recording from arterial chemoreceptors

The left carotid sinus nerve was sectioned at its junction with the glossopharyngeal trunk and the peripheral end desheathed. The nerve was immersed under paraffin oil and recordings of neural activity were made extracellularly from dissected nerve filaments using bipolar platinum-iridium electrodes connected to an amplifier (Neurolog NL104) and a computerized digital video recording system (Sony VCR EV-C500E; Dell 450/L PC). Individual action potentials from multi-unit (1–4) recordings were counted via a pulse height voltage discriminator (Digitimer D130) and quantified using the PC and computer software developed in-house. The contralateral carotid sinus nerve remained intact and the animals breathed room air in two experiments, in one of which the rat was anaesthetized with urethane. In two further experiments under pentobarbitone both carotid sinus nerves were cut and the rat was artificially ventilated and the neuromuscular system was blocked with gallamine.

Neural recording from vagal afferents

The right vagus nerve was sectioned at mid-cervical level and recordings of neural discharge (1–5 units) made from filaments dissected from the desheathed peripheral end in six rats, using the techniques described above. In some experiments gallamine was administered and the animals were artificially ventilated to prevent reflex cardiorespiratory changes from influencing the responses.

Drugs

The drugs used included: adenosine, adenosine 5'-triphosphate disodium (ATP), adenosine 5'-O-(3-thiophosphate) (ATP γ S), $\alpha\beta$ -MeATP (lithium salt) (all from Sigma); 2-methyl-5-hydroxy-tryptamine maleate (2-Me-5-HT) and suramin hexasodium (from RBI); MDL72222 (a gift from Merrell Dow); trichloroethylene, atropine sulphate and sodium cyanide (from BDH); gallamine triethiodide (from May & Baker); PPADS (tetrasodium salt; from Tocris Cookson). Drugs were dissolved in saline (0.9% w/v aqueous sodium chloride) and injected in volumes of 0.1 ml, washed in with 0.2 ml saline. The control was 0.3 ml saline and all injections were completed within 2 s, with the exception of antagonists which were slowly injected over 15–30 s. Injections were i.v. (jugular) or i.a. (femoral catheter in some experiments) and the minimum interval between successive doses was 5 min. In order to minimize the number of animals used, more than one procedure was performed wherever this was feasible (in a total of eight experiments involving atropine or vagotomy after recovery from PPADS, cutting carotid sinus nerves after vagotomy or PPADS), but at least one experiment in each group was done without any pre-treatment as a check that pre-treatment had not influenced the responses obtained.

Data and analysis

Half-maximum response (ED_{50}) values for bradycardia, apnoea and hyperventilation were calculated from individual log dose–response curves for the agonists before and after selective denervation or the administration of antagonist. As the concentration of the injected drug at the receptor site is not known, nor is it in equilibrium following a bolus injection, and because of difficulties in establishing a maximum response from receptors prone to desensitization, we minimized the number of doses used *in vivo* and the calculated ED_{50} was expressed as an apparent value, i.e. the best estimate. Results are presented as means \pm S.E.M. Statistical analysis (generally ANOVA, Student's *t* test, or the non-parametric Mann–Whitney test (two-tailed) – when variances were significantly different) was used to determine whether differences between group means were statistically significant. The Null hypothesis was rejected at $P \leq 0.05$.

RESULTS

Intravenous injection of $\alpha\beta$ -MeATP (0.6–600 nmol) in animals anaesthetized with pentobarbitone evoked a rapid dose-dependent bradycardia, hypotension and apnoea, the latter preceded by a transient hyperventilation (Fig. 1A). In approximately 20% of experiments there was a delayed secondary hyperventilation after the apnoea. Repeating the doses at intervals of less than 5–10 min led to a decrease in magnitude (total loss after high doses) of the apnoea, bradycardia, and hypotension, but not the hyperventilatory component of the response. ATP γ S (18–366 nmol) caused similar responses, although it was less potent than $\alpha\beta$ -MeATP in evoking bradycardia. Although we did not study the nucleotide in detail, ATP in doses approximately 100-fold greater than those of $\alpha\beta$ -MeATP also caused a slight hyperventilation, apnoea and bradycardia. Adenosine injected i.v. in high doses (4.7–11.2 μ mol) resulted in a bradycardia without hyperventilation or apnoea, which unlike the bradycardia evoked by $\alpha\beta$ -MeATP was slightly delayed in onset and unaffected by bilateral vagotomy or atropine. Similar cardiorespiratory effects to those of $\alpha\beta$ -MeATP were evoked by the 5-HT $_3$ agonist, 2-Me-5-HT (33–330 nmol).

Mean arterial BP was 106 ± 5 mmHg and respiratory minute volume 210 ± 27 ml min $^{-1}$ in rats ($n = 23$) anaesthetized with pentobarbitone; arterial blood gases and pH did not differ significantly between the groups. In control animals ($n = 23$) mean pH was 7.44 ± 0.01 , mean P_{a,CO_2} was 35.9 ± 1.4 mmHg and mean P_{a,O_2} was 71.8 ± 2.1 mmHg. Corresponding values for vagotomized rats ($n = 10$) were: 7.45 ± 0.02 , 31.4 ± 2.0 and 75.3 ± 1.9 ; after cutting the carotid sinus nerves they were 7.39 ± 0.05 , 34.8 ± 1.9 and 67.0 ± 6.2 ($n = 5$). Changes in reflex responses obtained after selective denervation were therefore not secondary to alterations in basal conditions, as indicated by the stable arterial blood gas tensions and pH.

In animals anaesthetized with urethane the reflex bradycardia, hypotension and apnoea in response to $\alpha\beta$ -MeATP (20–600 nmol) was attenuated or absent in four of five experiments although hyperventilation and vasopressor responses were present in them all, so pentobarbitone was used for the majority of our experiments.

Reflex bradycardia and hypotension

Injection of $\alpha\beta$ -MeATP in anaesthetized rats caused dose-dependent bradycardia within the first 5 s following i.v. injection which lasted for up to 5 s (Figs 1 and 4). ATP injected in high doses (2–5 μ mol) caused bradycardia, but this response was longer lasting in comparison with that evoked by $\alpha\beta$ -MeATP; adenosine in high doses also caused delayed long-lasting bradycardia. Experiments in pithed rats showed that i.v. $\alpha\beta$ -MeATP did not cause bradycardia, whereas high doses of ATP and adenosine caused similar responses to those observed in intact anaesthetized animals, namely bradycardia and hypotension (see Fig. 2).

There was a triphasic change in arterial BP following injection of $\alpha\beta$ -MeATP, comprising a transient hypotension associated with the bradycardia, and then a more prolonged (1–2 min) vasopressor phase (see Fig. 1) and after higher doses (100 nmol or more), a longer lasting (up to 10 min) delayed-depressor phase was obtained; but high doses were not routinely tested because of concerns over receptor desensitization. ATP γ S, ATP and adenosine (4.7–11.2 μ mol) caused only hypotension, there being no vasopressor component; slight hypertension occurred after 2-Me-5-HT, which also caused transient vasodepression secondary to the bradycardia. Intra-arterial injection of $\alpha\beta$ -MeATP also caused reflex cardiovascular effects, but had only about one-

tenth the potency of the same dose injected i.v., indicating that the sensory receptors were located in or near the heart and lungs. In pithed rats $\alpha\beta$ -MeATP caused only vasopressor responses, whereas injected ATP γ S, ATP and adenosine were exclusively vasodepressor responses and lacked any vasopressor component (Fig. 2). Blood pressure responses were not studied in detail because the primary reflex vasodepression was entirely secondary to the bradycardia, but we noted that the vasopressor response to $\alpha\beta$ -MeATP in anaesthetized rats was not significantly affected by suramin or PPADS (mean rise in BP evoked by 100 nmol $\alpha\beta$ -MeATP before suramin (14 μ mol (kg body wt)⁻¹) was 26 ± 2 mmHg, and after was 25 ± 3 mmHg,

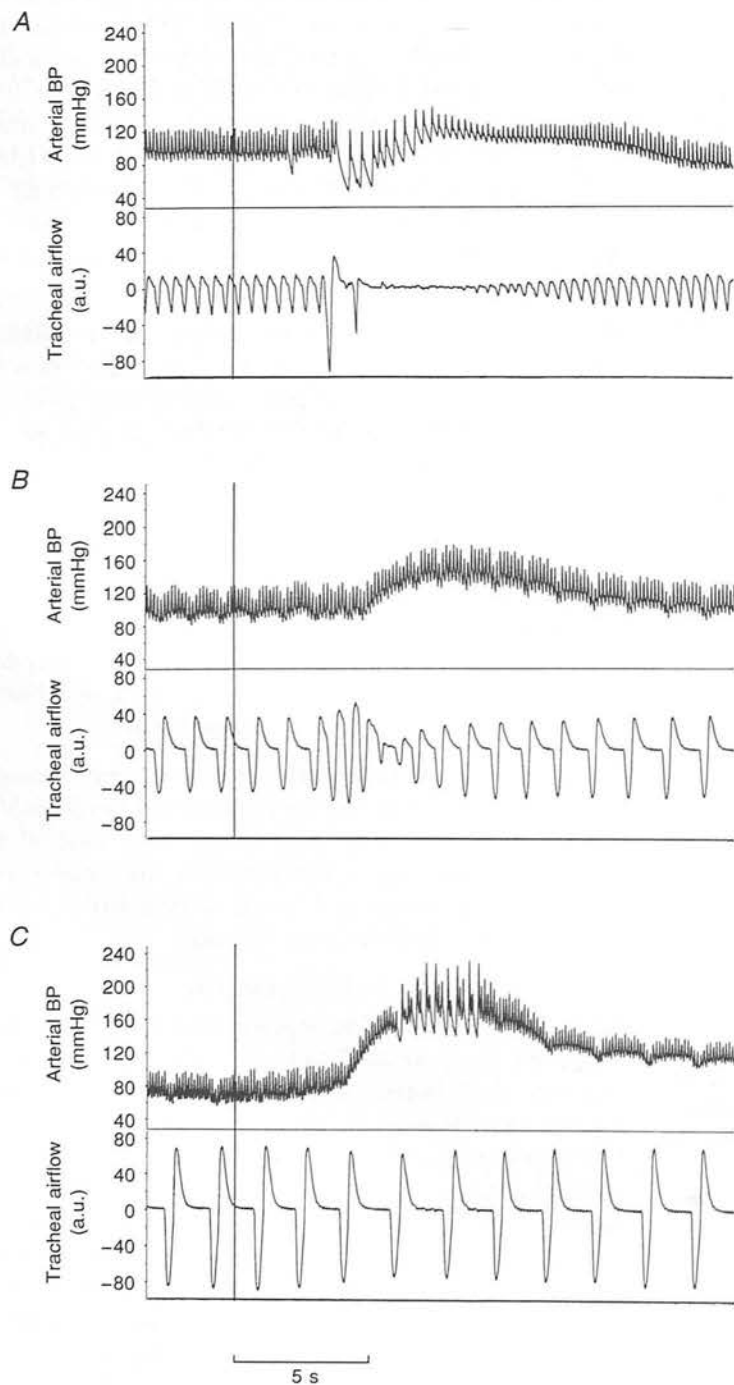


Figure 1. Cardiorespiratory effects of $\alpha\beta$ -MeATP

Cardiorespiratory effects of a dose of $\alpha\beta$ -MeATP (100 nmol i.v. at continuous vertical line) in a rat anaesthetized with pentobarbitone. In each panel the upper trace from the computerized chart recorder is arterial BP, and the lower trace is respiratory airflow (inspiration downwards, arbitrary units (a.u.), mV). Heart rate was measured by counting individual beats in the BP trace. During the control state (A) bradycardia, transient hyperventilation, vasodepression and apnoea were obtained in response to the rapid injection of $\alpha\beta$ -MeATP. After cutting both vagus nerves at mid-cervical level (B) the bradycardia, hypotension, and apnoea were abolished, leaving hyperventilation and a vasopressor response to the same dose of agonist. Cutting both carotid sinus nerves (C) abolished hyperventilation without reducing the vasopressor response. The delayed arrhythmia seen in C differed from the reflex bradycardia observed in A, and was associated with the substantial rise in systemic blood pressure; the arrhythmia may result from activation of C fibre sympathetic cardiac afferents innervating the left ventricle (see Hainsworth, 1991).

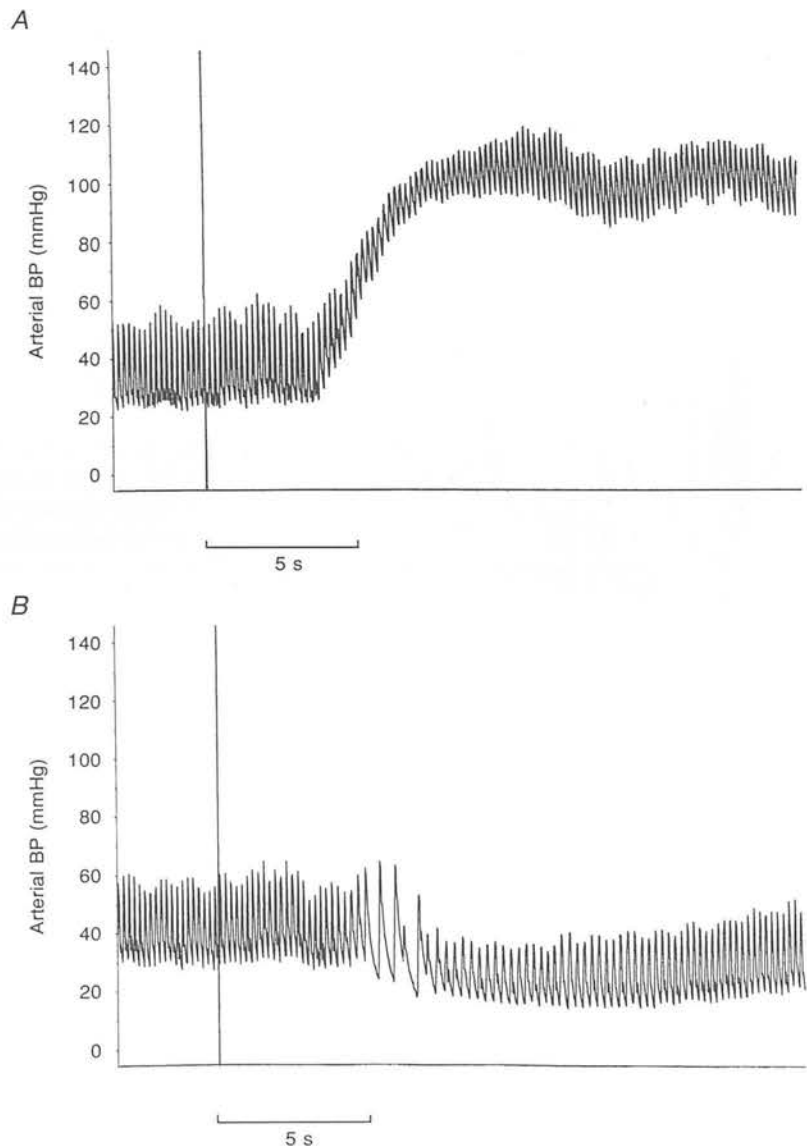
$n = 3$; BP caused by 40 nmol $\alpha\beta$ -MeATP before PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$) was $39 \pm 15 \text{ mmHg}$, and after was $39 \pm 8.5 \text{ mmHg}$, $n = 6$). The reflex bradycardia was attenuated when successive high doses of $\alpha\beta$ -MeATP were injected at intervals of less than 10 min, with a tendency to generate bell-shaped logdose-response curves, and some reduction was evident even after delays of 10–15 min. It was therefore necessary to avoid using high doses of P2 receptor agonist, and to accept that it would not be possible to establish the true maximal responses. The bradycardia was calculated from logdose-response (Fig. 3) and expressed as an apparent ED_{50} for each experiment.

Bradycardia evoked by $\alpha\beta$ -MeATP was significantly attenuated by atropine ($2.8 \mu\text{mol (kg body wt)}^{-1}$) and by bilateral vagotomy (Fig. 1B), as well as by the P2 purinoceptor antagonists suramin ($14 \mu\text{mol (kg body wt)}^{-1}$ i.v.) and PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.), but not by $1.7 \mu\text{mol (kg body wt)}^{-1}$. Pooled ED_{50} data from all the experiments are shown in Fig. 4A. In some instances it was not possible to calculate the dose required to match the

original ED_{50} for some responses after denervation or administration of an antagonist (e.g. after vagotomy the bradycardia was totally abolished, likewise after PPADS), so a minimum ED_{50} of 1×10^{-6} mol was entered to allow statistical analysis using non-parametric tests. The transient vasodepression which occurred at the same time as the bradycardia was absent when the reflex bradycardia was prevented, and PPADS also blocked the delayed prolonged hypotension previously evoked by $\alpha\beta$ -MeATP. $\text{ATP}\gamma\text{S}$ was less effective than $\alpha\beta$ -MeATP in evoking reflex bradycardia ($\text{ED}_{50} 78.1 \pm 6.8 \text{ nmol}$, $n = 4$; $P < 0.001$ versus $\alpha\beta$ -MeATP, t test). High doses of ATP or adenosine caused a bradycardia that was unaffected by vagotomy, atropine, suramin or PPADS. Antagonism caused by the single dose of PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.) was short lasting (10–15 min) in some experiments, but longer in others (45 min) – even though the same batch of drug was used – so an additional dose of antagonist was administered when it was apparent that its effects were waning; suramin ($14 \mu\text{mol (kg body wt)}^{-1}$) antagonism tended to last longer, up to about 60 min.

Figure 2. Cardiovascular effects in an artificially ventilated pithed rat in which reflex effects were abolished

Effects of $\alpha\beta$ -MeATP (60 nmol i.v. at vertical line) (A) and adenosine (375 nmol i.v.) in an artificially ventilated pithed rat (B) in which reflex effects were abolished. The P2X receptor agonist gave a vasopressor response with no bradycardia, whereas adenosine caused bradycardia and hypotension.



Activation of vagal 5-HT₃ receptors has been shown to elicit similar reflexes to those described above (Fozard, 1984), so the possibility that endogenous 5-HT might be involved in the reflexes was tested. We used the 5-HT₃ receptor agonist 2-Me-5-HT and found it gave responses similar to those caused by $\alpha\beta$ -MeATP. The reflex bradycardia and apnoea were antagonized by MDL72222, but not by the P2 antagonists suramin or PPADS (data not shown). The mean ED₅₀ for bradycardia evoked by $\alpha\beta$ -MeATP was not significantly affected by the 5-HT₃ antagonist (see Fig. 4).

Respiratory effects

Injections of $\alpha\beta$ -MeATP in rats anaesthetized with pentobarbitone evoked a complex dose-related change in ventilation, comprising a transient (2–3 breaths) hyperventilation of rapid onset (2–3 s post-injection) followed by

dose-related apnoea lasting from 1–20 s (see Fig. 1 and 3C). In some experiments a 5–30 s period of delayed hyperventilation followed the apnoea, but as it usually coincided with delayed hypotension, we did not investigate it further during this study. When high doses of agonist were repeated at short (< 5 min) intervals, apnoea was attenuated, so 10–15 min were allowed to elapse between successive high doses. The hyperventilation was less prone to desensitization. Intra-arterial injections of $\alpha\beta$ -MeATP (200 nmol) or 2-Me-5-HT (33 μ mol) evoked smaller respiratory responses to those obtained when the drugs were given i.v. in the same animal, and the i.v. to i.a. equipotent molar ratio for $\alpha\beta$ -MeATP was approximately 10 ($n = 2$). Reflex apnoea following i.v. $\alpha\beta$ -MeATP was usually attenuated or absent when urethane was used as the anaesthetic, although dose-related increases in ventilation were obtained.

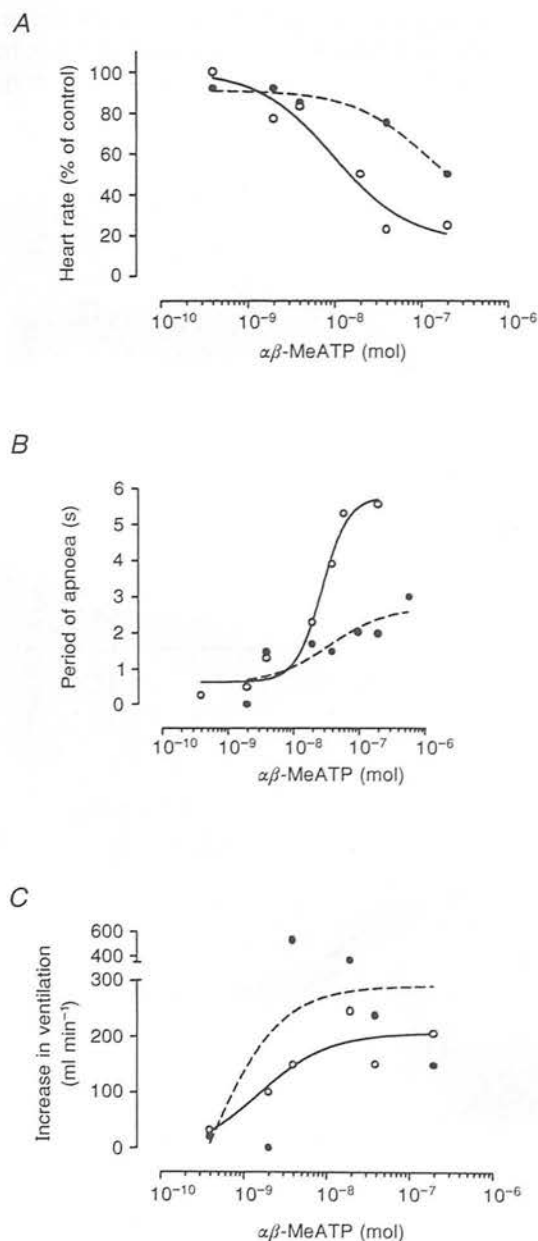


Figure 3. log dose-response curves showing the effects of $\alpha\beta$ -MeATP

Results from individual experiments illustrating the effects of $\alpha\beta$ -MeATP on heart rate (A), apnoea (B) and hyperventilation (C), before (○) and after (●) the purinoceptor antagonist PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.) which reduced the bradycardia and apnoea components of agonist-induced responses, whereas the hyperventilation component was unaffected or even enhanced by PPADS. Apparent ED₅₀ values were estimated from the log dose-response plots.

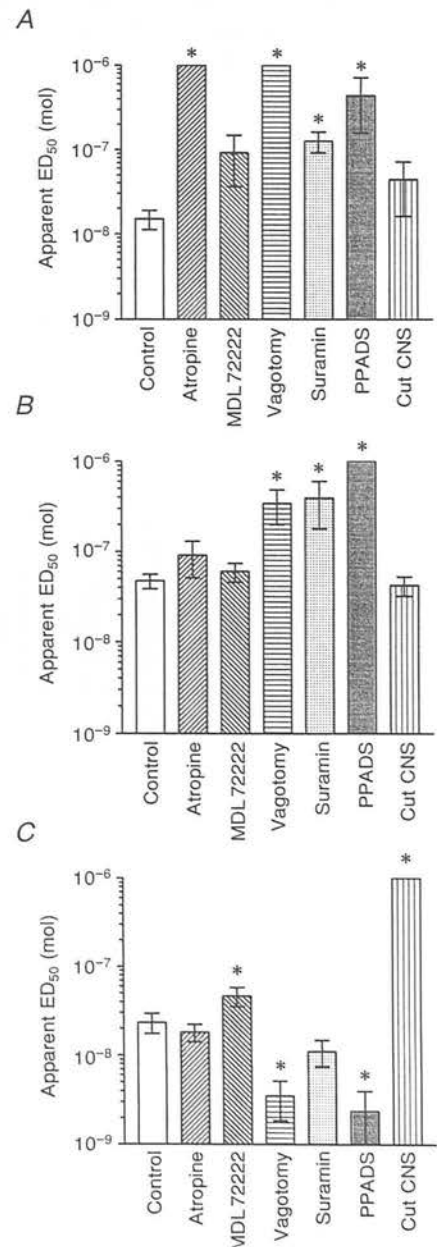
Apnoea. The ED_{50} for apnoea evoked by $\alpha\beta$ -MeATP was calculated from individual log dose-response plots (Fig. 3B). By pooling data from different experiments in which various procedures were performed we established that neither atropine ($2.8 \mu\text{mol (kg body wt)}^{-1}$ i.v.), MDL72222 ($0.24 \mu\text{mol (kg body wt)}^{-1}$ i.v.) nor cutting the carotid sinus nerves had any significant effect on the apnoea, as shown in Fig. 4B, although the dose of MDL72222 used, abolished apnoea and bradycardia caused by 2-Me-5-HT. Bilateral vagotomy significantly reduced the apnoea (Fig. 1B), but did not eliminate it in all experiments – high doses of $\alpha\beta$ -MeATP still elicited a reduced apnoeic response in some experiments post-vagotomy, an effect which was abolished by surgical removal of both nodose ganglia. The P2 purinoceptor antagonist suramin ($14 \mu\text{mol (kg body wt)}^{-1}$ i.v.) significantly reduced the apnoeic response to $\alpha\beta$ -MeATP (Fig. 5), and a similar effect was obtained with

PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.). Qualitatively similar respiratory responses were observed with ATP γ S, and the compound did not differ significantly from $\alpha\beta$ -MeATP in evoking apnoea ($ED_{50} (1.2 \pm 0.6) \times 10^{-7}$ nmol). ATP (maximum dose 5.4×10^{-6} mol caused apnoea, with a slight hyperventilation. Adenosine in doses of up to 3.7×10^{-6} mol did not cause apnoea, but the nucleoside was similar to ATP in that it caused a slight hyperventilation.

Hyperventilation. Increased ventilation within the first 5 s after an injection of $\alpha\beta$ -MeATP was a prominent feature of the response to the P2 agonist and was caused by an increase in depth and frequency of respiration (see Fig. 1). This response was generally followed by a period of apnoea (see above), depending on the dose of agonist administered: low doses usually only evoked hyperventilation. Injection of 2-Me-5-HT also evoked an increase in RMV. Delayed hyper-

Figure 4. Summary of pooled ED_{50} data for bradycardia, apnoea and hyperventilation evoked by $\alpha\beta$ -MeATP

Mean apparent ED_{50} values \pm S.E.M. for reflex changes evoked by i.v. $\alpha\beta$ -MeATP before (Control, $n = 14$) and after various procedures in rats anaesthetized with pentobarbitone. *A*, bradycardia was significantly reduced by atropine ($2.8 \mu\text{mol (kg body wt)}^{-1}$, $n = 4$), bilateral vagotomy ($n = 3$), suramin ($14 \mu\text{mol (kg body wt)}^{-1}$, $n = 3$) and by PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$, $n = 4$), but unaffected by MDL72222 ($0.24 \mu\text{mol (kg body wt)}^{-1}$, $n = 2$) or cutting the sinus nerves (Cut CNS, $n = 3$). *B*, apnoea was unaffected by atropine ($n = 4$), MDL72222 ($n = 3$), or cutting the carotid sinus nerves ($n = 4$), but was reduced after vagotomy ($n = 5$), suramin ($n = 4$) or PPADS ($n = 4$). *C*, hyperventilation was reduced by cutting the carotid sinus nerves ($n = 4$), and slightly but significantly by MDL72222 ($n = 4$). The response was potentiated by vagotomy ($n = 5$) or PPADS ($n = 4$), and unaffected by atropine or suramin (both $n = 4$). * $P < 0.05$ versus control values (Mann-Whitney test).



ventilation was observed following $\alpha\beta$ -MeATP, but this effect commenced 8–15 s after the injection, following a period of apnoea, and may have been secondary to the cessation of breathing or the hypotension. Cutting the carotid sinus nerves, thereby denervating the carotid bodies – confirmed by the lack of hyperventilation previously evoked by sodium cyanide ($2 \mu\text{mol i.v.}$) – significantly reduced the rapid-onset hyperventilation ($n = 4$), see Fig. 1C. Since the hyperventilatory response was substantially reduced by cutting the carotid sinus nerves it was not feasible to determine a post-denervation ED_{50} for $\alpha\beta$ -MeATP, so responses were expressed as being $> 1 \times 10^{-6}$ mol.

The primary increase in ventilation was measured during the 6 s period immediately following the injection and the mean apparent ED_{50} determined from individual logdose–response plots. The pooled data illustrated in Fig. 4C showed that suramin and PPADS tended to potentiate the reflex hyperventilation evoked by $\alpha\beta$ -MeATP, and vagotomy also increased the response. There was a slight but statistically significant decrease in responsiveness to $\alpha\beta$ -MeATP following the 5-HT_3 receptor antagonist MDL72222; atropine had no effect. Qualitatively similar responses were observed with $\text{ATP}\gamma\text{S}$, and its ED_{50} (2.8×10^{-8} mol, $n = 4$) was not different from that of $\alpha\beta$ -MeATP; vagotomy also potentiated the increase in ventilation obtained with $\text{ATP}\gamma\text{S}$. As described above, ATP caused a slight dose-related increase in ventilation when injected at a high dose of 5.5×10^{-6} mol (i.v.), but there was

also a delayed slight hyperventilation accompanying the profound hypotension which was unaffected by cutting the carotid sinus nerves. Adenosine lacked any fast onset stimulant action on ventilation when given in doses up to 3.7×10^{-6} mol i.v., but did cause a slight delayed hyperventilation that persisted after denervation of the carotid bodies.

ED_{50} values

Analysis of the mean ED_{50} values for $\alpha\beta$ -MeATP-induced bradycardia, apnoea and hyperventilation (see Fig. 4) showed that they differed significantly (ANOVA $P = 0.003$). Bonferroni's multiple comparison test indicated that the control ED_{50} value for apnoea was significantly higher than that for bradycardia ($P < 0.01$) and hyperventilation ($P < 0.05$), whereas those for bradycardia and hyperventilation did not differ from each other.

Neural recordings

Vagal afferents. Recordings were obtained mainly from vagal pulmonary inflation (slowly adapting stretch) receptors, whose discharge was in phase with inspiration and related to the stroke volume of the ventilator. Activity was also recorded from some spontaneously active vagal afferents which did not respond to pulmonary inflation, deflation, sodium cyanide, or to changes in mean BP. Overall, twenty-four multi-unit recordings were made and 62% of these (mainly pulmonary inflation receptors, but also unidentified vagal afferents) were rapidly (within 2–3 s of injection)

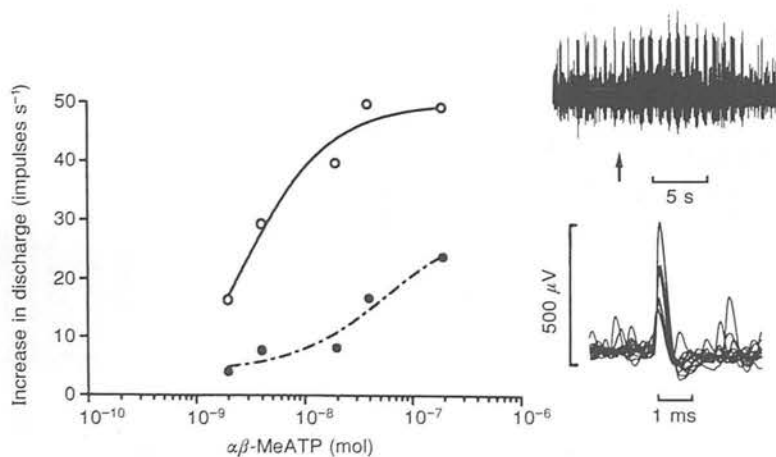


Figure 5. Activation of pulmonary vagal afferents by $\alpha\beta$ -MeATP

Multi-unit recording from vagal afferents which increased their discharge frequency without any rapid adaptation when the lungs were inflated, i.e. lung inflation receptors. Injection of the P2X agonist $\alpha\beta$ -MeATP increased firing of the three units counted by the spike voltage discriminator (O, continuous line) and PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.) antagonized the response (●, dashed line). The upper neurogram shows the neural discharge before and after a control i.v. injection of 2 nmol $\alpha\beta$ -MeATP at arrow – note that bursts of activity occurred during inspiration. The lower inset shows a sample of superimposed action potentials (3) triggered by the injection of $\alpha\beta$ -MeATP. The number of action potentials triggered during the 5 s period following onset of the response was plotted as the increase above basal values which averaged 14.1 ± 1.4 impulses s^{-1} before, and 16.3 ± 1.5 impulses s^{-1} after PPADS. The respiratory pump was set to a stroke volume of 3 ml with a frequency of 84 strokes min^{-1} .

excited by i.v. $\alpha\beta$ -MeATP (see Fig. 5), and also by 2-Me-5-HT, whereas 38% of the afferents were unresponsive to these drugs, including four recordings (17% of total population) that responded to lung inflation. The increase in discharge lasted from 2–10 s, the longer duration being associated with high doses. The mean ED_{50} for $\alpha\beta$ -MeATP-induced excitation was $(2.2 \pm 0.5) \times 10^{-8}$ mol ($n = 4$) before, and $(1.4 \pm 0.2) \times 10^{-7}$ mol ($n = 3$) after PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$; $P < 0.05$, paired t test $n = 3$). Vagal activation by 2-Me-5-HT (a single dose of $33 \mu\text{mol}$ was unaffected by PPADS, although it was abolished by the 5-HT₃ antagonist MDL72222 ($0.24 \mu\text{mol (kg body wt)}^{-1}$) which did not significantly affect the excitatory response to $\alpha\beta$ -MeATP. The ED_{50} for $\alpha\beta$ -MeATP-induced apnoea or bradycardia did not differ significantly from the vagal neural ED_{50} .

In one experiment under urethane anaesthesia two separate multi-unit recordings of vagal sensory discharge were made. In one of these recordings the afferents were unaffected by $\alpha\beta$ -MeATP, but were strongly excited by 2-Me-5-HT ($33 \mu\text{mol}$), whereas in the other they were activated by the purinoceptor agonist within 2 s of injection and the excitation lasted for 3–10 s. The ED_{50} for $\alpha\beta$ -MeATP was 1.5×10^{-7} mol, and in this recording the afferents showed only a very weak excitatory response to 2-Me-5-HT, either before or after excitation by $\alpha\beta$ -MeATP.

Carotid body chemoreceptors. Recordings from the peripheral end of sectioned carotid sinus nerves in four rats demonstrated that $\alpha\beta$ -MeATP injected i.v. caused a rapid dose-related increase in chemosensory discharge, as illustrated in Fig. 6. Intra-arterial injection of $\alpha\beta$ -MeATP

also increased chemosensory discharge, but with only one-tenth of the potency of the same dose injected i.v.; high doses of ATP ($(1.8\text{--}5.5) \times 10^{-6}$ mol) were also chemo-excitatory. The mean apparent ED_{50} for chemo-excitation evoked by $\alpha\beta$ -MeATP was $(2.3 \pm 0.9) \times 10^{-8}$ mol ($n = 4$), which was not significantly different from $(2.3 \pm 0.6) \times 10^{-8}$ mol ($n = 14$), obtained for hyperventilation. Neither suramin ($14 \mu\text{mol (kg body wt)}^{-1}$) nor PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$) antagonized the chemo-excitant action of $\alpha\beta$ -MeATP, nor did they affect the chemo-excitation evoked by sodium cyanide or asphyxia (stopping the respiratory pump for 30 s).

Fast onset chemo-excitation was also observed under urethane anaesthesia in the one experiment performed (ED_{50} for $\alpha\beta$ -MeATP 1.8×10^{-8} mol before and 4×10^{-8} mol after suramin ($14 \mu\text{mol (kg body wt)}^{-1}$); ED_{50} for ATP γ S was 3.7×10^{-8} mol before suramin).

DISCUSSION

The results obtained using $\alpha\beta$ -MeATP, an agonist which is relatively selective for particular subtypes of P2X receptor, together with subsidiary evidence from the rather weak and non-selective P2 receptor antagonists, PPADS and suramin (see Introduction), support our working hypothesis that activation of vagal P2X receptors in anaesthetized rats evokes a B–J reflex. Carotid body arterial chemoreceptors are also excited by P2X receptor agonists, causing reflex hyperventilation, so the overall cardiorespiratory response to i.v. injection of a P2X agonist such as $\alpha\beta$ -MeATP in

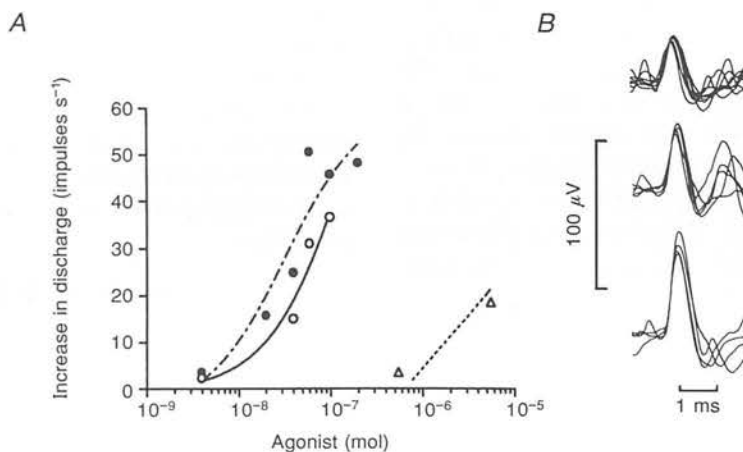


Figure 6. Activation of carotid body chemoreceptors by $\alpha\beta$ -MeATP and ATP, and the effect of P2 purinoceptor antagonist PPADS

Multi-unit recording from carotid chemoreceptor afferent fibres in an artificially ventilated rat anaesthetized with pentobarbitone. *A*, all three afferents counted by the voltage discriminator were activated by $\alpha\beta$ -MeATP (O, continuous line), and PPADS ($17 \mu\text{mol (kg body wt)}^{-1}$ i.v.) did not antagonize the response (●, dashed line). Responses evoked by ATP (Δ , dotted line) in the control state are also shown. *B*, three individual action potentials (successive triggered oscilloscope sweeps superimposed) sampled during their activation by $\alpha\beta$ -MeATP. The increase in total discharge above pre-injection basal frequency was plotted in *A*, and basal discharge from these afferents during ventilation with room air averaged 9.3 impulses s^{-1} before and 9.9 impulses s^{-1} after PPADS.

anaesthetized rats is quite complex, and will be considered in relation to the individual sensory elements involved. ATP had similar effects to those evoked by $\alpha\beta$ -MeATP, but we did not study the nucleotide in detail because it is rapidly inactivated by ecto-nucleotidases and it was not feasible to use selective ATPase inhibitors *in vivo*.

Anaesthetic

The vagal reflexes bradycardia and apnoea were seldom obtained under urethane anaesthesia, whereas both reflexes were invariably present during pentobarbitone anaesthesia. Other authors have noted that reflex responses to ATP are affected by the anaesthetic (e.g. Emmelin & Feldberg, 1948; comparing chloralose with decerebration in cats), and this could be due to block of central reflex pathways, or perhaps to some interaction of the anaesthetic agent with P2X receptors in the periphery. Neural recording from vagal afferents indicated a reduced responsiveness to $\alpha\beta$ -MeATP in one experiment under urethane, but more detailed studies would be required to establish the extent to which peripheral, as opposed to central, actions of the anaesthetic agent reduce responsiveness to P2X receptor agonists. We focused on studying the cardiorespiratory reflexes under pentobarbitone anaesthesia.

Vagal afferents

The rapid onset bradycardia and apnoea evoked by $\alpha\beta$ -MeATP was abolished by atropine or bilateral vagotomy, indicating that it was a vago-vagal reflex. In some experiments some reduction in ventilation persisted after vagotomy, but this could be eliminated by surgical removal of both nodose ganglia, suggesting that the P2X agonist, particularly in high doses, can activate purinoceptors located in the sensory ganglion (see Khakh *et al.* 1995) as well as those at the peripheral nerve terminals. The antagonists PPADS and suramin were both capable of antagonizing the bradycardia and apnoea induced by $\alpha\beta$ -MeATP. Desensitization to the reflex changes tended to occur when injections of the agonist were repeated at intervals of less than 10 min. Experiments in pithed rats, where reflexes were abolished by destruction of the brain and spinal cord, showed that only vasopressor effects, without bradycardia or vasodepression, were observed in response to $\alpha\beta$ -MeATP. This confirms that there is no direct cardiac action of the agonist. In contrast, ATP and adenosine caused bradycardia and hypotension which was still present after vagotomy in anaesthetized animals, and was also obtained in pithed rats. ATP is known to be predominantly vasodepressor, and $\alpha\beta$ -MeATP primarily vasopressor in rats, either when anaesthetized (Cox & Smits, 1996) or pithed (Schlicker, Urbanek & Gothert, 1989). Thus $\alpha\beta$ -MeATP causes a vagally mediated reflex fall in BP, but the drug also raises BP by mechanisms that do not involve vagal afferents. We found that, in doses which antagonized the reflex bradycardia and apnoea, neither PPADS nor suramin reduced the vasopressor response to $\alpha\beta$ -MeATP in anaesthetized animals. We did not study the antagonists in pithed rat, a preparation in which Schlicker *et al.* (1989)

reported that PPADS antagonized the vasopressor response to $\alpha\beta$ -MeATP. Further information on the vascular aspects of the actions of ATP can be obtained from a recent review (Rongen, Floras, Lenders & Smits, 1997).

Neural recordings confirmed what the reflex studies had suggested, namely that vagal afferents, particularly those associated with pulmonary function were activated by $\alpha\beta$ -MeATP, and the mean ED₅₀ value obtained for neural activation did not differ significantly from those for reflex bradycardia or apnoea. Not all vagal afferent fibres were excited by the P2X agonist, and it was not feasible in this study to establish whether cardiac as well as pulmonary vagal sensory nerves contribute to the reflex. More complex experiments involving local application of drugs and selective denervation of particular cardiac or pulmonary vagal branches would be necessary. Other workers have demonstrated that ventricular receptors, particularly those in the left ventricle, are activated by veratrine and are mainly responsible for the B-J reflex caused by this drug in cat (see Paintal, 1955). It has also been shown that vagal C fibre afferents from the lungs (Hurt, Wang, Xu, Strierious & Pelleg, 1994; Pelleg & Hurt, 1996) and the left ventricle (Taneyama, Benson, Hild & Goto, 1997) in dog can be activated by ATP, probably via P2X receptors (Pelleg & Hurt, 1996), and as reviewed in Introduction, rat nodose neurones and vagal afferent fibres possess P2X receptors. Ventricular afferents which travel to the spinal cord via sympathetic nerve tracts (see Hainsworth, 1991) may be affected by P2X agonists and so contribute to some of the cardiovascular changes observed, particularly following vagotomy and cutting the carotid sinus nerves (e.g. Fig. 1), but this possibility was not investigated during the present study. Thus, it is probable that cardiopulmonary vagal afferents in rats are similar to those in dog in having P2X receptors closely associated with at least part of the sensory fibre population, and the question of whether these purinoceptors have a discrete physiological role in activating or modulating input from particular sensory nerves is intriguing.

The reflexes evoked by $\alpha\beta$ -MeATP were not secondary to release of endogenous 5-HT because the B-J reflex evoked by 2-Me-5-HT was antagonized by the 5-HT₃ antagonist MDL 72222, which had no significant effect on the responses to the P2X agonist, and PPADS antagonized vagal responses to $\alpha\beta$ -MeATP without affecting those to the 5-HT₃ receptor agonist. This evidence confirmed that cardiorespiratory reflexes evoked by $\alpha\beta$ -MeATP resulted from selective actions at P2X receptors associated with vagal afferents.

Arterial chemoreceptors

Chemoreflexes were rapidly activated by intravenous $\alpha\beta$ -MeATP or, at 100 times the dose, ATP, and the mean ED₅₀s for neural activation and reflex hyperventilation evoked by $\alpha\beta$ -MeATP were not significantly different. Cardiorespiratory reflexes evoked in the anaesthetized rat were therefore not confined to excitation of vagal afferents. ATP

excites carotid body arterial chemoreceptors in cat and dog (Jarisch, Landgren, Neil & Zotterman, 1952), and McQueen & Ribeiro (1983) proposed the presence of P2 purinoceptors in the cat carotid body based on results obtained using $\alpha\beta$ -MeATP. Recent studies on cat carotid body chemoreceptors *in vitro* showed that ATP surface receptors are present that can be transiently activated by an infusion of ATP, although the receptors were subsequently desensitized (Spergel & Lahiri, 1993).

The respiratory reflex and neural data obtained during the present study strongly suggest that the rat carotid body contains P2X receptors which can activate arterial chemoreflexes. The rapid onset of chemo-excitation in response to P2X agonists shows that it is not secondary to vasoconstriction within the carotid body, since changes in discharge following injection of vasoconstrictors are sluggish, being much slower in onset (> 5 s) and tending to last longer – potent vasoconstrictors such as endothelin-1 only evoke small increases in rat carotid chemoreceptor discharge (McQueen, Dashwood, Cobb, Marr, Bond & Spyer, 1995) compared with those we obtained. The purinoceptors in the rat carotid body seem to be relatively insensitive to desensitization by $\alpha\beta$ -MeATP, and neither PPADS nor suramin acted as antagonists in the doses used. This means that these carotid body purinoceptors differ from other $\alpha\beta$ -MeATP-sensitive P2X receptors that are antagonized by these compounds. It is possible to speculate that the receptors comprise different subunits (e.g. P2X₄, whose responsiveness to ATP or $\alpha\beta$ -MeATP (weak agonist) is unaffected or potentiated by PPADS or suramin in recombinant expression of receptors in *Xenopus* oocytes *in vitro* (Bo, Zhang, Nassar, Burnstock & Schoepfer, 1995)), but it has yet to be established how the pharmacology of recombinant subunits correlates with that of the native receptors *in vivo*. Other actions of suramin or PPADS (e.g. inhibition of ecto-ATPase) may mask an antagonist action at P2 receptors in the carotid body, although this seems unlikely since the same argument would apply to the bradycardia and apnoea, which were antagonized. Also $\alpha\beta$ -MeATP is relatively resistant to breakdown so should be unaffected by enzyme inhibition (see Humphrey *et al.* 1995). The apparent potentiation of the respiratory response to $\alpha\beta$ -MeATP after PPADS or vagotomy is probably secondary to abolition or reduction in the apnoea which normally masks part of the hyperventilation, an interpretation which is supported by chemosensory discharge evoked by $\alpha\beta$ -MeATP being largely unaffected by PPADS.

Our conclusions concerning P2X receptor subtypes located on a variety of sensory nerves being involved in triggering cardiorespiratory reflexes are based on indirect evidence obtained using agonists and relatively non-selective antagonists (Humphrey *et al.* 1995) in functional experiments *in vivo*. It is tempting to surmise that, because of their sensitivity to $\alpha\beta$ -MeATP the receptors associated with vagal afferents must be of the P2X₁, P2X₂, P2X₃ or P2X_{2/3} subtype(s), but this needs further investigation.

Further information from studies involving, for example, selective P2X receptor antagonists or, more directly, receptor autoradiography and immunocytochemistry, would provide useful additional evidence concerning the presence of particular $\alpha\beta$ -MeATP-sensitive P2X receptor subtypes in the heart, lungs, and carotid body.

Vagally mediated bradycardia and apnoea evoked during the B–J reflex, and also the carotid chemoreflex hyperventilation in vagotomized rats, could be utilized for convenient functional characterization of compounds affecting P2X receptors, in much the same way as this reflex was utilized in the development of 5-HT₃ receptor antagonists (Fozard, 1984).

The physiological significance of the B–J reflex has been the subject of considerable debate during the 130 years since it was first described, and it is commonly regarded as being a pharmacological curiosity. However, the presence of fast ATP-gated cation channels on sensory terminals in vital structures (e.g. left coronary artery and/or ventricle; pulmonary system), together with the local presence of the endogenous ligand ATP, strongly suggests a functional signalling role for ATP (see Zimmermann, 1994) and these P2X receptors. Their presence in the cardiopulmonary system and the carotid body could have pathophysiological significance, particularly when hypoxaemia or tissue damage results in substantial release of ATP from cells and nerve terminals, such as during increased activity in adrenergic nerves (e.g. in stress) that leads to co-release of ATP with noradrenaline (see Burnstock, 1988). These conditions may be mimicked pharmacologically by a P2X agonist activating the B–J cardiac nociceptive reflex, even in the presence of an anaesthetic agent which will depress the reflex. Whether non-noxious low level neural activity in these sensory pathways is part of the interoceptive homeostatic mechanism regulating the cardiovascular (e.g. see Linden, 1973; Hainsworth, 1991) and respiratory systems physiologically, and whether particular peripheral sensory receptors possess sub-populations of specific P2X receptors that enable locally released ATP to influence their sensitivity and activity discretely, are intriguing questions that require investigation. P2X receptors in the heart and lungs are potential therapeutic targets for the treatment of cardiovascular and respiratory disorders.

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