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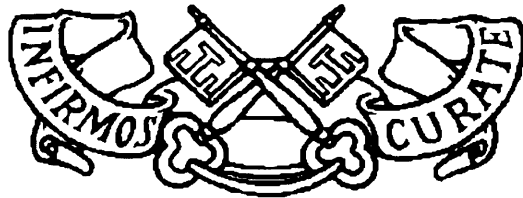# THE DETRUSOR ELECTROMYOGRAM AND PURINERGIC

MECHANISMS IN THE BLADDERS OF GUINEA-PIG AND MAN.

ANDREW BALLARO FRCS.

Institute of Urology

Royal Free and University College Medical School

University College London.

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ABSTRACT

It has been said that a technique capable of recording a urinary bladder electromyogram could be useful in the clinical evaluation of the detrusor neuropathies and myopathies implicated in the generation of lower urinary tract symptoms. However, in contrast to electromyography of skeletal and cardiac muscle, detrusor smooth muscle electromyography has remained in its infancy despite fifty years of scientific effort. The principal problems appear to be isolation of the real signal from artifacts, and uncertainties surrounding the existence of electromyographic activity during parasympathetically mediated muscle contraction. The discovery of purinergic neuromuscular transmission in overactive human bladder samples has renewed interest in detrusor electromyography as, in contrast to cholinergic mechanisms, purinergically mediated contractions can generate extracellular electrical activity. This thesis describes the development and validation of a novel technique for recording electrical activity from neurologically intact guinea-pig bladders and human detrusor *in vitro*. We characterise a purinergic electromyographic signal and show that detrusor taken from overactive human bladders has a greater propensity to generate electromyographic activity than normal by virtue of an aberrant purinergic mechanism. We discuss the potential role of electromyography in the clinical evaluation of a putative purinergic detrusor myopathy.

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GLOSSARY

α, β-mATP	Alpha, beta-methylene Adenosine tri-phosphate
Ach	Acetylcholine
ATP	Adenosine tri-phosphate
ANOVA	One way analysis of variance
CMG	Cystometrogram
ECG	Electrocardiogram
EJP	Excitatory junction potential
EFS	Electrical field stimulation.
EMG	Electromyogram
IOB	Idiopathic overactive bladder
mRNA	Messanger ribonucleic acid
NGF	Nerve growth factor
NOB	Neurogenic overactive bladder
OAB	Overactive bladder
OOB	Obstructed overactive bladder
P_{2x}	Subtype of ATP receptor
SD	Standard deviation
SEM	Standard error of the mean
TTX	Tetrodotoxin

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CHAPTER ONE. BLADDER ELECTRICAL ACTIVITY AND THE POSSIBLE UTILITY OF RECORDING IT IN PEOPLE.

1.1 Bladder electromyography.

Electromyography is the continuous recording of extracellular electrical activity generated during contractile activation of a muscular organ *in situ*. The technique relies on the existence of electrical currents in the milieu surrounding excitable cells, which generate voltage potentials that can be recorded between two suitably placed electrodes. These extracellular currents arise from ion movements across the muscle cell membrane during excitation contraction coupling.

In contrast to electromyography of cardiac and striated muscle, which have been developed over many years into advanced clinical sciences, no proven extracellular electrical activity has been recorded from human detrusor smooth muscle despite many reported attempts. In fact no electrical recordings from any smooth muscle containing human organ have been validated sufficiently for clinical use. By analogy to its utility in other muscles, detrusor electromyography was initially thought likely to give rise to detailed information into the mechanisms of excitation-contraction coupling in normal and diseased bladders, and their role in the generation of lower urinary tract symptoms [Corey et al, 1950]. Early experimental attempts at involved a variety of electrodes inserted blindly into the bladders of conscious volunteers during voluntary voiding. The increasing complexity of the methods subsequently employed reflects gradual appreciation of the difficulties involved.

1.1.1 The history of bladder electromyography.

In the early 1950s a group of investigators produced a series of reports describing a 'bio-electric bladder wave' recorded in humans using bi-polar silver ball electrodes pushed against the urothelium of the bladder wall (figure 1.1) [Corey et al,

1950, Boyce, 1952, Boyce et al, 1953, Vest et al, 1954]. This low frequency bi-phasic signal that was recorded from 105 subjects with normal and dysfunctional bladders, was several seconds in duration, coincident with intravesical pressure changes; an observation used to support its biological origin, and was said to vary characteristically in different bladder pathologies. The same authors also showed that a similar signal could be recorded from the excised, perfused bladders of a variety of animals, and increased in frequency and amplitude during pharmacostimulation of parasympathetic pathways.
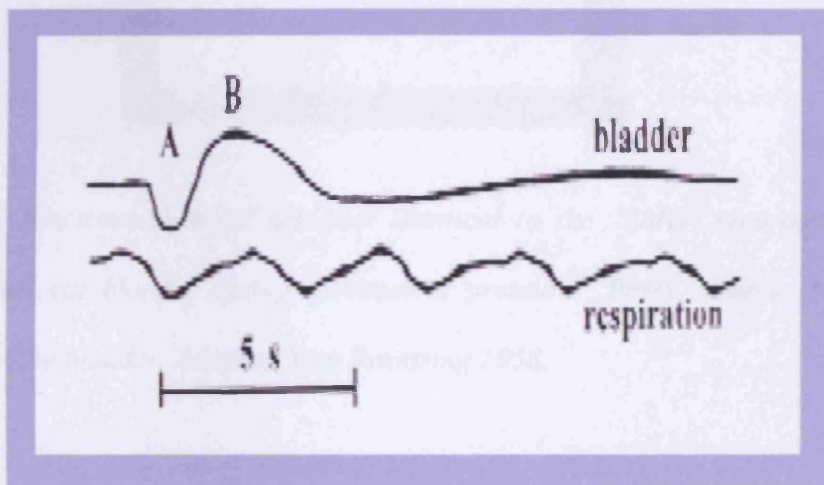
Figure 1.1 The first reported bladder electromyogram recorded from humans during voluntary voiding. Adapted from Corey et al. 1951. A and B denote the biphasic electromyographic signal.

The biological nature of the 'bladder wave' was corroborated by showing that the signal was independent of respiratory effort, the electrocardiogram, and surface potentials said to reflect skeletal muscle EMG recorded from the abdomen and limbs [Slater, 1953]. However, although initially accepted, later attempts to elicit electrical

signals from the exposed *in situ* bladders of anaesthetised cats revealed that an identical signal could be reproduced by pressure on the electrode and non-physiological movements of a dead bladder (figure 1.2) [Brunsting 1958]. The bladder wave was subsequently dismissed as artefact.
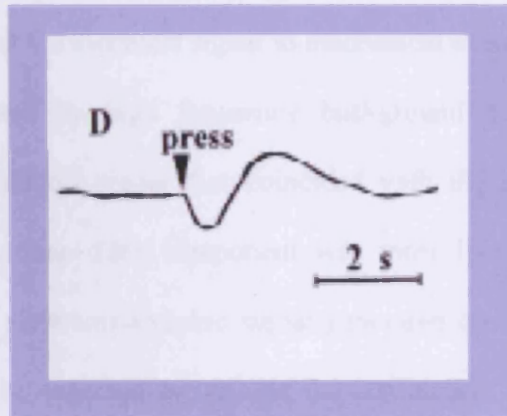
Figure 1.2 Electromechanical arterfact identical to the 'EMG' recorded from the anaesthetised cat bladder during mechanical pressure. 'Press' denotes mechanical pressure on the bladder. Adapted from Brunsting 1958.

In an approach designed to minimise gross movements of the recording electrode with relation to the bladder wall, Franksson and Petersen used bi-polar steel needle electrodes inserted through the urothelium into the underlying detrusor and recorded high frequency bursts of electrical activity from a number of conscious volunteers [Frankson and Petersen, 1953]. The signals persisted for some time after micturition, and were also evident during bladder filling. Nevertheless the authors reasoned that mechanical artefact had been excluded from their recordings as similar signals could be recorded using electrodes fixed to the bladder wall of an anaesthetised cat.

In later and more detailed clinical studies, flexible silver wire electrodes were inserted into the bladder wall using a hypodermic needle through the insensate anterior vaginal wall [Stanton et al, 1973, Jones et al, 1974]. Simultaneous recordings of the electrocardiogram, urethral sphincter EMG, and intravesical pressure changes were made in an attempt to account for extraneous physiological artefact, and to determine the real relationship of the electrical signal to mechanical events. The resulting 'bladder EMG' was dominated by high frequency background activity upon which was superimposed an electrical signal that coincided with the intravesical pressure rise during voluntary voiding. This component was most likely to reflect mechanical artefact as any real electromyographic signal generated by excitatory neuromuscular mechanisms would be expected to precede the contraction. Other studies conducted soon after suggested that high frequency signals originating from nearby skeletal musculature might also contaminate any real signal [Doyle et al, 1975].

In 1976 bladder electrical activity was investigated further in a carefully controlled study in anaesthetized cats designed to identify the 'real bladder electromyogram' by minimizing, with electronic filtering, the various artefacts contributing to the recordings (figure 1.3) [Craggs and Stephenson, 1976]. The authors found that electrical signals recorded in response to sacral ventral root stimulation (pre-ganglionic parasympathetic pathways) through platinum wire electrodes inserted into the bladder wall, were most clearly detected preceding a rise in intravesical pressure using a 10-40Hz-frequency band-pass filter. Furthermore, only activity in this same band was consistently related to intravesical pressure rises associated with reflex voiding [Craggs and Stephenson, 1982, 1985]. For signals above 40Hz the artefact was predominantly from nearby striated muscles, as evidenced by its elimination using succinylcholine. At frequencies below 10Hz the largest contaminant was movement of the bared platinum wire electrode tips in the bladder wall, causing electromechanical

artefact at least ten times greater in magnitude than the purported bladder EMG in the restricted band, that was almost certainly obscuring most of the real electromyographic signal. Interestingly, this large artefact could be replicated by artificial filling of an isolated, perfused but dead bladder, and was probably generated by the movement of fluid and tissue over the electrode during contraction.
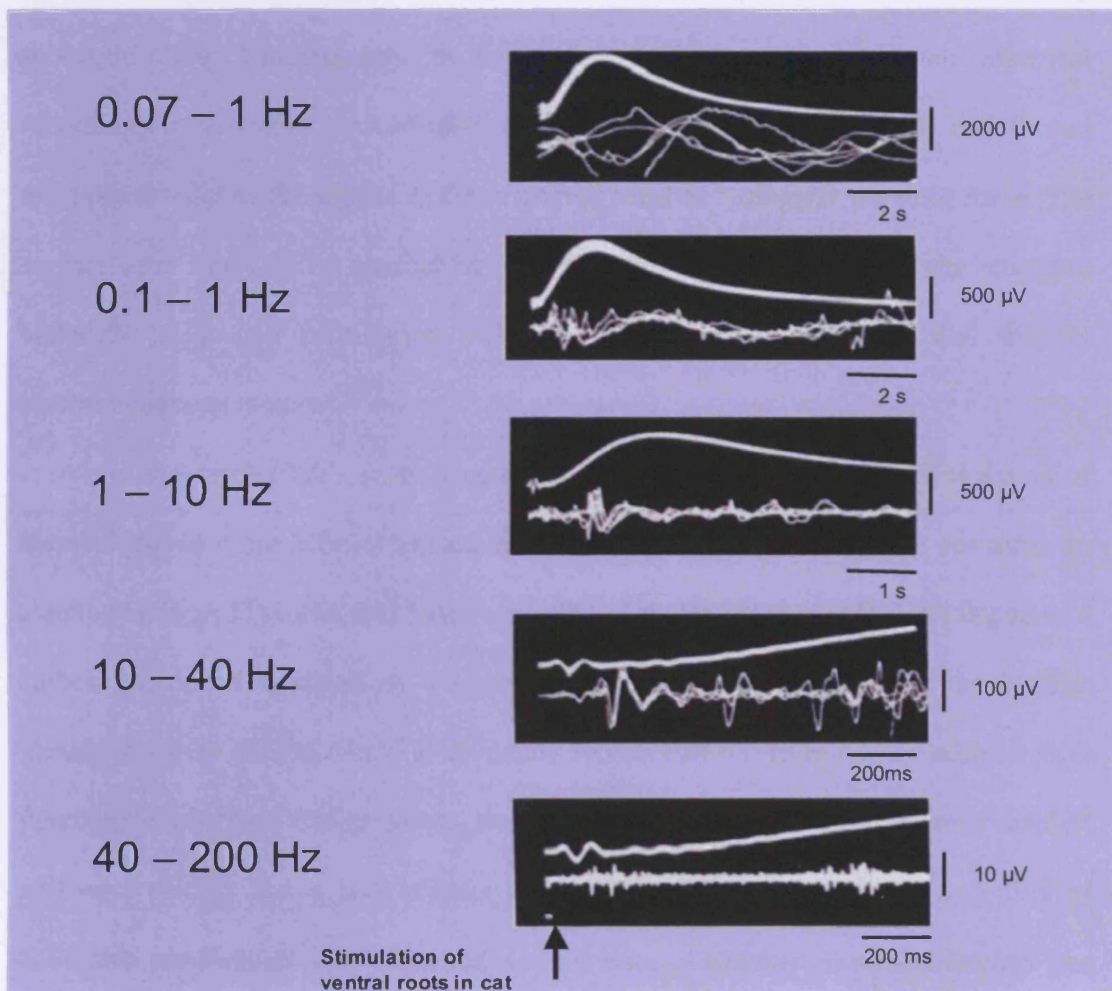
Figure 1.3 The 'real' bladder electromyogram. Filtered electrical recordings (lower traces) with intravesical pressure change (upper trace). Adapted from Craggs and Stephenson 1976.

Using these methods clinically, Nanninga and Kaplan attempted to validate signals recorded clinically from neuropathic bladders [Nanninga and Kaplan, 1978]. They excluded signals below 1Hz from their recordings, and found electrical activity in a 5-150 Hz range that appeared to vary characteristically between subjects with upper and lower motor detrusor neuropathies. In contrast to Craggs and Stephenson they concluded that signals of over 40Hz might still represent electromyographic activity from the detrusor not skeletal muscle, as in this case they could not be abolished by succinylcholine. Unfortunately in this study the relationship of their electrical recordings to the reflex-evoked bladder contractions was not described, and further attempts to validate the signals in the restricted band as biological were not made. The signals were likely to be artefact by virtue of the high impedence of the electrode materials used, and contributed little to the subject of recording real bladder electromyograms in man.

In the early 1980's studies were conducted to investigate whether isolation of the real signal from electromechanical artefact could be achieved by advances in electrode design [Takaiwa and Shiraiwa, 1984, Takaiwa et al, 1983]. Low impedence carbon electrodes mounted on a urethral catheter and pressed against the bladder urothelium were used to record a 'clinically relevant EMG' from twenty subjects with neuropathic bladders. Unfortunately, the signals appeared to be predominantly artefact and were evident during both bladder filling and voiding. Furthermore, only limited validation experiments were presented, and the issue of whether movement artefact was contaminating the signals, was not conclusively addressed.

In 1996 a further methodological attempt was made in the search for the detrusor electromyogram [Kinder et al, 1996]. This involved a novel electrode device consisting of an octagonal array of silver wire electrodes from which propagated spontaneous electrical activity was recorded from the serosal surface of a post-mortem

isovolumetric rabbit bladder preparation. Although the signals recorded in a 0.7-40Hz restricted frequency band were convincing in morphology, they were recorded from an unstimulated preparation independently from detrusor contraction. As a result their biological origins and relationship to mechanical events remained unclear. Furthermore, even if recordings from such an array of electrodes were real, it is unlikely that this device could be clinically useful.

Interestingly, a reversible mono-polar suction electrode incorporated into a urethral catheter was used by Shafik to record electrical signals from the dome and trigone of human bladders referenced an electrode fixed to the abdominal wall during voluntary voiding [Shafik, 1998]. 'Pacesetter potentials' and 'action potentials' were recorded from the dome at rest, and increased in frequency during voiding in contrast to trigonal action potentials, which did not change. From this work, a passive role for the trigone during micturition was inferred. Unfortunately, no effort to confirm the nature of the supposed EMG or to control for mechanical artefact, which was likely to be a major contributor to the recordings, was made. Furthermore, the duration of the monophasic 'action potentials' recorded was approximately four seconds, a significantly long component likely to be far in excess of any real biological electrical event, and the signals bore an inconsistent relationship to detrusor contractions. The potentials were almost certainly a direct reflection of gross bladder movements rather than detrusor smooth muscle depolarisation, and interestingly were not dissimilar in nature to the 'ureterogram' [Shafik, 1997a], 'vasogram' [Shafik, 1996], 'electrorenogram' [Shafik, 1997b] 'cholecystogram' [Shafik, 1998a] and 'electroorchidogram' [Shafik, 1998b] described by the same author.

In 1998 detrusor electromyography was further investigated in a more careful study [Scheepe et al, 1998]. Gold-plated needle electrodes attached to the exposed *in situ* bladders of anaesthetised rats were used to record electrical activity generated

during pharmacologically evoked isovolumetric detrusor contractions. Studies designed to identify and minimise physiological artefacts arising from diaphragmatic and intestinal muscle activity were conducted, and an electrical signal, that increased in amplitude and frequency on bladder filling, was identified as a likely real detrusor EMG. However, the signal could also be recorded preceding carbachol-evoked contractions, a finding contradicting their biological origin as cholinergic analogues probably initiate detrusor contractions without generating an EMG (see below). Furthermore, although the need for further investigation into the influence of local bladder contractions on the signal was recognised, the recordings appeared overwhelmed by artefact, undoubtably exacerbated by the use of high impedence gold electrodes, and bore an uncertain relationship to mechanical events.

In comment, Craggs emphasised the need to isolate the probably very small real electromyographic signal from the large electromechanical artefact generated as the tissue contracts [Craggs, 1998]. Furthermore, he described a series of criteria designed to validate any future electromyographic recordings as biological. These were; proof of elimination of, and accounting for all possible extraneous artefacts such as electrical activity generated by contracting striated and cardiac muscle, demonstration that the electrical changes always precede rather than coincide with the mechanical event, demonstration that the electrical signal reflects neuromuscular transmission, ie. is evoked by nerve-mediated stimulation rather than pharmacostimulation, and is not the neuronal action potential, and proof that the signals are not electromechanical in origin.

In response, a further attempt to record bladder electrical activity used low impedence Ag/AgCl electrodes implanted into the bladders of anaesthetised dogs [Scheepe et al, 1999]. Detrusor contractions were evoked by sacral anterior root stimulation, and attempts were made to correlate the evoked electrical signal with detrusor contractions. Electrical activity was recorded during isovolumetric bladder

contractions, and the signals 'spectral density' was analysed in an attempt to identify a frequency band that consistently preceded the onset of mechanical activity. This approach produced a high frequency 'real' electrical signal that preceded rises in intravesical pressure, contaminated by a constant lower frequency signal that was presumed to be artefact. Unfortunately no evidence of the biological nature of this high frequency signal other than its temporal relationship to the contraction was presented, and the recordings remained overwhelmed by artefact.

AUTHORS	SUBJECT	ELECTRODE	CONTROL EXPERIMENTS	SIGNAL
Corey & Boyce, Slater, 1950-53	humans	silver ball (urothelial)	none	mechanical artefact
Frankson 1953	humans	steel needle (embedded)	none	mechanical artefact (evident during filling)
Stanton 1973	humans	silver wire	ECG, striated EMG	mechanical artefact
Jones 1974	humans	silver wire	respiritory movement	movement artefact (respiratory)
Craggs & Stephenson 1976	cat	platinum wire	filtered, striated EMG artefact	possible real signal
Nanninger & Kaplan 1978	humans	platinum wire	none	mechanical artefact
Takaiwa & Shiraiwa 1984	humans	carbon wire	none	mechanical artefact (evident during filling)
Kinder et al. 1996	unstimulated live rabbit	silver multi-needle electrode	filtered	uncertain (no nerve-mediated contraction)
Shafik 1998	humans	reversible suction, (abdominal wall)	none	movement artefact (long signals)
Scheepe et al. 1998	rats	gold needle	skeletal EMG, carbachol	movement artefact
Scheepe et al. 1999	live dogs	reversible silver	spectral analysis	uncertain (high artefact)

Table 1.1 Summary of previously reported attempts at bladder electromyography.

1.1.2 The problem with detrusor electromyography.

Human detrusor has only been presumed to generate electromyographic activity by analogy to striated muscle, which in accordance with its more excitable function contracts by a different mechanism. Thus in contrast to striated muscle in which the tightly coupled relationship between electrical and mechanical events had been fully elucidated before the development of electromyography, the electrophysiological basis of detrusor smooth muscle contraction remains unclear.

The striated muscle EMG is a large readily recordable signal that reflects the net potentials generated by transmembrane sodium currents in geometrically aligned muscle fibres. These are arranged within discreet motor units and therefore depolarise synchronously leading to temporal summation of currents and a larger extracellular signal [Brading, 1987]. In contrast, the human detrusor smooth muscle action potential, which has only been recorded using intracellular electrodes in isolated single cells, is calcium dependent [Filo et al. 1965, Montgomery and Fry, 1992]. If action potentials are generated during detrusor contractions *in situ*, the resultant extracellular currents would be expected to be much smaller than those arising from striated muscle, as the detrusor cell membrane expresses a much lower population of calcium channels than striated muscle does sodium channels [Brading, 1987]. Furthermore, as detrusor fibres appear to be arranged randomly and activated asynchronously by *en passant* neuronal connections [Brading, 1987], extracellular currents would be less likely to summate and therefore smaller and more vulnerable to contamination by the relatively large electromechanical artefact generated at the tissue-electrode interface as the muscle contracts (figure 1.4). This artefact consists of fluctuating potentials caused by disturbance of the electrical double layer that develops around any pure metal electrode by movement at the tissue-electrode interface, and generates artefact in the recording [Cooper and Binnie, 1995].

Striated And Smooth Muscle

Pelvic (Parasympathetic)
Motor Pathway

?

Detrusor
Smooth
Muscle

Pudendal
(Somatic)
Motor Pathway

Sphincter
Striated
Muscle

10mV

100ms
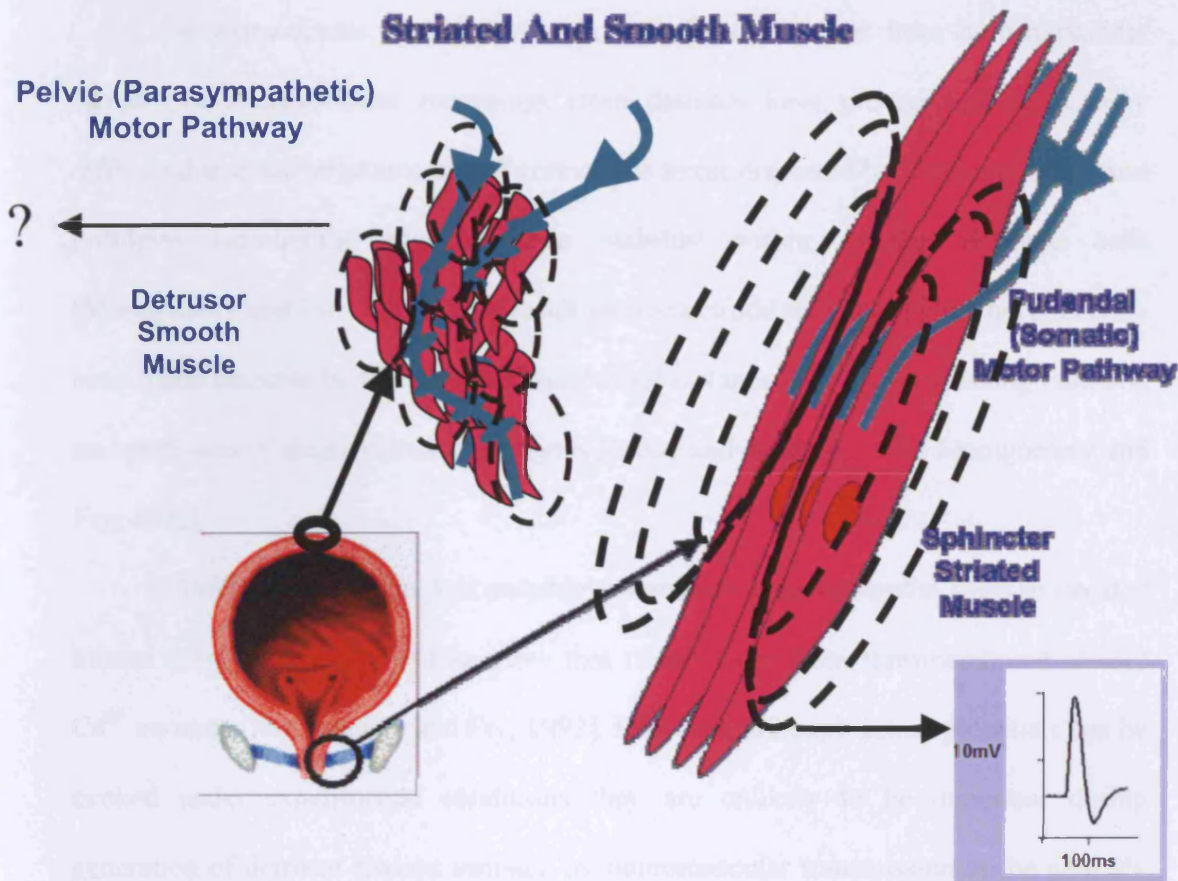
Figure 1.4 Schematic diagram showing anatomical arrangement and pattern of innervation in smooth and striated muscle with summed currents in striated muscle producing an extracellularly recordable electrical signal.

1.1.3 Does the bladder electromyogram exist?

That contraction of animal detrusor generates electrical activity was first shown by Ursillo, who described spontaneous and electrically evoked intracellularly recorded potentials from isolated rabbit detrusor smooth muscle strips [Ursillo, 1961]. Both intracellular and extracellular signals (using the double sucrose gap) were subsequently recorded in the guinea-pig *in vitro*. These contained an initial component comprising the excitatory junction potential (EJP) onto which was superimposed an action potential that

was later shown to be dependent on Ca^{2+} availability and Ca^{2+} channel activity [Creed et al., 1983, Mostwin 1986].

No extracellular electrical signals have been recorded from human bladder samples, and intracellular recordings from detrusor have proved technically very difficult due to the large amounts of connective tissue dispersed between myocytes, and problems maintaining microelectrode stability within moving detrusor cells [Montgomery and Fry, 1992]. As a result microelectrode recordings have only recently been made possible by the advent of single cell isolation techniques enabling isolation and perfusion of single detrusor myocytes [Inoue and Brading, 1991, Montgomery and Fry, 1992].

Under voltage clamp, it is possible to evoke an action potential from an isolated human detrusor myocyte and to show that it originates from transmembrane inward Ca^{2+} currents [Montgomery and Fry, 1992]. However, although action potentials can be evoked under experimental conditions they are unlikely to be important during generation of detrusor tension initiated by neuromuscular transmission in the neurally intact bladder; Action potentials cannot be recorded during stimulation of detrusor myocytes by the muscarinic analogue carbachol, and the carbachol-induced intracellular Ca^{2+} rise is unaffected by membrane Ca^{2+} channel blocking agents but sensitive to inactivation of the ryanodine receptors that regulate intracellular Ca^{2+} stores. This implies that the cytosolic Ca^{2+} needed for parasympathetically mediated contractile activation and that generates intracellularly recorded currents is mobilised from intracellular stores in the absence of membrane depolarisation rather than extracellularly [Wu et al., 1999]. If the same is true of cholinergic mechanisms in intact detrusor, contractions of normal human detrusor, which are exclusively generated by cholinergic neuromuscular transmission, are unlikely to be accompanied by membrane depolarisation or an EMG.

1.1.4 Summary.

In contrast to electromyography of skeletal muscle, which has been developed over many years into an advanced clinical science, detrusor smooth muscle electromyography has remained in its infancy. Many attempts to record an electromyogram from the bladders of conscious volunteers and animal bladders have been reported, however an artefact-free electromyogram has yet to be verified as real. The problems appear to be two fold: The anatomical differences between smooth and striated muscle indicate that any detrusor extracellular electrical activity is likely to be far smaller than that which can readily be recorded from striated muscle, and therefore vulnerable to contamination by the electromechanical artefact generated as the tissue contracts. Furthermore, normal human detrusor contracts solely as a result of cholinergic neurotransmission and if the implications of intracellular electrical recordings are applicable to the *in situ* organ, would not be expected to generate an electromyogram.

1.2 Purinergic activity in the bladders of guinea-pig and man.

1.2.1 Purinergic neuromuscular transmission in animal bladders.

In contrast to healthy human detrusor, bladder contractions in many small animals are normally co-mediated by purinergic (ATP acting on membrane bound P_{2x} receptors) and cholinergic neurotransmission [Langley and Anderson 1895, Ambrache and Zar 1970, Burnstock et al. 1972, Burnstock et al. 1978]. There is substantial evidence to suggest that acetylcholine and ATP are co-released by detrusor motor nerves; Small ATP-containing agranular vesicles have been visualised alongside the large acetylcholine-containing opaque vesicles in the same parasympathetic neurones [Hoyes et al. 1975], and detrusor excitatory neurotransmission can be completely abolished by the selective parasympatholytic agent botulinum toxin, an agent that only affects cholinergic nerves [MacKenzie et al. 1982].

However ATP appears to be more readily released than acetylcholine, and the ratio of the cholinergic to purinergic response to nerve stimulation increases with the intensity of the evoking stimulus *in vitro* [Brading and Williams 1990, Sibley 1984, Brading and Mostwin 1989]. As a result, a marked temporal separation of the post-synaptic effects of purinergic and cholinergic excitation occurs, resulting in a bi-phasic response to nerve stimulation. The initial response is fast acting, transient and mediated by ATP, the later phase slower, more tonic, and cholinergic [Chancellor et al. 1992, Krell et al. 1981, Levin et al. 1986, Bolegro et al. 1995]. The timecourse of these responses corresponds to the duration of the intracellular Ca^{2+} rise produced by the two transmitters [Oike et al. 1998, Hashitani et al. 2000], and may have functional relevance.

In small animals, purinergic neurotransmission is thought to initiate bladder contractions, while cholinergic mechanisms regulate their duration [Chancellor et al. 1992, Craggs et al. 1986, Maggi et al. 1987]. However, in the baboon and rhesus

monkeys (Old World primates) bladder contractions are mediated purely by cholinergic mechanisms (figure 1.5) [Craggs et al. 1986]. It has been proposed that this difference relates to the territorial marking behaviour displayed by many small animals, which requires a rapid, transient bladder contraction, in contrast with the tendency of more socially advanced Old World primates to practice urinary continence and more sophisticated methods of territorial marking [Craggs et al. 1986].
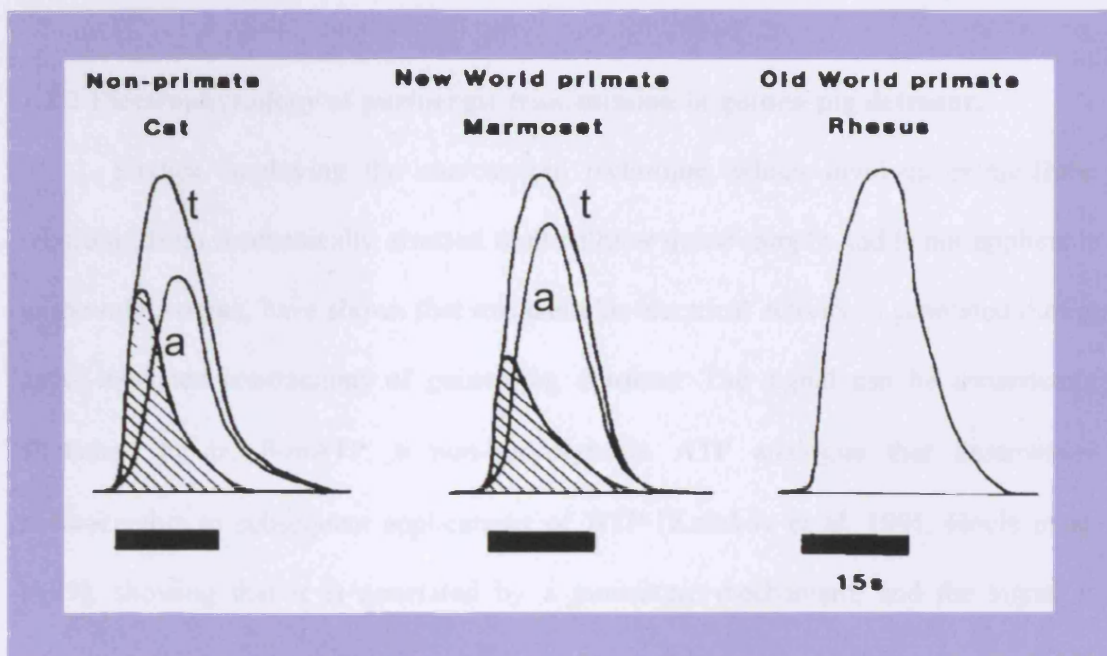
Figure 1.5 Putative non-cholinergic neuromuscular transmission in the bladders of new world but not old world primates. Adapted from Craggs et al. 1986. Traces represent: Total intravesical pressure (t), atropine-sensitive contraction (a) and atropine-resistant, presumed purinergic response (hatched area) evoked by sacral nerve stimulation. Bars represent stimulus.

Interestingly, changes in the relative contribution of purinergic and cholinergic neurotransmitter to the total bladder contraction in association with experimentally induced disease states have been observed in animal models. In detrusor taken from the

bladders of chronic spinal rats, an increase in the cholinergic and decrease in the purinergic components of the nerve-mediated contraction when compared with controls has been demonstrated, and a post-synaptic mechanism for this plasticity discounted as there was no difference in the responses to exogenous agonists [Yokota and Yamaguchi 1996]. In contrast, an increase in purinergic and associated decrease in cholinergic components has recently been reported in detrusor taken from partially obstructed guinea-pig bladders [Calvert et al. 2001].

1.2.2 Electrophysiology of purinergic transmission in guinea-pig detrusor.

Studies employing the sucrose-gap technique, which involves extracellular recording from mechanically arrested multicellular tissue sample and is not applicable to the intact organ, have shown that extracellular electrical activity is generated during nerve-mediated contractions of guinea-pig detrusor. The signal can be consistently abolished by α, β-mATP, a non-hydrolysable ATP analogue that desensitises purinoceptors to subsequent applications of ATP [Kasakov et al. 1991, Hoyle et al. 1989], showing that it is generated by a purinergic mechanism, and the signal is coupled to the early transient phase of the associated detrusor contraction. Atropine has no effect on the electrical signal, but abolishes the later phase of the contraction [Hoyle and Burnstock 1985, Fujii 1988, Brading and Mostwin 1989, Bramich and Brading 1996, Hashitani and Suzuki 1995, Hashitani et al. 2000]. Furthermore, microelectrode studies show that activation of guinea-pig detrusor purinoceptors by ATP initiates a voltage-gated transmembrane Ca^{2+} current and membrane depolarisation, while pharmaco-stimulation of muscarinic receptors triggers Ca^{2+} release from intracellular stores, independently from changes in membrane potential [Iacovou et al. 1990, Bo and Burnstock 1990, Nakayama, 1993, Wu et al. 2002].

1.2.3 Purinergic neuromuscular transmission in human derusor.

Although nerve-mediated contractions of human detrusor samples are reliably abolished by atropine, indicating their exclusively cholinergic origin [Hindmarsh et al. 1977, Sibley 1984, Palfrey et al. 1984, Kinder and Mundy 1985, Speakman et al. 1988, Bayliss et al. 1999], human detrusor strips contract vigorously in response to applications of ATP [Hustead et al. 1983, Palea et al. 1993, Sjorgren et al. 1982, Bayliss et al. 1999]. Human detrusor therefore appears equipped with the necessary apparatus to respond to purinergic neurotransmission, but which is presumably redundant under physiological conditions.

However, nerve-mediated atropine resistant contractions have recently been demonstrated in detrusor samples taken from overactive human bladders by several groups [Sjorgren et al. 1982, Palea et al. 1993, Bayliss et al. 1999, O'Reilley et al., 2000]. Indirect evidence is accumulating to suggest that these are mediated by purinergic neuromuscular transmission, and are likely to be important in the pathogenesis of detrusor overactivity. However, purinergic activity is not always found under experimental conditions [Kinder and Mundy 1987, Mills et al, 2000], has been disputed [Tagliani et al, 1997], and has only been demonstrated indirectly during tension generation experiments.

Atropine-resistant detrusor contractions were first recorded in detrusor samples taken from cystometrically characterised overactive bladders [Sjogren et al, 1982]. The same group suggested a purinergic mechanisms as the cause [Hustead et al. 1983], and this was supported by showing that atropine-resistant activity could be abolished by α, β-mATP. The existence of a purinergic contractile mechanism in dysfunctional human detrusor was further supported by recordings of atropine-resistant, α, β-mATP-sensitive nerve-mediated contractions in detrusor samples taken from patients with interstitial cystitis [Palea et al, 1993]. This was corroborated by a larger study that

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compared contractile responses of small detrusor strips taken from obstructed, neurogenic and idiopathically overactive bladders, with tissue from functionally normal controls [Bayliss et al, 1999]. Although the samples from both groups contracted with similar magnitude in response to exogenous ATP, a nerve-mediated atropine-resistant component that could be consistently abolished by α, β-mATP was found contributing up to a third of the total in the overactive but not the control groups (figure 1.6). With further analysis it was found that these experimentally evoked purinergically mediated contractions occurred only in the samples of idiopathically overactive and obstructed, but not neurogenic overactive detrusor.
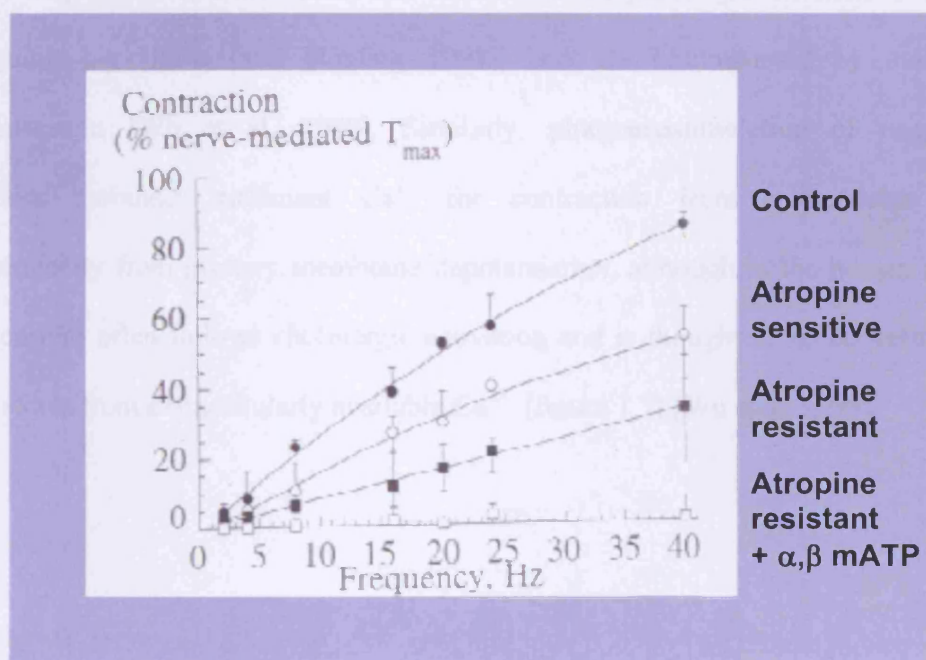
Figure 1.6 Atropine resistant α, β-mATP-sensitive contractions in overactive human detrusor. Adapted from Bayliss et al. 1999.

Excitatory purinergic neurotransmission has also been reported in aged human bladders in which the contribution of ATP-mediated contractions to the nerve-mediated total has been reported to increase significantly between the ages of forty and eighty, and is accompanied by an associated decrease in cholinergically mediated activity [Yoshida et al. 2001].

1.2.4 Electrophysiology of purinergic transmission in human detrusor.

Although no extracellular electrical recordings have been made from human smooth muscle, intracellular recordings in voltage-clamped isolated human detrusor myocytes show that applications of ATP generate large inward Ca^{2+} currents. These appear very similar in time course and intensity to the purinergic transients evoked in the guinea-pig [Inoue and Brading 1991], and are accompanied by membrane depolarisation [Wu et al. 1999]. Similarly, pharmacostimulation of muscarinic receptors mobilises sufficient Ca^{2+} for contraction from intracellular stores independently from primary membrane depolarisation, although in the human a small Ca^{2+} current often follows cholinergic activation and is thought to reflect refilling of these stores from extracellularly available Ca^{2+} (figure 1.7) [Wu et al. 1999].

Cholinergic

Carbachol

Purinergic

ATP
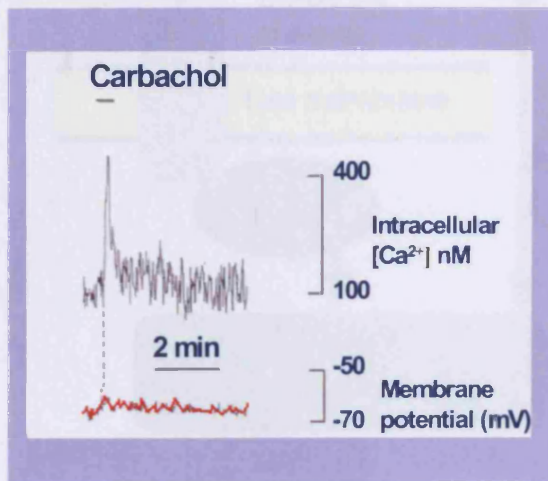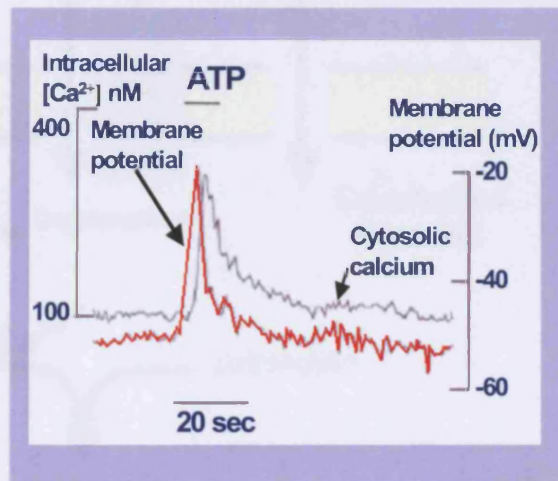
Figure 1.7 Cholinergic and purinergic effects on action potential generation in isolated human detrusor myocytes. Adapted from Wu et al. 1999.

These findings suggest that whereas normal human detrusor activated solely by muscarinic receptor excitation is likely to be electromyographically inert, the emergence of purinergic neuromuscular transmission in dysfunctional human detrusor may be associated with the generation of extracellular electrical activity, and therefore an electromyogram. Figure 1.8 shows a schematic representation of the probable mechanisms of cholinergic and purinergic detrusor contractile activation through Ca^{2+} mobilisation.
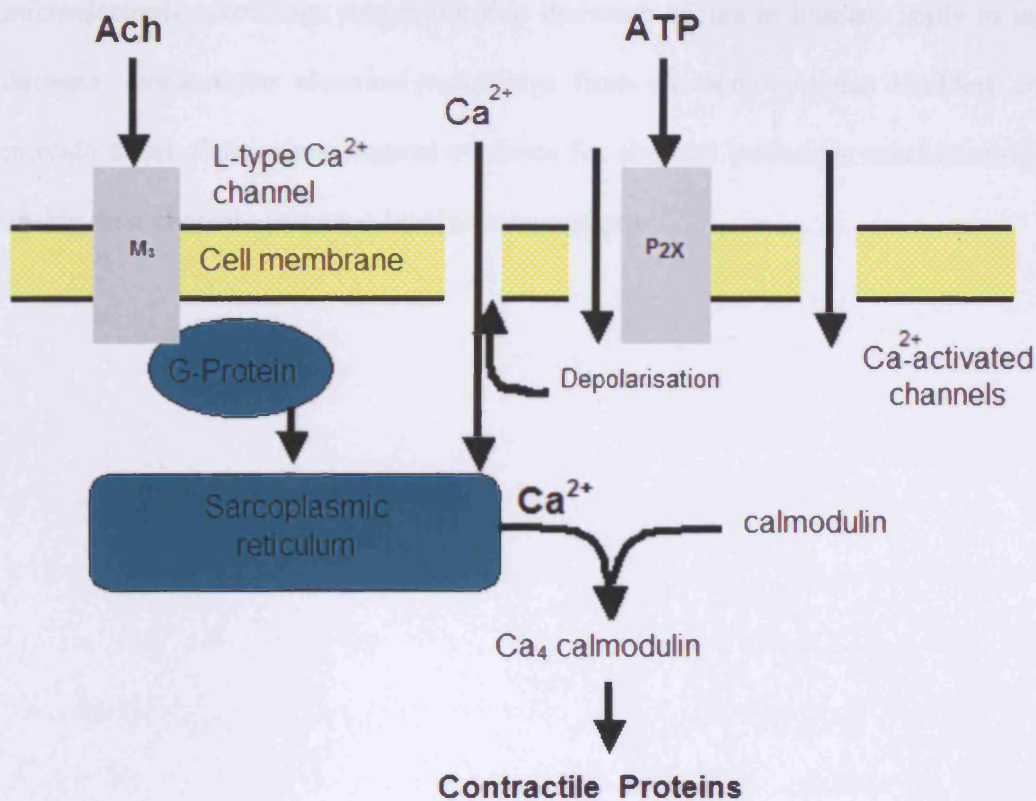
Figure 1.8 Schematic diagram showing probable mechanisms of cholinergic and purinergic excitation contraction-coupling in detrusor. Adapted from Wu et al. 1999.

1.2.5 Summary.

Purinergic neuromuscular transmission contributes to bladder emptying in small animals, and initiates a rapid transient contraction that may be important during territorial marking behaviour. In contrast, normal human detrusor is activated exclusively by a cholinergic mechanism, but is equipped with the cellular apparatus necessary to respond to ATP. Indirect evidence is accumulating to suggest that purinergic neurotransmission becomes pathologically expressed in the idiopathically and obstructed but not neuropathic overactive human bladder, and this may represent a vestigial mechanism. In animal bladders purinergic detrusor activation generates extracellular electrical activity wheras cholinergic mechanisms do not. If the results of

microelectrode recordings suggesting that the same occurs in humans apply to intact detrusor, extracellular electrical recordings from overactive human bladders could provide direct electrophysiological evidence for aberrant purinergic mechanisms, and enable their clinical evaluation by electromyography.

1.3 The basis for a purinergic myopathy in the overactive bladder.

1.3.1 Detrusor overactivity.

Detrusor overactivity is currently defined as the presence of involuntary bladder contractions detected during cystometric bladder filling [Abrams et al. 2002]. Overactive bladder contractions are associated with the symptom complex of urinary urgency, frequency and nocturia with or without urge incontinence, termed the overactive bladder syndrome (OAB) [Abrams et al. 2002], but may also occur in asymptomatic subjects [Van Doorn et al. 1990, Neal 1994, Digesu et al. 2003, Bradshaw et al., 2005]. There is evidence that no bladder is actually stable and that spontaneous low amplitude contractions of isolated areas of detrusor wall (micromotions) with coordinated relaxation of adjacent segments resulting in no overall pressure change normally exist, with contractions of higher amplitude and frequency associated with OAB symptoms [Van Duyl 1985, Coolsaet and Blaivas 1985, Drake et al. 2005]. In the absence of sensory disturbance, detrusor overactivity may be secondary to central neuropathy, bladder outflow obstruction, or as yet unknown insults when it is termed idiopathic.

The overactive bladder syndrome is one of the largest causes of morbidity in the community [Brocklehurst, 1993], affecting up to 2% of men and 9% of women under the age of 65, and 7% and 12% respectively in the older age group [Thomas et al. 1980]. Urinary urgency is the principal symptom associated with treatment seeking behaviour and is probably caused by activation of detrusor stretch receptors during bladder wall micromotions [Kuru, 1965, Coolsaet and Blaivas 1985, Coolsaet et al. 1993]. Behavioural modification and anti-muscarinic pharmacological agents form the mainstay of treatment. However, these have limited efficacy and anticholinergic drugs are poorly tolerated due to systemic side effects [Herbison et al. 2003]. Surgical bladder

augmentation is available for drug refractory severe cases, but carries high morbidity and is generally not suitable in the elderly.

Although indirect *in vitro* evidence suggests that aberrant purinergic neurotransmission is important in the pathogenesis of detrusor overactivity, the mechanisms involved remain unclear. No increase in the force or time-course of nerve-evoked contractions occurs in overactive detrusor *in vitro*, as would be expected if an additional neurotransmitter were contributing to them [Bayliss et al. 1999]. Furthermore the troublesome contractions of the *in situ* overactive bladder occur during urine storage in the apparent absence of motor nerve activity. The physiological changes that occur in samples of overactive detrusor and the possible role of purinergic mechanisms in its pathogenesis are discussed below.

1.3.2 Spontaneous detrusor activity.

A higher incidence of spontaneous contractile activity *in vitro* from animal models of bladder outflow obstruction [Speakman et al. 1987, Sibley 1987, Buckner et al. 2002], and human detrusor smooth muscle strips taken from neuropathic [Kinder and Mundy 1987, German et al. 1995], obstructed [Brading 1997], and idiopathically overactive [Kinder and Mundy 1987, Mills et al. 2000] bladders has been reported. This takes the form of fused tetanic contractions that are rarely seen in normal tissue [Brading 1997], and has obvious analogy to overactive bladder contractions *in situ*. However, Levin found that no amount of filling of whole, intact rabbit bladders produced spontaneous contractile activity which only occurred when the bladder wall was longitudinally stretched, and suggested that spontaneous activity *in vitro* was experimental artefact and unlikely to occur *in situ* [Levin and Ruggieri 1986].

Spontaneous detrusor contractions appear to be a myogenic phenomenon, as they are resistant to neurotoxins and to pharmacological blockade of muscarinic

receptors and adrenoceptors [Buckner et al 2002, Levin et al. 1986]. However, although independent of neuromuscular transmission, spontaneous detrusor contractions are sensitive to selective blockade of both membrane-bound L-type Ca^{2+} channels, and the ryanodine-sensitive receptors on the sarcoplasmic reticulum that control Ca^{2+} release from intracellular stores. This implies that Ca^{2+} mobilisation from both extracellular and intracellular stores is important in their generation [Buckner et al. 2002]. In guinea-pig at least, spontaneous contractions are generally associated with electrical potentials [Bramich and Brading 1996, Hashitani et al. 2000].

The relationship between spontaneous mechanical and electrical activity in human detrusor has yet to be determined and will require a technique that enables extracellular electrical recordings from intact moving detrusor samples. However, spontaneous Ca^{2+} transients have been recorded from single cell preparations, and are seen in a significantly greater numbers of cells isolated from samples of overactive human bladders compared to those from functionally normal controls. This suggests that abnormalities of Ca^{2+} signalling and spontaneous membrane electrical activity may be important in the genesis of overactive bladder activity [Wu et al. 1997, Fry and Wu 1998].

The human detrusor cell membrane potential normally exhibits small spontaneous fluctuations [Sui et al. 2001]. These probably reflect interaction between an inward Ca^{2+} current, and an outward K^+ current through Ca^{2+}-activated channels (BK channels). These are normally opened under conditions of raised intracellular Ca^{2+} [Wu et al. 2002]. BK channels are therefore likely to function as part of a negative feedback mechanism to limit Ca^{2+} influx [Herrera et al. 2000, Wu et al. 2002]. The frequency of spontaneous transient outward currents generated by BK channel activation appear to mirror increases in intracellular $[Ca^{2+}]$, and also levels of stored Ca^{2+} [Wu et al. 2002]. Intracellular Ca^{2+} store levels in turn are probably regulated via

L-Type Ca^{2+} channels, and also reflect changes in extracellular and intracellular $[Ca^{2+}]$ [Ganitkevich and Isenburg 1992, Wu et al. 2002]. A dynamic equilibrium between levels of cytosolic, extracellular and stored Ca^{2+} therefore appears to exist.

It has been proposed that spontaneous detrusor electrical and mechanical activity may result from disruption of this equilibrium under conditions that predispose to increased intracellular $[Ca^{2+}]$ such as an aberrant Ca^{2+}- mobilising neuromuscular transmitter such as ATP [Fry 1997]. The rapid and relatively large Ca^{2+} transients generated by purinoceptor activation would tend to saturate Ca^{2+} storage mechanisms and predispose to small abnormal intracellular $[Ca^{2+}]$ movements. These could activate voltage-gated membrane Ca^{2+} channels in the absence of a neuronal stimulus allowing further Ca^{2+} influx, and thereby generate spontaneous contraction of the affected cell [Fry 1997]. In support of this theory, spontaneous detrusor contractions are particularly evident under experimental conditions likely to raise intracellular $[Ca^{2+}]$ such as intracellular acidosis [Gallegos and Fry 1994], and a single application of either ATP or carbachol to cells taken from unstable bladders can initiate prolonged intracellular Ca^{2+} transients, which are not seen in normal cells [Wu et al. 1997]. Interestingly, a similar mechanism occurs in guinea-pig jejunal smooth muscle, in which increases in cytosolic Ca^{2+} initiate Ca^{2+} influx via a voltage-gated mechanism, and in cardiac muscle where Ca^{2+} overload induced by the administration of glycosides results in irregular electrical activity and arrythmias [Pacaud and Bolton 1991, Wendt 1989, Fry 1997].

1.3.3 Pathophysiology of purinergic activity in overactive detrusor.

Applications of ATP generate significantly greater contractions in detrusor strips taken from idiopathically unstable and obstructed human bladders than in stable controls indicating a myogenic mechanism [Harvey et al. 2002]. However, the half-maximal concentrations of ATP and carbachol required to generate the intracellular

Ca^{2+} transients necessary for contraction do not differ significantly in single myocytes originating from normal and overactive bladders [Wu et al. 1999]. This suggests that cells from healthy and overactive detrusor are equally sensitive to agonists acting at purinoceptors and muscarinic receptors, and implicates a pre-synaptic mechanism in the generation of purinergically-mediated contractions. Whether increased proportional pre-synaptic release of ATP, or reduced ATP breakdown in the synaptic cleft is responsible remains unclear. However evidence is accumulating to suggest that the latter is true.

Neurally released ATP in detrusor is rapidly hydrolysed in the synaptic cleft by a group of plasma membrane-bound ATPases (ecto-ATPases). Reduced activity of these enzymes in detrusor taken from overactive bladders compared with controls has recently been shown and may result from changes in the extracellular matrix in unstable detrusor [Harvey et al. 2002]. Detrusor from both obstructed and idiopathically overactive bladders and in animal models of obstruction exhibits marked structural abnormalities. These are found in a patchy distribution, and characterised by muscle cell hypertrophy, connective tissue infiltration between muscle bundles and reduced density of cholinergic intramural nerves [Gosling et al. 1986, Sibley 1987, Elbadawi 1995, Harrison et al. 1997, Harrison et al. 1990, Charlton et al. 1999]. These changes, in the absence of a corresponding increase in detrusor blood supply predispose to cellular hyopxia and extracellular acidosis [Gallegos and Fry 1994, Fry 1997, Nielsen 1995], and have been associated with reduced activity of cellular enzymes necessary for metabolism [Uvelius and Amer 1997, Kato et al. 1990]. Ecto-ATPase activity *in vitro* is pH dependent so that extracellular acidosis would tend to impair ATP hydrolysis [Tuana and Dhalla 1988, Harvey et al. 2002]. Any cellular hypoxia would be likely to be exacerbated by stretch of the bladder wall and compression of intramural blood vessels during urine storage [Kato et al. 1990].

1.3.4 Neural plasticity in detrusor.

Changes in detrusor that may predispose to increased ATP release from motor nerves in response to bladder outflow obstruction have also been reported. Experimentally induced bladder outflow obstruction in animal models is associated with an increase in the cross-sectional area of afferent and efferent neurons of the spinal micturition reflex [Steers et al. 1990], and shortening of its latency suggesting hypersensitivity [Steers and De Groat 1988]. This plasticity is independent of pre-ganglionic motor nerve activity [Steers et al. 1990], and has been attributed to increased local production of nerve growth factor (NGF) by detrusor myocytes [Steers et al. 1991]. Intramural excitatory post-ganglionic nerves have been shown to respond to NGF [Tuttle et al. 1994a, Tuttle et al. 1994b], and increased NGF levels correlate with the degree of increased urinary frequency that accompanies experimental bladder outflow obstruction [Steers et al. 1991]. Nerve growth factor secretion and up regulation of NGF mRNA in response to stretch has also been shown in cultured detrusor smooth muscle cells [Steers et al. 1996].

Nerve growth factor levels in detrusor are closely regulated by a number of smooth muscle mitogens, and there is evidence that these in turn may be influenced by levels of intracellular Ca^{2+}. Calcium channel antagonists reduce the NGF-dependent growth of pelvic nerves following experimental outflow obstruction [Steers et al. 1994c], and experimentally raised intracellular Ca^{2+} levels in nerve glioma cells induces binding of an activator protein to the NGF promotor and subsequent gene transcription [Colangelo et al. 1996]. Nerve growth factor production by smooth muscle cells appears to be more closely linked to the basal levels of intracellular Ca^{2+} rather than agonist associated Ca^{2-} transients [Sherer et al. 2000]. This gives rise to the possibility that intracellular Ca^{2-} overload could predispose to neural plasticity.

Hypersensitivity of the micturition reflex would tend to enhance the likelihood of reflex bladder contractions during filling, and a change in conduction properties of motor nerves could affect pre-synaptic neurotransmitter release mechanisms causing increased proportional ATP release to a level at which synaptic breakdown mechanisms are saturated allowing purinoceptor activation. However no studies comparing motor nerve ATP release in normal and overactive detrusor have been reported.

1.3.5 Propagation of spontaneous detrusor activity.

Ultrastructural changes within overactive detrusor suggest a mechanism by which localised spontaneous electrical activity could be propagated through the tissue. Normal detrusor muscle cells are interconnected predominantly by intermediate junctions. These conduct mechanical not electrical activity, which together with evidence suggesting that only a small proportion of detrusor cells are actually innervated, suggests mechanical rather than electrical conduction may coordinate generalised bladder contractions. In overactive detrusor, two other types of intracellular junctions not normally seen predominate: protrusion and ultra-close abutment junctions. It has been postulated that intercellular electrical coupling could be altered in overactive detrusor, and affect the spread of spontaneous electrical activity through the tissue [Elbadawi 1995, Fry 1997, Sui et al. 2003].

1.3.6 Summary.

Evidence from morphological, electrophysiological and tension generation studies of human detrusor muscle *in vitro* suggest that a purinergic detrusor myopathy may be important in the pathogenesis of overactive bladder contractions.

1.4 Aims of Thesis.

The importance of purinergic mechanisms in the generation of overactive bladder contractions can only be speculated on in the absence of a method with which to detect them *in situ*. The contrasting electrophysiological properties of purinergic and cholinergic detrusor activation in animal bladders, and the results of microelectrode studies on isolated myocytes indicating that human detrusor may be similar, suggest that the emergence of purinergic neuromuscular transmission in overactive human detrusor may generate extracellular electrical activity that could be detected clinically by electromyography. However, the only method by which extracellular electrical recordings from detrusor smooth muscle have been made is the sucrose-gap technique. This involves needle electrode recordings from mechanically isolated sections of bladder strips suspended in a multi-compartment organ bath and is not applicable to the intact organ.

The aims of this thesis were to develop and validate a new technique for recording purinergically generated extracellular electrical activity from neurally intact contracting detrusor smooth muscle, and using it to test the hypothesis that samples of overactive bladders have a greater propensity to generate extracellular electrical activity than normal by virtue of a purinergic mechanism. In doing so we aim to provide direct electrophysiological evidence of the expression of purinergic neurotransmission in overactive human detrusor, and to prove in principal a new technique for evaluating clinically the role of purinergic mechanisms in the generation of overactive bladder contractions.

1.4.1 Theoretical basis of methodology.

Analysis of the numerous attempts at detrusor electromyography suggested that no amount of electronic filtering or data processing, but only innovations in electrode

designs were likely to enable isolation of extracellular currents from electromechanical artefact and yield a real electromyographic signal [Ballaro 2000]. A possible solution is the use of bi-reversible suction electrodes. These comprise an active recording electrode enclosed within a field of suction, and a reference electrode positioned on the tissue outside the field. A small bleb of tissue is drawn into the lumen of the device by the suction force, and mechanically isolated from the remainder of the contracting organ. The suction force therefore both stabilises the electrode against the contracting tissue, minimising the movement of the tissue from which electrical recordings are made while allowing tension measurements from the rest. Suction electrodes have been used to record the cardiac action potential from the surface of the beating heart [Lab and Wollard 1978, Vigmond and Leon 1999] and, more relavently have also been used to record excitatory junction potentials initiated by sympathetic excitatory nerves in isolated guinea-pig vas deferens [Brock and Cunnane 1987, Cunnane and Manchanda 1989].

A reversible electrode system consists of a pure metal coated with a sparingly soluble salt of this metal, in contact with a conducting solution of a soluble salt of the same anion. In the case of electrodes for biological use platinum coated with platinum chloride immersed in the predominantly sodium chloride extracellular solution ($Pt \leftrightarrow PtCl \leftrightarrow Na^+Cl^-$). The interface between the sodium chloride and the electrode consists of platinum chloride ions at equilibrium with both the platinum electrode and the sodium chloride solution. When an external current passes such that the platinum electrode becomes positively charged, platinum atoms become ionised but immediately combine with chloride ions from the solution, producing neutral molecules of insoluble platinum chloride, which are deposited on the platinum. Platinum chloride ions therefore act as a bridge, helping the current to flow either way with minimum impairment. Reversible electrodes have lower impedence at low frequencies than pure

metal electrodes, and therefore reduce the degree of contamination by movement artefact [Cooper and Binnie 1995].

1.4.2 Objectives of thesis.

1. To design and construct a bi-polar reversible suction electrode and using it to test the hypothesis that the extracellular electrical activity known to accompany contraction of guinea-pig detrusor smooth muscle can be recorded from the intact organ stimulated through its nerve supply, in isolation from all artefacts.

2. To test the hypothesis using a more readily quantifiable detrusor strip preparation that this electrical signal is generated by purinergic and not cholinergic neurotransmission, and is coupled to atropine-resistant tension generation. By doing so we aim to validate an apparatus for recording purinergic extracellular electrical activity from human detrusor strips.

3. To perform simultaneous suction electrode and mechanical recordings from clinically characterised human detrusor samples, and to test the hypothesis that detrusor taken from overactive bladders has a greater propensity to generate extracellular electrical activity by virtue of a purinergic mechanism.

CHAPTER TWO: SUCTION ELECTRODE RECORDINGS FROM THE WHOLE EXCISED GUINEA-PIG BLADDER.

2.1 Introduction.

Guinea-pig detrusor is known to generate electrical activity during purinergic contractions that can be recorded extracellularly. The first part of this thesis describes the construction of a bi-polar reversible suction electrode and tests the hypothesis that this can be used to record biological electrical activity in isolation from all artefacts. Adaptation of the apparatus for evoking and recording an electromyographic signal from the mucosal aspect of the bladder using a catheter-mounted combined intravesical stimulation-recording device is also described.

2.2 Methods.

2.2.1 Suction electrode design and construction.

Serosal recordings.

Stage1: Active electrode.

A platinum/iridium needle electrode (1.5cm length, 0.4mm diameter, RS components, UK) connected to a length of insulated wire was placed within the lumen of a 1.5 cm length of polypropylene sheath (1.5mm diameter) and stabilised using three 1.2cm lengths of smaller polypropylene sheath (0.4mm diameter) glued to the inside wall of the larger sheath around the needle (figure 2.1). A 3mm length of needle was left exposed at its pointed end. This arrangement allowed the needle electrode to be mounted in the centre of the larger sheath, surrounded by the smaller sheaths through which a suction force could be applied.

A

polypropylene canulae

1.5mm

needle electrode

B

needle electrode

polypropylene canulae

1.5mm
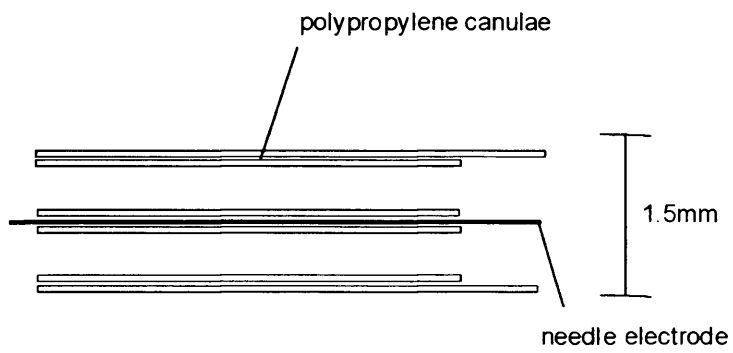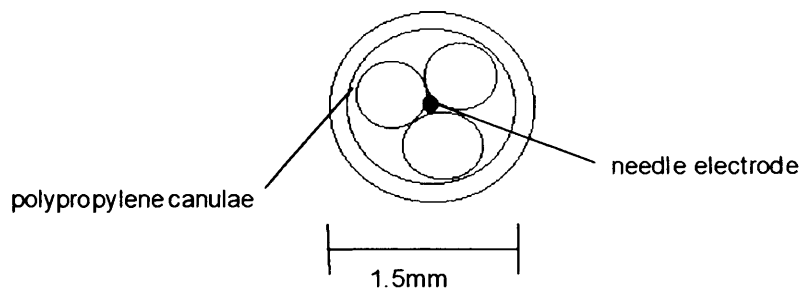
Figure 2.1. Schematic diagram showing stage 1 of the suction electrode construction.

Stage 2: Reference electrode.

Teflon-coated platinum wire (0.1mm diameter) was bared at one end and the uninsulated part wound around a 3mm length of silicone tubing (1.5mm internal diameter) to form a solenoid-type structure. The wire was secured by crimping, and the resulting structure was attached to the end of the stage 1 structure housing the pointed end of the needle, so that it was level with the needlepoint.

Stage 3: Electrode housing.

The stage 2 structure was secured inside a 3cm length of silicone tubing. The two portions of insulated wire attached to the two electrodes were brought out of the structure at the junction of the catheter and silicone tubing, and soldered to electric terminals. Figure 2.2 shows the completed suction electrode.

Intravesical recordings.

For intravesical electromyography the suction electrode was adapted to enable its passage into the guinea-pig bladder through the bladder neck. The stage 2 suction electrode structure was secured to a 12Fr Foley catheter so that the suction force could be delivered through the lumen of the catheter. A rigid polypropylene cannula (10cm length, 1.5 mm external diameter) was attached along side this structure to transmit the intravesical pressure, and attached to a pressure transducer. A tri-polar stimulation electrode was constructed by securing three loops of bared platinum wire (0.1mm diam) to the catheter/electrode structure at equal distances from each other (1mm), so that the middle wire was 1.5cm from the tip of the electrode. This formed a catheter mounted suction electrode with an incorporated bladder neck stimulator and intravesical pressure monitor (figure 2.3).

A

silicon tubing

insulated connecting wire

reference electrode

|— 3mm —|

1.5mm

suction

polypropylene canulae

active electrode

B

needle active electrode

Pt wire
reference electrode

polypropylene canula
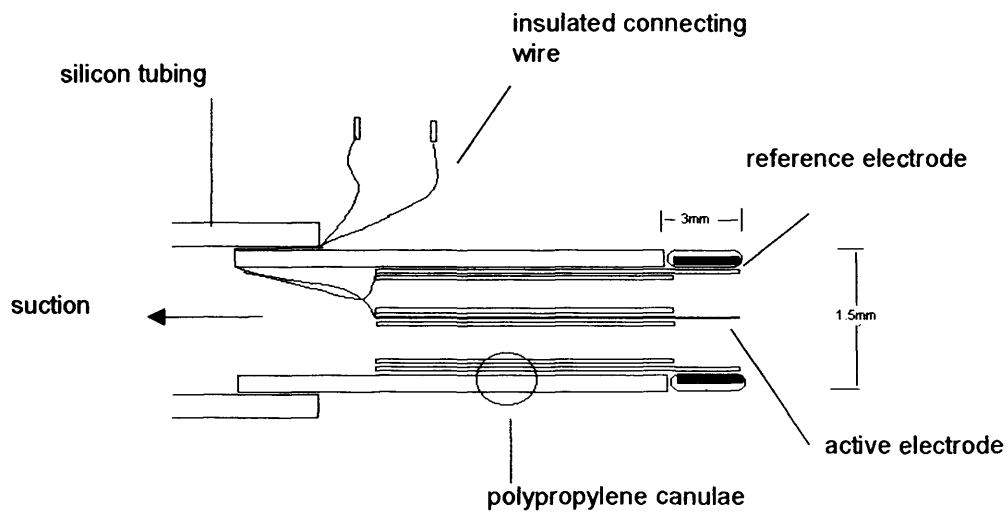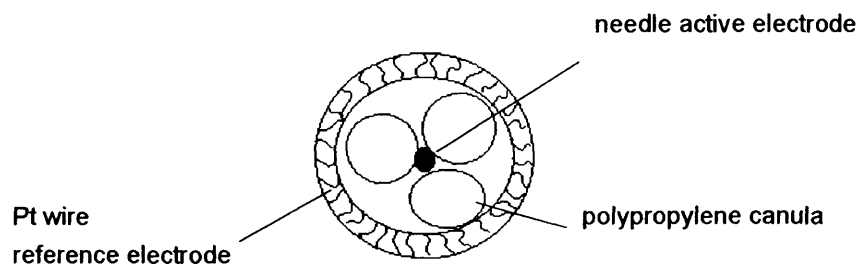
Figure 2.2. Schematic diagram showing A, longitudinal cross-section of suction electrode, B end view showing relationship between active and reference electrodes.
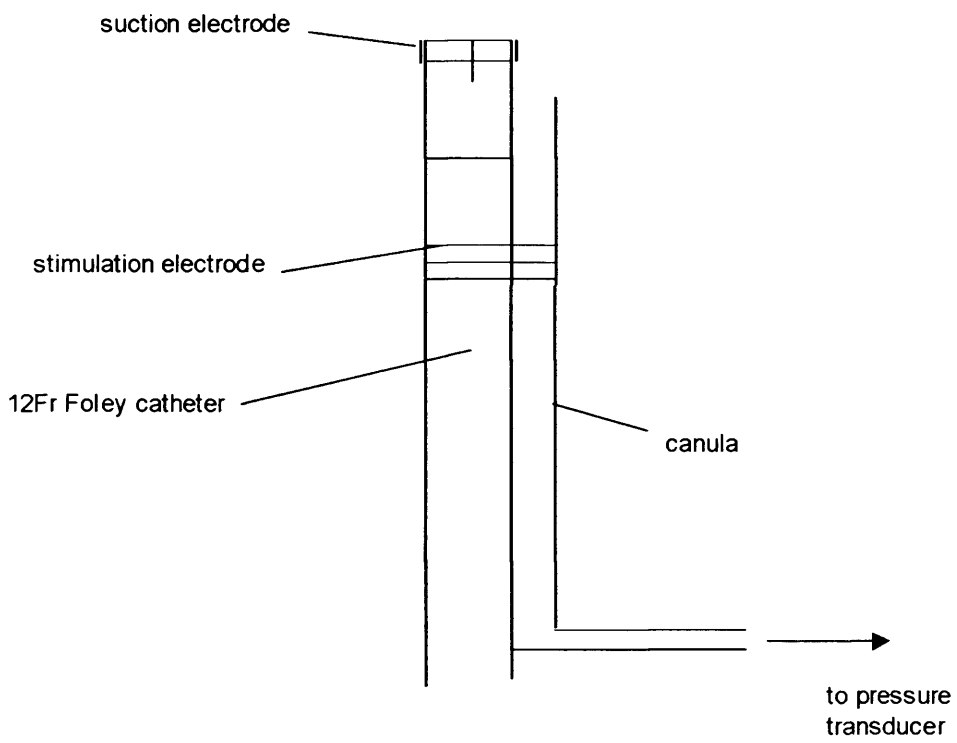
Figure 2.3. Schematic diagram showing intravesical catheter –mounted suction electrode.

A second intravesical electrode was constructed incorporating a platinum ball in place of the needle active electrode. The ball active electrode was fashioned by heating an insulated platinum wire using an oxyacetylene torch, resulting in a platinum sphere of 0.5mm diameter attached to insulated wire. The wire was inserted through the lumen of a 14 Gauge hypodermic needle, and crimped in place so that the platinum ball was secured against the end of the needle. The electrode was platinised as described below. The bared end of a length of Teflon-coated platinum wire was crimped within the other end of the needle, and the needle stabilised as before (figure 2.4). The reference electrode was constructed as previously described.

Electrode coating.

The active and reference electrodes were made reversible by coating with PtCl by electrolysis using a 9-volt battery and a platinum cathode in Kohlrausch solution (Appendix 1). The electrolysis circuit is shown in figure 2.5. The optimum resistance through which the electrolysing current was applied, and the optimum duration of electrolysis was determined by measuring the in series impedance of the electrode before and after electrolysis (Precision Component Analyser, Wayne Kerr Instruments). Electrolysis for 2 hours using a $10K\Omega$ resistor was found to be optimum. Figure 2.6A shows a representative graph of electrode impedance to recording signals at frequencies of 20Hz to 300KHz before, and after coating demonstrating reduced impedance, particularly to signals of low frequency, of the reversible electrodes. The impedance of the reversible electrode structure stabilised at 235Ω (figure 2.6B).
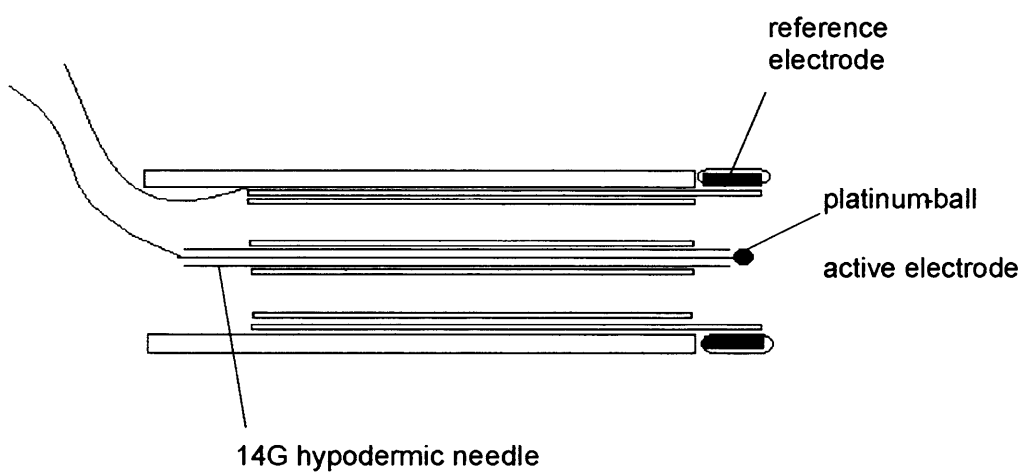
Figure 2.4. Schematic diagram showing suction electrode incorporating a platinum- ball active electrode.

10K resistor

9V battery

+ —

Kohlrausch
solution

platinum foil

suction electrode
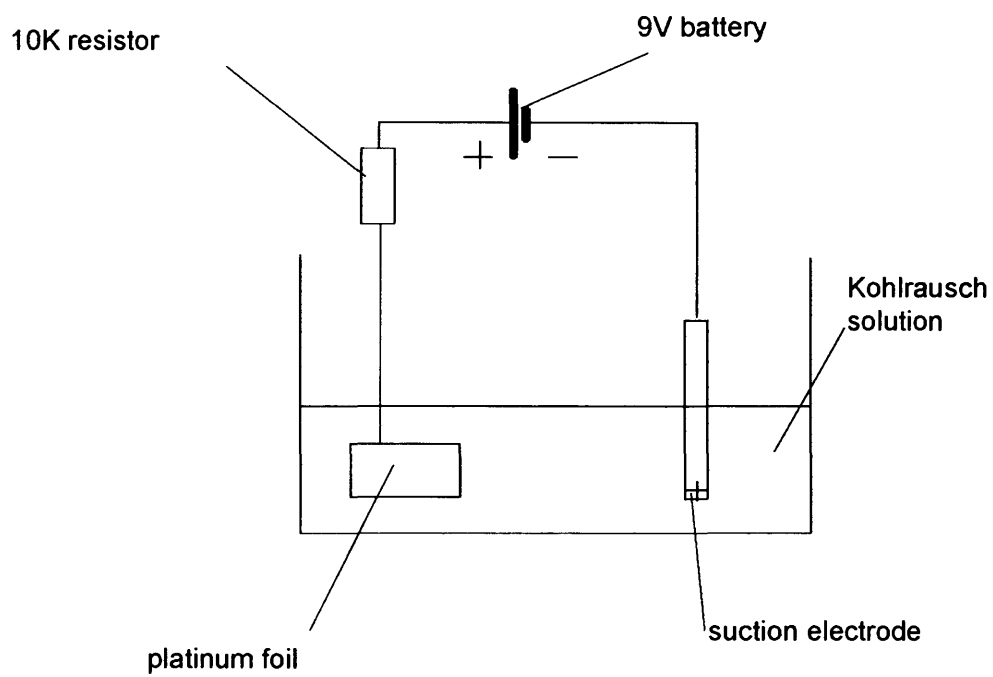
Figure 2.5. Schematic diagram showing apparatus for
Pt/PtCl coating of electrodes.

A
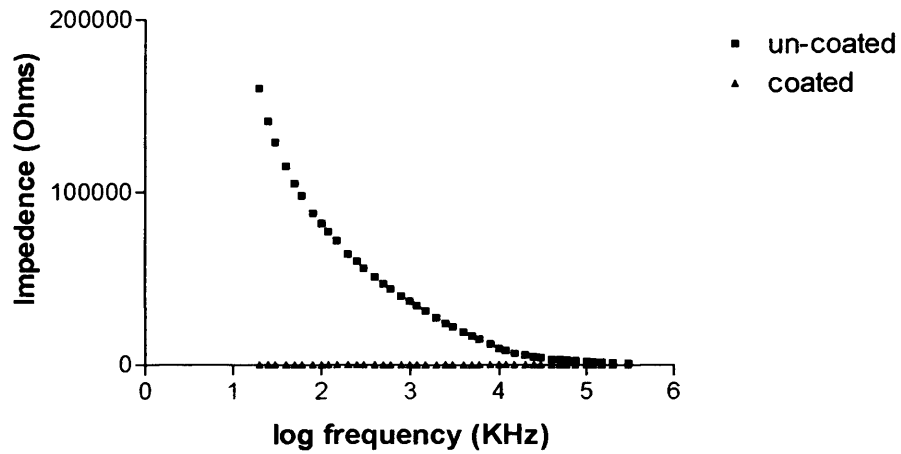
B
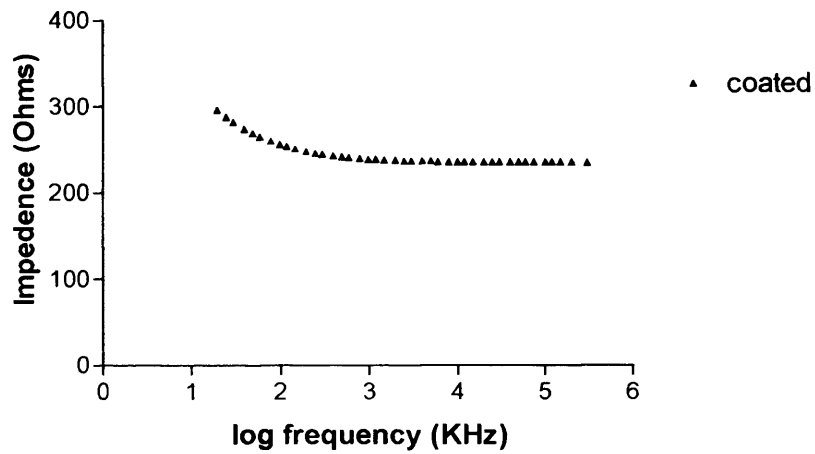
Figure 2.6. Graphs showing impedence of electrode system to recording signals of variable frequency before and after PtCl coating showing reduced impedence, particularly at low frequencies. A and B on different scales.

Suction Apparatus.

Suction force was provided by a mains-driven vacuum generator that generated a force of 100KPa. The generator was connected via non-collapsible tubing to the end of the electrode support tube (see figure 2.2) via a regulator (SMC-regulator, RS components).

2.2.2 Organ bath design and construction.

Serosal recordings.

No examples of an organ bath for the perfusion of a whole excised guinea-pig bladder preparation were found in the literature. A novel organ bath was therefore designed to enable mounting and superfusion of an intact guinea-pig bladder, stabilisation of the suction electrode on to the bladder, electrical stimulation of the bladder through its intrinsic nerves, and simultaneous recordings of the evoked electromyographic and mechanical activity. The organ bath was constructed from a standard polypropylene 50ml syringe.

Stage 1: Intravesical pressure monitor.

A rigid polypropylene canula (10cm length, 1.5mm external diameter) was inserted through the centre of the rubber washer of the syringe plunger. The end of the canula inside the organ bath, that was to be inserted into the bladder was blunted to prevent damage to the bladder wall. The canula was attached via non-distensible tubing to a standard electronic pressure monitor (figure 2.7A).

A

apperture for suction
electrode

perfusate outflow nozzle

perfusion chamber

perfusate inflow

nozzle

plug in

B

tri-polar stimulation
electrode ring

support for
stimulaton
electrode

rubber washer

intravesical canula

terminals for stimulation
electrode

to pressure monitor
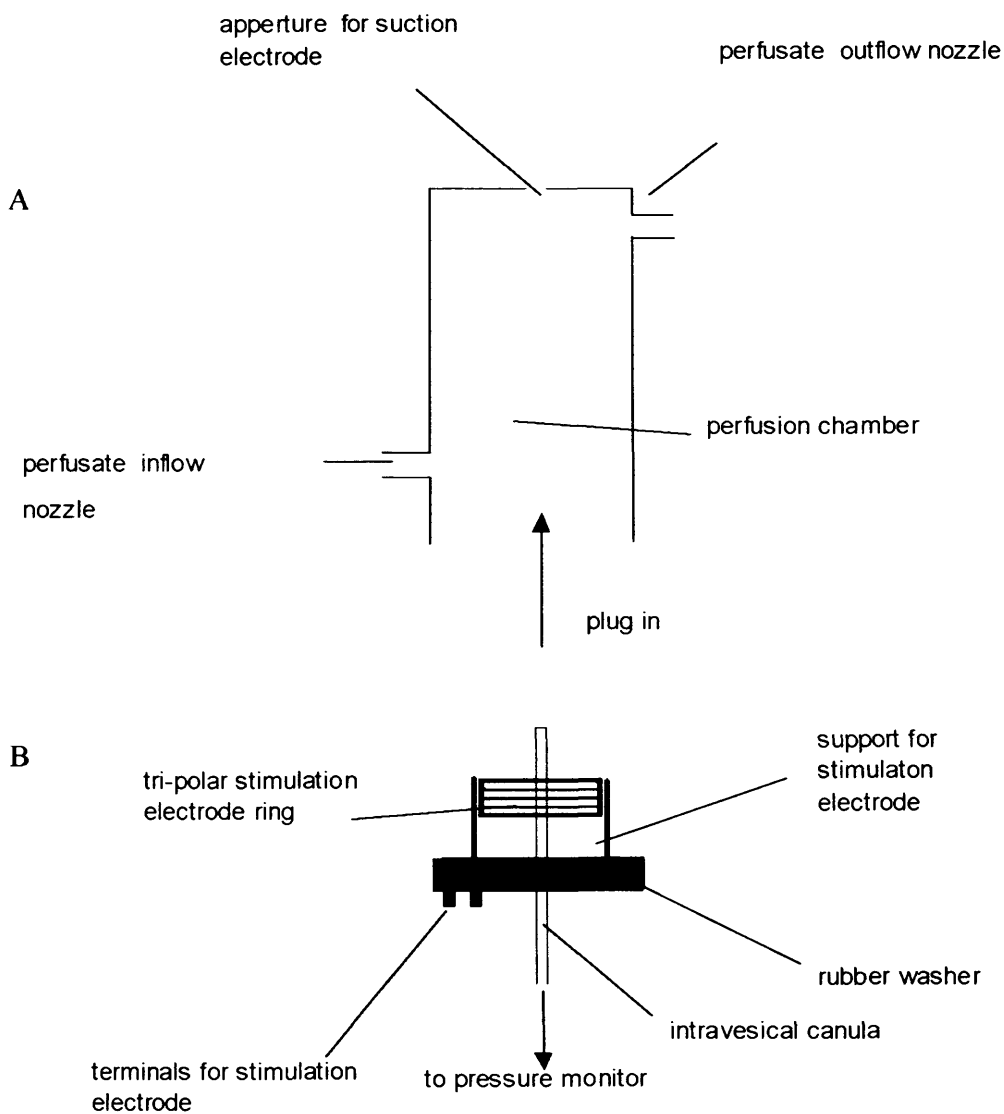
Figure 2.7. Schematic diagram showing
construction of organ bath perfusion chamber
(A). and base (B) incorporating stimulation
electrode.

Stage 2: Stimulation electrode.

A tri-polar stimulation electrode was used so as to minimise spread of the stimulus artefact. The electrode was constructed by embedding three lengths of bared platinum wire (0.1mm diameter), 1mm apart, into a circular cast of silicone adhesive (RS components). Care was taken to ensure that no bared wire protruded from the silicone ring, and that the three wires were equidistant throughout their circumference.

The tri-polar electrode was mounted on two supports that were secured to the syringe washer, and suspended approximately 2cm above it so that the open ends of the silicone ring lay in the horizontal plane (figure 2.7B). The insulated platinum wires leading from the three electrode wires were soldered to electric terminals so that the two outside wires were connected to a common terminal (anode) and the centre wire to another (cathode). The terminals were secured into the syringe washer so that they could be accessed from outside the perfusion chamber. The organ bath was electrically grounded using a mass of twisted platinum-chloride coated platinum which was secured to the washer on its inside surface and connected to a third silver terminal secured into the washer.

Stage 3: The perfusion chamber.

Inflow and outflow pipes were connected to the cylinder of the syringe, and the Luer nozzle removed. A hole of the same diameter as the silicone tubing housing the suction electrode was made in place of the nozzle, so that the suction electrode could be positioned through it. The washer section of the organ bath, containing the pressure monitoring canula, the stimulation electrode and electrical ground was inserted into the syringe to give the finished structure.

Intravesical recordings.

The organ bath for intravesical recordings was again fashioned from a 50ml polypropylene syringe. Inflow and outflow pipes were secured to the chamber of the syringe. The rubber washer of the syringe was removed from the handle and a hole of the same diameter as that of the suction electrode cut in its centre. The catheter mounted suction electrode was carefully inserted through the hole so that it extended into the chamber side of the washer by 2cm, and sealed in position with silicone adhesive.

2.2.3 Bladder excision and mounting.

Serosal recordings.

Male guinea-pig weighing 300-500grams were humanely killed with approval from the local ethical committee using section 1 methods. Their bladders were exposed via a midline abdominal incision and removed, after dividing the vasa differentia and vascular pedicles, with a small portion of the urethra. The ureters were ligated and divided close to the bladder wall, and the bladder immersed in warmed, gassed (95% O_2, 5% CO_2) Tyrode's solution (appendix 2) in a dissecting dish.

A 21guage plastic Venflon sheath attached to a syringe containing warmed Tyrode's solution was inserted into the bladder. The bladder was then carefully washed out with the Tyrode's solution so that no urine remained. The intravesical pressure monitor canula was inserted into the bladder through the bladder neck, and secured with silk ties so that a watertight seal was created. The canula and bladder was then quickly inserted through the washer, through the stimulation electrode ring, and the whole structure inserted into the perfusion chamber. The connecting line was filled with Tyrode's solution to enable accurate transmission of the intravesical pressure to the transducer. The organ bath was then mounted on a stand and warmed gassed Tyrode's

solution pumped (H.R Flow inducer, Watson-Marlow Ltd, Falmouth, Cornwall) through the organ bath at a rate of 3-5ml per minute. The bladder was filled with Tyrode's solution through a three-way tap, and pulled down until the bladder neck area was within the stimulation ring. The bladder was then filled with Tyrode's solution (1-3ml) until the outside bladder wall was in contact with the internal wall of the ring. The bladder was allowed to equilibrate.

Intravesical recordings.

The suction electrode was passed through the neck of excised guinea-pig bladders so that the electrode tip was in contact with the urothelium of the bladder dome, and the tri-polar stimulator was in contact with the mucosal aspect of the bladder neck. The bladder was then secured to the structure with a silk tie. Suction (60-80kPa) was applied through the catheter to cause the electrode to be sucked on to the dome urothelium. The whole structure was then inserted into the chamber of the organ bath, the bladder carefully filled with Tyrode's solution through the canula, and perfused with warmed gassed Tyrode's solution (figure 2.8).

2.2.4 Bladder stimulation protocol.

The tri-polar electrodes of both the serosal and intravesical recording apparatus were connected to a stimulator (SD-5 Stimulator, Grass, Quincy Mass. USA). The positive terminal of the stimulator was connected to the terminal connected to the two outside rings of the stimulation electrode, and the negative stimulation terminal to the inside ring. The bladder was stimulated using 2-10 electrical pulses of 200µs pulse width, at a frequency of 100 pulses s^{-1}, 40 – 80V, at 90 s intervals. The number of
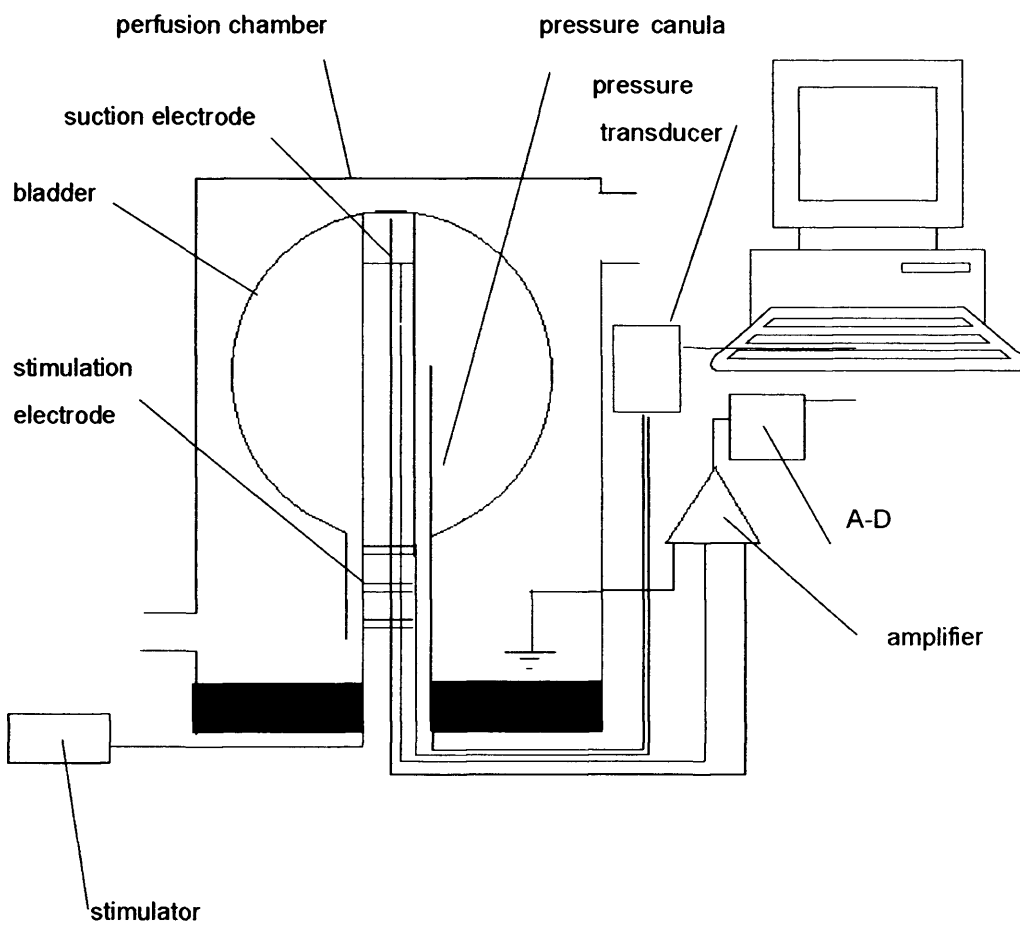
Figure 2.8. Schematic diagram showing experimental set   -up for recording evoked extracellular electrical activity and   intravesical pressure using the catheter   -mounted suction electrode. A   -D = analogue to digital converter.

pulses was adjusted within these limits to optimise the amplitude of the suction electrode recordings, and separation of the signal from the stimulus artefact.

2.2.5 Suction electrode attachment.

For serosal recordings, the organ bath was emptied and the bladder deflated. The suction electrode was inserted into the chamber through the hole in its top and applied to the serosa of the bladders dome. The suction electrode was placed as distant from the stimulation electrode as possible in order to further minimise the stimulus artefact (figure 2.9). A controlled suction force was gradually applied to a maximum of 80KPa. The bladder was then carefully refilled, taking care to move the suction electrode in tandem with the rising bladder dome to prevent loss of the suction seal. The effect of different levels of suction on evoked electrical signals recorded from the bladder was investigated by stimulating the bladder during graded increases in the suction pressure from 40 to 80 KPa by means of the suction regulator.

2.2.6 Signal processing.

The signals recorded from the suction electrode were filtered (1.6 Hz – 1 KHz), and amplified (x 10^4) (Preamp 50, Palmer Bioscience, Washington. USA). The active electrode was attached to the positive input of the amplifier, the reference electrode to the negative input, and the mass of platinum wire was connected to ground. The amplified signals were digitised (Pico ADC-11/22 driver, 10bits, 13 kilo-samples s^{-1}, Picotechnology Ltd), and displayed in real time, along with the digitised signals from the intravesical pressure monitor on a standard personal computer (Pentium III 450MHz processor, 64Mbytes RAM, 6 G-Byte hard drive) using proprietary (Picoscope for Windows, 1.6bits, copyright 1995-1998 Picotechnology Ltd) software. Figure 2.10 shows the experimental set-up for serosal recordings.
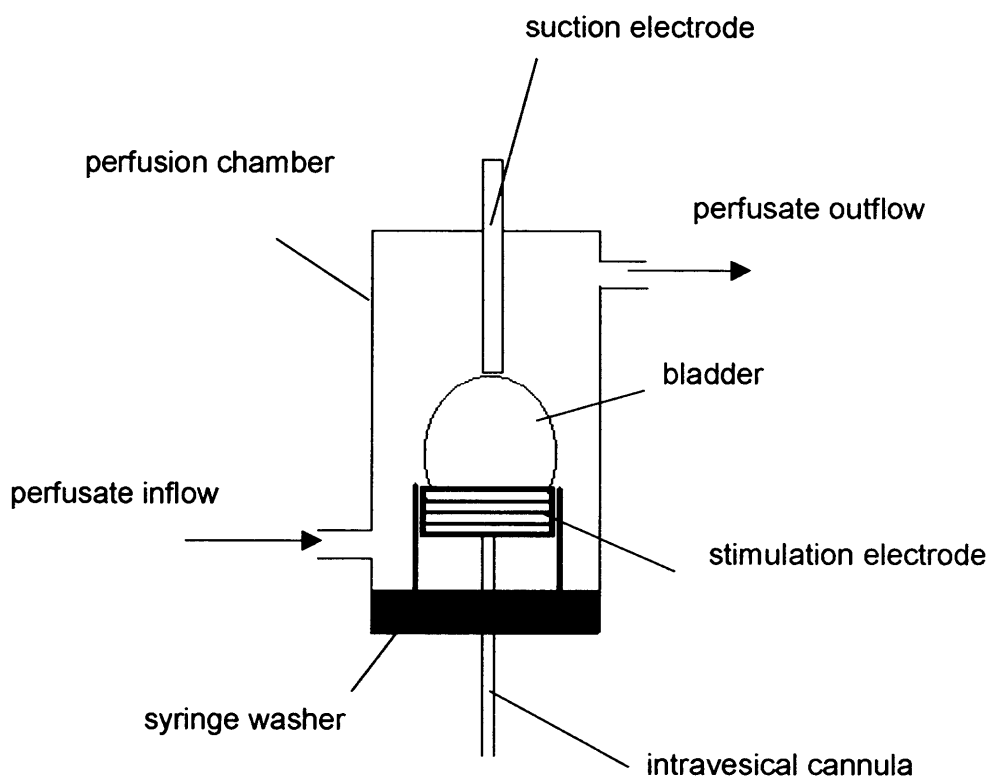
Figure 2.9. Schematic diagram showing whole guinea-pig bladder mounted in the organ bath with suction electrode and intravesical canula in recording positions.

amplifier

suction electrode

organ bath

bladder

A-D

stimulator

pressure transducer
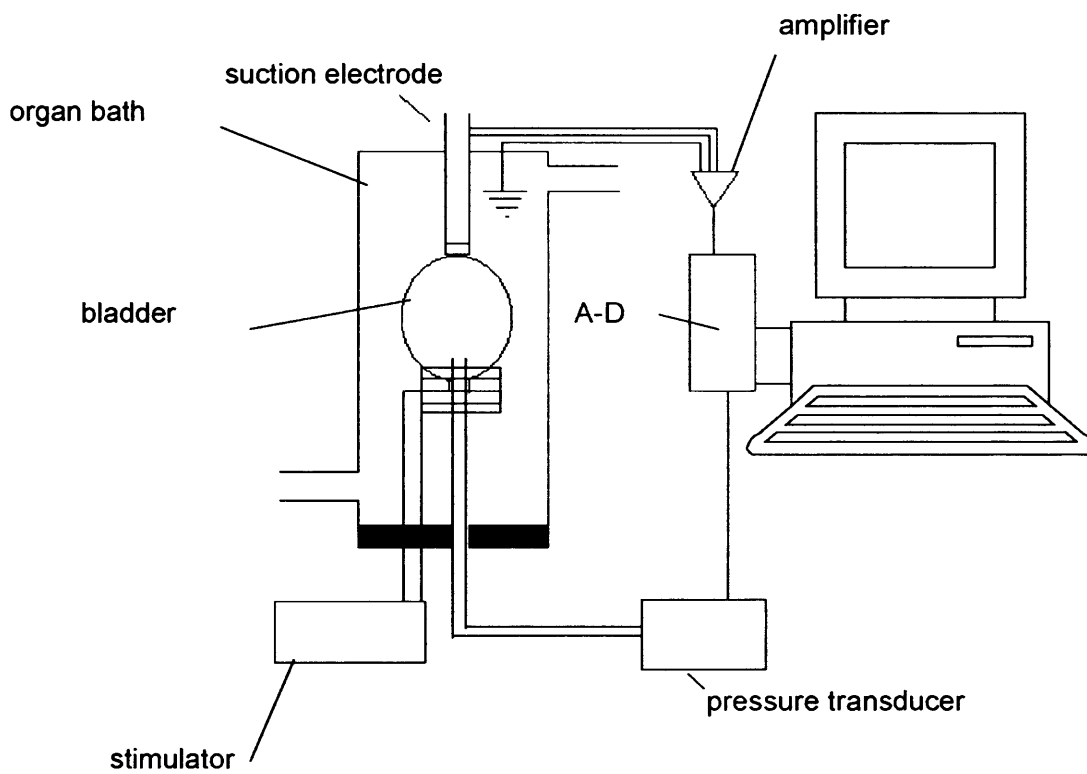
Figure 2.10. Schematic diagram showing experimental set-up for recording evoked electrical activity and intravesical pressure rises in a whole excised guinea-pig bladder preparation. A-D = analogue to digital converter.

2.2.7 Characterisation of the electrical signal.

To investigate the nature of the suction electrode recordings, and their relationship to stimulus and electromechanical artefact, experiments involving changes in stimulation and pharmacological interventions were conducted. Pharmacological agents were dissolved in gassed modified Tyrode's solution (37°C) and pumped into the organ bath at 3-5ml min^{-1}. The bladder was stimulated at interval of 90 s during experiments, and three recordings of the signals at each stage of the experiment were made. Post washout signals were recorded after each intervention.

Stimulation experiments.

The effect of increasing stimulus intensity on the evoked electrical signal was investigated by varying the numbers of stimulating pulses. The frequency and pulse width of stimulation was constant throughout the experiment. A real evoked electromyographic signal would be expected to increase in amplitude to a maximum in proportion to the stimulus intensity, and its temporal relationship to the stimulus artefact would also be expected to vary with the stimulus- signal interval expected to reduce with stimuli of increased intensity. The effects of repetitive stimulation at approximately 1 s intervals was also investigated. A biological electrical signal of equal amplitude would be expected to be evoked by each stimulus, and not change in morphology or parameters, in contrast to electromechanical artifact which would be expected to be accentuated during summed contractions resulting from repetitive stimulation.

Pharmacological interventions.

The sensitivity of the signals to tetrodotoxin 1µM (TTX), a neurotoxin that deactivates Na$^+$ channels on motor nerves, was determined. A biological electrical

signal originating from the transmembrane ion currents generated by neuromuscular transmission would be expected to be completely abolished, independently of the stimulus artefact, in the presence of TTX. In contrast, stimulus artefact and signals resulting from direct depolarisation of the muscle cell membrane by the stimulating current independently of neuromuscular transmission would not.

The effect on the evoked electrical signal of graded reduction in the $CaCl_2$ concentration of the superfusate was also investigated. As previously described, the detrusor smooth muscle action potential is completely dependent on the influx of Ca^{2+} alone, therefore a true extracellular recording, unlike any artefact, would also be expected to be Ca^{2+} dependent, and affected independently from the stimulus artefact by this intervention.

To investigate the origins of the signal in terms of the neuromuscular transmitter involved, the effects of bladder stimulation in the presence of atropine, α, β-mATP, and atropine and α, β-mATP together were determined. The ease with which atropine and α, β-mATP were likely to diffuse through the tissue from the serosal surface of the bladder alone was unknown. Increasing concentrations of atropine (1-100μM) and α, β-mATP (10-100μM) were applied until the maxium reduction in intravesical pressure evoked by electrical stimulation had stabilised, or the expected effect on the electrical signal had been observed.

2.2.8 Intravesical suction electrode recordings.

The bladder was stimulated using the same range of stimulation parameters as those used for the serosal recordings. The number of pulses was again varied to optimise the amplitude and separation of the suction electrode signal from stimulus artefact. The signals from the suction electrode were processed and displayed in real time with changes in intravesical pressure using the same experimental set-up that was

used for serosal recordings in the same manner as with the serosal recordings. The parameters of the signals were compared with those recorded from the bladders serosal surface. The stimulation parameters used to evoke the two sets of signals were also compared to control for differences in stimulation intensity. The parameters of the signals recorded from the bladders mucosa with needle and ball active electrodes were also compared. In one experiment the bladder was perfused with α, β-mATP to determine the sensitivity of the signal to purinergic desensitisaion.

2.2.9 Statistical analysis.

The two-tailed Student's t-test was used for all comparisons. Statistical significance was determined at 95% confidence limits ($P < 0.05$).

2.3 Results.

2.3.1 General observations.

The bladders remained viable in the organ bath for long periods (3-5 h), and simultaneous recordings of evoked detrusor electrical activity and intravesical pressure were consistently recorded. Spontaneous contractions appeared localised to sections of the bladder wall, rather than generalised, and were not associated with any electrical activity. A predominantly bi-phasic electrical signal was recorded from the serosal surface of six guinea-pig bladders in response to electrical stimulation (figure 2.11). The amplitude of the signal was constant over long periods in the absence of pharmacological intervention or temperature change, and the signal could be recorded from different sites on the same bladder provided that the platinum chloride coating remained intact. The amplitude of the signal did not vary according to the magnitude of the suction force applied through the electrode, but was lost when the suction seal with the bladder wall was broken.

2.3.2 Signal parameters.

The parameters of the electrical signals recorded from the bladders serosal surface are shown in table 2.1. The mean latency between the electrical signal and associated change in intravesical pressure was 279 ± 24ms. Both the evoked electrical signal and associated intravesical pressure rise were completely abolished by TTX (1μM) indicating that they were generated by stimulation of motor nerves. The electrical signal was clearly separate from the stimulus artefact and preceded any change in intravesical pressure in all cases.

Figure 2.11. Representative trace of evoked electrical signal (e) and intravesical pressure rise (p) recorded from the serosal surface of the whole excised guinea-pig bladder. St denotes stimulus artefact. Broken lines show measurement points for: a= amplitude, d=duration, t=time to maximum depolarisation.

Signal parameters	1	2	3	4	5	6	Mean ±SD
Amplitude (µV)	620	363	1130	533	342	830	647 ± 301
Duration (ms)	320	283	369	304	220	260	293 ± 51
Depolarisation time (ms)	51	96	100	50	29	43	62 ± 29

Table 2.1. Table showing parameters of evoked electrical signal recorded from the serosal surface of six whole guinea-pig bladders.

2.3.3 Effect of stimulus intensity on the electrical signal.

The amplitude of the evoked electrical signal, and its latency varied with the number of pulses used (figure 2.12). The amplitude was at a maximum when evoked using 20 pulses and decreased with increasing numbers of pulses. This decrease may have resulted from muscle fatigue, or because of nerve or muscle refractoriness. The latency of the signal decreased with an increasing stimulus intensity. Clear separation of the signal from the stimulus artefact was evident using lower numbers of pulses, whereas at higher stimulation intensities the artefact overwhelmed the signal. Repetitive stimulation evoked summated contractions and an electrical signal associated with each stimulus (figure 2.13). The parameters and morphology of the signal were unchanged in the presence of increased intravesical pressure.

2.3.4 Effect of $CaCl_2$ depletion of the perfusate on the electrical signal.

The amplitude of evoked electromyographic signals in the presence of superfusates containing reduced $[CaCl_2]$ were attenuated in a graded manner (figure 2.14). The latency of the signal increased with lower $[CaCl_2]$. Stabilisation of the effect of the intervention occurred after variable periods, generally between 20 and 40 minutes of exposure to the solutions. An electrical signal could still be evoked after prolonged exposure to calcium-free media.

2.3.5 Effect of atropine and α, β-mATP on the electrical signal.

Bladder stimulation in the presence of neuromuscular blockade with atropine 100μm and α, β-mATP 60μm together resulted in complete abolition of both the signal and the contraction (n=2) (figure 2.15). Bladder stimulation in the presence of atropine
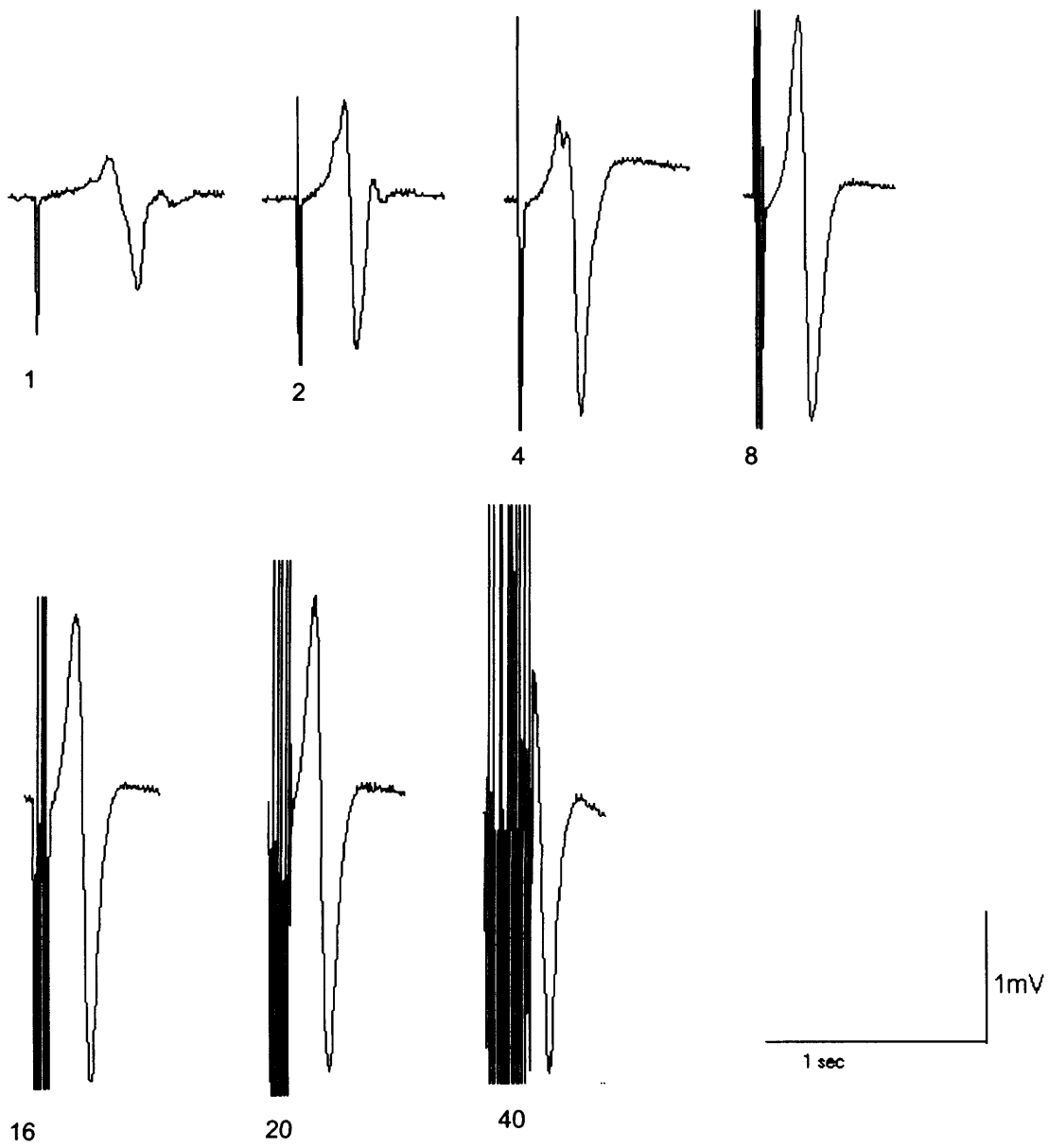
Figure 2.12. Representative traces showing electrical signal evoked by increasing numbers of electrical pulses recorded from the serosal surface of the whole guinea-pig preparation. Numbers indicate number of stimulating pulses of 0.1ms duration.
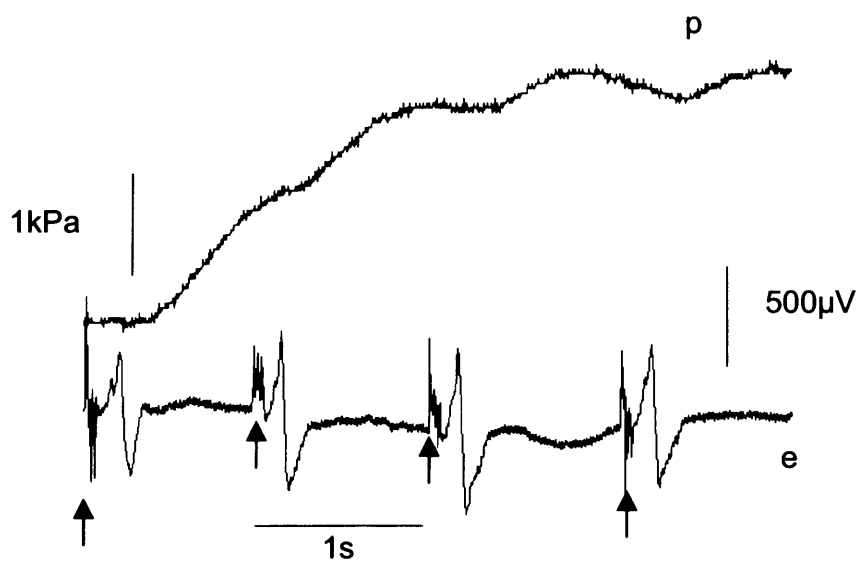
Figure 2.13. Trace of electrical signal (e) and intravesical pressure (p) recorded from the serosal surface of the whole guinea-pig bladder with repetative stimulation (arrows). Note no change in morphology of the electrical signal with summated contractions.
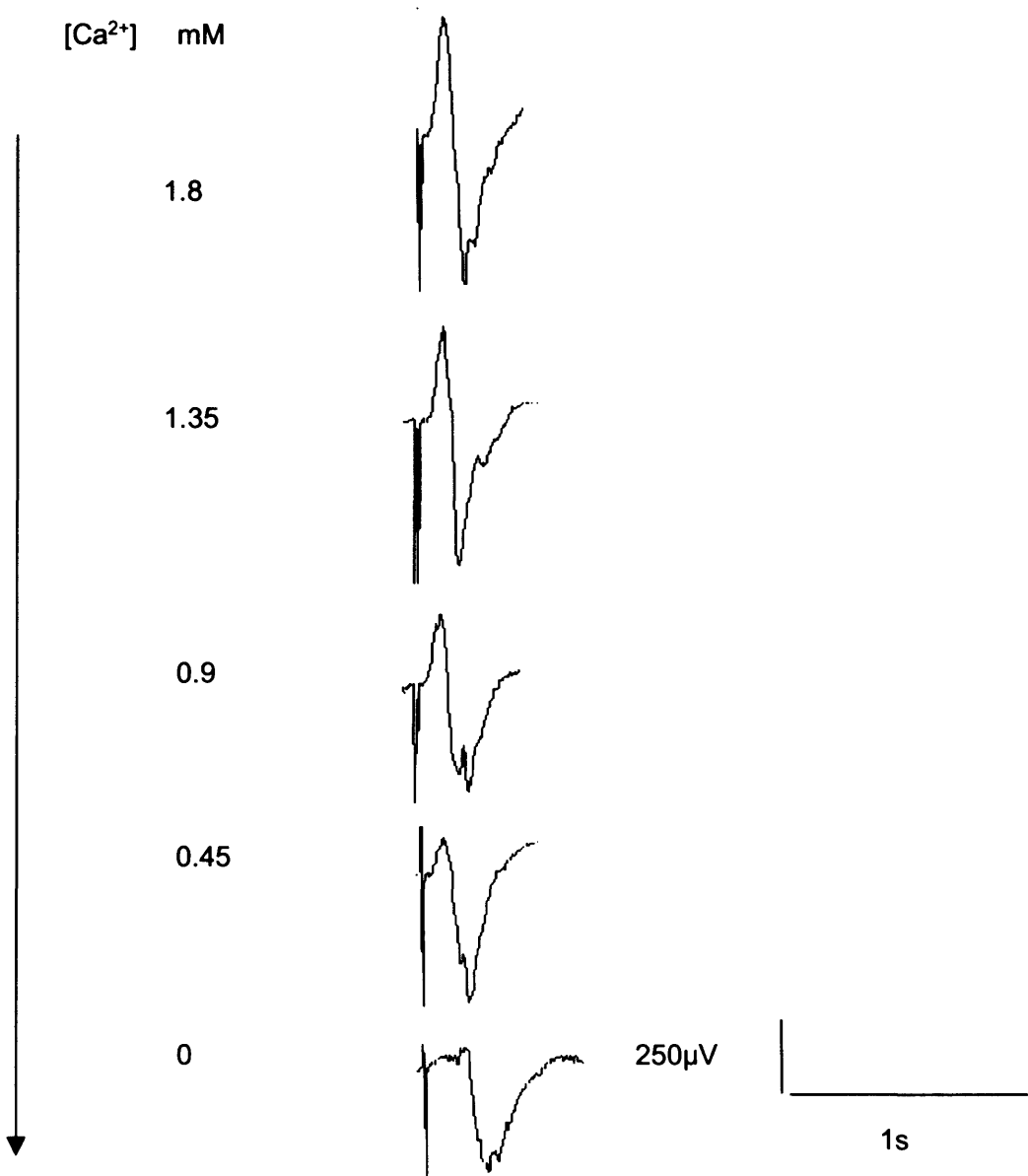
Figure 2.14. Representative traces of evoked electrical signals recorded from the serosal surface of the whole guinea-pig bladder perfused with $CaCl_2$- depleted solutions. Note reduced amplitude and increased latency of the signal.
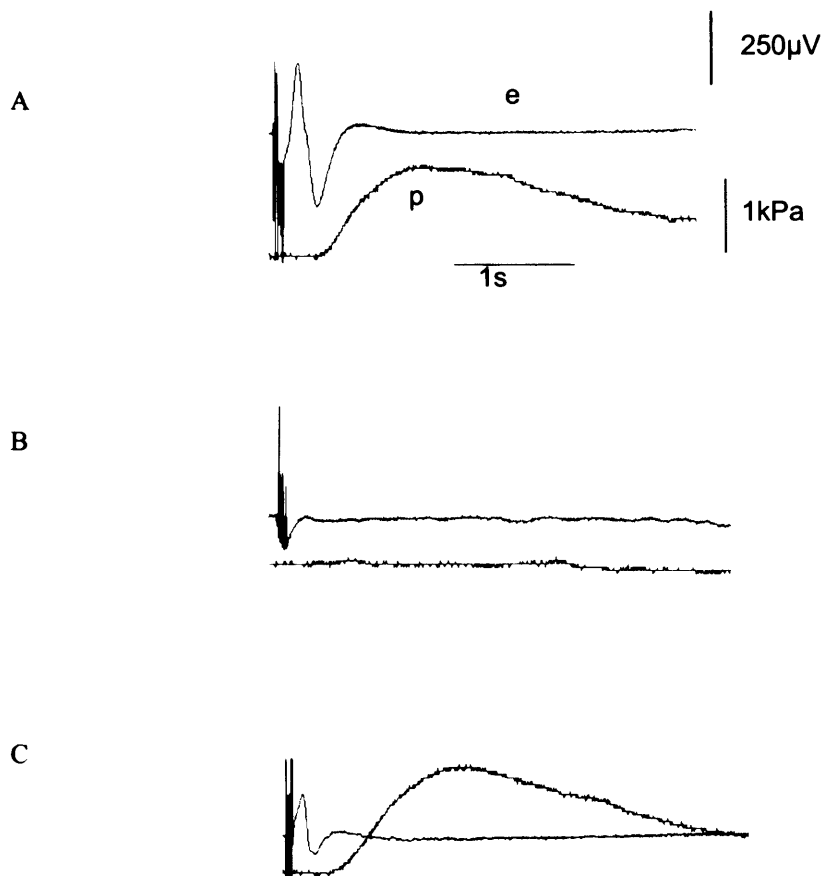
Figure 2.15. Evoked electrical (e) and intravesical pressure (p) traces recorded from the serosal surface of the whole guinea-pig bladder. A, control, B, with atropine + α, β-mATP, C, after washout.

100μm alone resulted in attenuation of the amplitude of the electrical signal and the contraction (figure 2.16). Continuous perfusion with α, β-mATP alone initially resulted in an intravesical pressure rise. Electrical stimulation of the bladder shortly after attenuation of the contraction evoked an attenuated mechanical response. Stimulation after prolonged exposure to the agent (1-2 h) resulted in the evoked electrical signal being almost completely abolished (n=2) (figure 2.17). Bladder stimulation in the presence of lower concentrations of atropine and α, β-mATP had variable effects on the bladder, with sub-maximal attenuation of the contraction, and bladder contraction could still be evoked in the presence of lower doses of neuromuscular blocking agents in combination, suggesting incomplete penetration into the detrusor.

2.3.6 Intravesical suction electrode recordings.

The catheter-mounted electrode-stimulation device enabled simultaneous recordings of electrically evoked contractile and electromyographic activity from the whole bladder preparation. Stimulation of the bladder by the intravesical bladder neck electrodes caused visible generalised bladder contractions that could be abolished by TTX 1μM. A predominantly bi-phasic electrical signal was consistently recorded from the mucosal aspect of six guinea-pig bladders in association with evoked contractions using the intravesical suction electrode (figure 2.18). The suction electrode containing the needle active electrode was used to record from four bladders, the electrode containing the ball active electrode was used to record from a further two different bladders.

The parameters of the signal recorded from all six bladders were (mean ± SD): amplitude 359 ± 430μV, duration 254 ± 22ms, time to depolarisation 46 ± 6ms (table 2.2). There was no statistical difference between the mean parameters of the signals

A

B
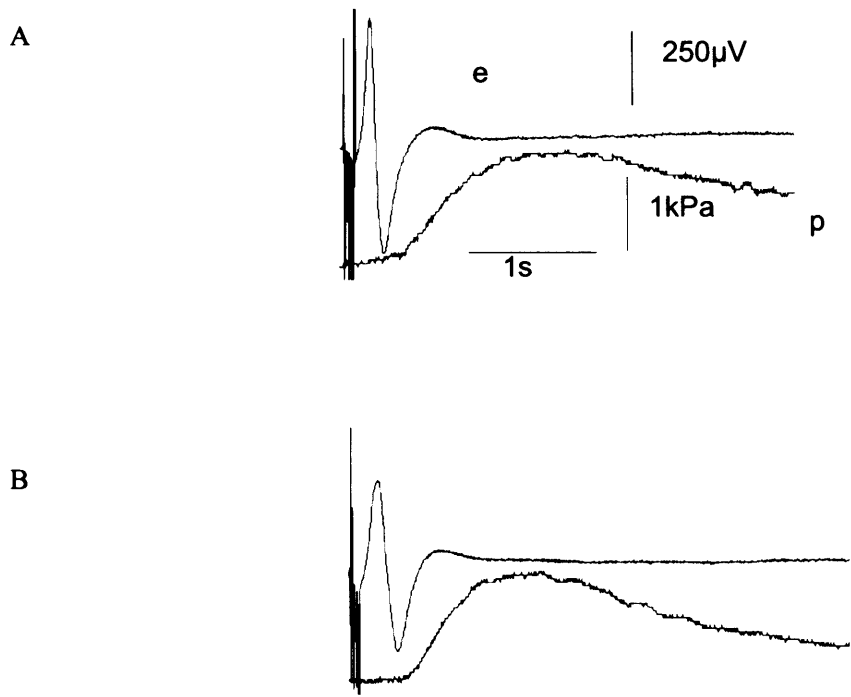
Figure 2.16. Evoked electrical (e) and intravesical pressure (p) signals recorded from the serosal surface of the whole guinea-pig bladder. A=control, B= superfused with atropine.
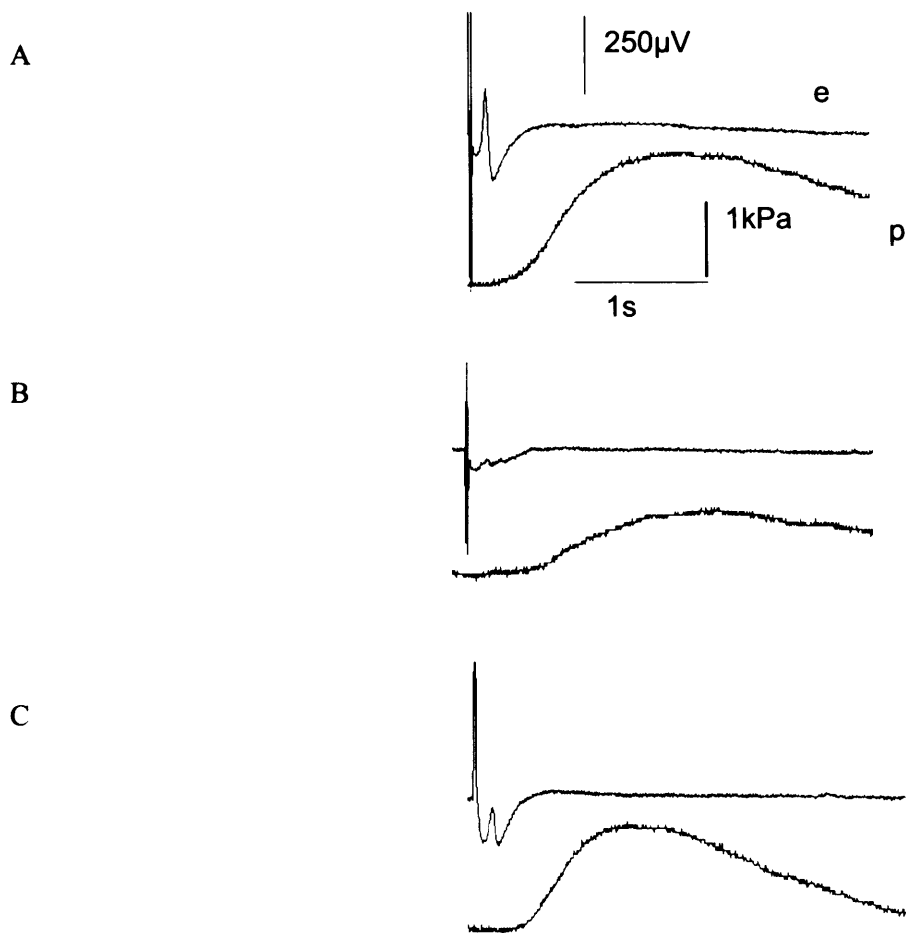
Figure 2.17. Evoked electrical signal (e) and intravesical pressure trace (p) recorded from the serosal surface of the whole guinea-pig bladder. A, control, B, with α, β-mATP, C, after washout. Note abolition of electrical signal with residual intravesical pressure rise, presumably cholinergic.
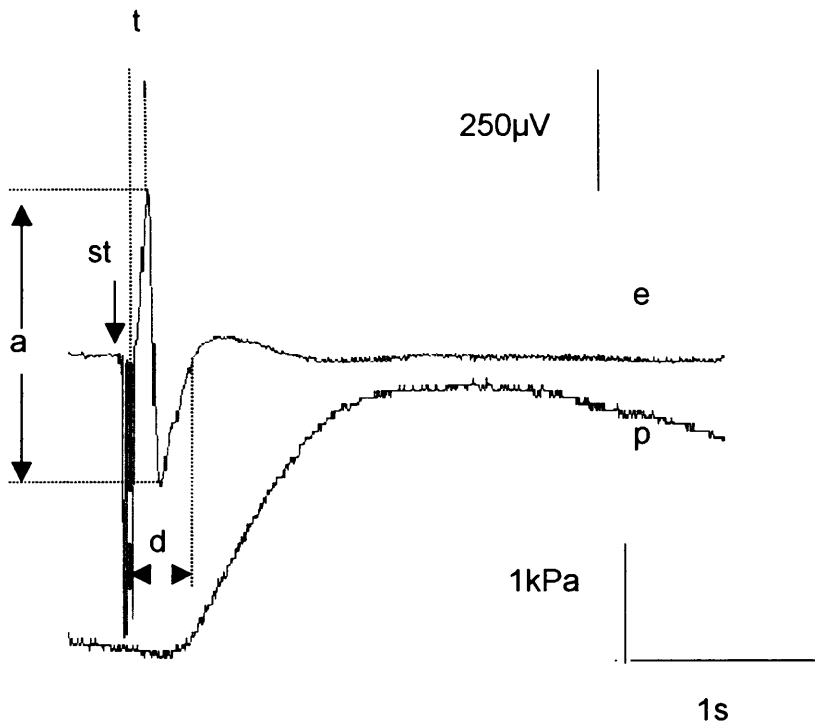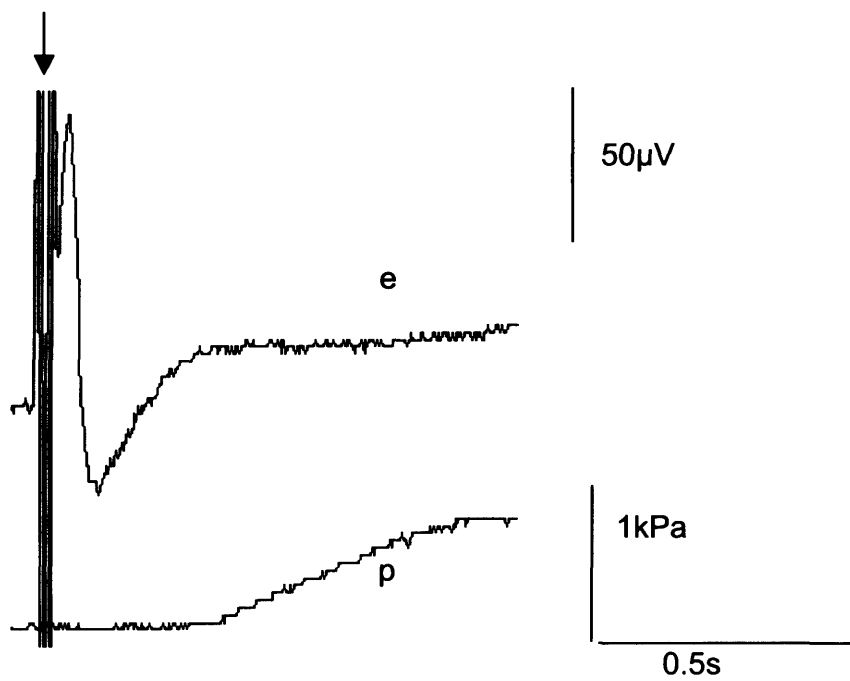
Figure 2.18. Representative electrical signal (e) and intravesical pressure (p) traces recorded from the urothelial surface of the whole guinea-pig bladder with intravesical suction electrode. Arrow marks stimulus artefact.

recorded from the bladder mucosal aspect using the needle electrode, P<0.32, P<0.05, P<0.14 respectively. There was no difference while in the mean of the amplitude of the electrical signals recorded from the bladder mucosal and serosal aspects using needle electrode [the position in the text unclear in places] and the duration, P<0.56. There was no obvious difference either in the morphology of the signals recorded from the bladder serosal or mucosal surfaces with the needle serosal electrode (Figure 2.19). As with the experiments where the signals recorded from the bladder serosal surface, these electrical transients were maximal in amplitude away from the stimulus gradient and could be abolished by perfusion with solution containing 0 mM ATP (n=1).

Signal parameter	1	2	3	4	5	6	Mean ± SD
Amplitude (μV)	78	502	40	1170	133	230	359 ± 430
Duration (ms)	201	296	202	324	292	209	209 ± 56
Depolarisation time (ms)	46	43	40	75	32	39	46 ± 15

Table 2.2 Table showing parameters of electrical signal recorded from the mucosal aspect of six whole guinea-pig bladders using the intravesical electrode.

recorded from the bladders mucosal aspect using ball or needle electrodes: P=0.32, P=0.86, P=0.31 respectively. There was no difference either in the means of the parameters of the electromyographic signals recorded from the bladders serosal and mucosal aspects using needle electrodes (table 2.3), or in the mean number of pulses used to generate them P=0.38. There was no obvious difference either in the morphology of the signals recorded from the bladders serosal or mucosal surface with ball or needle active electrode (figure 2.19). As with the serosal recordings, the signal recorded from the bladders urothelial surface preceded any intravesical pressure rises, was separate from the stimulus artefact and could be abolished during perfusion with solutions containing α, β-mATP 60μM, (n=1).

Signal parameters	Mucosa	Serosa	P- value
Amplitude (μV)	359 ± 176	647 ± 123	0.21
Duration (ms)	254 ± 22	293 ± 20	0.24
Depolarisation time (ms)	46 ± 6	62 ± 12	0.27

Table 2.3. Table showing comparison between parameters of electrical signal recorded from the whole guinea-pig bladders serosal and mucosal surfaces. Mean ± SEM, P value refers to unpaired t- test.

A

500ms

bladder

C

B

500ms
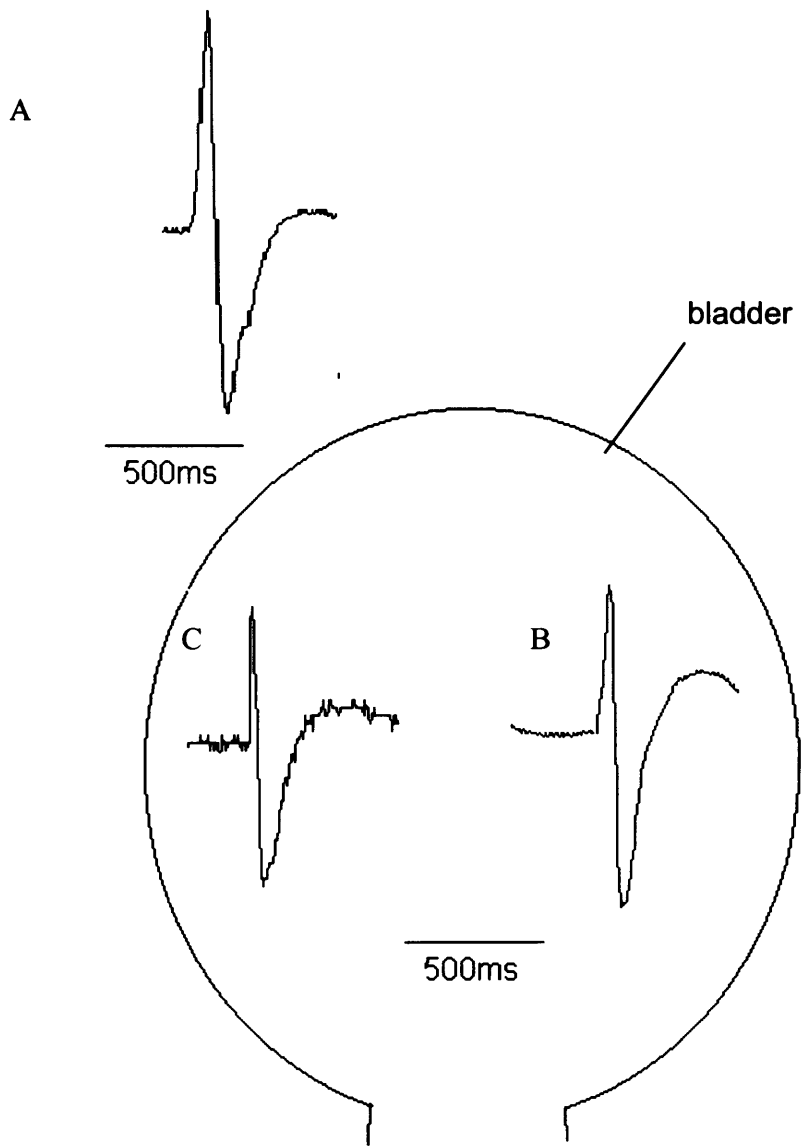
Figure 2.19. Morphology of electrical signal recorded from, A the whole guinea-pig bladdes serosal aspect, B the whole bladders mucosal aspect, C the whole bladders mucosal aspect using a ball active electrode. Different voltage scale but same time-scale used for all.

2.4 Discussion.

2.4.1 Summary of results.

The apparatus allowed consistent simultaneous electrical and mechanical recordings from a nerve-stimulated whole excised guinea pig bladder preparation. A predominantly bi-phasic electromyogaphic signal was recorded that varied with stimulus intensity, preceded the onset of contraction in all cases, and was independent of stimulus artefact. The signal was sensitive to reductions in the $CaCl_2$ of the superfusate, and to exposure to the neurotoxin TTX. Both the signal and contraction were abolished by combined muscarinic receptor and purinoceptor blockade. The signal was almost abolished, although the associated contraction was only attenuated, during prolonged exposure to high doses of α, β-mATP, but was also unexpectedly reduced in the presence of a high dose of atropine (100μM). The signal could also be recorded from the bladders mucosal aspect using an intravesical device, and did not differ obviously in morphology or significantly in parameters from that recorded from the serosa.

2.4.2 The origins of the electrical signal.

Several lines of evidence showed that the electrical signal was a reflection of biological electrical activity and not artefact.

Independence from electromechanical artefact.

The signal always preceded associated rises in intravesical pressure (eg. Figure 2.11). If the signal were electromechanical artefact it would be expected to coincide with the contraction. Recorded changes in intravesical pressure do not necessarily correlate with local detrusor tension, and the temporal relationship between the electrical and mechanical events in a whole bladder preparation may not always be

constant and reliable. However as the suction electrode was always placed at a maximum distance from the bladder neck stimulator in these experiments, any inaccuracy was likely to be in favour of recording an intravesical pressure rise before the electrical signal. Furthermore, repetitive stimulation resulting in summation of contractions caused no change in amplitude or morphology of the electrical signal as would be expected if an electromechanical contribution to the signal by virtue of movement artefact were present. Independence of the signal from electromechanical artefact was further shown by its consistent abolition, and dissociation from the contraction, during perfusion with α, β-mATP (see figure 2.17).

Independence from stimulus artefact.

The artefact in the recordings generated by the electrical stimulation was clearly identifiable and separate from the biological signal. This separation was increased with low numbers of stimulating pulses (see fig. 2.12) and during perfusion with $CaCl_2$-depleted solutions (see fig 2.14) showing independence of the two. Furthermore, the biological signal could be abolished independently of any change in the stimulus artefact by combined muscarinic receptor and purinoceptor blockade, exposure to α, β-mATP, and by exposure to solutions containing TTX.

Dependence on extracellular [CaCl2].

Attenuation of the evoked signals amplitude in the presence of $CaCl_2$ - depleted solutions was evidence that the signal is a biological event, as the detrusor smooth muscle action potential, unlike any artefact, is completely dependent on extracellular Ca^{2+} [Filo et al. 1965, Montgomery and Fry 1992]. However, pre-synaptic release of neurotransmitters is also a Ca^{2+} dependent process, as Ca^{2+} is required during fusion of neurotransmitter-containing vesicles with the synaptic membrane [Brading 1987].

Furthermore, the detrusor action potential (which is generated by opening of voltage-gated L-type calcium channels) is preceded by a post-synaptic excitatory junction potential (EJP) that is generated by the influx of cations through ligand-gated purinergic (P_{2X}) receptors. Accurate assessment of the contribution to the evoked electrical signal from these two components was not possible using the present preparation probably due to slow diffusion of calcium-channel blocking agents into the detrusor muscle. However the sensitivity of the signal to Ca^{2+} availability suggests a significant contribution from the action potential.

The persistence of the signal with calcium-free solutions may be due to several factors: Poor diffusion of the perfusate through the area of tissue being recorded from is likely as the bladder wall could be perfused on its serosal surface only, and the tissue bleb being recorded from was isolated from the perfusate by the suction seal around the electrode further hindering Ca^{2+} diffusion.

Neuromuscular origins of the signal.

Virtual abolition of the evoked electrical signal with prolonged exposure to α, β-mATP suggests predominant dependency of the signal on purinergic neuromuscular transmission. However in contrast to sucrose-gap studies, the signal also appeared to be reduced by atropine. Attenuation of the signal with the high dose of atropine used was surprising as muscarinic receptor blockade would not be expected to affect the electrical signal, and may have been due to several factors.

A proportion of the ganglia of excitatory parasympathetic motor nerves to the guinea-pig bladder are intra-mural and may be blocked by exposure to high doses of atropine acting on nicotinic receptors. This would prevent both cholinergic and purinergic neuromuscular transmission from the relevant post-ganglionic neurones. Furthermore a non-specific reduction in detrusor excitability has been shown to result

from exposure to high doses of atropine [Inoue and Brading 1991] and may also explain the atropine-induced reduction in signal amplitude.

Exposure to lower concentrations of atropine initially used resulted in sub-maximal attenuation of the evoked intravesical pressure rise, suggesting poor perfusion of the agent into the tissue (undescribed data). Similarly, lower concentrations of α, β-mATP than that used in these experiments failed to abolish the electrical signal. High concentrations of α, β-mATP may also have a direct inhibitory effect on the contractility of detrusor smooth muscle, therefore no further conclusions can be made from these data regarding the precise neuromuscular origins of the evoked electrical signal.

2.4.3 Intravesical recordings.

The finding that an α, β-mATP-sensitive electrical signal can be evoked by bladder neck stimulation and recorded using an intravesical catheter-mounted suction electrode has important implications as clinical electromyography would necessarily be performed using an intravesical device. No apparent change in the morphology of the suction electrode signals recorded from the bladders mucosal or serosal surface was seen, suggesting there is no contribution to the signal or increased interference to its recording due to the presence of urothelium. The similarity of the signal morphology and parameters when recorded from the bladders serosal and mucosal aspects under the same conditions, suggests that the bladder electromyogram could be recorded using an intravesical device. These studies therefore support the development of a *per-urethram* catheter mounted suction electrode for clinical use, should a potential application be demonstrated.

The observation that a very similar electrical signal could be recorded using a ball-shaped active electrode may also have clinical implications. The ball electrode is

unlikely to penetrate the mucosa and may therefore cause less discomfort than the needle in a conscious patient. Furthermore, a ball electrode is likely to be far safer for clinical use, reducing the risk of introducing infection and damage to the urethra during its placement.

2.5 Conclusions.

A biological electromyographic signal can be recorded from the guinea-pig bladder, and isolated from all artefacts using reversible suction electrodes. A similar signal can also be recorded from the bladders urothelial surface using an intravesical catheter-mounted suction ball-electrode. Precise determination of the neuromuscular and electrophysiological origins of this signal was not possible using the whole bladder preparation, due to uncertain perfusion of pharmacological agents in to the tissue. The following chapter describes suction electrode recordings from urothelially denuded detrusor strips, a more readily perfusable preparation and one more suitable for direct tension measurements.

CHAPTER THREE: SUCTION ELECTRODE RECORDINGS FROM ISOLATED GUINEA-PIG DETRUSOR STRIPS.

3.1 Introduction.

The electrophysiological origins of the electromyographic signal recorded from the intact guinea-pig bladder and its relationship to detrusor tension generation remain unclear. The second part of this thesis describes adaption of the apparatus and suction electrode to enable recordings from isolated detrusor muscle strips. We investigate the electrophysiological origins of the signal, and test the hypothesis that the suction electrode signal is generated by a purinergic and not a cholinergic mechanism

In contrast to guinea-pig detrusor strips which can be dissected with known orientation with relation to their innervation, and their intramural nerves stimulated focally with minimal artefact, human detrusor samples which were to be obtained during open bladder surgery were thought likely to be more difficult to orientate with respect to their intramural nerve supply. Stimulation of these nerves causing contraction of the entire muscle strip by focal direct stimulation might therefore be sub-optimal, and electrical field stimulation considered was more suitable. This section describes suction electrode recordings from both focally stimulated and field stimulated guinea-pig detrusor strips, and investigates methods of minimising stimulus artefact from the recordings. By doing so we aim to validate the suction electrode recording apparatus for the electrophysiological detection of human detrusor purinergic neurotransmission in a guinea-pig model.

3.2 Methods.

3.2.1 Adaptation of suction electrode for strip recordings.

The suction electrode was adapted to enable its positioning by a standard micromanipulator on small tissue samples. The stage two suction electrode structure was secured within a 15cm length of rigid plastic piping, and the wires leading from the active and reference electrodes fed through the side of the tube and secured to silver terminals. A hole (3mm diameter) was made in the side of the tubing 3cm from the tip of the electrode, over which a length of non-collapsible flexible plastic pipe was glued. The lumen of the plastic piping immediately distal from the electrode to the junction between the piping and tubing was sealed so that a suction force could be applied through the tubing to the suction electrode (figure 3.1). The active and reference electrodes were coated with platinum chloride as before.

3.2.2 Adaptation of the electrode for recording field-evoked electrical signals.

For field stimulated recordings the suction electrode structure was further modified in order to minimise its exposure to the stimulus, and to enable additional isolated recordings of the stimulus artefact to be made. The circumferential reference electrode was removed, and replaced by a ring of silicone rubber on to which were wound two sections of platinum wire of three coils each, at diametrically opposite points on the silicone ring (figure 3.2). The coils were coated with silicon adhesive on their outer surface so that only wire that was to be in contact with tissue was exposed, and platinised as before. The two reference electrodes were then individually wired to electrical terminals so that a separate signal could be recorded between the active and one reference electrode (A-R1 signal), and another between the two reference electrodes (R1-R2 signal).

micromanipulator holder

flexible tubing

electrode terminals

suction

rigid plastic piping
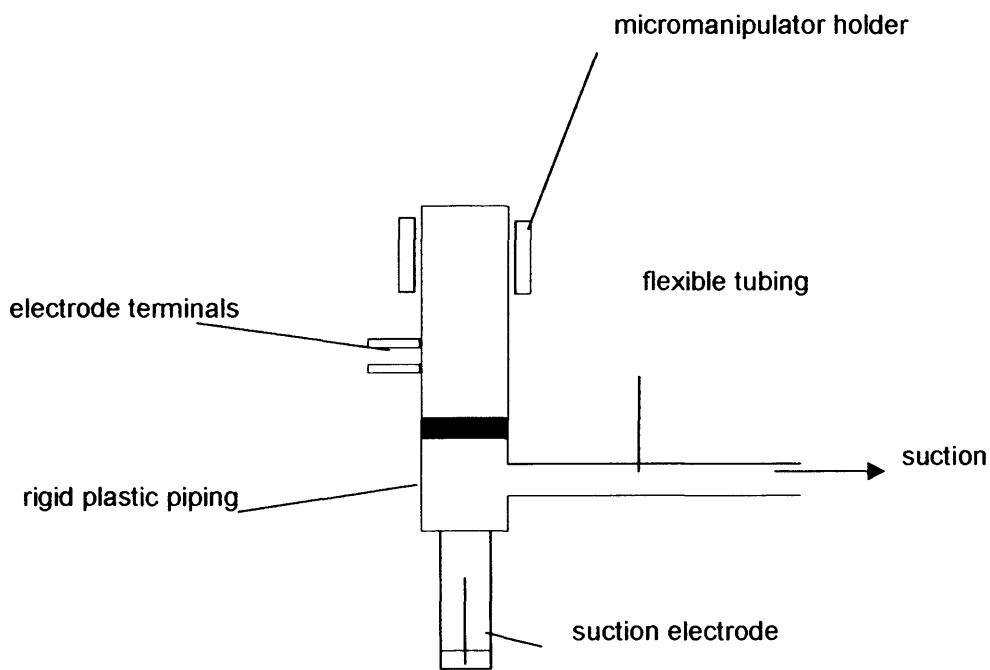
suction electrode

Figure 3.1.Schematic diagram showing suction electrode stucture adapted for positioning on detrusor strips.

A

B
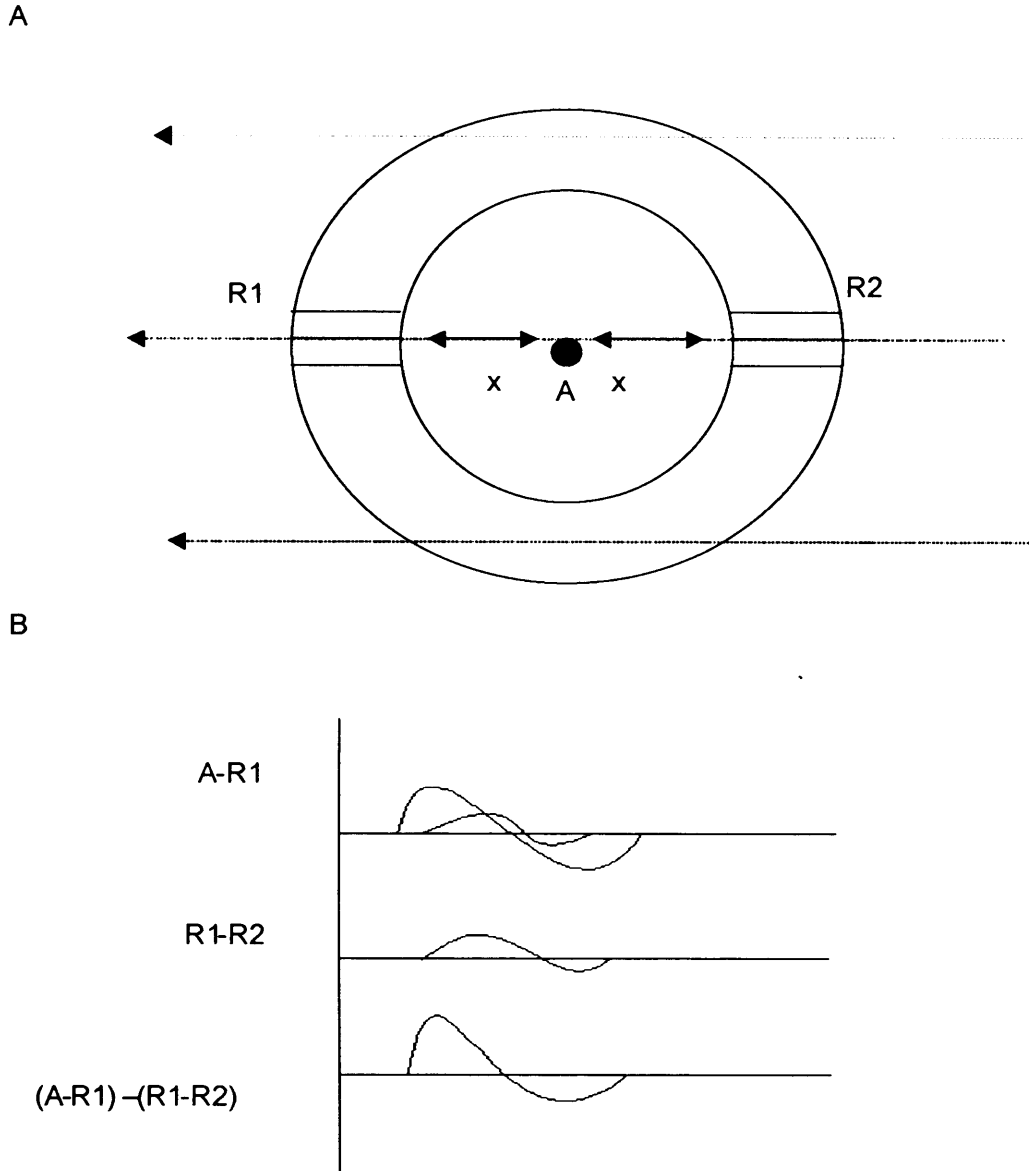
Figure 3.2. A. End view of modified suction electrode showing relationship of the three electrodes (A, R1 and R2). Arrows indicate field stimulus direction. B. Diagrammatic representation of theoretical signals recorded between different electrodes, with subtraction of the artifact recorded between R1 – R2 from the A –R1 signal producing the biological signal.

3.2.3 Construction of the organ bath and stimultor.

A perspex organ bath with a 5ml perfusion trough was adapted to enable mounting, superfusion and electrical stimulation of detrusor strips. A steel hook was positioned at one end, to which one end of the detrusor strip could be secured, and apparatus by which physiological solutions could be warmed, gassed and allowed to flow through the organ bath under gravity at a constant rate was set-up. A drainage system was attached to the organ bath.

An externally insulated tri-polar stimulation electrode was constructed by embedding three 1mm platinum strips in a trough-shaped silicone mould, 2mm apart. The electrode arrangement was secured within the trough of the organ bath so that the fixed end of the detrusor strip could be positioned within it, and focally stimulated by direct contact with the electrodes (figures 3.3- 3.6). The central electrode was connected to the cathode and the outside electrodes to the anode of the stimulator.

A standard Bridge tension transducer (Force-displacement transducer FT03, Grass Quincy, Mass. USA) was connected to a micromanipulator so that its hook could be precisely positioned and moved within the perfusion trough. The organ bath and micromanipulators were secured to a raised platform that was insulated against vibration.

3.2.4 Tissue acquisition and mounting.

Male guinea-pigs (Dunkin Hartley, 300-500g) were killed by cervical dislocation under Home Office licence, and their bladders excised. Full thickness detrusor strips (3 mm width x 8-10mm length) were dissected along the longitudinal axis of the bladder (neck to dome), along which intramural nerves were expected to run, so that nerve-mediated contraction of the whole strip could be evoked by focal stimulation of the strip at one end. The urothelium was carefully dissected away, and
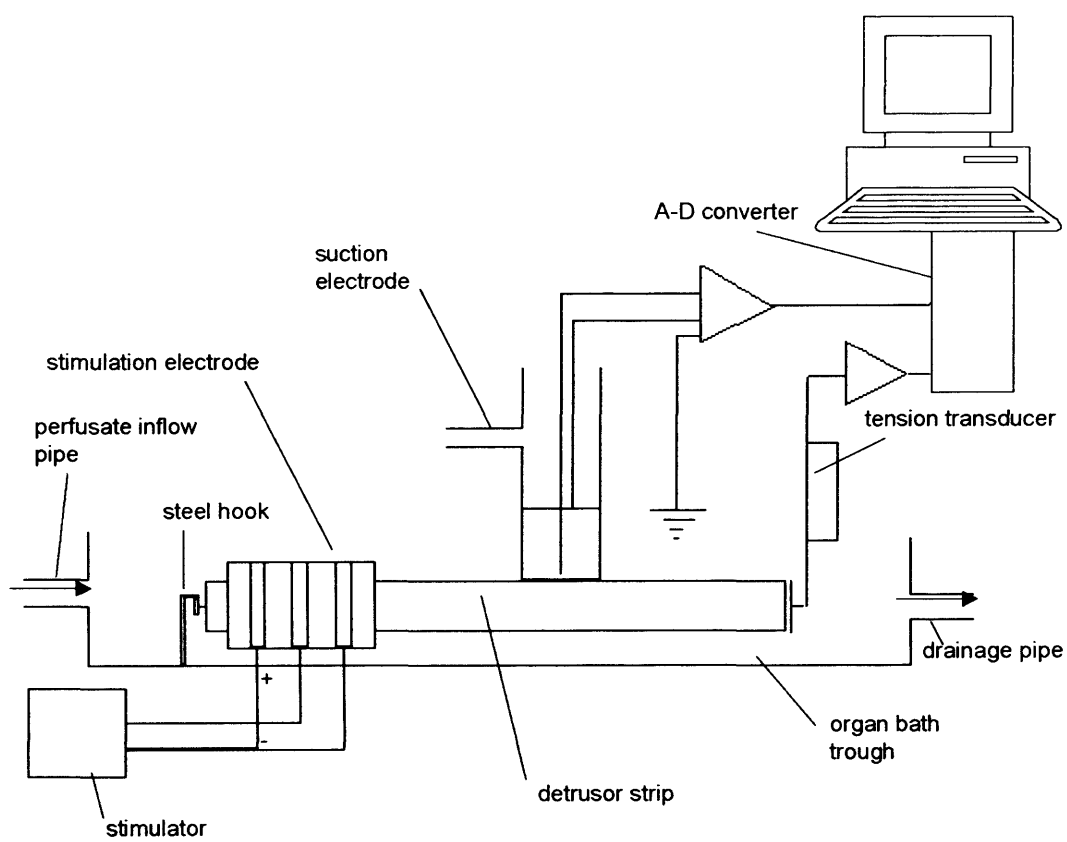
Figure 3.3. Experimental set-up for recording simultaneous extracellular electrical and mechanical activity from detrusor smooth muscle strips.
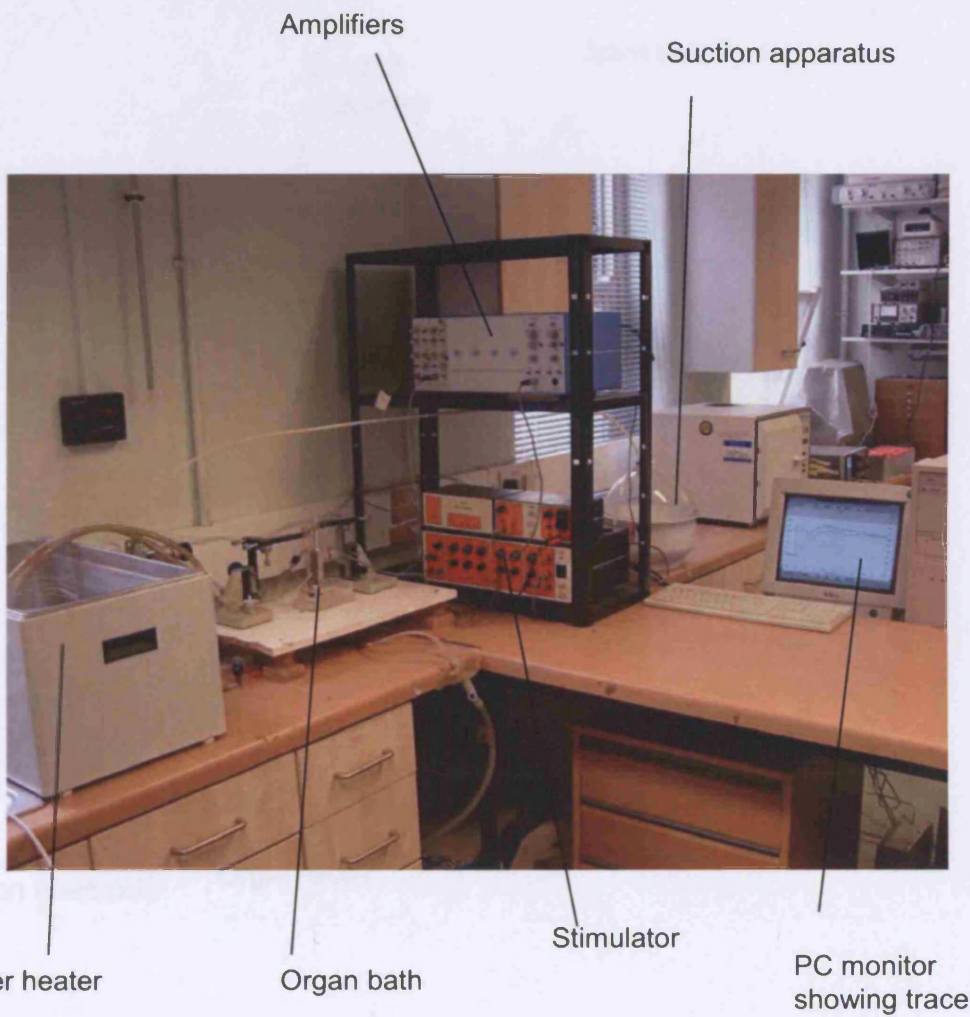
Figure 3.4. Photograph of experimental set-up for recording from detrusor strips.
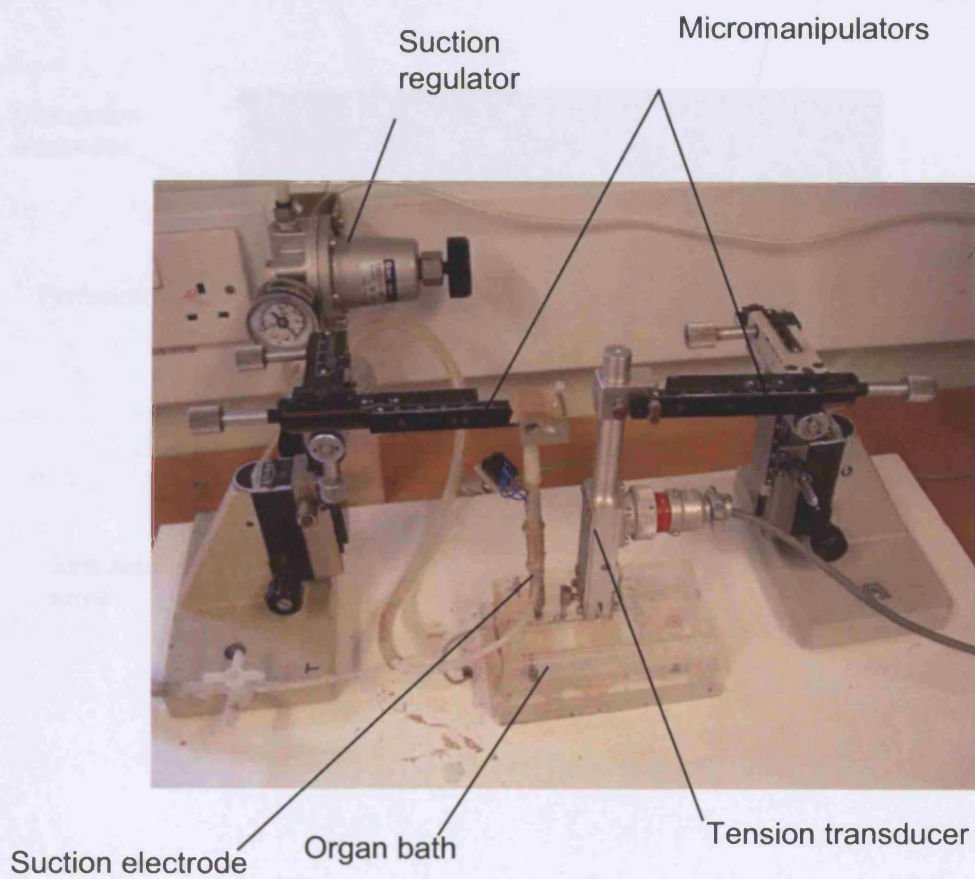
Figure 3.5. Photograph of experimental set-up for recording from detrusor strips.
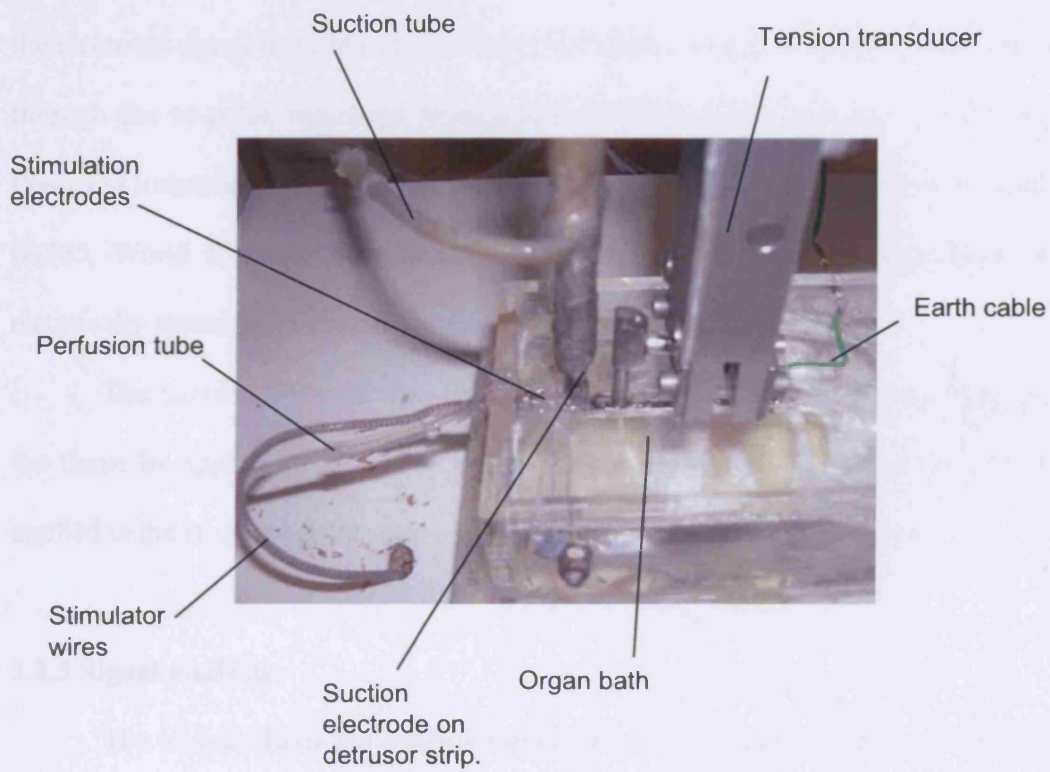
Figure 3.6. Close-up photograph of suction electrode applied to mounted detrusor strip.

the strips were mounted in the organ bath with the end originating closest to the bladder neck fixed to the hook in contact with the stimulation electrodes, and the other secured to the tension transducer.

The strips were superfused with gassed (95% O_2, 5% CO_2) modified Tyrode's solution at 36 (+/-1) °C, at 3-5ml min^{-1}. Electrical stimulation was delivered to the strips at the end originating from near the bladder neck with short trains (2-10 pulses) of 100μs pulses at frequencies optimized to produce adequate temporal separation of the electrical signal from stimulus artifact (50-100Hz). Direct stimulation was delivered through the tri-polar stimulator from a biological electrical stimulator (A310 World Precision Instruments, Sarasota, FL, USA) via a high current (100mA) stimulus isolator (A385, World Precision Instruments). The strips were allowed to equilibrate until electrically stimulated contractions of constant magnitude were observed.

The suction electrode was manipulated over the detrusor strip and attached to the tissue by applying a 60-80kPa suction force through its lumen. Tension was then applied to the strip to a limit determined by competency of the suction seal.

3.2.5 Signal analysis.

The signals from the tension transducer were amplified (Bridge 8 amplifier, World Precision Instruments) and digitized. The electrical signals from the suction electrode were filtered (1Hz – 1 KHz), amplified (Iso-Dam8a bio-amplifier, World Precision Instruments) and digitized. Both signals were displayed in real time on a standard personal computer (450MHz, 64Mb RAM) using proprietary software (Picoscope for Windows, Pico Technology Ltd, Hardwick, Cambs, UK).

3.2.6 Quantification of cholinergic and purinergic contractions at varying stimulus intenstities.

The relative contribution to the total nerve-mediated contraction from cholinergic and purinergic neurotransmission is known to be stimulus dependent, with the cholinergic component being more evident at higher frequencies [Brading and Williams 1991]. In these experiments low numbers of pulses delivered at high frequencies were necessary to optimise separation of the electrical signal from the stimulus artefact, therefore experiments were conducted to determine the relative contribution of purinergic and cholinergic neurotransmission to the contractile response at experimental stimulation intensities to ensure that both mechanisms were expressed.

For this purpose very small (3-4mm length, < 1mm diameter) urothelially denuded detrusor strips were prepared and mounted in a 5ml organ bath with one end fixed, the other attached to the tension transducer. The strips were perfused as before, and after a period of equilibration stimulated with 2, 4 and 8 pulses of 100μs pulse widths at pulse intervals of 125ms (8Hz), 31.25ms (32Hz) and 8ms (125Hz), at 90 s intervals before and during exposure to atropine 1μM or α, β-mATP 10 μM. Different strips were used for each intervention. The strips were stimulated using electrical field stimulation delivered through platinum electrodes positioned either side of the perfusion trough and connected to the stimulation apparatus previously described. The peak tension values generated during exposure to atropine and α, β-mATP were compared with controls. This apparatus was previously validated for the perfusion and nerve-mediated stimulation of small detrusor strips [Palfrey and Fry 1994]. The sensitivity of the contraction to TTX was determined at the highest intensity of stimulation (8 pulses at intervals of 8ms).

3.2.7 Experiments to determine electrophysiological characteristics of the signal.

The effect of pharmacological agents dissolved in Tyrode's solution on the amplitude of the electrical signal was determined. The tissue was stimulated at 90 s intervals, a period found to result in no deterioration of the signal or contraction over long periods (5-6 h). Recordings of restitution of the signal after post-intervention washout were performed to exclude errors from changes in tissue excitability and electrode failure.

Sensitivity of the electrical signal to extracellular $CaCl_2$ depletion.

The effect of $CaCl_2$ depletion (1.8 mM-0.2 mM in 0.2 mM gradations) of the superfusate on the amplitude of the electrical signal was determined. The total content of divalent ions in the superfusate was maintained at 2.8 mM by replacing $CaCl_2$ with $MgCl_2$ The elapsed time necessary for the reduction in signal amplitude to stabilize during perfusion with the initial $CaCl_2$-depleted solution (containing 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$) was recorded for each strip (10-15min), and subsequent measurements of the signal amplitude in the presence of subsequent $CaCl_2$-depleted solutions were performed after the same period of perfusion to avoid errors from variable delivery of the solution into the tissue.

Sensitivity of the electrical signal to atropine and α, β-mATP.

Having determined the contribution of purinergic and cholinergic mechanisms on the total detrusor contraction evoked by various stimulation intensities, the effect on the amplitude of the electrical signal of atropine α, β-mATP and atropine together with α, β-mATP using stimuli known to evoke purinergic and cholinergic mechanisms was determined. Comparison of the signal amplitude and peak tension generation before and during exposure to atropine was made after attenuation of the evoked peak tension

had stabilised with atropine as there was usually no effect on the electrical signal. This was within 20 min of exposure to the agent. The effectiveness of muscarinic receptor blockade after this period of exposure was tested by aplication of the cholinergic analogue carbachol 10µM, as there was often little atropine-sensitive contraction evident. Further analysis of the effect of atropine on the contraction was investigated using 3 s trains of pulses delivered at intervals of 50ms before and during exposure to the drug.

Sensitivity of the electrical signal to calcium channel blocking agents.

Depletion of the $CaCl_2$ content of the superfusate would in addition to reducing the amount of extracellular Ca^{2+} available for membrane depolarisation, be likely to inhibit calcium-dependent pre-synaptic neurotransmitter release mechanisms. The sensitivity of the electrical signal to tissue superfusion with nifedipine 30 µM, nifedipine 30 µM with $NiCl_2$ 0.5 mM, and nifedipine 30 µM with $CdCl_2$ 20µM, all judged to block detrusor membrane Ca^{2+} channels selectively (L and T-type), but not the pre-synaptic N-type Ca^{2+} channels responsible for neurotransmitter release, was also investigated to determine the dependency of the signal on transmembrane calcium currents.

3.2.8 Recording from field stimulated guinea-pig detrusor strips.

Full thickness 3 x 8mm guinea-pig detrusor strips were prepared as before, and mounted in an organ bath equipped with platinum field stimulating electrodes with one end of the strip fixed, the other attached to a tension transducer, and superfused with gassed, warmed Tyrode's solution. After a period of equilibration, the modified suction electrode was attached to the tissue which was then field stimulated using six pulses of 0.1ms width delivered at 10ms intervals in accordance with the stimulation equipment

described below. The electrical signals recorded from the suction electrode were processed as before.

3.2.9 Analysis of the signal evoked by field stimulation.

The A-R1 and R1-R2 signals recorded by the modified suction electrode were compared. The effects on the two signals of $CaCl_2$ –free superfusate, α, β-mATP 10-50μM and TTX 1 μM were determined to identify the biological component. The effect of altering the orientation of the reference electrodes with respect to the stimulating electrodes was also investigated, and the influence of filtering systems determined by comparing signals recorded with the electrode attached to dead tissue (stored in water for 48h) in a 1Hz-1KHz restricted band with those in a 0.1Hz-10KHz band. The effect of reversing the polarity of the stimulus on the signal was also examined to verify the real signal, which unlike any artefact would not be influenced by stimulus polarity.

3.2.10 Data analysis.

The effect of pharmacological interventions on the electrical signal was calculated from the mean of three consecutive recordings when the effect of the intervention on the signal was judged to have stabilised.

Analysis of differences between the signals and controls was conducted using Students't-test. Comparisons between the mean atropine and α, β-MATP resistant contractions produced by the different intensities of stimulation were performed using one-way analysis of variance (ANOVA). The null hypothesis was rejected at $P < 0.05$.

3.3 Results.

3.3.1 Signal parameters.

A predominantly bi-phasic electrical signal was recorded from 37 detrusor strips taken from 24 guinea-pig bladders. The parameters of the signal are shown in table 3.1. The mean amplitude and time to depolarization of the signal were significantly smaller than that of the signal previously recorded from the whole bladder, the signals duration was not significantly different (table 3.1).

3.3.2 Relationship between atropine and α, β-mATP - resistant components of the contraction and stimulus intensity.

The evoked contractions of detrusor strips during exposure to atropine $1\mu M$ and α, β-mATP $10\mu M$ at various intensities of stimulation are shown in figures 3.7 and 3.8. The mean atropine- resistant component was 75 ± 9 % of control (range 55-85, n=4) and was significantly attenuated in association with stimuli of increasing intensities (P=0.017). However, this effect did not reach statistical significance when calculated for increasing numbers of pulses or pulse intervals independently (table 3.2). The mean α, β-mATP - resistant component was 31 ± 17 % control (range 12-50, n=6), and was significantly increased when evoked by stimuli of increasing intensities (p=0.03). This increase was also statistically significant when calculated for the number of stimulating pulses (table 3.2B), but not for pulse interval (table 3.2A). Tetrodotoxin $1\mu M$ abolished the contraction evoked by 8 pulses at all frequencies (n = 2).

The results suggest that whereas the component of the contraction generated by purinergic neurotransmission is independent of the stimulation intensity and at a maximum when evoked by only two pulses, the cholinergically mediated component varies with stimulus intensity, and contributes very little to the total at low intensities

Signal parameters	Bladder (n=6)	Strip (n=37)	P- value
Amplitude (µV)	646 ± 123	222 ± 238	< 0.001
Duration (ms)	293 ± 20	236 ± 139	0.33
Depolarisation time (ms)	61 ± 12	43 ± 16	0.029

Table 3.1 Parameters (mean ± SD) of the evoked electrical signal recorded from guinea-pig detrusor strips and whole bladders. P- values refer to the probability of a difference between the two.
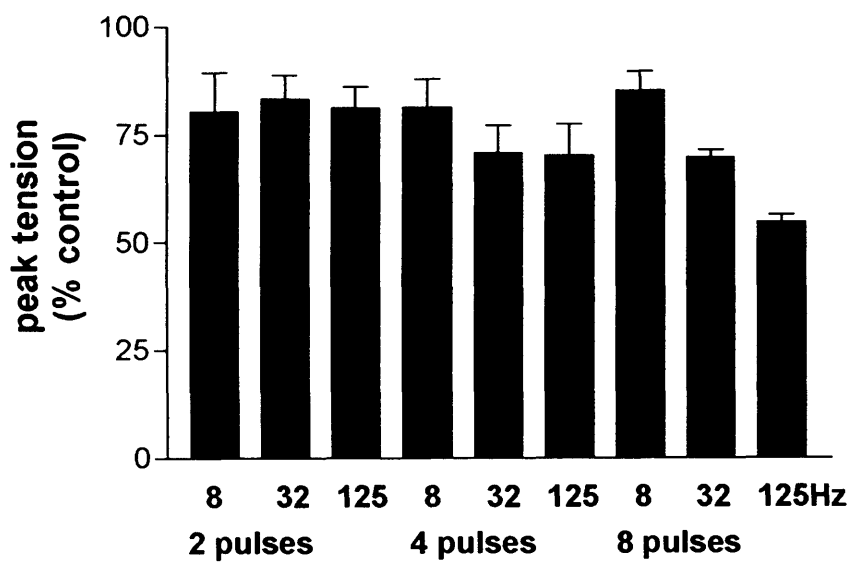
Figure 3.7. Bar chart showing residual peak tension evoked from guinea-pig detrusor strips by increasing stimulation during perfusion with atropine.Mean ± SD (n = 4).
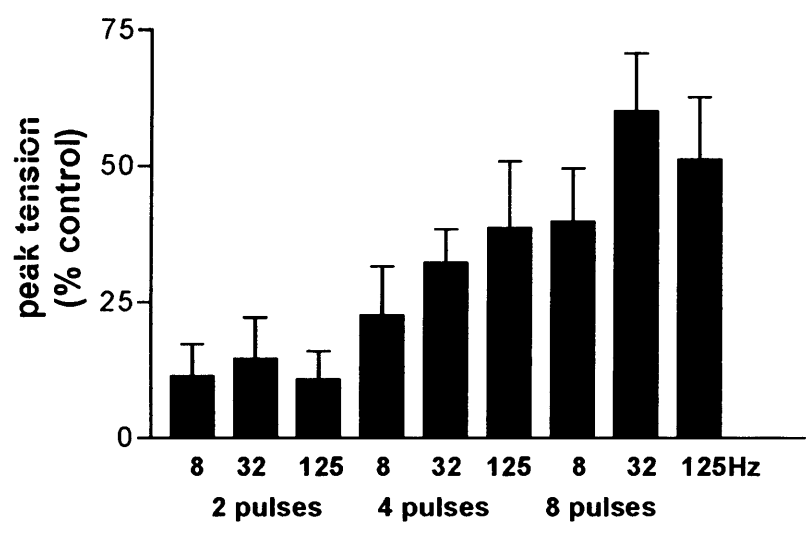
Figure 3.8. Bar chart showing residual peak tension evoked in guinea-pig detrusor strips by increasing stimulus parameters during perfusion with α. β-mATP. Mean ± SD (n = 6).

Pulse interval (ms)	Atropine	α, β-mATP
125	82 ± 3	25 ± 14
31.25	75 ± 7	36 ± 23
8	69 ± 13	34 ± 21
P- value	0.25	0.75

Table 3.2A Peak evoked tension of guinea-pig detrusor strips at various pulse intervals during perfusion with atropine and α, β-mATP. Mean ± SD, % nerve-mediated control. P- values refer to the probability of a difference between the residual peak tension between the three groups (ANOVA).

Number of pulses	Atropine	α, β-mATP
2	81 ± 1.5	12 ± 2
4	74 ± 6	31 ± 8
8	70 ± 15	50 ± 10
P- value	0.38	0.003

Table 3.2B Peak evoked tension of guinea-pig detrusor strips evoked various numbers of pulses during perfusion with atropine and α, β-mATP. Mean ± SD, % nerve-mediated control. P- values refer to the probability of a difference between the residual peak tension between the three groups (ANOVA).

implying that ATP is more readily released from motor nerves than acetylcholine. In light of these results, experiments to determine the effect of atropine on the electrical signal were performed using at least 4 pulses.

3.3.3 Charaterisation of the signal.

Effect of extracellular $CaCl_2$ - depletion of the superfusate on the signal.

The electrical signal was sensitive to graded depletion of the [$CaCl_2$] of the superfusate in a concentration dependent manner. Responses to $CaCl_2$ depletion of the superfusate stabilized within 15 min of exposure to each solution. Figure 3.9 shows a typical individual experiment and demonstrates a gradually reduced amplitude and increased duration of the electrical signal with this intervention. A small contraction remained, and was presumably generated by calcium release from residual intracellular Ca^{2+} stores. The effects of [$CaCl_2$] depletion on detrusor strips taken from six different bladders is shown in figure 3.10. The signal was abolished by solutions containing < 0.4mM $CaCl_2$ in all experiments. Restitution of the signal occurred rapidly in response to superfusion with normal Tyrodes solution.

Effect of α, β-mATP on the signal.

The electrical signal was abolished in the presence of α, β-mATP (30 or 50 μM) in association with an attenuated contraction (16 ± 9% control, n=6). A representative trace is shown in figure 3.11. Abolition of the signal by α, β-mATP took considerably longer than with $CaCl_2$ depleted superfusate. Both mechanical and electrical signals partly recovered after a prolonged washout period, controlling against electrode failure or tissue death. All electrical and mechanical activity was subsequently abolished by
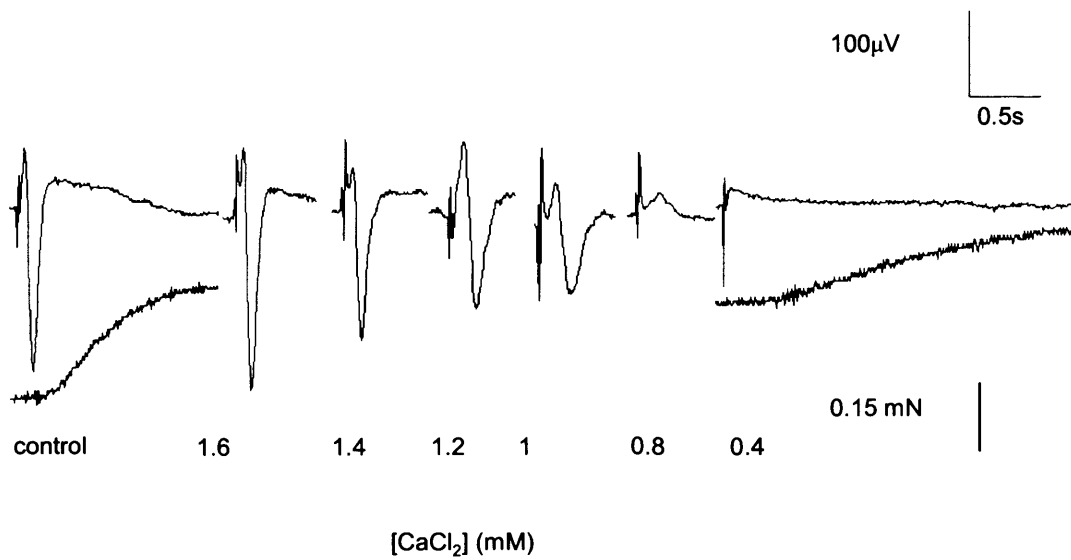
Figure 3.9. Representative traces showing effect of graded $CaCl_2$-depletion of the superfusate on the evoked electrical signal recorded from guinea-pig detrusor strips. Mechanical traces also shown for highest and lowest values.
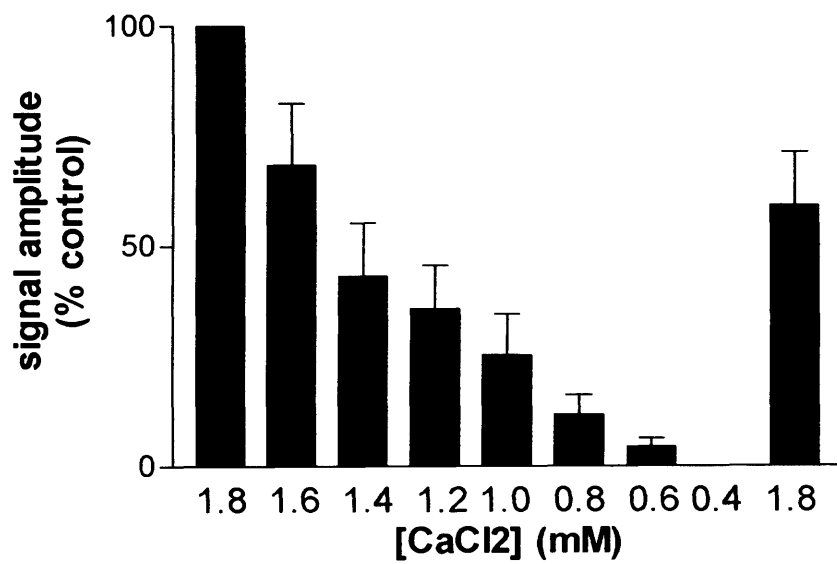
Figure 3.10. Bar chart showing effect of $CaCl_2$ -depleted superfusate on the amplitude of the electrical signal evoked from guinea-pig detrusor strips. Mean ± SD, (n = 6).

Control

α,β-mATP

Washout

50μV

0.5s

0.3mN
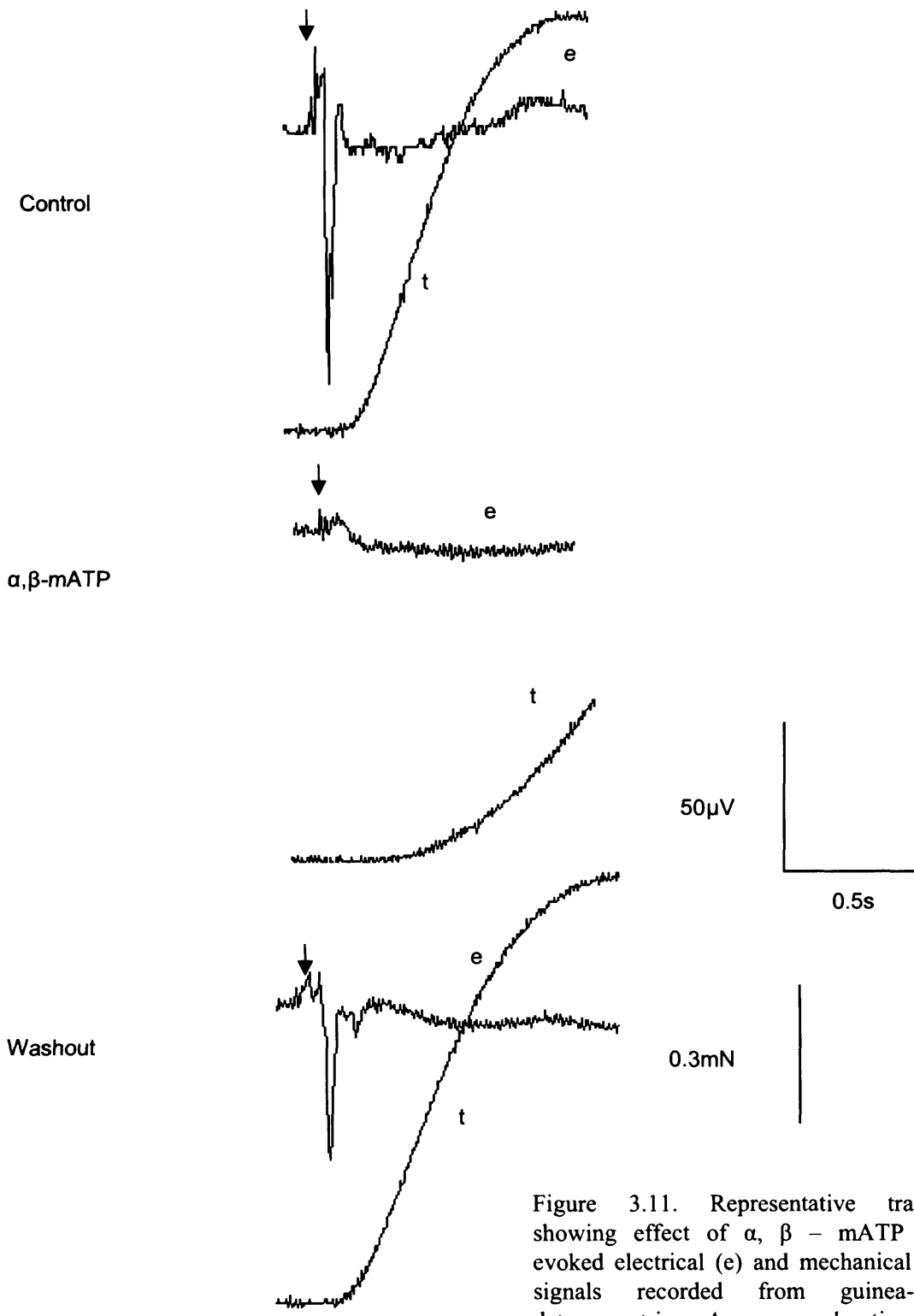
Figure 3.11. Representative traces showing effect of α, β – mATP on evoked electrical (e) and mechanical (t) signals recorded from guinea-pig detrusor strips. Arrows mark stimulus artifact.

tetrodotoxin 1 μM. Figure 3.12 shows the combined results of experiments performed on detrusor strips taken from six different bladders.

Effect of atropine on the signal.

The efficacy of atropine 1μM in producing complete muscarinic receptor blockade was tested. Application of carbachol to the preparation after 20 minutes of exposure to atropine resulted in no contracture (n = 2). With atropine 1μM, nerve evoked contractions were significantly reduced to 74 $\pm$ 14 % control in 12 strips (P<0.0001), and were changed in response to supramaximal stimulation with the later portion attenuated. In 10 of these the amplitude of the electrical signal was not significantly reduced, a representative trace is shown in figure 3.13. In two preparations, however, the electrical signal was reduced to a mean 71 % of control when the contraction was reduced to an average of 68% of control. Overall, the mean amplitude of the electrical signal was not reduced by exposure to atropine while the peak contraction was attenuated (P=0.77) (figure 3.14). No change was observed change in signal morphology with atropine, and no additional effect on the electrical or mechanical signal of an increased concentrations (10μM) or prolonged atropine exposure was observed.

Effect of atropine and α, β-mATP on the signal.

In 6 preparations exposed to atropine, α, β-mATP 50μM was added to the atropine-containing superfusate. In all cases both the signal and the residual contraction were abolished.

Effect of Ca^{2+} channel blocking agents on the signal.

Table 3.3 shows the effect of nifedipine, nifedipine plus $NiCl_2$, and nifedipine plus $CdCl_2$ on the amplitude of the evoked electrical and mechanical signals. Both were

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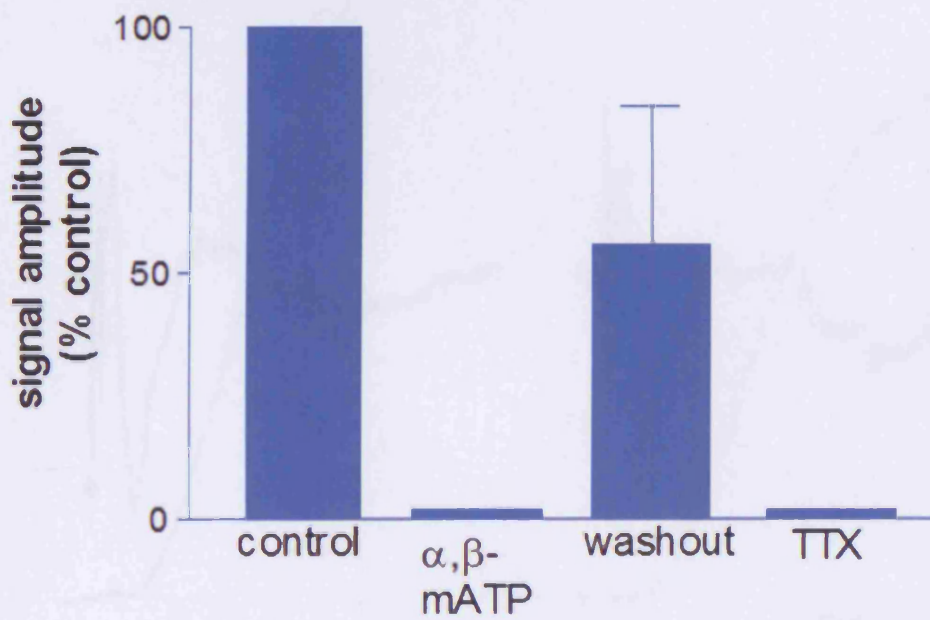
Figure 3.12. Bar chart showing effect of α, β – mATP on the mean amplitude of the evoked electrical signal recorded from guinea-pig detrusor strips and of TTX 1μM on the washout signal. Mean ± SD (n = 6).
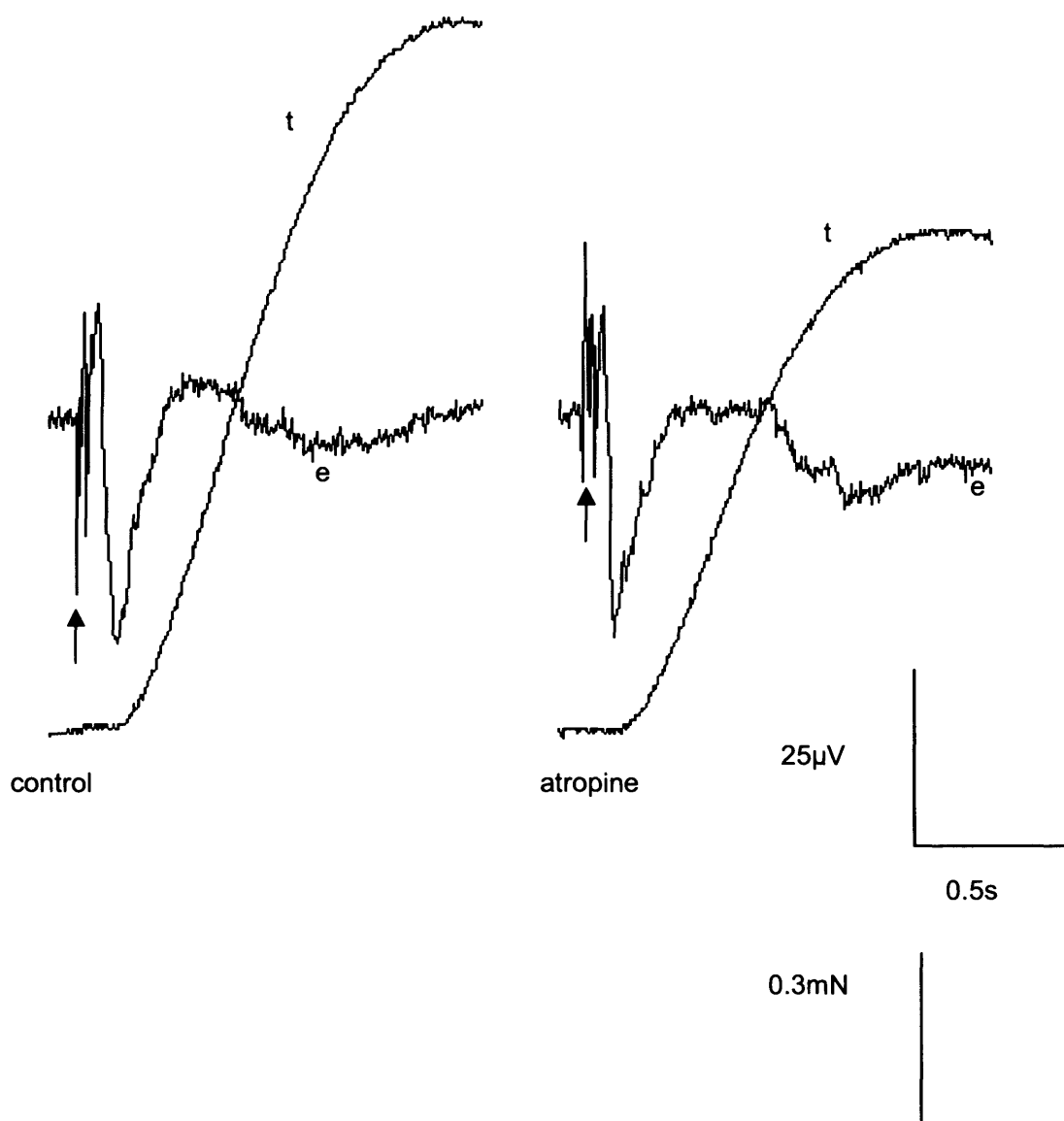
Figure 3.13. Representative traces showing effect of atropine 1μM on evoked electrical (e) and mechanical (t) signals recorded from guinea-pig detrusor strips. Arrows mark stimulus. Note no change in amplitude or morphology of the electrical signal but attenuation of the peak tension.

116
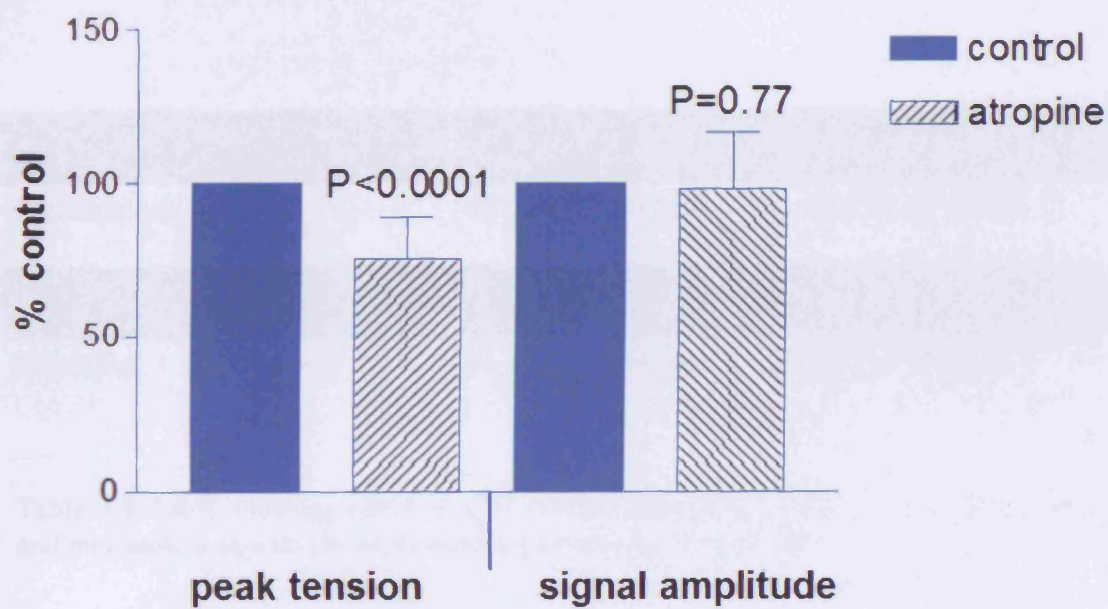
Figure 3.14. Bar chart showing effect of atropine 1μM on evoked electrical signal and mean peak tension recorded from guinea-pig detrusor strips. Mean ± SD (n = 12).

significantly disrupted by nifedipine. However, the electrical signal was further reduced by application of pharmacological agent. Figures 3.18c, d, e show that contraction was almost abolished. In two experiments the evoked electrical and mechanical signals were abolished by application of ... β-toxTP500 µM (figure 3.18...

3.3.5 Spontaneous activity

Spontaneous activity, qualitatively but similar in morphology to those evoked by electrical stimulation, was sporadically observed soon after electrode placement on the detrusor strip (figure 3.19)... muscle were prominent injury potentials and afterdepolarisations seen in myolized 3.19b). Rhythmic slow component activity was

Intervention	Signal amplitude	Peak tension
Nifedipine (n = 3)	40 ± 28 (P = 0.033)	38 ± 28 (P = 0.03)
Nifedipine + CdCl$_2$ (n = 4)	12 ± 8 (P < 0.001)	< 1 (P< 0.001)
Nifedipine + NiCl$_2$ (n = 5)	11 ± 8 (P< 0.001)	2 ± 2 (P< 0.001)

Table 3.3 Table showing effect of Ca^{2+} channel blocking agents on evoked electrical and mechanical signals (% nerve-mediated control). Mean ± SD.

An evoked electrical signal was recorded from local electrode bundle in three detrusor strips from three rats ... Two NiCl$_2$ and CdCl$_2$ were different in morphology. The first injury component ... contained components that were identical to those reaction with CdCl$_2$ application abolished with pure ... in ... figure ... CdCl$_2$ and TTX (figure 3.21) showing a signal similar in morphology to that recorded in the TTX system that was prevented artefact. However, the NiCl$_2$ signal was also subsequently altered by the later intracellular component that it also abolished its mechanical component

significantly attenuated by nifedipine alone, and the electrical signal was further reduced by a combination of pharmacological agents (figures 3.15-3.16), when the contraction was almost abolished. In two experiments the residual electrical and mechanical signals were abolished by application of α, β-mATP 100 μM (figure 3.17).

3.3.4 Spontaneous activity.

Signals of smaller amplitude but similar in morphology to those evoked by electrical stimulation were occasionally observed soon after electrode placement on the detrusor strip (figure 3.18A), which were presumably injury potentials, and spontaneously during recordings (figure 3.18B). Although some contractile activity was evident, the degree of coupling between spontaneous electrical and mechanical activity was inconsistent. Signals of similar amplitude were noted during initial exposure to α, β-mATP in two experiments. No electrical activity was observed during applications of carbachol.

3.3.5 Recordings from field-stimulated detrusor strips and optimisation of the stimulus artefact.

An evoked electrical signal was recorded from both electrode systems in three detrusor strips taken from two animals. The A-R1 and R1-R2 signals differed in morphology. The A-R signal contained a bi-phasic component that was abolished during perfusion with $CaCl_2$ depleted superfusate (figure 3.19) α, β-mATP (figure 3.20), and TTX (figure 3.21), leaving a signal similar in morphology to that recorded by the R1-R2 system that was presumed artefact. However, the R1-R2 signal was also inconsistently altered by the same interventions suggesting that it may also contain a small biological component.
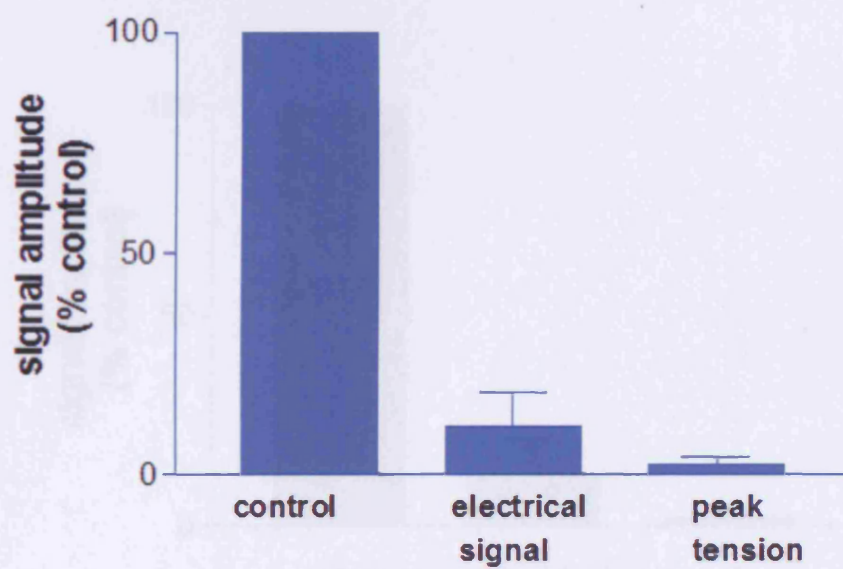
Figure 3.15. Bar chart showing effect of nifedipine 30μM plus NiCl 0.5mM on evoked electrical and mechanical signals recorded from guinea-pig detrusor strips. Mean ± SD (n = 5).
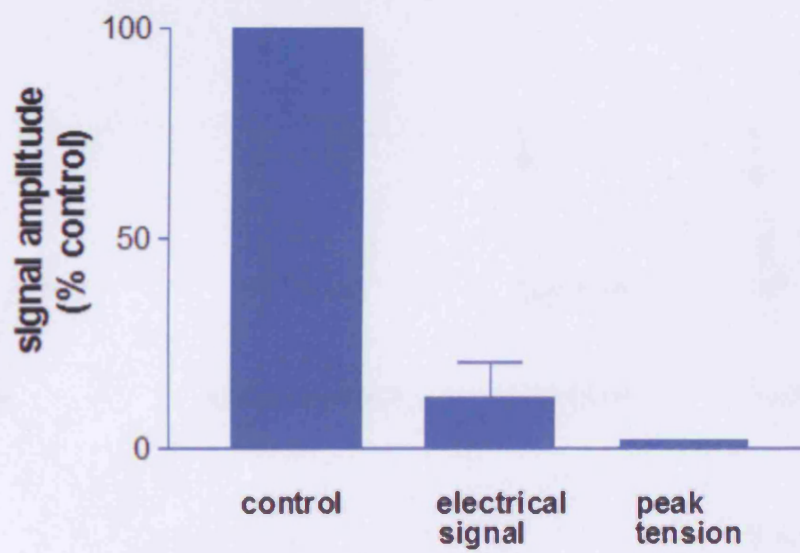
Figure 3.16. Bar chart showing effect of nifedipine 30µM plus CdCl 20µM on evoked electrical and mechanical signals. Mean ± SD (n = 4).

control   nifedipine+NiCl$_2$ α,β-mATP   washout

50μV

0.5s
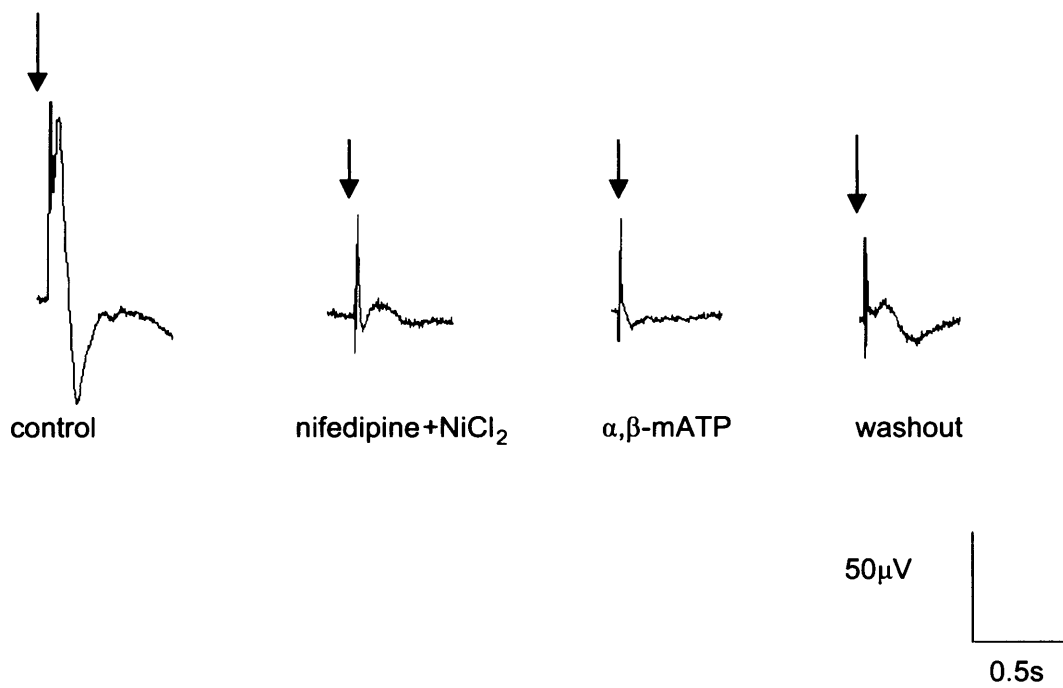
Figure 3.17. Representative trace showing effect on the electrical signal recorded from guinea-pig detrusor strips of nifedipine and NiCl$_2$ and abolition of the residual signal by α, β-mATP. Arrows mark stimulus.

A

B

25µV

0.14mN

0.5s
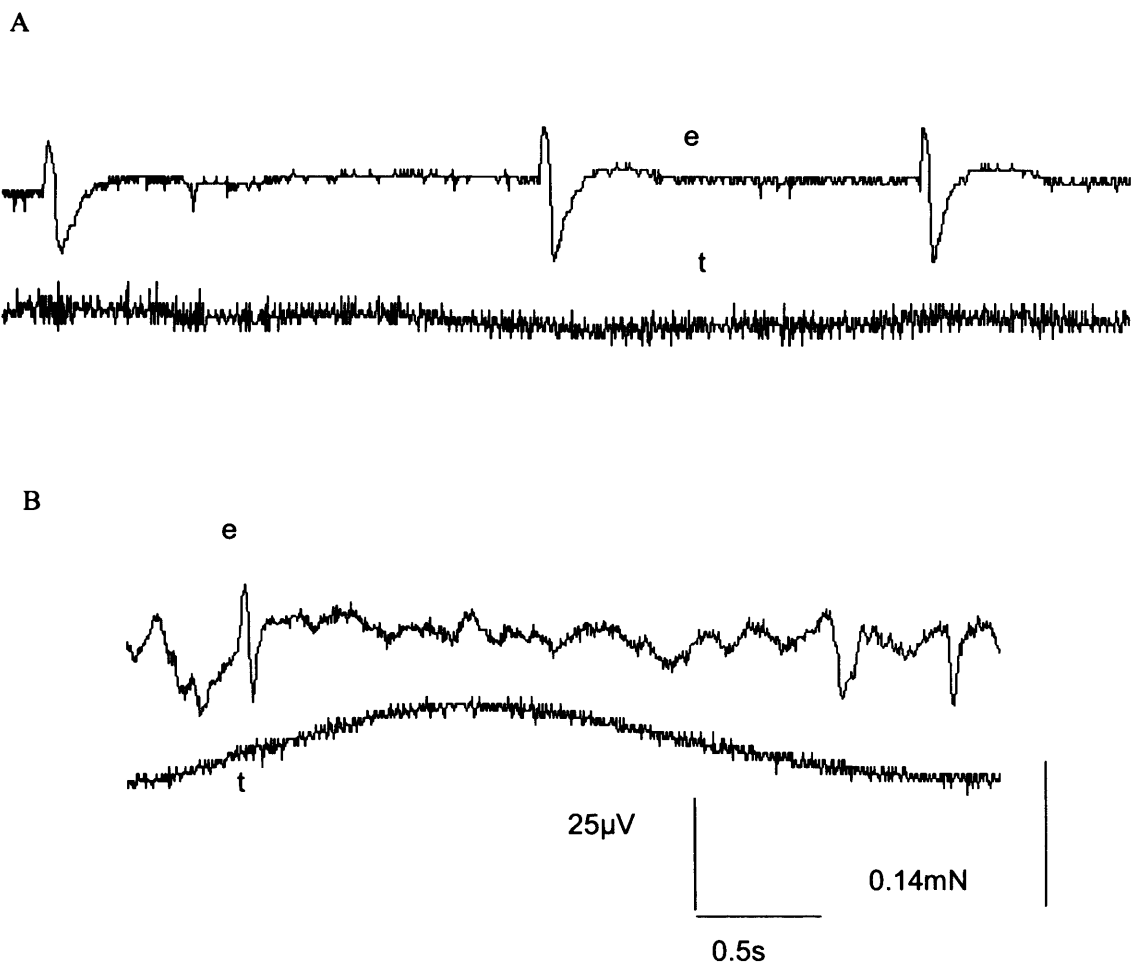
Figure 3.18. Examples of non-stimulated electrical (e) and mechanical (t) activity recorded from guinea-pig detrusor strips. A= Soon after electrode placement, B= In between regular stimuli.
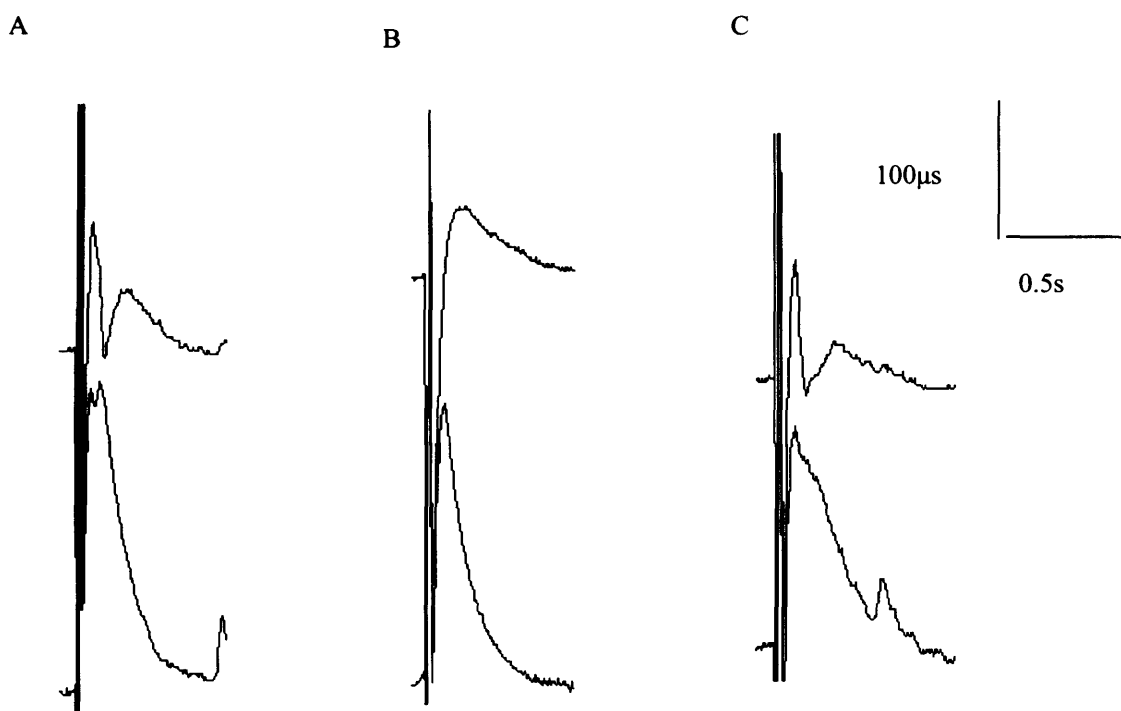
Figure 3.19. Representative A-R (top) and R-R (bottom) electrical traces showing effect of $CaCl_2$ - depletion of the superfusate. A = control, B = zero $CaCl_2$, C = washout.
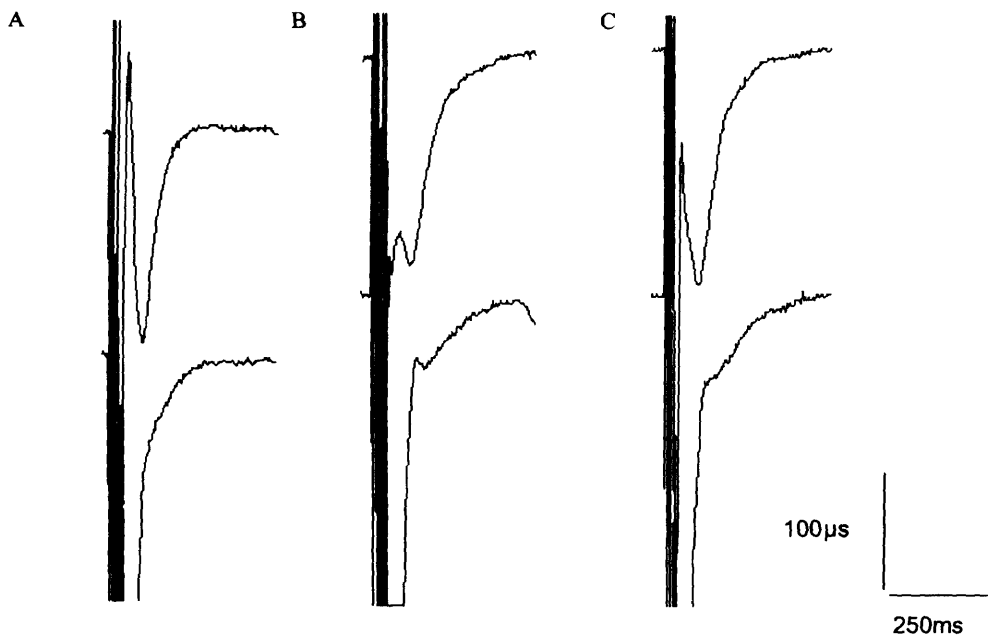
Figure 3.2. Traces of A –R (top) and R – R (bottom) signals showing effect of α, β – mATP. A = control, B = partial effect, C = abolition of signal.

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A   B
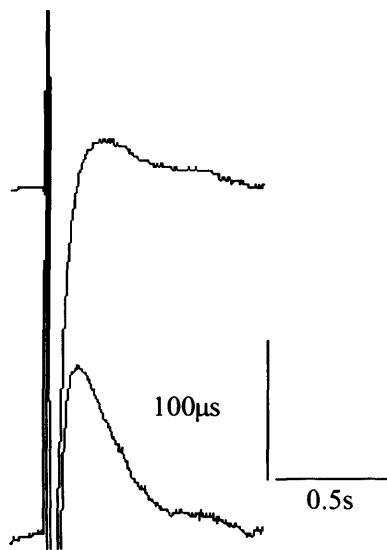
Figure 3.21. Traces of A – R (top) and R – R (bottom) electrical
signals. A = control signal, B = with TTX showing stimulus artefact.

The stimulus artefact recorded in the absence of a biological signal with the electrode attached to a dead detrusor strip was reduced when processed using the wider electronic bandwidth (figure 3.22). The morphology of the stimulus artefact was also affected by rotating the suction electrode with relation to the field stimulus, and could be optimally minimised by positioning both the reference and active electrodes equidistant from the stimulating electrodes. The independence of the biological signal from artifact was verified by recording using reversed polarity stimulus. This generated an unaltered biological signal but reversed the polarity of the artefact (figure 3.23).

A   B

100μs

0.5s
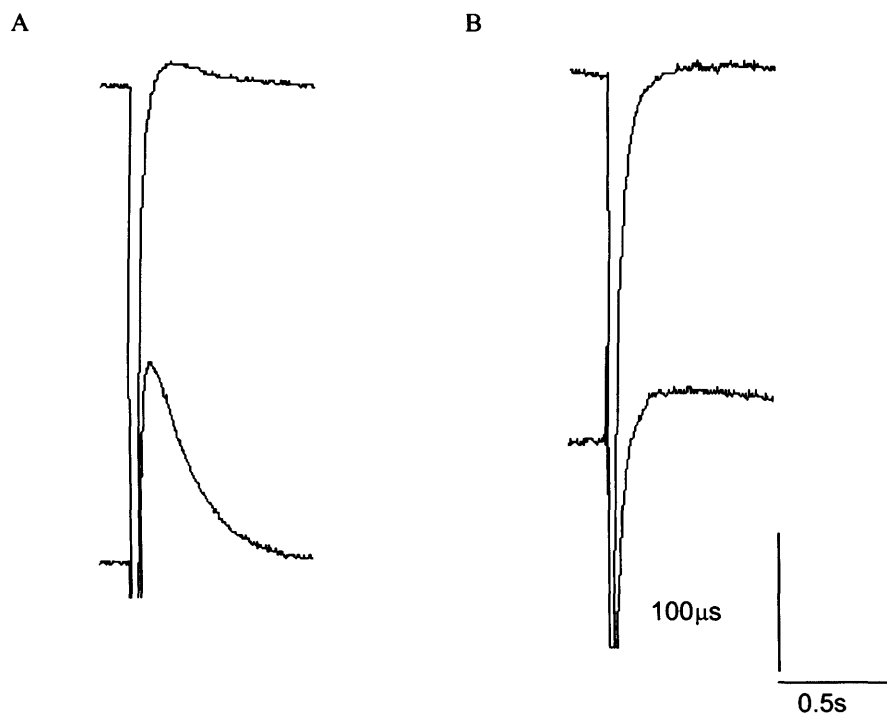
Figure 3.22. Representative A – R (top) and R – R (bottom) electrical traces recorded from dead tissue in 1Hz – 1KHz restricted band (A), and 0.1Hz – 10KHz band (B). Note reduced stimulus artefact in the later.
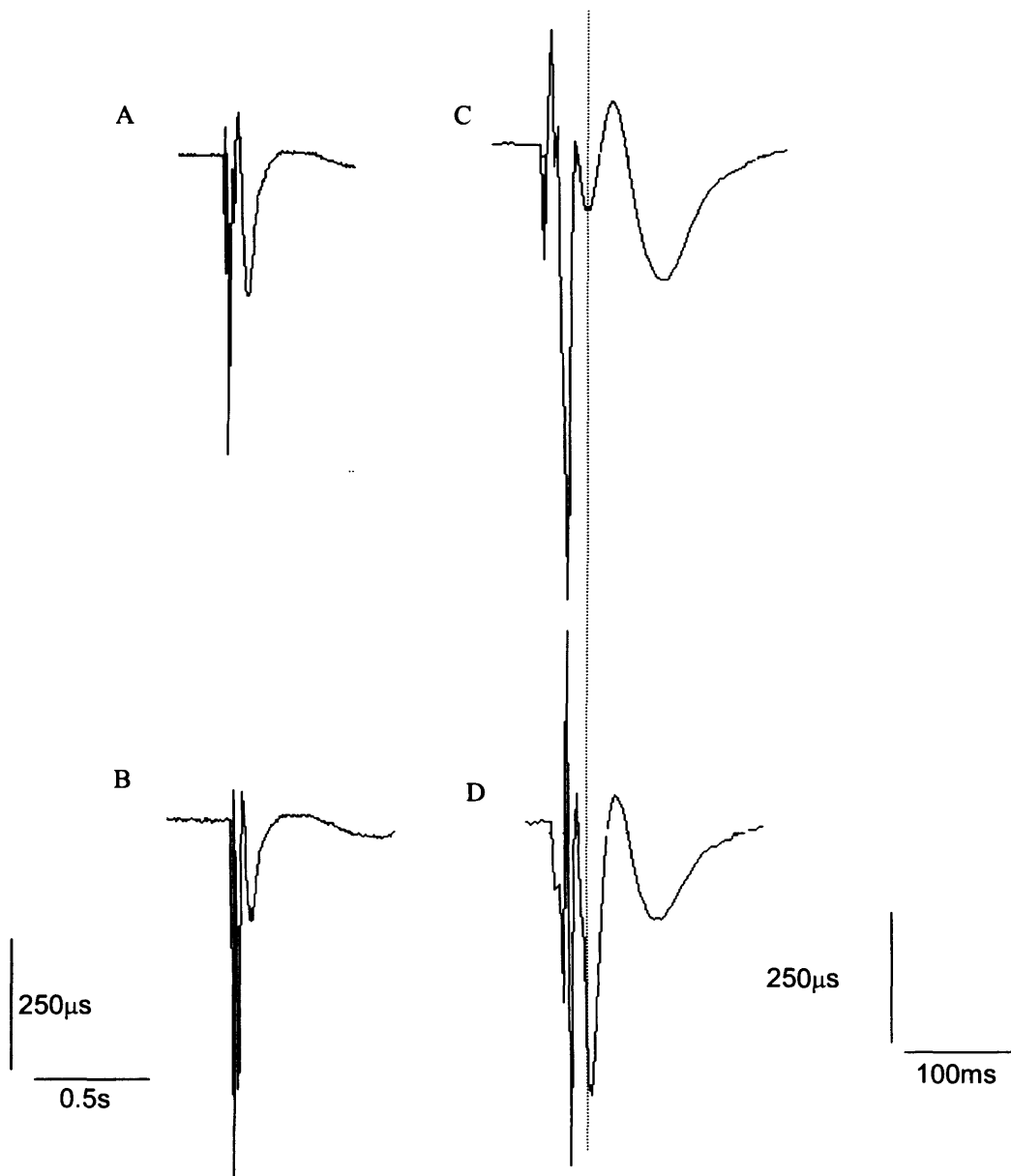
Figure 3.23. Electrical traces recorded from detrusor strips evoked by three pulses of 100μs width. A = Standard polarity, B = reversed polarity. Figures shown on expanded timebase in C and D to demonstrate unchanged polarity of the evoked signal. End of stimulus artefact marked with dotted line.

3.4 Discussion.

3.4.1 Summary of results.

The results show that an artefact free extracellular electrical signal could be recorded in association with contractions evoked by both focal direct, and field stimulation of motor nerves in guinea-pig detrusor strips using suction electrodes. The signals were of lower amplitude but similar in morphology to those recorded electromyographically from the whole bladder. The difference in amplitude of the signals recorded from the two preparations probably reflected the reduced mass of activated tissue in electrical contact with the electrode.

The signal was consistently abolished by purinoceptor desensitisation but not by exposure to atropine, indicating that it originated exclusively from a purinergic mechanism. Attenuation of the signal in the presence of various Ca^{2+} channel blocking agents suggested that it was generated predominantly by Ca^{2+} influx through membrane bound channels.

3.4.2 Technical considerations.

A mucosa-free detrusor strip preparation is in principle more suitable than the whole bladder for experiments investigating the effects of pharmacological agents on the electromyographic signal and its relationship to mechanical events, as it is more amenable to perfusion. This was evidenced by shorter periods of exposure to $CaCl_2$-depleted solutions being necessary for stabilisation of the signal, and abolition of the signal by solutions containing. $\leq 0.4mM$ $CaCl_2$ in contrast to the intact bladder preparation in which the signal persisted in $CaCl_2$ free solutions (see figures 2.14 and 3.6). Furthermore, tension measurements were more direct, as unlike the whole bladder, the strips contracted linearly and the distance between the electrode and tension

transducer was shorter enabling more accurate correlation between electrical and mechanical activity.

Nevertheless, several hours of exposure to α, β-mATP and Ca^{2+} channel blocking agents at increasing concentrations was often required for stabilisation of the effect on the signal indicating poor perfusion of complex molecules through the preparation. As a result, quantification of the residual electrical signal during exposure to these agents may be inaccurate, and under-estimate real Ca^{2+} channel dependent activity. It may be argued that the relatively high doses of α, β-mATP could have inhibited the excitability of the preparation directly. It will later be shown that in human tissue expressing no atropine-resistant activity α, β-mATP $50\mu M$ had no effect on contractions (see figure 4.6).

A disadvantage of the strip preparation was the relatively short distance between the stimulating and recording electrodes, giving rise to a stimulation artefact that could overwhelm the biological signal using field stimulation. This was overcome by the use of bladder strips dissected along the bladders longitudinal axis and stimulated at one end, which minimized the stimulus artefact and achieved temporal separation of stimulus artefact from the biological signal. However, the rigid attachment of the suction electrode to the mid-portion of the muscle strip prevented tension generated proximally (ie. by the portion of the strip situated between the stimulation and suction electrodes) from being transmitted to the tension transducer, with only tension generated 'distally' being recorded. This compromised analysis of the temporal relationship between electrical and mechanical signals, as the electrical event was likely to be evoked momentarily before recordable contractions. The experiments also showed that the biological electrical signal could also be recorded from field stimulated detrusor strips, and that the resulting stimulus artefact could be minimised by

optimising the orientation of the electrodes and by lowering high pass electronic filtering.

3.4.3 Electrophysiological origins of the signal.

Intracellular electrical recordings from nerve-stimulated guinea-pig detrusor show that purinergic, not cholinergic excitatory neurotransmission is associated with membrane depolarisation (see section 1.2.4). Neurally released Ach initiates detrusor contractions independently of primary changes in membrane potential by secondary-messenger mediated mobilization of intracellular Ca^{2+} stores (see figure 1.8) [Wu et al. 1999]. Sucrose-gap recordings have shown that this purinergic electrical activity occurs and can be recorded extracellularly. In these studies we have shown that the electrical signal recorded with suction electrodes could be consistently abolished by desensitisation of purinoceptors, confirming that the signal originated from a purinergic mechanism. Furthermore, although a small atropine-sensitive component to the signal may have been present, the electrical signal recorded with suction electrodes was not dependent on cholinergic neurotransmission, and was dissociated from the contraction during muscarinic receptor blockade.

Purinergic excitation-contraction coupling in detrusor involves activation of ligand-gated, membrane bound P_{2X} receptors allowing the influx of cations (principally sodium ions) into the cell and generating an excitatory junction potential (EJP). This potential opens voltage-gated membrane Ca^{2+} channels, enabling the rapid influx of calcium-ions down their electrochemical gradient into the cell which generates the action potential and activates intracellular contractile proteins (figure 1.8) [Wu et al, 1999, Brading 1987]. The signal recorded with suction electrodes was readily abolished by extracellular Ca^{2+}-depletion of the superfusate. However, the persistence of a small electrical component during pharmacological blockade of membrane Ca^{2+} channels,

which could be subsequently abolished by α, β-mATP, suggested that the suction electrode signal was an admixture of the purinergic EJP and the inward Ca^{2+} current (although the caveat relating to uncertain diffusion of pharmacological agents into the tissue applies). We therefore believe that suction electrodes enable true extracellular recordings of the net trans-membrane currents initiated by purinergic neurotransmission leading to contraction in detrusor smooth muscle.

The precise nature of the spontaneous electrical signals is uncertain. Although associated with spontaneous tension development their degree of coupling to mechanical events was difficult to quantify using the current apparatus, furthermore as they were seldom apparent, and inconsistent and ephemeral when they were, analysis of their nature using pharmacological interventions was not possible.

3.4.4 Implications of results.

The experiments described here show that the biological electromyographic signal recorded from the guinea-pig bladder is generated by purinergic and not cholinergic neuromuscular transmssion. Normal human detrusor is activated exclusively by cholinergic signalling and, if the mechanisms of excitatory neurotransmission in animal and human detrusor are similar, would not be expected to generate extracellular electrical activity or an electromyogram. However, as previously discussed purinergically-generated contractions in detrusor taken from overactive bladders have been recorded (see section 1.2.3), and the studies described here suggest that this is likely to generate recordable extracellular electrical activity.

However, several differences in the nature of human and animal detrusor tissue samples are likely to make suction electrode recordings from human detrusor samples difficult. The purinergic component of the contraction in dysfunctional human detrusor is rarely above 30% of the total (eg. Bayliss et al. 1999), and the resulting electrical

signal is therefore likely to be of a lower intensity than that recorded from the guinea-pig, in which the purinergic component contributes more [Brading 1987]. Furthermore, human detrusor has larger amounts of connective tissue dispersed throughout the tissue than in the guinea-pig, and this is increased in dysfunctional and obstructed bladders [Charlton et al. 1999]. The presence of this presumably electrically inert material is likely to dilute the electromyographic signal further, and may also interfere with the quality of the recordings.

3.5 Conclusion.

The biological electromyographic signal recorded from the guinea-pig bladder can also be recorded from both direct and field stimulated isolated detrusor strips, and correlated with nerve-evoked tension changes. In accordance with the implications of microelectrode and sucrose-gap studies, the signal is generated by a purinergic and not a cholinergic mechanism, and probably represents an admixture of the purinergic EJP and transmembrane inward Ca^{2+} current. These experiments validated the suction electrode and recording apparatus for detecting purinergic extracellular electrical activity in human detrusor smooth muscle strips.

CHAPTER FOUR: SUCTION ELECTRODE RECORDINGS FROM HUMAN DETRUSOR SAMPLES.

4.1 Introduction.

The final part of this thesis describes suction electrode recordings from contracting human detrusor smooth muscle strips. An electrical signal is characterised and the relationship between its expression, atropine-resistant contractions and clinically evident bladder dysfunction is investigated. We test the hypothesis that detrusor samples taken from overactive bladders have a greater propensity than normal to generate an extracellular electrical signal by virtue of purinergic contractile mechanisms.

4.2 Methods.

4.2.1 Tissue acquisition.

Detrusor smooth muscle samples were obtained from patients undergoing open bladder surgery. Informed consent was obtained in writing using a standardised form, with the knowledge and approval of relevant local Ethics Committees. Detrusor samples of approximately 1 x 0.5-1cm were taken from readily accessible areas of the open bladder by the operating surgeon, or from cystectomy specimens immediately after their removal. The tissue was placed in gassed Tyrode's solution and transported to the laboratory. The specimen was pinned out in a dissecting dish, and detrusor muscle bundles of approximately 3 x 8mm for suction electrode recordings, and two additional smaller strips (<0.5mm width) for use during quantification of atropine resistance were dissected from immediately adjacent areas of the sample. Efforts were made to obtain samples containing bundles of aligned muscle fibres, with a minimum

of non-muscular tissue, and to minimise tissue trauma by careful tissue handling and limited exposure to air.

4.2.2 Experimental set-up.

The experimental set-up was identical to the field-stimulated set-up used for recording from the guinea-pig. The larger detrusor strips were mounted in the trough of the organ bath with one end fixed to a hook, the other to the tension transducer and superfused with warmed ($36 \pm 1^\circ C$), gassed (95% O_2, 5% CO_2) Tyrode's solution. The strips were stimulated using three-second trains of 0.1ms pulses with 50ms between pulses (20 pulses s^{-1}) at intervals of 90 s until the peak evoked contraction had stabilised. The suction electrode was then manipulated over the strip, and gently attached to it using a suction force of 60-80KPa. Care was taken to ensure that the orientation of the electrode was central with relation to the stimulating electrodes in order to minimise stimulus artefact. Tension was then gently applied to the strip to a limit determined by the competency of the suction seal around the electrode. After a further period of equilibration the strip was stimulated using 6-10 0.1ms pulses at 10ms intervals, and 90 s between pulse trains. The signal from the suction electrode was filtered (0.1Hz-10KHz) amplified and digitised, and displayed in real time with that from the tension transducer. If no signal was evident after 20 minutes the electrode was removed, and reattached onto the strip at a different position. Three attachments were undertaken for each strip. When a putative electrical signal was recorded, its electrophysiological origins were investigated by determining its sensitivity to α, β-mATP 50μM, TTX 1μM, and atropine 1μM dissolved in Tyrode's solution.

4.2.3 Clinical characterisation of tissue.

The medical records were reviewed and the results of any urodynamic investigations and the presence of reported lower urinary tract symptoms during patient interview prior to surgery were recorded. Urodynamic evaluation was not performed specifically for this study, and was only available when undertaken for clinical purposes. Using these variables the samples were categorised into 'normal' and 'overactive' groups. The overactive group was further subdivided into idiopathic, neurogenic and obstructed groups.

4.2.4 Quantification of atropine-resistant contractions.

Nerve-mediated atropine-resistant contractions of both the larger detrusor strips, and the two smaller muscle strips taken from each detrusor sample were quantified. With the larger strips from which no electrical signal was recorded, atropine-resistant contractions were quantified using a supramaximal stimulus of 3 s trains of pulses delivered at intervals of 50ms (20Hz) every 90 s before and during exposure to atropine 1 μM with the electrode unattached. When atropine-resistant activity was recorded, TTX 1 μM was added to the superfusate in addition to atropine and any residual contractions recorded subtracted from the total atropine-resistant component. With the large strips from which an electrical signal was recorded, which had been superfused with atropine and α, β-mATP during signal charaterisation experiments, atropine-resistance was inferred from the effect of α, β-mATP on the contractions evoked in association with the electrical signal using the experimental stimulus protocol with the electrode in the recording position.

Atropine-resistant contractions of the two smaller strips taken from each sample were quantified using a validated experimental set-up [Palfrey et al. 1984]. A separate investigator performed these experiments independently and without knowledge of the

results of suction electrode recordings. Similarly, the results of the experiments on the small strips were not known by myself until after the electrical recordings had been performed. The two strips were mounted in paired organ baths, and field stimulated until evoked contractions had stabilised. Atropine-resistant contractions were quantified using standard supramaximal stimulation parameters (3 s trains of pulses at 50ms pulse intervals) and also shorter trains of 10 pulses at 10ms pulse intervals to mimic the experimental stimulation protocol before and after exposure to atropine 1μM. When atropine-resistant activity was recorded TTX 1μM was added to the perfusate and stimulation continued. The nerve-mediated atropine resistant component of the contraction was calculated for each strip by subtracting the TTX resistant component from the atropine resistant component.

To control for a non-specific inhibitory effect of α, β- mATP 50μM on tissue excitability and cholinergically mediated contractions, which could account for an artefactual reduction in tension, α, β- mATP 50μM was applied to small detrusor strips that had been previously shown to express no atropine resistance.

4.2.5 Data analysis.

The effect of pharmacological agents on the electrical signal and peak contraction was calculated from the mean of three consecutive recordings taken immediately before the intervention and after the effect had stabilised. The magnitude of atropine-resistant contractions for a group was expressed as median and interquartiles, and statistically compared using non-parametric tests.

4.3 Results.

4.3.1 Characteristics of tissue samples.

Bladder samples were obtained from twenty-four patients. Eight were undergoing clam ileocystoplasty for urodynamically confirmed idiopathic detrusor overactivity, and five suffered from neurogenic detrusor overactivity and were undergoing bladder augmentation or urinary diversion procedures. Nine samples were from patients with functionally normal bladders undergoing cystectomy or continent diversion and were classified as controls. Two further samples were taken during open cystolithotomy. One of these was from a patient with recurrent urethral stricture and bladder stone and was very likely to have bladder outflow obstruction. The other had cystometrically evident overactive bladder contractions (likely to reflect bladder hypersensitivity) in the absence of outflow obstruction and was classified as a control (table 4.1).

4.3.2 Suction electrode recordings and characterisation of the signal.

A stable and quantifiable bi-phasic electrical signal was recorded from five human detrusor strips originating from four bladders (figure 4.1). The signal parameters were (mean $\pm$ SD): amplitude 56 ± 14 μV, duration 212 ± 58ms, and time to depolarisation 60 ± 10ms, and were not significantly different (P=0.127, 0.71, 0.32 respectively, two-tailed Student's t-test), than those of the signal recorded from guinea-pig detrusor strips. The signal was closely related to the stimulus artefact, but was not altered by changing the polarity of the stimulating pulses indicating its independence (figure 4.2), and always preceded the onset of recorded tension rises. The signal and contraction were both reversibly abolished by TTX (n = 3, figure 4.3), and the electrical signal was abolished by α, β-mATP 50μM in all cases (figure 4.4-5). With α, β-mATP the contraction was reduced by $39 \pm 12\%$ in three strips and the residual contraction

Patient	Diagnosis	CMG	Symptoms	AR large strips	AR small strips	Signal present
MB 31m	IOB	U	Y	-	12, 17	N
HF 72m	Control (TCC)	-	N	16	-	N
FJ 68m	Control (TCC)	-	Y	0	-	N
JB 31m	OOB	-	N	28	28, 38	Y
MW 62n	IOB	U	Y (i)	37	35, 33	Y
IB 27m	Control (pain)	-	N	12	0	N
DM 20m	NOB	U	Y (i)	0	13, 0	N
DH 73m	Control (stone)	U	Y (i)	0	0, 0	N
MN 69f	Control (TCC)	-	Y	19	0, 0	N
HG 76m	Control (TCC)	-	Y	8	0, 0	N
HS 40f	NOB	S	Y	Y**	30, 0	Y*
MS 84m	Control (TCC)	-	N	0	0, 0	N
WH 71m	Control (TCC)	-	N	0	0, 0	N
MS 55f	IOB	U	Y (i)	5	0, 0	N
JA 27f	IOB	U	Y (i)	16	7, 0	Y*
CG 27f	NOB	U	Y (i)	35	0, 0	N
JP 57f	IOB	U	Y (i)	18	13, 19	Y
JP 57f	IOB	U	Y (i)	43	13, 19	Y
AA 39f	IOB	U	Y (i)	52	22, 0	Y
PC 48f	Control	S	N	0	0	N
MT 39m	NOB	U	N	0	0, 0	N
MW 39f	NOB	U	Y	0	0, 0	N
JA 44f	Control (pain)	S	Y	4	7, 0	N
AS 58m	IOB	U	Y	12	0, 0	N
PC 52f	IOB	U	Y	0	20, 0	N

Table 4.1 Results from human detrusor strips. IOB= idiopathic overactive bladder, NOB = neurogenic overactive bladder, OOB = obstructed overactive bladder, TCC= bladder cancer. CMG = cystometry, U = unstable, S = stable, (i) = incontinent, * = uncharacterisable signal, AR = atropine-resistant contractions (% nerve-mediated total), ** = atropine resistance present but unquantifiable. For patient 'JP' recordings were made from two large strips, and two small strips from same sample.
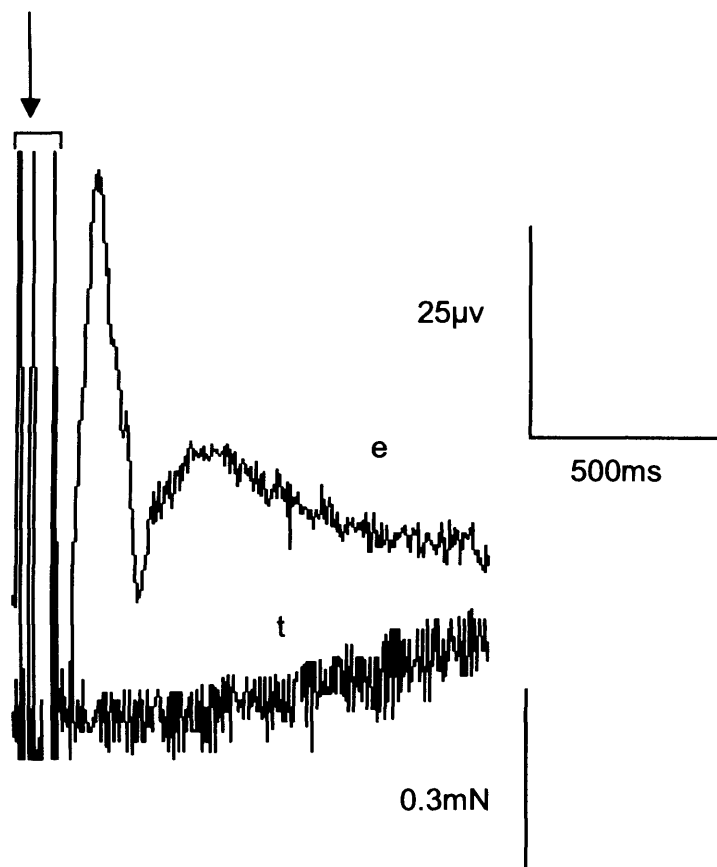
Figure 4.1. Representative trace of evoked electrical (e) and mechanical (t) signals recorded from human detrusor strips. Arrow marks stimulus artefact.
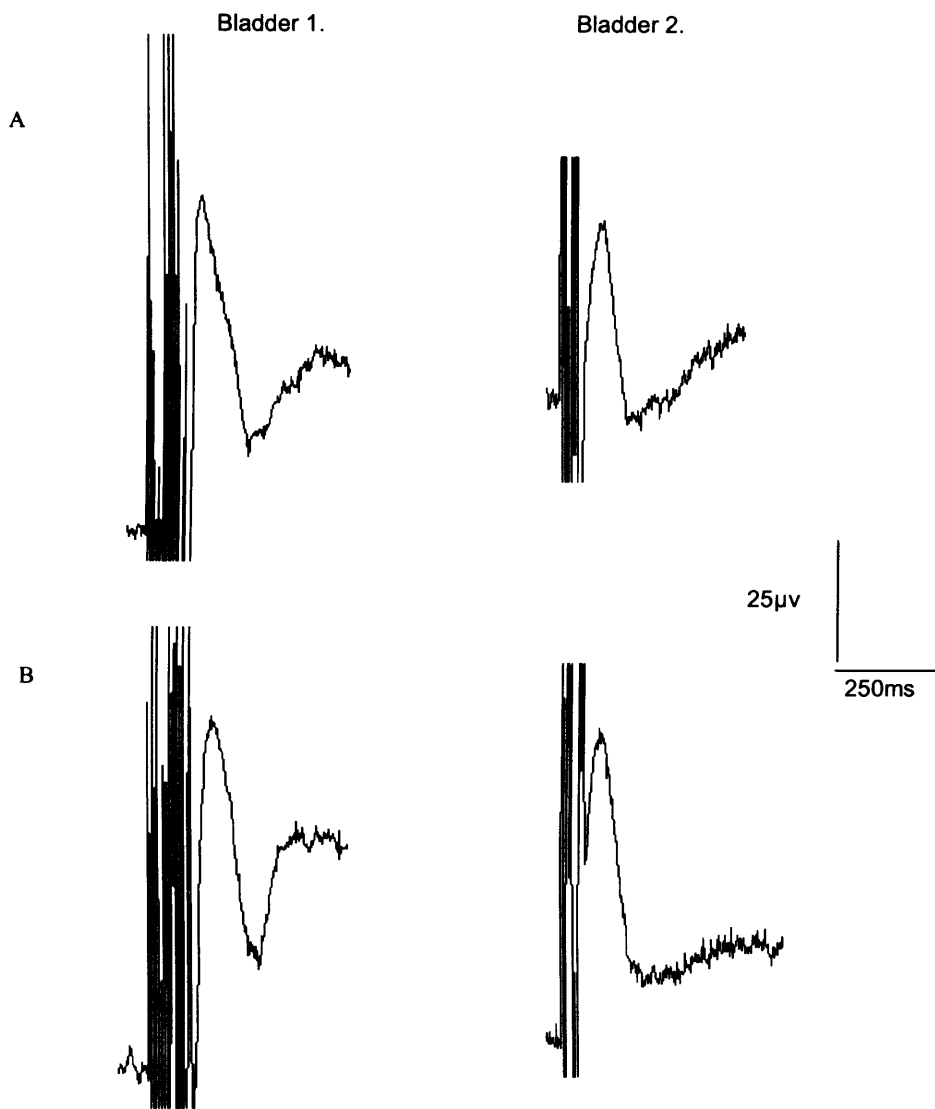
Figure 4.2. Traces of consecutive recordings of electrical signals evoked by ten pulses of opposite polarity (A and B) from two human detrusor strips.
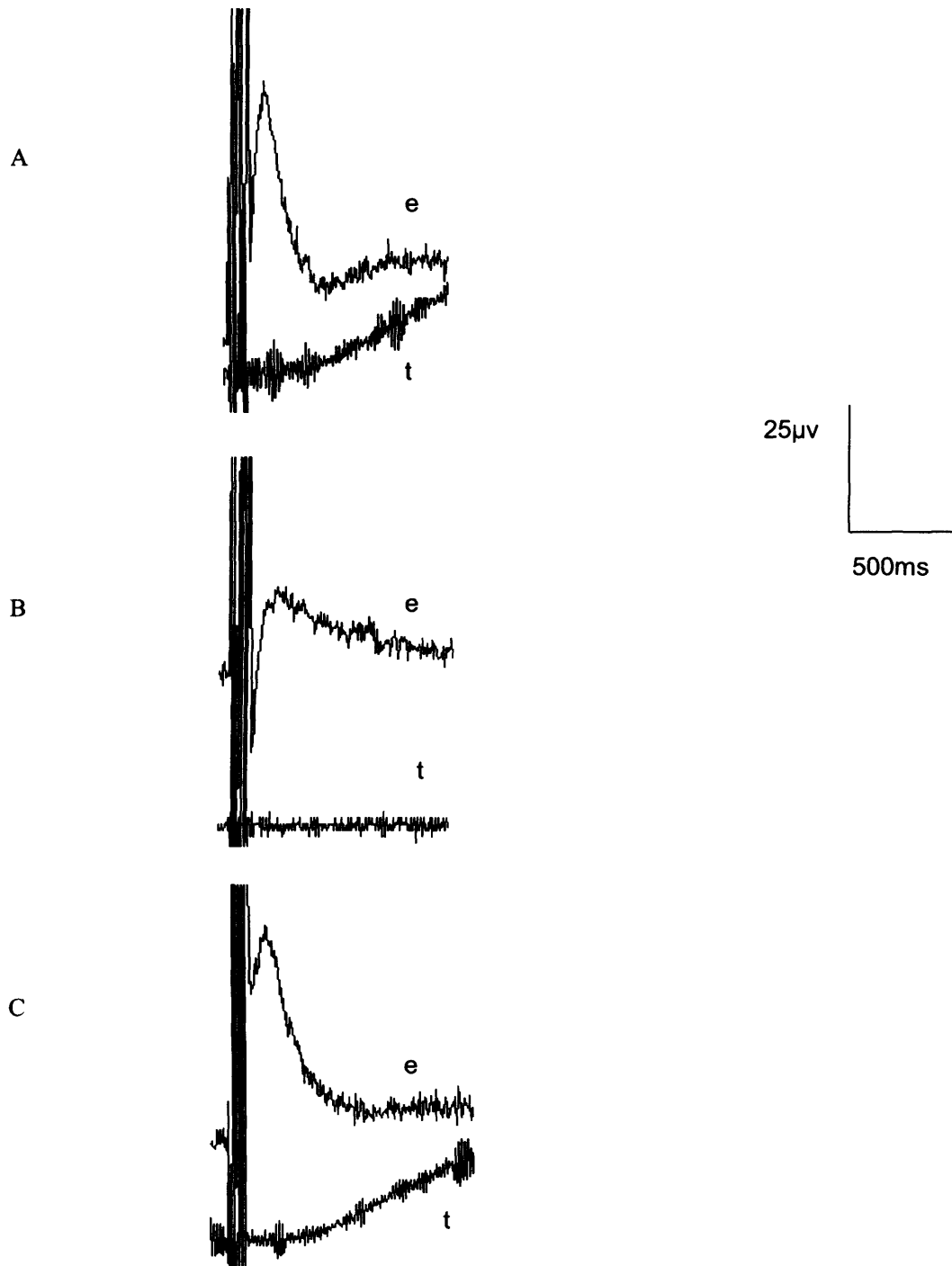
Figure 4.3. Representative traces showing effect of TTX 1μM on electrical (e) and mechanical (t) traces recorded from human detrusor. A = control, B = with TTX, C = washout.
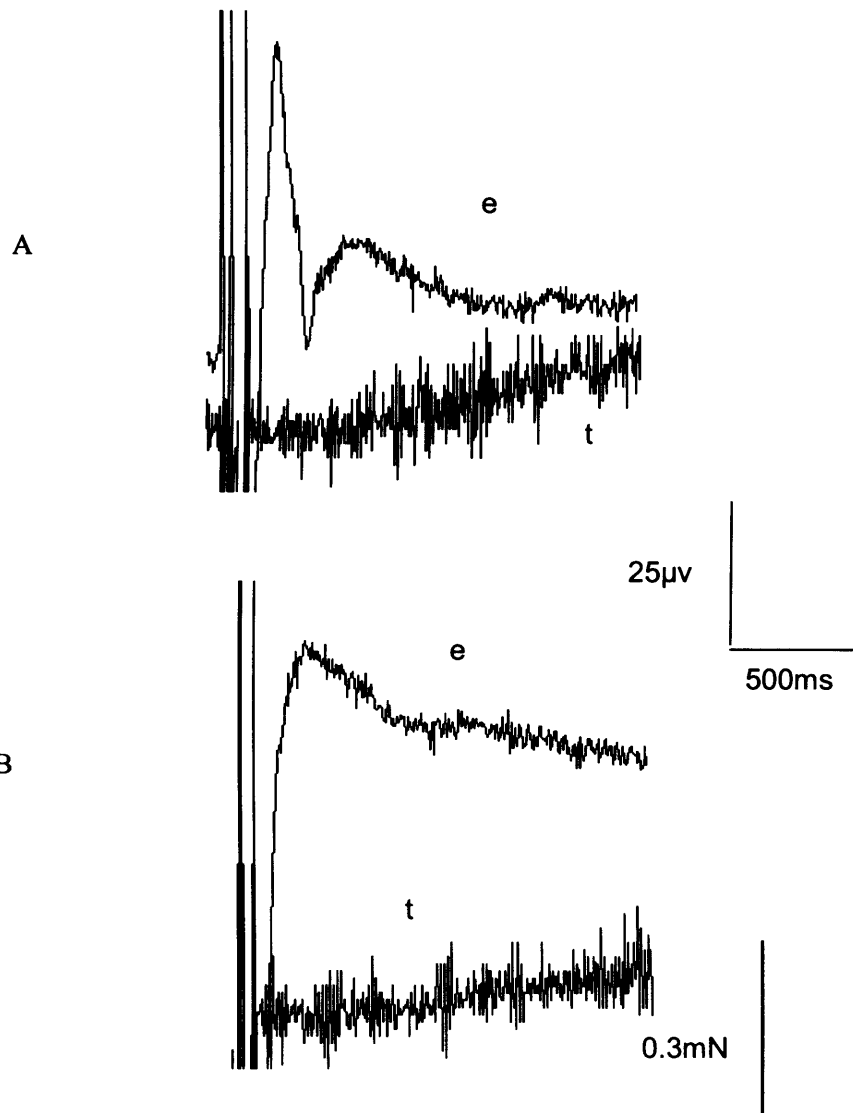
Figure 4.4. Representative traces of electrical (e) and mechanical (t) signals recorded from human detrusor strips showing abolition of the control electrical signal (A) with α, β-mATP (B).
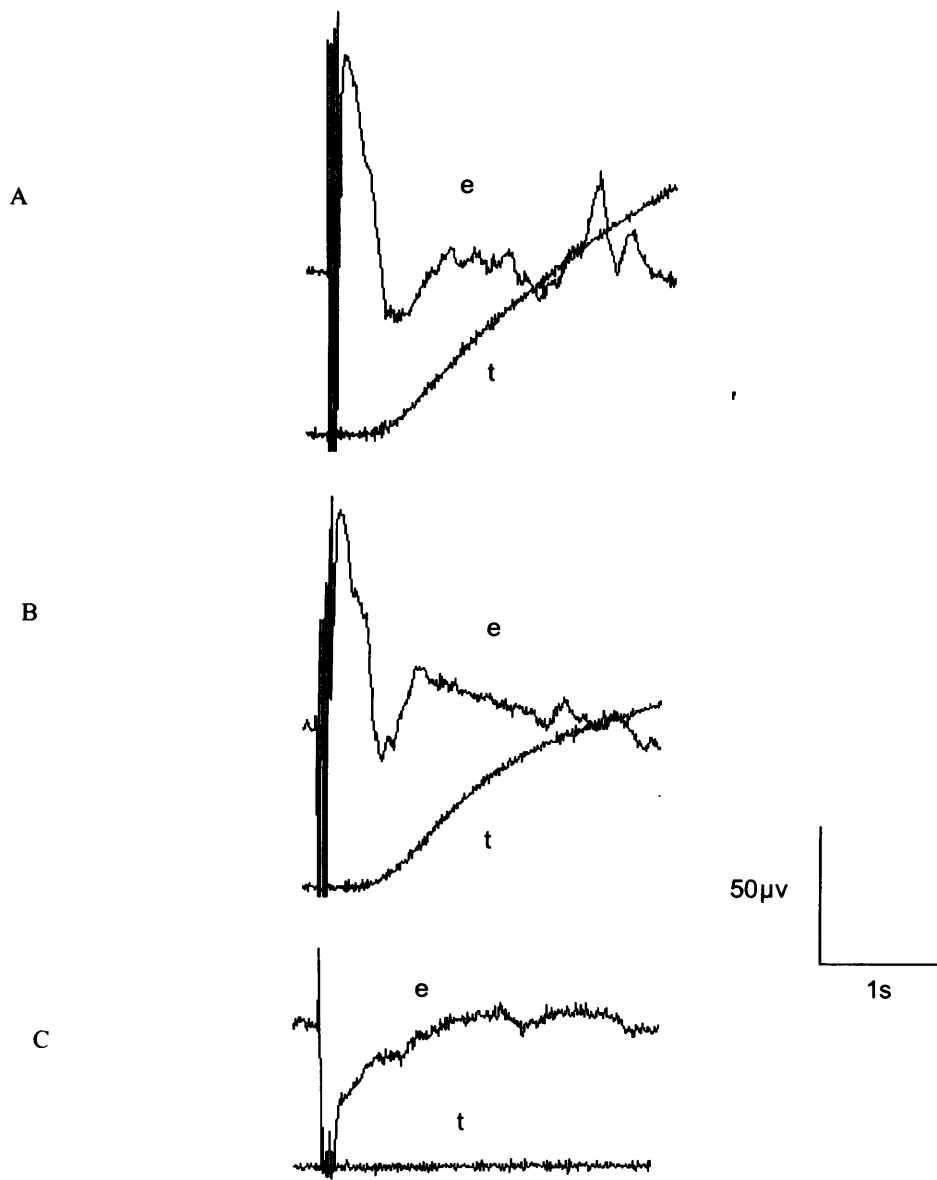
Figure 4.5. Electrical (e) and mechanical (t) traces recorded from human detrusor strip showing effect of atropine (B), and atropine + α, β – mATP (C), on control signals (A).

virtually abolished by atropine. In two experiments, suction electrode recordings were conducted during exposure to atropine 1μM, which attenuated the contraction by an average 40% of control, but had no effect on the electrical signals. Subsequent application of α, β-mATP abolished both the atropine resistant electrical and mechanical signals (figure 4.5). Restitution of the signal could not be recorded despite several hours washout after α, β-mATP.

In detrusor strips from two bladder samples an electrical signal was recorded that was similar in morphology to the quantifiable signal but was unstable, and not evoked consistently enough to enable its pharmacological characterisation. Although both signals originated from tissue later found to express true atropine-resistant activity, one from neurogenic the other from idiopathically overactive bladder, these signals were not presumed to be purinergic, and were not included in the data analysis.

Effect of α, β-mATP 50μM on cholinergically mediated contractions.

Peak evoked contractions of tissue samples that had been shown to express no atropine resistant contractile activity were unchanged during exposure to α, β-mATP 50μM (n=2) (figure 4.6), showing that the ATP analogue had no inhibitory effect on non-purinergic contractile mechanisms.

Control with 3s train
stimulus

Control with
10 pulses
stimulus

α, β-mATP 50μM

3s train

Atropine 10μM

TTX 1 μM
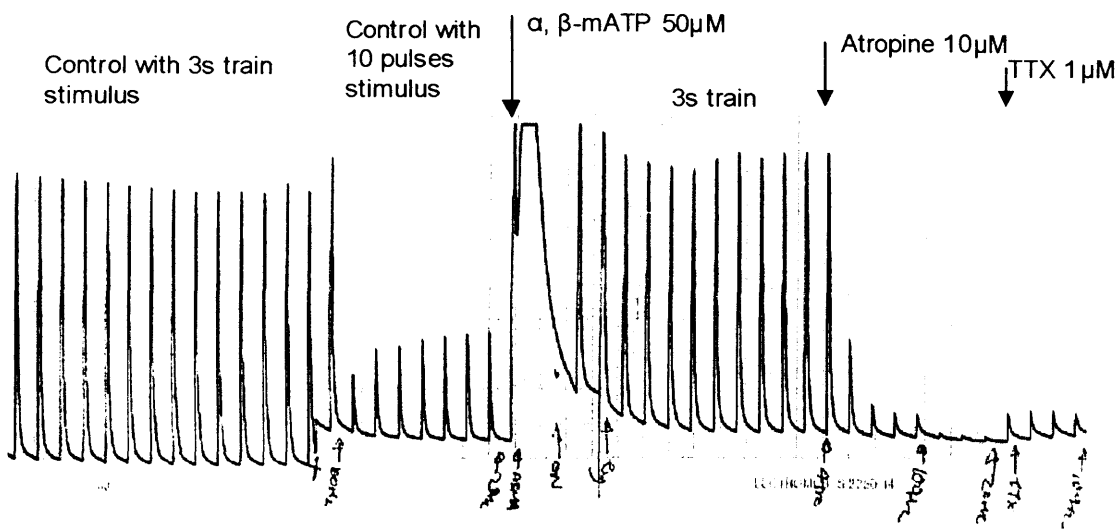
Figure 4.6. Trace of evoked contractions at 90s intervals of small human detrusor strip showing no effect of α, β – mATP on atropine-sensitive contractions.

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4.3.3 Quantification of atropine-resistant contractions.

Atropine-resistant, TTX sensitive contractions were recorded from 14 of the small strips from 10 bladder samples, and from 14 of the larger strips from 13 samples from which the suction electrode recordings were made. In one sample two large strips were recorded from, both which expressed an electrical signal and atropine-resistant activity. In the small strips atropine-resistant activity appeared to be heterogeneously distributed, with some samples expressing atropine-resistance in only one of the two strips, while in others a similar degree was seen in both. (see table 4.1). Atropine-resistant contractions were significantly greater (P= 0.017, Wilcoxon Matched pairs test) in the larger (median, interquartiles; 8%, 0–23.5%) than in the smaller (0%, 0–8.5%) strips, and in four samples was recorded from the larger strips when none was evident in the smaller strips. Atropine-resistant activity was recorded from the smaller but not the larger strip in only one sample. These discrepancies are consistent with overestimation of atropine-resistance in larger strips due to their incomplete perfusion with atropine. Quantification of atropine-resistant activity for each bladder sample was as a result made from the small strip data.

4.3.4 Relationship between the expression of electrical and atropine-resistant activity.

A quantifiable and α, β-mATP sensitive electrical signal was recorded from four of the ten strips taken from bladder samples that expressed real atropine resistance. The associated contraction was attenuated by α, β-mATP in all indicating that purinergically-mediated activity was present in the tissue used for electrical recordings. Of the six bladder samples from which atropine-resistant contractions but no electrical signal was recorded, contractions of the large strip were abolished by atropine in three,

indicating that only cholinergic neurotransmission was expressed, and an unquantifiable electrical signal was recorded from two of the others. In the remaining sample from which atropine-resistant activity but no electrical signal was recorded, atropine-resistant contractions of the larger strip were not quantified. This was one of the first human bladder samples tested and failure to record an electrical signal could reasonably be attributed to technical aspects. No electrical signal was recorded from any bladder sample from which no atropine-resistant contractions were detected.

Atropine resistant contractions contributed a median 22%, 0-34% (n=8) in the small strips from samples expressing an electrical signal and were significantly greater than those from which no signal was recorded (7%, range 0-20%) in 5 of the 31 strips (P<0.01, Mann-Whitney U) (figure 4.7).

4.3.5 Relationship between the expression of electrical and atropine-resistant activity and clinical variables.

Involuntary detrusor contractions were detected by cystometry in all eight patients with urge incontinence. There appeared to be poor association between the expression of an electrical signal and clinical variables. An electrical signal was recorded from only four out of the eight bladder samples from incontinent patients, and four out of ten samples from cystometrically verified overactive bladders.

Atropine-resistant contractions were detected from 7 of 13 samples taken from cystometrically confirmed overactive bladders. Of the 6 remaining samples not expressing atropine-resistant activity, three were from neurogenically overactive bladders, two from bladders characterised as idiopathically unstable, and one from a patient with a bladder stone, which would have been likely to have caused hypersensitive bladder contractions.
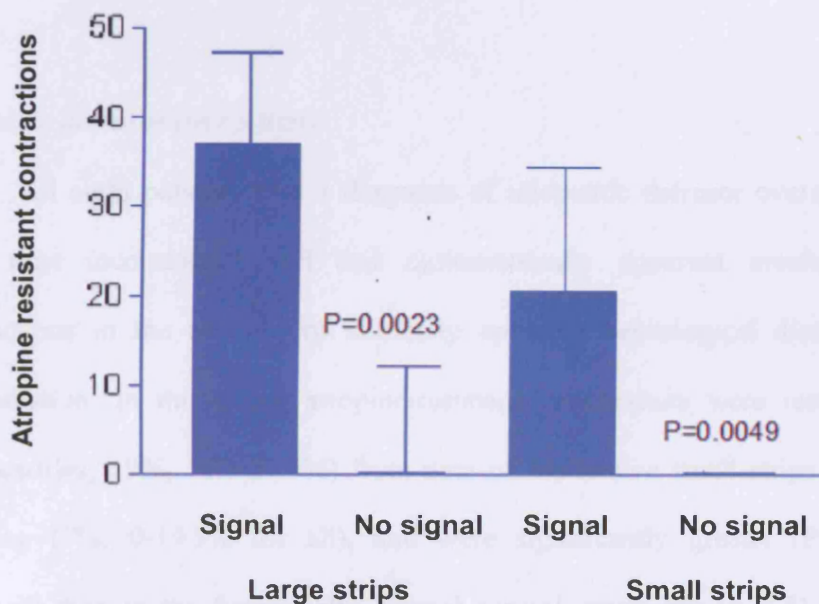
Figure 4.7. Bar chart showing atropine-resistant contractions (median, 75th centile % nerve-mediated total) recorded from large and small strips taken from human detrusor samples that expressed and electrical signal and those that did not. P values refer to difference in magnitude of atropine-resistant contractions between strips expressing an electrical signal and no signal (Mann – Whitney U).

4.3.6 Relationship between the expression of electrical and atropine-resistant activity in detrusor from overactive and functionally normal bladders.

Controls.

The bladder samples characterised as functionally normal were taken from patients undergoing cystectomy. Several of these patients described mild lower urinary tract symptoms but none reported urinary urgency or urge incontinence and only the patient with bladder stone had been cystometrically characterised. Atropine-resistant contractions were detected in only one (7% of nerve-mediated total) of 14 small strips taken from patients in this group, and no electrical signal was recorded.

Idiopathic detrusor overactivity.

All eight patients with a diagnosis of idiopathic detrusor overactivity suffered from urge incontinence and had cystometrically apparent involuntary detrusor contractions in the absence of clinically apparent neurological disease or bladder inflammation. In this group atropine-resistant contractions were recorded (median, interquartiles; 19%, 12.5-27.5%) from nine of the twelve small strips taken from six samples, (7%, 0-19.5% for all), and were significantly greater (P=0.021, Mann-Whitney) than in the functionally normal control group (figure 4.8). A quantifiable electrical signal was recorded from four strips taken from three of these samples. Of the three idiopathically overactive bladder samples that expressed atropine-resistant activity from which no electrical signal was recorded, two expressed very little atropine resistance, (0%, 0-14.5%) in two of four strips. In one of these contractions of the larger strip were abolished by atropine indicating that no atropine-resistant activity was expressed by the tissue from which suction recordings were made. In the other an
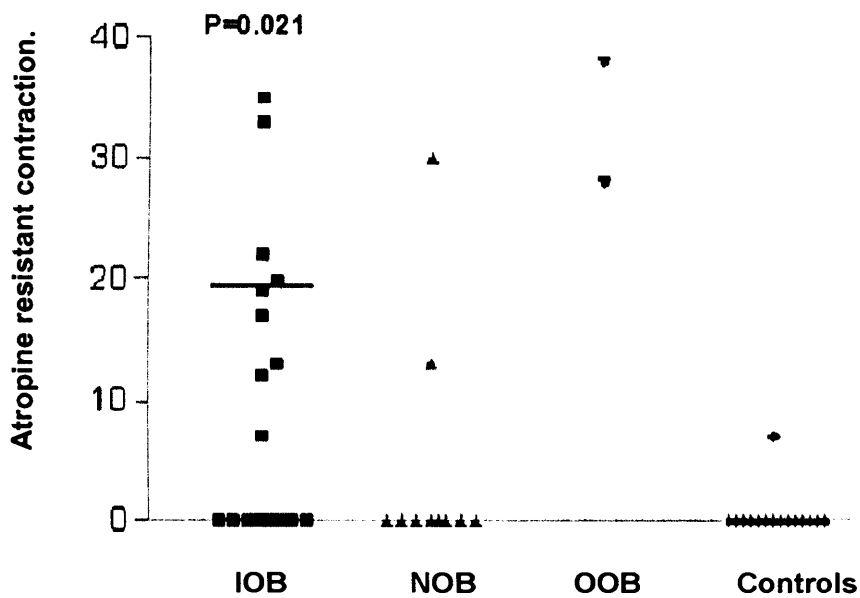
Figure 4.8. Scatter diagram showing degree of atropine resistant contractile activity (% nerve mediated total) detected in individual small detrusor strips taken from clinically characterised human bladders. IOB = idiopathic overactive bladders, NOB =neurogenic overactive bladders, OOB = obstructed overactive bladder. Horizontal line indicates median value of atropine resistance in those strips in which it was expressed. P value relates to comparison with controls (Mann Whitney U).

non-characterisable electrical signal was recorded. In one sample expressing atropine-resistant activity no electrical signal was recorded. In tissue taken from two samples clinically characterised as idiopathically unstable, no atropine resistance or electrical signal was found.

Neurogenic detrusor overactivity.

All five patients diagnosed with neurogenic detrusor overactivity had central nervous system pathology. Three of these had overactive bladders demonstrated by cystometry, and reported urge incontinence. One patient who practiced self-catheterisation exhibited reduced bladder compliance but no involuntary detrusor contractions on cystometry and was continent. Atropine-resistant contractions were found contributing a median 21% in only 2 of 11 small strips examined (0%, 0-6.5% for all), and were not significantly different than those recorded from stable controls (P=0.58, Mann Whitney U) (figure 4.8). No quantifiable electrical signal was recorded from any sample in this group.

Bladder outlow obstruction.

Only one tissue sample was obtained from an obstructed bladder. This was from a young man with recurrent urethral strictures who was undergoing open cystolithotomy. Although not cystometrically characterised this bladder was likely to have been chronically obstructed. Both an electrical signal and a high degree of atropine resistance (mean 33%), was recorded from this sample (figure 4.8).

4.3.7 Effect of stimulation protocols on atropine-resistant contractions.

In the small strips, contractions evoked by 10 pulses delivered at 10ms intervals were generally too weak to be recorded by our equipment. Atropine-resistant

contractions evoked by supra-maximal, (20%, 0-31%), and experimental, (21%, 0-34%) stimulation proptocols are shown in table 4.2. Although some variability was present, no significant difference in the median atropine-resistant contractions evoked by the two protocols was found (P= 0.58, Wilcoxon matched pairs).

Supramaximal stimulus (60 x 50ms)	Expermimental stimulus (6x 10ms)
30	18
0	35
32	34
19	21
34	33
0	0
0	0
22	44
20	0

Table 4.2 Atropine-resistant contractions (% nerve-mediated total) evoked by supramaximal (60 pulses at 50ms intervals) and experimental (6 pulses at 10ms intervals) stimulation protocols in the same human detrusor strips. P= 0.58 for a difference between the two (Wilcoxon matched pairs test).

4.4 Discussion.

4.4.1 Electrophysiological origins of the signal.

The electrical signal recorded from human detrusor always preceded the onset of associated tension rise, and was not evident during the contraction (eg. Figure 4.1). In contrast to the experimental arrangement using focal electrical stimulation, which may have created temporal artefact in recording the electrical signal before the contraction, the field-stimulated set-up would be expected to accurately reflect the temporal relationship between the two. The effects of purinergic desensitisation using α, β-mATP, which abolished the signal but not the contraction (see figure 4.4 and 4.5), and expression of the signal only in association with atropine-resistant contractions (see figure 4.7) and never with atropine-sensitive contractions of greater magnitude, showed that the signal was not mechanical artefact.

Several lines of evidence show that the signal was independent from stimulus artefact. The signal was not altered when evoked by pulses of opposite polarity, and was abolished independently of the stimulus by TTX and α, β-mATP (see figures 4.3-4.5). Furthermore, the signal could only be evoked from the small numbers of samples expressing atropine-resistant contractions despite a similar stimulus being applied to all.

Consistent abolition of the electrical signal during exposure to α, β-mATP, which had no effect on atropine sensitive contractions (see figure 4.6) but no amplitude reduction with atropine, is strong evidence that it was generated by a purinergic mechanism.

4.4.2 Relationship to previous detrusor electrical recordings.

Detailed comparisons between the parameters of intracellular and associated extracellular electrical signals are not possible, as the extracellular signal represents the summed intracellular potentials generated by an unspecified number of cells occurring

over an unspecified period of time. However, the duration and depolarisation time of the suction electrode signals were of a similar order of magnitude as the intracellularly recorded Ca^{2+} transients evoked in isolated human detrusor cells by ATP suggesting that they reflected the same mechanism [Inoue and Brading 1991, Montgomery and Fry 1992]. No significant difference was found in parameters between the signals recorded from human and guinea-pig detrusor strips. If the amplitude of the signal was proportional to the degree of atropine-resistant activity, the amplitude of the signal recorded from human detrusor might be expected to be approximately half that recorded from guinea-pig, as atropine resistant activity contributes approximately 60% of the total in guinea-pig and 30% in man. However, differences in tissue architecture and the ratio of muscle to connective tissue in the two samples render this comparison innacurate. The similarity in the duration and time to depolarisation of the signals recorded from guinea-pig and human detrusor was unsurprising as intracellular electrical studies have shown that the timecourse of the ATP generated transmembrane currents in human and pig detrusor myocytes are the same [Inoue and Brading, 1991].

4.4.3 Physiological relevance of experimental stimulation methods.

A limitation of this study is the physiological revalence of the experimental stimulus. In contrast to the short trains of high-current pulses delivered at high frequency used to evoke an electrical signal, voluntary bladder contractions *in situ* are probably evoked by long trains of low-current pulses at lower frequencies. A variable effect on purinergic and cholinergic mechanisms of different stimuli is possible, it is known that purinergic mechanisms are preferentially expressed at higher frequencies [Inoue and Brading, 1991]. Mechanisms involved in neuromuscular transmitter release may be differentially affected by the intervals between stimulating pulses, and the enzymes involved in acetylcholine and ATP breakdown may be overwhelmed at

different rates by repetitive quanta of neurotransmitter released at increasingly shorter intervals. However, this is not supported by analysis of previous studies in which force-frequency curves constructed using stimulation frequencies of 2-40Hz, demonstrated no frequency dependent effect in the relative contributions of Ach or ATP to the total nerve mediated contraction [Bayliss et al. 1999]. Furthermore the experiments comparing the magnitude of atropine-resistant contractions evoked by experimental and supramaximal stimuli here showed no difference between the two. The atropine-resistant contractile activity and associated purinergic electrical signal recorded in these experiments are therefore likely to reflect a physiologically revalent phenomenon.

4.4.4 Quantification of atropine-resistant activity.

Atropine-resistant contractions were recorded more commonly and were of a greater magnitude from the large detrusor strips used for electrical recordings than the smaller strips. This may have reflected heterogeneous distribution of the propensity to generate atropine-resistant activity, with larger strips more likely to contain affected detrusor bundles. Alternatively atropine-resistance may have been overestimated in larger strips due to poor diffusion of atropine. Furthermore, the magnitude of purinergically mediated contractions identified by attenuation of the peak contraction with α, β-mATP in the larger strips that generated an electrical signal was also significantly greater than the peak atropine-resistant tension recorded in small strips from the same samples, was not due to any effect of α, β-mATP on atropine sensitive activity or difference in stimulation protocols, and could not have been caused by impaired diffusion, which would tend to underestimate the magnitude of purinergically mediated contractions. However, quantification of atropine-resistant contractions by this method was unlikely to be accurate, as the optimal resting length of the tissue was not determined, and was limited by competency of the suction seal around the

electrode. Furthermore, the sensitivity of these contractions to TTX was not determined, and the damping effect of the attached suction electrode were unquantified. In view of these issues quantification of atropine-resistant activity for each bladder sample was calculated using data generated by the smaller strips using the validated experimental set-up. Qualitative confirmation of the presence of purinergic activity in the tissue used for suction electrode recordings was inferred from the effect of α, β-mATP on the contractions of the larger strips. However, when contractions of the larger strips were abolished by atropine, the strip was assumed to express no atropine resistance.

4.4.5 Purinergic electrical and mechanical activity in overactive and functionally normal detrusor samples.

The results are in agreement with previous studies, in finding significantly greater nerve-evoked atropine-resistant contractions in detrusor samples taken from idiopathically overactive bladders, compared with neurogenically overactive and functionally normal bladders. Figure 4.9 shows the relationship between atropine resistant contractions, expression of an electrical signal, and clinical bladder dysfunction for all samples.

There was close association between the expression of a purinergic electrical signal and atropine-resistant contractions, and in only one sample in which atropine resistant activity but no electrical signal was found could its absence not be explained by the lack of atropine resistance in the larger strip. In the idiopathically overactive group the electrical signal was recorded from samples expressing the largest degree of atropine-resistance, and not in association with contractions that were abolished by atropine. Demonstration that the contractions of all strips from which an electrical
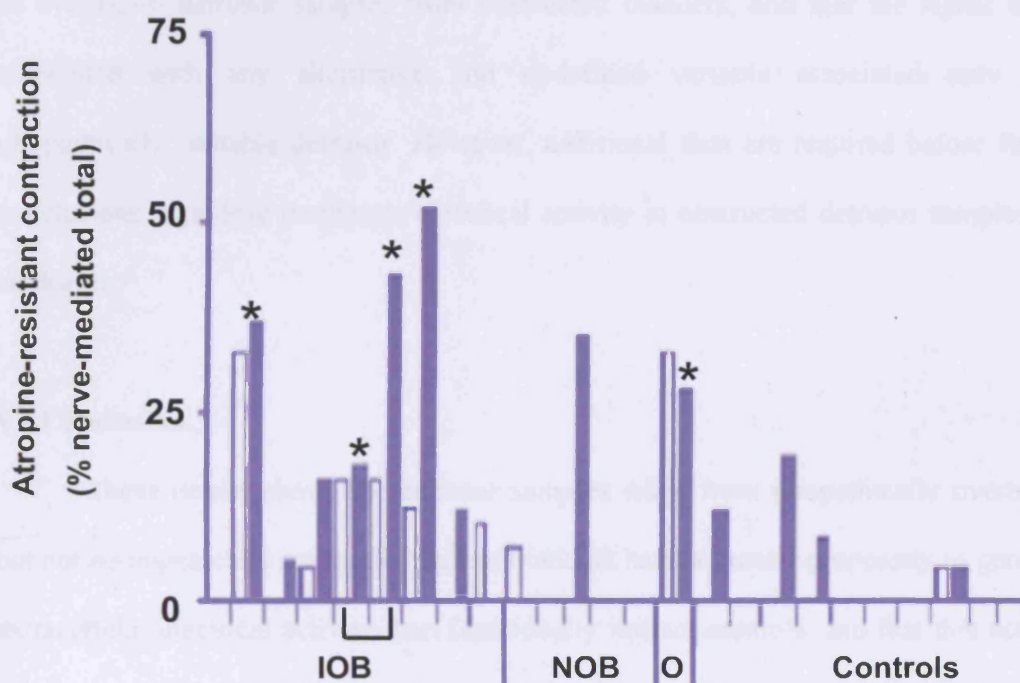
Figure 4.9. Bar chart showing the relationship between mean atropine-resistant contractions recorded from the two small (clear bars on left) and single large (solid bars on right) human detrusor strips from clinically characterised bladder samples (each division on x axis represents one sample) and expression of a purinergic electrical signal marked with *. Gaps indicate zero atropine – resistant activity. IOB = idiopathic overactive bladder, NOB = neurogenic overactive bladder, O = obstructed overactive bladder. Grouped samples from same patient.

signal was recorded were attenuated by α, β-mATP, and that this agent had no effect on atropine-sensitive activity, is qualitative evidence of the expression of purinergic contractile mechanisms in the tissue that generated a signal and suggests coupling between the electrical signal and atropine-resistant tension generation.

The finding of an electrical signal in conjunction with atropine-resistant contractions in the sample from a patient with recurrent urethral stricture and bladder stone suggests that atropine-resistant activity and a purinergic electrical signal can also be evoked in detrusor samples from obstructed bladders, and that the signal is not associated with any alternative and undefined variable associated only with idiopathically unstable detrusor. However, additional data are required before further conclusions regarding purinergic electrical activity in obstructed detrusor samples can be drawn.

4.5 Conclusion.

These results show that detrusor samples taken from idiopathically overactive but not neurogenically overactive human bladders have a greater propensity to generate extracellular electrical activity than functionally normal controls, and that this activity is generated by a purinergic mechanism. These studies demonstrate for the first time validated, nerve-evoked extracellular electrical recordings from contracting human detrusor smooth muscle strips and provide direct electrophysiological evidence for the expression of purinergic neurotransmission in conjunction with atropine-resistant tension generation in the overactive human bladder.

CHAPTER FIVE: GENERAL DISCUSSION

5.1 Summary of results.

In the first part of this thesis an electromyographic signal was recorded from the whole guinea-pig bladder during nerve-stimulation and shown to be biological and free from the artefacts that have hindered previous attempts at detrusor electromyography. The signal was also recorded from the bladders mucosal aspect, indicating the potential for the suction electrode technique for recording bladder electrical activity clinically using a catheter-mounted electrode inserted *per urethram*. The whole bladder preparation was found unsuitable for investigating the signals electrophysiological origins and precise relationship to purinergic and cholinergic tension generation.

By adapting the apparatus to enable recording from urothelially-denuded detrusor strips, the second part of the thesis describes investigations into the electrophysiological origins of the signal and its relationship to evoked tension changes. The results showed that the signal originated from a purinergic but not a cholinergic mechanism, thus validating the suction electrode method for recording purinergic electrical activity from contracting human detrusor smooth muscle strips.

The final part of this thesis describes suction electrode recordings from human detrusor smooth muscle strips. The results show significantly greater atropine-resistant activity in samples of overactive detrusor than controls. Furthermore, they show that in contrast to samples taken from functionally normal bladders which appeared electrically inert, a purinergically mediated extracellular electrical signal could be recorded coupled to nerve-evoked atropine-resistant tension generation by suction electrodes from samples of idiopathically overactive bladders.

5.2 Limitations of study.

Experimental numbers.

Although providing strong evidence in support of the hypothesis that detrusor samples from overactive bladders can generate purinergic extracellular electrical activity the experimental data is limited in numbers due to the scarcity of tissue samples and the heterogeneity of atropine-resistant activity in affected detrusor. Furthermore, these studies are not sufficient to allow implications regarding the existence of purinergic electrical activity in overactive detrusor taken from obstructed bladders.

The possibility of electromechanical artefact.

Purinergically mediated contractions in detrusor strips differ mechanically from those generated by cholinergic mechanisms; purinoceptor activation initiating a rapid rise in tension, while muscarinic mechanisms produce a slower more tonic rise. It could be argued that this diffrence could lead to an artefactual electromechanical signal that was not controlled for by experiments in this thesis, the faster twitch of purinergically mediated contractions perhaps generating tissue movements around the electrode within the suction field that were not generated during cholinergic activation.

However, it is unlikely that the environment within the lumen of the suction electrode would allow any tissue movement at all due to the suction field. Furthermore, a mechanical signal would be expected to be mono-phasic and related to the time-course of the duration of the contraction in contrast to the suction electrode signal, which was bi-phasic, and always recorded preceding any change tension changes. The field-stimulated preparation used for human detrusor recordings would be expected to reflect the correct temporal relationship between evoked electrical and mechanical activity, and to have recorded the two signals simultaneously if the electrical signal was mechanical artefact. The experiments using repetitive stimulation in the guinea-pig

whole bladder demonstrated no morphological change in the signal with increasing tension generation, and it is highly unlikely that the signal would not be attenuated at all with atropine if it originated from a mechanical event.

Limitations of experimental method.

An additional limitation to this thesis is the physiological relevance of the stimulation parameters used. The contribution of purinergic and cholinergic contractile components to the total are subject to a considerable degree of plasticity [Calvert et al. 2002] and vary with the intensity of evoking stimulus [Brading and Williams 1990, Sibley 1984, Brading and Mostwin 1989]. Experiments in this thesis confirmed that in the guinea-pig ATP is more readily released and contributes maximally at low stimulation intensities, whereas the cholinergic contribution develops with increasing stimulation. If this phenomenon also occurs in human detrusor, the purinergic electrical and mechanical signals evoked in the large strips used for suction electrode recordings could be an experimental artefact and not physiologically relevant.

However, previously constructed force frequency curves showing atropine resistant contractions as a percentage of the total nerve-mediated component showed no variation with increasing stimulus frequencies [Bayliss et al. 1999], and although some variability was present, control experiments in this thesis comparing the atropine resistant contractions evoked by nerve-stimulation at supra-maximal and experimental intensities demonstrated no difference between the two (see table 4.2). Furthermore, although it may be argued that atropine-resistant activity may have been overestimated, any error caused by experimental stimulation intensities is likely to be quantitative rather than qualitative, as the electrical signal and atropine-resistant contractions were recorded only from overactive bladders. The implications of this thesis are therefore likely to be relevant at physiological stimulus intensities.

5.3 Implications and applications of thesis.

5.3.1 Previous attempts at detrusor electromyography.

Human detrusor.

Despite the availability of sucrose-gap technology, no extracellular electrical recordings from human detrusor have previously been reported. The experiments described here are in accordance with microelectrode recordings from isolated detrusor myocytes and show that a purinergic electrical signal can be recorded from isolated human detrusor smooth muscle only in association with purinoceptor-mediated tension generation. Failure to record an electrical signal during completely atropine-sensitive contractions and the insensitivity of the signal to atropine suggests that contraction of healthy human detrusor does not generate an electromyogram. The absence of previously reported extracellular electrical recordings from human detrusor may therefore reflect the absence of such activity in most detrusor samples, which are most readily acquired from cancerous but functionally normal bladders.

Animal detrusor.

In contrast, many attempts have been made to record both evoked and spontaneous electromyographic activity from both excised and *in situ* animal bladders, the majority of which express varying degrees of purinergic transmission and would as a result be expected to generate an electrical signal. However, only the signals recorded by Craggs and Stephenson from anaethesised cats bore any morphological resemblance to those recorded in the present study. All other electromyographic recordings consisted mainly of electromechanical artefact, a hurdle overcome in this thesis by the use of suction electrodes.

5.3.2 The potential utility of suction electrodes in the evaluation of a purinergic detrusor myopathy.

Current evidence supporting the existence of purinergic neuromuscular transmission in overactive human detrusor originates solely from quantitative studies of atropine resistant detrusor contractions and is therefore indirect. Although the results presented here are tentative, demonstration that an extracellular purinergic electrical signal can be recorded and is coupled to purinoceptor-mediated tension generation in samples of overactive but not functionally normal detrusor provides direct electrophysiological evidence in support of a functional purinergic myopathy in the overactive bladder.

Mechanisms by which purinergic neurotransmission may become expressed and generate spontaneous detrusor contractions have been proposed (see section 1.3). Detrusor structural changes secondary to bladder outflow obstruction or associated with as yet unknown pathologies in idiopathically overactive detrusor would be likely to hinder gaseous exchange, perhaps more so in muscle fibres of particular orientation with relation to their blood supply. The resulting hypoxia may impair ecto-ATPase activity reducing ATP breakdown. The additional Ca^{2+} mobilised as a result would be likely to disrupt homeostatic mechanisms leading to spontaneous intracellular Ca^{2+} transients and 'unstable' contractions in affected areas [Fry, 1997]. Detrusor hypoxia may also affect the function of detrusor motor nerves, and conrtibute to plasticity in the differential release of neurotransmitters and increasing ATP release.

Suction electrode recordings *in vitro* from human detrusor strips could be a useful experimental tool for investigating the pathobiology of purinergic detrusor activity. Detection of an electrical signal from normal human detrusor after administration of ecto-ATPase inhibiting agents, and abolition of a signal evoked from overactive detrusor by ecto-ATPase analogues would support the hypothesis that

reduced enzyme activity is involved. These studies would be difficult to conduct using tension generation experiments during pharmacological neuromuscular blockade. Furthermore, as it is evident that detrusor neurotransmitter release mechanisms are subject to plasticity, it would be interesting to test the hypothesis that a purinergic electrical signal can be evoked from functionally normal human detrusor by varying the parameters of nerve stimulation.

The propensity to generate purinergically-mediated activity appears to be heterogeneously distributed in samples of overactive bladders (see table 4.1 and Bayliss et al. 1999], and is therefore likely to be patchily distributed around the bladder. This suggests that the patchy morphological changes seen in overactive bladders and the propensity to generate purinergic activity may be causally related and predispose to clinically important contractions localised to affected areas of the bladder wall. Although no spontaneous activity was recorded from human detrusor in this study, it would be interesting to determine whether spontaneous electrical signals could be recorded using refined recording apparatus and whether they are generated more frequently in structurally altered atropine-resistant samples from overactive bladders. Furthermore, ultrastructural analysis of detrusor samples from which a purinergic electrical signal had previously been recorded would provide morphological evidence of a structural purinergic myopathy.

Evidence is accumulating to suggest that the symptoms of urinary urgency, frequency and urge incontinence may result from activation of stretch receptors during contractions of small localised areas of bladder wall with more generalised or higher amplitude contractions causing incontinence [Coolsaet and Blaivas 1985, Coolsaet et al. 1993, Drake et al. 2005]. If the patchy expression of a purinergic myopathy in overactive detrusor is associated with an increased propensity to generate spontaneous action potentials *in situ*, bladder electromyography using a catheter-mounted electrode

attached to relavent areas of bladder wall in conjunction with cystometric techniques, could be a valuable tool with which to investigate the relationship between OAB symptoms, bladder activity and purinergic mechanisms.

Pressure flow cystometry is currently the only clinical tool available for identification and evaluation of the overactive bladder. This detects only the presence of gross intravesical pressure rises and gives no information regarding their aetiology. Involuntary contractions of detrusor generated by disruption of the bladders central nervous regulation produce the same cystometric data as those resulting from presumed detrusor myopathies. An alternative potential method for characterising diseased detrusor is by biopsy [Elbadawi et al. 1997, Hailemariam et al. 1997]. This is invasive and has not been adequately validated for this purpose. If pathological purinergic neurotransmission is expressed *in situ* only in idiopathicaly and obstructed but not neurogenic overactive bladders as implied by Bayliss et al., detrusor electromyography could enable clinical differentiation of myopathy from central neuropathy as a cause of bladder overactivity.

A targeted drug model.

Current drug treatments for overactive bladder are based on muscarinic receptor blockade. These have poor efficacy, and have a wide range of systemic side effects that limit patient compliance and are particularly troublesome in the elderly. By recording signals that directly reflect purinergic neurotransmitter activity, without the need for concomitant neuromuscular blocking agents, suction electrode recordings *in vitro* from overactive detrusor samples could be useful in the development of novel pharmacological agents directed against purinergic mechanisms. Furthermore, *in situ* detrusor electromyography could be used during drug evaluation studies, and for monitoring disease response to drug treatment.

5.4 Future study.

It is not known whether extracellular electrical recordings from human detrusor have been attempted using established recording techniques, none have been reported. It is hoped that the work presented in this thesis will provoke new attempts to record purinergic electrical activity from overactive human detrusor using established extracellular recording techniques. Although not suitable for development as a clinical tool, successful sucrose gap recordings of purinergic activity would corroborate the results presented here, and may be more sensitive for detecting purinergic electrical activity *in vitro*. Furthermore, suction electrode recordings from overactive bladder samples should be repeated, and the technique refined to improve the quality and consistency of the recordings, and to determine whether a purinergic electrical signal can reliably be recorded from overactive obstructed human bladder samples.

Future work should incorporate development of the suction electrode for clinical use. This will entail replacement of the electrode coating with a more stable reversible coating, and re-construction of the electrode structure using biocompatible materials. The effect of suction on the bladder mucosa will require investigation, and suction electrode electropmyography may require co-application of local anaesthetic agents with unknown effects on the signal. The principal difficulty however, will arise from the likely differences in the intensity of the physiological nerve stimulus, which is likely to be much lower than that used in these experiments and likely to generate a smaller electrical signal. Furthermore, the signal will be more difficult to detect as exclusion of electrically inert material from under the suction electrode will not be possible, and human urothelium is thicker and more robust than that of the guinea-pig, and likely to provide a greater barrier to extracellular detrusor recordings. Despite these potential difficulties, this thesis provides electrophysiological evidence for the

existence of a purinergic myopathy in overactive human bladder, and proof in principal

of a technique with which to detect and evaluate it clinically by electromyography.

APPENDICES.

APPENDIX 1.

Manufacture of Kohlrausch solution (Millar, 1992)

1. Dissolve 1.0g hydrogen hexachloroplatinate (chloroplatinic acid ($H_2PtCl_6.6H_2O$) in 50ml distilled water.

2. Mix 1.125ml concentrated hydrochloric acid with 200ml distilled water. Take 100ml of this HCL solution and add 0.625g lead acetate. Take 10ml of this and remix with another 90ml of the hydrochloric acid solution; this gives a mixture of lead acetate and lead chloride.

3. To make stock Kohlrausch solution add 2 parts of this lead acetate-lead chloride solution to 3 parts of the chloroplatinic acid solution (step 1).

4. The plating solution is 1 part Kohlrausch solution with 1 part 0.17% aqueous gelatine solution.

APPENDIX 2.

Tyrode's solution.

Constituent	Concentration (mM)
NaCl	118
$NaHCO_3$	24
KCL	4
$MgCl_2.6H2O$	1
$NaH_2PO_4.H_2O$	0.4
$CaCl_2.6H_2O$	1.8
Glucose	6.1
Sodium pyruvate	5

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