Purinergic receptor expression in neuronal, bladder smooth muscle and urothelial cells: characterization and inhibition by low molecular weight antagonists

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I, Joel Robert Gever, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

P2 purinoceptors comprise ionotropic (P2X) and metabotropic (P2Y) receptor families, responsive to nucleotide ligands and diversely distributed on virtually every mammalian cell. Most cells and tissues co-express multiple subtypes of purinoceptor; thus, unraveling the functional role – and pharmacological potential – of any subtype is a complex task. Additionally limiting is the paucity of potent, selective antagonists, particularly those with suitable physicochemical and pharmacokinetic properties for animals models and clinical development.

These studies address questions initially debated >10 years ago, following the successful cloning of purinoceptor families. First, given the large, polyanionic or nucleotide chemical probes available for pharmacology, are these receptors medicinally tractable? Secondly, given the admixture of purinoceptor expression in mammalian cells, would selective interference impact pathophysiology and disease burden; or would redundancy dominate?

Through the current investigations some answers can be offered. First, a resounding "yes", second, a more equivocal "possibly". Importantly, in addressing these queries, our investigations – and others - have furnished both important data on biological relevance of P2 subtype expression and function, as well as excellent chemical and biological tools for future investigators, so that more answers can be found.

Meanwhile, the pharmacological characteristics of two novel prototype antagonists have been detailed: for $P2X_1$ (RO-1) and $P2X_3$-containing receptors (RO-4). Additionally, the potential value of these compounds for the study of P2X signaling *in vitro* and *in vivo*, as well as templates for candidate medicines with a wide variety of potential therapeutic uses are demonstrated. It has also been possible to elucidate the potential of selective interference in certain target tissues – urological and sensory – and increasing the apparent therapeutic potential.

We can indeed conclude that P2X channels of focus in this work, $P2X_1$, $P2X_3$ and $P2X_{2/3}$, are druggable; the true therapeutic value of antagonists of these channels is awaited.

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Key co-authors deserve additional acknowledgement, particularly Renee Martin, David Hackos, Bikramjit Chopra, Wilhelm Lachnit, and all of the medicinal chemists at Roche who have worked their magic over the years and spun gold from straw (I hope they won't be offended that I've just referred to their precious chemical library as "straw").

Many friends and family have offered unflagging faith in me over the years, none more than my sister, Debbie. She deserves much of the credit for the fact that 200 + pages later, I'm sitting here finally writing the acknowledgements for this thesis.

And then, there are two major contributors of "good" insanity in my life to balance out the "bad" insanity. The first is "Hxfo" (aka Mignone). She knows who she is and for never doubting me, she has earned my eternal love. The second is my 5 year old daughter, Sophia, for which sanity is a relative term and certainly not one to be taken too seriously. She's cornered the world's market for silliness and affection and no father could ever wish for a better daughter. It's not an exaggeration to say I did this for her.

Publications arising from this thesis

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2. Gever, J.R., Jaime-Figueroa, S., Knight, G.E., Dunn, P.M., Mandel, D.A., Hegde, S.S., Greenhouse, R.J., Padilla, F., Dillon, M.P., Burnstock, G., Ford, A.P.D.W. Pharmacological characterization of RO-1, a selective $P2X_1$ antagonist (Manuscript in preparation for submission to *J. Pharmacol. Exp. Ther*. in 2009)

3. Chopra, B., Gever, J.R., Barrick, S.R., Hanna-Mitchell, A.T., Beckel, J.M., Ford, A.P.D.W., Birder, L.A. Expression and function of rat urothelial P2Y receptors (2008) *Am. J. Physiol. Renal Physiol.* 294(4):F821-9

4. Gever, J.R., Cockayne, D.A., Dillon, M.P., Burnstock, G., Ford, A.P.D.W. (2006) Pharmacology of P2X channels *Plugers Arch.* 452(5):513-37

5. Dunn, P.M., Gever, J.R., Ruan, H.Z., Burnstock, G. Developmental changes in heteromeric $P2X_{2/3}$ receptor expression in rat sympathetic ganglion neurons (2005) *Dev. Dyn.* 234(3):505-11

6. Lachnit, W.G., Oglesby, I.B., Gever, J.R., Gever, M., Huang, C., Li, X.C., Jin, H., McGivern, J.G., Ford, A.P.D.W. (2000) *J. Auton. Nerv. Syst.* 81(1-3):75-81

Table of Contents

Prologue

Although the practical application of pharmacological interventions derives ultimately from the earliest efforts by humans to alter physiological function for therapeutic (e.g. willow bark for pain and fever), recreational (consumption of fermented products or naturally hallucinogenic plants and fungi) and even aesthetic purposes (e.g. *Atropa belladonna* extracts for pupil dilation), the scientific origin of pharmacology derives primarily from the incremental (but significant) advances by such visionary scientists as Claude Bernard (the action of curare on neuromuscular junction), Oswald Schmiedeberg (the effect of muscarine on the heart and its similarities to direct electrical stimulation of the vagus nerve) and Thomas Renton Elliott, whose underappreciated work comparing sympathetic nerve stimulation and the pharmacological effects of epinephrine was first published in 1904 and recounted by Ronald Rubin a century later (Rubin, 2007). Later, Sir Henry Dale studied the effects of acetylcholine (extracted from the rye fungus, ergot) on cat blood pressure and rat smooth muscle and in this way set the groundwork for the ensuing decades of research into the fundamental mechanisms of chemical synaptic transmission. To this day, the progress of pharmacology has been driven largely through the discovery and characterization of chemicals with unique physiological properties and selectivity for specific molecular targets in living organisms.

It has been my goal throughout my postgraduate studies to be an active participant in the unfolding discovery of chemical signaling in peripheral nervous function. The chapters in this thesis, I hope, will comprehensively detail several related, but distinct studies undertaken within the context of this theme. I have been fortunate to have been intimately associated with a world leading academic team at

UCL while simultaneously playing a major part in applying pharmacological research specifically for the advancement of medicinal chemistry. This interface has provided a rich seam of opportunity to develop and showcase our own contributions to the evolving research exploring the nature of autonomic physiology.

The organization of chapters contained in this thesis is as follows: the first chapter provides an introductory review of the current understanding of the pharmacological characteristics of ATP-gated ion channels, known as P2X receptors, and includes a brief summary of the localization and function of each homomeric and heteromeric subtype. Chapter two contains the details of the wide range of experimental methods used and developed in these studies. Chapters three and four focus on new chemical tools that have emerged from our efforts: RO-4 and RO-1, unique and selective antagonists of P2X receptors with highly desirable pharmacological characteristics, which were revealed through the application of models ranging from single cells expressing recombinant receptors to whole tissues and anesthetized animals expressing native P2X receptors. In each case, characteristics are described of novel chemical antagonists for $P2X_1$ and $P2X_3$ receptors that represent the very first medicinal prototypes: chemicals that satisfy basic criteria to allow for their optimization into candidate medicines. In fact, one of the "theses" of this dissertation is the unforeseen but important finding that it is possible to find selective, low molecular weight, chemically attractive antagonists of receptors of a ligand (ATP) for which there are many binding sites, even within and on the surface of a single cell (e.g. kinases, transporters, ligand gated ion channels, G protein-coupled receptors…etc.).

Subsequent chapters (five and six) describe the study of "native" P2 receptor expression and function (both P2X and G protein-coupled P2Y receptors) in the bladder epithelium and nerve ganglia of rats and how this may provide insight to the role of analogous purinergic receptors in human physiology and pathology. These two types of tissue, bladder epithelium and sympathetic and sensory nerve ganglia, exemplify an apparent dichotomy of purinergic receptor signaling; in some cases, as with urothelium, there appears to be a plethora of receptors present which can be activated by ATP or its breakdown products, whereas in others (e.g. nerve ganglia such as cervical, trigeminal and dorsal root ganglia), $P2X_3$-containing receptors seem to dominate the transmission of signals between nerves and on sensory neurons relaying information from the periphery to the central nervous system. This dichotomy contrasting redundancy of chemical signaling with specificity is another major theme of this thesis upon which I hope I have successfully shed a little light.

The concluding chapter describes the creation of an engineered cell line using a "trick" of cell biology: the use of an exogenous agent, tetracycline, to control $P2X_3$ receptor expression via the insertion of a regulatable gene promoter sequence. This ingenious artifact allowed for the generation of a cell line for the study of P2X receptors in *in vitro* models of channel function without needing to consider the negative selection pressure that their expression may otherwise engender. Cell lines such as these were a great advance that allowed us to so effectively and successfully conduct lead finding activities that led directly to the discoveries described in chapters three and four (regarding RO-4 and RO-1).

The common thread tying these chapters together is the use of unique, recently discovered tools, both chemical and biological, to increase our understanding of the

function and pharmacological characteristics of several distinct purinergic receptors that are important primarily in the function of the smooth muscle and nerves of visceral organs. The use of these tools has produced experimental results that I hope have advanced our understanding of the important role that receptors activated by extracellular ATP play in living organisms.

Chapter 1: Introduction

Abstract

Significant progress in understanding the pharmacological characteristics and physiological importance of homomeric and heteromeric P2X channels has been achieved in recent years. P2X channels, gated by ATP and most likely trimerically assembled from seven known P2X subunits, are present in a broad distribution of tissues and are thought to play an important role in a variety of physiological functions, including peripheral and central neuronal transmission and inflammation. The known homomeric and heteromeric P2X channels can be distinguished from each other on the basis of pharmacological differences when expressed recombinantly in cell lines, but whether this pharmacological classification holds true in native cells and *in vivo* is less well established. Nevertheless, several potent and selective P2X antagonists have been discovered in recent years and shown to be efficacious in various animal models including those for visceral organ function, chronic inflammatory and neuropathic pain and inflammation. The recent advancement of drug candidates targeting P2X channels into human trials confirms the "druggability" of this novel target family and provides hope/optimism that safe and effective medicines for the treatment of disorders involving P2X channels may be identified in the near future.

Acknowledgement

The following chapter was originally published in Pfluger's Archiv – European Journal of Physiology as an invited review (Gever et al., 2006) and has been updated to include advances in the field since publication. The author of this thesis wrote the review with assistance from his co-authors: Debra A. Cockayne, Michael P. Dillon, Geoffrey Burnstock and Anthony P.D.W. Ford.

Introduction

Receptors activated by adenosine 5'-triphosphate (ATP), and related di- and tri-phosphate nucleotides, were originally named P2 receptors to differentiate them from P1 receptors, activated most potently by adenosine (Burnstock, 1978). In 1985, Burnstock and Kennedy further proposed dividing P2 receptors into P2X and P2Y receptor families, initially on the basis of differences in agonist and antagonist potencies, and later on the basis of differences in receptor structure and signal transduction mechanism (Burnstock and Kennedy, 1985;Abbracchio and Burnstock, 1994). Accordingly, it is now widely accepted that the terms P2X and P2Y describe ligand-gated ion channels and G-protein coupled receptors, respectively (Fredholm et al., 1994;Ralevic and Burnstock, 1998).

Our understanding of P2X channels emerged gradually at first from pharmacological investigations of native excitable tissues, and then exploded with great interest following their molecular cloning and characterization in the mid-1990s. Seven P2X receptor subunits have been identified that share less than 50% identity and range in length from 379 to 595 amino acids. P2X receptor subunits share a similar structural topology consisting of two transmembrane domains connected by a large extracellular loop containing the putative ATP binding site, and intracellular N- and C-termini of various lengths (Valera et al., 1994;Brake et al., 1994;Newbolt et al., 1998;Torres et al., 1998a;Ennion et al., 2000;Jiang et al., 2000b;Khakh, 2001;Roberts and Evans, 2004;Vial et al., 2004). In the last decade, the subunit composition of functional P2X channels has been elucidated, especially in recombinant systems, along with an understanding of their biophysical characteristics, such as ion selectivity, permeability, and kinetics of activation and inactivation. Data from a variety of experimental techniques including chemical cross-linking followed by native polyacrylamide gel electrophoresis (PAGE), mutagenesis, and atomic force and electron microscopy support the idea that P2X channels exist as homomeric and heteromeric trimers (Nicke et al., 1998;Jiang et al., 2003;Aschrafi et al., 2004;Barrera et al., 2005;Mio et al., 2005;Nicke, 2008;Young et al., 2008;Barrera et al., 2008). These channels are selectively permeable to cations (p_{Ca2+} ~ 2- to 5-fold greater than p_{Na+} and p_{K+}) (Valera et al., 1994;Lewis et al., 1995;Evans et al., 1996;Buell et al., 1996b; Egan and Khakh, 2004), and different trimers display unique

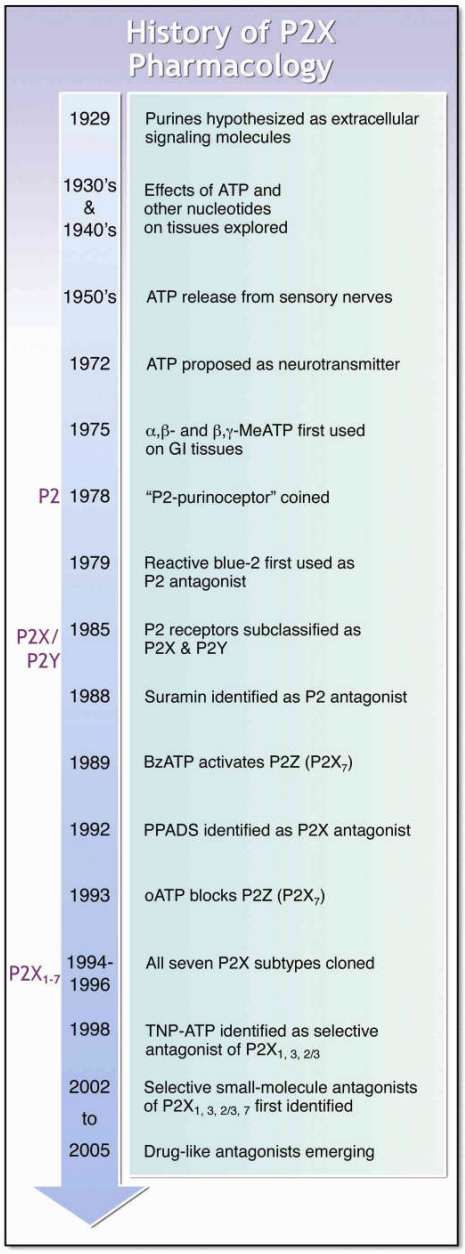

Figure 1. Timeline of the discovery of P2 receptors and the highlights of their pharmacological characterization. References used to construct timeline:
(Drury and Szent-Györgyi, 1929;Holton, 1959;Burnstock, 1972;Satchell and Maguire, 1975;Burnstock, 1978;Kerr and Krantis, 1979;Burnstock and Kennedy, 1985;Dunn and Blakeley, 1988;Gonzalez et al., 1989;Lambrecht et al., 1992;Murgia et al., 1993;Brake et al., 1994;Valera et al., 1994;Chen et al., 1995;Lewis et al., 1995;Bo et al., 1995;Collo et al., 1996;Garcia-Guzman et al., 1996;Surprenant et al., 1996;Virginio et al., 1998b;Jarvis et al., 2002;Alcaraz et al., 2003;Baxter et al., 2003) (Jaime-Figueroa et al., 2005;Merriman et al., 2005;Ford et al., 2006).

pharmacological properties (Lewis et al., 1995;Radford et al., 1997;Le et al., 1998;Torres et al., 1998b;King et al., 2000;Brown et al., 2002;Nicke et al., 2005). Significant progress has also been made in ascribing functions to various mammalian P2X subtypes in both physiological and pathological settings, in virtually every cell type and organ system.

Despite these advances, progress has been less impressive in certain regards. First, in many tissues and cells it remains to be established which homomeric or heteromeric form(s) of P2X channels transmit ionotropic responses to ATP, a discrepancy that may be attributable to the failure of recombinant expression systems to fully elaborate the characteristics of native P2X channels. Secondly, there remains a paucity of potent and selective pharmacological tools. Agonists that can selectively activate distinct members of this family have not been found, and with the exception of two notable family members, progress has been slower than perhaps anticipated in identifying selective inhibitors. Thus, exploration of therapeutic potential remains still very superficial.

The focus of this review is on the pharmacology of P2X receptors, with the aim of reviewing each *reasonably* established channel trimer, and a goal of capturing a) pharmacological characteristics that reflect the greatest distinctiveness and b) properties that have been identified more recently (over the last 3-5 years). The reader should be aware that many recognized properties of P2X receptors are based on data from recombinant channels, expressed heterologously in either oocytes or mammalian cells, and the degree to which these properties deviate from the functional characteristics of native channels is not entirely clear. A second caveat is that as a general guiding rule, robust pharmacological classification depends heavily on the determination of 'constants' that are derived under conditions closely approximating thermodynamic equilibrium. However, the nature of P2X channels, especially varying rates of desensitization, makes it very difficult (if not impossible) to ensure thermodynamic equilibrium has been established. Accordingly, a review of the literature will reveal many "dependent" variables – EC_{50} and IC_{50} estimates– dependent on the experimental conditions employed. In many cases, because of the difficulty or impossibility in attaining steady-state conditions (e.g. in standard electrophysiological or calcium flux studies), or in clearly establishing "simple,

reversible competition", one essentially cannot estimate equilibrium dissociation constants. This means that a clear fingerprint can not yet be established for many of the P2X channels, and until truly selective antagonists are developed, it will probably remain a challenge. The arrival of novel antagonists will provide a greater opportunity to study channels under conditions that more closely approximate true equilibrium - for example using radioligand binding approaches. Until then, one must remain cautious with claiming unequivocal characterizations based on agonist EC_{50} or antagonist IC_{50} estimates.

Homomeric $P2X_1$ channels

Key Messages

1. $P2X_1$ channels are predominantly expressed in smooth muscle and platelets, where they regulate smooth muscle contractility and various prothrombotic functions.
2. Pharmacologically, $P2X_1$ is almost identical to $P2X_3$ in terms of agonist and kinetic properties. However, β,γ-MeATP has a higher potency for $P2X_1$ versus $P2X_3$.
3. Many $P2X_1$ selective antagonists are available but drug-likeness is low. The only non-acidic small molecule $P2X_1$ antagonist is RO1.

Localization and Function of $P2X_1$ Channels

The gene encoding the $P2X_1$ protein subunit was first cloned from rat vas deferens (Valera et al., 1994), and although $P2X_1$ messenger ribonucleic acid (mRNA) and protein have a fairly broad tissue distribution, most notable is its dense localization within the smooth muscle lining a variety of hollow organs including the urinary bladder, intestines, arteries, and vas deferens (Valera et al., 1994;Valera et al., 1995;Collo et al., 1996;Mulryan et al., 2000;Burnstock and Knight, 2004). A role for $P2X_1$ in smooth muscle contractility emerged from early studies demonstrating that ATP was the neurotransmitter involved in atropine-resistant, nonadrenergic, noncholinergic contractions of the guinea pig detrusor smooth muscle (Burnstock, 1972). These neurogenic contractions could be mimicked by ATP, and suppressed by desensitization following exposure to the hydrolytically stable ATP analogue, alpha,

beta-methylene ATP (α,β-MeATP) (Burnstock et al., 1972;Burnstock et al., 1978;Kasakov and Burnstock, 1983). Electrophysiological recordings also showed that ATP and α,β-MeATP elicited dose-dependent membrane depolarization and inward currents in isolated detrusor smooth muscle cells that showed rapid desensitization (Fujii, 1988;Inoue and Brading, 1990;Inoue and Brading, 1991). It is now well-established that $P2X_1$ channels mediate the purinergic component of sympathetic and parasympathetic nerve-mediated smooth muscle contraction in a variety of tissues including urinary bladder (Hoyle et al., 1989;Palea et al., 1995;Vial and Evans, 2000), vas deferens (Lambrecht et al., 1992;Trezise et al., 1995;Mulryan et al., 2000), saphenous vein (von Kugelgen et al., 1995), and the renal microvasculature (Inscho et al., 2003). Consistent with this, $P2X_1$-mediated inward currents are abolished in the detrusor smooth muscle, vas deferens and mesenteric arteries of mice lacking the gene encoding $P2X_1$ protein subunits (Mulryan et al., 2000;Vial and Evans, 2000;Vial and Evans, 2002). Nerve-mediated vasoconstriction and contraction of the urinary bladder and vas deferens are also reduced by ~50-70 % in these mice (Mulryan et al., 2000;Vial and Evans, 2000;Vial and Evans, 2002). ATP-mediated vasodilation of mesenteric arteries appears to also involve $P2X_1$ channels, though evidence suggests this is mediated primarily via endothelial cells rather than through direct action on smooth muscle(Harrington et al., 2007).

$P2X_1$ is also present on blood platelets (MacKenzie et al., 1996) and ATP activation of $P2X_1$ receptors has been implicated in the regulation of various platelet functions including shape change (Rolf et al., 2001) and aggregation under increased sheer stress conditions (Erhardt et al., 2003;Hechler et al., 2003). Platelets from $P2X_1$-deficient mice have deficits in aggregation, secretion, adhesion and thrombus growth under certain in vitro conditions (Hechler et al., 2003). $P2X_1$-deficient mice also have reduced mortality and thrombus formation in models of systemic thromboembolism and laser-induced vessel wall injury, respectively (Hechler et al., 2003). Conversely, transgenic mice overexpressing human $P2X_1$ protein subunits in the megakaryocytic cell lineage exhibit hypersensitive platelet responses in vitro, and increased mortality in a model of systemic thromboembolism (Oury et al., 2003). Taken together, these data suggest that $P2X_1$ channels may play an important role in platelet physiology and hemostasis (Gachet, 2008).

Activation of $P2X_1$ Channels

Two defining characteristics of the homomeric $P2X_1$ channel are its rapid desensitizing kinetics and its sensitivity to activation by α,β-MeATP (Valera et al., 1994;Evans et al., 1995). In cells expressing recombinant rat or human $P2X_1$, α,β-MeATP is generally less potent than ATP and 2-(methylthio) ATP (2-MeSATP) (pEC_{50} ≈ 6-7), and somewhat more potent than adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) (pEC_{50} ≈ 5.5) (Valera et al., 1994;Evans et al., 1995;Valera et al., 1995;Torres et al., 1998b;Bianchi et al., 1999). These characteristics are shared by the homomeric $P2X_3$ channel, and therefore cannot be used to uniquely define $P2X_1$. However, beta, gamma-methylene ATP (β,γ-MeATP) is reported to be equipotent to α,β-MeATP at $P2X_1$, but approximately 30- to 50-fold less potent at $P2X_3$, and >100-fold less potent at $P2X_{2,4,5,7}$ (Evans et al., 1995;Buell et al., 1996b;Garcia-Guzman et al., 1996;Surprenant et al., 1996;Garcia-Guzman et al., 1997b;King et al., 1997). Consequently, β,γ-MeATP has been used as a selective agonist in some studies investigating $P2X_1$-mediated smooth muscle contraction (e.g. urinary bladder, vas deferens, saphenous veins) (O'Connor et al., 1990;Trezise et al., 1995;von Kugelgen et al., 1995;Theobald, Jr., 1996;Mok et al., 2000;Mulryan et al., 2000;Knight and Burnstock, 2004). Adenosine 5'-diphosphate (ADP) was originally reported to be an agonist at $P2X_1$ with moderate potency (pEC_{50} = 4.1-5) (Evans et al., 1995;Bianchi et al., 1999), however it has since been shown that this activity was imparted by impurities. Indeed, purified ADP at concentrations as high as 1 mM fail to elicit currents in oocytes expressing human $P2X_1$ (Mahaut-Smith et al., 2000). One report further suggests that 3'-O-(4-benzoyl)benzoyl ATP (BzATP) may be the most potent agonist at $P2X_1$ with a reported pEC_{50} of 8.74, approximately 100-fold more potent than α,β-MeATP (Bianchi et al., 1999). Recently, a recombinant chimeric rat $P2X_2/P2X_1$ receptor, incorporating the N-terminus and first transmembrane domain of $P2X_2$ (conferring non-desensitizing kinetics) with the extracellular loop, second transmembrane domain and C-terminus of $P2X_1$ (retaining $P2X_1$ pharmacology), was used to unmask nanomolar potency of ATP (pEC_{50} = 8.5) and other nucleotide agonists (Rettinger and Schmalzing, 2004). The deactivation rate of currents (τ)

through the rat $P2X_2/P2X_1$ chimera following washout of agonist was inversely related to potency (e.g. for ATP, τ = 63 s and pEC_{50} = 8.5, while for α,β-MeATP, τ = 2.5 s and pEC_{50} = 7.2), leading the authors to conclude that the rate-limiting step in the recovery from desensitization was the rate of agonist unbinding. A similar finding has recently been reported for the rapidly desensitizing $P2X_3$ channel (see $P2X_3$ section below) (Rettinger and Schmalzing, 2004;Pratt et al., 2005).

Diadenosine polyphosphates are also known to be agonists at $P2X_1$ with potencies similar to ATP, and selectivity for rat $P2X_1$ over rat $P2X_2$, $P2X_3$ and $P2X_4$. Only Ap_6A is a full agonist (pEC_{50} = 6.1 at $P2X_1$, $\geq$ 5.8 at $P2X_3$, >> 4 at $P2X_2$ and $P2X_4$), whereas Ap_5A (pEC_{50} = 6.0 at $P2X_1$, $\approx$ 5.9 at $P2X_3$, >> 4 at $P2X_2$ and $P2X_4$) and Ap_4A (pEC_{50} = 7.4 at $P2X_1$, > 6.4 at $P2X_4$, $\geq$ 6.1 at $P2X_3$, > 4.8 at $P2X_2$) are partial agonists, with Ap_4A being at least 10-fold selective for $P2X_1$ over the other P2X channels tested (Wildman et al., 1999b). Conversely, diinosine polyphosphates (synthesized through the deamination of Ap_nAs by the AMP-deaminase of *Aspergillus sp.*) are potent $P2X_1$ antagonists (see below) (King et al., 1999).

Recently, evidence for the regulation of P2X channels by phosphoinositides has surfaced (Zhao et al., 2007). $P2X_1$-mediated contractions of rat mesenteric artery were decreased by depletion of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] by the phosphatidyl 3- and 4-kinase inhibitor, wortmannin, and it was further shown that phosphoinositides probably interacted at a specific residue on the intracellular C-terminus of $P2X_1$, Lys_{364} (Bernier et al., 2008b). A similar phenomenon has been shown for other P2X channels, including $P2X_2$(Fujiwara and Kubo, 2006) and $P2X_4$(Bernier et al., 2008a).

Inhibition of $P2X_1$ Channels

The first antagonists shown to block $P2X_1$ channels were the non-selective P2 antagonist, suramin (Dunn and Blakeley, 1988), and the non-selective P2X antagonist, pyridoxal-5'-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS) (Lambrecht et al., 1992;Valera et al., 1994). Subsequently, several analogues of both suramin and PPADS were synthesized that had increased $P2X_1$ potency and selectivity (Ziyal et al., 1997;Jacobson et al., 1998;Rettinger et al., 2000). NF023 is a suramin analogue that was first identified as a P2X selective antagonist based on inhibition of α,β-MeATP-

evoked vasoconstriction in pithed rats (Urbanek et al., 1990). Following a thorough pharmacological characterization using two electrode voltage-clamp recordings in oocytes expressing recombinant P2X channels, NF023 was shown to be a $P2X_1$ antagonist (pIC_{50} = 6.6) with selectivity over $P2X_3$ and $P2X_{2/3}$ (~35- to 100-fold) and $P2X_2$ and $P2X_4$ (~400-fold or greater) (Soto et al., 1999). Even greater potency was achieved with the discovery of another suramin analogue, NF279, which has a pIC_{50} of 7.7, and increased selectivity over rat $P2X_3$ (85-fold) and human $P2X_4$ (>15,000-fold)(Rettinger et al., 2000). Unlike NF023, NF279 is a reasonably potent rat $P2X_2$ antagonist with a pIC_{50} of 6.1 (40-fold less potent than at rat $P2X_1$). The mechanism of antagonism of NF279 and NF023 was further investigated using non-desensitizing $P2X_2$ channels ($P2X_2$ for NF279 and the chimeric $P2X_2/P2X_1$ for NF023) to avoid the agonist-antagonist hemi-equilibrium conditions present in rapidly desensitizing channels. Incubation with either NF023 or NF279 resulted in parallel, surmountable shifts in the concentration-response curves to ATP, consistent with competitive antagonism (Rettinger et al., 2000;Rettinger and Schmalzing, 2004).

PPADS analogues with increased potency and selectivity have also emerged. MRS2220 was the first PPADS analogue identified with modest selectivity for rat $P2X_1$ (pIC_{50} = 5) over rat $P2X_3$ (pIC_{50} = 4.2) and $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ (inactive up to 100 μM) (Jacobson et al., 1998). Pyridoxal-5'-phosphate-6-azo-naphthyl-5-nitro-3,7-disulfonate (PPNDS), another PPADS analogue, inhibited α,β-MeATP-induced isometric contractions of rat vas deferens with a pK_B = 7.43 (vs. 6.59 for PPADS), and inward currents of rat $P2X_1$-expressing oocytes with pIC_{50} = 7.84 (vs. 7.06 for PPADS). PPNDS also blocked guinea pig ileum smooth muscle contractions evoked by adenosine 5'-O-(2-thiodiphosphate) (ADPβS) with a pA_2 = 6.13 (vs. 6.2 for PPADS) (Lambrecht et al., 2000).

Certain nucleotides have also been shown to be potent and selective $P2X_1$ antagonists. 2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) and other related trinitrophenyl ATP analogues (e.g. TNP-ADP, TNP-AMP and TNP-GTP) are 300- to 4000-fold selective for $P2X_1$ (pIC_{50} = 8.22), $P2X_3$ (IC_{50} = 8.5-9.0) and $P2X_{2/3}$ (IC_{50} = 7.4-8.2) over $P2X_2$, $P2X_4$ and $P2X_7$ ($pIC_{50} \leq 5.9$) (Virginio et al., 1998b). As mentioned previously, diinosine polyphosphates are also potent $P2X_1$ antagonists, possibly acting via stabilization of the desensitized state of the channel

(see $P2X_3$ section below). Ip_5I is the most potent and selective for rat $P2X_1$ (pIC_{50} = 8.5), being 900-fold selective over $P2X_3$ (pIC_{50} = 5.6) and > 100-fold selective over $P2X_2$ (inactive up to 30 μM) (King et al., 1999).

As is the case with all P2X receptors, agonist-evoked currents through $P2X_1$ are altered by extracellular pH, being reduced at pH 6.3 but unaffected at pH 8.3 (Evans et al., 1996;Haines et al., 1999). Although extracellular calcium has been shown to reduce currents through most P2X channels, $P2X_1$ is unaffected up to concentrations as high as 100 mM (Stoop et al., 1997).

Homomeric $P2X_2$ channels

Key Messages

1. $P2X_2$ channels are widely distributed throughout the peripheral and central nervous system, and on many non-neuronal cell types, where they play a role in sensory transmission and modulation of synaptic function.
2. $P2X_2$ channels exhibit agonist activity and slow desensitization kinetics similar to $P2X_4$ and $P2X_5$.
3. $P2X_2$ channels are the only P2X subtype potentiated by acidic conditions; they are also potentiated by Zn^{2+}, but inhibited by other divalent cations at high concentrations.

Localization and Function of $P2X_2$ Channels

The gene encoding the $P2X_2$ subunit was first cloned from neuronally-derived rat pheochromocytoma PC12 cells (Brake et al., 1994), and subsequent localization studies have demonstrated a broad tissue distribution. $P2X_2$ is expressed within the peripheral and central nervous systems (CNS) where it plays a role in ATP-mediated fast synaptic transmission at both nerve terminals and at interneuronal synapses. Within the CNS, $P2X_2$ receptors are localized within the cortex, cerebellum, hypothalamus, striatum, hippocampus, nucleus of the solitary tract, as well as in the dorsal horn of the spinal cord (Kidd et al., 1995;Collo et al., 1996;Kanjhan et al., 1996;Vulchanova et al., 1996;Simon et al., 1997;Vulchanova et al., 1997;Pankratov et al., 1998;Kanjhan et al., 1999;Khakh et al., 1999b;Wong et al., 2000;Burnstock and Knight, 2004;Scheibler et al., 2004). Accordingly, $P2X_2$ channels may have wide

ranging functions in the regulation of many CNS processes including memory and learning, motor function, autonomic coordination and sensory integration. Several studies have proposed a role for homomeric $P2X_2$, and possibly heteromeric $P2X_2$-containing channels, in ATP-mediated facilitation of inhibitory γ-amino butyric acid-mediated (GABAergic) synaptic transmission in the hippocampus and dorsal horn (Bardoni et al., 1997;Hugel and Schlichter, 2000;Khakh et al., 2003;Boue-Grabot et al., 2004). $P2X_2$ is also heavily expressed in the peripheral nervous system on both sensory and autonomic ganglion neurons (Collo et al., 1996;Simon et al., 1997;Vulchanova et al., 1997;Xiang et al., 1998;Zhong et al., 1998;Zhong et al., 2000b;Zhong et al., 2000a;Zhong et al., 2001;Calvert and Evans, 2004;Ma et al., 2004;Cockayne et al., 2005;Ma et al., 2005) signifying roles in afferent and efferent signaling pathways, and in the enteric nervous system where homomeric $P2X_2$ channels are thought to mediate fast synaptic excitation on S-type myenteric neurons (Zhou and Galligan, 1996;Castelucci et al., 2002;Ren et al., 2003;Galligan, 2004;Ohta et al., 2005; Bornstein 2008). Numerous recent studies have implicated both peripheral and central $P2X_2$ channels in chemosensory transduction in a variety of physiological systems including the regulation of respiratory control in response to hypoxia and hypercapnia (via sensory neurons within neuroepithelial bodies and the carotid body and the ventrolateral medulla) (Zhang et al., 2000;Prasad et al., 2001;Gourine et al., 2003;Rong et al., 2003;Mason et al., 2004;He et al., 2006), and in the detection of chemical stimuli such as odorants (via trigeminal neurons in the nasal epithelium)(Spehr et al., 2004), and taste (by gustatory nerves) (Finger et al., 2005).

$P2X_2$ protein subunits are also expressed on many non-neuronal cell types including cells of the anterior pituitary (Vulchanova et al., 1996) and adrenal medulla (Vulchanova et al., 1996), endothelial and epithelial cells (King et al., 1998b;Hansen et al., 1999b;Birder et al., 2004), epithelial and other support cells within the cochlea (King et al., 1998b;Housley et al., 1999;Jarlebark et al., 2002), skeletal, cardiac and smooth muscle (Hansen et al., 1998;Hansen et al., 1999a;Lee et al., 2000;Ryten et al., 2001;Jiang et al., 2005b), interstitial cells of Cajal (Burton et al., 2000;Burnstock and Lavin, 2002), and lymphocytes (Di Virgilio et al., 2001). A role for $P2X_2$ in many of these tissues has yet to be defined, but may involve functions of ATP such as

autocrine/paracrine regulation of hormone release, exocytosis/endocytosis, regulation of sound transduction, smooth muscle contractility, and pacemaker activity.

$P2X_2$ is unique among other P2X receptor subunits in that multiple splice variants of the human, rat and guinea pig $P2X_2$ mRNA have been identified that are capable of producing channels with different functional properties (see below) (Housley et al., 1995;Brandle et al., 1997;Simon et al., 1997;Koshimizu et al., 1998b;Lynch et al., 1999;Chen et al., 2000). Given the ability of full-length $P2X_2$ protein subunits to form heteromeric assemblies with truncated $P2X_2$ splice variants or other P2X subunits (e.g. $P2X_3$ or $P2X_6$, see corresponding sections below), $P2X_2$-containing channels in whole tissues or animal studies may function in a manner not entirely predicted by *in vitro* studies utilizing recombinant full-length $P2X_2$ subunits expressed in cell lines.

Activation of $P2X_2$ Channels

On the basis of a similar rank order of agonist potencies and slow desensitization kinetics following activation, $P2X_2$ can be grouped with $P2X_4$ and $P2X_5$. ATP, ATP-γ-S and 2-MeSATP are the most potent agonists with similar pEC_{50}s that are commonly reported as ranging from 5.1 to 6.3 (Evans et al., 1995;King et al., 1996;King et al., 1997;Bianchi et al., 1999;Lynch et al., 1999;Neelands et al., 2003). Bz-ATP has been reported to be a less potent partial agonist (Evans et al., 1995;Michel et al., 1996a), and α,β-MeATP, β,γ-MeATP, ADP and uridine 5'-triphosphate (UTP) are inactive up to 100-300 μM (Brake et al., 1994;Evans et al., 1995;King et al., 1996;King et al., 1997;Bianchi et al., 1999). The only diadenosine phosphate capable of gating $P2X_2$ channels is Ap_4A (pEC_{50} = 4.8); Ap_2A, Ap_3A, Ap_5A and Ap_6A are all inactive up to 100 μM (Pintor et al., 1996;Wildman et al., 1999a).

One property that differentiates $P2X_2$ from all other homomeric P2X channels is the ability of acidic pH to potentiate ATP-evoked currents (King et al., 1996;Stoop et al., 1997). ATP concentration-response curves at recombinant rat $P2X_2$ channels expressed in oocytes are facilitated by protons, with a maximal potentiation at pH 6.5 (pKa = 7.05 for potentiation), producing a shift of the pEC_{50} from 5.3 (pH = 7.4) to 5.9 (pH = 6.5) and no change in the maximal response (King et al., 1997).

Conversely, ATP-evoked currents are reduced under basic conditions (pEC_{50} = 4.5 at pH 8.0) (King et al., 1997). The ATP binding site of the $P2X_2$ channel is likely to include a histidine residue within the extracellular loop, and mutation of this residue to an alanine (H319A) significantly reduces the pH sensitivity of $P2X_2$ expressed in oocytes (Clyne et al., 2002). Extracellular histidine residues (His^{120} and His^{213} in rat) may also be important in mediating the potentiation of currents through $P2X_2$ by Zn^{2+} (1-10 μM) (Brake et al., 1994;Nakazawa and Ohno, 1997;Wildman et al., 1998;Clyne et al., 2002;Clyne et al., 2003) although human $P2X_2$-mediated currents in oocytes have been shown to be inhibited by Zn^{2+}(Tittle and Hume, 2008). It has also been hypothesized, based on results from mutational studies, that the Zn^{2+} binding site resides at the interface between $P2X_2$ subunits on homomeric channels (Nagaya et al., 2005). Until recently, this was the only evidence for an intersubunit binding site for any factor on a P2X channel, although intersubunit binding sites have been demonstrated to be present in other ion channels including $GABA_A$, glycine, and nicotinic receptors (for discussion, see (Sigel, 2002;Cascio, 2004;Grutter et al., 2004)). However, evidence has emerged for an intersubunit binding site for ATP based on models derived from similarities between P2X and acid-sensing ion channel subtype 1 (ASIC1), for which a crystallographic structure is now known (Guerlet et al., 2008).

$P2X_2$ channels are known to dilate after prolonged agonist activation, a characteristic shared by homomeric $P2X_4$ and $P2X_7$ channels (Khakh et al., 1999a;Virginio et al., 1999). Recent studies using fluorescence resonance energy transfer (FRET) have shown that the increased permeability of $P2X_2$ channels following pore dilation is due to the movement of subunit cytosolic domains, resulting in a transition from a state of high to lower ionic selectivity (measured as permeability to N-methyl-D-glucamine; NMDG) over the course of ~13 seconds (Fisher et al., 2004). Although in recent years certain evidence has suggested that the ATP-evoked cellular uptake of some large molecular weight fluorescent dyes such as quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]-diiodide (YO-PRO-1) may not pass exclusively through a pore intrinsic to P2X channels, the FRET work with $P2X_2$ (Fisher et al., 2004) and additional patch-clamp coordinated spectroscopy (Chaumont and Khakh, 2008), and other evidence from experiments with $P2X_7$ channels, suggests that NMDG most

likely does pass directly through a dilated P2X channel (see $P2X_7$ section below) (Jiang et al., 2005a).

Inhibition of $P2X_2$ Channels

There are no known selective or highly potent $P2X_2$ antagonists. PPADS, TNP-ATP and reactive blue-2 are approximately equipotent inhibitors of ATP-evoked currents through human or rat $P2X_2$ channels (pIC_{50}s range from 5.4-6.4), clearly less potent than at the homomeric $P2X_1$ or $P2X_3$ channels (King et al., 1997;Virginio et al., 1998b;Bianchi et al., 1999;Lynch et al., 1999). Suramin has been reported to be of similar potency (pIC_{50} = 5.4-6.0) as PPADS and TNP-ATP at $P2X_2$ (Evans et al., 1995;Miller et al., 1998;Wildman et al., 1998) in some studies, while others have reported suramin as having a 3-10 fold lower potency (pIC_{50} = 4.5 to 5.0) than these antagonists (King et al., 1997;Bianchi et al., 1999).

As stated above, currents through $P2X_2$ channels are potentiated by Zn^{2+}, whereas other divalent cations (e.g. Mn^{2+}, Mg^{2+}, Ca^{2+} and Ba^{2+}), at extracellular concentrations of 1-7 mM, have all been shown to reduce ATP-evoked currents through rat $P2X_2$ channels expressed in oocytes. It is speculated that this inhibition may occur through open channel blockade (King et al., 1997;Ding and Sachs, 1999).

Several splice variants of the wild-type rat $P2X_2$ channel ($rP2X_{2a}$) have been identified, but only one variant (rat $P2X_{2b}$; containing a 69 amino acid deletion in the C-terminus) has been shown to form functional channels (Brandle et al., 1997;Simon et al., 1997). The key difference is that the homomeric rat $P2X_{2b}$ channel expressed in oocytes has a more rapid desensitization ($P2X_{2b}$ τ = 12 - 27.5 s; $P2X_{2a}$ τ = 56 – 115 s), and reduced sensitivity to antagonists such as PPADS and suramin (Brandle et al., 1997;Simon et al., 1997;Lynch et al., 1999). A human splice variant ($hP2X_{2b}$) with a similar amino acid deletion in the C-terminus has also been isolated from pituitary tissue, but had identical desensitization characteristics and sensitivity to agonists and antagonists as the wild-type human $P2X_{2a}$ channel (Lynch et al., 1999). Thus, regions in the C-terminus thought to be important in controlling the desensitization kinetics of the rat $P2X_2$ channel (e.g. Val^{370}, Pro^{373} – Pro^{376}) apparently do not regulate the kinetics of the human $P2X_2$ channel in the same manner (Koshimizu et al., 1998a;Smith et al., 1999).

Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels

Key Messages

1. $P2X_3$ and $P2X_{2/3}$ channels are predominantly localized on peripheral and central terminals of unmyelinated C-fiber and thinly myelinated Aδ sensory afferents, where they mediate sensory neurotransmission.
2. $P2X_3$ and $P2X_{2/3}$ channels are pharmacologically similar, and like $P2X_1$ are selectively gated by α,β-MeATP. These channels differ, however, in their desensitization kinetics and in their sensitivity to extracellular ions.
3. Non-acidic, "drug-like" $P2X_3$/$P2X_{2/3}$ antagonists have been identified

Localization and Function of $P2X_3$ and $P2X_{2/3}$ Channels

Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels have become increasingly recognized as playing a major role in mediating the primary sensory effects of ATP (Burnstock, 1999;Burnstock, 2001a;North, 2002;Burnstock, 2003;Jarvis, 2003;Ford et al., 2006). The gene encoding the $P2X_3$ protein subunit was originally cloned from dorsal root ganglion (DRG) sensory neurons (Chen et al., 1995;Lewis et al., 1995) and, in the adult, $P2X_3$ and $P2X_{2/3}$ channels are predominantly localized on small-to-medium diameter C-fiber and Aδ sensory neurons within the dorsal root, trigeminal, and nodose sensory ganglia (Vulchanova et al., 1997;Bradbury et al., 1998;Dunn et al., 2001;Burnstock and Knight, 2004). Electrophysiological studies on sensory neurons from $P2X_2$ and $P2X_3$-deficient mice have confirmed that $P2X_3$ and $P2X_{2/3}$ channels account for nearly all ATP responses in DRG sensory neurons (Rae et al., 1998;Burgard et al., 1999;Cockayne et al., 2000;Cockayne et al., 2005), while $P2X_2$ and $P2X_{2/3}$ channels are predominant in nodose sensory neurons (Thomas et al., 1998;Virginio et al., 1998b;Cockayne et al., 2005). $P2X_3$ and $P2X_{2/3}$ channels are present on both the peripheral and central terminals of primary sensory afferents projecting to a number of somatosensory and visceral organs including the skin, joint, bone, lung, urinary bladder, ureter, and gastrointestinal tract (Vulchanova et al., 1997;Vulchanova et al., 1998;Kirkup et al., 1999;Brouns et al., 2000;Cockayne et al., 2000;Lee et al., 2000;Yiangou et al., 2001a;Yiangou et al., 2001b;Brouns et al., 2003;Wynn et al., 2003;Rong and Burnstock, 2004;Wynn et al., 2004;Ichikawa et al.,

2004;Gilchrist et al., 2005;Ishikawa et al., 2005). Accordingly, central $P2X_3$ and $P2X_{2/3}$ channels are present within the dorsal horn of the spinal cord and within the nucleus tractus solitarius (NTS), where they appear to play a role in the presynaptic modulation of glutamate release (Gu and MacDermott, 1997;Nakatsuka and Gu, 2001;Nakatsuka et al., 2003;Jin et al., 2004). $P2X_3$ and $P2X_{2/3}$ channels are also present within the enteric nervous system where they are thought to mediate excitation of AH-type intrinsic sensory neurons (Van Nassauw et al., 2002;Poole et al., 2002;Bian et al., 2003;Galligan, 2004). Recent studies have demonstrated that epithelial tissues, including the bladder uroepithelium, airway epithelial cells, and pulmonary neuroepithelial bodies express $P2X_3$ and $P2X_{2/3}$ channels, where they may modulate certain mechanosensory or chemosensory responses (Fu et al., 2004;Wang et al., 2005).

Several studies have shown that $P2X_3$ is expressed during development in various regions of the brain and in regions of the spinal cord outside of the dorsal horn; however, a role for $P2X_3$ during development of the nervous system has not been clearly established (Kidd et al., 1998;Cheung and Burnstock, 2002;Cheung et al., 2005;Studeny et al., 2005).

$P2X_3$ and $P2X_{2/3}$ channels have been characterized as fulfilling a role in nociceptive transmission and mechanosensory transduction within visceral hollow organs (Jarvis, 2003;Galligan, 2004;Ford et al., 2006). Studies using pharmacological agents, such as the $P2X_1$, $P2X_3$ and $P2X_{2/3}$ selective antagonist TNP-ATP (Tsuda et al., 1999b;Tsuda et al., 1999a;Jarvis et al., 2001;Honore et al., 2002b;Ueno et al., 2003), and the $P2X_3$, $P2X_{2/3}$ selective antagonist A-317491 (Jarvis et al., 2002;McGaraughty et al., 2003;Wu et al., 2004)(see below), have shown that peripheral and spinal $P2X_3$ and $P2X_{2/3}$ channels are involved in transmitting persistent, chronic neuropathic, and inflammatory pain. $P2X_3$-deficient mice (Cockayne et al., 2000;Souslova et al., 2000), and animals treated with $P2X_3$-selective antisense(Barclay et al., 2002;Honore et al., 2002a;Inoue et al., 2003) or small interfering RNA (siRNA)(Dorn et al., 2004) have revealed similar findings.

$P2X_3$ receptors also play a role in visceral mechanosensory transduction where according to the “tubes and sacs” hypothesis proposed by Burnstock, ATP released from the epithelial lining of visceral hollow organs can activate $P2X_3$ and/or $P2X_{2/3}$

channels on adjacent primary sensory afferents (Burnstock, 1999). Within the urinary bladder (Ferguson et al., 1997;Vlaskovska et al., 2001;Sun and Chai, 2002) and ureter (Knight et al., 2002;Calvert et al., 2008) for example, ATP is released from the urothelium upon distension. Distension leads to increased afferent nerve activity that is mimicked by ATP and α,β-MeATP, and attenuated in $P2X_3$-deficient mice (Vlaskovska et al., 2001;Rong et al., 2002). ATP and α,β-MeATP can directly stimulate the micturition reflex in conscious rats, and this is inhibited by TNP-ATP (Pandita and Andersson, 2002). Moreover, $P2X_3$-and $P2X_2$-deficient mice have reduced urinary bladder reflexes (Cockayne et al., 2000;Cockayne et al., 2005). A similar role has been postulated in gastrointestinal tissues where α,β-MeATP excites extrinsic (Kirkup et al., 1999;Wynn et al., 2003) and intrinsic(Burnstock, 2001a;Bertrand and Bornstein, 2002;Bian et al., 2003) afferents, and $P2X_3$-deficient mice have impaired peristalsis (Bian et al., 2003).

Activation of $P2X_3$ and $P2X_{2/3}$ Channels

Like $P2X_1$, native and recombinantly expressed homomeric $P2X_3$ channels respond to α,β-MeATP with a rapidly desensitizing inward current (typically described as bi-exponential decay with a fast component of $\tau_{d1} \approx$ 30-100 ms and a slow component of $\tau_{d2} \approx$ 250-1000 ms) at concentrations (pEC_{50} = 5.7-6.3) approximately 100-fold lower than those required to activate other homomeric P2X channels (Chen et al., 1995;Lewis et al., 1995;Robertson et al., 1996;Garcia-Guzman et al., 1997b;Virginio et al., 1998a;Bianchi et al., 1999;Burgard et al., 1999;Grubb and Evans, 1999;Neelands et al., 2003;Ford et al., 2005). When tested side-by-side in the same assay systems, ATP and 2-MeSATP (pEC_{50} = 6.1-6.9) have been consistently shown to be slightly more potent than α,β-MeATP (Robertson et al., 1996;Garcia-Guzman et al., 1997b;Bianchi et al., 1999;Neelands et al., 2003). Most studies have determined that ATP-γ-S is of similar potency as α,β-MeATP (pEC_{50} = 6.2-6.3) (Bianchi et al., 1999;Neelands et al., 2003), although it was originally reported to be less potent (Chen et al., 1995). Again, as at $P2X_1$, BzATP is the most potent agonist at homomeric $P2X_3$ channels, with the concentration required to elicit half-maximal responses (pEC_{50} = 7.1-7.5) being ~5-fold lower than that required for ATP or 2-MeSATP (Bianchi et al., 1999;Neelands et al., 2003). Overall, the distinguishing

pharmacological features between $P2X_3$ and $P2X_1$ include lower sensitivity of $P2X_3$ to L-β,γ-MeATP (pEC_{50} < 4 at $P2X_3$; pEC_{50} ~ 5.5 at $P2X_1$) (Chen et al., 1995;Evans et al., 1995;Garcia-Guzman et al., 1997b;Rae et al., 1998) and Ap_4A (pEC_{50} = 6.1-6.3 at $P2X_3$; pEC_{50} = 7.4 at $P2X_1$) (Bianchi et al., 1999;Wildman et al., 1999a). Conversely, Ap_3A appeared to be a $P2X_3$-selective agonist in one report (pEC_{50} = 6.0 at rat $P2X_3$; pEC_{50} < 4 at $P2X_1$, $P2X_2$ and $P2X_4$) (Wildman et al., 1999a), but it has since been reported to be a significantly weaker partial agonist at human $P2X_3$ (pEC_{50} = 4.7, 53% of ATP-evoked maximal response) and inactive at rat $P2X_3$ (pEC_{50} < 4) (Bianchi et al., 1999); this finding remains controversial. It has also recently been suggested that desensitized $P2X_3$ channels bind some agonists (e.g. ATP) with very high affinity (<1 nM), and that the subsequent rate of recovery from desensitization is primarily dependent on the rate of agonist unbinding (Pratt et al., 2005).

The heteromeric $P2X_{2/3}$ channel shares many of the activation characteristics of homomeric $P2X_3$ including selective gating by α,β-MeATP and a similar rank order of agonist potencies (Lewis et al., 1995;Bianchi et al., 1999;Liu et al., 2001). However, the key difference is that α,β-MeATP-evoked inward currents through recombinant or natively expressed (nodose ganglion neurons) $P2X_{2/3}$ channels are slowly desensitizing (Lewis et al., 1995;Burgard et al., 1999). In fact, the relatively sustained agonist-evoked cation influx through $P2X_{2/3}$ channels has enabled the use of mechanism of action studies requiring agonist-antagonist equilibrium (i.e. Schild-style curve shift experiments) to better understand putative antagonist binding sites (see below).

Another fundamental way in which $P2X_{2/3}$ differs from $P2X_3$ is in their opposite response to changes in pH. Like $P2X_2$, inward currents through $P2X_{2/3}$ channels (recombinantly expressed in oocytes or natively expressed in rat nodose ganglion neurons) are strongly increased under acidic conditions by as much as 250% at pH = 6.3, and strongly decreased under basic conditions by about 75% at pH 8.0. In contrast, currents through $P2X_3$ channels are much less sensitive to variations in pH, being unaffected at modestly basic (pH 8.0) or acidic (pH 6.5) conditions, and only significantly reduced in a much more acidic environment (pH 5.5) (Li et al., 1996a;Li et al., 1996b;Stoop et al., 1997;Wildman et al., 1999b), although this effect may be dependent on the concentration used(Gerevich et al., 2007) and on the N-

glycosylation state of the channel(Wirkner et al., 2008). In fact, the agonist-evoked response of $P2X_{2/3}$ channels is extremely sensitive to small changes in extracellular pH (pK_a = 7.1-7.2) (Li et al., 1996a;Li et al., 1996b), a factor that must be taken into consideration when comparing the potency estimates of competitive antagonists from studies conducted under different assay conditions (e.g. TNP-ATP and A-317491; see below).

Channels containing $P2X_3$ subunits appear to be sensitive to positive allosteric modulation by agents such as cibacron blue, ethanol and Zn^{2+}. Cibacron blue elicited a 3- to 7-fold increase in the maximal ATP-evoked Ca^{2+} influx through recombinant homomeric human $P2X_3$ channels (but not $P2X_1$, $P2X_2$ or $P2X_7$) expressed in 1321N1 astrocytoma cells (pEC_{50} for potentiation = 5.9), and pre-incubation with 3 μM cibacron blue increased the pEC_{50} of ATP from 6.4 to 7.3 (Alexander et al., 1999). Because the actions of cibacron blue were independent of ATP concentration, and mediated both a leftward shift of the agonist concentration-effect curve and a rightward shift of the concentration-effect curve of a non-competitive antagonist (PPADS), it was concluded that cibacron blue positively modulates ATP activation of $P2X_3$-mediated inward currents via an allosteric binding site (Alexander et al., 1999). ATP-evoked currents through $P2X_3$ channels are also potentiated by high concentrations of ethanol (5-200 mM), but unlike cibacron blue ethanol produces only a modest increase in ATP potency (from pEC_{50} = 5.6 to 6.0 in the presence of 100 mM ethanol) with no change in the maximal response (Davies et al., 2005). Neither ethanol nor cibacron blue have been tested on $P2X_{2/3}$ channels so it is unknown if the heteromer retains the sensitivity to these agents exhibited by the homomeric $P2X_3$ channel. Agonist-evoked inward currents through both homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels are sensitive to positive modulation by Zn^{2+} (pEC_{50} for potentiation = 4.9-5.0) (Li et al., 1993;Li et al., 1996b;Wildman et al., 1999b). For example, in oocytes expressing recombinant rat $P2X_3$ channels, 100 μM Zn^{2+} increased the potency of ATP from pEC_{50} = 5.3 to 6.1, with no change in the maximal response (Davies et al., 2005). Furthermore, because the potentiation of $P2X_3$-mediated inward currents in oocytes by ethanol and Zn^{2+} were synergistic, not additive, and the maximal potentiation by Zn^{2+} was increased in the presence of

ethanol, the authors concluded that ethanol and Zn^{2+} are acting on different sites or by different mechanisms (Davies et al., 2005).

Inhibition of $P2X_3$ and $P2X_{2/3}$ Channels

As with $P2X_1$, the activation of $P2X_3$ and $P2X_{2/3}$ channels by α,β-MeATP is sensitive to inhibition by TNP-ATP. Nanomolar concentrations of TNP-ATP can inhibit α,β-MeATP-evoked inward currents and Ca^{2+} influx in cell lines expressing recombinant rat $P2X_3$ (pIC50 = 9.0) and $P2X_{2/3}$ (pIC50 = 8.3-8.5) channels (Virginio et al., 1998b;Burgard et al., 2000), representing an ~1000-fold or greater selectivity over other homomeric P2X channels. Similarly, α,β-MeATP evoked currents through natively expressed rat $P2X_3$ (DRG neurons) and $P2X_{2/3}$ (nodose ganglion neurons) channels are also inhibited by TNP-ATP with pIC_{50}s of 9.1-9.5 and 7.7, respectively (Grubb and Evans, 1999;Dunn et al., 2000). Not surprisingly, based on the structural similarity to ATP, TNP-ATP is thought to be a competitive antagonist of ATP-mediated responses at $P2X_3$ and $P2X_{2/3}$ channels. In a manner consistent with competitive antagonism, pre-incubation with increasing concentrations of TNP-ATP produced parallel and surmountable rightward shifts (slope of Schild plot ≈ 1) of α,β-MeATP concentration-effect curves in 1321N1 cells expressing the heteromeric $P2X_{2/3}$ channel, or a $P2X_{2\text{-}3}$ chimeric channel composed of subunits incorporating the N-terminus and first transmembrane domain of $P2X_2$ (conferring non-desensitizing kinetics) with the extracellular loop, second transmembrane domain and C-terminus of $P2X_3$ (retaining $P2X_3$ pharmacology). In these experiments the affinity estimates (pA_2) of TNP-ATP were 8.7 (human $P2X_{2\text{-}3}$), 8.2 (rat $P2X_{2/3}$) and 8.7 (human $P2X_{2/3}$) (Burgard et al., 2000;Neelands et al., 2003). A similar affinity estimate ($K_D \approx 2$ nM) was determined in experiments measuring the on- and off-rates of TNP-ATP on rat $P2X_{2/3}$ channels, where it was illustrated that the high affinity of TNP-ATP derives primarily from fast binding ($k_{+1} \approx 100\ \mu M^{-1}s^{-1}$) and not slow unbinding ($k_{-1} \approx 0.3\ s^{-1}$) (Spelta et al., 2002). Further evidence that TNP-ATP acts at the ATP binding site is the observation that pre-incubation of rat DRG neurons (natively expressing homomeric $P2X_3$ channels) with approximately pIC_{80} concentrations of TNP-ATP (10 nM) significantly reduced the rate of desensitization of α,β-MeATP -evoked currents, as would be expected for a competitive antagonist (Ford et al., 2005).

In addition to TNP-ATP, both suramin and PPADS are antagonists of rat $P2X_3$- and $P2X_{2/3}$-mediated responses. Antagonism occurs at concentrations (pIC50 = 5.4-6.5) similar to those required to block activation of $P2X_1$ and $P2X_5$ channels, and lower than those required to block $P2X_2$, $P2X_4$ and $P2X_7$ channels (Bianchi et al., 1999;Grubb and Evans, 1999), although the human $P2X_3$ channel has been reported to be somewhat less sensitive to suramin ($pIC_{50} \leq 4.8$) than the rat $P2X_3$ channel (pIC_{50} = 6.1) (Garcia-Guzman et al., 1997b;Bianchi et al., 1999). As discussed previously in the $P2X_1$ section, Ip_5I is a $P2X_1$-selective antagonist that has moderate potency as an antagonist of inward currents through native or recombinantly expressed $P2X_3$ channels (pIC_{50} = 5.6-6.9) (King et al., 1999;Dunn et al., 2000). Recently, it was observed that Ip_5I inhibited $P2X_3$-mediated inward currents in rat DRG neurons only when pre-exposed to desensitized receptors, suggesting that this antagonist inhibits $P2X_3$ (and presumably $P2X_1$) activity through stabilization of the desensitized state of the channel (Ford et al., 2005).

High extracellular concentrations of calcium inhibit α,β-MeATP-evoked currents through rat $P2X_3$ (pEC_{50} = 1.1) and $P2X_{2/3}$ (pEC_{50} = 1.8) channels (Virginio et al., 1998a). Additionally, increasing the extracellular but not the intracellular concentration of Ca^{2+} from 1 to 10 mM has been shown to speed the recovery of $P2X_3$ channels from the desensitized state, and this was true even if the increase was reversed several minutes before activating the channels. These data suggest that Ca^{2+} (and other polyvalent cations like Gd^{3+} and Ba^{2+}) bind to an extracellular site to alter channel recovery (Cook et al., 1998).

Homomeric $P2X_4$ channels

Key Messages

1. $P2X_4$ subunits are widely distributed within neuronal and non-neuronal tissues.
2. $P2X_4$ channels localized on activated microglia have been implicated in chronic inflammatory and neuropathic pain.
3. Species differences exist in the responses of $P2X_4$ channels to α,β-MeATP and PPADS.
4. $P2X_4$ channels can be differentiated from $P2X_2$ and $P2X_5$ channels by differing activation sensitivity to pH and Zn^{2+}.

Localization and Function of $P2X_4$ Channels

The gene encoding the $P2X_4$ protein subunit was originally cloned from rat brain (Bo et al., 1995), and $P2X_4$ may be the most widely distributed of the P2X channels. mRNA and protein localization studies indicate that the $P2X_4$ subunit is expressed in several regions of the rat brain (particularly cerebellar Purkinje cells) and spinal cord (Bo et al., 1995;Buell et al., 1996b;Collo et al., 1996;Soto et al., 1996;Wang et al., 1996;Garcia-Guzman et al., 1997a;Rubio and Soto, 2001;Bo et al., 2003b;Burnstock and Knight, 2004), autonomic and sensory ganglia (Buell et al., 1996b;Xiang et al., 1998;Bo et al., 2003b), arterial smooth muscle (Nori et al., 1998;Glass et al., 2000;Lewis and Evans, 2001;Bo et al., 2003b), osteoclasts (Naemsch et al., 1999;Hoebertz et al., 2000), parotid acinar cells (Collo et al., 1996;Tenneti et al., 1998), kidney (Garcia-Guzman et al., 1997a;McCoy et al., 1999;Bo et al., 2003b), lung (Buell et al., 1996b;Soto et al., 1996;Bo et al., 2003b), heart (Soto et al., 1996;Garcia-Guzman et al., 1997a;Bo et al., 2003b), liver (Garcia-Guzman et al., 1997a;Bo et al., 2003b), pancreas (Bo et al., 2003b), and human B lymphocytes (Sluyter et al., 2001). The functional role of $P2X_4$ in most of these tissues is still unclear. However, several recent studies have demonstrated that $P2X_4$ receptor expression is increased on activated spinal cord microglia following spinal nerve injury, spinal cord injury, or formalin-induced inflammatory pain (Inoue et al., 2004;Tsuda et al., 2003;Guo et al., 2005;Schwab et al., 2005). Moreover, intraspinal administration of $P2X_4$ antisense oligonucleotides decreased the induction of $P2X_4$ receptors on spinal microglia, and suppressed the development of tactile allodynia after spinal nerve injury (Tsuda et al., 2003). Intraspinal administration of TNP-ATP and PPADS also suppressed tactile allodynia in this study; however these antagonists are not selective for $P2X_4$ channels and may mediate reversal of chronic pain through other P2X channels. Recent data supports the notion that $P2X_4$ channels are expressed on activated microglia in the spinal cord regulating BDNF release and $P2X_4$-deficient mice did not display mechanical hyperalgesia after peripheral nerve injury (Ulmann et al., 2008) and further evidence suggests that extracellular matrix molecules like fibronectin and integrin may be involved (Tsuda et al., 2008). These findings suggest that ATP and $P2X_4$ may be important in the modulation of chronic

inflammatory and neuropathic pain by spinal cord microglia, a topic that has received considerable recent attention (Inoue et al., 2004).

Activation of $P2X_4$ Channels

Homomeric $P2X_4$ channels generally produce a slowly-desensitizing inward current in response to ATP (Bo et al., 1995;Buell et al., 1996b;Garcia-Guzman et al., 1997a). $P2X_4$ channels are activated most potently by ATP, with pEC_{50}s in recombinant systems ranging from 4.7 to 5.5 for rat (Bo et al., 1995;Buell et al., 1996b;Seguela et al., 1996;Soto et al., 1996;Miller et al., 1998;Khakh et al., 1999b) and 5.1 to 6.3 for human (Garcia-Guzman et al., 1997a;Bianchi et al., 1999;Jones et al., 2000). $P2X_4$ can also be activated by 2-MeSATP and CTP, but in most cases these compounds were observed to be $\geq$ 10-fold less potent partial agonists (Seguela et al., 1996;Soto et al., 1996;Garcia-Guzman et al., 1997a). There may be species differences regarding the sensitivity of $P2X_4$ channels to activation by α,β-MeATP. α,β-MeATP is a weak partial agonist at recombinant mouse and human $P2X_4$ expressed in human embryonic kidney (HEK293) cells or oocytes, (Garcia-Guzman et al., 1997a;Bianchi et al., 1999;Jones et al., 2000) whereas at rat $P2X_4$ it has been shown to behave as a moderately potent antagonist of ATP-evoked inward currents (pIC_{50} = 5.3) (Jones et al., 2000). β,γ-MeATP has consistently failed to activate rat or human $P2X_4$ channels at concentrations up to 300 μM (Buell et al., 1996b;Garcia-Guzman et al., 1997a). To summarize, $P2X_4$ channels respond to ATP and 2-MeSATP with slowly desensitizing currents at ~10-fold or higher concentrations than is required to activate $P2X_1$ and $P2X_3$. $P2X_4$ channels are also generally insensitive to activation by methylene-substituted ATP analogues, a pattern of agonist activity shared by $P2X_2$ and $P2X_5$.

As is the case for $P2X_2$, ATP-evoked currents through $P2X_4$ channels can also be positively modulated by Zn^{2+}, with up to a 3-fold increase in the potency of ATP and no change in the maximal response at physiologically relevant concentrations (0.1-10 μM) (Garcia-Guzman et al., 1997a;Wildman et al., 1999b;Colvin et al., 2003). However, unlike $P2X_2$, ATP-evoked currents through rat $P2X_4$ are also potentiated by ivermectin, as has been previously shown for $GABA_A$ and α_7 nicotinic channels (Krusek and Zemkova, 1994;Krause et al., 1998;Dawson et al., 2000). In oocytes expressing recombinant rat $P2X_4$, ivermectin increased the potency of ATP 10-fold,

and increased the maximal response by 50-300% with a pEC_{50} for potentiation of 6.6, but had no effect on $P2X_2$, $P2X_3$, $P2X_{2/3}$ or $P2X_7$ (Khakh et al., 1999b). Recently, single-channel recordings of ATP-evoked currents through human $P2X_4$ expressed in HEK293 cells suggested that ivermectin increases maximal channel currents after binding to a high affinity site (pEC_{50} = 6.6), and may also bind to a low affinity site (pEC_{50} = 5.7) to increase the affinity of ATP by stabilizing the open channel conformation (Priel and Silberberg, 2004), possibly through interactions in the transmembrane domains (Jelinkova et al., 2008).

Inhibition of $P2X_4$ Channels

An unusual property of the rat $P2X_4$ receptor which differentiates it from other P2X channels is its relative insensitivity to classic, non-selective P2X antagonists, such as suramin and PPADS, at concentrations as high as 100 - 500 μM (Buell et al., 1996b;Soto et al., 1996;Jones et al., 2000). Indeed, there have even been reports that suramin, PPADS and cibacron blue at some concentrations can potentiate ATP-evoked currents in rat and mouse $P2X_4$ (Bo et al., 1995;Miller et al., 1998;Townsend-Nicholson et al., 1999). However, the *rat* $P2X_4$ may be uniquely insensitive as moderate sensitivity of the human $P2X_4$ has been reported for several antagonists, including PPADS (human $P2X_4$ pIC_{50} = 4.6 – 5.0; rat $P2X_4$ pIC_{50} < 3.3), suramin (human $P2X_4$ pIC_{50} = 3.7; rat $P2X_4$ pIC_{50} < 3.3), bromphenol blue (human $P2X_4$ pIC_{50} = 4.1; rat $P2X_4$ pIC_{50} < 3.5), and cibacron blue (human $P2X_4$ pIC_{50} = 4.4; rat $P2X_4$ pIC_{50} = 3.9) and the mouse $P2X_4$ has also been reported to be inhibited by PPADS (pIC_{50} = 5.0) with potency similar to that seen at the human $P2X_4$ (Garcia-Guzman et al., 1997a;Jones et al., 2000). It has been hypothesized that PPADS acts in part by forming a Schiff base with a lysine residue in $P2X_1$ and $P2X_2$ which in $P2X_4$ is replaced by a glutamate at the analogous position (Glu^{249}); indeed, when this residue is replaced by a lysine, the resultant $P2X_4$ mutant is sensitive to inhibition by PPADS (Buell et al., 1996b). However, the human $P2X_4$ has only one lysine (Lys127) not present in the rat $P2X_4$ in the region of the ectodomain (between residues 81 and 183) shown to confer sensitivity to PPADS and mutation of this residue to a lysine in the rat $P2X_4$ (N127K) did not produce a PPADS-sensitive channel (Garcia-Guzman et al., 1997a). Consequently, the increased sensitivity of the human $P2X_4$ to inhibition by

PPADS can not be simply explained by a difference in the ability of PPADS to form a Schiff base via lysine residues.

As with $P2X_3$ channels, acidic conditions (pH 6.3 – 6.5) decrease currents through $P2X_4$ but basic conditions (pH 8.0 – 8.3) have little or no effect (Stoop et al., 1997;Wildman et al., 1999b). This is another key difference from $P2X_2$ where ATP-evoked inward currents are increased at low pH and decreased at high pH (King et al., 1996;King et al., 1997). ATP-evoked currents through rat $P2X_4$ can also be inhibited by high concentrations of ethanol (5 – 500 mM) and mutant studies have suggested that histidine 241 in the extracellular loop is probably involved (Davies et al., 2005;Xiong et al., 2005).

Homomeric $P2X_5$ and heteromeric $P2X_{1/5}$ channels

Key Messages

1. Expression of $P2X_5$ has been most closely linked with differentiating tissues, particularly skeletal muscle and skin.
2. Recombinantly expressed $P2X_5$ channels from some species (human, chick, bullfrog) respond to activation with robust currents, whereas others (rat, zebrafish) respond much more weakly.
3. $P2X_5$ channels have unusually high chloride permeability and unusually slow recovery from desensitization.
4. Unlike $P2X_5$, $P2X_{1/5}$ can be activated by α,β-MeATP and blocked by TNP-ATP with reasonable potency.

Localization and Function of $P2X_5$ and $P2X_{1/5}$ Channels

$P2X_5$ mRNA and immunoreactivity are found in a variety of tissues including brain, spinal cord, heart, and eye (Collo et al., 1996;Garcia-Guzman et al., 1996;Bo et al., 2000;Jensik et al., 2001;Ruppelt et al., 2001;Burnstock and Knight, 2004); moreover, it has become apparent in recent years that $P2X_5$ expression is most evident in differentiating tissues, including skeletal muscle (Meyer et al., 1999;Collet et al., 2002;Ryten et al., 2002) and epithelial cells of the nasal mucosa (Gayle and Burnstock, 2005), gut (Groschel-Stewart et al., 1999), bladder and ureter (Lee et al., 2000) and skin (Groschel-Stewart et al., 1999;Greig et al., 2003a;Inoue et al., 2005).

It has been shown that activation of $P2X_5$–containing channels by ATP inhibits proliferation and increases differentiation of rat skeletal muscle satellite cells through phosphorylation of a mitogen-activated protein kinase (MAPK) signaling pathway (Ryten et al., 2002). Additionally, $P2X_5$ protein subunits are expressed in squamous cell carcinomas of the skin and prostate and may play a regulatory role in the proliferation and differentiation of certain types of cancer cells (Greig et al., 2003b;Calvert et al., 2004).

In human, mRNA expression has been reported to be low in many of the tissues mentioned previously, and instead appears to be expressed at the highest levels in tissues related to the immune system such as thymus, spleen, lymph node, leukocytes, appendix and bone marrow (Le et al., 1997). Additionally, both mRNA and immunohistochemical localization studies indicate that $P2X_5$ is present in cultured human epidermal keratinocytes (Greig et al., 2003a;Inoue et al., 2005). However, due to the scarcity of published data involving human tissues, the expression and function of $P2X_5$ channels in humans is still unclear.

An important recent development is the identification of functional evidence for the presence of $P2X_{1/5}$ channels in mouse cortical astrocytes (Lalo et al., 2008). ATP is known to be released in the brain and to mediate excitation of a small portion (estimated to be between 5 and 15%; Pankratov et al., 2003) of central neurons and can be released alone or as a cotransmitter with glutamate and GABA (Pankratov et al., 2006). ATP is also involved in neuronal-glial and glial-glial signaling, probably acting on both P2Y (Bowser and Khakh, 2004; Shigetomi et al., 2008) and P2X receptors (Fields and Burnstock, 2006; Pankratov et al., 2009), although which specific subtypes are involved is still unclear. The recent work identifying the $P2X_{1/5}$ channel as being perhaps the primary mediator of ATP action by P2X channels in glial cells (Lalo et al., 2008) is also important because it marks one of the very few examples where there is functional evidence of native heteromeric P2X expression, the other being the $P2X_{2/3}$ channel in nodose ganglia (Lewis et al., 1995).

Activation of $P2X_5$ and $P2X_{1/5}$ Channels

The initial pharmacological characterization of the homomeric rat $P2X_5$ channel was impaired by the inability to detect a robust functional response when expressed in

recombinant cell lines (Collo et al., 1996;Garcia-Guzman et al., 1996); however, subsequent work has highlighted some potentially important interspecies differences. For example, recombinant chick, bullfrog and human $P2X_5$ channels respond to ATP with large, rapidly activating, slowly desensitizing inward currents, whereas recombinant rat and zebrafish $P2X_5$ respond very poorly to ATP (Collo et al., 1996;Garcia-Guzman et al., 1996;Le et al., 1997;Bo et al., 2000;Jensik et al., 2001;Diaz-Hernandez et al., 2002;Bo et al., 2003a). ATP and 2-MeSATP are typically full agonists with similar pEC_{50}s ranging from 4.8 to 5.7 in all species tested (Collo et al., 1996;Garcia-Guzman et al., 1996;Ruppelt et al., 2001;Bo et al., 2003a). In most species, methylene-substituted ATP analogues (i.e. α,β-MeATP and β,γ-MeATP) are weak or inactive agonists (Garcia-Guzman et al., 1996;Jensik et al., 2001), although in one recent study using rat $P2X_5$ expressed in oocytes, α,β-MeATP was a partial agonist (pEC_{50} = 6.0, ~50% of maximal ATP-evoked current) with a potency comparable to ATP (pEC_{50} = 6.4) (Wildman et al., 2002). Only the chick $P2X_5$ channel appears to be consistently sensitive to activation by α,β-MeATP with currents as large as 80% of the maximum evoked by ATP (Bo et al., 2000;Ruppelt et al., 2001).

Additionally, both the chick and human $P2X_5$ channels have been reported to have relatively high chloride permeability ($p_{Cl^-}/p_{Na^+} = p_{Cl^-}/p_{Cs^+} = 0.5$), an unusual property for P2X channels and one of the few traits differentiating $P2X_5$ from $P2X_2$ (Ruppelt et al., 2001;Bo et al., 2003a). Although ATP-evoked currents through $P2X_5$ channels are slowly desensitizing, recovery from desensitization is also very slow requiring 20-60 minutes to recover to 30-65% (Bo et al., 2000;Jensik et al., 2001;Ruppelt et al., 2001;Wildman et al., 2002). Human, chick and bullfrog $P2X_5$ have also been reported to dilate to a large pore upon prolonged exposure to ATP in a fashion classically seen with $P2X_7$, although also seen with $P2X_2$ and $P2X_4$ (Bo et al., 2000;Jensik et al., 2001;Bo et al., 2003a).

The $P2X_{1/5}$ channel, as with other heteromeric P2X channels (e.g. $P2X_{2/3}$), uniquely combines some of the pharmacological and biophysical characteristics observed for the individual homomeric channels constructed from the constituent subunits. For example, whereas $P2X_1$ channels respond to ATP with a rapidly desensitizing current, and $P2X_5$ channels respond with a relatively slowly

desensitizing current, $P2X_{1/5}$ channels have a characteristic biphasic response to ATP consisting of a transient peak current followed by a sustained plateau current (Torres et al., 1998b;Haines et al., 1999;Le et al., 1999). In addition, a rebound inward current after the plateau current has been observed when large inward peak currents are elicited (Haines et al., 1999), possibly suggesting passage from the desensitized state to a closed state through an intermediate open state (North, 2002). The calcium permeability of $P2X_{1/5}$ (p_{Ca2+}/p_{Na+} = 1.1) more closely resembles $P2X_5$ (p_{Ca2+}/p_{Na+} = 1.5) than $P2X_1$ (p_{Ca2+}/p_{Na+} = 3.9 – 5.0), but unlike the $P2X_5$ receptor there is no evidence that the $P2X_{1/5}$ receptor can dilate to a large pore upon prolonged exposure to ATP (Valera et al., 1994;Evans et al., 1996;Surprenant et al., 2000;Bo et al., 2003a).

Pharmacologically, $P2X_{1/5}$ channels more closely resemble $P2X_1$ than $P2X_5$. The rank order of agonist potencies acting on recombinant rat $P2X_{1/5}$ channels has been reported as ATP ≥ 2-MeSATP > ATP-γ-S ≥ α,β-MeATP ≥ β,γ-MeATP > ADP, a rank order similar to the homomeric $P2X_1$ channel, although only ATP and 2-MeSATP were reported to be full agonists while ATP-γ-S, α,β-MeATP, β,γ-MeATP and ADP were partial agonists (Valera et al., 1994;Evans et al., 1995;Haines et al., 1999;Surprenant et al., 2000). In studies where recombinant rat $P2X_1$ and $P2X_{1/5}$ channels expressed in HEK293 cells or oocytes were tested side by side, ATP and α,β-MeATP were approximately equipotent at $P2X_{1/5}$ (pEC_{50} = 6.2-6.4 for ATP; pEC_{50} = 5.3-6.0 for α,β-MeATP) and $P2X_1$ (pEC_{50} = 6.2 for ATP; pEC_{50} = 5.6-5.8 for α,β-MeATP) (Torres et al., 1998b;Haines et al., 1999;Le et al., 1999).

The magnitude of ATP-evoked inward currents through homomeric rat $P2X_5$ channels is approximately doubled by moderate concentrations of Zn^{2+} (1-100 μM), but high concentrations (1 mM) block currents (Wildman et al., 2002); the effect of Zn^{2+} on $P2X_{1/5}$ channels has not been published. With regard to positive modulation of agonist activity, $P2X_{1/5}$ is unlike either homomeric $P2X_1$ or $P2X_5$ channels. Thus, whereas high concentrations of extracellular calcium inhibits ATP-evoked currents through rat $P2X_5$ and have no effect on rat $P2X_1$, a potentiation of currents through rat $P2X_{1/5}$ is reported with a maximal increase of 40-60% at 50 mM Ca^{2+} (Haines et al., 1999;Surprenant et al., 2000;Wildman et al., 2002).

Inhibition of $P2X_5$ and $P2X_{1/5}$ Channels

Like $P2X_2$, but unlike $P2X_4$, PPADS and suramin are effective antagonists of ATP-evoked currents through $P2X_5$ channels at concentrations as low as 1 μM (Collo et al., 1996;Garcia-Guzman et al., 1996;Bo et al., 2000;Jensik et al., 2001). In HEK293 cells expressing human $P2X_5$, PPADS (pIC_{50} = 6.7) and suramin (pIC_{50} = 5.5) are moderately potent antagonists but TNP-ATP is barely effective (1 μM producing 11% inhibition) (Bo et al., 2003a). $P2X_5$-mediated inward currents are also reduced in an acidic extracellular environment (pH ≤ 6.5), but basic conditions have no effect (Wildman et al., 2002). The only ion shown to inhibit currents through $P2X_5$ channels is calcium, which exhibits a half-maximal effect at an extracellular concentration of 6.7 mM (Haines et al., 1999).

No selective antagonists of the $P2X_{1/5}$ channel have been described, so it is difficult to distinguish this channel from other P2X channels on the basis of antagonist potencies. PPADS and suramin block ATP-evoked currents through recombinant rat $P2X_{1/5}$ channels with potencies (pIC_{50} = 6.2 and 5.8, respectively) similar to those seen using recombinant rat homomeric $P2X_1$ and $P2X_5$ channels (Bianchi et al., 1999;Haines et al., 1999). However, the potency of TNP-ATP (pIC_{50}s range from 6.1 to 7.2) is intermediate between $P2X_1$ (pIC_{50} = 8.2) and $P2X_5$ (pIC_{50} < 5) (Virginio et al., 1998b;Haines et al., 1999;Le et al., 1999;Surprenant et al., 2000;Bo et al., 2003a). In fact, TNP-ATP may be a weak partial agonist at the rat $P2X_{1/5}$ channel (Surprenant et al., 2000). Also, unlike either $P2X_1$ or $P2X_5$ (and in common only with the homomeric $P2X_7$ channel), both low (6.3) and high (8.3) pH reduce ATP-evoked currents through $P2X_{1/5}$ channels, whereas only low pH has been reported to inhibit currents through $P2X_1$ or $P2X_5$ homomers (Stoop et al., 1997;Surprenant et al., 2000;Wildman et al., 2002).

In summary, homomeric $P2X_5$ channels can be distinguished from the other rapidly activating, slowly desensitizing, α,β-MeATP-insensitive P2X channels (e.g. $P2X_2$ and $P2X_4$) primarily on the basis of differential modulation by pH and sensitivity to potentiation by ivermectin ($P2X_4$ only). Recombinant heteromeric $P2X_{1/5}$ channels behave in some respects like $P2X_1$ (agonist activity and lack of pore dilation), and in other respects like $P2X_5$ (calcium permeability and presence of sustained current), but in many essential ways they are unique (sensitivity to TNP-

ATP and pH and kinetic response). The physiological relevance of the heteromeric $P2X_{1/5}$ channel is unknown. However, it has been hypothesized that $P2X_{1/5}$ may mediate excitatory junction potentials at arterial neuroeffector junctions in guinea pig (Surprenant et al., 2000). In light of the relatively small currents through homomeric $P2X_5$ channels and the fairly widespread distribution of mRNA and immunoreactivity for $P2X_5$ (see above), it seems reasonable that $P2X_5$ may function in some tissues in heteromeric form. For example, functional evidence for the expression of heteromeric $P2X_{1/5}$ channels in mouse cortical astrocytes has been published and may be an important part of ATP-mediated signal transduction in glial networks (Lalo et al., 2008).

Homomeric $P2X_6$ and heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels

Key Messages

1. $P2X_6$ is present throughout the CNS where it often colocalizes with $P2X_2$ and/or $P2X_4$.
2. $P2X_6$ does not form functional homomeric channels without extensive glycosylation, at which point they can be activated by α,β-MeATP and blocked by TNP-ATP.
3. Heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels retain many characteristics of homomeric $P2X_2$ and $P2X_4$, respectively, and it is difficult to distinguish between these channels.
4. Homomeric $P2X_6$ channels differ from heteromeric channels containing $P2X_6$ subunits on the basis of sensitivity to α,β-MeATP, pH, ivermectin and/or antagonists such as TNP-ATP, PPADS, and suramin.

Localization and Function of $P2X_6$, $P2X_{2/6}$ and $P2X_{4/6}$ Channels

$P2X_6$ mRNA expression and immunoreactivity are expressed throughout the CNS, particularly in portions of the cerebellum (Purkinje cells) and hippocampus (pyramidal cells) (Collo et al., 1996;Norenberg and Illes, 2000;Bobanovic et al., 2002;Burnstock and Knight, 2004;Xiang and Burnstock, 2005;Rubio and Soto, 2001). Additionally, expression of $P2X_6$ has been reported in sensory ganglia (Xiang et al., 1998), thymus (Glass et al., 2000), skeletal muscle (Meyer et al., 1999;Ryten et al.,

2001), gland cells of the uterus, granulose cells of the ovary and bronchial epithelia (Collo et al., 1996), and human salivary gland epithelial cells (Worthington et al., 1999). Recently, $P2X_6$ was shown to be the only P2X subtype to be upregulated in human heart tissue (cardiac fibroblasts and in a cardiomyocyte-enriched cell population) from patients with congestive heart failure (CHF) compared to normal human hearts (Banfi et al., 2005). Since $P2X_6$ does not form functional homomeric channels under most circumstances, it has been hypothesized that $P2X_6$ functions *in vivo* primarily as a heteromeric channel in combination with other P2X subunits known to be expressed in the same regions (e.g. $P2X_2$ and $P2X_4$).

Activation of $P2X_6$, $P2X_{2/6}$ and $P2X_{4/6}$ Channels

Until recently, $P2X_6$ was thought to be largely incapable of forming functional homomeric channels when expressed in either oocytes or HEK293 cells (Collo et al., 1996;Le et al., 1998;Khakh et al., 1999b), primarily due to a failure to even form homo-oligomers (Torres et al., 1999;Barrera et al., 2005). One study found that $P2X_6$ was retained in the endoplasmic reticulum of oocytes as tetramers and high molecular mass aggregates, and failed to be exported to the membrane surface (Aschrafi et al., 2004). However, recent data suggests that non-functional $P2X_6$ channels can be expressed on the plasma membrane of HEK293 cells if they are partially glycosylated, and that further glycosylation leads to a functional homomeric $P2X_6$ channel (Jones et al., 2004). In this case, the rat $P2X_6$ channel can be differentiated from $P2X_2$ or $P2X_4$ by an increased sensitivity to activation by ATP (pEC_{50} = 6.3 at $P2X_6$; 5.3 and 4.5 at $P2X_4$ and $P2X_2$, respectively) and α,β-MeATP (pEC_{50} = 6.2 at $P2X_6$; < 4.5 at $P2X_4$ and $P2X_2$) (Brake et al., 1994;Evans et al., 1995;Jones et al., 2000;Jones et al., 2004). ATP induced rapid inward currents through rat $P2X_6$ channels, but the rate of current decay after agonist was removed was significantly slower than the current decay through $P2X_{2/3}$ channels expressed in the same HEK293 cell line (Jones et al., 2004).

When co-expressed with $P2X_2$ or $P2X_4$ in oocytes, $P2X_6$ can also form heteromeric $P2X_{2/6}$ or $P2X_{4/6}$ channels, respectively (Le et al., 1998;King et al., 2000). The heteromeric $P2X_{4/6}$ channel is pharmacologically similar to the homomeric $P2X_4$ channel, and may differ only slightly in the potencies of 2-MeSATP (pEC_{50} = 5.1 at rat $P2X_{4/6}$; pEC_{50} = 4.6 at rat $P2X_4$) and α,β-MeATP (pEC_{50} = 4.9 at rat $P2X_{4/6}$; pEC_{50}

= 4.3 at rat $P2X_4$), but not ATP (pEC_{50} = 5.4 at rat $P2X_{4/6}$; pEC_{50} = 5.2 at rat $P2X_4$) (Le et al., 1998). ATP-evoked currents in oocytes expressing $P2X_{4/6}$ or $P2X_4$ channels behave virtually identically in the presence of 10 μM Zn^{2+} where currents are potentiated by a factor of 1.8, or under basic conditions, where at pH 8.0 currents are slightly increased to 121 and 106% of pH 7.5 control responses for $P2X_{4/6}$ and $P2X_4$, respectively. As with the homomeric $P2X_4$ channel, ivermectin marginally potentiates agonist-evoked currents in oocytes expressing $P2X_{4/6}$ channels, shifting the pEC_{50} of α,β-MeATP from 4.6 to 4.8 in the presence of 3 μM ivermectin (Khakh et al., 1999b). Similarly, the heteromeric $P2X_{2/6}$ and homomeric $P2X_2$ channels are also virtually identical in their rank order of agonist activation (ATP = ATP-γ-S = 2-MeSATP>>BzATP, α,β-MeATP, β,γ-MeATP, ADP, Ap_nA), and when expressed in oocytes they were similarly responsive to ATP (pEC_{50} = 4.7 and 4.5 for $P2X_2$ and $P2X_{2/6}$, respectively) (King et al., 2000). Both heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels differ from the homomeric $P2X_6$ channel primarily by their significantly lower sensitivity to α,β-MeATP, and by the greater sensitivity to pH ($P2X_{2/6}$) or ivermectin ($P2X_{4/6}$) imparted by the other P2X subunits comprising the heteromeric channel.

Inhibition of $P2X_6$, $P2X_{2/6}$ and $P2X_{4/6}$ Channels

ATP-evoked currents through the functional glycosylated homomeric $P2X_6$ channel can be blocked by TNP-ATP (pIC_{50} = 6.1) and PPADS (pIC_{50} = 6.1), but not suramin (27% reduction at 100 μM) (Jones et al., 2004). The sensitivity to inhibition by TNP-ATP and PPADS is in marked contrast to the heteromeric $P2X_{4/6}$ channels which, like the homomeric $P2X_4$ channel, is relatively insensitive to inhibition by 10 μM PPADS (38% inhibition), suramin (41% inhibition) or reactive blue-2 (26% inhibition but >45% potentiation in rat $P2X_4$) (Le et al., 1998). The heteromeric $P2X_{2/6}$ channel is similarly sensitive to inhibition by suramin (pIC_{50} = 5.2) as the homomeric $P2X_2$ channel (pIC_{50} = 5.0), but more sensitive than the homomeric $P2X_6$ channel (see above) (King et al., 2000). However $P2X_2$ and $P2X_{2/6}$ channels can be distinguished on the basis of their differing responses to activation under acidic conditions. Under moderately acidic conditions (pH 6.5), the potency of ATP at both $P2X_2$ and $P2X_{2/6}$ channels increases relative to responses evoked at pH 7.5 (from pEC_{50} = 4.8 to 5.9 at

$P2X_2$; from 4.5 to 5.1 at $P2X_{2/6}$). Under more strongly acidic conditions (pH 5.5) the potency of ATP at $P2X_2$ increases further (to pEC_{50} = 6.3) with no change in the maximal response, whereas at $P2X_{2/6}$ the maximal ATP-evoked response is dramatically decreased (76% reduction) (King et al., 2000).

To summarize, the homomeric $P2X_6$ channel differs from the heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels primarily on the basis of their relative sensitivities to α,β-MeATP, pH and/or ivermectin, and additionally by their differing sensitivity to inhibition by TNP-ATP, PPADS and suramin. The differences are more subtle between the heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels than between the homomeric $P2X_2$ and $P2X_4$, but a potential way to distinguish them is on the basis of different responses to pH (at pH 5.5, maximal response to ATP unaffected at $P2X_2$ but reduced at $P2X_{2/6}$) or reactive blue-2 (potentiates $P2X_4$ but slightly inhibits $P2X_{4/6}$).

Homomeric $P2X_7$ channels

Key Messages

1. $P2X_7$ channels are predominantly localized on immune cells and glia, where they mediate proinflammatory cytokine release, cell proliferation and apoptosis.
2. $P2X_7$ protein subunits form only homomeric channels, and activation requires unusually high concentrations of agonist.
3. $P2X_7$ channels allow passage of larger molecular weight molecules upon prolonged agonist exposure.
4. Potent and selective antagonists, some with drug-like properties, have been identified in recent years.

Localization and Function of $P2X_7$ Channels

The $P2X_7$ receptor, formerly known as the cytolytic P2Z receptor (Blanchard et al., 1995;Falzoni et al., 1995;Baricordi et al., 1996), is predominantly expressed on cells of the immune system such as macrophages/monocytes, dendritic cells, lymphocytes, and mast cells, as well as on various types of glia within the peripheral and central nervous system, including microglia, astrocytes, oligodendrocytes, and Schwann cells (Collo et al., 1997;Rassendren et al., 1997;Buell et al., 1998;Di

Virgilio et al., 2001;Franke et al., 2001;Burnstock and Knight, 2004;Bulanova et al., 2005;Chessell et al., 2005). $P2X_7$ receptors are also expressed on epithelial cells, fibroblasts, osteoblasts, and some neuronal populations (Groschel-Stewart et al., 1999;Solini et al., 1999;Deuchars et al., 2001;Gartland et al., 2001;Sim et al., 2004).

Activation of the $P2X_7$ channel has been associated with multiple cellular functions (Watters et al., 2001;North, 2002). However, it is best characterized for its role in mediating the processing and release of mature, biologically active interleukin-1β (IL-1β) and interleukin-18 (IL-18) from immune cells and glia (Ferrari et al., 1997;Grahames et al., 1999;Perregaux et al., 2000;Mehta et al., 2001;Chakfe et al., 2002;Rampe et al., 2004; Ferrari et al., 2006; Qu et al., 2007; Wewers and Sarkar, 2009). Macrophages and microglia pretreated with the $P2X_7$ receptor antagonists KN-62 or periodate-oxidized ATP (oATP) (see below), or from $P2X_7$-deficient mice, fail to release IL-1β when challenged with ATP or BzATP (Grahames et al., 1999;Mehta et al., 2001;Solle et al., 2001;Rampe et al., 2004). $P2X_7$-mediated IL-1β release may be mediated in part through interactions with the pannexon channel (Pelegrin and Surprenant, 2006) although there appears to be a mechanism of $P2X_7$-mediated cytokine release independent of pannexin (Pelegrin et al., 2008; Pelegrin et al., 2009). Consistent with this, $P2X_7$-deficient mice have a decreased incidence and severity of disease in a model of monoclonal anti-collagen-induced arthritis (Labasi et al., 2002), and deficits in models of chronic inflammatory and neuropathic pain (Chessell et al., 2005; Hughes et al., 2007). A role for $P2X_7$ in neurodegeneration and CNS inflammation has also been postulated based on its role in cytokine, reactive oxygen species, and neurotransmitter release from microglia and astrocytes, induction of cell death, and its upregulation around β-amyloid plaques in a transgenic mouse model of Alzheimer's disease (Chakfe et al., 2002;Le Feuvre et al., 2002;Duan et al., 2003;Parvathenani et al., 2003). Priming of macrophages or microglia with β-amyloid peptide is a potent co-stimulus for $P2X_7$-mediated cytokine release (Rampe et al., 2004), and $P2X_7$ channels appear to play a role in microglial-dependent neurotoxicity in a rat co-culture system of microglia and embryonic cortical neurons (Skaper et al., 2005). The role of $P2X_7$ has also been investigated in models of spinal cord injury or cerebral ischemia to further assess the role of $P2X_7$ in neurodegeneration and cell death (Le Feuvre et al., 2003;Wang et al., 2004). Most

recently, $P2X_7^{-/-}$ mice exhibited an anti-depressant like profile in several behavioral models of depression, possibly indirectly through suppression of pro-inflammatory cytokines or directly through neurochemical changes in the central nervous system (Basso et al., 2009).

$P2X_7$ channels are also expressed on osteoblasts and osteoclasts (Gartland et al., 2001;Naemsch et al., 2001;Jorgensen et al., 2002), but the physiological role of $P2X_7$ channels in bone development and remodeling is not entirely clear. $P2X_7$ does not appear to be critical for multinucleated osteoclast formation (Gartland et al., 2003a;Gartland et al., 2003b;Li et al., 2005), and one recent study has suggested that $P2X_7$ channels may be important for osteoblastic responses to mechanical loading (Li et al., 2005) as opposed to early suggestions of $P2X_7$-mediated osteoblast apoptosis (Gartland et al., 2001). In addition, studies of bone formation and resorption in two different strains of $P2X_7$-deficient mice have generated conflicting results, with one study demonstrating a phenotype of suppressed periosteal bone formation and excessive trabecular bone resorption (Ke et al., 2003), and the other showing no skeletal alterations (Gartland et al., 2003b).

A recent finding that should be mentioned is that $P2X_7$ has been shown to form a heteromeric receptor for the first time (with $P2X_4$), combining characteristics of both receptor subtypes, but it is still unclear if this is an important functional receptor in native cells (Guo et al., 2007).

Activation of $P2X_7$ Channels

$P2X_7$ channels are the least sensitive among P2X channels to activation by nucleotides. It has generally been established that BzATP is the most potent agonist at the rat $P2X_7$ channel (pEC_{50} = 5.2-5.7). BzATP is ~10- to 30-fold more potent than ATP (pEC_{50} = 3.7-4.1) when measuring inward currents in recombinantly expressed $P2X_7$ channels, while other common P2X agonists such as 2-MeSATP, ATP-γ-S, α,β-MeATP and β,γ-MeATP are even less potent or inactive altogether (Surprenant et al., 1996;Rassendren et al., 1997;Chessell et al., 1998c;Hibell et al., 2000). By comparison, ATP has typically been reported to be 10- to 100-fold more potent at the other homomeric P2X channels (Bo et al., 1995;Evans et al., 1995;Collo et al., 1996;Bianchi et al., 1999). However, $P2X_7$ channels do show species differences in

agonist potencies. BzATP at concentrations of 10-30 μM can evoke maximal inward currents or Ba^{2+} influx through rat $P2X_7$ channels, whereas at least 10-fold higher concentrations are required to evoke similar responses through human or mouse $P2X_7$ channels (Wiley et al., 1994;Rassendren et al., 1997;Hibell et al., 2000;Duan et al., 2003). In one study measuring inward currents through native $P2X_7$ channels in mouse NTW8 microglial cells, or through recombinant rat, human, or mouse $P2X_7$ channels expressed in HEK293 cells under identical conditions, the pEC_{50}s for BzATP were 5.7 (rat $P2X_7$), 4.3 (human $P2X_7$), 4.0 (mouse $P2X_7$) and 4.2 (NTW8) (Chessell et al., 1998a).

The $P2X_7$ protein is unique in the P2X receptor family for having an unusually long intracellular C-terminus, increased in length by approximately 120-200 amino acids (Suprenant et al., 1996). The C-terminus of $P2X_7$ appears to interact with several intracellular and membrane-bound proteins, including cytoskeletal elements, heat shock proteins and enzymes (Kim et al., 2001; Wilson et al., 2002), and specific basic amino acid residues, Arg^{578} and Lys^{570}, are putative lipopolysaccharide binding sites linked to inflammatory mediator production in macrophages (Denlinger et al., 2001; Denlinger et al., 2003). The C-terminus is also essential for proper channel gating as channels constructed from carboxy tail-truncated $hP2X_7$ protein subunits ($P2X_7^{1-436}$) resulted in currents only 5% of wild type currents ($P2X_7^{1-595}$; Becker et al., 2008). Furthermore, the currents through the truncated $P2X_7$ could be recovered by simply expressing the missing section of the C-terminus as a discrete protein, suggesting that there are important functional interactions between the C-terminus and the rest of the $P2X_7$ protein (Becker et al., 2008). Accordingly, the C-terminus may be an important site of action for negative modulators of $P2X_7$ channel function.

$P2X_7$ was also the first P2X channel that was shown to allow passage of larger molecular weight (≤ 900 Da) molecules, such as the fluorescent dyes YO-PRO-1 and ethidium bromide, after prolonged exposure to agonist (Surprenant et al., 1996). This phenomenon may occur by dilation of the channel pore(Yan et al., 2008), although this has recently become somewhat controversial (North, 2002;Liang and Schwiebert, 2005). It has been shown that pore formation and dye uptake in mouse macrophages involves second messengers such as Ca^{2+} and MAP kinases (Faria et al., 2005), and in rat retinal microvascular cells, activation of $P2Y_4$ inhibits $P2X_7$-mediated pore

formation (Sugiyama et al., 2005). Additionally, either alteration of the extracellular sodium concentration or deletion of an 18-amino acid domain in the C-terminus of rat $P2X_7$ subunits expressed in HEK293 cells, resulted in markedly different permeabilities to NMDG and YO-PRO-1. These studies suggested that these molecules enter the cell through different pathways, and the authors concluded that NMDG probably enters through a pore intrinsic to the channel whereas YO-PRO-1 most likely enters through a distinct, non-$P2X_7$ related pore (Jiang et al., 2005a), quite possibly the pannexon channel (Pelegrin and Surprenant, 2006). Although the mechanism(s) of pore dilation are still unclear, BzATP tends to be more potent at evoking intracellular YO-PRO-1 accumulation than inward currents, with pEC_{50}s ranging from 6.6-7.1 at rat $P2X_7$, 6.0-6.3 at human $P2X_7$ and 4.7-4.9 at mouse $P2X_7$, again most potent at the rat ortholog (Chessell et al., 1998c;Hibell et al., 2000;Michel et al., 2000;Hibell et al., 2001).

Inhibition of $P2X_7$ Channels

As with most of the other homomeric and heteromeric P2X channels, PPADS is an inhibitor of rat, human, and mouse $P2X_7$-mediated inward currents and Ca^{2+} influx with moderate, variable potencies (pIC50 = 4.2-6.0) (Surprenant et al., 1996;Rassendren et al., 1997;Chessell et al., 1998b;Bianchi et al., 1999;Duan et al., 2003). However, PPADS may be a more potent antagonist of BzATP-stimulated YO-PRO-1 accumulation, with reported pIC_{50}s of 7.8-7.9 and 6.9-7.1 in HEK293 cells expressing human and rat $P2X_7$, respectively (Chessell et al., 1998c;Hibell et al., 2001). Interestingly, in the same studies, the mouse $P2X_7$ channel was significantly less sensitive to PPADS (pIC_{50} = 5.0-5.2) (Chessell et al., 1998c;Hibell et al., 2001). Suramin, another non-selective P2X (and P2Y) antagonist, has been reported to be a weak or inactive antagonist ($pIC_{50} \leq 4.1$) at $P2X_7$ channels of all species tested (Surprenant et al., 1996;Bianchi et al., 1999;Duan et al., 2003). Oxidized ATP is an irreversible antagonist of $P2X_7$-mediated fluorescent dye uptake, but it requires long incubation times (1 to 3 hours) and high concentrations (100-300 μM) to be effective (Murgia et al., 1993;Surprenant et al., 1996;Michel et al., 2000;Hibell et al., 2001). However, oATP may have utility for exploring the mechanism of action of various antagonists. For example, pre-incubation of HEK293 cells expressing human $P2X_7$

with either PPADS or suramin attenuated the irreversible antagonism of oATP, supporting the notion that these agents may be acting at the ATP binding site or a site that excludes this binding (Michel et al., 2000). On the other hand, in curve shift experiments, increasing concentrations of PPADS results in a significant suppression of the BzATP concentration-response curve maxima, suggesting that it may be behaving as a non-competitive antagonist of the $P2X_7$ channel (Chessell et al., 1998a;Michel et al., 2000). However, this finding could also be explained by inadequate agonist-antagonist equilibrium at the receptor since PPADS is known to be very slowly reversible (Chessell et al., 1998a;Michel et al., 2000). Brilliant Blue G has been reported to be a $P2X_7$-selective antagonist of agonist-evoked inward currents in recombinant cell lines with pIC_{50}s of 8.0 and 6.6 at rat and human $P2X_7$ channels, respectively, compared to pIC_{50}s of 5.9 (rat $P2X_2$), 5.5 (human $P2X_4$) or > 5.3 (rat $P2X_4$, rat $P2X_1$, human $P2X_1$, human $P2X_3$, rat $P2X_{2/3}$ and human $P2X_{1/5}$) (Jiang et al., 2000a).

Another class of $P2X_7$ antagonists are the large cationic inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), including calmidazolium, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), and related compounds. Calmidazolium inhibits BzATP-evoked inward currents, but not YO-PRO-1 accumulation, in HEK293 cells expressing rat $P2X_7$ with a pIC_{50} of 7.9 (Virginio et al., 1997), and has also been reported to inhibit inward currents through human $P2X_7$ (Chessell et al., 1998a). KN-62 is among the most potent inhibitors of both inward currents and fluorescent dye uptake through human (pIC_{50} = 7.0-8.0) and mouse (pIC_{50} = 7.0-8.0) $P2X_7$ channels (pIC_{50} = 6.7), but is inactive at rat $P2X_7$ (pIC_{50} < 5.5) (Gargett and Wiley, 1997;Chessell et al., 1998b;Chessell et al., 1998a;Humphreys et al., 1998;Hibell et al., 2001;Baraldi et al., 2003). Although KN-62 is an inhibitor of CaMKII, a closely related compound, KN-04, also potently inhibits $P2X_7$-mediated Ba^{2+} uptake and ethidium influx but is inactive at CaMKII, thereby suggesting that these compounds do not inhibit $P2X_7$ function through the involvement of CaMKII (Gargett and Wiley, 1997;Humphreys et al., 1998). Many synthetic analogues of KN-62 have been tested, with the most potent being the fluoride derivative of KN-62 with a pIC_{50} of 8.9, almost 40-fold more potent than KN-62 in the same study (Baraldi et al., 2003).

$P2X_7$ channels are also very sensitive to their extracellular ionic environment. BzATP-evoked inward currents and YO-PRO-1 uptake have been shown to increase when extracellular concentrations of either monovalent or divalent cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) or anions (Cl^-) are decreased (Rassendren et al., 1997;Virginio et al., 1997;Chessell et al., 1998a;Michel et al., 1999;Gudipaty et al., 2001; Jiang 2009). The most potent negative modulator of BzATP-evoked inward currents through rat $P2X_7$ among the divalent cations is Cu^{2+} (pIC_{50} = 6.3), followed by Zn^{2+} (pIC_{50} = 5.0), Mg^{2+} (pIC_{50} = 3.3) and Ca^{2+} (pIC_{50} = 2.5) (Virginio et al., 1997), although as shown for $P2X_2$(Tittle and Hume, 2008), the effects of Zn^{2+} is species- and agonist-specific(Moore and MacKenzie, 2008). Site-directed mutagenesis studies suggest that Zn^{2+} and Cu^{2+} probably interact at specific residues in the ectodomain, His^{62} and Asp^{197} (Liu et al., 2008). Both acidic and basic conditions inhibit $P2X_7$-mediated inward currents (Virginio et al., 1997;Michel et al., 1999), but increasing the pH from 5.5 to 9.0 resulted in a progressive increase in the maximum YO-PRO accumulation in HEK293 cells expressing human $P2X_7$ (Michel et al., 1999).

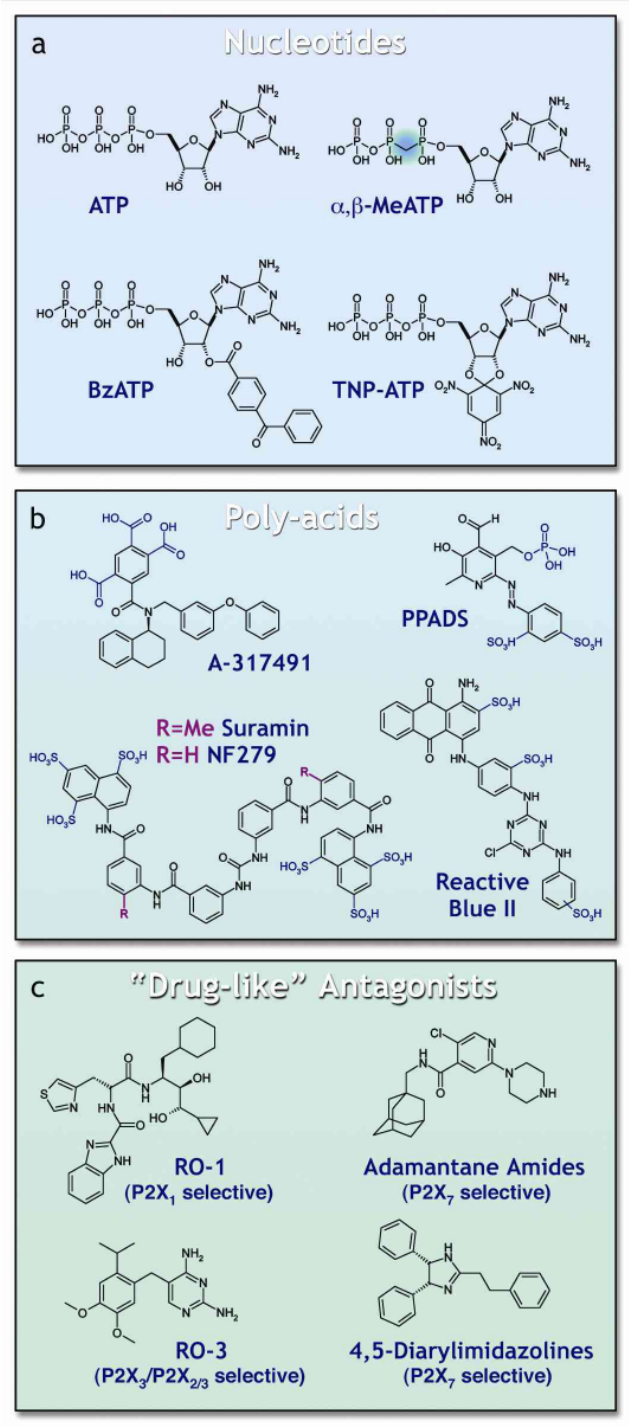

Figure 2. Commonly used P2X agonists and antagonists a) Nucleotides related to the structure of ATP: ATP, α,β-MeATP and BzATP are agonists, TNP-ATP is an antagonist. b) Antagonists with multiple acidic functional groups imparting poor *in vivo* pharmacokinetic properties c) Selective antagonists with improved "drug-like" properties (e.g. oral bioavailability, improved metabolic stability).

Recent Advances

In recent years some of the most significant advances in purinergic pharmacology have been in the development of more potent and selective antagonists at certain P2X receptor subtypes, most notably $P2X_1$, $P2X_3$, $P2X_{2/3}$, and $P2X_7$. Some of these advances are limited to increases in potency and selectivity and not related to improving the other physicochemical characteristics required for a molecule to be advanced as a medicinal candidate. For example, suramin analogues with extremely high potency and selectivity for $P2X_1$-containing channels have been described in recent years. NF449 has pIC_{50}s of 9.5 and 9.2 (>3000-fold more potent than suramin) at rat $P2X_1$ and $P2X_{1/5}$, respectively (expressed in oocytes) with 400-1,000,000-fold selectivity over rat $P2X_2$, $P2X_{2/3}$, $P2X_3$, and $P2X_4$ (Rettinger et al., 2005). NF864 has been shown to inhibit α,β-MeATP-evoked human platelet shape change and intracellular calcium increase with pA_2 estimates of 8.49 and 8.17, respectively; approximately 5-7 fold more potent than NF449 and 200-540-fold more potent than suramin (Horner et al., 2005). Although these compounds are potentially very useful as *in vitro* tools, their utility *in vivo* would be expected to be limited by poor pharmacokinetic properties.

All of the antagonists described above and in previous sections are either nucleotides that are highly acidic and rapidly degraded, or large polyanionic molecules (suramin, dyes, PPADS). None of these are ideal starting points for medicinal optimization. However, one report has described a small molecule $P2X_1$ antagonist with drug-like properties, RO-1, derived from the optimization of dipeptide compounds synthesized originally as potential renin inhibitors (Jaime-Figueroa et al., 2005). Although it is moderately potent (pIC_{50} = 5.5 at human $P2X_1$), it is selective over other homomeric and heteromeric P2X receptors (pIC_{50} < 4 at $P2X_2$, $P2X_3$ and $P2X_{2/3}$) and effectively reduces rat detrusor smooth muscle contractions evoked by β,γ-MeATP or electrical field stimulation (Gever et al., 2004;Ford et al., 2006).

In 2002, data were published for the first time on a selective $P2X_3$/$P2X_{2/3}$ "small molecule" antagonist from Abbott, A-317491 (Jarvis et al., 2002); a tricarboxylic acid identified from random screening and originally patented in racemic form as an inhibitor of squalene synthetase and protein farnesyltransferase. Activation of recombinant and native $P2X_3$ and $P2X_{2/3}$ channels was inhibited by

submicromolar concentrations of A-317491 (in human clones, pIC_{50} = 7.0 at $P2X_3$, 6.8 at $P2X_{2/3}$, 5.0 at $P2X_1$, 4.3 at $P2X_2$, < 4 at $P2X_4$ and $P2X_7$), and antinociceptive efficacy was demonstrated in several rodent models of chronic inflammatory and neuropathic pain. A-317491 was later shown to inhibit ATP-evoked Ca^{2+} influx through slowly-desensitizing chimeric human $P2X_{2-3}$ channels (see above) in a manner consistent with competitive antagonism (pA_2 = 7.3) (Neelands et al., 2003). Long plasma half-life and demonstrated usefulness in some *in vivo* models make this a significant advance in this area. However, the poor pharmacokinetic properties of A-317491 (poor oral bioavailability, high protein binding, and poor tissue distribution) would likely make it unattractive for medicinal development.

More recently, the identification of a series of $P2X_3$/$P2X_{2/3}$ antagonists structurally related to the diaminopyrimidine antibacterial drug trimethoprim, exemplified by RO-3 (see Figure 2), represents a step toward discovery of drug-like P2X antagonists (Ford et al., 2006). RO-3 is a potent inhibitor of human homomeric $P2X_3$ (pIC_{50} = 7.0) and heteromeric $P2X_{2/3}$ (pIC_{50} = 5.9) channels with selectivity over other P2X channels (pIC_{50} < 5 at $P2X_{1,2,4,5,7}$). Furthermore, RO-3 has moderate to high metabolic stability in rat and human hepatocytes and liver microsomes, and is highly permeable, orally bioavailable (14%), and has a reasonable *in vivo* plasma half-life ($t_{1/2}$ = 0.41 h) in rats. The synthesis of other chemical analogues with improved drug-like properties has more recently been described (Carter et al., 2009; Jahangir et al., 2009).

An interesting recent finding is the discovery that spinorphin, an endogenous anti-nociceptive peptide, can block ATP-evoked inward currents in oocytes expressing human $P2X_3$ with low picomolar potency (Jung et al., 2007). Also, surprisingly, a bisphosphonate developed for the treatment of osteoporosis, minodronic acid, is a $P2X_{2/3}$ antagonist, though not very potent (IC_{50} = 62.7 μM of α,β -MeATP-induced [^{14}C]-guanidine hydrochloride in CHO cells expressing recombinant $rP2X_{2/3)}$ and was shown to be effective in several models of inflammatory pain (Kakimoto et al., 2008).

Several chemical series of $P2X_7$ antagonists with improved "drug-like" properties have also been reported (Guile et al., 2009; Gunosewoyo et al., 2009). Aventis and AstraZeneca have published the syntheses of 4,5-diarylimidazolines (the

most potent having a pIC_{50} of 8.0 versus BzATP-evoked YO-PRO-1 influx) and cyclic imides (the most potent having a pA_2 of 7.7 versus BzATP-evoked ethidium influx), respectively; selectivity or mechanism of action data were not provided in either case (Merriman et al., 2005;Alcaraz et al., 2003). Subsequent to the publication of the first synthesis of cyclic imide $P2X_7$ antagonists, a more detailed characterization was described of AZ11645373, a non- competitive, potent and selective cyclic imide $P2X_7$ antagonist with low nanomolar affinity for the human, but not the rat, $P2X_7$ receptor (Stokes et al., 2006). Another class of $P2X_7$ antagonists reported by AstraZeneca is based on a series of adamantanes with affinity estimates (pA_2) as high as 8.8 (Baxter et al., 2003). The adamantane chemical series of $P2X_7$ antagonists was initially plagued with poor metabolic characteristics (high rat hepatocyte and human microsomal clearance) but this was reportedly overcome by the synthesis of an indazole amide derivative which was deemed suitable for further lead optimization (Baxter et al., 2003). Further optimization to identify less lipophilic compounds with greater *in vitro* metabolic stability as well as chemical analogues with potency at rodent $P2X_7$ to facilitate *in vivo* studies resulted in improved adamantine-based antagonists (Furber et al., 2007). In fact, AstraZeneca have advanced a $P2X_7$ antagonist, AZD9056, into Phase II clinical trials for rheumatoid arthritis although neither the structure nor the efficacy of this compound in humans has been announced up to the time of this writing. Not to be left out, Neurogen has recently published the characterization of their own adamantine small molecule inhibitor of $P2X_7$ (AACBA or GSK314181A), showing efficacy in acute *in vivo* models of pain and inflammation but no efficacy in animal models of arthritic or neuropathic pain unless the drug was administered prophylactically(Broom et al., 2008). Abbott has recently published data showing the preclinical efficacy of a $P2X_7$ antagonist, A-740003, in rodent models of neuropathic pain (Jarvis et al., 2005;Honore et al., 2006). A-740003 is reported to be a selective, competitive antagonist of agonist-evoked intracellular calcium flux with affinity estimates (pK_i) of 7.7, 8.0 and 6.8 at recombinant human, rat and mouse $P2X_7$ channels. This compound was also reported to reduce nociception in models of neuropathic pain produced by spinal nerve ligation (ED_{50} = 41 μmol/kg, i.p.), chronic constriction injury of the sciatic nerve (54% effect at 300 μmol/kg, i.p.), and vincristine-induced neuropathy

(51% reduction at 300 μmol/kg, i.p.) (Jarvis et al., 2005) and reduced thermal hyperalgesia following intraplantar administration of carrageenan or complete Freund's adjuvant (ED_{50} = 38 – 54 mg/kg, i.p.)(Honore et al., 2006). The synthesis and optimization of cyanoguanidine $P2X_7$ antagonists has been further described in additional papers published by Abbott scientists (Betschmann et al., 2008; Morytko et al., 2008). Another chemical series of P2X7 antagonists developed by Abbott are those containing tetrazole functional groups (Nelson et al., 2006), of which A-438079 is an example (McGaraughty et al., 2007). A-438079 has been reported to reduce nocifensive behaviors in three different models of neuropathic pain after intraperitoneal doses ranging from 10 to 300 μmol/kg (McGaraughty et al., 2007). Additional chemical series of $P2X_7$ antagonists discovered by Abbott derive from modifications of the tetrazoles to triazoles (Carroll et al., 2007; Florjancic et al., 2008) and the identification of *N'*-aryl carbohydrazides (Nelson et al., 2008). A series of syntheses to explore the structure-activity relationship of KN-62 analogues resulted in modest gains in potency recently (Lee et al., 2008). The same group conducted a random screen of the Korea Chemical Bank and found a novel series of iminium quaternary protoberberine alkaloid antagonists of $P2X_7$ (Lee et al., 2009); the suitability of either of these two chemical series as *in vivo* tools or as the basis for optimization to medicinal candidates is unknown.

Antagonists with improved drug-like properties have only been identified for $P2X_1$, $P2X_3$, $P2X_{2/3}$ and $P2X_7$ channels. So why is this? The most parsimonious explanation is that these channels have been more clearly linked to specific pathological conditions (e.g. platelet and smooth muscle function, nociception and inflammation), and are not as broadly localized as other P2X channels. Consequently, they may have garnered the most attention as attractive targets for drug discovery and received greater focus from screening of compound libraries. The "druggability" of the other homomeric and heteromeric P2X channels remains unknown for now.

In the decade since the seven known P2X subtypes were cloned, significant advances have been made in our understanding of their physiological roles, in part through the use of non-selective pharmacological agents in relevant animal models. As the selectivity and potency of these pharmacological tools have improved, so has our understanding of the biological function of the channels at which they act. For

example, the role of $P2X_3$ and $P2X_{2/3}$ channels in the detection of noxious stimuli through sensory neurons has been elucidated, in part, through blockade of these stimuli in animal models by selective $P2X_3/P2X_{2/3}$ antagonists (Jarvis et al., 2002;Ford et al., 2006). Similarly, preclinical experiments using selective $P2X_7$ antagonists have supported the hypothesis that this channel may have an important role in inflammatory processes (Jarvis et al., 2005). The challenge remains to advance candidate medicines targeting P2X channels through human clinical trials, and judging by recent progress we are optimistic that safe and effective medicines for the treatment of disorders involving P2X channels will be reported in the coming years.

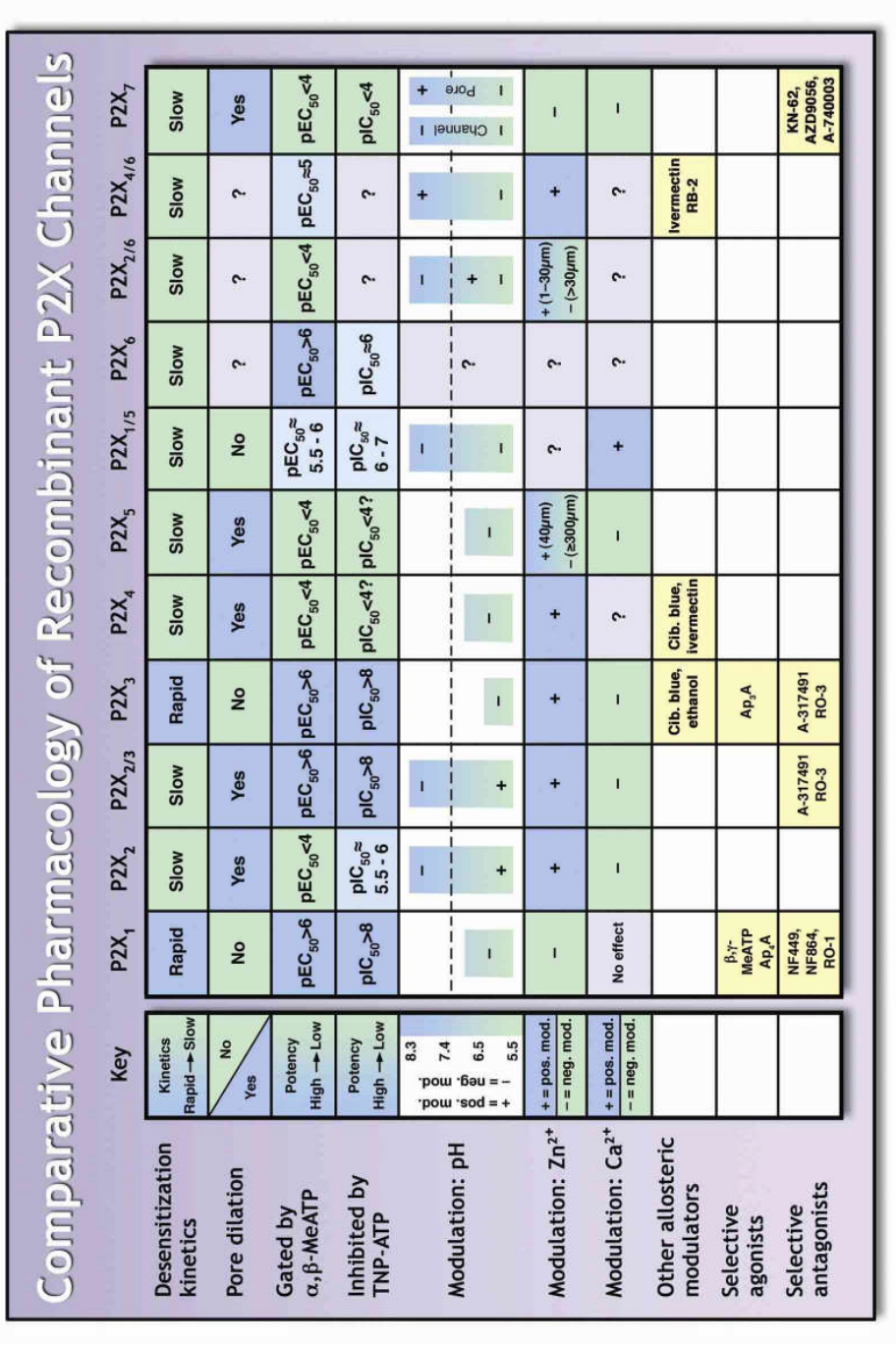

Figure 3. Comparison of pharmacological and biophysical factors distinguishing homomeric and heteromeric P2X channels. The key on the left side of the table explains the use of color: blue boxes denote more rapid desensitization kinetics, ability of the channel to dilate to a larger pore, higher potency to agonism or antagonism or positive modulation of channel activation by an extracellular ion; green boxes denote slower desensitization kinetics, the inability of the channel to dilate to a larger pore, lower potency to agonism or antagonism or negative modulation of channel activation by an extracellular ion. A "?" appears where there is no published data. See text for references.

Chapter 2: Methods

Drug substances and Materials

RO-4 (5-(5-Iodo-2-isopropyl-4-methoxy-phenoxy)-pyrimidine-2,4-diamine and RO-10 (5-(2-Isopropyl-4,5-dimethoxy-phenoxy)-pyrimidine-2,4-diamine were synthesized and characterized in the Department of Medicinal Chemistry at Roche Palo Alto. The anhydrous mono-hydrochloride salt of RO-4 is a white crystalline solid with a melting point of 260°C; in the solid state it is stable at 40°C with 75% relative humidity for at least 2 months. Solutions (2 mg/mL) and suspensions of RO-4 in acidic media are physically and chemically stable for at least 4 weeks at room temperature.

RO-1 (1*H*-Benzoimidazole-2-carboxylic acid [1-(*R*)-1-(*S*)-cyclohexylmethyl-3-cyclopropyl-2-(*R*),3(*S*)-dihydroxy-propylcarbamoyl]-2-thiazol-4-yl-ethyl)-amide) was synthesized and characterized in the Department of Medicinal Chemistry at Roche Palo Alto. RO-1 crystallizes from ether as a white solid, with a melting point of 121 – 126 °C and is stable at room temperature. See Jaime et al for further details regarding the synthesis and chemical properties of RO-1 (Jaime-Figueroa et al., 2005).

ATP, α,β-MeATP, ATP, ADP, AMP, ATPγS, adenosine, reactive blue-2, tetracycline, 1,1-dimethyl-4-phenylpiperazinium (DMPP) bulk chemicals and reagents were obtained from Sigma. TNP-ATP was from Molecular Probes Europe (Leiden, The Netherlands). Ham's F-12 Nutrient Media, Hank's Balanced Salt Solution, phosphate-buffered saline, geneticin (G418), fetal bovine serum and versene (EDTA) were obtained from Gibco (Gaithersburg, MD, USA). 2MeSATP, βγMeATP, suramin and PPADS were obtained from Research Biochemicals (Natick, MA, USA). Hygromycin was obtained from Calbiochem (La Jolla, CA, USA). Fluo 3-AM was obtained from Molecular Probes (Eugene, OR, USA). All solutions were prepared in deionized water or dimethylsulfoxide.

Cell Culture

Recombinant Cell Lines

Chinese Hamster Ovary (CHO-K1) cells stably expressing recombinant human $P2X_4$, rat $P2X_5$ or human $P2X_7$ were cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 250 μg/mL G418 (Invitrogen), 100 μg/mL hygromycin B. Expression of recombinant human $P2X_1$ and rat $P2X_3$ in CHOK-K1 cells was regulated through the use of a tetracycline-controlled transactivator (tTA) gene expression system such that cells grown in the presence of 0.1 μg/mL tetracycline did not express $P2X_1$ or $P2X_3$ protein subunits, but upon removal of tetracycline from the growth medium, abundant expression of $P2X_1$ or $P2X_3$ was achieved within 7 days (Lachnit et al., 2000). 1321N1 human astrocytoma cells expressing $hP2X_2$, $hP2X_3$ and $hP2X_{2/3}$ were cultured in a base medium of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and either 275 μg/mL G418 ($hP2X_2$), 0.25 μg/mL puromycin ($hP2X_3$) or 300 μg/mL G418 and 0.25 μg/mL puromycin ($hP2X_{2/3}$). 1321N1 astrocytoma cells expressing chimeric human $P2X_{2-3}$ were prepared as described previously (Neelands et al., 2003).

Cells Dissociated From Animal Tissues

Bladder smooth muscle from 17 day old rats was removed, cut into small pieces, and incubated in Ca^{2+} and Mg^{2+} free Hanks Balanced Salt Solution (HBSS) at 37 °C for 20 minutes. This was followed by two sequential, 15 minute long, incubation in HBSS containing 30 μM Ca^{2+}, 1.5 mg ml^{-1} collagenase (Worthington CLS 2) and 4 mg ml^{-1} dispase (Type II, Boehringer-Manheim). After the second incubation, the tissue was rinsed in HBSS and re-suspended in HBSS containing 30 μM Ca^{2+}, 5 mM Mg^{2+} and 2 mg ml^{-1} BSA. The tissue was triturated gently to release single cells. The resultant cell suspension was plated in 35 mm culture dishes for electrophysiological recording. Smooth muscle cells were used for voltage clamp electrophysiology experiments within 10 hours of dissociation.

Preparation of rat urothelial cultures has been described previously (Birder et al., 1998; Truschel, 1999; Birder, 2001). In brief, urinary bladders were rapidly excised from euthanized Sprague-Dawley rats, gently stretched (urothelial side up),

and incubated overnight in DMEM containing penicillin/streptomycin/fungizone and dispase (2.5 mg/ml, Invitrogen, Carlsbad, CA). The urothelium was then gently scraped, treated with trypsin-EDTA (0.25%, Invitrogen), and, after gentle pipetting, resuspended in serum-free keratinocyte medium (Invitrogen). The cell suspension was plated onto either collagen-coated black-walled 96-well fluorometric imaging plate reader (FLIPR) plates (30,000 cells/well) or onto collagen-coated glass coverslips (50,000 cells/coverslip) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. The majority of cultured urothelial cells was cytokeratin 17 positive (Dako, Carpentaria, CA) and regarded as from epithelial origin, as reported previously (Birder et al., 2002).

Superior cervical ganglion neurons were cultured from E18, P1, P7, and P17 rats. Post-natal rats and pregnant females were killed by inhalation of a rising concentration of CO_2 and death was confirmed by cardiac hemorrhage. Embryos were removed from pregnant females and placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK). Neonatal animals were killed by cervical dislocation followed by decapitation. Superior cervical ganglia were rapidly dissected out, and placed in L-15 medium. The ganglia were then desheathed, cut, and incubated in 4 ml Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS; Life Technologies, Bethesda, MD) with 10 mM Hepes buffer (pH 7.4) containing 1.5 mg ml^{-1} collagenase (Class II, Worthington Biochemical Corporation, UK) and 6 mg ml^{-1} bovine serum albumin (Sigma Chemical Co., Poole, UK) at 37°C for 45 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg ml^{-1} trypsin (Sigma) at 37°C for 15 min. The solution was replaced with 1 ml growth medium comprising L-15 medium supplemented with 10% bovine serum, 50 ng ml^{-1} nerve growth factor, 2 mg ml^{-1} $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 i.u. ml^{-1} penicillin, and 200 μg ml^{-1} streptomycin. The ganglia were dissociated into single neurons by gentle trituration. The cell suspension was diluted to 8 ml, and centrifuged at 160*g* for 5 min. The pellet was resuspended in 0.8 ml growth medium and plated onto 35-mm Petri dishes coated with 10 μg ml^{-1} laminin (Sigma). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and used on the following day.

Cloning and transfection

Poly (A+) RNAs were extracted from dorsal root ganglia from L4–L5 spinal levels of Sprague–Dawley rats using a Micro-FastTrack Kit (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was carried out with 1 μg of rat dorsal root ganglia poly (A+) RNA, 10 pmol of oligo (dT), and 200 units of SuperScript II (GIBCO BRL, Gaithersburg, MD, USA) followed by reverse transcription-coupled polymerase chain reaction (PCR) using oligos X3P1-2: 5'-GACTCCGCGGCTGTGAGCAGTTTCTCAGTATG and X3P2-2: 5'-TCATGAATTCTGCAGCCTAAGGGTGAGCATG. PCR was performed and the resulting PCR product contained the entire coding region of the rat P2X3 gene as well as 21 nucleotides from the 5' untranslated sequence and 42 nucleotides from the 3' untranslated sequence. This cDNA fragment was inserted into the pCRII vector (Invitrogen), fully sequenced and then digested with restriction enzymes *Sac*II and *Eco*RI, respectively. The insert was isolated and directionally cloned into the mammalian expression vector pTRE (CLONTECH Labs., Palo Alto, CA, USA) at *Sac*II and *Eco*RI sites. pTRE is a tetracycline (tet)-regulatable expression vector developed by Gossen and Bujard (1992). The resulting construct was used for transfection to generate stably transformed CHO cell lines.

To establish stably transfected cell lines, 5.0 μg of $P2X_3$-pTRE DNA and 1.0 μg of pTK-Hygromycin DNA were incubated with 5 μg of CLONfectin (CLONTECH Labs.) for 30 min and overlaid onto 10^6 CHO-K1 tTA cells for 1 h in serum-free medium. Cells were then selected in the presence of 200 μg/ml of hygromycin. Positive individual colonies were selected by PCR following propagation of cells in the absence of tetracycline. Following selection, the cells were maintained in Ham's F-12 nutrient media supplemented with 10% fetal bovine serum, G418 (250 μg/ml), hygromycin (200 μg/ml) and 1 μg/ml tetracycline at 37°C in 7% CO_2. Immunoblots were performed on cell membranes as previously reported by (Oglesby et al., 1999).

Cytosolic Calcium Measurements

FLIPR

Receptor-evoked changes in intracellular calcium were measured using Ca^{2+}-selective fluorescent dyes quantitated with a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). CHO-K1 cells (transfected with recombinant human $P2X_1$, rat $P2X_3$, human $P2X_4$, human $P2X_5$ or human $P2X_7$ receptor subunits) and 1321N1 astrocytoma cells (transfected with cloned human P2X2, human $P2X_3$ or human $P2X_{2/3}$) were passaged in flasks in commercial media containing and lacking Phenol Red (CHOK1 in Ham's F-12 and 1321N1 in DMEM; Invitrogen). 18-24 hours before the FLIPR experiment, cells were released from their flasks, centrifuged, and resuspended in nutrient medium at 2.5 x 10^5 cells/mL. The cells were aliquoted into black-walled 96-well plates at a density of 50,000 cells/well and incubated overnight in 5% CO_2 at 37°C. On the day of the experiment, cells were washed in FLIPR buffer (FLIPR buffer: calcium- and magnesium-free Hank's balanced salt solution, 10 mM HEPES, 2 mM CaCl, 2.5 mM probenecid; FB) and loaded with fluorescent dye Fluo-3 AM [2 μM final conc.]. After a 1 hour dye loading incubation at 37°C, the cells were washed and test compounds (dissolved with DMSO at 10 mM and serially diluted with FB) or vehicle were added to each well and allowed to equilibrate for 20 or 60 minutes at room temperature. The plates were then placed in the FLIPR and a baseline fluorescence measurement (excitation @ 488 nm and emission @ 510-570 nm) was obtained for 10 seconds before agonist or vehicle addition. The agonist was α,β-MeATP added to produce a final concentration ranging from 3 nM to 10 μM. Fluorescence was measured for an additional 2 minutes at 1 second intervals after agonist addition. A final addition of ionomycin (5 μM, final concentration) was made to each well of the FLIPR test plate to establish cell viability and maximum fluorescence of dye-bound cytosolic calcium. Peak fluorescence in response to the addition of α,β-MeATP (in the absence and presence of test compounds) was measured and inhibition curves generated using nonlinear regression (Prism v.4, GraphPad Software, San Diego, CA) employing a four parameter logistic equation (Y = Bottom + (Top – Bottom)/(1 + 10^(LogEC50 - X) * HillSlope). PPADS, a standard P2X antagonist, was used as a positive control.

Urothelial cells plated onto collagen-coated black-walled 96-well FLIPR plates were grown to 90% confluence and washed in FLIPR buffer, composed of Ca^{2+}/Mg^{2+}-free HBSS supplemented with HEPES (10 mM), $CaCl_2$ (2 mM), and probenecid (2.5 mM). Fluo-3 (2 µM; Molecular Probes, Eugene, OR) in FLIPR buffer (final volume = 200 µl) was added to each well and incubated for 1 h at 37°C, and the cells were washed four times with FLIPR buffer. Purinergic receptor agonists were diluted and added to additional assay plates (agonist plates) at concentrations twice those needed to construct E/[A] curves, ranging from 100 nM to 1 mM final. In studies in which antagonist profiles were studied, urothelial cells were pretreated for at least 10 min before agonist application. The agonist, antagonist, and FLIPR cell plates were placed in the FLIPR incubation chamber. A baseline fluorescence measurement (excitation wavelength 488 nm; emission wavelength 530 nm) was obtained, and reactions were started with the addition to FLIPR cell plates of 100 µl/well from the agonist plates. Fluorescence was measured for 3–5 min at 1- to 5-s intervals, with readings taken until a plateau phase was reached. Ionomycin (5 µM) was added at the end of each experiment to determine cell viability and maximum fluorescence of dye-bound cytosolic calcium.

Fura-2

Cultured rat urothelial cells (18–72 h after plating) were incubated with the fluorescent Ca^{2+} indicator fura-2-acetoxymethyl (5 µM, Molecular Probes, Eugene, OR) in HBSS containing bovine serum albumin (5 mg/ml) for 30 min at 37°C in an atmosphere containing 5% CO_2. Cells were washed in HBSS (containing in mM; 138 NaCl, 5 KCl, 0.3 KH_2PO_4, 4 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose mosmol/kgH_2O titrated to pH 7.35 with 1 N NaOH), transferred to a perfusion chamber, and mounted onto an epifluorescence microscope (Olympus IX70). In Ca^{2+}-free HBSS, the Ca^{2+} was substituted with additional NaCl (2 mM) and EGTA (0.5 mM). Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) was performed by ratiometric imaging of fura-2 at 340 and 380 nm (100 Hz), and the emitted light was monitored at 510 nm. The fluorescence ratio (F340/F380) was calculated and acquired by C-Imaging systems (Compix, Cranberry, PA), and background fluorescence was subtracted. All test agents were bath applied (flow rate = 1.5 ml/min). Thapsigargin

(Sigma-Aldrich, St. Louis, MO) and U73122 (Tocris Bioscience) were preincubated with urothelial cells for at least 10 min before agonist application. Data were obtained from at least three independent urothelial cultures and from at least four sets of experiments from each culture. Data were analyzed using Student's unpaired *t*-test and expressed as a mean percentage of the maximum response ± SE to ionomycin (5 µM).

Pharmacological Selectivity

The selectivity of RO-4 for $P2X_3$ and $P2X_{2/3}$ channels over other homomeric P2X channels was established by measuring the potency of antagonism by RO-4 of agonist-evoked intracellular calcium flux in cell lines expressing recombinant P2X channels (see above). Additionally, RO-4 was examined in two broad commercially available panels of selectivity, one covering 75 receptors, channels, enzymes and transporters (Cerep, Poitiers, France) and a second one covering more than 100 kinases (Ambit, San Diego, California, USA).

Radioligand Binding

Radioligand binding experiments were conducted in membranes derived from Chinese hamster ovary cells (CHO) expressing the rat $P2X_3$ (CHO-r$P2X_3$) ion channel using a tetracycline-off expression vector (Lachnit et al., 2000) or 1321N1 human astrocytoma cells expressing h$P2X_3$ or h$P2X_{2/3}$. Cells were harvested in 1X Versene (Invitrogen) and homogenized by a Polytron (Kinematica, Switzerland) in ice cold 50 mM Tris pH 7.4 with 1X Complete^{TM} protease inhibitor cocktail (Roche Molecular Systems, USA). Plasma membranes were isolated by a two step centrifugation. Homogenized membranes are centrifuged at 1000x gravity (g) for 15 min. at 4^{o}C. The 1000x g pellet was discarded and the supernatant was centrifuged at 43,000x g for 30 min. at 4^{o}C. The 43,000x g supernatant was discarded and the pellet was stored at -70^{o}C until assayed. . Tritium-labeled RO-4 (81.2 Ci/mmol) was synthesized by the Radiochemistry Department at Roche (Palo Alto, CA, USA); purity was confirmed by HPLC to be >97%. The ligand affinities at $P2X_3$ and $P2X_{2/3}$ membranes were determined under equilibrium binding conditions in 50 mM Tris pH 7.4. [^{3}H]-RO-4 (1.7 – 140 nM for homomeric and 1.3 – 660 nM for heteromeric ion channels) was incubated with membranes (200-350 µg.mL^{-1}) in the absence or

presence of 10 μM of an unlabelled RO-4 analogue, RO-10, (to define non-specific binding) for 2-5 hours at 22°C to determine its equilibrium dissociation affinity constant (K_d) as well as the receptor expression level (B_{max}) of the membranes. For unlabelled molecules, dissociation affinity constants (K_B) were determined by co-incubating unlabelled molecule (serially diluted over a million-fold concentration range) with [^{3}H] RO-4 (1-5 nM) and CHO-rP2X$_3$ membranes. In all cases, incubation was ended by filtration with ice cold 50 mM Tris (pH 7.4) on GF/B filters. Filters were soaked in MicroScint-20 scintillation cocktail (PerkinElmer Life Sciences, Boston, MA) for at least 3 hours prior to quantification of filter-trapped radioactivity using a Perkin Elmer TopCount plate reader. Competition binding data were analyzed by non-linear regression to a 4-parameter hyperbolic function to estimate all of max, min, Hill slope and IC_{50}; K_B estimates were calculated from observed IC_{50}s using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Affinities are presented as the mean and standard deviations determined over 2-4 repeat experiments.

Initial experiments to characterize cell lines regulated by the tet-off system employed radiolabelled ATPγS. Saturation binding isotherms were conducted by incubating membranes (5–10 μg protein) at room temperature for 90 min with 0.05–30 nM [^{35}S]ATPγS in the absence and presence of 10 μM ATPγS (200–500 μl) in 50 mM Tris, 1 mM EDTA buffer (pH 7.4). For competition experiments, displacement of 0.25 nM [^{35}S]ATPγS by various nucleotides as well as suramin, PPADS and reactive blue-2 was determined over a range of concentrations. The assays were terminated by vacuum filtration through wet, 20 mM $Na_4P_2O_7$ pretreated GF/B glass fiber filters and washed for 10 s with 4°C buffer.

Assessment of competition in the binding modes between [^{3}H] RO-4 and other unlabelled test ligands was based on the expectations of Cheng-Prusoff relationship that describes binding of two ligands in a mutually exclusive manner (Cheng and Prusoff, 1973). This relationship is described by the equation $IC_{50} = K_B \times \left(\frac{[A^*]}{K_d} + 1\right)$ where [A*] represents the radioligand concentration, K_d the equilibrium dissociation constant of A*, K_B the equilibrium dissociation constant of the unlabelled test compound, and IC_{50} is the test compound concentration that displaces 50% of the binding of A*. Radioligand binding studies were conducted using the conditions

described above by incubating CHO-rP2X$_3$ membranes with [^{3}H]-RO-4 in the absence or presence of competing agents. For this analysis, IC_{50} values were determined for each unlabeled test compound over a range of 5-8 different radioligand concentrations ([^{3}H]-RO-4 0.1 – 60 nM). The observed IC50 values were plotted as a ratio of IC_{50}/K_B vs $[A^*]/K_d$ for all concentrations of radioligand (A*) tested. From the Cheng-Prusoff equation, a competitive interaction between unlabeled ligand and [^{3}H] RO-4 will plot as a line with a slope of unity and a y-intercept of 1.

Whole Cell Voltage Clamp Electrophysiology

Standard giga-seal patch clamp technique was employed to study all channels for these experiments. The patch clamp rig consisted of the following components: Anti-vibration table (TMC), microscope (Zeiss Axiovert 100), micromanipulator (Sutter Instruments MP285), patch-clamp amplifier (Molecular Devices Axopatch 200B), digitizer (Molecular Devices Digidata 1200), drug perfusion system (Cellectricon Dynaflow ProII Perfusion system), acquisition software (Molecular Devices PClamp9). All experiments were conducted at room temperature.

For recordings, the bath solution consisted of (in mM) 147 - 155 NaCl, 2 - 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 -10 D-glucose, 10 HEPES, pH 7.4 with NaOH, 310 mOsM and the pipette intracellular solution consisted of (in mM) 120 - 130 CsF, 10 NaCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.2 with CsOH, 290mOsM (for recordings from smooth muscle cells the intracellular solution also contained 10 mM tripotassium citrate; for recordings from the initial set of experiments characterizing the tet-regulated cell lines, the intracellular solution contained, in mM, 140 K-aspartate, 20 NaCl, 5 HEPES, 10 EGTA and adjusted to pH 7.3 with KOH). Standard wall borosilicate glass electrodes (OD 1.50mm, ID 0.87mm, with filament) were pulled with a Sutter Instruments P-87 pipette puller. The average resistance of the electrodes used was 3.5 MOhm. To activate the P2X$_{2/3}$ heteromeric channel, 10 μM α,β-MeATP solution (pH adjusted with NaOH) was used. This value is approximately the EC_{50} for the channel under the conditions of the experiment. The channel was activated at a regular intervals of approximately 30 sec. for a duration of 2 sec. Test compound was added when the current from the channel was consistent for at least 3 agonist applications (about 90 sec). The block due to the compound was monitored until

equilibrium was achieved, and then compound was washout out to determine the off rate kinetics. Data were analyzed using Molecular Devices ClampFit as well as Microcal Origin.

For experiments examining the effects of RO-4, drugs were delivered using the Dynaflow Cellectricon system; for all other experiments drugs were applied rapidly through a 4- or 6-barrel manifold comprising capillaries made of fused silica coated with polyimide 250 μm internal diameter (SGE Milton Keynes, UK) connected to a single outlet made of the same tubing, which was placed about 200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs with solution flow controlled by computer driven solenoid valves. One barrel was used to apply agonist free solution to enable rapid termination of agonist application. Solution exchange measured by the change in open tip current on switching from 150 mM NaCl to 150 mM KCl solution was complete in 20 ms; however, complete exchange of solution around an intact cell was considerably slower (≤ 100 ms). Antagonists were present for 2 min before and during the reapplication of agonists.

Tissue Bath Studies

Male Sprague-Dawley rats (250-300g) were killed by increasing CO_2 according to Home Office (UK) regulations. The urinary bladder and tail artery were removed and placed in physiological saline (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$16.3, $MgSO_4$ 0.61, glucose 7.8, $CaCl_2$ 2.52, pH 7.2). Detrusor muscle strips, approximately 15 x 2 mm, and tail artery rings of approximately 4 mm in length were suspended in 10 ml organ baths containing continuously gassed (95 % O_2 / 5 % CO_2) Krebs solution and maintained at 37 ± 1°C, one end of the bladder strip or vascular ring attached to a rigid support and the other end to a FT03C force-displacement transducer. An initial tension of 1g was applied to both tissues which were allowed to equilibrate for 1 hour. Mechanical activity was recorded using the software PowerLab Chart for Windows (Version 4; ADInstruments, Australia).

Electrical field stimulation (EFS; 75 V, 0.1 msec, 1-32 Hz) for 10 seconds at 5 min intervals was applied and frequency-response curves constructed in the presence of either pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 30 μM) or prazosin (3 μM). The curves were repeated in the presence of RO-1 (0.1, 1 and 10

μM). On vascular preparations, concentration-response curves were constructed to ATP (1 μM - 1 mM) and NA (0.3 – 300 μM) in the absence and presence of RO-1. On bladder preparations, concentration-response curves to β,γ-meATP (0.3 – 300 μM) and CCh (30 nM – 1 mM) were constructed in the absence and presence of RO-1. Electrical stimulation frequency-response and agonist concentration-effect curves in the absence and presence of RO-1 were compared using a 2-way analysis of variance (ANOVA) followed by a *post hoc* test (Tukeys) using GraphPad Prism (GraphPad software, Inc., San Diego, CA). $P < 0.05$ was taken as significant for all tests.

RNA extraction and quantitative real-time PCR

Sprague-Dawley rats were euthanized by inhalation of medical grade CO_2 followed by thoracotomy and cardiac puncture, and urinary bladders were excised. Bladders were cut open longitudinally and pinned urothelial side up in sylgard coated dishes and covered with oxygenated Krebs solution. The urothelium was then gently teased away from the underlying tissue using fine forceps and scissors under a dissecting microscope. The urothelium and remaining smooth muscle tissue were placed separately into Trizol (Invitrogen). RNA was extracted according to manufacturer's guidelines and contaminating genomic DNA was removed using Turbo DNA-free (Ambion, Austin, TX). First-strand synthesis was performed using Omniscript RT kit (Qiagen, Valencia, CA), using 1 μg of RNA and random hexamer primers. Quantitative PCR was performed using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) using an iCycler thermal cycler with the MyiQ optical attachment. The primers used were as follows (100 nM, each): $P2Y_2$: (left) AGCTCTGTCATGCTGGGTCT, (right) GTAATAGAGGGTGCGGGTGA; $P2Y_4$: (left) GCAAGTTTGTCCGCTTTCTC, (right) AGGCAGCCAGCTACTACCAA; and β-actin: (left) ATGGTGGGTATGGGTCAGAA, (right) GCTGTGGTGGTGAAGCTGTA. The experimental protocol was 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. For each sample, serial dilutions of 1 μg cDNA (1/10) were used to generate a standard curve, and run in triplicate. Results are expressed as a ratio of the threshold cycle of each receptor to the threshold cycle of β-actin.

Western blotting

Sprague-Dawley rats were euthanized by inhalation of medical grade CO_2 followed by thoracotomy and cardiac puncture, and urinary bladders were excised. Bladders were cut open longitudinally and pinned urothelial side up in sylgard-coated dishes and covered with oxygenated Krebs solution containing protease inhibitor cocktail (Roche, Indianapolis, IN). The urothelium was then gently teased away from the underlying tissue using fine forceps and scissors under a dissecting microscope. Thereafter, urothelial, underlying smooth muscle, and whole bladder tissues were cut into smaller pieces using dissecting scissors. Tissue samples were then placed into a lysis buffer containing Tris·HCl (125 mM pH 7.4), glycerol (20% vol/vol), SDS (2% wt/vol), sodium fluoride (50 mM), sodium orthovanadate (2 mM), tetra-sodium pyrophosphate (30 mM), dithiothreitol (0.2% vol/vol), and protease inhibitor cocktail (Roche). Protein lysates were homogenized and sonicated before centrifugation at 4,500 rpm for 30 min at 4°C. Protein concentrations were determined by the Coomassie Plus protein assay (Pierce, supplied by Fisher Scientific, Pittsburgh, PA). Whole rat brain cell lysate (5 µg/lane; Abcam, Cambridge, MA) was used as a positive control for antibody binding. Cell extracts were resolved electrophoretically on NuPage 4–12% bis-Tris acrylamide gels using 3-(*n*-morpholino)-propanesulphonic acid buffer (Invitrogen) and transferred electrophoretically onto 0.45 µm polyvinylidene fluoride membrane (GE Healthcare, Piscataway, NJ) in 25 mM Tris base containing 192 mM glycine at 4°C, 25 V for 90 min. Membranes were probed with primary antibodies overnight at 4°C, and bound antibody was detected with either goat anti-rabbit or rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase (GE Heathcare). Immunolabeled proteins were analyzed using chemiluminescence (ECL-plus detection kit, GE Healthcare).

Immunocytochemistry

Adult Sprague-Dawley rats (250–350 g) were euthanized by inhalation of medical grade CO_2 followed by thoracotomy and cardiac puncture. Urinary bladders were excised, embedded in OCT Tissue-Tek (Sakura Finetek, Torrance, CA), rapidly frozen over liquid nitrogen, and stored at –80°C before use. Frozen urinary bladder

sections (10 µm) were sectioned using a cryostat (Hacker-Bright Instruments, Fairfield, NJ), mounted onto microscope slides, and air-dried. Tissue sections were then fixed using 4% paraformaldehyde and washed in PBS. Tissue sections were placed in a tissue permeabilizing solution (0.5% Triton X-100 and 10% goat serum) and washed in PBS before incubation in primary antisera. Superior cervical ganglia were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then they were rapidly frozen by immersion in isopentane at -70°C for 2 min, cut into 10-µm sections using a cryostat, thaw-mounted on gelatin-coated poly-L-lysine-coated slides, and air-dried at room temperature. For immunohistochemistry on cultured neurons, ganglia were dissociated as above, plated in chamber slides and maintained in culture for 24 hr. They were fixed in 4% formaldehyde (in 0.1 M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 120 min, then washed with distilled water three times.

For the urinary bladder expiriments, tissue sections were incubated in rabbit-polyclonal anti-rat $P2Y_2$ or $P2Y_4$ receptor antibodies (5 µg/ml, 4°C overnight, Alomone Labs, Jerusalem, Israel). Colocalization studies were also conducted with P2Y receptor antibodies and the urothelial cell markers cytokeratin 17 (basal cells) and cytokeratin 20 (apical cells) and the neuronal marker PGP 9.5. Mouse anti-human cytokeratin 17 (1:2,000) and cytokeratin 20 (1:1,000) were obtained from Dako Cytomation (Carpinteria, CA). Mouse monoclonal PGP 9.5 antibody (1:50) was obtained from Abcam. Primary antibodies were removed, and tissue sections were washed in PBS before incubation in FITC-conjugated anti-rabbit IgG and/or Texas-red-conjugated anti-mouse IgG (1:500, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. For studies examining $P2Y_2$ colocalization with PGP 9.5, Cy3-conjugated anti-rabbit IgG (1:500, Jackson ImmunoResearch) and FITC-conjugated anti-mouse IgG (1:500, Jackson ImmunoResearch) were used. Tissue sections were then washed in PBS and mounted with glass coverslips using a glycerol-based aqueous antifade mountant, Citifluor (Ted Pella, Redding, CA). Background immunofluorescence was assessed in the absence of primary antibodies and secondary only.

For the superior cervical ganglia experiments, antibodies against rat $P2X_2$ and $P2X_3$ subunits (Oglesby et al., 1999) were used in this study with an indirect three-layer immunofluorescent method. Primary antibody to P2X subunits were raised in rabbits, detected with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) and visualised with Streptavidin-Texas Red (red fluorophore, Sigma). Briefly, the sections or cells were incubated overnight with the primary antibodies diluted to 3 µg/ml with 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate and 0.2% Triton X-100. Subsequently, the slides were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 1 hr, followed by incubation in Streptavidin-Texas Red diluted 1:200 in PBS containing 0.05% Merthiolate for 1 hr. All incubations were held at room temperature and separated by three 5-min washes in PBS. Slides were mounted with citiflour and examined with fluorescence microscopy. Control experiments were performed both by using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and by omission of the primary antibody to confirm the specificity of the immunoreaction.

Measurement of ATP release

Cultured rat urothelial cells (18–72 h after plating onto glass coverslips) were transferred into a perfusion chamber and superfused with an oxygenated Krebs solution (containing in mM; 4.8 KCl, 120 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, and 10 HEPES, pH 7.4) at room temperature (flow rate = 0.5 ml/min) until a stable baseline level of ATP release was measured; all test agents were bath applied. Perfusate was collected (100 µl) at 30-s intervals after agonist stimulation, ATP levels were quantified using a luciferin-luciferase reagent, and ATP concentrations were extrapolated from a standard-curve (ATP assay, Sigma-Aldrich). Only selected purinergic receptor agonists could be tested in this system to evaluate release of ATP from cultured urothelial cells. ATP γS, ADP, UDP, 2-meSADP, suramin, and pyridoxal-phosphate-6-azophenyl-2,4-disulfonate (PPADS) were all found to interfere with the luciferin-luceriferase based ATP assay mix and were not tested further. Data were obtained from at least three independent cultures and at least $n = 3$ from each

culture. Data are expressed as mean ± SE and analyzed using Student's unpaired *t*-test, and statistical significance was accepted when $P < 0.05$.

Spinal Electrical Stimulation-Evoked Intravesical Pressure Change in Pithed Rats

Intravesical pressure changes in the urinary bladders of pithed rats evoked by spinal (L6-S2) electrical stimulation were measured as described previously (Hegde et al., 1998).

Pharmacokinetics

Animals

Protocols for the pharmacokinetic studies were approved by the Institutional Animal Care and Use Committee at Roche, LLC. Male Hannover Wistar rats (240-300 g) with a single catheter inserted into the jugular vein were obtained from Charles River Laboratories, Hollister, CA. Intraveneous dosing was into the tail vein and oral administration was via disposable teflon gavage needle.

For brain harvesting, rats are euthanized with isoflurane, the skin was removed from the skull and the skull opened with sharp-sharp scissors. The skull flaps were removed, the brain was carefully removed with a spatula and gently blotted dry with Kim Wipes before weighing in tared scintillation vials and stored frozen at -80º C until used.

Blood and Urine Collection

Rats were housed individually in metabolic cages. Blood was collected at pre-determined time points using lithium heparin as anticoagulant from the jugular vein. After centrifugation at 3000 x g for 5 min., plasma was obtained and stored at -80º C until analysis. Urine is funneled into cups attached to the cages. At time points specified by the study protocol urine was removed from the collection cup and the volume was determined. A 1 mL aliquot was transferred into a 96 well collection tube and frozen until analysis

Plasma Protein Binding

Heparinized rat plasma was obtained from Pel-Freez® Biologicals (Rogers, AR) and stored -80º C until use. Centrifree Micropartition Devices (Millipore, Bedford, MA) were used to separate unbound from protein-bound material. Briefly,

RO-4 was spiked into heparinized and heparinized ultrafiltrated plasma (N = 3) to yield a final concentration between 200 and 5000 ng/mL. 1 mL of the plasma solutions and 0.3 mL of the ultrafiltrate solution were added to the filtration device and centrifuged (fixed angle) for 20 min at 2000 x rpm. Protein binding was calculated according to:

% bound = [(mean filtrate conc – mean plasma conc)/mean plasma conc]x100

Determination of Brain to Plasma Ratio

Three parts (weight) of saline were added to one part of brain. The brain was minced and subsequently homogenized (2 x 10 sec) on ice using a sonicator (Ultrasonic Processor XL, Heat Systems, Inc., Farmingdale, NY). Sample preparation was the same as for plasma samples. Brain to plasma ratios were calculated according to:

Brain/plasma ratio = Cbrain(ng/g)/Cplasma(ng/mL)

Pharmacokinetic Analysis

Non-compartmental analysis using WinNonlin, v 5.2 (Pharsight Corporation, Mountain View, CA) was applied to estimate pharmacokinetic parameters. The maximum drug concentration in the plasma (Cmax) and time (Tmax) thereof was determined from the observed values. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule and extrapolation to infinity using the elimination rate constant. The bioavailability (% F) was calculated as % F = (AUCoral/Dose) / (AUCiv/Dose) x 100.

Data analysis

All responses were normalized with respect to cell capacitance, to give a current density in pA/pF, unless otherwise stated. All data are expressed as the means ± S.E.M. Statistical analysis (Student's *t*-test) was performed using Origin 4.1 (Microcal, Northampton, MA). Concentration-response data were fitted with the Hill equation:

$$Y = A/[1 + K/X]^{nH}$$

where A is the maximum effect, K is the EC_{50}, and nH is the Hill coefficient. The combined data from the given number of cells were fitted, and the results are presented as values ± S.E., determined by the fitting routine. Traces were acquired using Fetchex (pCLAMP software) and plotted using Origin 4.1.

Chapter 3: RO-4, A Potent Orally Bioavailable $P2X_3/P2X_{2/3}$ Antagonist

Abstract

$P2X_3$ and $P2X_{2/3}$ receptors are subtypes of the P2X family of ligand gated ion channels gated by ATP and are thought to be of particular importance in pain. The current work describes the *in vitro* pharmacological characteristics of RO-4, a novel, orally bioavailable, highly potent and selective $P2X_3$ /$P2X_{2/3}$ antagonist. The potency (pIC_{50}) of antagonism of RO-4 for rat and human $P2X_3$ and human $P2X_{2/3}$ receptors was determined using a combination of radioligand binding, intracellular calcium flux and whole cell voltage-clamp electrophysiology. The pIC_{50} was found to range from 7.3 to 8.5, while concentrations 300-fold higher had little or no effect on other P2X channels or on an assortment of receptors, enzymes and transporter proteins. In contrast to A317491 and TNP-ATP, competition binding and intracellular calcium flux experiments suggested that RO-4 acts in a non-competitive fashion with ATP. Several favorable pharmacokinetic parameters in rat are reported, including oral bioavailability (%F=32.9), half-life ($t_{1/2}$ = 1.63 hrs) and *in vitro* protein binding (98.2%), which in combination with the antagonist potency and selectivity of RO-4 for $P2X_3$ and $P2X_{2/3}$ illustrate that RO-4 is an excellent *in vivo* tool compound and may serve as the basis of valuable therapeutics for the treatment of a wide variety of pain conditions.

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Introduction

ATP, a ubiquitous energy donor and receptor ligand present in every living cell, gates a family of ion channels known as P2X receptors which, not surprisingly, are themselves localized widely in cell types of nearly every origin, including neuronal, muscular, epithelial, and immune (Burnstock and Knight, 2004). The functional P2X channel is assembled as hetero- or homo-trimers from protein subunits encoded by one or more of seven genetically distinct subtypes, $P2X_{1-7}$ (Gever et al., 2006;North and Surprenant, 2000). The distribution of one protein subunit, $P2X_3$, is somewhat more limited than the other subtypes, with mRNA and/or protein found largely in small diameter, unmyelinated C-fiber sensory neurons, as well as in some epithelial cells and enteric neurons (Chen et al., 1995;Jin et al., 2004;Lewis et al., 1995;Vulchanova et al., 1998;Wang et al., 2005;Burnstock, 2008a). Since C-fiber sensory neurons have been shown to be important for the detection of noxious stimuli in damaged or sensitized tissues, $P2X_3$ and heteromeric $P2X_{2/3}$ channels, expressed in sensory neurons, are targets of great interest for the treatment of certain types of pain and disorders of visceral sensory function (e.g. overactive bladder, IBS) (Burnstock, 2007).

Accordingly, $P2X_3$ receptor gene deletion results in a markedly attenuated nocifensive phenotype in mice, including reduced sensitivity to thermal stimuli and decreased pain-related behaviors after intraplantar injection of carageenan or formalin (Cockayne et al., 2000). Reduction of $P2X_3$ expression through intrathecal administration of $P2X_3$-selective antisense or siRNA also causes a significant decrease in behavioral signs of chronic inflammatory and neuropathic pain in mice (Barclay et al., 2002;Dorn et al., 2004;Honore et al., 2002a). The role of the $P2X_3$ protein in the function of visceral organs, such as the urinary bladder and small intestine, is suggested by the sensory deficits observed in $P2X_3$-KO mice, leading to bladder and intestinal hyporeflexia (Cockayne et al., 2000;Bian et al., 2003;Ren et al., 2003). Thus it appears that $P2X_3$ protein subunits are involved in certain types of nociception as well as in sensory transmission from some visceral organs.

Many of the earlier studies linking $P2X_3$ with nociception relied on the use of molecules with poor potency and selectivity (e.g. PPADS, suramin, Reactive Blue 2)

and/or low metabolic stability (e.g. TNP-ATP) making them less than ideal for *in vivo* studies (Jarvis et al., 2001;Honore et al., 2002b;Ueno et al., 2003). A more selective and potent, low molecular weight, dual $hP2X_3/hP2X_{2/3}$ antagonist, A-317491 (K_i = 9 – 22 nM; > 1000-fold selective over other P2X channels), has been described (Jarvis et al., 2002). However, less appealing characteristics, including very high protein binding (>99.9%), low oral bioavailability and poor CNS penetration, limit its use as an *in vivo* tool. A-317491 was reported to behave as a competitive antagonist on the basis of functional curve shift experiments using the slowly desensitizing $P2X_{2/3}$ receptor. Several research groups have created chimeric P2X protein subunits by combining the N-terminus and first transmembrane domain of the slowly desensitizing $P2X_2$ channel with the remaining extracellular portion, second transmembrane domain and intracellular C-terminus of $P2X_3$ (denoted as $P2X_{2-3}$) in order to confer slow desensitization kinetics on the rapidly desensitizing P2X channels (Werner et al., 1996;Neelands et al., 2003). In this manner, TNP-ATP tested in curve shift experiments at the chimeric $P2X_{2-3}$ receptor behaved in a manner consistent with competitive antagonism, as seen previously with the heteromeric $P2X_{2/3}$ receptor, whereas it had previously appeared to exhibit non-competitive behavior using the wildtype $P2X_3$ channel (Virginio et al., 1998b;Neelands et al., 2003).

In an effort to identify novel, drug-like antagonists, we conducted a series of chemical library screens at recombinant $P2X_3$ and $P2X_{2/3}$ channels, and identified several interesting chemical leads with activity. Chemical optimization has been successful in the generation of high affinity, selective pharmacological and clinical tools. In the current work, we present data on a compound of unique chemical structure, RO-4, demonstrating: 1) high antagonist potency at and selectivity for $P2X_3$ and $P2X_{2/3}$ channels 2) moderate protein binding and attractive pharmacokinetic profile suitable for *in vivo* studies 3) evidence suggesting RO-4 behaves as an allosteric antagonist.

Results

RO-4 was synthesized following the optimization from an active, but weaker, screening hit derived from the bacterial dihydrofolate reductase inhibitor trimethoprim (Figure 1) discovered by high through-put screening of the Roche

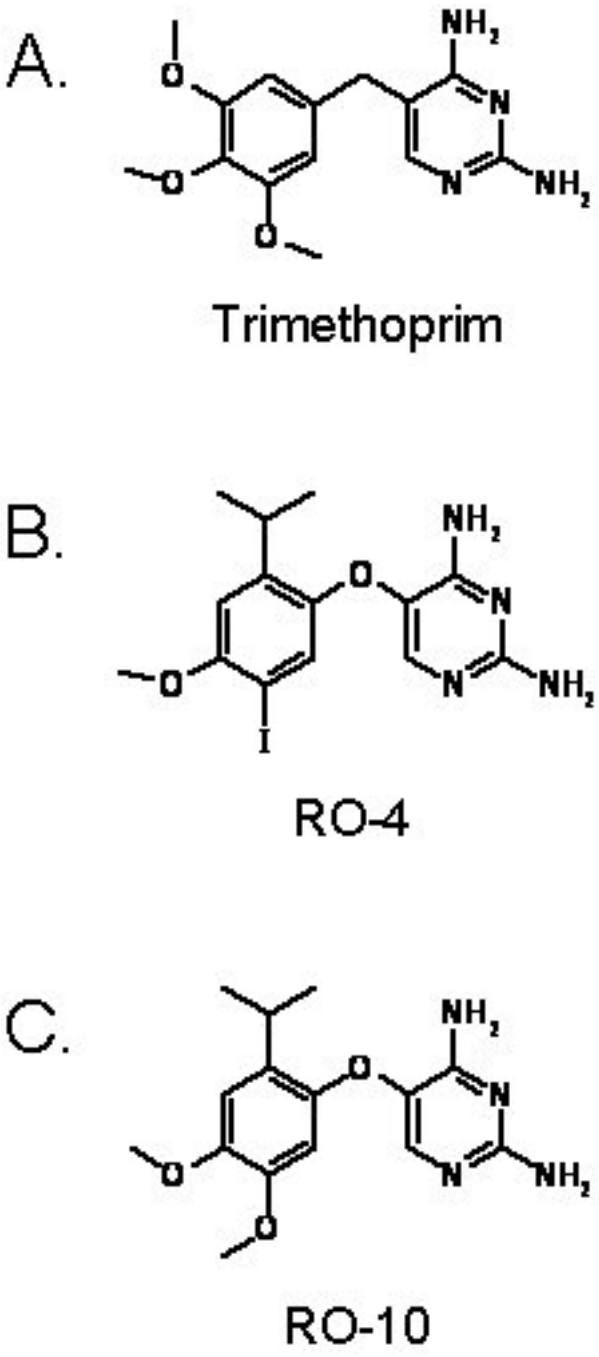

Figure 1. Chemical structures of A) trimethoprim; MW = 290.32, B) RO-4; MW = 400.21, and C) RO-10; MW = 304.35.

compound collection. A close structural analog, RO-10, was also used in the current work for some mechanistic experiments. Chemical synthesis information can be found in the patent literature (Dillon, M.P.; Broka, C.A.; Carter, D.S.;; Hawley, R.C.; Jahangir, A.; Lin, C.J.J.; Parish, D.W.: Preparation of diaminopyrimidines as $P2X_3$ and $P2X_{2/3}$ antagonists. US 2005/209260 A1).

RO-4 is a highly potent inhibitor of α,β-MeATP-evoked intracellular calcium flux in cell lines expressing recombinant rat and human $P2X_3$ and human $P2X_{2/3}$ channels (Figure 2A, Table 1). It is an equally potent inhibitor of human and rat $P2X_3$ (pIC_{50} = 8.0) and can also block human and rat $P2X_{2/3}$ channel function with marginally reduced potency (pIC_{50} = 7.3). The functional potency of RO-4 was confirmed by whole cell voltage clamp recordings using the same recombinant cell lines employed for the calcium flux experiments (see Figure 2B, Table 1). Additionally, electrophysiological recordings were obtained from acutely dissociated rat dorsal root and nodose ganglion neurons which are known to endogenously express $P2X_3$ and $P2X_{2/3}$ channels, respectively (Rae et al., 1998;Virginio et al., 1998a). Inhibition by RO-4 of α,β-MeATP-evoked inward currents was of similar potency in cells expressing either recombinant or native channels with potency estimates (pIC_{50}) of 8.4 and 8.5 for recombinant and native $rP2X_3$, respectively and 7.7 and 7.6 for recombinant human and native rat $P2X_{2/3}$, respectively.

The kinetics of the interaction between RO-4 and the $hP2X_{2/3}$ channel were determined using manual patch clamp electrophysiology coupled with the Cellectricon Dynaflow high-speed perfusion system. Inhibition of α,β-MeATP-evoked inward currents was concentration-dependent and complete, and reversible upon washout of the antagonist (Figure 3A and 3B). Each recording consisted of a k_{obs} measurement at some particular concentration of RO-4 as well as an off rate (k_{off}) measurement. The values of these parameters were determined by fitting a simple exponential function to the data (Figure 3A and 3B). k_{obs} was found to be linearly dependent on the concentration of RO-4, whereas k_{off} was found to be independent of antagonist concentration, consistent with a simple, 2nd-order kinetic model (Fig. 3C). We determined the value of k_{on} by fitting the following linear equation:

$$k_{obs} = k_{on} * [\text{Antagonist}] + k_{off}$$

Based on this fit, we found k_{on} = 7.49 x 10^{-5} ± 0.41 x 10^{-5} s^{-1} nM^{-1} and k_{off} = 3.5 x 10^{-3} ± 0.29 x 10^{-3} s^{-1}.

The selectivity of RO-4 for $P2X_3$ and $P2X_{2/3}$ over other P2X channels was established by testing the ability of RO-4 to block agonist-evoked intracellular calcium flux in cell lines expressing recombinant human $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$ or $P2X_7$ receptors (see Table 2). In all cases, RO-4 produced no inhibition up to a concentration of 10 μM. Additionally, two general screens of selectivity, one comprising 75 receptors, channels, enzymes and transporters (Cerep, Poitiers, France) and a second one covering more than 100 kinases (Ambit, San Diego, California, USA) established RO-4 to be a highly selective molecule (see Table 2). The only target in the panel at which RO-4 had submicromolar affinity was the melatonin ML1 receptor (pK_i = 6.2). Affinity estimates (pK_i) were also calculated for histamine H_2, serotonin $5HT_3$ and Na^+ channel (site 2) and found to be 5.3, 5.6 and 5.2, respectively. Finally, because of the structural similarity of RO-4 to well-known bacterial dihydrofolate reductase inhibitors, RO-4 was also tested for the ability to inhibit the human isoform of this enzyme and found to be weakly active (pIC_{50} = 6.0 compared to the positive control, pyrimethamine, pIC_{50} = 7.1).

In order to measure the affinity for the $P2X_3$ or $P2X_{2/3}$ channels directly using radioligand binding displacement studies, RO-4 was tritium-labeled on the methoxy group at the 4 position of the phenyl ring. Saturation binding experiments were conducted using [^{3}H]-RO-4 and cell membrane homogenates prepared from the same cell lines employed for intracellular calcium flux experiments; the specific binding window was significant for all three cell lines and best fitted a one-site binding model (Figure 4A - C). The affinity of RO-4 was equal at human and rat $P2X_3$ (K_D = 15 and 14 nM, respectively) and was marginally lower at the heteromeric human $P2X_{2/3}$ receptor as well (K_D = 30 nM, see Table 1). In competition binding experiments, [^{3}H]-RO-4 binding was completely eliminated by concurrent incubation with either RO-10 or α,β-MeATP, but the inhibition potency (IC_{50}) of RO-10 alone was sensitive to the concentration of [^{3}H]-RO-4 (Figure 5A). A graph relating the ratio of IC_{50}/K_b of RO-10 to the ratio of [RO-4]/K_D of RO-4 resulted in a straight line following

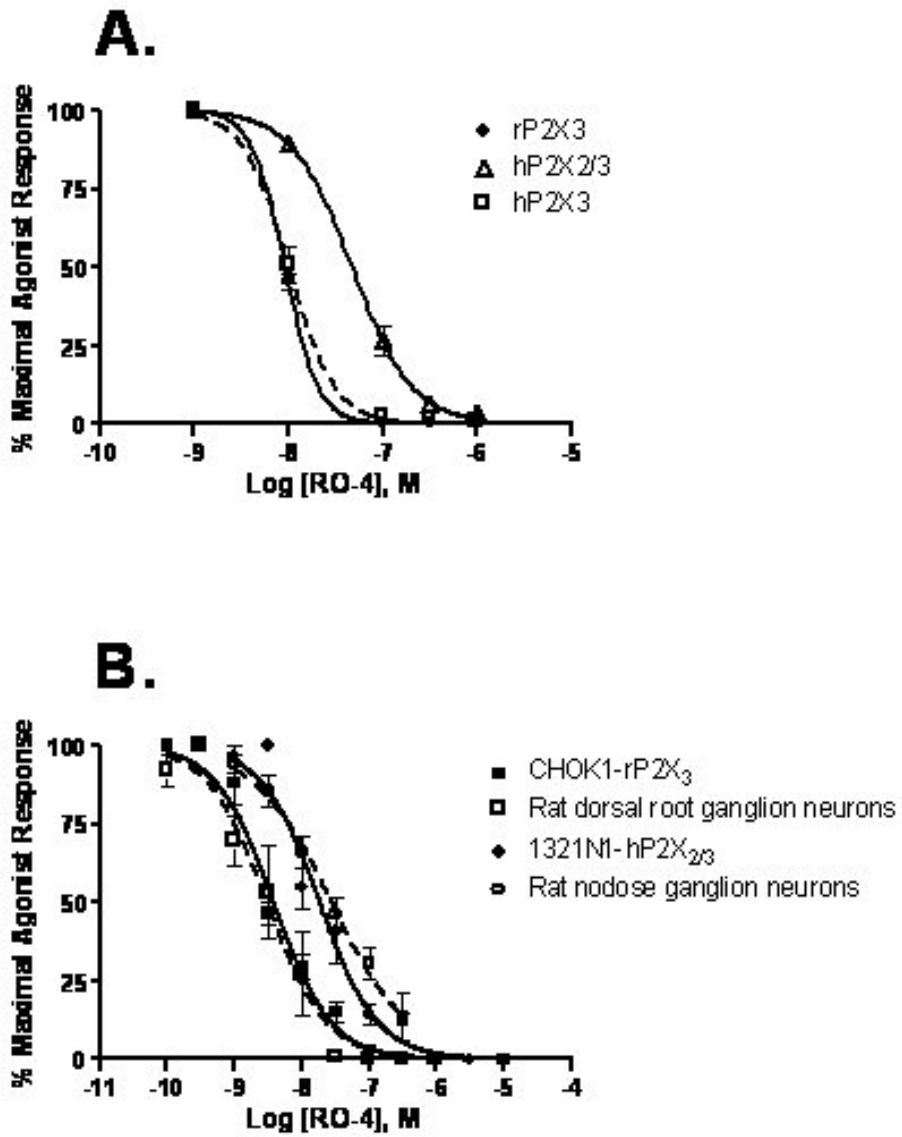

Figure 2. In vitro potency estimates of RO-4 antagonism at native and recombinant $P2X_3$ and $P2X_{2/3}$ receptors. A) Inhibition by RO-4 of α,β-MeATP-evoked intracellular calcium flux through recombinantly expressed rat $P2X_3$ (CHOK1 cells) or human $P2X_3$ and $P2X_{2/3}$ (1321N1 cells). Potency estimates (pIC_{50}) were 8.06, 8.05 and 7.41 for human $P2X_3$, rat $P2X_3$ and human $P2X_{2/3}$, respectively. Inhibition curves were constructed in the presence of approximately EC_{80} concentrations of α,β-MeATP: 1 μM ($P2X_3$) and 5 μM ($P2X_{2/3}$) B) Inhibition by RO-4 of α,β-MeATP-evoked inward currents through recombinantly expressed rat $P2X_3$ (CHOK1 cells) or human $P2X_{2/3}$ (1321N1 cells) and through isolated rat dorsal root ($P2X_3$) or nodose ($P2X_{2/3}$) ganglion neurons. Potency estimates (pIC_{50}) were 8.42 and 7.73 for recombinantly expressed rat $P2X_3$ and human $P2X_{2/3}$, respectively and 8.51 and 7.56 for acutely dissociated rat dorsal root ($P2X_3$) and nodose ($P2X_{2/3}$) ganglion neurons, respectively.

Table 1: Potency and affinity estimates of RO-4

Assay	$hP2X_3$		$rP2X_3$		$hP2X_{2/3}$	
	n	pIC_{50} ± s.e.m.	n	pIC_{50} ± s.e.m.	n	pIC_{50} ± s.e.m.
Intracellular calcium flux (FLIPR)	3	8.06 ± 0.15	9	8.05 ± 0.03	9	7.41 ± 0.08
Voltage clamp electrophysiology (recombinant cell lines)		n.d.	3 - 5	8.42	3 - 4	7.73
Voltage clamp electrophysiology (rat sensory neurons)		n.d.	3 - 8	8.51	3 - 8	7.56
	n	K_D ± s.e.m.	n	K_D ± s.e.m.	n	K_D ± s.e.m.
Radioligand binding	3	15 ± 1 nM	3	30 ± 1 nM	3	14 ± 2 nM

Table 2: Pharmacological selectivity of RO-4

Receptor	N	$pIC_{50} \pm$ s.e.m.
$hP2X_3$	3	8.06 ± 0.15
$rP2X_3$	3	8.05 ± 0.03
$hP2X_{2/3}$	3	7.41 ± 0.08
$hP2X_1$	3	< 5
$hP2X_2$	3	< 5
$hP2X_4$	3	< 5
$rP2X_5$	3	< 5
$hP2X_7$	3	< 5
ML_1	1*	6.0
$5\text{-}HT_3$	1*	5.3
H_2	1*	5.2
Na^+ Channel (site 2)	1*	5.2
α_{1A}	3	5.08 ± 0.10
α_{1B}	3	5.18 ± 0.17
α_{1D}	3	5.20 ± 0.09

closely that expected of a purely competitive agent, as would be expected for such a close structural analogue of RO-4 (Figure 5B). However, the IC_{50}/K_b ratio for α,β-MeATP was insensitive to radioligand concentration, suggesting RO-4 and α,β-MeATP do not behave in a purely competitive manner.

To better understand the mechanism of antagonism, a series of functional curve shift experiments, based on the original work of Arunlakshana and Schild (Arunlakshana and Schild, 1959), were conducted using intracellular calcium flux as the functional readout. As previously stated, a slowly desensitizing, chimeric $P2X_{2-3}$ channel was used in place of the wild type homomeric $P2X_3$ channel. Figures 6A and 6B show the rightward, parallel, fully surmountable shifts of the agonist concentration-effect curves to α,β-MeATP by TNP-ATP using cell lines expressing either heteromeric $P2X_{2/3}$ or chimeric $P2X_{2-3}$ receptors, confirming previously published conclusions that TNP-ATP is a competitive antagonist of these channels (Neelands et al., 2003). Relating the dose ratios for the half-maximal calcium influxes (EC_{50}) to the concentration of antagonist required for these dose ratios (i.e. Schild plot) produced a linear relationship with a slope of 1.2 (in both cases) and pA_2 estimates of 7.4 and 8.3 for $P2X_{2/3}$ and $P2X_{2-3}$, respectively (Figure 6A and 6B). However, in the same cell lines, RO-4 produced non-surmountable shifts of the agonist concentration-effect curve consistent with the possibility (though not proving) that RO-4 does not act as a purely competitive antagonist (Figure 6C and 6D).

To further explore if TNP-ATP and RO-4 blocked channel function through independent or common mechanisms, α,β-MeATP concentration-effect curves were constructed after incubation with both antagonists in combination. Figures 7A shows that both 32 nM RO-4 and 10 nM TNP-ATP produced approximately 3-fold shifts in the α,β-MeATP concentration-effect curves in cells expressing $hP2X_{2-3}$. When the same cells expressing $hP2X_{2-3}$ were equilibrated with a combination of 32 nM RO-4 and 10 nM TNP-ATP, the α,β-MeATP pEC_{50} was shifted 10-fold, a multiplicative effect. RO-10, a close structural analog of RO-4 (Figure 1) which would be expected to act at precisely the same binding site (as shown in displacement studies), produces rightward shifts that are only marginally greater in combination with RO-4 than alone in either the chimeric $P2X_{2-3}$ or the heteromeric $P2X_{2/3}$ receptor (Figure 7B and 7C). From these experiments it would be reasonable to conclude that molecules from the

chemical series represented by RO-4 and RO-10 do not act through the same mechanism as TNP-ATP and therefore bind allosterically to the ATP binding site.

To assess the utility of RO-4 as a tool compound to investigate antagonism of $P2X_3$ and $P2X_{2/3}$ receptors *in vivo* rats were dosed with 2 mg/kg of RO-4 intravenously or orally as a suspension. The relevant pharmacokinetic parameters were determined (Table 3). RO-4 is orally bioavailable (F = 32.9%) with a T_{max} of 30 minutes and half-life of 1.63 hours. CNS penetration was determined by measuring the brain to plasma ratio (B/P); RO-4 is highly CNS penetrant with a B/P ratio ≈ 6 (total brain extracted concentration/total plasma concentration). In addition; the *in vitro* protein binding was determined to be 98.2% in rat plasma.

Discussion

Here we present a detailed pharmacological characterization of RO-4, a selective, dual $P2X_3/P2X_{2/3}$ receptor antagonist with pharmacokinetic and pharmacodynamic properties that allow for use in *in vivo* models. At the initiation of our medicinal chemistry efforts, the majority of the known $P2X_3$ ligands were either nucleotides or high molecular weight, polyacidic dyes, all of which offered poor selectivity of action. In an attempt to discover novel more drug-like chemotypes a high-throughput screen of the Roche chemical library was performed looking for inhibitors of α,β-MeATP-evoked intracellular calcium flux employing cell lines expressing recombinant forms of $P2X_3$ or $P2X_{2/3}$ receptors. One of several initial hits was structurally related to trimethoprim, a bacterial dihydrofolate reductase inhibitor used as an antibiotic, and was chemically optimized, yielding several chemical analogues; the current work describes the characterization of a representative molecule from this series, RO-4.

RO-4 is a dual $P2X_3/P2X_{2/3}$ receptor antagonist capable of inhibiting agonist-evoked intracellular calcium flux and inward currents at concentrations in the mid to low nanomolar range in cell lines recombinantly expressing rat or human $P2X_3$ and human $P2X_{2/3}$ receptors and also natively expressed $P2X_3$ and $P2X_{2/3}$ receptors naturally present in the dorsal root and nodose ganglia of rat. Although RO-4 may be marginally selective for the homomeric $P2X_3$ receptor over the heteromeric $P2X_{2/3}$ based on functional potency estimates, it does not appear to be selective for species

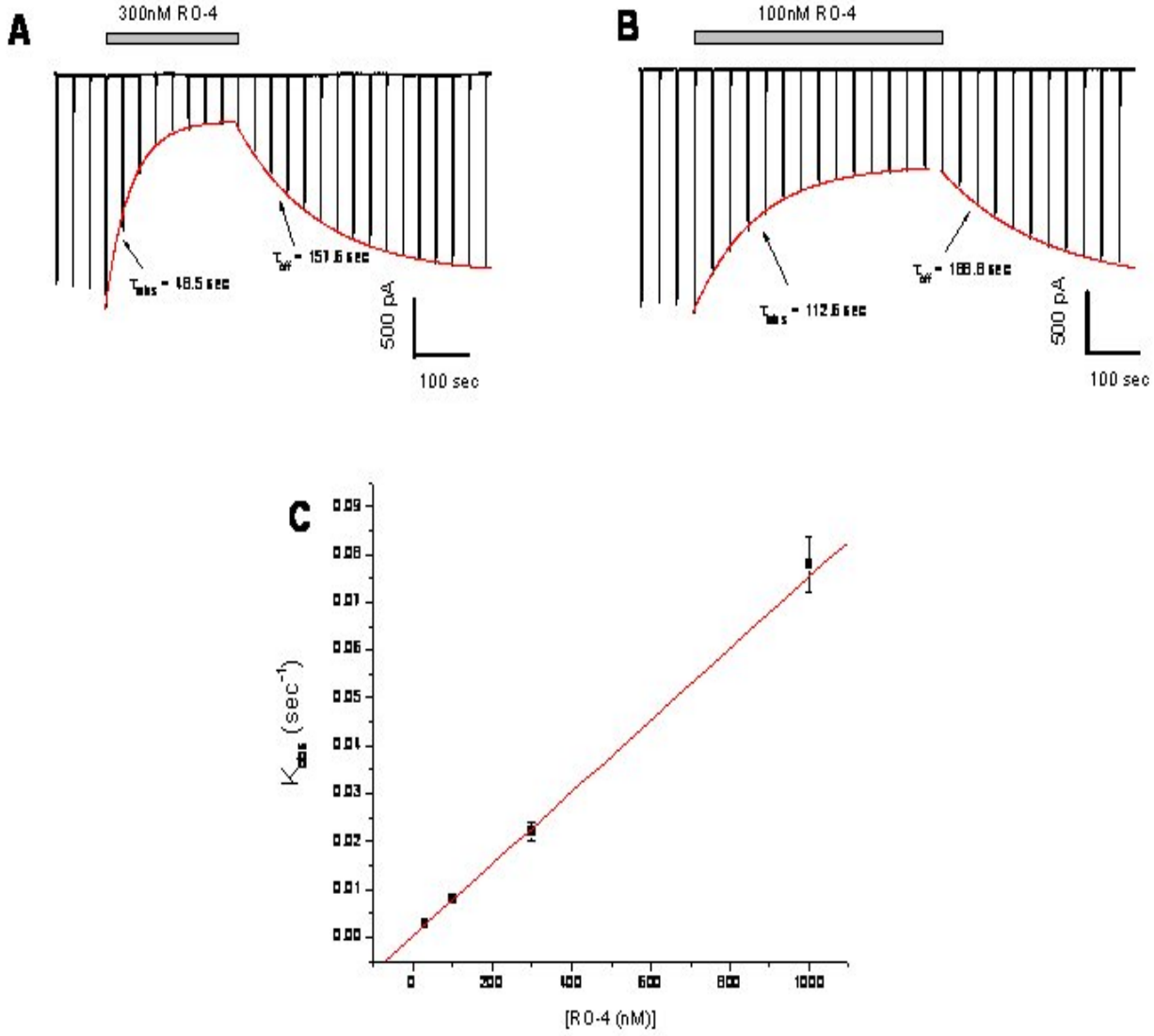

Figure 3. Antagonism by RO-4 of α,β-MeATP-evoked inward currents in 1321N1 astrocytoma cells expressing $hP2X_{2/3}$. Agonist pulses consist of 10 μM α,β-MeATP at 30 second intervals, 2 seconds of agonist exposure per pulse. Application of A) 300 nM RO-4 or B) 100 nM RO-4 produced τ_{obs} (association kinetics) of 48.5 and 112.6 seconds, respectively and τ_{off} (dissociation kinetics) of 157.6 and 188.8 seconds, respectively. C, Plotting the relationship of RO-4 concentration and k_{obs} shows there is a linear dependence, consistent with a simple, second order kinetic model.

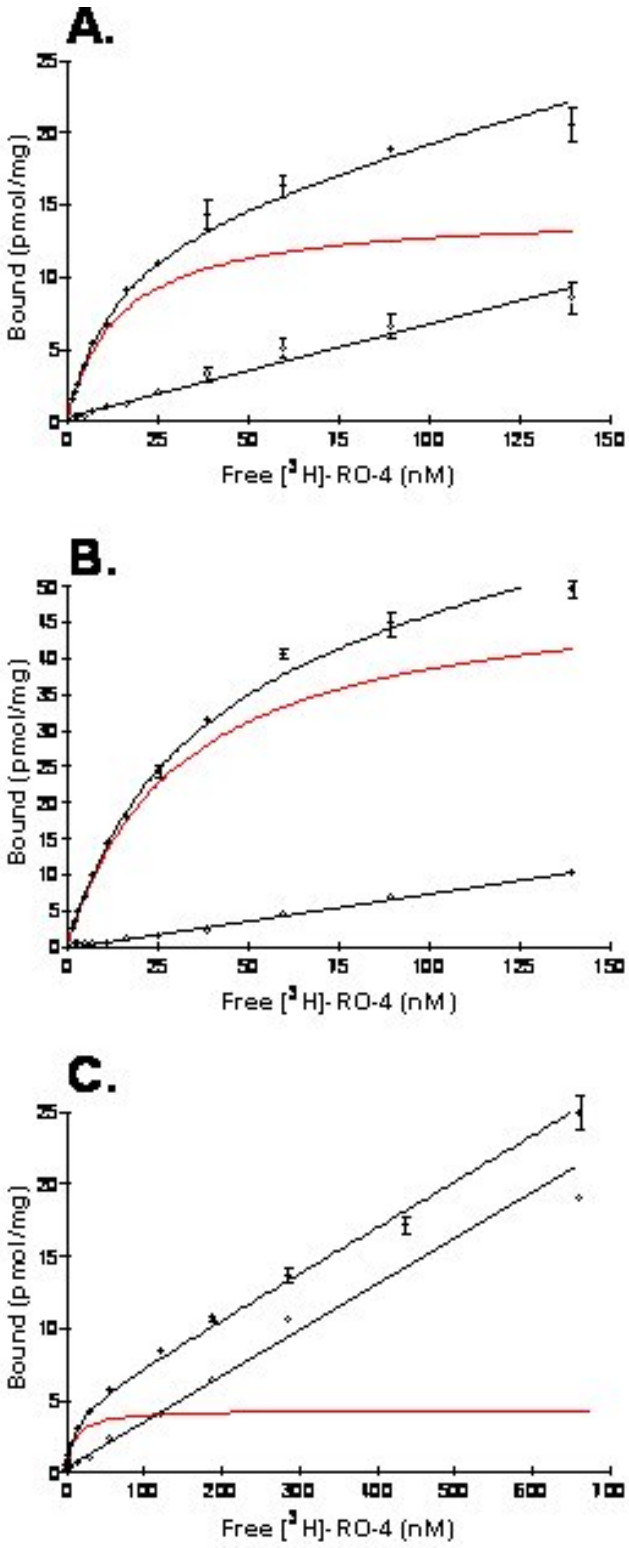

Figure 4. Specific binding (red curve) of [^{3}H]-RO-4 to CHOK1 membranes expressing A) $hP2X_3$ or B) $rP2X_3$ receptors or C) 1321N1 astrocytoma cells expressing $hP2X_{2/3}$ receptors. Specific binding was fit simultaneously using total binding (solid symbols) and non-specific binding (open symbols; determined using unlabelled RO-10).

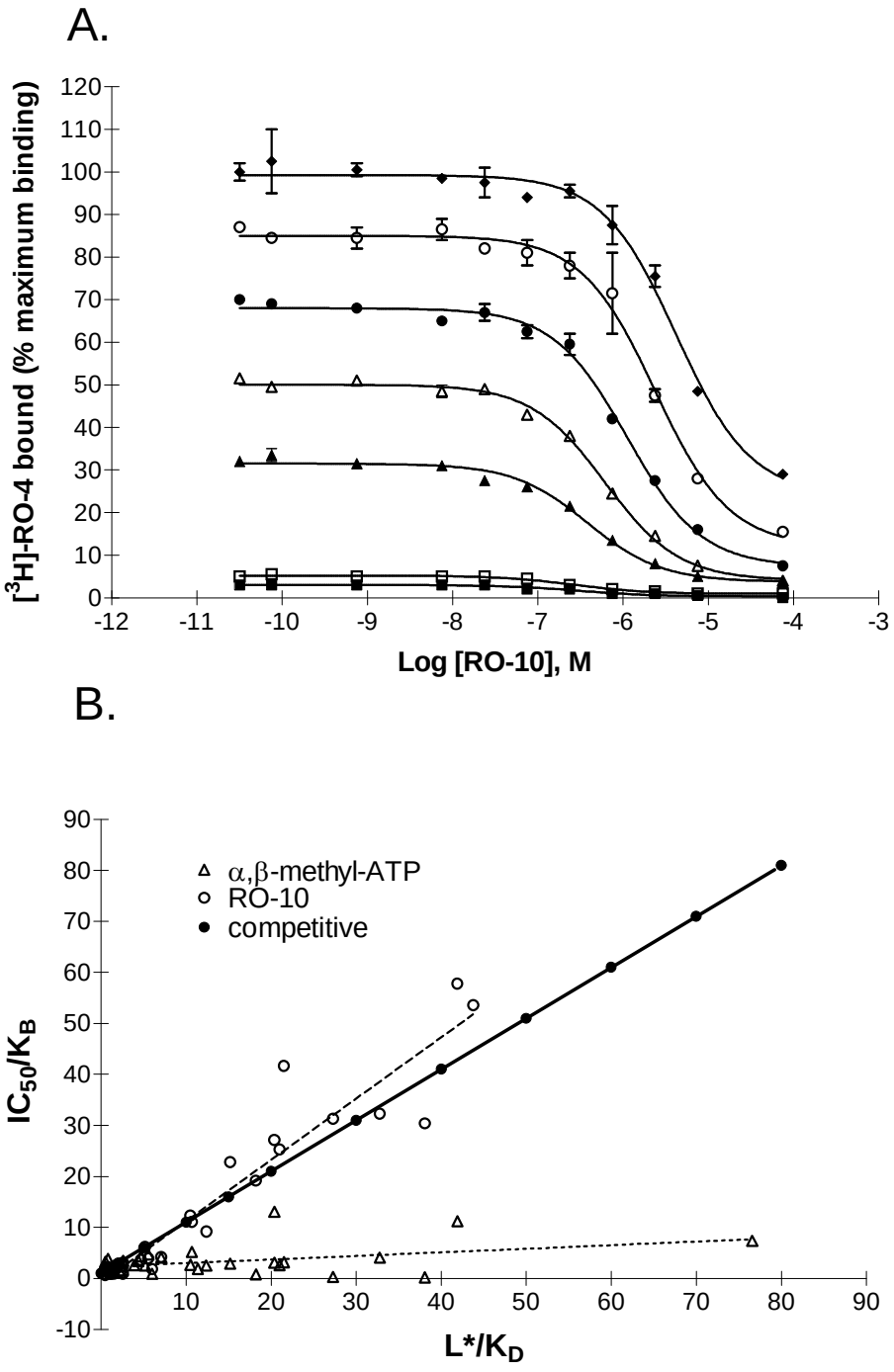

Figure 5. Competition binding experiments between [^{3}H]-RO-4 and unlabelled RO-10 or α,β-MeATP in employing membrane homogenates from CHOK1 cells expressing recombinant rat $P2X_3$. A) Inhibition of binding by RO-10 of varying concentrations of [^{3}H]-RO-4 (0.65, 1.24, 13, 33.9, 74.5, 158, 296; in nM). B) Relationship of inhibition potencies (normalized to affinity estimates for each compound)

homologues, nor does it differentiate between native or recombinantly expressed receptors. Further confirmation was obtained by using radiolabelled RO-4 to directly measure its affinity for P2X protein subunits in membrane homogenates of the same cell lines used for the functional intracellular calcium flux experiments; it was found to be in good agreement (radioligand binding K_D estimates ranged from 14 – 30 nM, intracellular calcium flux and inward current IC_{50} estimates ranged from 3 – 50 nM).

Although RO-4 is a potent inhibitor of $P2X_3$ and $P2X_{2/3}$ channels, it is equally important to establish selectivity in order to anticipate and understand potential off-target effects. Accordingly, RO-4 was tested at most of the known homomeric P2X channels ($P2X_6$ does not readily form functional, homomeric channels) for the ability to inhibit intracellular calcium flux and further tested in a broad profile of receptors, channels and enzymes (75 targets total; Cerep) as well as a screen of over 100 kinases (Ambit). RO-4 showed no submicromolar potency at any of these targets except the melatonin MT1 receptor ($pIC_{50} = 6$). Nevertheless, even in this case, RO-4 was 30- to 100-fold selective for $P2X_3$ and $P2X_{2/3}$ and at the majority of targets tested selectivity was greater than 1000-fold.

Having established both potency at and selectivity for $P2X_3$ and $P2X_{2/3}$ channels, we conducted a series of experiments to better understand the mechanism of inhibition of RO-4. A classical experiment which provides evidence (though not proof) for whether an agent is acting orthosterically or allosterically is the curve shift experiment pioneered by Arunlakshana and Schild (Arunlakshana and Schild, 1959) in which the concentration-effect relationship of an agonist acting on a receptor or tissue is constructed in the presence and absence of an antagonist. A competitive antagonist will be very sensitive to agonist concentration and will typically move the agonist concentration-effect curve in parallel, fully surmountable shifts (if rapid equilibration can be achieved) to a higher agonist concentration range whereas the effect of allosteric antagonists may frequently deviate from this behavior (e.g. by not shifting the C/E curve any further despite increasing antagonist concentrations or by insurmountability of inhibition, though this latter property may also be a reflection of slow reversibility of a competitive interaction). Modifications of this experiments were carried out using both radioligand binding and intracellular calcium flux as the readouts for this effect.

First, using a concentration of [^{3}H]-RO-4 ranging from 0.04 – 20 times K_D binding at membrane homogenates from cell lines recombinantly expressing $hP2X_3$ channels, inhibition curves were constructed using α,β-MeATP and a close structural analogue, RO-10 (Figure 1). When the IC_{50}s of RO-10 are related to radioligand concentration (normalized to their own affinities for $hP2X_3$), a linear relationship very close to the theoretical relationship of a purely competitive agent was observed, as expected given the chemical similarity of these two compounds. In contrast, although binding of α,β-MeATP was mutually exclusive with RO-4, the resulting IC_{50}s did not follow the relationship expected of a purely competitive agent and in fact was relatively insensitive to radioligand concentration. These data suggest RO-4 does not behave as a competitive antagonist with the nucleotide agonists.

We wanted to confirm this result with functional evidence but one of the ideal requirements for these types of mechanistic experiments is the establishment of an agonist-antagonist equilibrium. Consequently, the slowly desensitizing heteromeric $P2X_{2/3}$ receptor was used, as well as a chimeric $P2X_{2-3}$ receptor modified to desensitize slowly much like the $P2X_{2/3}$ receptor, while retaining the extracellular portion (and perhaps agonist and antagonist binding sites) of the homomeric $P2X_3$ receptor. This approach has been used successfully for both rapidly desensitizing P2X channels, $P2X_1$ and $P2X_3$, to demonstrate competitive antagonism in chimeric versions of these receptors (Werner et al., 1996;Neelands et al., 2003). The $hP2X_{2-3}$ chimera was recombinantly expressed in 1321N1 astrocytoma cells (devoid of native expression of any P2X channels) and found to have slowly desensitizing kinetics similar to the homomeric $P2X_2$ channel as previously published (data not shown). Confirming previously published results, TNP-ATP, a nucleotide analog of ATP, inhibited α,β-MeATP-evoked intracellular calcium flux in cell lines expressing either the chimeric $hP2X_{2-3}$ or the heteromeric $P2X_{2/3}$ channels and shifted the agonist concentration-effect relationship to the right in a fully surmountable manner with no change in the Hill slopes. Relating the agonist "dose-ratios" (DR) resulting in half-maximal increases in intracellular calcium flux (log(DR – 1)) with the corresponding antagonist concentration produced a linear plot with slopes close to unity (n = 1.2 for both channels) and pA_2 estimates of affinity, represented by the x-intercept, of 8.3 and 7.4 for $hP2X_{2-3}$ and $P2X_{2/3}$, respectively. However, for both the chimeric $hP2X_{2-3}$

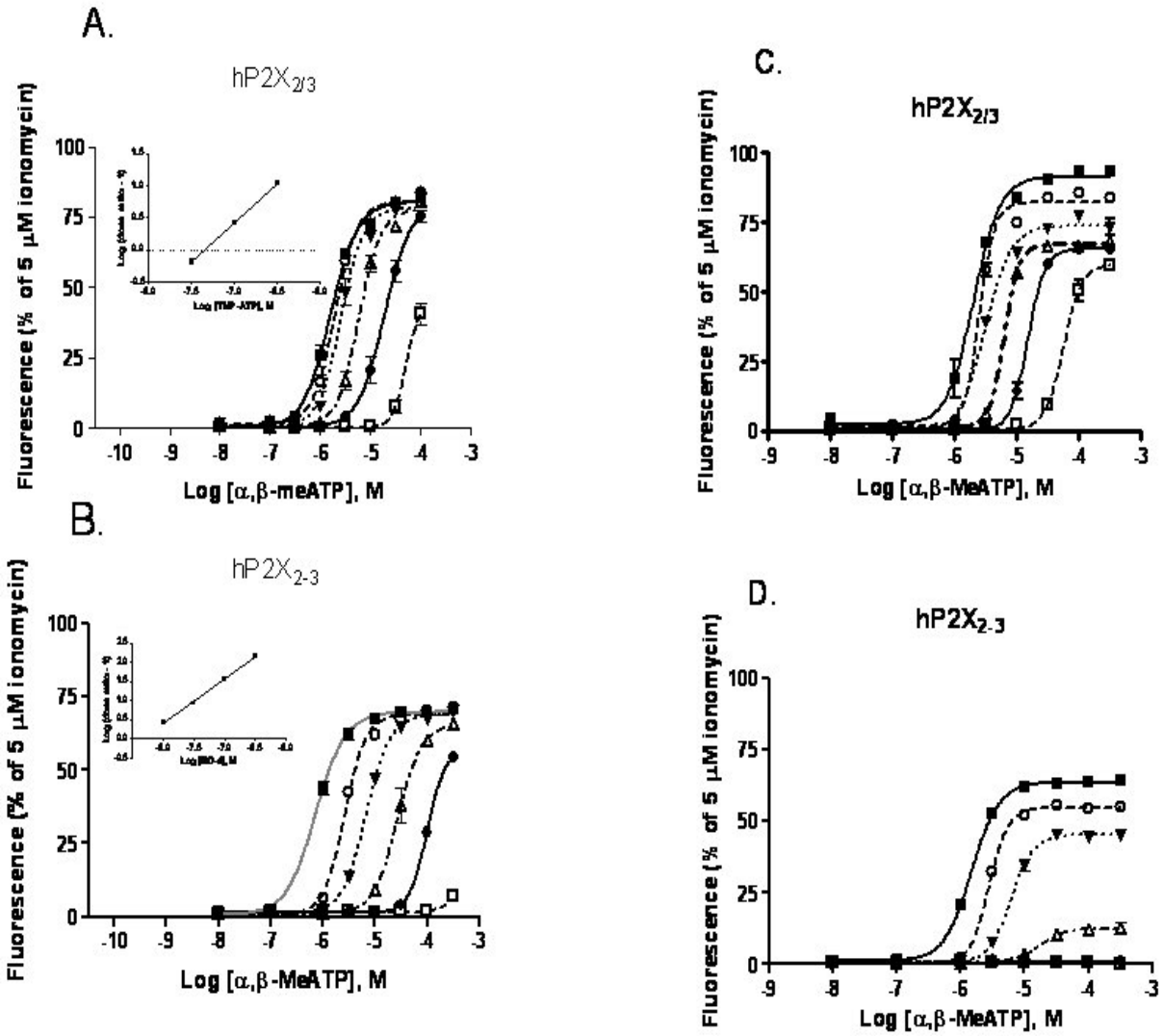

Figure 6. Shifts of α,β-MeATP-evoked intracellular calcium flux by varying concentrations of antagonist in $hP2X_{2/3}$ (A and C) and $hP2X_{2-3}$ (B and D). Concentrations of antagonists are represented by the following symbols: filled square (vehicle; no antagonist), open circle (10 nM), filled upside-down triangle (32 nM), open triangle (100 nM), filled circle (320 nM), open square (1000 nM). Insets in A and B represent the relationship of the logarithm (dose ratio – 1) to the antagonist concentration (i.e. "Schild plot"). The dose ratio is defined as $EC_{50(antagonist)}/EC_{50(vehicle)}$. The slopes of these lines were 1.2 in both cases and the x-intercepts were 7.4 and 8.3 for $hP2X_{2/3}$ and $hP2X_{2-3}$, respectively.

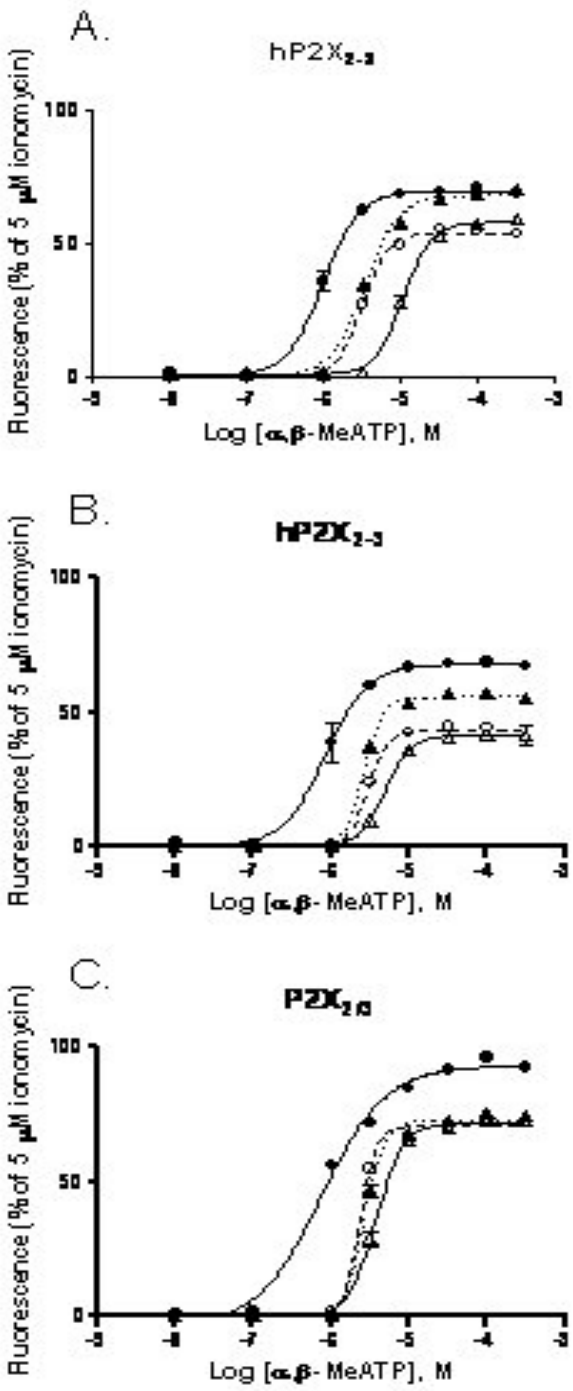

Figure 7. Shifts of α,β-MeATP-evoked intracellular calcium flux by varying concentrations of antagonist in $hP2X_{2-3}$ (A and B) and $hP2X_{2/3}$ (C). Concentrations of antagonists are represented by the following symbols: filled circle (vehicle; no antagonist), filled triangle (A: 10 nM TNP-ATP; B: 320 nM RO-10; C: 1000 nM RO-10), open circle (32 nM RO-4), open triangle (A: 10 nM TNP-ATP + 32 nM RO-4; B: 320 nM RO-10 + 32 nM RO-4; C: 1000 nM RO-10 + 32 nM RO-4)

(used as a surrogate of wild-type $P2X_3$ pharmacology) and the heteromeric $P2X_{2/3}$ channels, RO-4 shifted the agonist-effect curves in a non-parallel, insurmountable fashion, suggesting that RO-4 does not behave in a purely competitive manner. It should be noted that even though peak α,β-MeATP-evoked intracellular calcium flux was measured after 5 minutes of equilibration between agonist and antagonist, it is possible that complete equilibrium was not established due to the slow off-rate of RO-4 and may have caused, in part, the observed insurmountability of the response.

To further address this, the concentration-effect relationship of α,β-MeATP was observed in the presence of RO-4 and TNP-ATP, an antagonist already established to be acting at the orthosteric binding site. If two antagonists are acting through entirely independent mechanisms, the shift of the agonist concentration-effect curve in the presence of both antagonists might be expected to be multiplicative relative to their individual shifts, whereas two antagonists acting at the same binding site will result in an additive shift. Concentrations of RO-4 and TNP-ATP individually resulting in a half-log increase of the pEC_{50} of α,β-MeATP at the chimeric $hP2X_{2-3}$ channel produced a full-log increase when used in combination, close to a multiplicative rather than additive increase, suggesting that RO-4 does not block $P2X_{2-3}$-mediated intracellular calcium increases through the same mechanism. Since TNP-ATP acts at the ATP binding site, these data provide further evidence that RO-4 acts allosterically. Supporting this notion, the combination of RO-4 and RO-10 resulted in a much less than multiplicative (essentially additive) effect on agonist pEC_{50} shifts, consistent with the presumption that these structurally similar chemical analogues bind at precisely the same site. Thus, when the radioligand binding and functional curve shift experiments are viewed in their entirety, it is reasonable to conclude that RO-4 binds allosterically to the ATP binding site and consequently behaves as a non-competitive antagonist.

$P2X_3/P2X_{2/3}$ antagonism that is relatively insensitive to the concentration of the endogenous agonist, ATP, may be a desirable characteristic for a drug targeting these channels. ATP is present in the cytoplasm of most cells at millimolar concentrations and stored in synaptic vesicles at even higher concentrations, perhaps as high as 200 mM (Pankratov et al., 2006;Burnstock, 2007). Since it has been hypothesized that the peak ATP concentration in the synaptic cleft

could approach 500 μM (Pankratov et al., 2006), inhibition of P2X channel function through a mechanism relatively insensitive to ATP concentrations might be a desirable attribute.

The only other novel, small molecule $P2X_3$ antagonist for which published data are available, A-317491, has been reported to be fully surmountable in curve shift experiments(Burgard et al., 2000;Neelands et al., 2003) and consequently may prove to be less effective when ATP concentrations are very high. Furthermore, A-317491 is plagued by very high protein binding (resulting in a negligible free plasma fraction), low oral bioavailability and no CNS penetration, therefore its utility as an *in vivo* tool is limited. For example, in the chronic constriction injury and L5-L6 nerve ligation models of neuropathic pain, A-317491 is effective only if dosed intrathecally, highlighting the poor CNS penetration of A-317491 (McGaraughty et al., 2003;Sharp et al., 2006). In models where the peripheral role of P2X channels is presumed to be important, such as rat Freund's complete adjuvant model of inflammatory pain or in the rat cyclophosphamide-induced bladder cystitis model, A-317491 is effective, but only if administered intravenously (Wu et al., 2004;Ito et al., 2008). In contrast RO-4 may be a significantly superior *in vivo* tool compound demonstrating high oral bioavailability and CNS penetration in addition to high $P2X_3$/$P2X_{2/3}$ antagonist potency.

When the $P2X_3$ protein was first cloned and characterized, both as a homomeric channel as well as a heteromeric channel formed with $P2X_2$ subunits (Lewis et al., 1995;Chen et al., 1995), it generated a lot of interest as a therapeutic target because it appeared to be located almost exclusively on small diameter, nociceptive neurons. Over the ensuing years, the evidence linking $P2X_3$ and/or $P2X_{2/3}$ channels with different types of pain, particularly neuropathic and chronic inflammatory pain, as well as sensory function of hollow viscera, has grown dramatically. For example, when $P2X_3$ expression in mice is reduced by intrathecal administration of antisense oligonucleotides (Honore et al., 2002a), alloying responses in models of neuropathic pain are significantly reversed. Furthermore, topical application of A-317491 directly to the spinal cord reduced neuronal excitability in the dorsal horn of rats after chronic constriction injury of the sciatic nerve, a well-established model of neuropathic pain (Bennett and Xie, 1988), whereas intravenous

administration (presumed to be acting peripherally due to the very poor CNS penetrance of this compound) had no effect (Sharp et al., 2006). It is estimated that as much as 8% of the population in the developed world suffer from neuropathies (produced by nerve injury, diabetes or viral infections, for example), which are treated very poorly with the current medicines (Dworkin et al., 2007). Additionally, a medicine targeting $P2X_3$ and $P2X_{2/3}$ receptors may prove beneficial for patients suffering from other types of pain as well as disorders of the lower urinary and gastrointestinal tracts (Bian et al., 2003;Ford et al., 2006;Brederson and Jarvis, 2008). A potent inhibitor of $P2X_3$ and $P2X_{2/3}$ receptors will hopefully prove to be a useful tool in the physician's arsenal of therapeutic medicines but this will have to await for the arrival of potent inhibitors with the proper metabolic and toxicological characteristics to test in clinical trials.

Table 3: Pharmacokinetic parameters of RO-4

Parameter	Result	
	RO-4	A-317491
Bioavailability (F)	32.9%	0%
Peak plasma concentration (T_{max})	0.5 hr	NA
Plasma half-life ($T_{1/2}$)	1.63 hr	NA
CNS penetration (brain/plasma ratio)	6	0
Rat Plasma protein binding (bound)	98.2%	99.9%

Chapter 4: Pharmacological Characterization of RO-1, A Selective $P2X_1$ Antagonist

Abstract

The presence of $P2X_1$ receptors has been demonstrated in both human and rat bladder smooth muscle based on immunocytochemical and PCR analysis. Contraction of bladder detrusor is also known to be mediated in part by $P2X_1$ receptors. Consequently, a $P2X_1$ antagonist may have therapeutic value for the treatment of detrusor hyperreflexia. We have identified a novel and selective antagonist, RO-1, with micromolar affinity for the $P2X_1$ receptor. Measuring intracellular calcium flux in recombinant cell lines as a functional readout (Fluorometric Imaging Plate Reader; FLIPR), RO-1 dose-dependently inhibited activation of $P2X_1$ receptors by α,β-methyleneATP (α,β-MeATP) with an pIC_{50} of 5.5 ; the pIC_{50} for other P2X receptors was greater than 4. This compound behaved in a manner consistent with non-competitive antagonism because inhibition of α,β-MeATP-induced calcium flux was not surmountable and IC_{50}s were independent of agonist concentration. Additionally, RO-1 blocked α,β-MeATP-evoked currents in voltage-clamped, dissociated rat bladder smooth muscle cells. 10 μM RO-1 reduced ATP-induced contractions of isolated rat caudal artery smooth muscle in tissue baths by about 50% and reduced contractions of isolated rat bladder smooth muscle tissue strips evoked by β,γ-methyleneATP (β,γ-MeATP). However, it failed to inhibit sacral-stimulated bladder responses in pithed rats whereas pre-treatment with α,β-MeATP or PPADS greatly inhibited bladder responses in the same model. In summary, RO-1 selectively inhibits activation of homomeric $P2X_1$ channels in *in vitro* experiments so it may find utility as a pharmacological tool. It is not clear why inhibition is observed in isolated cell or tissue experiments but not in *in vivo* experiments.

Acknowledgements

The following chapter will be submitted to the Journal of Pharmacology and Experimental Therapeutics in 2008 (modified to fulfill the editing requirements of the publisher) The author of this thesis contributed at an intellectual level for all aspects of the work and conducted all of the FLIPR experiments.

Introduction

A fundamental intercellular signaling mechanism in most mammalian species is mediated via the release into the extracellular space of ATP, and the concomitant or subsequent appearance of its breakdown products, ADP, AMP and adenosine, leading to activation of P2X ligand-gated ion channels ($P2X_{1-7}$) and the P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11-14}$) and P1 (A_1, A_{2A}, A_{2B}, A_3) G-protein coupled receptors. ATP is present in the cytosol of most cells at high (approaching millimolar) concentrations and has been shown to be released exocytotically after cell damage or shear stress (Burnstock, 2007). Furthermore, ATP is also stored in vesicles in nerve terminals, either alone or as a cotransmitter in combination with other neurotransmitters (e.g., norepinephrine) and can be released to produce a depolarization of the post-synaptic membrane, which may be neuronal, secretory or muscular in identity (Zimmermann, 2008).

It was established decades ago that there exists a non-adrenergic non-cholinergic (NANC) component of smooth muscle contraction which could not be blocked by either muscarinic or adrenergic receptor antagonism, (Burnstock et al., 1964). In the late 1960s and early 1970s, the laboratory of Burnstock generated evidence for this NANC component being mediated largely by ATP. This inspired the naming of a group of receptors as the purinergic receptor family, after the purine nucleoside base of the only endogenous ligand known at the time, ATP (Burnstock, 1978). The evidence included data showing that ATP is formed and stored in nerve terminals, released and degraded in the extracellar space and that the effects of transmitter released from nerve stimulation were mimicked by exposure to exogenous ATP (Burnstock, 1972). Later, the gene encoding the $P2X_1$ protein subunit was cloned and shown to be present and responsible for a significant portion of smooth muscle contraction of vas deferens, bladder and gut (Vial and Evans, 2000;Lee et al., 2000;Elneil et al., 2001;Vial and Evans, 2001;O'Reilly et al., 2001) as well as vascular smooth muscle (Lewis and Evans, 2001;Vial and Evans, 2002). These findings, mostly consistent with chemical neurotransmission principles, gave rise to new concepts in our understanding of cell signaling and autonomic physiology and pharmacology (Burnstock, 2009).

It remains possible, given the diversity of receptors for ATP described, that the effects of ATP on visceral and vascular smooth muscle are not restricted solely to activation of a single purinergic receptor subtype. Using the lower urinary tract as an example, $P2X_2$ is also present in rat detrusor smooth muscle, urothelium and nerve fibers (Studeny et al., 2005) and appears to be upregulated in human detrusor smooth muscle tissue samples from patients with idiopathic detrusor instability (O'Reilly et al., 2002). Bladder epithelials cells from many species, including human, express $P2X_3$ receptors (Elneil et al., 2001;Wang et al., 2005) and rat urothelium has recently been shown to express $P2Y_2$ and/or $P2Y_4$ receptors (Chopra et al., 2008), all of which are activated by ATP. Many other systems and processes are mediated by multiple purinergic receptor subtypes including thrombus formation (Gachet, 2008) and gut motility (Galligan and North, 2004) while ATP-mediated changes in vascular tone produce either vasoconstriction through direct action on smooth muscle or vasodilation, most likely acting on endothelial cells, involving $P2X_1$ (Lamont et al., 2006;Harrington et al., 2007), $P2X_4$ (Yamamoto et al., 2000;Yamamoto et al., 2006) or $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors (Gitterman and Evans, 2000;Vial and Evans, 2002). Consequently, selective antagonists for these distinct receptors would be useful for unraveling the complex and interwoven web of purinergic signaling in a variety of tissues.

There are several molecules with moderate to high selectivity for $P2X_1$ over other P2X channels, but they are structurally related to suramin (e.g. NF023, NF279) or PPADS (e.g MRS2220) and due to low oral bioavailability and/or low metabolic stability are not ideal as tools for *in vivo* experiments. A selective $P2X_1$ antagonist with greater metabolic stability in both tissues and whole blood, would be useful to elucidate the specific role of $P2X_1$ channels relative to other purinergic receptors activated by ATP and ADP, particularly in the function of vascular, gastrointestinal and bladder smooth muscle as well as platelet aggregation and thrombus formation. RO-1, identified from a series of chemical library screens at recombinant human $P2X_1$, is an antagonist of moderate potency at $P2X_1$ and is at least 30-fold selectivity over other P2X channels as well as $P2Y_1$. This paper describes its pharmacological characterization, selectivity of action and its use in studies employing isolated rat tissues (detrusor and tail artery smooth muscle contraction) as well as the effect on

spinal electrical stimulation-evoked increases of intravesical bladder pressure in pithed rats after intravenous dosing of RO-1.

Results

RO-1 (Figure 1A), a novel compound originally synthesized as a potential renin inhibitor but shown subsequently to be inactive, was identified as a "hit" from a comprehensive screen of the Roche compound library. RO-1 was observed to inhibit two types of $P2X_1$-mediated functional responses: α,β-MeATP-evoked intracellular calcium flux in CHOK1 cells expressing recombinant human $P2X_1$ channels and ATP-evoked inward currents in acutely dissociated rat detrusor smooth muscle cells natively expressing $P2X_1$ channels (Figure 1B). Inhibition potency estimates (pIC_{50}) were 5.5 and 5.2 for recombinant human and natively expressed rat $P2X_1$, respectively. Although RO-1 is not a highly potent compound, it is at least 30-fold selective for $P2X_1$ over other P2X channels as well as $P2Y_1$ (Table 1).

Having established RO-1 as a selective $P2X_1$ antagonist, experiments were conducted to examine the effect of RO-1 on isolated tissue strips in organ bath studies. Figure 2A shows the dose-dependent inhibition by RO-1 of β,γ-MeATP-evoked contractions of rat bladder smooth muscle tissue strips. Concentrations of RO-1 as low as 0.1 μM significantly inhibited β,γ-MeATP -evoked contractions which were largely abolished by 10 μM RO-1 (approximately 70% reduction of contractile force). In contrast, when carbachol was used to evoke tissue contractions, RO-1 inhibited the tissue contraction to a lesser extent (approximately 30% reduction of contractile force), even at the highest concentration of 10 μM (Figure 2B). Similarly, RO-1 produced concentration-dependent inhibitions of ATP-evoked rat tail artery contractions whereas similar concentrations of RO-1 (1 and 10 μM) had significantly less effect on norepinephrine-evoked contractions.

Furthermore, contractions evoked by electrical field stimulations under conditions designed to isolate purinergic from non-purinergic components of muscle contraction produced similar results. In the presence of 30 μM PPADS (to isolate the adrenergic component; Figure 3B), 1 μM RO-1 inhibited contractions of tail artery evoked by 32 and 64 Hz by 9 and 12%, respectively, while in the presence of 3 μM

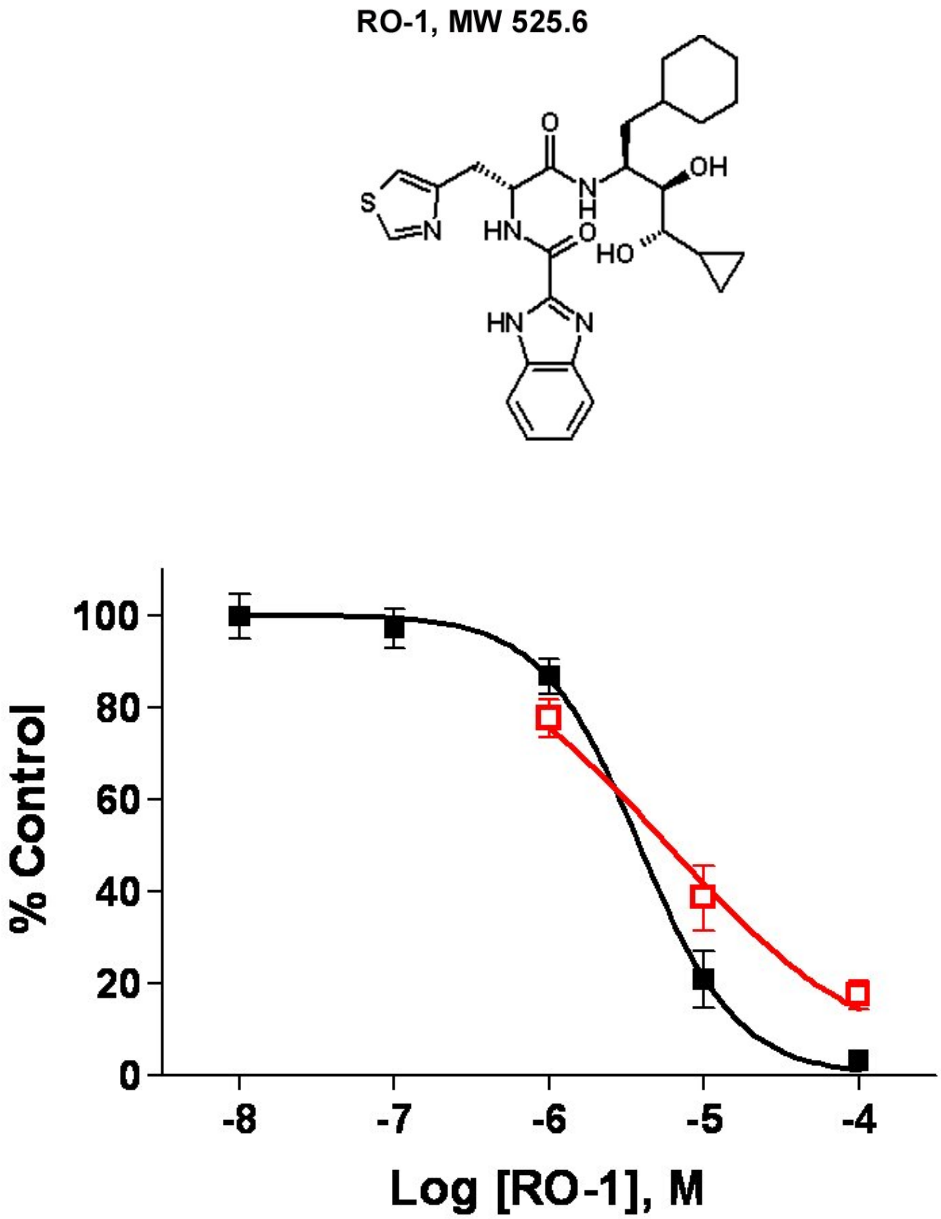

Figure 1. Structure and *in* vitro potency estimates of RO-1 A) Chemical structure of RO-1; MW = 525.6. B) Concentration-effect curves showing the inhibition of cytosolic calcium flux evoked by 0.1 μM α,β-MeATP in CHO-K1 cells expressing recombinant human $P2X_1$ receptors (filled black squares; pIC_{50} = 5.5) or currents evoked by 1 μM ATP in voltage-clamped, dissociated rat bladder smooth muscle cells (open red squares; pIC_{50} = 5.2)

Table 1: Pharmacological selectivity of RO-1

Receptor	N	pIC_{50}
DSM	3	5.2
$hP2X_1$	3	5.5
$hP2X_2$	3	<4
$hP2X_3$	3	<4
$hP2X_{2/3}$	3	<4
$hP2X_4$	3	< 4
$rP2X_5$	3	< 4
$hP2X_7$	3	< 4
$hP2Y_1$	3	< 4

prazosin (to isolate the purinergic component; Figure 3A), 1 μM RO-1 was about four-fold more effective at inhibiting 32 and 64 Hz-evoked contractions (39 and 43%, respectively). A lower concentration of RO-1 (0.1 μM) was also far more effective at blocking the purinergic component of 32 and 64 Hz-evoked contractions (15 and 13%, respectively) than the adrenergic component (6 and 0%). Tissue contractions evoked by lower frequencies were too small to differentiate the effect of RO-1 on purinergic- and adrenergic-mediated contractions.

The plasma profile of RO-1 after a single 10 mg/kg intravenous dose in rats (n = 6) is shown in Figure 4. The maximal average plasma concentration (Cmax) of 9587 ng/mL (18.2 μM) was measured at the first time point of 5 minutes and a plot of the subsequent decline in plasma concentration produced a half-life ($t_{1/2}$) estimate of 1.1 hrs (Figure 4; Table 2). Since 98.5 % of RO-1 was determined to be bound to plasma proteins, the free plasma concentration of RO-1 at the C_{max} was estimated to be 144 ng/mL (0.27 μM).

Figure 5 shows the effect of RO-1 (10 mg/kg, i.v.) and PPADS (10 mg/kg, i.v.) on segmental (L6-S2) spinal electrical stimulation-evoked increases of intravesical bladder pressure in pithed rats. PPADS reduced the pressure increase by > 64 - 72 % at all frequencies tested while RO-1 only slightly reduced pressure increases at 4, 8 and 16 Hz.

Discussion

Progression in the field of purinoceptor pharmacology has been slowed by the relative lack of selective antagonists useful as tools for both in vitro and in vivo experimental purposes. Although some potent and selective antagonists of $P2X_3/P2X_{2/3}$ and $P2X_7$ have been identified in recent years (Ford et al., 2006;Donnelly-Roberts and Jarvis, 2007;Broom et al., 2008;Furber et al., 2007), primarily as a consequence of drug discovery efforts targeting P2X channels of particular therapeutic interest, truly selective and useful antagonists for many of the other P2X receptor subtypes are still largely lacking. Since the expression of purinergic receptor subtypes is quite widespread, the value of selective tools to identify the specific function of specific receptor subtypes in tissues and organ systems of interest can not be overstated.

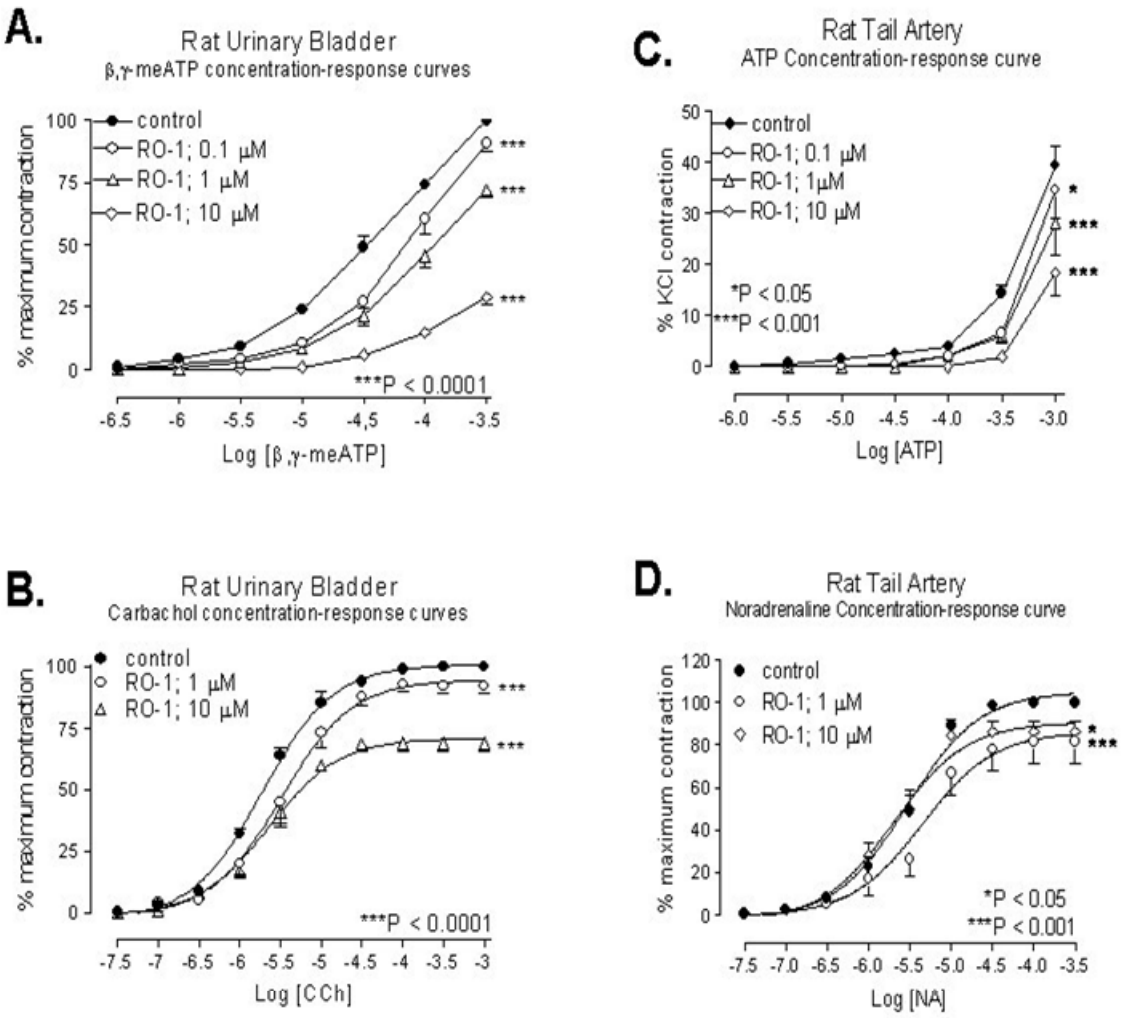

Figure 2. Concentration-response curves for agonist-evoked rat tissue contractions A) Contractions of rat urinary bladder tissue strips evoked by increasing concentrations of β,γ-MeATP in the presence of vehicle or 0.1 – 10 μM RO-1 (normalized to the maximal contraction evoked by β,γ-MeATP) B) Contractions of rat urinary bladder tissue strips evoked by increasing concentrations of carbachol in the presence of vehicle or 1 – 10 μM RO-1 (normalized to the maximal contraction evoked by carbachol) C) Contractions of rat tail artery tissue evoked by increasing concentrations of ATP in the presence of vehicle or 0.1 – 10 μM RO-1 (normalized to the maximal contraction evoked by KCl) D) Contractions of rat tail artery tissue evoked by increasing concentrations of noradrenaline in the presence of vehicle or 1 – 10 μM RO-1 (normalized to the maximal contraction evoked by noradrenaline); asterisks denote statistical significance.

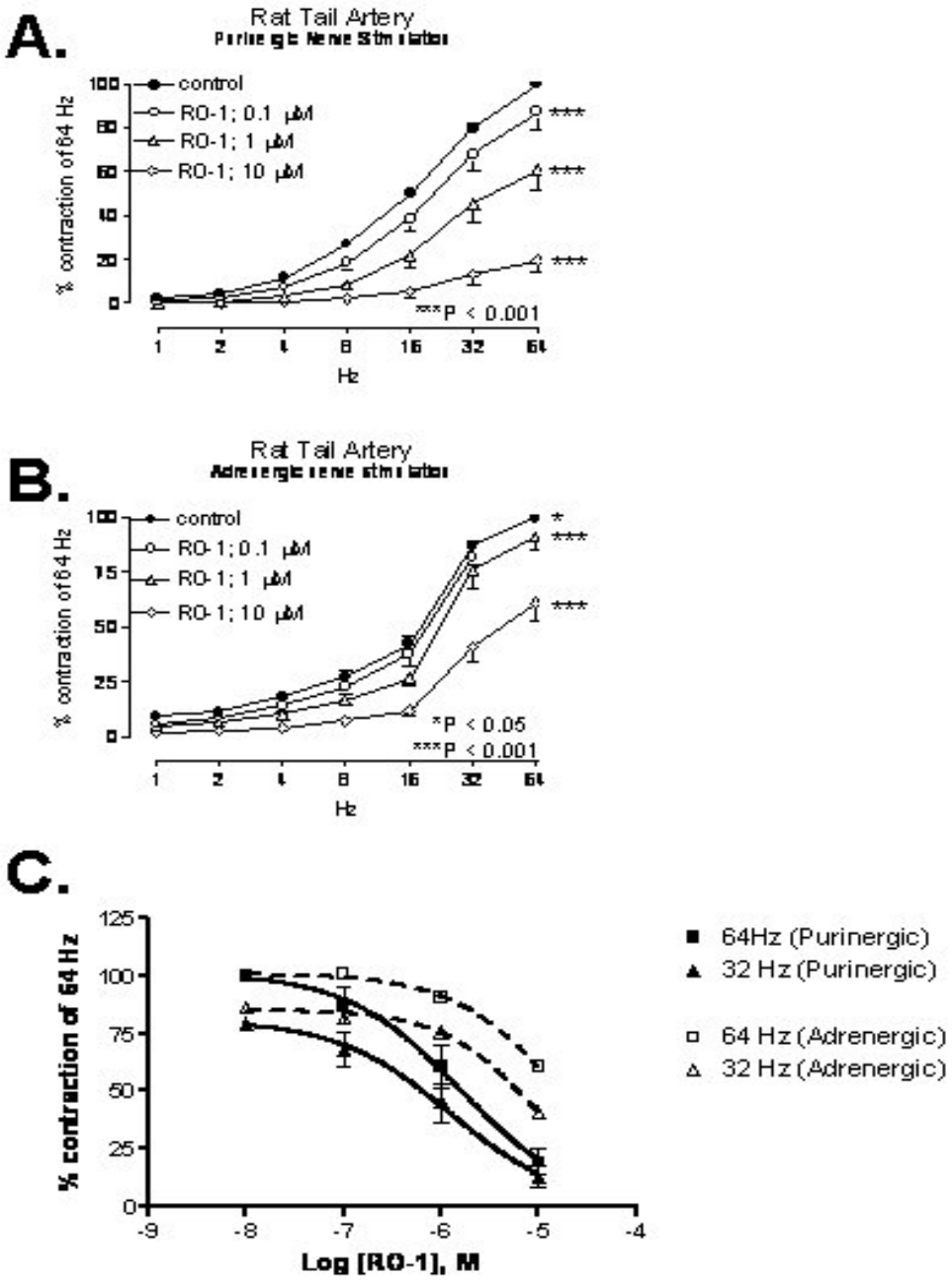

Figure 3. Frequency-response curves for electrical field stimulation-evoked rat tail artery tissue contractions A) Frequency-response curves (1 – 64 Hz, 75 V, 0.1 msec) in the presence of 3 μM prazosin and vehicle or 0.1 – 10 μM RO-1 B) Frequency-response curves (1 – 64 Hz, 75 V, 0.1 msec) in the presence of 30 μM PPADS and vehicle or 0.1 – 10 μM RO-1 C) Concentration-response relationships of 32 or 64 Hz-evoked rat tail artery contractions in the presence of increasing concentrations of RO-1. Curves were constructed in the presence of 3 μM prazosin (filled symbols) or 30 μM PPADS (open symbols)

RO-1 is an early (i.e. not chemically optimized) lead identified during a comprehensive screen of the Roche chemical library for antagonists of recombinant human $P2X_1$. Although RO-1 is only a moderately potent $P2X_1$ antagonist, it retains at least 30 fold selectivity for $P2X_1$ over other P2X subtypes as well as $P2Y_1$ (the only P2Y subtype tested) and is therefore potentially useful for selectively blocking this receptor in tissues containing multiple purinergic receptor subtypes. In fact, concentrations of RO-1 as low as 100 nM (representing 1000-fold selectivity over other P2X subtypes; see Table 1) selectively blocked agonist-evoked purinergic contractions of rat urinary bladder and tail artery while having no effect on cholinergic or adrenergic agonist-evoked contractions, respectively (see Figure 2). Although higher concentrations of RO-1 (10 μM) reduced carbachol- and norepinephrine-evoked contractions of rat urinary bladder and tail artery tissues, respectively, the magnitude of inhibition was significantly less than the inhibition of the corresponding purinergic receptor-mediated tissue contractions (see Figure 2). One reasonable explanation is that a purinergic component of tissue contraction is elicited upon activation of cholinergic receptors. Indeed, recent work suggests that activation of cholinergic receptors in rat urinary bladder tissue strips results in the partial desensitization of the purinergic (NANC) component of tissue contraction, possibly through the postjunctional release of ATP mediated by muscarinic receptors (Lai et al., 2008). It has also been shown in rat vas deferens that stimulation of P2X receptors facilitates the nerve-mediated release of norepinephrine (Queiroz et al., 2003), so if an analogous process is occurring in rat urinary bladder, it is possible that a P2X antagonist could appear to block cholinergic-mediated contraction directly.

The selectivity of RO-1 for blocking purinergic-mediated rat tail artery contraction (isolated through incubation of tissues with 3 μM atropine) over adrenergic-mediated contraction (isolated through incubation of tissues with 30 μM PPADS) was also seen when contractions were elicited by electrical field stimulation (1 – 64 Hz), with the greatest selectivity seen at the higher frequencies (32 and 64 Hz). However, it should be pointed out that relatively high concentrations of RO-1 (10 μM) also reduced adrenergic-mediated EFS-evoked arterial contractions. This observation may indicate simply that 30 μM PPADS was not sufficient to completely

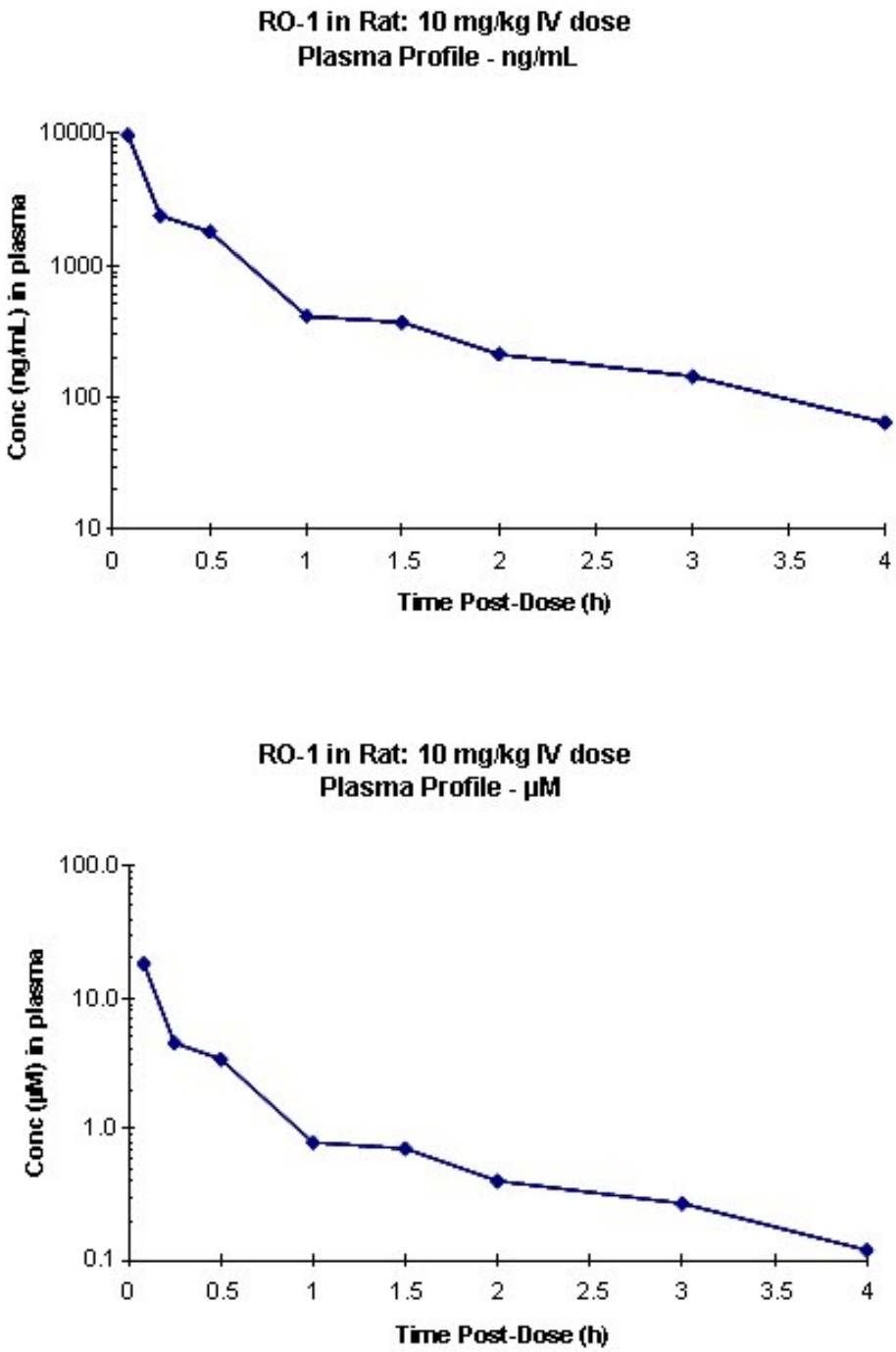

Figure 4. Plasma profile in rats of RO-1 after a single, bolus intravenous dose (10 mg/kg) A) data represented as ng/mL, total plasma concentration B) data represented as µM, total plasma concentration

Table 2: Pharmacokinetic properties of RO-1

	Plasma Concentration of RO-1 in rat (10 mg/kg bolus i.v. dose)					
	Total			Free		
Time (hr)	Mean (ng/mL)	SD (ng/mL)	Mean (μM)	Mean (μM)	**T1/2 (hr)**	**1.10**
0.0833	9587	6708	18.2	0.273	**AUC (0-4hr; h·ng/mL)**	**3096**
0.25	2367	974	4.5	0.067	**AUC(total;h·ng/mL)**	**3197**
0.5	1805	955	3.4	0.051	**Cl (mL/min/kg)**	**3.13**
1	418	131	0.8	0.012	**VdB (L/kg)**	**4.96**
1.5	370	1	0.7	0.011		
2	212	132	0.4	0.006		
3	144	55	0.3	0.004		
4	63	27	0.1	0.002		

block all P2X receptors that would be activated by prejunctional EFS-evoked release of ATP. Alternatively, the possibility of direct antagonism of adrenergic receptors can not be ruled out because RO-1 was not tested for the ability to inhibit α_1 adrenergic receptor subtypes, though even if this were the case, RO-1 would still be significantly selective for the purinergic component of tissue contraction.

Another possible explanation for the modest suppression of EFS-evoked adrenergic-mediated contractions of rat tail artery by RO-1 is that there exists an underlying synergistic relationship between purinergic and adrenergic receptors in this tissue. Purinergic cotransmission, first proposed in 1976 (Burnstock, 1976), was shown early on to occur in many tissues including cat nictitating membrane (Langer and Pinto, 1976), guinea pig vas deferens (Fedan et al., 1981) and rat tail artery (Sneddon and Burnstock, 1984). Not only was cotransmission shown to occur in vascular tissue but synergistic effects on the contraction of rat mesenteric arteries mediated through postjunctional α_1 and P2X receptors were observed as well (Ralevic and Burnstock, 1990).

The study of the role of purinergic receptors in the efferent control of urinary bladder function derived initially from observations of NANC contractions in guinea pig urinary bladder (Burnstock et al., 1978) and has continued to this day (for a recent review, see (Ford et al., 2006). In many species, ATP released from parasympathetic nerves originating from the sacral and lower lumbar regions of the spinal cord is an important excitatory neurotransmitter producing smooth muscle contraction in the urinary bladder (Andersson and Wein, 2004). The pithed rat segmental (L6-S2) spinal electrical stimulation-evoked intravesical pressure change model was developed to isolate parasympathetic-mediated effects on bladder function without confounding effects from central pathways (Hegde, 1998). That work, as well as the current work, shows there is a significant purinergic component which can be blocked by the non-selective P2X antagonist, PPADS, or desensitized through pre-incubation with α,β-MeATP (Hegde et al., 1998). Although a single intravenous bolus dose of RO-1 (10 mg/kg) resulted in only modest inhibition of intravesical bladder pressure increases, it is likely this dose was too small to produce a profound effect. Adjusting for protein binding (98.5%), the free plasma concentration of RO-1 is 270 nM after 5 minutes and has dropped to only 68 nM after 15 minutes (see Table

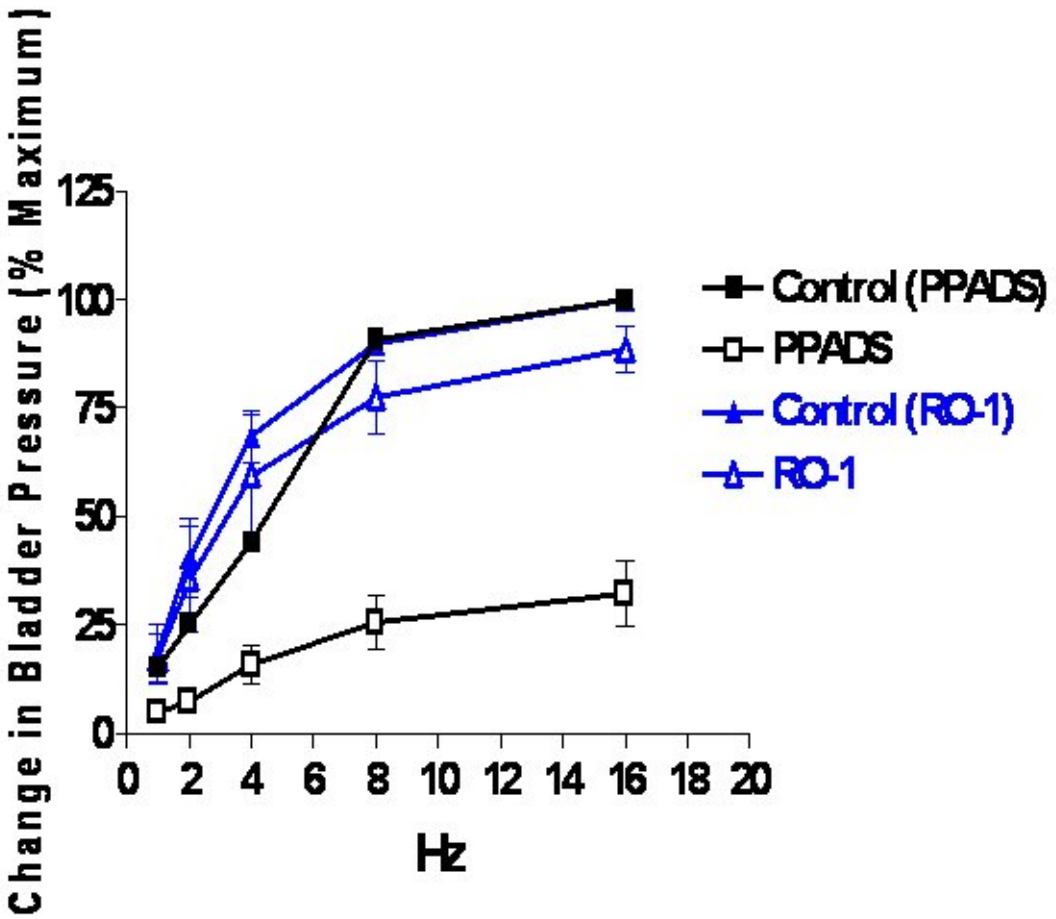

Figure 5. Frequency-response relationship of PPADS (10 mg/kg, i.v.) or RO-1 (10 mg/kg, i.v.) on segmental spinal stimulation-evoked (NANC) increases of intravesical bladder pressure in pithed rats (n = 3)

2). Further studies would call for an increased dose, although the low solubility of RO-1 in saline could pose technical difficulties. Nevertheless, even at these very low free plasma concentrations, there appeared to be a small reduction of 8 and 16 Hz-evoked intravesical bladder pressure increases.

In addition to potential therapeutic effects on lower urinary tract function, a selective $P2X_1$ antagonist could serve as a target for a male contraceptive. The fertility of male $P2X_1^{-/-}$ mice were reduced by at least 90% through the elimination of the purinergic-mediated component of sympathetic nerve stimulated contraction of the vas deferens (Mulryan et al., 2000). Nerve-mediated contractions of human vas deferens are also mediated partially by purinergic receptors; EFS-evoked contractions of human vas deferens tissue strips were reduced by 40% in the presence of the non-selective P2X antagonist, PPADS (Banks et al., 2006). Platelet aggregation and thrombus formation is another process where at least three subtypes of purinergic receptor, $P2Y_1$, $P2Y_{12}$ and $P2X_1$, each with a distinct function. $P2Y_1$, activated by ADP, is important for ADP- and collagen-induced shape change and platelet aggregation while $P2Y_{12}$, the target of the antiplatelet drugs clopidogrel and prasugrel, is involved in the amplification of platelet aggregation induced by a variety of agonists, including ADP as well as collagen, thrombin, serotonin and many others (Gachet, 2008). The putative role of $P2X_1$ channels in thrombus formation has been suggested by gene deletion (Hechler et al., 2003) or overexpression (Oury et al., 2003) studies and to a lesser extent through the use of $P2X_1$ selective suramin analogues, such as NF449 (Kassack et al., 2004). Activation of $P2X_1$ evokes transient shape change (Rolf et al., 2001) and appears to play a role in collagen- and shear-induced aggregation, but further elucidation of the role of $P2X_1$ could be assisted through the use of a selective antagonist. Although RO-1 is not fully optimized, it is selective for $P2X_1$ over other P2X receptors and at least one P2Y receptor ($P2Y_1$) and has been shown both previously (King et al., 2004) and in the current work to block purinergic-mediated contractions of visceral and vascular smooth muscle. Further optimization would be expected to improve efficacy further and with the right physicochemical and metabolic attributes, potentially serve as a valuable therapeutic for the regulation of vascular tone, thrombus formation and genitourinary function.

Chapter 5: Expression And Function Of Rat Urothelial P2Y Receptors

Abstract

The control and regulation of the lower urinary tract are partly mediated by purinergic signaling. This study investigated the distribution and function of P2Y receptors in the rat urinary bladder. Application of P2Y agonists to rat urothelial cells evoked increases in intracellular calcium; the rank order of agonist potency (pEC_{50} ± SE) was ATP (5.10 ± 0.07) > UTP (4.91 ± 0.14) > UTPγS (4.61 ± 0.16) = ATPγS (4.70 ± 0.05) > 2-methylthio adenosine 5'-diphosphate = 5'-(*N*-ethylcarboxamido)adenosine = ADP (<3.5). The rank order potency for these agonists indicates that urothelial cells functionally express $P2Y_2$/$P2Y_4$ receptors, with a relative lack of contribution from other P2Y or adenosine receptors. Real-time PCR, Western blotting, and immunocytochemistry confirmed the expression of $P2Y_2$ and to a lesser extent $P2Y_4$ in the urothelium. Immunocytochemical studies revealed expression of $P2Y_2$ staining in all layers of the urothelium, with relative absence of $P2Y_4$. $P2Y_2$ staining was also present in suburothelial nerve bundles and underlying detrusor smooth muscle. Addition of UTP and UTPγS was found to evoke ATP release from cultured rat urothelial cells. These findings indicate that cultured rat urothelial cells functionally express $P2Y_2$/$P2Y_4$ receptors. Activation of these receptors could have a role in autocrine and paracrine signaling throughout the urothelium. This could lead to the release of bioactive mediators such as additional ATP, nitric oxide, and acetylcholine, which can modulate the micturition reflex by acting on suburothelial myofibroblasts and/or pelvic afferent fibers.

Acknowledgements

The following chapter was published in Am. J. Physiol. Renal Physiol. in 2008 (Chopra et al., 2008). The author of this thesis contributed equally to the first author (Bikramjit Chopra) at an intellectual level for all aspects of the work and conducted all of the FLIPR experiments.

Introduction

The control and regulation of lower urinary tract functions are regulated by the complex integration of sympathetic, parasympathetic, and afferent pathways(de Groat, 2006). These highly regulated processes are mediated by neural controls involving many neurotransmitters, including acetylcholine, amino acids, nitric oxide, neuropeptides, and monoamines, as well as ATP acting on purinergic receptors (de Groat, 2006). Kasakov and Burnstock(Kasakov and Burnstock, 1983) initially demonstrated that parasympathetic neural contractions of the bladder were in part mediated by nonadrenergic, noncholinergic atropine resistant purinergic transmission. Purinergic transmission is also involved in transducing bladder mechanosensation and other forms of afferent information to the central nervous system(de Groat, 2004;de Groat, 2006;Ford et al., 2006). For example, intravesical administration of ATP or α,β-methylene ATP into the bladder evokes bladder hyperactivity, an effect that is blocked with selective purinergic receptor antagonists (Namasivayam et al., 1999;Rong et al., 2002;Vlaskovska et al., 2001).

P2 purinergic and pyrimidinergic receptors can be divided into two major families, ionotropic ligand-gated P2X and metabotropic G-protein coupled P2Y receptors. To date, seven P2X receptors have been identified ($P2X_{1–7}$) and eight P2Y receptors have been recognized as molecularly distinct proteins that can produce functional responses ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). Urinary bladders of a number of species, such as human (O'Reilly et al., 2001), rat (Lee et al., 2000), and cat(Birder et al., 2004), are known to express purinergic receptors, including $P2X_1$ on detrusor smooth muscle (Lee et al., 2000;Vial and Evans, 2000) and $P2X_3$ on suburothelial nerve plexi and urothelium (Birder et al., 2004;Cockayne et al., 2000;Lee et al., 2000).

As with many hollow organs and sacs, distention or mechanical stretch evokes the release of ATP from the urothelium lining the urinary bladder (Ferguson et al., 1997;Sun et al., 2001;Vlaskovska et al., 2001). Urothelial ATP release in response to distention/mechanical stimulation occurs from both mucosal and serosal compartments (Lewis and Lewis, 2006). Urothelial-released ATP is thought to

activate $P2X_3$ receptors expressed on suburothelial nerves in a paracrine manner, which convey afferent information to the central nervous system, leading to altered micturition reflexes. Indeed, $P2X_3$-deficient mice exhibit normal distention-evoked urothelial ATP release but marked urinary bladder hyporeflexia, characterized by decreased voiding frequency and increased bladder capacity (Cockayne et al., 2000;Vlaskovska et al., 2001). The ability of the urothelium to sense mechanical distention and convey information to afferent nerves supports the notion that the urothelium plays an important sensory role in the urinary bladder (Birder, 2001;Birder, 2005;Birder, 2006;de Groat, 2006;Lazzeri, 2006;Wein, 2005).

The pyrimidine nucleotide UTP and the dinucleotides ADP and UDP bind to the P2Y family of metabotropic heptahelical G-protein coupled receptors. Birder et al. (Birder et al., 2004) reported the constitutive expression of $P2Y_1$, $P2Y_2$, and $P2Y_4$ in feline urothelium and reduction of $P2Y_2$ in a naturally occurring model of feline interstitial cystitis (FIC), suggesting that P2Y receptors may play a role in urothelial function. $P2Y_6$ receptors have also been reported to be expressed on the guinea-pig urothelium (Sui et al., 2006). Relatively little, however, is known about the distribution and function of P2Y receptors in the rat bladder. This study investigated the expression of P2Y receptors on the rat urothelium.

Results

FLIPR. FLIPR analysis of cultured rat urothelial cells after stimulation with purinergic receptor agonists revealed that these agents evoke increases in intracellular calcium. These responses typically reached peak within 30 s and fully recovered to baseline levels between 2–3 min after application (Figure 1D). The rank order of agonist potency (pEC_{50} ± SE) was ATP (5.10 ± 0.07) ≥UTP (4.91 ± 0.14) > UTPγS (4.61 ± 0.16) = ATP γ S (4.70 ± 0.05) >> 2-MeSADP = 5-(*N*-ethylcarboxamido)adenosine (NECA) = ADP = UDP (<3.5; see Figure 1A). Curve shift analysis with a number of P2 receptor antagonists revealed that suramin (30 μM) and the selective $P2Y_1$ receptor antagonist MRS2179 (30 μM) had little or no effect on either UTP- or UTPγS-evoked increases in intracellular calcium (Figure 1*B* and 1*C*). In contrast, PPADS (30 μM) produced a rightward shift in UTP- and UTPγS-

evoked changes in intracellular calcium (Figure 1*B–D*). Furthermore, PPADS (30 μM) produced an inhibition of cytosolic calcium increases evoked by UTP (30 μM) and/or UTPγS (30 μM), with a pIC_{50} value of 4.8 (data not shown). These findings demonstrate that UTP- and UTPγS-evoked responses in cultured rat urothelial cells are sensitive to PPADS.

Fura-2. Bath application of either UTP (10 μM) or UTPγS (10 μM) evoked a rapid increase in $[Ca^{2+}]_i$ in cultured rat urothelial cells. These responses typically reached peak within 1 min and fully recovered to baseline levels between 2–3 min after application. The mean UTP and UTPγS (10 μM) responses were 30 ± 3% (n = 30) and 31.6 ± 3.0% (n = 32) of the peak ionomycin (5 μM) response, respectively (Figure 2A and 2D). In the absence of extracellular calcium, the amplitudes of UTP (10 μM; 31.6 ± 1.9%)- and UTPγS (10 μM; 38.8 ± 3.0%)-evoked responses were not significantly different than those evoked in medium containing normal (2 mM) extracellular calcium (Figure 2B and 2E). Thapsigargin (10 μM) was used to deplete intracellular calcium stores by inhibiting intracellular calcium reuptake by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump. Both UTP (10 μM)- and UTPγS (10 μM)-evoked responses were significantly attenuated under these conditions (Figure 2C and 2F). UTP and UTPγS responses after pretreatment with thapsigargin (10 μM) were 3.5 ± 1.1% (n = 30; $P < 0.01$) and 2.1 ± 0.8% (n = 30; $P < 0.01$) of the peak ionomycin (5 μM) response, respectively. These findings indicate that UTP- and UTPγS-evoked changes in intracellular calcium in cultured rat urothelial cells result from the release of calcium from intracellular stores. Inhibition of phospholipase C (PLC) with U73122 (10 μM) significantly attenuated UTP (10 μM) and UTPγS (10 μM); the evoked responses were 7.3 ± 2.7% (n = 14; $P < 0.01$) and 14.4 ± 1.5% (n = 17; $P < 0.01$) of the peak ionomycin (5 μM) responses, respectively (Figure 2G, 2H and 2I), suggesting UTP- and UTPγS-evoked responses are mediated by PLC-linked processes.

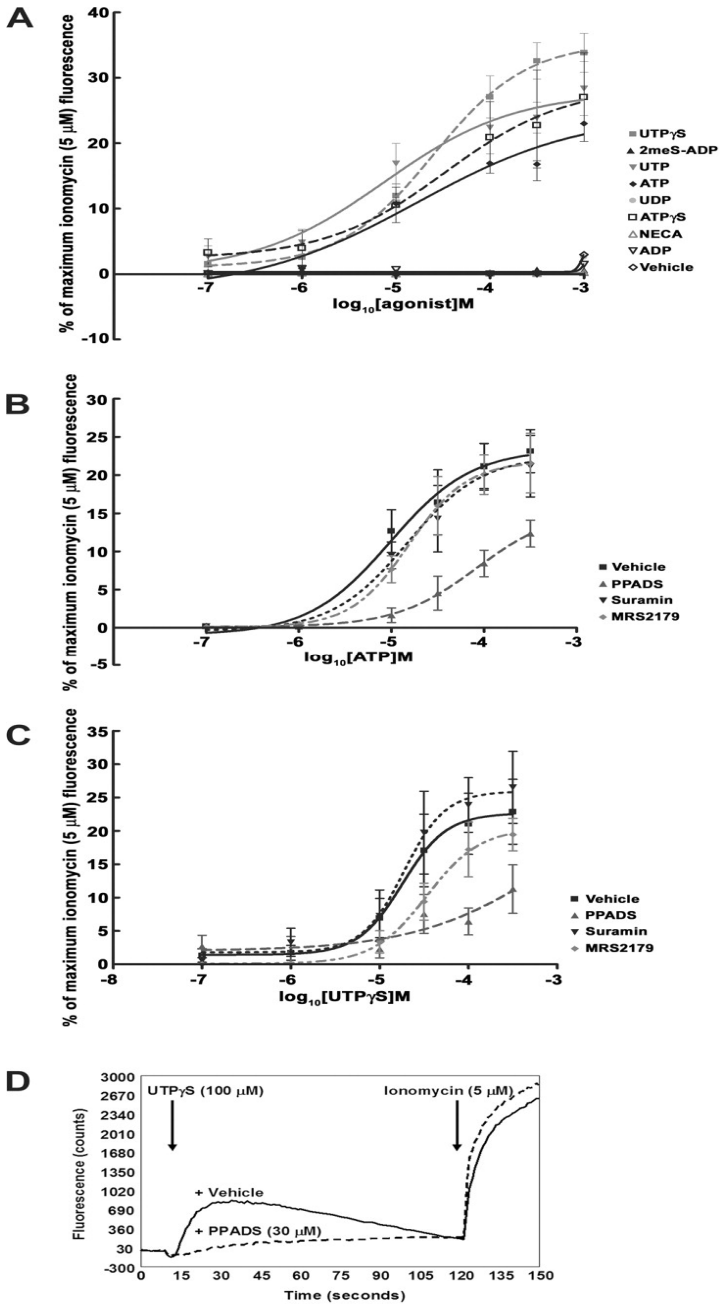

Figure 1: FLIPR analysis of changes in intracellular calcium in cultured rat urothelial cells by purinergic receptor agonists and antagonists. *A*: concentration response (100 nM to 1 mM) of a range of purinergic receptor agonists in cultured rat urothelial cells. *B*, *C*: effect of the P2 receptor antagonists pyridoxal-phosphate-6-azophenyl-2,4-disulfonate (PPADS; 30 μM), suramin (30 μM), and MRS2179 (30 μM) on ATP- and UTPγS-evoked changes in intracellular calcium in cultured rat urothelial cells. *D*: representative continuous traces of changes in fluo-3 fluorescence in response to UTPγS (100 μM) alone and in the presence of PPADS (30 μM). NECA, 5-(*N*-ethylcarboxamido)adenosine.

Western blotting. Expression of P2Y receptors in urothelial, detrusor smooth muscle, and whole bladder protein lysates was assessed using Western blotting; whole rat brain protein lysates were used as positive controls for antibody binding. Strong $P2Y_2$ immunoblotting (60 kDa) was also present in all tissue samples assessed. $P2Y_4$ immunoblotting (80–85 kDa) was observable to a lesser extent relative to the other P2Y subtypes. In one of three rats assessed, $P2Y_4$ immunoblotting was observable in the rat urothelium and detrusor smooth muscle and absent in the other two rats assessed (Figure 3A and 3B, top).

Expression of P2Y mRNA in the urothelium and smooth muscle. The relative expression of P2Y receptor mRNA compared with β-actin in the urothelium and detrusor smooth muscle was assessed using quantitative real-time PCR. The rank order for expression of urothelial P2Y mRNA was $P2Y_2 > P2Y_4$ (Figure 3A and 3B, bottom). However, no statistical significance was found between the levels of the two receptors. Levels of P2Y receptor mRNA was between two and seven times lower in the detrusor smooth muscle compared with the urothelium (Figure 3A and 3B, bottom). The rank order for expression of P2Y mRNA in the detrusor was $P2Y_2 > P2Y_4$.

Immunocytochemistry. Immunocytochemical studies provided evidence for the expression of $P2Y_2$ with very little or no detectable $P2Y_4$ receptor staining in the normal rat urinary bladder (Figure 4). $P2Y_2$ immunoreactivity was present in the urothelium (Figure 4A), putative nerve fibers/plexi, as indicated by PGP 9.5 staining (Figure 4J – O), and detrusor smooth muscle (data not shown). Colocalization experiments with putative markers of the urothelium, cytokeratin 17 (basal cells) and cytokeratin 20 (apical cells), revealed $P2Y_2$ immunoreactivity to be present in all layers of the urothelium and restricted primarily to the plasma membrane and cytoplasm (Figure 4F and 4I). $P2Y_4$ receptor staining was not present in the normal rat urinary bladder (Figure 4B). P2Y1 receptor expression was not assessed in the present study due to concerns about antibody binding specificity. Omission of primary antibodies from the incubation buffer completely attenuated secondary antibody labeling (Figure 4C).

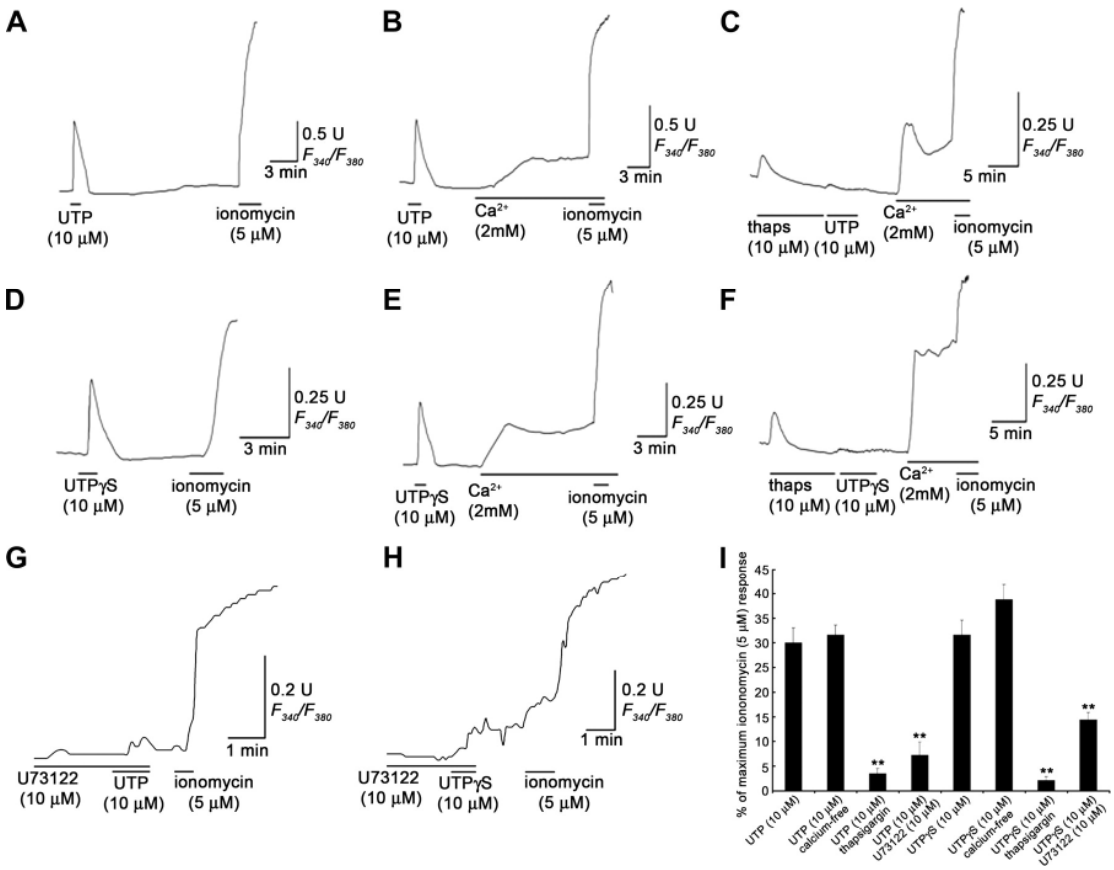

Figure 2: UTP- and UTP‴S-evoked changes in intracellular calcium in cultured rat urothelial cells are significantly attenuated by inhibition of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump and phospholipase C. *A*, *D*: UTP (10 μM)- and UTP‴S (10 μM)-evoked changes in intracellular calcium concentration ($[Ca^{2+}]_i$) normal physiological calcium (2 mM). *B*, *E*: in the absence of extracellular calcium, the amplitudes of UTP (10 μM)- and UTP‴S (10 μM)-evoked responses were not significantly different that those in normal extracellular calcium. *C*, *F*: thapsigargin (10 μM) was used to deplete intracellular calcium stores by inhibiting the SERCA pump. Both UTP (10 μM) and UTP‴S (10 μM) responses were abolished under these conditions. *G*, *H*: pretreatment of rat urothelial cells with the phospholipase C inhibitor U73122 (10 μM) significantly attenuated both UTP (10 μM)- and UTP‴S (10 μM)-evoked changes in $[Ca^{2+}]_i$. *I*: histograms illustrating the mean changes in $[Ca^{2+}]_i$ evoked by UTP (10 μM) and UTP‴S (10 μM) as a percentage of the maximum ionomycin (5 μM) response in rat urothelial cells alone and after pretreatment with thapsigargin (10 μM) and U73122 (10 μM; $^{**}P < 0.01$).

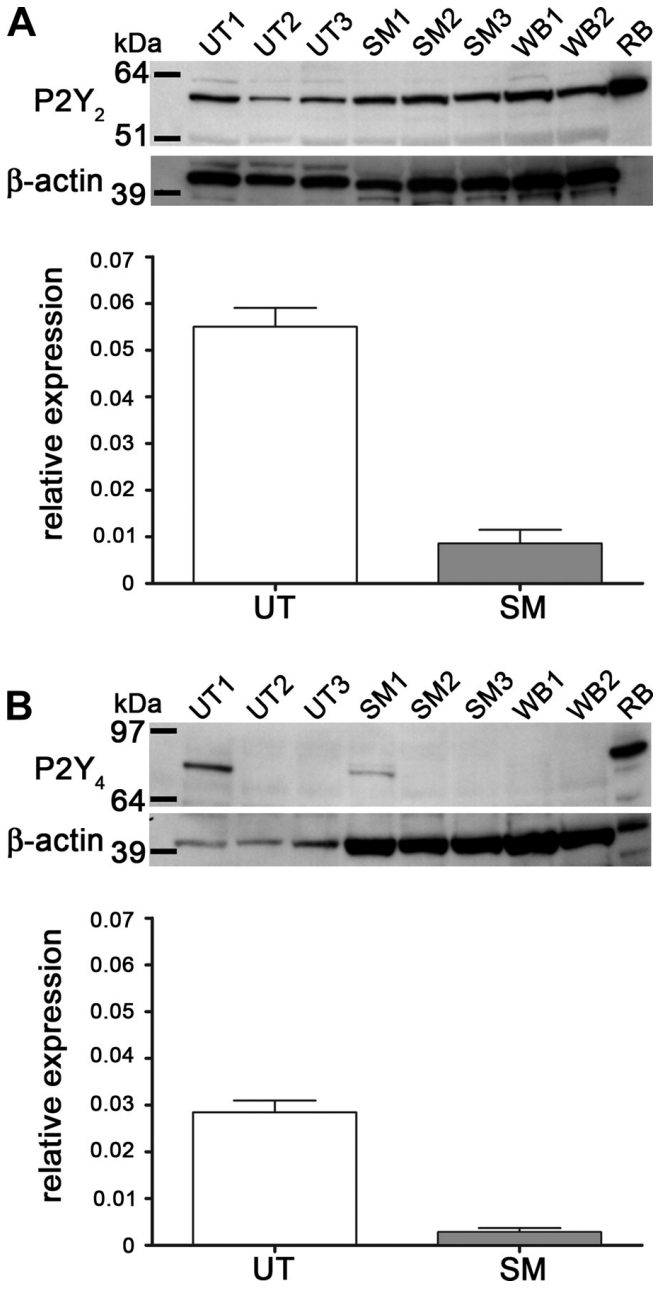

Figure 3: Gene and protein expression of $P2Y_{2,4}$ receptors in the rat urinary bladder. Expression of $P2Y_2$ (*A*, *top*) and $P2Y_4$ (*B*, *bottom*) receptors in urothelial (UT1–3), detrusor smooth muscle (SM1–3), and whole bladder (WB1–2) protein lysates was assessed using Western blotting; whole rat brain (RB) protein lysate was used as positive control for antibody binding. Immunoblotted proteins corresponding to $P2Y_2$ (60 kDa) and $P2Y_4$ (85 kDa) were detected. $P2Y_4$ immunoblotting was only detected in one of three rat urinary bladders tested. Relative expressions of $P2Y_2$ (*A*, *bottom*) and $P2Y_4$ (*B*, *bottom*) RNA in the urothelium and detrusor smooth muscle compared with β-actin; $n = 3$ rats was assessed using quantitative PCR. UT, urothelium; SM, smooth muscle.

P2Y Receptor Evoked ATP Release From Cultured Rat Urothelial Cells

Serosal release of ATP from the urothelium has been reported to activate underlying pelvic afferent fibers in a paracrine manner (Cockayne et al., 2000;Ferguson et al., 1997;Vlaskovska et al., 2001). We assessed whether activation of P2Y receptors can evoke the release of ATP from cultured rat urothelial cells.

Due to interference with the luciferin-luceriferase based ATP assay mix, numerous purine nucleotides were not assessed, including ATP, ATPγS, ADP, UDP, and 2-MeSADP; in addition, PPADS and suramin were also not tested. Agonists were bath applied for 60 s, and typical responses reached a peak between 10–30 min after application and returned to baseline levels 15 min postapplication (Figure 5A). In many cases, the ATP release evoked from rat urothelial cells after addition of agonists exhibited an oscillatory release profile over the time period assessed (Figure 5A). UTP (10 μM) consistently and reproducibly evoked the release of ATP from cultured rat urothelial cells; average ATP release evoked was 268 ± 47 nM/100 μl; n = 3 independent cultures (Figure 5A and 5B). The selective $P2Y_{2/4}$ receptor agonist UTPγS also evoked ATP release from cultured rat urothelial cells in a dose-related manner; the average levels of ATP released were 534 ± 56 nM/100 μl, 473 ± 62 nM/100 μl, and 91 ± 12 nM/100 μl after application of 10, 5, and 1 μM UTPγS, respectively (Figure 5A and 5B).

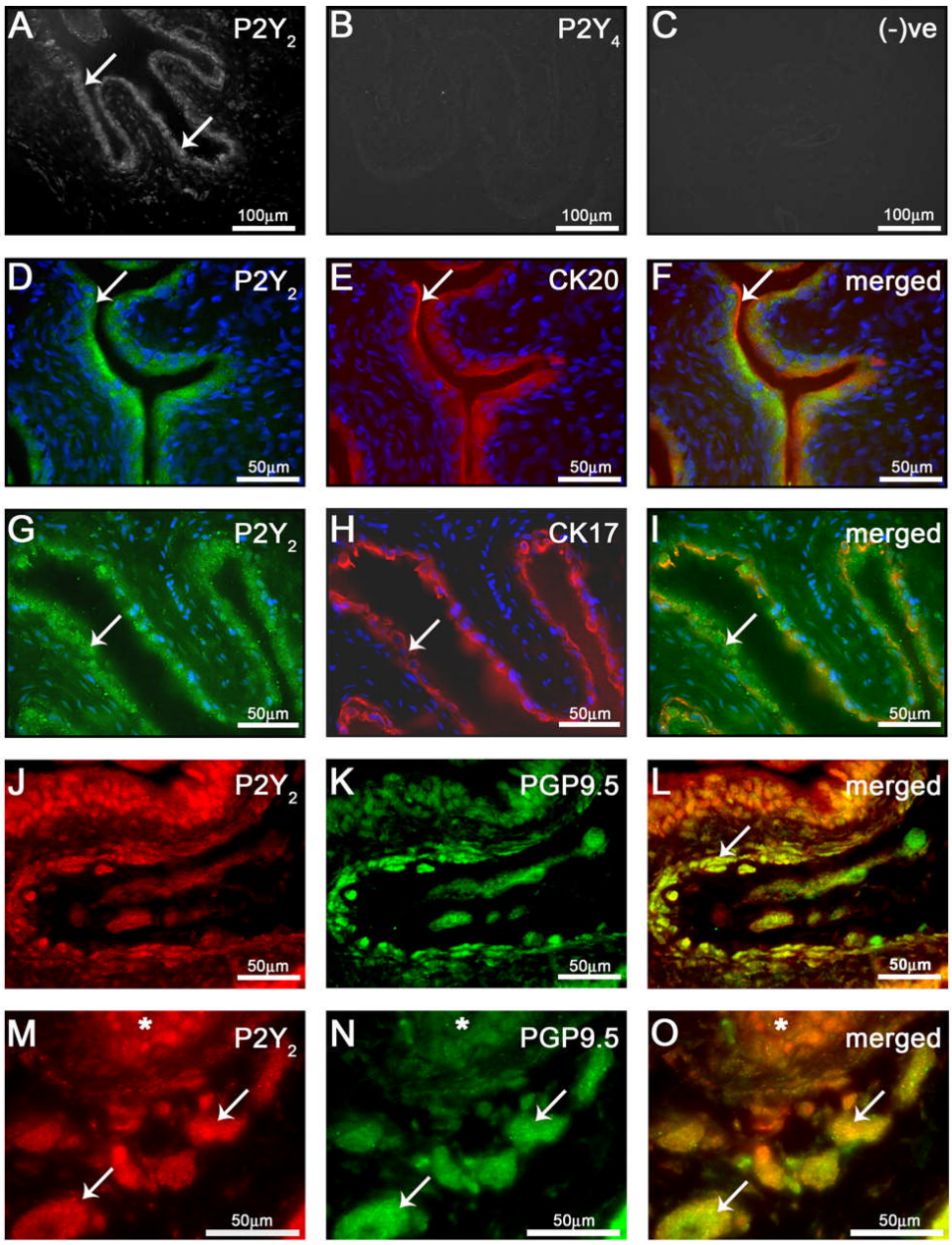

Figure 4: Expression of $P2Y_{2,4}$ in the rat urinary bladder. *A*: $P2Y_2$ immunoreactivity was present in the bladder urothelium (arrows). *B*: little or no $P2Y_4$ immunoreactivity was detected in the rat bladder. *C*: background immunofluorescence was assessed in the absence of primary antibodies and secondary only. Colocalization of $P2Y_2$ receptor with cytokeratin 20 (*D–F*) and cytokeratin 17 (*G–I*) revealed $P2Y_2$ expression in both apical and basal cells of the urothelium. Further colocalization studies with $P2Y_2$ with PGP9.5 revealed $P2Y_2$ receptor expression within submucosal nerve fibers (*J–L*) and nerve bundles (*M–O*; *denotes localization of the urothelium).

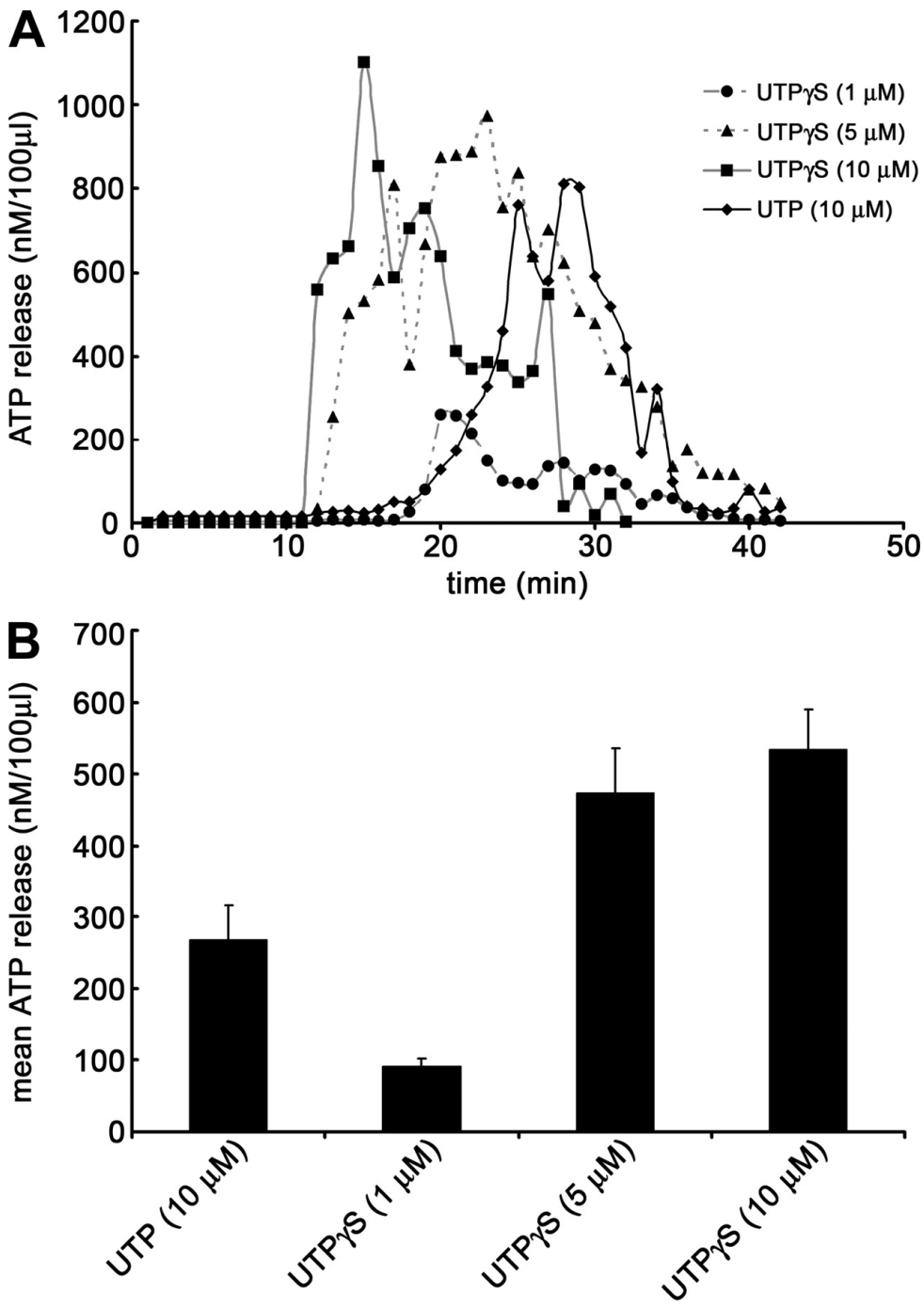

Figure 5: UTP and UTPγS evoke ATP release from cultured rat urothelial cells. *A*: representative time-course recordings illustrating ATP release evoked from cultured rat urothelial cells after stimulation with varying concentrations of UTPγS (1, 5, and 10 µM) and UTP (10 µM). Agonists were applied at $t = 0$. *B*: histograms illustrating mean release of ATP from cultured rat urothelial cells as described above. Data were obtained from at least 3 independent cultures and at least $n = 3$ from each culture.

Discussion

The data presented in this study demonstrate the functional presence and distribution of P2Y receptors in the rat urothelium. Based on data obtained with intracellular calcium imaging techniques and assessment of ATP release using a luciferin-luciferase assay, we have demonstrated that cultured rat urothelial cells are responsive to exogenously applied P2Y receptor agonists. The agonist profile of these responses provides functional evidence for the presence of $P2Y_{2/4}$ receptors in cultured rat urothelial cells. Furthermore, PCR, Western blotting, and immunocytochemical studies of the rat bladder indicate the constitutive expression of $P2Y_2$ receptors with relatively little $P2Y_4$ receptor expression in the normal rat bladder.

Purinergic receptors have previously been demonstrated to be expressed on the urinary bladders of a number of species (Birder et al., 2004;Lee et al., 2000;O'Reilly et al., 2001). In the present study, we demonstrate the functional presence of metabotropic P2Y receptors on cultured rat urothelial cells. FLIPR analysis revealed the rank order of P2 agonist potency to be ATP ≥UTP > UTPγS = ATPγS >> 2-MeSADP = NECA = ADP = UDP. Based on current pharmacological profiling, this rank order is not consistent with that reported for $P2Y_1$ (ADP > UTP), $P2Y_6$ (UDP > UTP), $P2Y_{11}$ (ATP >> UTP), $P2Y_{12}$ (ADP > UTP), $P2Y_{13}$ (2-MeSADP = ADP > UTP, ATP) or $P2Y_{14}$ (UDP-glucose >> UTP, ATP, ADP) (Abbracchio et al., 2003;Brunschweiger and Muller, 2006;Sak and Illes, 2005). Only the rat $P2Y_2$ and $P2Y_4$ receptors have been reported to be activated preferentially and equipotently by UTP and ATP (Brunschweiger and Muller, 2006;Jacobson et al., 2006). Closer analysis revealed that UTP- and UTPγS-evoked increases in cytosolic calcium in rat urothelial cells were mediated by the release of calcium from intracellular stores and via PLC-linked mechanisms, consistent with the mode of action of putative $P2Y_{2/4}$ receptors (Barnard et al., 1994;Boarder et al., 1995). Therefore, the rank order of these agonists indicates that rat urothelial cells functionally express $P2Y_2$ and/or $P2Y_4$ receptors with a relative lack of functional contribution from other P2Y or adenosine receptors. The expression of adenosine receptors has recently been reported in the rat urinary bladder urothelium (Yu et al., 2006); however, results obtained from the

present study did not reveal the presence of functional adenosine receptors, as putative A1 and A2 receptor agonists, adenosine, and NECA, at the concentrations tested did not induce changes in levels of intracellular calcium in cultured rat urothelial cells. Nevertheless, since the function of adenosine receptors is perhaps better assessed through a more direct measure of receptor activation (e.g., through quantification of cAMP accumulation via these G_s- and $G_{i/o}$-protein coupled receptors), the presence of adenosine receptors cannot be ruled out.

Antagonist studies revealed that PPADS (30 µM), which blocks most P2X and some P2Y receptors significantly attenuated ATP- and UTP-evoked responses in cultured rat urothelial cells. Recombinant rat $P2Y_2$ or $P2Y_4$ receptors expressed in oocytes have been reported to be relatively insensitive to antagonism by PPADS (IC_{50} > 1 mM and 10 mM, respectively; (Wildman et al., 2003)). However, other studies have demonstrated that PPADS can antagonize UTP-evoked Ca^{2+} responses in human astrocytoma cells expressing recombinant $P2Y_2$ receptors (IC_{50} = 24 µM; (Fam et al., 2003)). Suramin (30 µM), which is a general blocker of purinergic receptors and is effective at most P2Y receptors, but reported to have selectivity for $P2Y_2$ receptors (IC_{50} = 8.9 µM; (Wildman et al., 2003)), had relatively little or no effect on ATP-, UTP-, or UTPγS-evoked $[Ca^{2+}]_i$ transients, as assessed by FLIPR. The findings from these antagonist studies provide somewhat stronger evidence for the functional presence of $P2Y_4$ than $P2Y_2$ receptors. This variance in the data, however, may be due to the relative lack of specificity of these P2 receptor antagonists. In the present study, PPADS produced a significant shift of UTP- and ATP-evoked calcium transients at a test concentration of 30 µM, consistent with these previous findings. Given the lack of specificity of PPADS and suramin, but convergence from immunocytochemical, PCR, Western blotting, and P2 agonist profiles, it is likely that $P2Y_2$ is the predominant P2 receptor subtype functionally expressed in cultured rat urothelial cells.

Immunofluorescence studies of rat urinary bladder revealed the presence of $P2Y_2$ receptors in the rat urothelium, with little or no expression of $P2Y_4$. $P2Y_2$ immunoreactivity was present throughout the rat urinary bladder, including detrusor smooth muscle, underlying nerve fibers/plexi, and urothelium. Previous studies (Birder et al., 2004) conducted in cat urinary bladder have revealed constitutive

expression of $P2Y_1$, $P2Y_2$, and $P2Y_4$ in the urothelium. $P2Y_6$ receptor expression has also been reported in the guinea pig urothelium (Sui et al., 2006). Additionally, the presence of $P2Y_2$ was strongly indicated in the urothelium, and to a lesser extent in detrusor smooth muscle, by measurement of both mRNA and protein levels. In contrast, $P2Y_4$ had lower mRNA expression in both urothelium and detrusor smooth muscle relative to $P2Y_2$, and when assessed by Western blot, only one of three rat bladders tested provided evidence for the presence of $P2Y_4$. Functional $P2Y_1$ receptors, as well as mRNA transcripts, have previously been reported in the rat urinary bladder detrusor smooth muscle (King et al., 2004;Obara et al., 1998). In the current study, we observed $P2Y_1$ receptor mRNA in the bladder urothelium and detrusor smooth muscle (data not shown).

Activation of P2Y receptors with UTP and UTPγS in cultured rat urothelial cells evoked the release of ATP. Distention of the urinary bladder evokes ATP release from both mucosal and serosal sides of the urothelium (Lewis and Lewis, 2006). These findings have important implications for the action of urothelial ATP release in the urinary bladder. Mucosal release of ATP has the potential to act in a paracrine/juxtacrine manner on urothelial cells. This could lead to activation of purinergic receptors expressed on urothelial cells which may further evoke the release of other bioactive mediators, such as nitric oxide (Birder et al., 1998), prostacyclin (Downie and Karmazyn, 1984;Maggi, 1992), bradykinin(Chopra et al., 2005), acetylcholine (Beckel et al., 2005), neurokinins (Ishizuka et al., 1995), and additional ATP from urothelial cells. Furthermore, urothelially expressed P2Y receptors may be involved in regulating changes in urothelial membrane capacitance after bladder distention. Wang et al. (Wang et al., 2005) demonstrated that application of UTP onto the mucosal side of the rabbit urothelium increased urothelial membrane capacitance by stimulating exocytosis and fusion of discoidal/fusiform vesicles but not on the serosal side. In addition, application of 2-MeSADP and 2-MeSATP to the serosal urothelium also caused an increase in membrane capacitance, suggesting involvement of $P2Y_1$ receptors.

Serosal release of ATP from the urothelium after bladder distention may act on underlying pelvic afferent fibers. Indeed, in $P2X_3$-deficient mice, distention of the

urinary bladder resulted in decreased afferent nerve activity, suggesting a role for urothelially derived ATP release acting as a sensor and conveying information to afferent fibers (Cockayne et al., 2000;Vlaskovska et al., 2001). Strong immunolabeling of $P2Y_2$ was evident on suburothelial PGP9.5-positive nerve bundles. $P2Y_{2/4}$ receptors have been reported to activate and regulate capsaicin-sensitive cutaneous afferent nerve activity with relatively little effect on thinly myelinated A-mechanoreceptors (Stucky et al., 2004). These findings suggest that endogenous ATP release from serosal urothelium may contribute to regulating pelvic afferent activity by acting at $P2Y_{2/4}$ receptors.

Moreover, a recently discovered cell type in the human and guinea pig bladder, myofibroblasts, have emerged as a potential modulator of sensory signaling between the urothelium and pelvic afferents (Sui et al., 2006;Wiseman et al., 2003). These cells are small, spindle-shaped cells, which are responsive to ATP, express connexin 43, and may serve to transfer information between urothelium and bladder sensory nerves. Recent studies (Sui et al., 2006) in guinea pig bladder revealed strong expression of $P2Y_6$ and weaker labeling of $P2X_3$, $P2Y_2$, and $P2Y_4$ on the surface of myofibroblasts (Sui et al., 2006). Myofibroblasts are also found in the gastrointestinal tract and have been demonstrated to evoke ATP release in response to mechanical stimulation (Furuya et al., 2005;Powell et al., 1999). The released ATP activates P2Y receptors on the surrounding cells and propagated calcium waves with a concomitant transient contraction (Furuya et al., 2005). These findings suggest that distention of the urinary bladder and subsequent release of ATP from the serosal side of the urothelium may activate myofibroblasts in addition to pelvic nerve afferents. Potential cross talk between these cell types via the release of bioactive mediators, such as further ATP, or release of other neurogenic compounds may provide a local circuit which could regulate bladder tone.

The expression of purinergic receptors is known to be altered in a range of debilitating urological conditions such as interstitial cystitis (Palea et al., 1995;Tempest et al., 2004), idiopathic detrusor instability (Apostolidis et al., 2005;Brady et al., 2004;O'Reilly et al., 2002), and urge incontinence (Moore et al., 2001). Histologically, there is increased $P2X_2$ and $P2X_3$ receptor expression levels in

human bladder urothelium obtained from patients diagnosed with interstitial cystitis compared with control patients (Tempest et al., 2004), and urothelial cells isolated from patients with interstitial cystitis and cats diagnosed with a comparable disorder (FIC) release significantly greater amounts of ATP after stretch/mechanical stimuli (Birder et al., 2003) (Sun et al., 2001;Sun and Chai, 2006). In the model of FIC, there is also a concomitant reduction of $P2X_1$ and loss of $P2Y_2$ expression in the urothelium (Birder et al., 2004). The combination of these physiological and molecular changes may contribute to the underlying symptoms associated with interstitial cystitis.

The emerging profile of purinergic receptors expressed on the urothelium in a range of species suggests that these receptors play an important role in bladder function. The data presented in this study demonstrate the expression of P2Y receptors in the rat urothelium. These findings further confirm that the urothelium, which was commonly perceived to act as a passive barrier in the urinary bladder, is a dynamic tissue that has sensory properties conferred by the expression of a wide range of receptors and its ability to release bioactive mediators in response to changes in its local environment.

Chapter 6: Developmental Changes In Heteromeric $P2X_{2/3}$ Receptor Expression In Rat Sympathetic Ganglion Neurons

Abstract

We have used whole cell patch clamp recording and immunohistochemistry to investigate the expression of $P2X_{2/3}$ receptors in rat superior cervical ganglion neurons during late embryonic and early post-natal development. Neurons from E18 and P1 animals responded to the nicotinic agonist dimethylphenylpiperazinium (DMPP), and the purinoceptor agonists ATP and α,β-MeATP with sustained inward currents. Responsiveness to DMPP was maintained at P17, while that to ATP declined dramatically, and responses to α,β-MeATP were rarely detected. Immunohistochemistry for the $P2X_3$ subunit revealed widespread staining in superior cervical ganglia from P1 rats, but little immunoreactivity in ganglia from P17 animals. In neurons from P1 animals, the response to α,β-MeATP exhibited pharmacological properties of the heteromeric $P2X_{2/3}$ receptor. In conclusion, sympathetic neurons of the rat superior cervical ganglion are more responsive to ATP and α,β-MeATP at birth and during the early post-natal period, due largely to the expression of the $P2X_3$ subunit, but these responses are much reduced in mature rats

Acknowledgements

The following chapter was published in Developmental Dynamics in 2005 (Dunn et al., 2005). The author of this thesis contributed at an intellectual level equally to the first author (Phil Dunn) for all aspects of the work and conducted approximately 50% of the electrophysiological experiments.

Introduction

Neuronal release of ATP was first demonstrated from the peripheral terminals of primary afferent fibers (Holton, 1959). Since then, co-storage and release of ATP from the terminals of both central and peripheral neurons has been widely described, and its role as a neurotransmitter is well established (see (Burnstock, 1972;Burnstock, 1976;Burnstock, 2003)). When released from nerve terminals, ATP can produce both rapid effects through the activation of a family of ligand gated ion channels (the P2X receptors), and slower and longer-lasting actions, which are usually mediated via the G protein-coupled P2Y receptors (Ralevic and Burnstock, 1998). To date, seven P2X receptor sub-units have been identified, which can assemble to form either homomeric or heteromeric receptors (North, 2002). In the periphery, the activation of P2X receptors is important for neuromuscular transmission in the vas deferens (Mulryan et al., 2000) and for the activation of primary afferent fibers in the urinary bladder (Cockayne et al., 2000;Vlaskovska et al., 2001) and carotid body (Prasad et al., 2001;Rong et al., 2003). P2X receptors are also found on most autonomic ganglion neurons (Dunn et al., 2001). Since ATP is released from preganglionic nerve terminals (Vizi et al., 1997), these receptors may play a role in ganglionic neurotransmission.

In rat and mouse sympathetic ganglia, immunohistochemical and molecular biology suggest the presence of a variety of P2X subunits. However, most neurons respond to ATP, but not to α,β-MeATP, which in combination with other pharmacological data indicates the presence of homomeric $P2X_2$ receptors (Dunn et al., 2001).

Nevertheless, a few neurons in these ganglia do respond to α,β-MeATP (Khakh et al., 1995;Schadlich et al., 2001;Calvert and Evans, 2004). This agonist is considered to be selective for P2X receptors containing $P2X_1$ or $P2X_3$ subunits (North, 2002), although some activation of receptors containing $P2X_5$ or $P2X_6$ subunits has been reported (Wildman et al., 2002;Jones et al., 2004). Studies using $P2X_1$ knockout mice have led to the suggestions that at least some α,β-MeATP-evoked responses in mouse superior cervical ganglion neurons are mediated by receptors containing the

$P2X_1$ subunit (Calvert and Evans, 2004). However, autonomic ganglion neurons share the same embryological origins as dorsal root ganglion neurons, which express predominantly $P2X_3$ homomeric or $P2X_{2/3}$ heteromeric receptors. It is therefore possible that some α,β-MeATP responses in sympathetic neurons might be mediated by $P2X_3$-containing receptors.

There are many examples of changes in receptor expression during development due to the expression of different receptor subunits, for example, the γ to ε switch in nicotinic acetylcholine receptors at the neuromuscular junction (Mishina et al., 1986) and changes in GABA and NMDA receptors in the cerebellum (Gutierrez et al., 1997;Cathala et al., 2000). Changes in expression of P2 receptors also occur during embryonic and postnatal development (Burnstock, 2001b). For example, P2X receptors are expressed transiently in developing rat and chick skeletal muscle (Wells et al., 1995;Meyer et al., 1999;Ryten et al., 2001), and transient expression of the $P2X_3$ subunit has been observed in the brain and spinal cord during rat(Cheung and Burnstock, 2002) and mouse (Boldogkoi et al., 2002) embryogenesis. Dynamic expression of P2Y receptor subtypes has also been described in the embryonic rat brain (Cheung et al., 2003).

In this study, we have used whole cell patch clamp recording and immunohistochemistry to investigate the expression of the $P2X_3$ receptor subunit and the formation of heteromeric $P2X_{2/3}$ receptors in rat superior cervical ganglion neurons during late embryonic and early post-natal development.

Results

Responses of P1 and P17 Superior Cervical Ganglion Neurons

We initially compared the responsiveness of superior cervical ganglion neurons from early (P1) and late (P17) post-natal rats to ATP and α,β-MeATP. All neurons tested responded to the nicotinic agonist DMPP. All superior cervical ganglion neurons from P17 animals responded to 100 μM ATP with a sustained inward current, with current densities ranging from 3 to 19 pA/pF (Figure 1A and1B). In keeping with previous studies on adult neurons (Khakh et al., 1995;Calvert and

Evans, 2004), 100 μM α,β-MeATP failed to evoke any response in most neurons from P17 animals. However, in one culture, two neurons did give significant responses to α,β-MeATP (see Fig. 2Bi). In superior cervical ganglion neurons from P1 animals, the responsiveness to ATP was significantly greater than that in P17 animals, and the majority of neurons also responded to α,β-MeATP with an inward current (Figure 1A and 1B).

Immunohistochemistry

In P17 ganglia, most neurons failed to show any significant staining for $P2X_3$. However a small sub-population of neurons did exhibit distinct immunoreactivity (Fig. 2Ai). In ganglia from P1 animals, many neurons were immunopositive for $P2X_3$, and the strongest staining was considerably more intense than that seen in P17 ganglia (Fig. 2Aii). This pattern of staining was consistent with the responses to α,β-meATP of neurons from P17 and P1 superior cervical ganglion (Fig. 2B). In contrast, neurons from both P1 (Fig. 2Ci) and P17 (Fig. 2Cii) superior cervical ganglia exhibited quite uniform immunoreactivity for the $P2X_2$ subunit.

Temporal Change in Agonist Responses

Having established that in contrast to adult neurons, the majority of neurons in P1 superior cervical ganglion respond to α,β-MeATP, we investigated the time dependence of the change in agonist responsiveness, by looking at neurons from E18, P1, P7, and P17 animals. Neurons from E18 embryos all responded to 100 μM α,β-MeATP with small inward currents, while more robust currents were produced by 100 μM ATP and 10 μM DMPP (Fig. 3). Response to all three agonists was maximal between birth and seven days. Following this, the current density to the nicotinic agonist DMPP was maintained, but there was a marked reduction in the response to ATP and few neurons responded to α,β-MeATP (Fig. 3).

Pharmacological Properties

To further investigate the nature of the receptors responsible for the response to α,β-MeATP in P1 sympathetic ganglia, we carried out pharmacological characterization of this receptor. The response to α,β-MeATP was concentration

dependent. Fitting the data with the Hill equation yielded an EC_{50} value of 29.9 ± 5 μM, with a Hill coefficient of 1.1. (Fig. 4A).

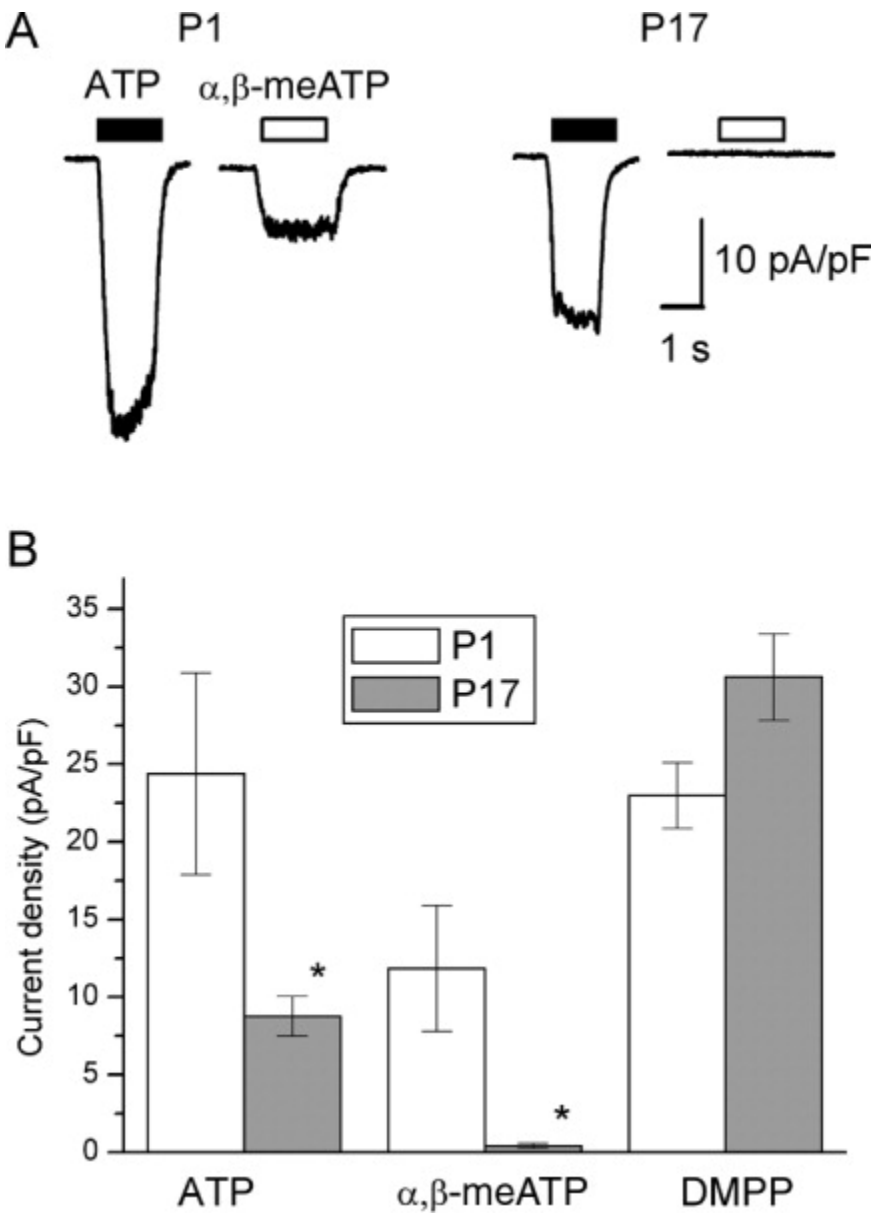

Figure 1: Responsiveness of postnatal superior cervical ganglion neurons, from P1 and P17 rats. A: Representative whole cell patch clamp recordings (holding potential -60 mV) of responses to ATP and α,β-MeATP (both at 100 μM). While P1 neurons respond to both agonists, neurons from older animals (P17) respond to ATP but not α,β-MeATP. B: Histogram comparing chemosensitivity of P1 and P17 neurons to ATP (100 μM), α,β-MeATP (100 μM), and the nicotinic agonist DMPP (10 μM). Columns represent the mean ± S.E. from 10 to 15 neurons, from three separate cultures.*Significantly different by unpaired Student's *t*-test.

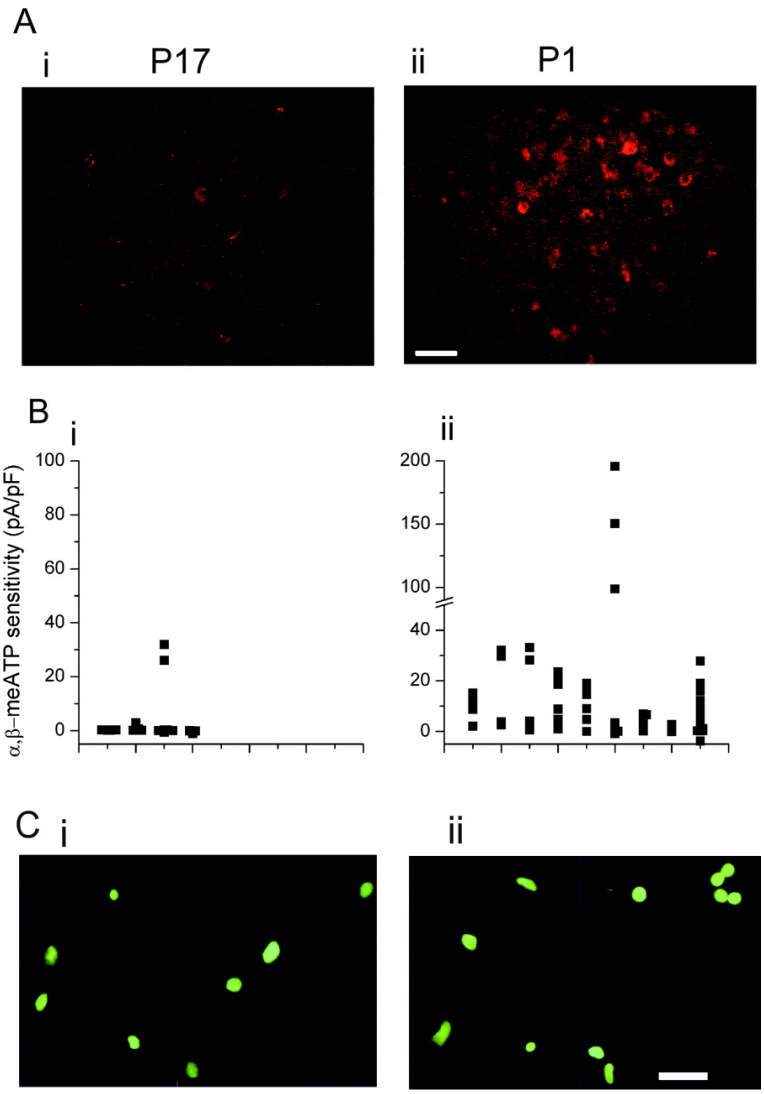

Figure 2: Comparison of $P2X_2$ and $P2X_3$ immunoreactivity and α,β-MeATP sensitivity in superior cervical ganglion neurons. A: Immunohistochemistry for the $P2X_3$ subunit in sections of superior cervical ganglia from P17 (i) and P1 (ii) rats. In P17 ganglia, few neurons are immunoreactive for the $P2X_3$ subunit, while in ganglia from newborn animals, the staining is widespread, and in some neurons intense. Calibration bar = 50 μm. B: Comparison of the responsiveness of neurons from P17 (i) and P1 (ii) superior cervical ganglia to α,β-MeATP. Each point represents a single neuron, and each vertical column represents a single experiment. While few neurons from P17 ganglia gave any detectable response to α,β-MeATP, most neurons from newborn animals responded, and in some cases the responses were very large. C: Immunohistochemistry for the $P2X_2$ subunit in cultured superior cervical ganglion neurons revealed strong and uniform staining in cells from both P1 (i) and P17 (ii) rats. Calibration bar = 50 μm.

Trinitrophenyl-ATP is a subtype selective antagonist with nanomolar affinity for P2X receptors containing the $P2X_1$ and $P2X_3$ subunits (Virginio et al., 1998b). TNP-ATP produced a reversible concentration-dependent antagonism of the response to α,β-MeATP. Fitting the Hill equation to this data gave an IC_{50} of 13 ± 8 nM, with a Hill coefficient close to unity (Fig. 4B).

A characteristic of P2X receptors involving the $P2X_2$ sub-unit is that they exhibit positive allosteric modulation by Zn^{2+} and H^+ ions (North, 2002). We, therefore, investigated the effects of these two ions on response of P1 superior cervical ganglion neurons to α,β-MeATP. Lowering the pH from 7.4 to 6.8 produced a dramatic increase in the response to 20 μM α,β-meATP (Fig. 4C). However co-application of 10 μM Zn^{2+} produced no significant change in the response to α,β-MeATP. In contrast, this concentration of Zn^{2+} more than doubled the response to 20 μM ATP in these neurons (Fig. 4C). Adult nodose ganglion neurons respond to α,β-MeATP with a sustained inward current due to the presence of heteromeric $P2X_{2/3}$ receptors (Lewis et al., 1995). We therefore investigated the effect of Zn^{2+} on responses to α,β-MeATP on nodose ganglion neurons taken from newborn rats. On these neurons, Zn^{2+} produced a small but significant increase in the amplitude of the α,β-MeATP response (Fig. 4C).

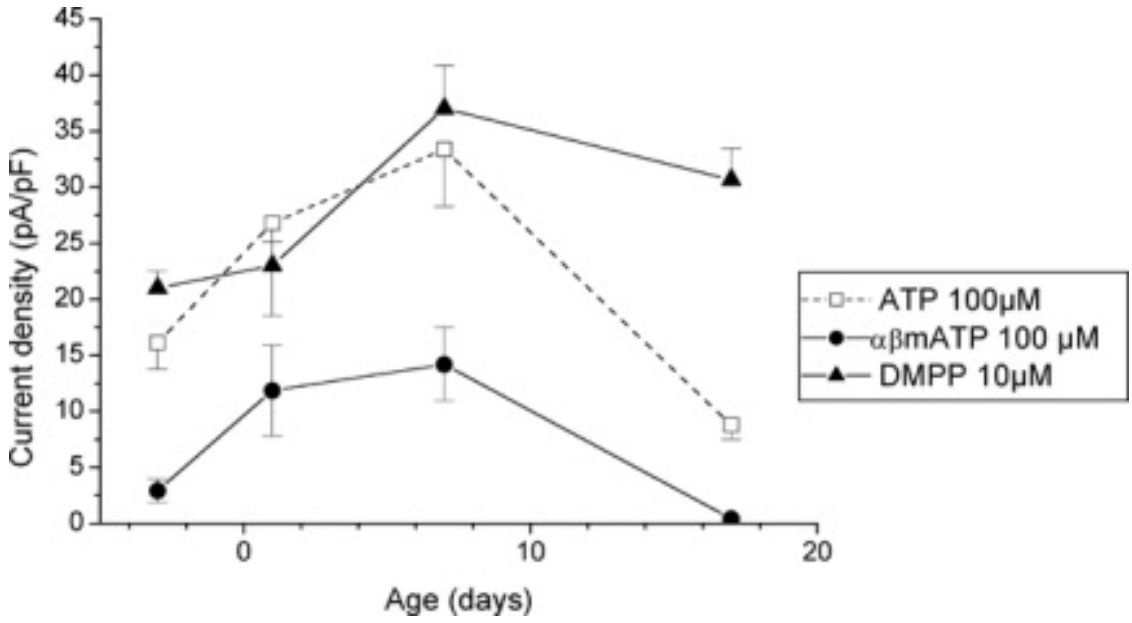

Figure 3: Temporal change in chemosensitivity of rat superior cervical ganglia. The graph shows the sensitivity of neurons to ATP, α,β-MeATP, and DMPP at 4 different developmental ages from late embryonic to weaning. Each point represents the mean ± S.E. for 6 to 34 neurons. Sensitivity to the nicotinic agonist DMPP increased from E18 to P7, and was then maintained at P17. In contrast, sensitivity to the purinergic agonists peaked at about P8 and then declined, so that by P17 responses to α,β-meATP were rarely detectable.

Discussion

The main finding of this study is that there is a marked change in the expression of α,β-MeATP-sensitive $P2X_{2/3}$ receptors in sympathetic neurons of the rat superior cervical ganglion. The levels of this receptor peak soon after birth, then decline to very low levels by the time animals are about 17 days old. The expression of P2X receptors may be altered by dissociation and cell culture (Smith et al., 2001), possibly as a result of ATP release due to metabolic stress, ischemia, or trauma (see (Volonte et al., 2003)). Although our results show agreement between functional experiments and immunohistochemistry, we cannot rule out the possibility of changes resulting from the use of cell culture.

P2X receptors responding to α,β-MeATP are believed to require the presence of either the $P2X_1$ or $P2X_3$ (North, 2002), although there is some evidence that $P2X_5$ or $P2X_6$ receptors may also respond to this agonist (Wildman et al., 2002;Jones et al., 2004). Much of our data suggests that the α,β-MeATP-sensitive receptor expressed in sympathetic neurons from newborn rats is the heteromeric $P2X_{2/3}$ receptor. Firstly, we observed considerable levels of $P2X_3$ immunoreactivity in ganglia from P1 animals, which was virtually absent in ganglia from animals more than 17 days old. The sustained nature of the responses would argue against the involvement of homomeric $P2X_1$ or $P2X_3$ receptors, which give rapidly desensitizing responses (North, 2002). The EC_{50} value we obtained for α,β-MeATP (30 μM) is similar to the value of 39 μM reported for nodose ganglion neurons (Dunn et al., 2000), but slightly greater than the value of 9 μM reported for heteromeric $P2X_{2/3}$ receptors expressed in Xenopus oocytes (Liu et al., 2001). The sensitivity of this receptor to the antagonist TNP-ATP, with an IC_{50} of 13 nM, is quite similar to the values of 7 and 11 nM reported for recombinant $P2X_{2/3}$ receptors (Virginio et al., 1998b;Liu et al., 2001) and 21 nM for receptors in the rat nodose ganglion (Dunn et al., 2000). The potency of this antagonist is, however, considerably less than the low nanomolar values reported for the homomeric $P2X_3$ receptor (North, 2002). The potentiation of α,β-MeATP responses by low pH is also in keeping with the properties of the heteromeric

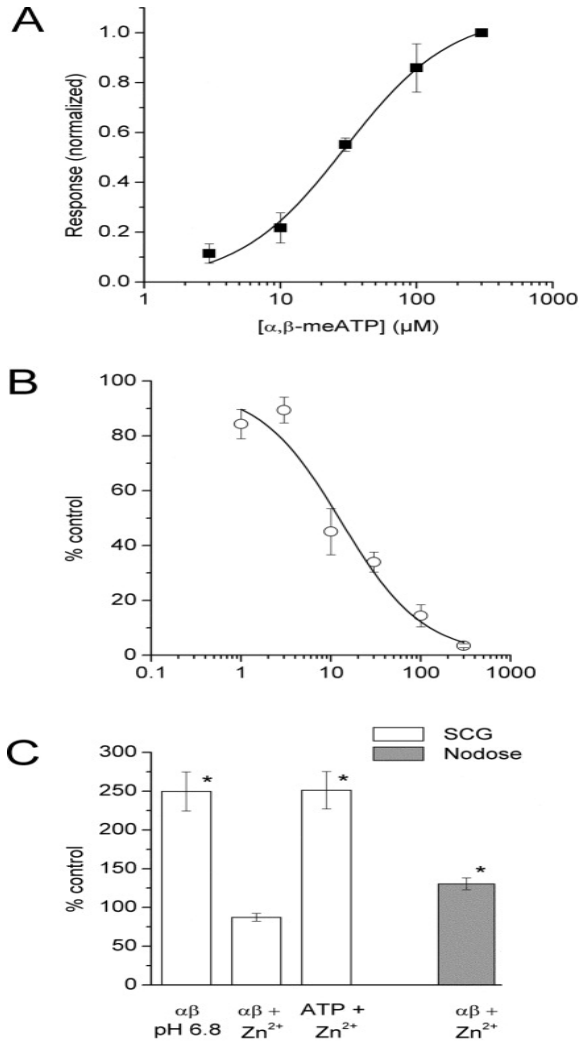

Figure 4: Pharmacological properties of the α,β-MeATP sensitive receptor in superior cervical ganglion neurons from newborn rats. A: Concentration response curve for the inward current evoked by α,β-MeATP. Points represent mean ± SE from 5 neurons. The curve shows a least squares fit of the Hill equation to the data, with an EC_{50} of 29.9 ± 5 μM and a Hill coefficient of 1.1 ± 0.15. B: Concentration response curve for the inhibition of the response by TNP-ATP. Fitting the Hill coefficient to the data gave an IC_{50} of 13 ± 8 nM and a Hill coefficient of 0.95 ± 0.4. C: Effect of allosteric modulators on the response of P1 SCG neurons to purinergic agonists. While lowering the pH to 6.8 significantly potentiated the response to 20 μM α,β-MeATP, the response was unaffected by 10 μM Zn^{2+}. In contrast, the response to 10 μM ATP was significantly increased. Similarly, the response of nodose ganglion neurons from P1 rats was significantly potentiated by 10 μM Zn^{2+}. Columns represent the mean ± S.E. from 7 neurons. *Significantly different from 100% by one sample *t*-test

$P2X_{2/3}$ receptor (Liu et al., 2001), and contrasts with the negative allosteric action of protons at the homomeric $P2X_3$ receptor (North, 2002). Responses at the $P2X_{2/3}$ receptor are also potentiated by Zn^{2+}, although this effect is less pronounced than at the homomeric $P2X_2$ receptor (Liu et al., 2001). In our experiments, Zn^{2+} enhanced responses to ATP in P1 superior cervical ganglion neurons, and also increased responses to α,β-MeATP in nodose ganglion neurons from neonatal rats. However, we failed to observe potentiation of α,β-MeATP responses in P1 superior cervical ganglion neurons. The reason for this is at present unclear, but might indicate the involvement of other P2X subunits or spliced variants. Studies using $P2X_1$ knockout mice have indicated that a small percentage of superior cervical ganglion neurons respond to α,β-MeATP through activation of $P2X_1$ receptors (Calvert and Evans, 2004). Although we cannot exclude involvement of $P2X_1$ subunits in a heteromeric receptor, the kinetic and pharmacological properties of the response we observed do not match those of the homomeric $P2X_1$ receptor.

ATP is co-released with acetylcholine from pre-ganglionic nerve terminals (Vizi et al., 1997), and may thus play a role in synaptic transmission. This notion is supported by observation of synaptic responses, which are resistant to nicotinic receptor antagonists in some ganglia (Seabrook et al., 1990;Callister et al., 1997). P2X receptors are also present on the terminals of postganglionic sympathetic neurons, where they can modulate the release of noradrenaline (Sperlagh et al., 2000;Queiroz et al., 2003). In the central nervous system, many $P2X_3$-containing receptors are localized to presynaptic terminals. Thus, the loss of the $P2X_3$ subunit in P17 SCG neurons may reflect the targeting of these subunits to the nerve terminal.

$P2X_{2/3}$ receptors exhibit a higher affinity for ATP than the homomeric $P2X_2$ receptors present on adult SCG neurons. This is likely to account for the high responsiveness to ATP, which we observed in embryonic and P1 ganglion neurons. Interestingly, this change in P2X receptor expression occurs at a time when synaptogenesis is taking place in the superior cervical ganglion (Smolen and Raisman, 1980;Mills and Smith, 1983), which might indicate a role for purinergic receptors in this process.

In conclusion, we have shown that sympathetic neurons of the rat superior cervical ganglion exhibit larger responses to ATP and α,β-meATP at birth and during the early post-natal period. This appears to be due, at least in part, to the expression of the $P2X_3$ subunit, giving rise to the presence of heteromeric $P2X_{2/3}$ receptors. Sensitivity to purinergic agonists then declines. It is tempting to speculate that the role of these receptors may be in some way related to synapse formation, which occurs during the early post-natal period.

Chapter 7: Regulated Expression Of The Rat Recombinant $P2X_3$ Receptor In Stably Transfected CHO-K1 tTA Cells

Abstract

In this report, the regulatible expression by tetracycline of the rat recombinant $P2X_3$ receptor in stably transfected Chinese hamster ovary (CHO-K1) expressing the tetracycline-controlled transactivator (tTA) is described. cDNA encoding the rat $P2X_3$-receptor was subcloned into pTRE (a tetracycline-repressible expression vector) which was used to transfect stably CHO-K1 tTA cells. Using whole cell patch clamp techniques, 100 μM ATP evoked inward currents of 2.9±1.6 nA in transfected cells grown in the absence of tetracycline (*tet*−). The $P2X_3$ receptor protein was detectable by immunoblot as early as 24 h and protein expression levels continued to increase as much as 192 h following activation of tTA by the removal of the antibiotic. Saturation binding isotherms using [^{35}S]ATPγS yielded a pK_d of 8.2±0.1 and a B_{max} of 31.9±3.5 pmol/mg protein in *tet*− cell membranes and a pK_d of 8.1±0.1 and a B_{max} of 5.8±0.8 pmol/mg protein in *tet*+ cell membranes. The agonist ligands 2MeSATP and αβMeATP displaced the binding of [^{35}S]ATPγS in *tet*− cell membranes with very high affinity, yielding pIC_{50} values of 9.4±0.2 and 7.5±0.2, respectively. In *tet*+ cell membrane, displacement of [^{35}S]ATPγS by 2MeSATP and αβMeATP was of much lower affinity (pIC_{50} values of 7.8 and 6.2, respectively). ATP, ADP and UTP showed similar displacement of [^{35}S]ATPγS binding in *tet*− and *tet*+ cell membranes. In other experiments, cytosolic Ca^{2+} was monitored using the fluorescent indicator, fluo-3. Increases in cytosolic Ca^{2+} were elicited by 100 nM αβMeATP in *tet*− cells while no increases in cytosolic Ca^{2+} were detected below 100 μM αβMeATP in either *tet*+ cells or untransfected cells. These calcium responses to αβMeATP had a pEC_{50} of 6.7 and were transient, returning to baseline within 120 s. Suramin produced concentration-dependent, parallel, dextral shifts of $E/[A]$ curves to αβMeATP yielding a pK_B of 5.6. PPADS produced non-parallel, dextral shifts of $E/[A]$ curves to αβMeATP which were insurmountable. These results show for the first time, expression of a functional, homomeric recombinant rat $P2X_3$ receptor which is under regulated expression in a stably transfected mammalian cell line.

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Introduction

ATP has long been known as a ubiquitous intracellular source of energy in metabolism. Yet it was not until 1972 that Burnstock proposed that ATP was an active substance in the autonomic nervous system (Burnstock, 1972). Since then, ATP has been established as a neurotransmitter (Burnstock, 1976;Burnstock, 1978). Extracellular ATP has been shown to activate P2 receptors which are divided into two families according to their structure and transduction mechanism: P2Y receptors which are metabotropic and are coupled to G-proteins (review: (King et al., 1998a) and P2X receptors which are cation-selective ligand-gated ion channels (Abbracchio and Burnstock, 1994). Currently, seven P2X subunits, $P2X_1$ through $P2X_7$, have been isolated from the rat (review: (Buell et al., 1996a). The deduced rat proteins, 379–595 amino acids long, share 35–59% identity with each other. They are predicted to contain two transmembrane domains, M1 and M2, connected by a large extracellular loop of ~270 amino acids that contain 10 conserved cysteine residues, and intracellular amino and carboxyl termini (North, 1996). cDNA sequences for each of these subunits form functional ATP-gated, cation-selective channels with electrophysiological and pharmacological profiles similar to that of P2X receptors in native tissues when heterologously expressed in cells such as *Xenopus* oocytes, HEK 293 cells and CHO-K1 cells (review: (Buell et al., 1996a). The $P2X_3$ receptor was originally cloned from a rat dorsal root ganglion cDNA library and, when expressed in *Xenopus* oocytes, is characterized by its sensitivity to αβMeATP and its rapid rate of desensitization (Chen et al., 1995;Lewis et al., 1995). The mRNA for the $P2X_3$ receptor is reported to be expressed in sensory neurons from trigeminal, dorsal root, and nodose ganglia (Lewis et al., 1995;Chen et al., 1995;Collo et al., 1996). Furthermore, recent immunohistochemical observations clearly demonstrate that $P2X_3$ is principally expressed by small- or medium-sized neurons in sensory ganglia(Vulchanova et al., 1997;Bradbury et al., 1998;Novakovic et al., 1999). These observations have led to the suggestion that this receptor plays a role in nociception (Kennedy and Leff, 1995;Burnstock and Wood, 1996;Burnstock, 1996). Indeed, recently, (Cook et al., 1997) reported ATP-evoked action potentials in nociceptive

(tooth-pulp afferents) rat sensory neurons which had $P2X_3$ immunoreactivity in their sensory endings and cell bodies.

The pharmacological characterization of these receptors in native systems has been difficult because of the lack of suitable pharmacological probes. Therefore, expression of P2X receptors in heterologous expression systems in high density is critical for functional characterization of these receptors and the identification of novel ligands. To this end, we sought a method to prepare a cell line in which the $P2X_3$ receptor would be stably transfected and could be expressed at high levels without introducing selection pressure/resistance to expression that would delimit the usefulness of such a recombinant cell line. In order to achieve this, we used a tetracycline-controlled gene expression system developed by (Gossen and Bujard, 1992) which has been demonstrated to give a higher level of gene expression than wild-type CMV-promoter/enhancer systems (Yin et al., 1996). Here, we report the regulated expression by tetracycline of the rat recombinant $P2X_3$ receptor in stably transfected CHO-K1 tTA cells (transfected with the tetracycline-controlled transactivator) and pharmacological analysis by radioligand binding using [^{35}S]ATPγS which has been reported, in the absence of divalent cations, to specifically label the P2X receptor (Michel et al., 1996b;Michel and Humphrey, 1996). Functional pharmacological characterization was also conducted by measuring changes in intracellular calcium.

Results and Discussion

In order to characterize the $P2X_3$ receptor fully, we sought an expression method that would produce the $P2X_3$ receptor at very high density in a stably transfected cell line. We chose the tetracycline-controlled transactivator (tTA) gene expression system as it has been reported previously that the maximal gene expression level is higher than that of the wild-type CMV promoter/enhancer-driven system (Yin et al., 1996). In this expression system, a modified CMV promoter with a tet-repressor binding element is regulated by a fusion of the tet-repressor and the herpes simplex virus VP-16, designated tTA (Gossen and Bujard, 1992), which is constitutively expressed in CHO-K1 tTA cells. Therefore, this strategy also allows controlled

regulation of gene expression by the addition or removal of tetracycline in the growth media.

Following transfection with the $P2X_3$ receptor into the CHO-K1 tTA cell line, a wide range of clonal variation in $P2X_3$ receptor expression levels was observed (data not shown). However, several clonal cell lines that were under tet-regulation with very high expression levels were identified. These cells were continuously maintained in the presence of 0.1 μg/ml tetracycline to prevent the expression of the $P2X_3$ receptor. Fig. 1A shows that following the removal of tetracycline from the growth media, the $P2X_3$ receptor protein was detectable by immunoblot as early as 24 h and protein expression levels continued to increase as much as 192 h following activation of tTA by the removal of the antibiotic. Maximal [^{35}S]ATPγS binding density obtained from saturation binding isotherms using cell membrane preparations also increased in a time dependent manner and were much greater than $P2X_3$ transfected CHO-K1 tTA cells grown in the presence of tetracycline (Fig. 1B). From these initial results, further characterization of the $P2X_3$ transfected cell line was conducted using cells that were cultured for 192 h following the removal of tetracycline (*tet*−).

Saturation binding isotherms using [^{35}S]ATPγS yielded a pK_d of 8.2±0.1 and a B_{max} of 31.9±3.5 pmol/mg protein in *tet*− transfected cell membranes and a pK_d of 8.1±0.1 and a B_{max} of 5.8±0.8 pmol/mg protein in *tet*+ transfected cell membranes (Table 1). The maximal binding densities (up to 40 pmol/mg protein) observed in this study were higher than expected for heterologous expression of a membrane receptor protein in a mammalian system. However, these binding densities were similar in $P2X_3$ and $P2X_4$ infected CHO cells using the Semliki forest virus (SFV) expression system previously reported (Michel et al., 1996b;Michel et al., 1997). The SFV infection system has also been shown to generate very high levels of gene expression in mammalian cells (Lundstrom et al., 1994). However, unlike the regulatible gene expression system used in this study, the SFV expression system is a transient expression system.

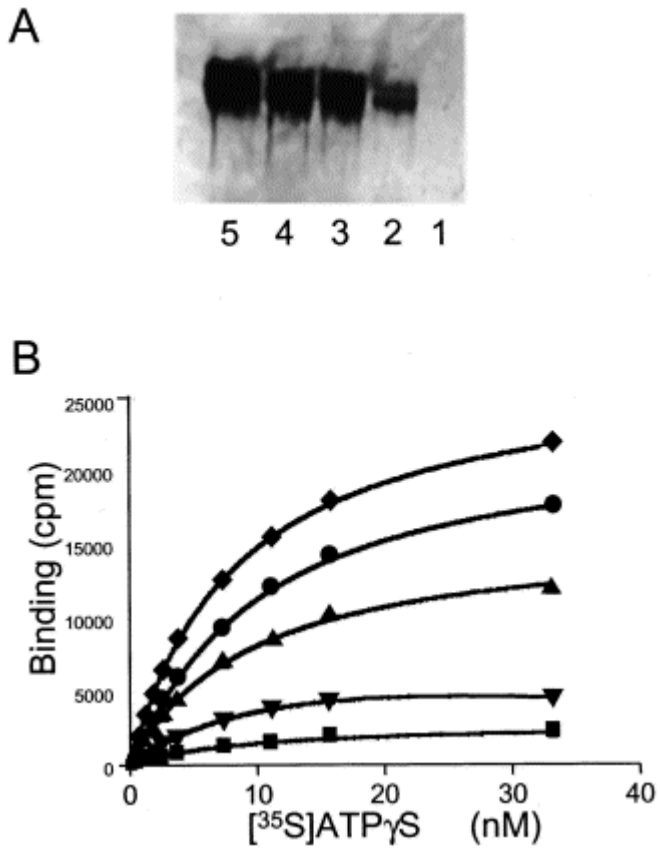

Figure 1: Regulatible expression of the $P2X_3$ receptor in $P2X_3$-pTRE transfected CHO-K1 tTA cells. (A) Immunoblot analysis of $P2X_3$ transfected CHO-K1 tTA cell membranes grown in the presence (1) and 24 h (2), 48 h (3), 96 h (4) and 192 h (5) in the absence of 0.1 μg/ml tetracycline. Saturation binding isotherms of [^{35}S]ATPγS to $P2X_3$ transfected CHO-K1 tTA cell membranes grown in the presence (▪) and 24 h (▼), 48 h (▲), 96 h (•), and 192 h (♦) in the absence of 0.1 μg/ml tetracycline. Representative values presented are from single experiment performed in duplicate

The agonist ligands 2MeSATP and αβMeATP displaced the binding of 0.25 nM [^{35}S]ATPγS with high affinity, yielding pIC_{50} values of 9.3±0.2 and 7.7±0.2, respectively (Table 1). In *tet*+ transfected cell membranes, displacement of 0.25 nM [^{35}S]ATPγS by 2MeSATP and αβMeATP was of much lower affinity (pIC_{50} values of 8.1 and 5.4, respectively) (Table 1). The relatively high affinity of αβMeATP is consistent with its agonist activity at this receptor (Chen et al., 1995;Lewis et al., 1995). Other nucleotide agonists, such as ATP, ADP and UTP showed similar displacement of [^{35}S]ATPγS binding in *tet*− and *tet*+ transfected cell membranes (Table 1). Although agonist ligand affinities using [^{35}S]ATPγS were consistently 5–10-fold lower than previously reported (Michel et al., 1996b;Michel et al., 1997), the rank order of agonist affinities was consistent with the previous report. Moreover, the rank order of agonist potencies originally reported for this receptor (Chen et al., 1995;Lewis et al., 1995) are similar to those observed in competition binding studies. The affinity differences observed in this study and those previously reported by (Michel et al., 1996b) may be due to a number of factors such as experimental binding conditions which could lead to changes in the affinity state of the receptor.

	$P2X_3$ (*tet*−)		Mock (*tet*+)	
	B_{max} (pmol/mg protein)	K_D (nM)	B_{max} (pmol/mg protein)	K_D (nM)
ATPγS	31.9 ± 3.5	8.2±0.1	5.8 ± 0.8	8.1 ± 0.1
	pIC_{50}	n_H	pIC_{50}	n_H
	8.5	1.1	8.2	0.8
ATP				
ATPγS	8.4	0.8	8.5	0.9
αβMeATP	7.7/4.9[a]		5.4	0.5
βγMeATP	6.5	0.8	7.0	1.0
2MeSATP	9.3/5.0[a]		8.1	0.7
ADP	6.9	1.0	7.3	1.0
AMP	< 4		< 4	
UTP	4.0	1.0		

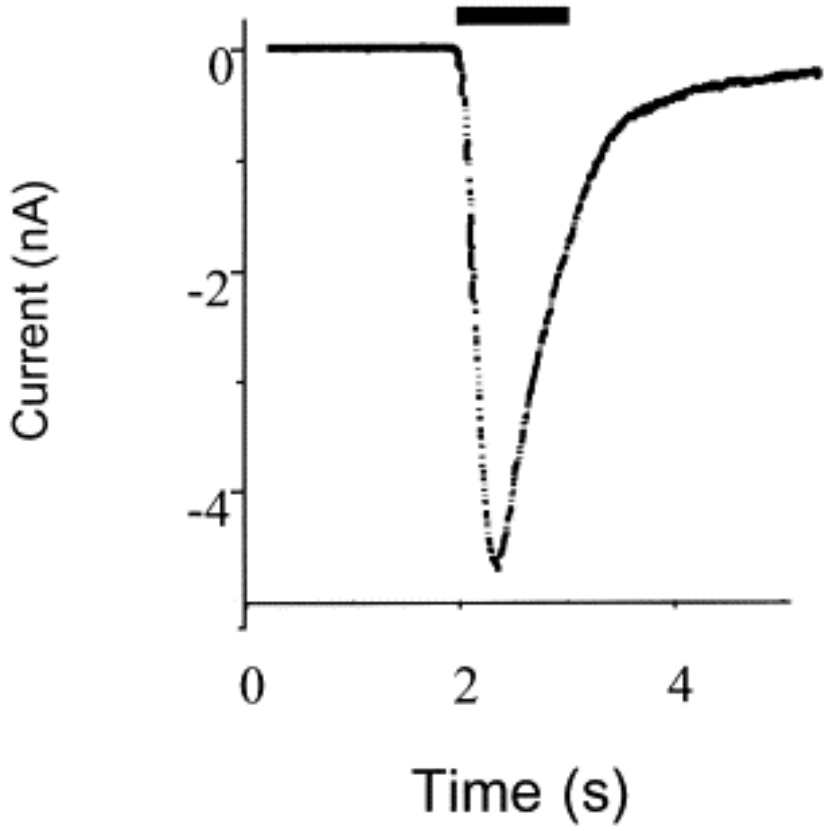

Figure 2: Representative trace showing inward current in response to 100 μM ATP in (*tet*−) $P2X_3$ transfected CHO-K1 tTA cells.

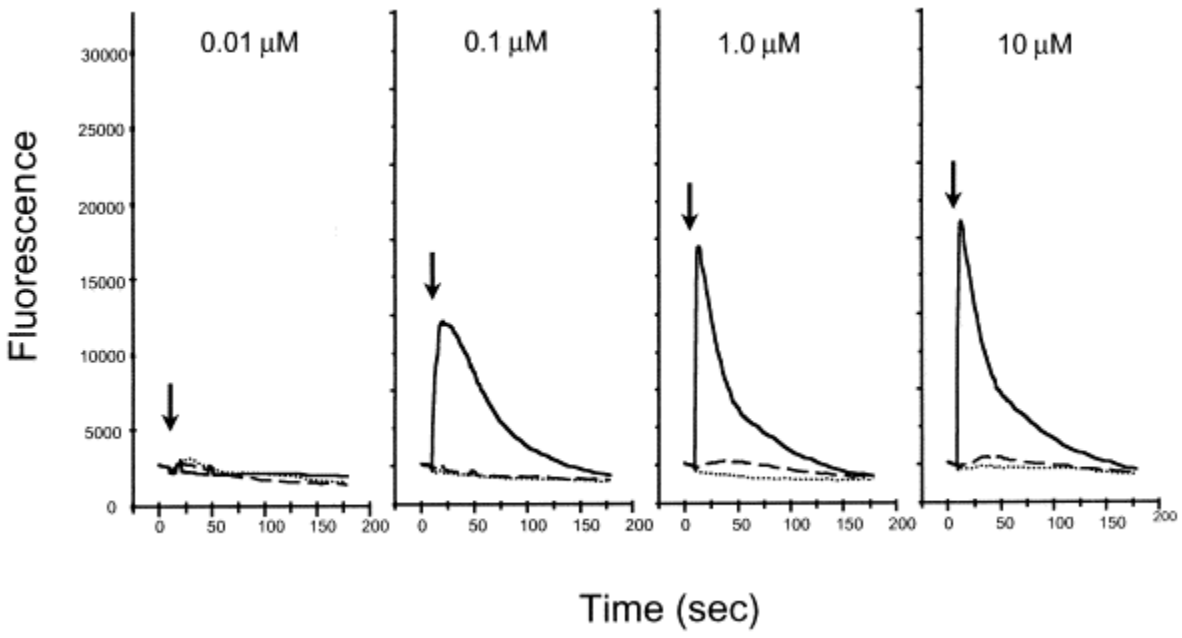

Figure 3: Representative fluorescence traces showing intracellular calcium responses to αβMeATP (0.01–10 µM) in untransfected (……), *tet*+ $P2X_3$ transfected (– – –), and *tet*− $P2X_3$ transfected (———) CHO-K1 tTA cells. Arrows indicate addition of to αβMeATP.

In whole cell patch clamp experiments, 100 μM ATP evoked quickly rising, rapidly desensitizing, large inward currents ranging from 0.1 to 10 nA (2.9±1.6 nA; *n*=7) in *tet*− transfected cells (Fig. 2). No currents were observed in *tet*+ transfected cells or in untransfected cells (data not shown). These currents which desensitized rapidly were consistent with what has been reported by other laboratories using *Xenopus* oocyte expression systems (Chen et al., 1995;Lewis et al., 1995) as well as transient mammalian expression systems such as $P2X_3$ SFV infected CHO cells (Evans et al., 1995) and $P2X_3$ transfected HEK 293 cells (Stoop et al., 1997).

αβMeATP (0.1–100 μM) elicited concentration-dependent increases in fluorescence in fluo 3 loaded *tet*− transfected cells yielding a $p[A]_{50}$ of 6.7 while no increases in cytosolic Ca^{2+} were detected below 100 μM αβMeATP in either *tet*+ transfected cells or in untransfected cells (Fig. 3). The increase in fluorescence following the addition of αβMeATP occurred rapidly (peaking within 3 to 4 s) and was transient, returning to baseline within 2–3 min (Fig. 3). In *tet*− transfected cells, suramin (1–100 μM) produced concentration-dependent, parallel, dextral shifts of *E*/[*A*] curves to αβMeATP without a change in maxima, consistent with simple competitive antagonism (Fig. 4A). Schild regression analysis yielded a pK_B estimate of 5.6 with a slope that was not significantly different from unity. The functional affinity estimate for suramin is consistent with the affinity estimate obtained from competition studies with [^{35}S]ATPγS (data not shown). In contrast to suramin, PPADS (0.1–3 μM) produced dextral insurmountable concentration-dependent shifts in the *E*/[*A*] curves to αβMeATP (Fig. 4B) and thereby appearing as pseudoirreversible as described previously(Evans et al., 1995). A functional agonist profile was not possible as CHO-K1 cells express an endogenous P2 receptor linked to increases in intracellular calcium that is activated by both ATP and UTP but not αβMeATP (Iredale and Hill, 1993)(Lachnit et al., unpublished observations).

In summary, we have demonstrated that a high expression level of the $P2X_3$ receptor in a stably transfected mammalian cell line is achievable using a regulatible gene expression system and that the pharmacological properties of this homomeric receptor are consistent with what has been reported previously. Furthermore, this tetracycline controlled gene expression system has been shown to be a powerful tool

in the quantitative analysis of P2X receptor function. Whether or not these gene expression systems can unrestrictedly generate mammalian cell lines with expression levels of this magnitude with other membrane receptors such as G-protein coupled receptors, remains to be investigated. If so, this system will be very useful in the critical and quantitative evaluation of such receptors.

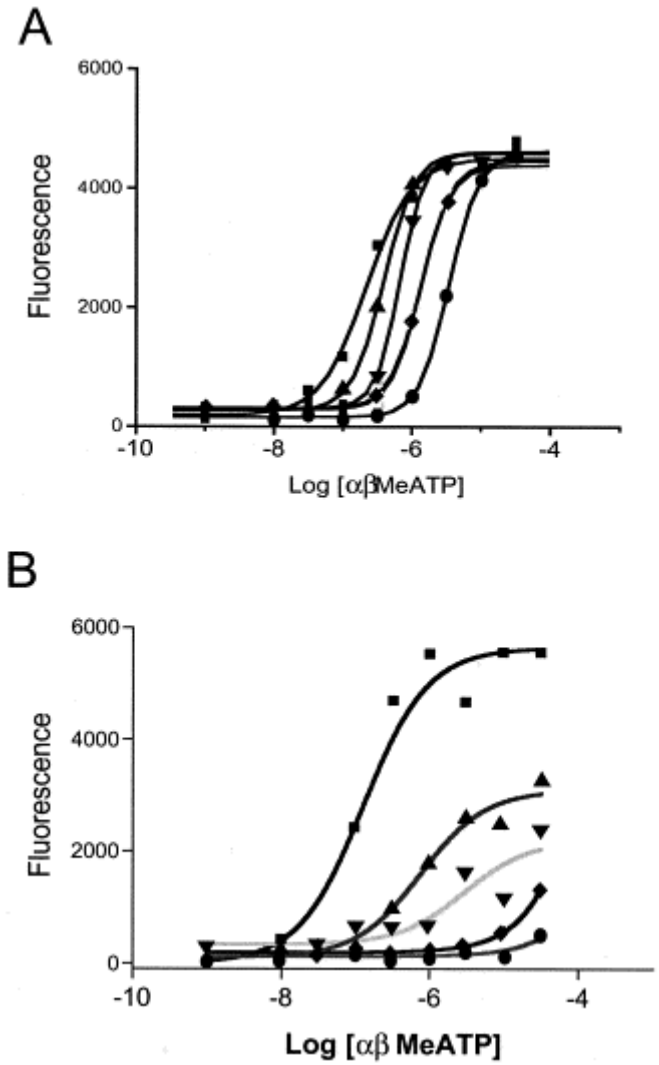

Figure 4: (A) The effect of suramin on intracellular calcium changes to αβMeATP in *tet*− $P2X_3$ transfected CHO-K1 tTA cells. A representative concentration–effect curve is shown for αβMeATP which was repeated four to five times in the absence and presence of 1 μM (▪), 3 μM (▲), 10 μM (▼) 30 μM (♦), and 100 μM (•) suramin. (B) The effect of PPADS on intracellular calcium changes to αβMeATP in *tet*− $P2X_3$ transfected CHO-K1 tTA cells. A representative concentration–effect curve is shown for αβMeATP which was repeated four to five times in the absence and presence of 0.1 μM (▪), 0.3 μM (▲), 1 μM (▼) and 3 μM (♦) PPADS. Values presented are expressed as change in fluorescence units.

Chapter 8: Closing Discussion and Conclusions

The work published in this thesis is part of a wider effort undertaken over several years to increase our knowledge of the pharmacology of P2X receptors and was conducted in the laboratories of both Roche Palo Alto and the Autonomic Neuroscience Institute at UCL. In it are described advances in the development and characterization of tools, both chemical and biological, useful for the study of purinergic receptor function in a variety of *in vitro* and *in vivo* models. The specific receptors and tissues studied are of particular relevance in the function of nerves and muscle cells that are present in or are connected to visceral organs, such as those located in the gastrointestinal and lower urinary tracts, but the tools employed will likely find future use for research spanning a wide spectrum of physiological processes and organ systems.

Chapters two and three of this thesis described the pharmacological characterization of two chemically novel, selective P2X antagonists: RO-4, a chemically optimized antagonist of $P2X_3$-containing receptors and RO-1, an un-optimized antagonist of $P2X_1$ receptors. However, despite the difference in the levels of chemical optimization of these two compounds, both were demonstrated to be useful for the study of native and recombinantly expressed P2X receptors. There are no previously published examples of small molecular weight antagonists, that fulfill all the criteria necessary for medicinal optimization (e.g. favorable pharmacokinetic characteristics, low molecular mass, good solubility), of $P2X_1$, $P2X_3$ or $P2X_{2/3}$ receptors; the current work represents the first such examples in a field previously represented only by relatively non-selective, large polyanions (e.g. PPADS, suramin, dyes) or nucleotide congeners (e.g. TNP-ATP), none of which form the foundation for medicinal development. Accordingly, both RO-4 and RO-1 would be expected to be useful for the study of purinergic signaling in tissues beyond those presented here. RO-4 embodies key advances beyond high potency and selectivity of antagonism for

$P2X_3$ and $P2X_{2/3}$ receptors, including a non-competitive mechanism of action, insensitive to agonist concentration and therefore potentially more effective when ATP concentrations are very high (e.g. under conditions of severe inflammation).

$P2X_3$ and $P2X_{2/3}$ receptors are predominantly located in small diameter sensory neurons innervating a variety of somatosensory and visceral organs and appear to be of particular importance for the transmission of nociceptive and mechanosensory information from the periphery to the central nervous system. The importance of signaling through $P2X_3$ and $P2X_{2/3}$ receptors has been shown in several models of chronic inflammatory and neuropathic pain (Wirkner et al., 2007), but never through the use of an orally bioavailable, CNS-penetrant $P2X_3/P2X_{2/3}$ antagonist (such as represented by RO-4). Furthermore, there is much work yet to be done to elucidate the role of $P2X_3$ and $P2X_{2/3}$ receptors in tissues for which there is genetic (mRNA) or protein evidence of their localization but little or incomplete understanding of their function, such as in the lens (Suzuki-Kerr et al., 2008), retina (Shigematsu et al., 2007), pancreatic beta cells (Silva et al., 2008) and chondrocytes (Varani et al., 2008), just to give some of the most recently published examples. A selective, widely distributed and orally bioavailable $P2X_3/P2X_{2/3}$ antagonist like RO-4 could be of great potential value for the study of these and many other tissues.

In a similar vein, although RO-1 has been used primarily in the present work to study the smooth muscle function *in vitro* of vascular and urinary bladder smooth muscle, $P2X_1$ receptors are also present (both alone and in mixed populations of multiple purinergic receptor subtypes) in a variety of tissues, including astrocytes, platelets, several types of white blood cells and sympathetic and sensory neurons (Burnstock and Knight, 2004), for which their function is poorly characterized; the

use of a selective, $P2X_1$ antagonist (perhaps developed from optimization of RO-1) could be useful to unweave the "web" of P2 receptors present in these and many other tissues (Volonte et al., 2006).

In chapter five, it has been possible to detail just such a web of purinergic receptors present on the urothelium of the rat urinary bladder. These studies, along with work published previously by other investigators (Ruggieri, 2006), illustrates that both P2X and P2Y receptors are present in the urothelium and these P2 receptors are critically involved in what appears to be a complex network of purinergic signaling mediated by ATP, released both through tissue damage and exocytosis. Not only can ATP act on multiple purinergic receptor subtypes to elicit functional responses, but it was shown that a $P2Y_{2/4}$ selective agonist, UTP-γ-S, can evoke further ATP release and may therefore serve as a positive feedback mechanism. Although this is only one example of a tissue containing multiple receptors for extracellular ATP and multiple, varied responses evoked by activation of these receptors, it is likely that this phenomenon is more the rule than the exception. It is difficult to find a tissue or cell type that doesn't have multiple receptor subtypes capable of being activated by ATP and its breakdown products ADP and adenosine (Burnstock and Knight, 2004). Making the situation even more complex, the expression of purinergic receptors in some cell types appears to change during post-natal development for some species, as described in chapter six where there is clear functional evidence that sympathetic ganglion neurons isolated from rats become progressively less sensitive to α,β-MeATP during the weeks after birth. Purinergic signaling in embryological and postnatal development is an active field of study and

one that may be highly relevant for cell regeneration and wound healing (Burnstock, 2008b).

The final chapter uses an account of the development and characterization of a novel cell line used for the *in vitro* study of purinergic receptors where a gene promoter regulated by an exogenously applied agent, tetracycline, is used to control the expression of $P2X_3$ receptors in CHO-K1 cells. This cell line has been, and likely will continue to be, put to good use to study the pharmacology and mechanism of action of selective $P2X_3$/$P2X_{2/3}$ antagonists and this method for regulating gene translation is potentially useful wherever tight control of receptor expression is required (e.g. when overexpression of a receptor is detrimental to the viability of a cell line). Tightly regulated, robust cell lines such as these were invaluable as we undertook lead identification efforts targeting several different P2X channels, including the rapidly desensitizing $P2X_1$ and $P2X_3$ subtypes.

The work contained in this thesis allows us to conclude:

1. P2X channels not previously known to be druggable (i.e. $P2X_3$-containing receptors and $P2X_1$) are in fact quite suitable targets for chemical leads possessing all of the necessary physicochemical attributes required for them to be optimized into candidate medicines.
2. Selective antagonists of P2X channels (e.g. $P2X_3$-containing receptors and $P2X_1$) can be used to reveal the functional mosaic of ATP-activated receptors present in many cells and tissues and in so doing clarify complex and fundamental intercellular signaling mechanisms.
3. The expression and functional interactions of receptors activated by ATP (and its breakdown products: ADP and adenosine), co-localized on the same cells

or within the same tissues (as described for rat urothelium and bladder smooth muscle in chapter five) is more likely the rule rather than the exception in most vertebrates.

4. A possible exception to the above "rule" may be the sensory ganglia of dorsal root and cranial nerves. In small cells in these nerves, it appears that despite evidence for multiple P2X subunit expression, the $P2X_3$-containing channels do contribute a large proportion of ATP-evoked currents.

In summary, I have presented work spanning several fronts of purinoceptor pharmacology and I have endeavored to describe the key tools used and to capture the salient conclusions derived from each series of experiments. This work discusses specific examples in which key advances in our understanding of purinergic function and signaling in several organ systems were achieved. More importantly it further describes the discovery and applicability of chemical and cellular tools of potentially widespread value in the study of purinergic receptor pharmacology. It has only been a little over a decade since the cloning of P2X receptors was completed and there is still much to do to advance the study of purinergic receptors; this work represents one step intended not only to further our understanding of purinergic receptor pharmacology but additionally to supply the tools required by others to elucidate the complex signaling evoked by receptors activated by ATP.

References

Abbracchio MP, Boeynaems J M, Barnard E A, Boyer J L, Kennedy C, Miras-Portugal M T, King B F, Gachet C, Jacobson K A, Weisman G A and Burnstock G (2003) Characterization of the UDP-Glucose Receptor (Re-Named Here the P2Y14 Receptor) Adds Diversity to the P2Y Receptor Family. *Trends Pharmacol Sci* **24**:52-55.

Abbracchio MP and Burnstock G (1994) Purinoceptors: Are There Families of P2X and P2Y Purinoceptors? *Pharmac Ther* **64**:445-75.

Alcaraz L, Baxter A, Bent J, Bowers K, Braddock M, Cladingboel D, Donald D, Fagura M, Furber M, Laurent C, Lawson M, Mortimore M, McCormick M, Roberts N and Robertson M (2003) Novel $P2X_7$ Receptor Antagonists. *Bioorg Med Chem Lett* **13**:4043-4046.

Alexander K, Niforatos W, Bianchi B, Burgard E C, Lynch K J, Kowaluk E A, Jarvis M F and Van Biesen T (1999) Allosteric Modulation and Accelerated Resensitization of Human $P2X_3$ Receptors by Cibacron Blue. *J Pharmacol Exp Ther* **291**:1135-1142.

Andersson KE and Wein A (2004) Pharmacology of the Lower Urinary Tract: Basis for Current and Future Treatments of Urinary Incontinence. *Pharmacol Rev* **56**:581-631.

Apostolidis A, Popat R, Yiangou Y, Cockayne D, Ford A P D W, Davis J B, Dasgupta P, Fowler C J and Anand P (2005) Decreased Sensory Receptors $P2X_3$ and TRPV1 in Suburothelial Nerve Fibers Following Intradetrusor Injections of Botulinum Toxin for Human Detrusor Overactivity. *Journal of Urology, The* **174**:977-983.

Arunlakshana, O. and Schild, H. O. (1959) Some quantitative uses of drug antagonists. *Brit.J.Pharmacol*. **14:**48-58.

Aschrafi A, Sadtler S, Niculescu C, Rettinger J and Schmalzing G (2004) Trimeric Architecture of Homomeric $P2X_2$ and Heteromeric P2X1+2 Receptor Subtypes. *J Mol Biol* **342**:333-343.

Banfi C, Ferrario S, De Vincenti O, Ceruti S, Fumagalli M, Mazzola A, D'Ambrosi N, Volonte C, Fratto P, Vitali E, Burnstock G, Beltrami E, Parolari A, Polvani G, Biglioli P, Tremoli E and Abbracchio M P (2005) P2 Receptors in Human Heart: Upregulation of $P2X_6$ in Patients Undergoing Heart Transplantation, Interaction With TNFα and Potential Role in Myocardial Cell Death. *J Mol Cell Cardiol* **39**:929-939.

Banks FCL, Knight G E, Calvert R C, Thompson C S, Morgan R J and Burnstock G (2006) The Purinergic Component of Human Vas Deferens Contraction. *Fertility and Sterility* **85**:932-939.

Baraldi PG, Nunez M.C., Morelli A, Falzoni S, Di Virgilio F and Romagnoli R (2003) Synthesis and Biological Activity of *N*-Arylpiperazine-Modified Analogues of KN-62, a Potent Antagonist of the Purinergic $P2X_7$ Receptor. *J Med Chem* **46**:1318-1329.

Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, Eunson L, Abdel'al S, Natt F, Hall J, Winter J, Bevan S, Wishart W, Fox A and Ganju P (2002) Functional Downregulation of $P2X_3$ Receptor Subunit in Rat Sensory Neurons Reveals a Significant Role in Chronic Neuropathic and Inflammatory Pain. *J Neurosci* **22**:8139-8147.

Bardoni R, Goldstein P A, Lee C J, Gu J G and MacDermott A B (1997) ATP P2X Receptors Mediate Fast Synaptic Transmission in the Dorsal Horn of the Rat Spinal Cord. *J Neurosci* **17**:5297-5304.

Baricordi OR, Ferrari D, Melchiorri L, Chiozzi P, Hanau S, Chiari E, Rubini M and Di Virgilio F (1996) An ATP-Activated Channel Is Involved in Mitogenic Stimulation of Human T Lymphocytes. *Blood* **87**:682-690.

Barnard, E. A., Burnstock, G., and Webb, T. E. (1994) G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol Sci* **15:**67-70.

Barrera N, Henderson R and Edwardson J (2008) Determination of the Architecture of Ionotropic Receptors Using AFM Imaging. *Pflugers Archiv European Journal of Physiology* **456**:199-209.

Barrera NP, Ormond S J, Henderson R M, Murrell-Lagnado R D and Edwardson J M (2005) Atomic Force Microscopy Imaging Demonstrates That $P2X_2$ Receptors Are Trimers but That $P2X_6$ Receptor Subunits Do Not Oligomerize. *J Biol Chem* **280**:10759-10765.

Basso AM, Bratcher N A, Harris R R, Jarvis M F, Decker M W and Rueter L E (2009) Behavioral Profile of P2X7 Receptor Knockout Mice in Animal Models of Depression and Anxiety: Relevance for Neuropsychiatric Disorders. *Behavioural Brain Research* **198**:83-90.

Baxter A, Bent J, Bowers K, Braddock M, Brough S, Fagura M, Lawson M, McInally T, Mortimore M, Robertson M, Weaver R and Webborn P (2003) Hit-to-Lead Studies: The Discovery of Potent Adamantane Amide $P2X_7$ Receptor Antagonists. *Bioorg Med Chem Lett* **13**:4047-4050.

Beckel, J. M., Meyers, S., Giesselman, B. R., de Groat, W. C., and Birder, L. A. (2005) Acetylcholine release from rat bladder epithelial cells and cholinergic modulation of bladder reflexes. *Exp Biol Abstracts* 863, 2.

Becker D, Woltersdorf R, Boldt W, Schmitz S, Braam U, Schmalzing G and Markwardt F (2008) The $P2X_7$ Carboxyl Tail is a Regulatory Module of $P2X_7$ Receptor Channel Activity. *J Biol Chem* **283**:25725-25734.

Bennett, G. J. and Xie, Y. K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33:**87-107.

Bernier LP, Ase A R, Chevallier S, Blais D, Zhao Q, Boue-Grabot E, Logothetis D and Seguela P (2008a) Phosphoinositides Regulate P2X4 ATP-Gated Channels Through Direct Interactions. *J Neurosci* **28**:12938-12945.

Bernier LP, Ase A R, Tong X, Hamel E, Blais D, Zhao Q, Logothetis D E and Seguela P (2008b) Direct Modulation of P2X1 Receptor-Channels by the Lipid Phosphatidylinositol 4,5-Bisphosphate. *Molecular Pharmacology* **74**:785-792.

Bertrand PP and Bornstein J C (2002) ATP As a Putative Sensory Mediator: Activation of Intrinsic Sensory Neurons of the Myenteric Plexus Via P2X Receptors. *J Neurosci* **22**:4767-4775.

Betschmann P, Bettencourt B, Donnelly-Roberts D, Friedman M, George J, Hirst G, Josephsohn N, Konopacki D, Li B, Maull J, Morytko MJ, Moore NS, Namovic M, Rafferty P,

Salmeron-Garcia JA, Tarcsa E, Wang L and Woller K (2008) Synthesis and Activity of N-Cyanoguanidine-Piperazine $P2X_7$ Antagonists. *Bioorg Med Chem Lett* **18**:3848-3851.

Bian X, Ren J, DeVries M, Schnegelsberg B, Cockayne D A, Ford A P D W and Galligan J J (2003) Peristalsis Is Impaired in the Small Intestine of Mice Lacking the $P2X_3$ Subunit. *J Physiol* **551**:309-322.

Bianchi BR, Lynch K J, Touma E, Niforatos W, Burgard E C, Alexander K M, Park H S, Yu H, Metzger R, Kowaluk E, Jarvis M F and van Biesen T (1999) Pharmacological Characterization of Recombinant Human and Rat P2X Receptor Subtypes. *Eur J Pharmacol* **376**:127-138.

Birder, L. A. (2001) Involvement of the urinary bladder urothelium in signaling in the lower urinary tract. *Proc.West.Pharmacol.Soc.* 44, 85-86.

Birder, L. A. (2005) More than just a barrier: urothelium as a drug target for urinary bladder pain. *AJP - Renal Physiology* **289:**F489-F495.

Birder, L. A. (2006) Role of the urothelium in urinary bladder dysfunction following spinal cord injury. *Prof.Brain Res.* **152:**135-146.

Birder, L. A., Apodaca, G., de Groat, W. C., and Kanai, A. J. (1998) Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. *AJP - Renal Physiology* **275:**F226-F229.

Birder LA, Barrick S R, Roppolo J R, Kanai A J, de Groat W C, Kiss S and Buffington C A (2003) Feline Interstitial Cystitis Results in Mechanical Hypersensitivity and Altered ATP Release From Bladder Urothelium. *Am J Physiol Renal Physiol* **285**:F423-F429.

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G and Kanai AJ (2002) Beta-Adrenoceptor Agonists Stimulate Endothelial Nitric Oxide Synthase in Rat Urinary Bladder Urothelial Cells. *J Neurosci* **22**:8063-8070.

Birder LA, Ruan H Z, Chopra B, Xiang Z, Barrick S, Buffington C A, Roppolo J R, Ford A P D W, de Groat W C and Burnstock G (2004) Alterations in P2X and P2Y Purinergic Receptor Expression in Urinary Bladder From Normal Cats and Cats With Interstitial Cystitis. *Am J Physiol Renal Physiol* **287**:F1084-F1091.

Blanchard DK, Wei S, Duan C, Pericle F, Diaz J I and Djeu J Y (1995) Role of Extracellular Adenosine Triphosphate in the Cytotoxic T- Lymphocyte-Mediated Lysis of Antigen Presenting Cells. *Blood* **85**:3173-3182.

Bo X, Jiang L H, Wilson H L, Kim M, Burnstock G, Surprenant A and North R A (2003a) Pharmacological and Biophysical Properties of the Human $P2X_5$ Receptor. *Mol Pharmacol* **63**:1407-1416.

Bo X, Kim M, Nori S L, Schoepfer R, Burnstock G and North R A (2003b) Tissue Distribution of $P2X_4$ Receptors Studied With an Ectodomain Antibody. *Cell Tissue Res* **313**:159-165.

Bo X, Schoepfer R and Burnstock G (2000) Molecular Cloning and Characterization of a Novel ATP P2X Receptor Subtype From Embryonic Chick Skeletal Muscle. *J Biol Chem* **275**:14401-14407.

Bo X, Zhang Y, Nassar M, Burnstock G and Schoepfer R (1995) A P2X Purinoceptor cDNA Conferring a Novel Pharmacological Profile. *FEBS Lett* **375**:129-133.

Boarder, M. R., Weisman, G. A., Turner, J. T., and Wilkinson, G. F. (1995) G protein coupled P2 purinoceptors: from molecular biology to functional responses. *Trends Pharmacol Sci* **16:**133-139.

Bobanovic LK, Royle S J and Murrell-Lagnado R D (2002) P2X Receptor Trafficking in Neurons Is Subunit Specific. *J Neurosci* **22**:4814-4824.

Boldogkoi Z, Schntz B, Sallach J and Zimmer A (2002) P2X3 Receptor Expression at Early Stage of Mouse Embryogenesis. *Mechanisms of Development* **118**:255-260.

Bornstein JC (2008) Purinergic Mechanisms in the Control of Gastrointestinal Motility. *Purinergic Signalling* **4**:197-212.

Boue-Grabot E, Emerit M B, Toulme E, Seguela P and Garret M (2004) Cross-Talk and Co-Trafficking Between P1/GABA Receptors and ATP-Gated Channels. *J Biol Chem* **279**:6967-6975.

Bowser DN and Khakh BS (2004) ATP Excites Interneurons and Astrocytes to Increase Synaptic Inhibition in Neuronal Networks. *J Neuroscience* **24**:8606-8620.

Bradbury EJ, Burnstock G and McMahon S B (1998) The Expression of $P2X_3$ Purinoreceptors in Sensory Neurons: Effects of Axotomy and Glial-Derived Neurotrophic Factor. *Mol Cell Neurosci* **12**:256-268.

Brady CM, Apostolidis A, Yiangou Y, Baecker P A, Ford A P, Freeman A, Jacques T S, Fowler C J and Anand P (2004) $P2X_3$-Immunoreactive Nerve Fibres in Neurogenic Detrusor Overactivity and the Effect of Intravesical Resiniferatoxin. *Eur Urol* **46**:247-253.

Brake AJ, Wagenbach M J and Julius D (1994) New Structural Motif for Ligand-Gated Ion Channels Defined by an Ionotropic ATP Receptor. *Nature* **371**:519-523.

Brandle U, Spielmanns P, Osteroth R, Sim J, Surprenant A, Buell G, Ruppersberg J P, Plinkert P K, Zenner H P and Glowatzki E (1997) Desensitization of the $P2X_2$ Receptor Controlled by Alternative Splicing. *FEBS Lett* **404**:294-298.

Brederson, J. D. and Jarvis, M. F. (2008) Homomeric and heteromeric P2X3 receptors in peripheral sensory neurons. *Curr Opin Investig Drugs* **9:**716-725.

Broom DC, Matson D J, Bradshaw E, Buck M E, Meade R, Coombs S, Matchett M, Ford K K, Yu W, Yuan J, Sun S H, Ochoa R, Krause J E, Wustrow D J and Cortright D N (2008) Characterization N-(Adamantan-1-Ylmethyl)-5-[(3R-Amino-Pyrrolidin-1-Yl)Methyl]-2-Chloro-Benzamide (AACBA), a P2X7 Antagonist in Animal Models of Pain and Inflammation. *J Pharmacol Exp Ther*jpet.

Brouns I, Adriaensen D, Burnstock G and Timmermans J P (2000) Intraepithelial Vagal Sensory Nerve Terminals in Rat Pulmonary Neuroepithelial Bodies Express $P2X_3$ Receptors. *Am J Resp Cell Mol Biol* **23**:52-61.

Brouns I, Van Genechten J, Hayashi H, Gajda M, Gomi T, Burnstock G, Timmermans J P and Adriaensen D (2003) Dual Sensory Innervation of Pulmonary Neuroepithelial Bodies. *Am J Resp Cell Mol Biol* **28**:275-285.

Brown SG, Townsend-Nicholson A, Jacobson K A, Burnstock G and King B F (2002) Heteromultimeric $P2X_{1/2}$ Receptors Show a Novel Sensitivity to Extracellular PH. *J Pharmacol Exp Ther* **300**:673-680.

Brunschweiger, A. and Muller, C. E. (2006) P2 receptors activated by uracil nucleotides - an update. *Curr Med Chem* **13:**289-312.

Buell G, Chessell I P, Michel A D, Collo G, Salazzo M, Herren S, Gretener D, Grahames C, Kaur R, Kosco-Vilbois M H and Humphrey P P A (1998) Blockade of Human $P2X_7$ Receptor Function With a Monoclonal Antibody. *Blood* **92**:3521-3528.

Buell, G., Collo, G., and Rassendren, F. (1996a) P2X receptors: an emerging channel family. *Eur J Neurosci* **8:**2221-2228.

Buell G, Lewis C, Collo G, North R A and Surprenant A (1996b) An Antagonist-Insensitive P2X Receptor Expressed in Epithelia and Brain. *EMBO J* **15**:55-62.

Bulanova E, Budagian V, Orinska Z, Hein M, Petersen F, Thon L, Adam D and Bulfone-Paus S (2005) Extracellular ATP Induces Cytokine Expression and Apoptosis Through $P2X_7$ Receptor in Murine Mast Cells. *J Immunol* **174**:3880-3890.

Burgard EC, Niforatos W, Van Biesen T, Lynch K J, Kage K L, Touma E, Kowaluk E A and Jarvis M F (2000) Competitive Antagonism of Recombinant $P2X_{2/3}$ Receptors by 2', 3'-*O*-(2,4,6-Trinitrophenyl) Adenosine 5'-Triphosphate (TNP-ATP). *Mol Pharmacol* **58**:1502-1510.

Burgard EC, Niforatos W, Van Biesen T, Lynch K J, Touma E, Metzger R E, Kowaluk E A and Jarvis M F (1999) P2X Receptor-Mediated Ionic Currents in Dorsal Root Ganglion Neurons. *J Neurophysiol* **82**:1590-1598.

Burnstock, G and Wood, J. N. (1996) Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Curr Opin Neurobiol* **6:**526-532.

Burnstock G (1972) Purinergic Nerves. *Pharmacol Rev* **24**:509-581.

Burnstock, G. (1976)Do some nerve cells release more than one transmitter? *Neuroscience* **1:**239-248.

Burnstock G (1978) A basis for distinguishing two types of purinergic receptor, in *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach* (Straub RW and Bolis L eds) pp 107-118, Raven Press, New York.

Burnstock, G. A unifying purinergic hypothesis for the initiation of pain. Lancet 347, 1604-1605. 1996.

Burnstock G (1999) Release of Vasoactive Substances From Endothelial Cells by Shear Stress and Purinergic Mechanosensory Transduction. *J Anat* **194**:335-342.

Burnstock G (2001a) Purine-Mediated Signalling in Pain and Visceral Perception. *Trends Pharmacol Sci* **22**:182-188.

Burnstock, G. Purinergic signalling. 151, 89-127. (2001b) Berlin, Springer-Verlag. Handbook of experimental pharmacology, I.Purinergic and pyrimidinergic signalling. Abbracchio, M. P. and Williams, M.

Burnstock, G. (2003) Purinergic receptors in the nervous system. Schwiebert, E. M. Extracellular nucleotides and nucleosides: Release, receptors, and physiological and pathophysiological effects. **54:**307-368. USA, Elsevier Science. *Current topics in membranes*. Benos, D. J. and Simon, S. A.

Burnstock G (2007) Physiology and Pathophysiology of Purinergic Neurotransmission. *Physiol Rev* **87**:659-797.

Burnstock, G. (2008a) The journey to establish purinergic signalling in the gut. *Neurogastroenterol Motil* **20 (Suppl. 1):**8-19.

Burnstock G (2008b) Unresolved Issues and Controversies in Purinergic Signalling. *J Physiol (Lond)* **586**:3307-3312.

Burnstock, G. (2009) Autonomic neurotransmission: 60 years since Sir Henry Dale. *Annu.Rev.Pharmacol.Toxicol.* **49:**1-30.

Burnstock, G., Campbell, G., Bennett M., and Holman, M. E. (1964) Innervation of the guinea pig taenia coli: are there intrinsic inhibitory nerves which are distinct from sympathetic nerves? *International Journal of Neuropharmacology* **3:**163-166.

Burnstock G, Cocks T, Crowe R and Kasakov L (1978) Purinergic Innervation of the Guinea-Pig Urinary Bladder. *Br J Pharmacol* **63**:125-138.

Burnstock G, Dumsday B and Smythe A (1972) Atropine Resistant Excitation of the Urinary Bladder: the Possibility of Transmission Via Nerves Releasing a Purine Nucleotide. *Br J Pharmacol* **44**:451-461.

Burnstock G and Kennedy C (1985) Is There a Basis for Distinguishing Two Types of P_2-Purinoceptor? *Gen Pharmacol* **16**:433-440.

Burnstock G and Knight G E (2004) Cellular Distribution and Functions of P2 Receptor Subtypes in Different Systems. *Int Rev Cytol* **240**:31-304.

Burnstock G and Lavin S (2002) Interstitial Cells of Cajal and Purinergic Signalling. *Auton Neurosci* **97**:68-72.

Burton LD, Housley G D, Salih S G, Jarlebark L, Christie D L and Greenwood D (2000) $P2X_2$ Receptor Expression by Interstitial Cells of Cajal in Vas Deferens Implicated in Semen Emission. *Auton Neurosci* **84**:147-161.

Callister RJ, Keast J R and Sah P (1997) Ca(2+)-Activated K+ Channels in Rat Otic Ganglion Cells: Role of Ca2+ Entry Via Ca2+ Channels and Nicotinic Receptors. *J Physiol* **500**:571-582.

Calvert JA and Evans R J (2004) Heterogeneity of P2X Receptors in Sympathetic Neurons: Contribution of Neuronal $P2X_1$ Receptors Revealed Using Knockout Mice. *Mol Pharmacol* **65**:139-148.

Calvert RC, Shabbir M, Thompson C S, Mikhailidis D P, Morgan R J and Burnstock G (2004) Immunocytochemical and Pharmacological Characterisation of P2-Purinoceptor-Mediated Cell Growth and Death in PC-3 Hormone Refractory Prostate Cancer Cells. *Anticancer Res* **24**:2853-2859.

Calvert, R. C., Thompson, C. S., and Burnstock, G. (2008) ATP release from the human ureter on distension and P2X(3) receptor expression on suburothelial sensory nerves. Purinergic Signal. **4:**377-381.

Carroll WA, Kalvin D M, Medrano A P, Florjancic A S, Wang Y, Donnelly-Roberts D L, Namovic M T, Grayson G, Honore P and Jarvis M F (2007) Novel and Potent 3-(2,3-Dichlorophenyl)-4-(Benzyl)-4H-1,2,4-Triazole P2X7 Antagonists. *Bioorg Med Chem* Lett **17**:4044-4048.

Carter DS, Alam M, Cai H, Dillon MP, Ford APDW, Gever JR, Jahangir A, Lin C, Moore AG, Wagner PJ and Zhai Y (2009) Identification and SAR of Novel Diaminopyrimidines. Part 1: The Discovery of RO-4, a Dual $P2X_3/P2X_{2/3}$ Antagonist for the Treatment of Pain. *Bioorg Med Chem Lett* **19**:1628-1631.

Cascio M (2004) Structure and Function of the Glycine Receptor and Related Nicotinicoid Receptors. *J Biol Chem* **279**:19383-19386.

Castelucci P, Robbins H L, Poole D P and Furness J B (2002) The Distribution of Purine $P2X_2$ Receptors in the Guinea-Pig Enteric Nervous System. *Histochem Cell Biol* **117**:415-422.

Cathala L, Misra C and Cull-Candy S (2000) Developmental Profile of the Changing Properties of NMDA Receptors at Cerebellar Mossy Fiber-Granule Cell Synapses. *J Neurosci* **20**:5899-5905.

Chakfe Y, Seguin R, Antel J P, Morissette C, Malo D, Henderson D and Seguela P (2002) ADP and AMP Induce Interleukin-1β Release From Microglial Cells Through Activation of ATP-Primed $P2X_7$ Receptor Channels. *J Neurosci* **22**:3061-3069.

Chaumont S and Khakh BS (2008) Patch-Clamp Coordinated Spectroscopy Shows $P2X_2$ Receptor Permeability Dynamics Require Cytosolic Domain Rearrangements but not Panx-1 Channels. *PNAS* **105**:12063-12068.

Chen C, Parker M S, Barnes A P, Deininger P and Bobbin R P (2000) Functional Expression of Three $P2X_2$ Receptor Splice Variants From Guinea Pig Cochlea. *J Neurophysiol* **83**:1502-1509.

Chen CC, Akopian A N, Sivilotti L, Colquhoun D, Burnstock G and Wood J N (1995) A P2X Purinoceptor Expressed by a Subset of Sensory Neurons. *Nature* **377**:428-31.

Chessell IP, Michel A D and Humphrey P P (1998a) Effects of Antagonists at the Human Recombinant P2X7 Receptor. *Br J Pharmacol* **124**:1314-1320.

Chessell IP, Simon J, Hibell A D, Michel A D, Barnard E A and Humphrey P P (1998b) Cloning and Functional Characterisation of the Mouse P2X7 Receptor. *FEBS Lett* **439**:26-30.

Chessell IP, Simon J, Hibell A D, Michel A D, Barnard E A and Humphrey P P A (1998c) Cloning and Functional Characterisation of the Mouse $P2X_7$ Receptor. *FEBS Lett* **439**:26-30.

Chessell IP, Hatcher J P, Bountra C, Michel A D, Hughes J P, Green P, Egerton J, Murfin M, Richardson J, Peck W L, Grahames C B A, Casula M A, Yiangou Y, Birch R, Anand P and Buell G N (2005) Disruption of the P2X7 Purinoceptor Gene Abolishes Chronic Inflammatory and Neuropathic Pain. *Pain* **114**:386-396.

Cheung KK and Burnstock G (2002) Localization of $P2X_3$ Receptors and Coexpression With $P2X_2$ Receptors During Rat Embryonic Neurogenesis. *J Comp Neurol* **443**:368-382.

Cheung KK, Chan W Y and Burnstock G (2005) Expression of P2X Purinoceptors During Rat Brain Development and Their Inhibitory Role on Motor Axon Outgrowth in Neural Tube Explant Cultures. *Neuroscience* **133**:937-945.

Cheung, K. K., Ryten, M., and Burnstock, G. (2003) Abundant and dynamic expression of G protein-coupled P2Y receptors in mammalian development. *Dev Dyn* **228:**254-266.

Chopra B, Barrick S R, Meyers S, Beckel J M, Zeidel M L, Ford A P D W, de Groat W C and Birder L A (2005) Expression and Function of Bradykinin B1 and B2 Receptors in Normal and Inflamed Rat Urinary Bladder Urothelium. *J Physiol* **562**:859-871.

Chopra B, Gever J R, Barrick S R, Hanna-Mitchell A T, Beckel J M, Ford A P D W and Birder L A (2008) Expression and Function of Rat Urothelial P2Y Receptors. *Am J Physiol Renal Physiol* **294**:F821-F829.

Clyne JD, Brown T C and Hume R I (2003) Expression Level Dependent Changes in the Properties of $P2X_2$ Receptors. *Neuropharmacology* **44**:403-412.

Clyne JD, LaPointe L D and Hume R I (2002) The Role of Histidine Residues in Modulation of the Rat $P2X_2$ Purinoceptor by Zinc and PH. *J Physiol* **539**:347-359.

Cockayne DA, Dunn P M, Zhong Y, Rong W, Hamilton S G, Knight G E, Ruan H Z, Ma B, Yip P, Nunn P, McMahon S B, Burnstock G and Ford A P D W (2005) $P2X_2$ Knockout Mice and $P2X_2/P2X_3$ Double Knockout Mice Reveal a Role for the $P2X_2$ Receptor Subunit in Mediating Multiple Sensory Effects of ATP. *J Physiol* **567**:621-639.

Cockayne DA, Hamilton S G, Zhu Q M, Dunn P M, Zhong Y, Novakovic S, Malmberg A B, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit W G, Burnstock G, McMahon S B and Ford A P (2000) Urinary Bladder Hyporeflexia and Reduced Pain-Related Behaviour in P2X3-Deficient Mice. *Nature* **407**:1011-1015.

Collet C, Strube C, Csernoch L, Mallouk N, Ojeda C, Allard B and Jacquemond V (2002) Effects of Extracellular ATP on Freshly Isolated Mouse Skeletal Muscle Cells During Pre-Natal and Post-Natal Development. *Pflugers Arch* **443**:771-778.

Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North R A and Buell G (1997) Tissue Distribution of the $P2X_7$ Receptor. *Neuropharmacology* **36**:1277-1284.

Collo G, North R A, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A and Buell G (1996) Cloning of $P2X_5$ and $P2X_6$ Receptors and the Distribution and Properties of an Extended Family of ATP-Gated Ion Channels. *J Neurosci* **16**:2495-2507.

Colvin RA, Fontaine C P, Laskowski M and Thomas D (2003) Zn^{2+} Transporters and Zn^{2+} Homeostasis in Neurons. *Eur J Pharmacol* **479**:171-185.

Cook SP, Rodland K D and McCleskey E W (1998) A Memory for Extracellular Ca^{2+} by Speeding Recovery of P2X Receptors From Desensitization. *J Neurosci* **18**:9238-9244.

Cook SP, Vulchanova L, Hargreaves K M, Elde R and McCleskey E W (1997) Distinct ATP Receptors on Pain-Sensing and Stretch-Sensing Neurons. *Nature* **387**:505-8.

Davies DL, Kochegarov A A, Kuo S T, Kulkarni A A, Woodward J J, King B F and Alkana R L (2005) Ethanol Differentially Affects ATP-Gated $P2X_3$ and $P2X_4$ Receptor Subtypes Expressed in Xenopus Oocytes. *Neuropharmacology* **49**:243-253.

Dawson GR, Wafford K A, Smith A, Marshall G R, Bayley P J, Schaeffer J M, Meinke P T and McKernan R M (2000) Anticonvulsant and Adverse Effects of Avermectin Analogs in Mice Are Mediated Through the Γ-Aminobutyric $Acid_{A\ Receptor}$. *J Pharmacol Exp Ther* **295**:1051-1060.

de Groat, W. C. (2004) The urothelium in overactive bladder: passive bystander or active participant. *Urology* **64:**7-11.

de Groat WC (2006) Integrative Control of the Lower Urinary Tract: Preclinical Perspective. *Br J Pharmacol* **147**:S25-S40.

Denlinger LC, Fisette PL, Sommer JA, Watters JJ, Prabhu U, Dubyak GR, Proctor RA and Bertics PJ (2001) The Nucleotide Receptor $P2X_7$ Contains Multiple Protein- and Lipid-Interaction Motifs Including a Potential Binding Site for Bacterial Lipopolysaccharide. *J Immunol* **167**:1871-1876.

Denlinger LC, Sommer JA, Parker K, Gudipaty L, Fisette PL, Watters JW, Proctor RA, Dubyak GR and Bertics PJ (2003) Mutation of a Dibasic Amino Acid Motif Within the C Terminus of the $P2X_7$ Nucleotide Receptor Results in Trafficking Defects and Impaired Function. *J Immunol* **171**:1304-1311.

Deuchars SA, Atkinson L, Brooke R E, Musa H, Milligan C J, Batten T F C, Buckley N J, Parson S H and Deuchars J (2001) Neuronal $P2X_7$ Receptors Are Targeted to Presynaptic Terminals in the Central and Peripheral Nervous Systems. *J Neurosci* **21**:7143-7152.

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz J M, Morelli A, Torboli M, Bolognesi G and Baricordi O R (2001) Nucleotide Receptors: an Emerging Family of Regulatory Molecules in Blood Cells. *Blood* **97**:587-600.

Diaz-Hernandez M, Cox J A, Migita K, Haines W, Egan T M and Voigt M M (2002) Cloning and Characterization of Two Novel Zebrafish P2X Receptor Subunits. *Biochem Biophys Res Commun* **295**:849-853.

Ding S and Sachs F (1999) Ion Permeation and Block of $P2X_2$ Purinoceptors: Single Channel Recordings. *J Membr Biol* **172**:215-223.

Donnelly-Roberts DL and Jarvis M F (2007) Discovery of P2X7 Receptor-Selective Antagonists Offers New Insights into P2X7 Receptor Function and Indicates a Role in Chronic Pain States. *Br J Pharmacol* **151**:571-579.

Dorn G, Patel S, Wotherspoon G, Hemmings-Mieszczak M, Barclay J, Natt F J C, Martin P, Bevan S, Fox A, Ganju P, Wishart W and Hall J (2004) SiRNA Relieves Chronic Neuropathic Pain. *Nucleic Acids Res* **32**:e49.

Downie, J. W. and Karmazyn, M. (1984) Mechanical trauma to bladder epithelium liberates prostanoids which modulate neurotransmission in rabbit detrusor muscle. *Journal of Pharmacology And Experimental Therapeutics* **230:**445-449.

Drury AN and Szent-Györgyi A (1929) The Physiological Activity of Adenine Compounds With Especial Reference to Their Action Upon Mammalian Heart. *J Physiol* **68**:213-237.

Duan S, Anderson C M, Keung E C, Chen Y, Chen Y and Swanson R A (2003) $P2X_7$ Receptor-Mediated Release of Excitatory Amino Acids From Astrocytes. *J Neurosci* **23**:1320-1328.

Dunn PM and Blakeley A G (1988) Suramin: a Reversible P2-Purinoceptor Antagonist in the Mouse Vas Deferens. *Br J Pharmacol* **93**:243-245.

Dunn, P. M., Gever, J. R., Ruan, H. Z., and Burnstock, G. (2005) Developmental changes in heteromeric P2X2/3 receptor expression in rat sympathetic ganglion neurons. *Dev Dyn* **234:**505-511.

Dunn PM, Liu M, Zhong Y, King B F and Burnstock G (2000) Diinosine Pentaphosphate: an Antagonist Which Discriminates Between Recombinant $P2X_3$ and $P2X_{2/3}$ Receptors and Between Two P2X Receptors in Rat Sensory Neurones. *Br J Pharmacol* **130**:1378-1384.

Dunn PM, Zhong Y and Burnstock G (2001) P2X Receptors in Peripheral Neurons. *Prog Neurobiol* **65**:107-134.

Dworkin RH, O'Connor A B, Backonja M, Farrar J T, Finnerup N B, Jensen T S, Kalso E A, Loeser J D, Miaskowski C, Nurmikko T J, Portenoy R K, Rice A S C, Stacey B R, Treede R D, Turk D C and Wallace M S (2007) Pharmacologic Management of Neuropathic Pain: Evidence-Based Recommendations. *Pain* **132**:237-251.

Egan TM and Khakh BS (2004) Contribution of Calcium Ions to P2X Channel Responses. *J Neurosci* **24**:3413-20.

Elneil S, Skepper J N, Kidd E J, Williamson J G and Ferguson D R (2001) Distribution of $P2X_1$ and $P2X_3$ Receptors in the Rat and Human Urinary Bladder. *Pharmacology* **63**:120-128.

Ennion S, Hagan S and Evans R J (2000) The Role of Positively Charged Amino Acids in ATP Recognition by Human $P2X_1$ Receptors. *J Biol Chem* **275**:35656.

Erhardt JA, Pillarisetti K and Toomey J R (2003) Potentiation of Platelet Activation Through the Stimulation of $P2X_1$ Receptors. *J Thromb Hemostasis* **1**:2626-2635.

Evans RJ, Lewis C, Buell G, Valera S, North R A and Surprenant A (1995) Pharmacological Characterization of Heterologously Expressed ATP-Gated Cation Channels (P_{2X} Purinoceptors). *Mol Pharmacol* **48**:178-183.

Evans RJ, Lewis C, Virginio C, Lundstrom K, Buell G, Surprenant A and North R A (1996) Ionic Permeability of, and Divalent Cation Effects on, Two ATP-Gated Cation Channels (P2X Receptors) Expressed in Mammalian Cells. *J Physiol* **497**:413-422.

Falzoni S, Munerati M, Ferrari D, Spisani S, Moretti S and Di Virgilio F (1995) The Purinergic P2Z Receptor of Human Macrophage Cells. Characterization and Possible Physiological Role. *J Clin Invest* **95**:1207-1216.

Fam SR, Gallagher C J, Kalia L V and Salter M W (2003) Differential Frequency Dependence of P2Y1- and P2Y2- Mediated Ca 2+ Signaling in Astrocytes. *J Neurosci* **23**:4437-4444.

Faria RX, DeFarias F P and Alves L A (2005) Are Second Messengers Crucial for Opening the Pore Associated With P2X7 Receptor? *Amer J Physiol Cell Physiol* **288**:C260-C271.

Fedan, J. S., Hogaboom, G. K., O'Donnell, J. P., Colby, J., and Westfall, D. P. (1981) Contribution by purines to the neurogenic response of the vas deferens of the guinea pig. *Eur.J.Pharmacol.* **69:**41-53.

Ferguson DR, Kennedy I and Burton T J (1997) ATP Is Released From Rabbit Urinary Bladder Epithelial Cells by Hydrostatic Pressure Changes - a Possible Sensory Mechanism? *J Physiol* **505**:503-511.

Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi O R and Di Virgilio F (1997) Extracellular ATP Triggers IL-1 Beta Release by Activating the Purinergic P2Z Receptor of Human Macrophages. *J Immunol* **159**:1451-1458.

Ferrari D, Pizzirani C, Adinolfi E, Lemoli M, Curti A., Idzko M, Panther E and Di Virgilio F (2006) The P2X7 Receptor: a Key Player in IL-1 Processing and Release. *J Immunol* **176**:3877-3883.

Fields RD and Burnstock G (2006) Purinergic Signalling in Neuron-Glia Interactions. *Nat Rev Neuroscience* **7**:423-436.

Finger TE, Danilova V, Barrows J, Bartel D L, Vigers A J, Stone L, Hellekant G and Kinnamon S C (2005) ATP Signaling Is Crucial for Communication From Taste Buds to Gustatory Nerves. *Science* **310**:1495-1499.

Fisher JA, Girdler G and Khakh B S (2004) Time-Resolved Measurement of State-Specific $P2X_2$ Ion Channel Cytosolic Gating Motions. *J Neurosci* **24**:10475-10487.

Ford APDW, Gever J R, Nunn P A, Zhong Y, Cefalu J S, Dillon M P and Cockayne D A (2006) Purinoceptors As Therapeutic Targets for Lower Urinary Tract Dysfunction. *Br J Pharmacol* **147**:S132-S143.

Ford KK, Matchett M, Krause J E and Yu W (2005) The $P2X_3$ Antagonist P^1, P^5-Di[Inosine-5'] Pentaphosphate Binds to the Desensitized State of the Receptor in Rat Dorsal Root Ganglion Neurons. *J Pharmacol Exp Ther* **315**:405-413.

Franke H, Bringmann A, Pannicke T, Kr U, Grosche J, Reichenbach A and Illes P (2001) P2 Receptors on Macroglial Cells: Functional Implications for Gliosis. *Drug Devel Res* **53**:140-147.

Fredholm BB, Abbracchio M P, Burnstock G, Daly J W, Harden T K, Jacobson K A, Leff P and Williams M (1994) Nomenclature and Classification of Purinoceptors. *Pharmacol Rev* **46**:143-156.

Fu XW, Nurse C A and Cutz E (2004) Expression of Functional Purinergic Receptors in Pulmonary Neuroepithelial Bodies and Their Role in Hypoxia Chemotransmission. *Biol Chem* **385**:275-284.

Fujii K (1988) Evidence for Adenosine Triphosphate As an Excitatory Transmitter in Guinea-Pig, Rabbit and Pig Urinary Bladder. *J Physiol* **404**:39-52.

Fujiwara Y and Kubo Y (2006) Regulation of the Desensitization and Ion Selectivity of ATP-Gated P2X2 Channels by Phosphoinositides. *J Physiol (Lond)* **576**:135-149.

Furber M, Alcaraz L, Bent JE, Beyerbach A, Bowers K, Braddock M, Caffrey MV, Cladingboel D, Collington J, Donald DK, Fagura M, Ince F, Kinchin EC, Laurent C, Lawson M, Luker TJ, Mortimore MMP, Pimm AD, Riley RJ, Roberts N, Robertson M, Theaker J, Thorne PV, Weaver R, Webborn P and Willis P (2007) Discovery of Potent and Selective Adamantane-Based Small-Molecule $P2X_7$ Receptor Antagonists/Interleukin-1β Inhibitors. *J Med Chem* **50**:5882-5885.

Furuya K, Sokabe M and Furuya S (2005) Characteristics of Subepithelial Fibroblasts As a Mechano-Sensor in the Intestine: Cell-Shape-Dependent ATP Release and P2Y1 Signaling. *J Cell Sci* **118**:3289-3304.

Gachet, C. (2008) P2 receptors, platelet function and pharmacological implications. *Thrombosis and Haemostasis* **99:**466-472.

Galligan JJ (2004) Enteric P2X Receptors As Potential Targets for Drug Treatment of the Irritable Bowel Syndrome. *Br J Pharmacol* **141**:1294-1302.

Galligan JJ and North R A (2004) Pharmacology and Function of Nicotinic Acetylcholine and P2X Receptors in the Enteric Nervous System. *Neurogastroenterol Motil* **16 Suppl 1**:64-70.

Garcia-Guzman M, Soto F, Gomez-Hernandez J M, Lund P E and Stuhmer W (1997a) Characterization of Recombinant Human $P2X_4$ Receptor Reveals Pharmacological Differences to the Rat Homologue. *Mol Pharmacol* **51**:109-118.

Garcia-Guzman M, Soto F, Laube B and Stuhmer W (1996) Molecular Cloning and Functional Expression of a Novel Rat Heart P2X Purinoceptor. *FEBS Lett* **388**:123-127.

Garcia-Guzman M, Stuhmer W and Soto F (1997b) Molecular Characterization and Pharmacological Properties of the Human $P2X_3$ Purinoceptor. *Mol Brain Res* **47**:59-66.

Gargett CE and Wiley J S (1997) The Isoquinoline Derivative KN-62 a Potent Antagonist of the P2Z-Receptor of Human Lymphocytes. *Br J Pharmacol* **120**:1483-1490.

Gartland A, Buckley K A, Bowler W B and Gallagher J A (2003a) Blockade of the Pore-Forming $P2X_7$ Receptor Inhibits Formation of Multinucleated Human Osteoclasts *in Vitro*. *Calcif Tissue Int* **73**:361-369.

Gartland A, Buckley K A, Hipskind R A, Perry M J, Tobias J H, Buell G, Chessell I, Bowler W B and Gallagher J A (2003b) Multinucleated Osteoclast Formation *in Vivo* and *in Vitro* by $P2X_7$ Receptor-Deficient Mice. *Crit Rev Eukaryot Gene Expr* **13**:243-253.

Gartland A, Hipskind R A, Gallagher J A and Bowler W B (2001) Expression of a $P2X_7$ Receptor by a Subpopulation of Human Osteoblasts. *J Bone Miner Res* **16**:846-856.

Gayle S and Burnstock G (2005) Immunolocalisation of P2X and P2Y Nucleotide Receptors in the Rat Nasal Mucosa. *Cell Tissue Res* **319**:27-36.

Gerevich Z, Zadori Z S, Koles L, Kopp L, Milius D, Wirkner K, Gyires K and Illes P (2007) Dual Effect of Acid PH on Purinergic P2X3 Receptors Depends on the Histidine 206 Residue. *J Biol Chem* **282**:33949-33957.

Gever JR, Cockayne D A, Dillon M P, Burnstock G and Ford A P D W (2006) Pharmacology of P2X Channels. *Pflugers Archiv European Journal of Physiology*1-25.

Gever, J. R., Padilla, F., Knight, G. F., Dunn, P. M., Tran, A., Mandel, D. A., Hegde, S. S., Jiame-Figueroa, S., Greenhouse, R. J., Lachnit, W. G., Burnstock, G., and Ford, P. D. W. (2004) In vitro and in vivo characterization of RO0437626, a novel and selective $P2X_1$ antagonist. Purines 2004 : 4th International Symposium on Nucleosides and nucleotides ; Chapel Hill, NC; June 6-9 2004, p.86.

Gilchrist LS, Cain D M, Harding-Rose C, Kov A N, Wendelschafer-Crabb G, Kennedy W R and Simone D A (2005) Re-Organization of $P2X_3$ Receptor Localization on Epidermal Nerve Fibers in a Murine Model of Cancer Pain. *Brain Res* **1044**:197-205.

Gitterman DP and Evans R J (2000) Properties of P2X and P2Y Receptors Are Dependent on Artery Diameter in the Rat Mesenteric Bed. *Br J Pharmacol* **131**:1561-1568.

Glass R, Townsend A and Burnstock G (2000) P2 Receptors in the Thymus: Expression of P2X and P2Y Receptors in Adult Rats, an Immunohistochemical and in Situ Hybridisation Study. *Cell Tissue Res* **300**:295-306.

Gonzalez FA, Ahmed A H, Lustig K D, Erb L and Weisman G A (1989) Permeabilization of Transformed Mouse Fibroblasts by 3'-O-(4-Benzoyl)Benzoyl Adenosine 5'-Triphosphate and the Desensitization of the Process. *J Cell Physiol* **139**:109-115.

Gossen, M. and Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci 89, 5547-5551. 1992.

Gourine AV, Atkinson L, Deuchars J and Spyer K M (2003) Purinergic Signalling in the Medullary Mechanisms of Respiratory Control in the Rat: Respiratory Neurones Express the $P2X_2$ Receptor Subunit. *J Physiol* **552**:197-211.

Grahames CBA, Michel A D, Chessell I P and Humphrey P P A (1999) Pharmacological Characterization of ATP- and LPS-Induced IL-1β Release in Human Monocytes. *Br J Pharmacol* **127**:1915-1921.

Greig AV, James S E, McGrouther D A, Terenghi G and Burnstock G (2003a) Purinergic Receptor Expression in the Regenerating Epidermis in a Rat Model of Normal and Delayed Wound Healing. *Exp Dermatol* **12**:860-871.

Greig AV, Linge C, Healy V, Lim P, Clayton E, Rustin M H, McGrouther D A and Burnstock G (2003b) Expression of Purinergic Receptors in Non-Melanoma Skin Cancers and Their Functional Roles in A431 Cells. *J Invest Dermatol* **121**:315-327.

Groschel-Stewart U, Bardini M, Robson T and Burnstock G (1999) Localisation of $P2X_5$ and $P2X_7$ Receptors by Immunohistochemistry in Rat Stratified Squamous Epithelia. *Cell Tissue Res* **296**:599-605.

Grubb BD and Evans R J (1999) Characterization of Cultured Dorsal Root Ganglion Neuron P2X Receptors. *Eur J Neurosci* **11**:149-154.

Grutter T, Le Novere N and Changeux J P (2004) Rational Understanding of Nicotinic Receptors Drug Binding. *Curr Top Med Chem* **4**:645-650.

Gu JG and MacDermott A B (1997) Activation of ATP P2X Receptors Elicits Glutamate Release From Sensory Neuron Synapses. *Nature* **389**:749-753.

Gudipaty L, Humphreys B D, Buell G and Dubyak G R (2001) Regulation of $P2X_7$ Nucleotide Receptor Function in Human Monocytes by Extracellular Ions and Receptor Density. *Am J Physiol Cell Physiol* **280**:C943-C953.

Guerlet G, Taly A, Prado de Carvalho L, Martz A, Jiang R, Specht A, Le Noviere N and Grutter T (2008) Comparative Models of P2X2 Receptor Support Inter-Subunit ATP-Binding Sites. *Biochemical and Biophysical Research Communications* **375**:405-409.

Guile SD, Alcaraz L, Birkinshaw TN, Bowers KC, Ebden MR, Furber M and Stocks MJ (2009) Antagonists of the $P2X_7$ Receptor. From Lead Identification to Drug Development. *J Med Chem* **52**:3123-3141.

Gunosewoyo H, Coster MJ, Bennett MR and Kassiou M (2009) Purinergic $P2X_7$ Receptor Antagonists: Chemistry and Fundamentals of Biological Screening. *Bioorg Med Chem* **17**:4861-4865.

Guo C, Masin M, Qureshi O S and Murrell-Lagnado R D (2007) Evidence for Functional P2X4/P2X7 Heteromeric Receptors. *Mol Pharmacol* **72**:1447-1456.

Guo LH, Trautmann K and Schluesener H J (2005) Expression of P2X4 Receptor by Lesional Activated Microglia During Formalin-Induced Inflammatory Pain. *J Neuroimmunol* **163**:120-127.

Gutierrez A, Khan Z U, Miralles C P, Mehta A K, Ruano D, Araujo F, Vitorica J and De Blas A L (1997) GABAA Receptor Subunit Expression Changes in the Rat Cerebellum and Cerebral Cortex During Aging. *Molecular Brain Research* **45**:59-70.

Haines WR, Torres G E, Voigt M M and Egan T M (1999) Properties of the Novel ATP-Gated Ionotropic Receptor Composed of the $P2X_1$ and $P2X_5$ Isoforms. *Mol Pharmacol* **56**:720-727.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth F J (1981) Improved Patch-Clamp Techniques for High-Resolution Current Recording From Cells and Cell-Free Membrane Patches. *Pfl++gers Archiv European Journal of Physiology* **391**:85-100.

Hansen MA, Balcar V J, Barden J A and Bennett M R (1998) Localisation of P2X Subtypes in Heart, Artery and Bladder. *Drug Dev Res* **43**:5.

Hansen MA, Bennett M R and Barden J A (1999a) Distribution of Purinergic P2X Receptors in the Rat Heart. *J Auton Nerv Syst* **78**:1-9.

Hansen MA, Dutton J L, Balcar V J, Barden J A and Bennett M R (1999b) P2X (Purinergic) Receptor Distributions in Rat Blood Vessels. *J Auton Nerv Syst* **75**:147-155.

Harrington LS, Evans R J, Wray J, Norling L, Swales K E, Vial C, Ali F, Carrier M J and Mitchell J A (2007) Purinergic 2X1 Receptors Mediate Endothelial Dependent Vasodilation to ATP. *Molecular Pharmacology* **72**:1132-1136.

He L, Chen J, Dinger B, Stensaas L and Fidone S (2006) Effect of Chronic Hypoxia on Purinergic Synaptic Transmission in Rat Carotid Body. *J Appl Physiol* **100**:157-162.

Hechler B, Lenain N, Marchese P, Vial C, Heim V, Freund M, Cazenave J P, Cattaneo M, Ruggeri Z M, Evans R and Gachet C (2003) A Role of the Fast ATP-Gated $P2X_1$ Cation Channel in Thrombosis of Small Arteries in Vivo. *J Exp Med* **198**:661-667.

Hegde SS, Mandel D A, Wilford M R, Briaud S, Ford A P D W and Eglen R M (1998) Evidence for Purinergic Neurotransmission in the Urinary Bladder of Pithed Rats. *Eur J Pharmacol* **349**:75-82.

Hibell AD, Kidd E J, Chessell I P, Humphrey P P A and Michel A D (2000) Apparent Species Differences in the Kinetic Properties of $P2X_7$ Receptors. *Br J Pharmacol* **130**:167-173.

Hibell AD, Thompson K M, Xing M, Humphrey P P A and Michel A D (2001) Complexities of Measuring Antagonist Potency at $P2X_7$ Receptor Orthologs. *J Pharmacol Exper Ther* **296**:947-957.

Hoebertz A, Townsend-Nicholson A, Glass R, Burnstock G and Arnett T R (2000) Expression of P2 Receptors in Bone and Cultured Bone Cells. *Bone* **27**:503-510.

Holton P (1959) The Liberation of Adenosine Triphosphate on Antidromic Stimulation of Sensory Nerves. *J Physiol* **145**:494-504.

Honore P, Kage K, Mikusa J, Watt A T, Johnston J F, Wyatt J R, Faltynek C R, Jarvis M F and Lynch K (2002a) Analgesic Profile of Intrathecal $P2X_3$ Antisense Oligonucleotide Treatment in Chronic Inflammatory and Neuropathic Pain States in Rats. *Pain* **99**:11-19.

Honore P, Mikusa J, Bianchi B, McDonald H, Cartmell J, Faltynek C and Jarvis M F (2002b) TNP-ATP, a Potent $P2X_3$ Receptor Antagonist, Blocks Acetic Acid-Induced Abdominal Constriction in Mice: Comparison With Reference Analgesics. *Pain* **96**:99-105.

Honore P, Donnelly-Roberts D, Namovic M T, Hsieh G, Zhu C Z, Mikusa J P, Hernandez G, Zhong C, Gauvin D M, Chandran P, Harris R, Medrano A P, Carroll W, Marsh K, Sullivan J P, Faltynek C R and Jarvis M F (2006) A-740003 [N-(1-{[(Cyanoimino)(5-Quinolinylamino) Methyl]Amino}-2,2-Dimethylpropyl)-2-(3,4-Dimethoxyphenyl)Acetamide], a Novel and Selective P2X7 Receptor Antagonist, Dose-Dependently Reduces Neuropathic Pain in the Rat. *J Pharmacol Exp Ther* **319**:1376-1385.

Horner S, Menke K, Hildebrandt C, Kassack M U, Nickel P, Ullmann H, Mahaut-Smith M P and Lambrecht G (2005) The Novel Suramin Analogue NF864 Selectively Blocks $P2X_1$ Receptors in Human Platelets With Potency in the Low Nanomolar Range. *Naunyn Schmiedebergs Arch Pharmacol* **372**:1-13.

Housley GD, Greenwood D, Bennett T and Ryan A F (1995) Identification of a Short Form of the P2xR1-Purinoceptor Subunit Produced by Alternative Splicing in the Pituitary and Cochlea. *Biochem Biophys Res Commun* **212**:501-508.

Housley GD, Kanjhan R, Raybould N P, Greenwood D, Salih S G, Jarlebark L, Burton L D, Setz V C, Cannell M B, Soeller C, Christie D L, Usami S, Matsubara A, Yoshie H, Ryan A F and Thorne P R (1999) Expression of the $P2X_2$ Receptor Subunit of the ATP-Gated Ion Channel in the Cochlea: Implications for Sound Transduction and Auditory Neurotransmission. *J Neurosci* **19**:8377-8388.

Hoyle CHV, Chapple C and Burnstock G (1989) Isolated Human Bladder: Evidence for an Adenine Dinucleotide Acting on P2X-Purinoceptors and for Purinergic Transmission. *Eur J Pharmacol* **174**:115-8.

Hugel S and Schlichter R (2000) Presynaptic P2X Receptors Facilitate Inhibitory GABAergic Transmission Between Cultured Rat Spinal Cord Dorsal Horn Neurons. *J Neurosci* **20**:2121-2130.

Hughes JP, Hatcher JP and Chessell IP (2007) The Role of $P2X_7$ in Pain and Inflammation. *Purinergic Signalling* **3**:163-169.

Humphreys BD, Virginio C, Surprenant A, Rice J and Dubyak G R (1998) Isoquinolines As Antagonists of the $P2X_7$ Nucleotide Receptor: High Selectivity for the Human Versus Rat Receptor Homologues. *Mol Pharmacol* **54**:22-32.

Ichikawa H, Fukunaga T, Jin H W, Fujita M, Takano-Yamamoto T and Sugimoto T (2004) VR1-, VRL-1- and P2X3 Receptor-Immunoreactive Innervation of the Rat Temporomandibular Joint. *Brain Res* **1008**:131-136.

Inoue K, Denda M, Tozaki H, Fujishita K, Koizumi S and Inoue K (2005) Characterization of Multiple P2X Receptors in Cultured Normal Human Epidermal Keratinocytes. *J Invest Dermatol* **124**:756-763.

Inoue K, Tsuda M and Koizumi S (2003) ATP Induced Three Types of Pain Behaviors, Including Allodynia. *Drug Dev Res* **59**:56-63.

Inoue K, Tsuda M and Koizumi S (2004) ATP- and Adenosine-Mediated Signaling in the Central Nervous System: Chronic Pain and Microglia: Involvement of the ATP Receptor $P2X_4$. *J Pharmacol Sci* **94**:112-114.

Inoue R and Brading A F (1990) The Properties of the ATP-Induced Depolarization and Current in Single Cells Isolated From the Guinea-Pig Urinary Bladder. *Br J Pharmacol* **100**:619-625.

Inoue R and Brading A F (1991) Human, Pig and Guinea-Pig Bladder Smooth Muscle Cell Generate Similar Inward Currents in Response to Purinoceptor Activation. *Br J Pharmacol* **103**:1840-1841.

Inscho EW, Cook A K, Imig J D, Vial C and Evans R J (2003) Physiological Role for $P2X_1$ Receptors in Renal Microvascular Autoregulatory Behavior. *J Clin Invest* **112**:1895-1905.

Iredale, P. A. and Hill, S. J. Increases in intracellular calcium via activation of an endogenous P2-purinoceptor in cultured CHO-K1 cells. Br.J Pharmacol 110, 1305-1310. 1993.

Ishikawa T, Miyagi M, Ohtori S, Aoki Y, Ozawa T, Doya H, Saito T, Moriya H and Takahashi K (2005) Characteristics of Sensory DRG Neurons Innervating the Lumbar Facet Joints in Rats. *Eur Spine J* **14**:559-564.

Ishizuka, O., Mattiasson, A., and Andersson, K. E. Tachykinin effects on bladder activity in conscious normal rats. J Urol 154, 257-261. 1995.

Ito K, Iwami A, Katsura H and Ikeda M (2008) Therapeutic Effects of the Putative P2X3/P2X2/3 Antagonist A-317491 on Cyclophosphamide-Induced Cystitis in Rats. *Naunyn-Schmiedeberg's Archives of Pharmacology* **377**:483-490.

Jacobson KA, Costanzi S, Ivanov A A, Tchilibon S, Besada P, Gao Z G, Maddileti S and Harden T K (2006) Structure Activity and Molecular Modeling Analyses of Ribose- and Base-Modified Uridine 5'-Triphosphate Analogues at the Human P2Y2 and P2Y4 Receptors. *Biochemical Pharmacology* **71**:540-549.

Jacobson KA, Kim Y C, Wildman S S, Mohanram A, Harden T K, Boyer J L, King B F and Burnstock G (1998) A Pyridoxine Cyclic Phosphate and Its 6-Azoaryl Derivative Selectively Potentiate and Antagonize Activation of $P2X_1$ Receptors. *J Med Chem* **41**:2201-6.

Jahangir A., Alam M, Carter DS, Dillon MP, Dubois DJ, Ford APDW, Gever JR, Lin C, Wagner PJ, Zhai Y, Zira J (2009) Identification and SAR of Novel Diaminopyrimidines. Part 2: The Discovery of RO-51, a Potent and Selective, Dual $P2X_3$/$P2X_{2/3}$ Antagonist for the Treatment of Pain. *Biorg Med Chem Lett* **19**:1632-1635.

Jaime-Figueroa S, Greenhouse R, Padilla F, Dillon M P, Gever J R and Ford A P D W (2005) Discovery and Synthesis of a Novel and Selective Drug-Like $P2X_1$ Antagonist. *Bioorg Med Chem Lett* **15**:3292-3295.

Jarlebark LE, Housley G D, Raybould N P, Vlajkovic S and Thorne P R (2002) ATP-Gated Ion Channels Assembled From $P2X_2$ Receptor Subunits in the Mouse Cochlea. *Neuroreport* **13**:1979-1984.

Jarvis MF, Burgard E C, McGaraughty S, Honore P, Lynch K, Brennan T J, Subieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer C T, Zhu C Z, Chu K, Lee C H, Stewart A O, Polakowski J, Cox B F, Kowaluk E, Williams M, Sullivan J and Faltynek C (2002) A-317491, a Novel Potent and Selective Non-Nucleotide Antagonist of $P2X_3$ and $P2X_{2/3}$ Receptors, Reduces Chronic Inflammatory and Neuropathic Pain in the Rat. *Proc Nat Acad Sci* **99**:17179-17184.

Jarvis MF, Donnelly-Roberts D D, Honore P, Harris R, Namovic M M, Hernandez G., Zhong C, Zhu C, Gauvin D M, Chandran P, Hsieh G, Perez-Medrano A, Carroll W, Sullivan J P and Faltynek C R (2005) A-740003, a Novel and Selective $P2X_7$ Receptor Antagonist Produces Dose-Dependent Analgesia in Models of Neuropathic Pain. *Abstract Viewer/Itinerary Planner Washington, DC: Society for Neuroscience* **2005.Online**:Program No. 514.5.

Jarvis MJ (2003) Contributions of $P2X_3$ Homomeric and Heteromeric Channels to Acute and Chronic Pain. *Exp Opin Ther Targets* **7**:513-522.

Jarvis MF, Wismer C T, Schweitzer E, Yu H, van Biesen T, Lynch K J, Burgard E C and Kowaluk E A (2001) Modulation of BzATP and Formalin Induced Nociception: Attenuation by the P2X Receptor Antagonist, TNP-ATP and Enhancement by the P2X3 Allosteric Modulator, Cibacron Blue. **132**:259-269.

Jelinkova I, Vavra V, Jindrichova M, Obsil T, Zemkova HW, Zemkova H and Stojilkovic SS (2008) Identification of $P2X_4$ Receptor Transmembrane Residues Contributing to Channel Gating and Interaciton With Ivermectin. *Pflugers Arch - Eur J Physiol* **456**:939-950.

Jensik PJ, Holbird D, Collard M W and Cox T C (2001) Cloning and Characterization of a Functional P2X Receptor From Larval Bullfrog Skin. *Am J Physiol Cell Physiol* **281**:C954-C962.

Jiang LH (2009) Inhibition of $P2X_7$ Receptors by Divalent Cations: Old Action and New Insight. *Eur Biophys J* **38**:339-346.

Jiang LH, Kim M, Spelta V, Bo X, Surprenant A and North R A (2003) Subunit Arrangement in P2X Receptors. *J Neurosci* **23**:8903-8910.

Jiang LH, MacKenzie A B, North R A and Surprenant A (2000a) Brilliant Blue G Selectively Blocks ATP-Gated Rat $P2X_7$ Receptors. *Mol Pharmacol* **58**:82-88.

Jiang LH, Rassendren F, MacKenzie A, Zhang Y H, Surprenant A and North R A (2005a) N-Methyl-D-Glucamine and Propidium Dyes Utilize Different Permeation Pathways at Rat $P2X_7$ Receptors. *Am J Physiol Cell Physiol* **289**:C1295-C1302.

Jiang LH, Rassendren F, Surprenant A and North R A (2000b) Identification of Amino Acid Residues Contributing to the ATP-Binding Site of a Purinergic P2X Receptor. *J Biol Chem* **275**:34190-34196.

Jiang T, Yeung D, Lien C F and Gorecki D C (2005b) Localized Expression of Specific P2X Receptors in Dystrophin-Deficient DMD and Mdx Muscle. *Neuromuscul Disord* **15**:225-236.

Jin Y-H, Bailey T W, Li B, Schild J H and Andresen M C (2004) Purinergic and Vanilloid Receptor Activation Releases Glutamate From Separate Cranial Afferent Terminals in Nucleus Tractus Solitarius. *J Neurosci* **24**:4709-4717.

Jones CA, Chessell I P, Simon J, Barnard E A, Miller K J, Michel A D and Humphrey P P (2000) Functional Characterization of the $P2X_4$ Receptor Orthologues. *Br J Pharmacol* **129**:388-394.

Jones CA, Vial C, Sellers L A, Humphrey P P, Evans R J and Chessell I P (2004) Functional Regulation of $P2X_6$ Receptors by *N*-Linked Glycosylation: Identification of a Novel Aβ-Methylene ATP-Sensitive Phenotype. *Mol Pharmacol* **65**:979-985.

Jorgensen NR, Henriksen Z, Sorensen O H, Eriksen E F, Civitelli R and Steinberg T H (2002) Intercellular Calcium Signaling Occurs Between Human Osteoblasts and Osteoclasts and Requires Activation of Osteoclast P2X7 Receptors. *J Biol Chem* **277**:7574-7580.

Jung KY, Moon H D, Lee G E, Lim H H, Park C S and Kim Y C (2007) Structure-Activity Relationship Studies of Spinorphin As a Potent and Selective Human P2X3 Receptor Antagonist. *J Med Chem* **50**:4543-4547.

Kakimoto S, Nagakura Y, Tamura S, Watabiki T, Shibasaki K, Tanaka S, Mori M, Sasamata M and Okada M (2008) Minodronic Acid, a Third-Generation Bisphosphonate, Antagonizes Purinergic $P2X_{2/3}$ Receptor Function and Exerts an Analgesic Effect in Pain Models. *Eur J Pharmacol* **589**:98-101.

Kanjhan R, Housley G D, Burton L D, Christie D L, Kippenberger A, Thorne P R, Luo L and Ryan A F (1999) Distribution of the $P2X_2$ Receptor Subunit of the ATP-Gated Ion Channels in the Rat Central Nervous System. *J Comp Neurol* **407**:11-32.

Kanjhan R, Housley G D, Thorne P R, Christie D L, Palmer D J, Luo L and Ryan A F (1996) Localization of ATP-Gated Ion Channels in Cerebellum Using P2x2R Subunit-Specific Antisera. *Neuroreport* **7**:2665-2669.

Kasakov L and Burnstock G (1983) The Use of the Slowly Degradable Analog, α,β-Methylene ATP, to Produce Desensitisation of the P2-Purinoceptor: Effect on Non-Adrenergic, Non-Cholinergic Responses of the Guinea-Pig Urinary Bladder. *Eur J Pharmacol* **86**:291-294.

Kassack MU, Braun K, Ganso M, Ullmann H, Nickel P, Boing B, Muller G and Lambrecht G (2004) Structure-Activity Relationships of Analogues of NF449 Confirm NF449 As the Most Potent and Selective Known P2X1 Receptor Antagonist. *European Journal of Medicinal Chemistry* **39**:345-357.

Ke HZ, Qi H, Weidema A F, Zhang Q, Panupinthu N, Crawford D T, Grasser W A, Paralkar V M, Li M, Audoly L P, Gabel C A, Jee W S S, Dixon S J, Sims S M and Thompson D D (2003) Deletion of the $P2X_7$ Nucleotide Receptor Reveals Its Regulatory Roles in Bone Formation and Resorption. *Mol Endocrinol* **17**:1356-1367.

Kennedy C and Leff P (1995) Painful Connection for ATP. *Nature* **377**:385-6.

Kerr DIB and Krantis A (1979) A New Class of ATP Antagonist. *Proc Austr Physiol Pharm Soc* **10**:156P.

Khakh BS (2001) International Union of Pharmacology. XXIV. Current Status of the Nomenclature and Properties of P2X Receptors and Their Subunits. *Pharmacol Rev* **53**:107-118.

Khakh BS, Bao X R, Labarca C and Lester H A (1999a) Neuronal P2X Transmitter-Gated Cation Channels Change Their Ion Selectivity in Seconds. *Nature Neurosci* **2**:322-330.

Khakh BS, Gittermann D, Cockayne D A and Jones A (2003) ATP Modulation of Excitatory Synapses Onto Interneurons. *J Neurosci* **23**:7426-7437.

Khakh BS, Humphrey P P and Surprenant A (1995) Electrophysiological Properties of P2X-Purinoceptors in Rat Superior Cervical, Nodose and Guinea-Pig Coeliac Neurones. *J Physiol* **484**:385-395.

Khakh BS, Proctor W R, Dunwiddie T V, Labarca C and Lester H A (1999b) Allosteric Control of Gating and Kinetics at $P2X_4$ Receptor Channels. *J Neurosci* **19**:7289-99.

Kidd EJ, Grahames C B, Simon J, Michel A D, Barnard E A and Humphrey P P (1995) Localization of P2X Purinoceptor Transcripts in the Rat Nervous System. *Mol Pharmacol* **48**:569-573.

Kidd EJ, Miller K J, Sansum A J and Humphrey P P A (1998) Evidence for $P2X_3$ Receptors in the Developing Rat Brain. *Neuroscience* **87**:533-539.

Kim M, Jiang LH, Wilson HL, North RA and Surprenant, A (2001) Proteomic and Functional Evidence for a $P2X_7$ Receptor Signalling Complex. *The EMBO Journal* **20**;6347-6358

King BF, Knowles I D, Burnstock G and Ramage A G (2004) Investigation of the Effects of P2 Purinoceptor Ligands on the Micturition Reflex in Female Urethane-Anaesthetized Rats. *Br J Pharmacol* **142**:519-530.

King BF, Liu M, Pintor J, Gualix J, Miras-Portugal M T and Burnstock G (1999) Diinosine Pentaphosphate (IP_5I) Is a Potent Antagonist at Recombinant Rat $P2X_1$ Receptors. *Br J Pharmacol* **128**:981-988.

King BF, Townsend-Nicholson A and Burnstock G (1998a) Metabotropic Receptors for ATP and UTP: Exploring the Correspondence Between Native and Recombinant Nucleotide Receptors. *Trends in Pharmacological Sciences* **19**:506-514.

King BF, Townsend-Nicholson A, Wildman S S, Thomas T, Spyer K M and Burnstock G (2000) Coexpression of Rat $P2X_2$ and $P2X_6$ Subunits in *Xenopus* Oocytes. *J Neurosci* **20**:4871-4877.

King BF, Wildman S S, Ziganshina L E, Pintor J and Burnstock G (1997) Effects of Extracellular PH on Agonism and Antagonism at a Recombinant $P2X_2$ Receptor. *Br J Pharmacol* **121**:1445-1453.

King BF, Ziganshina L E, Pintor J and Burnstock G (1996) Full Sensitivity of P_{2X2} Purinoceptor to ATP Revealed by Changing Extracellular PH. *Br J Pharmacol* **117**:1371-1373.

King M, Housley G D, Raybould N P, Greenwood D and Salih S G (1998b) Expression of ATP-Gated Ion Channels by Reissner's Membrane Epithelial Cells. *Neuroreport* **9**:2467-2474.

Kirkup AJ, Booth C E, Chessell I P, Humphrey P P A and Grundy D (1999) Excitatory Effect of P2X Receptor Activation on Mesenteric Afferent Nerves in the Anaesthetised Rat. *J Physiol* **520**:551-563.

Knight GE, Bodin P, De Groat W C and Burnstock G (2002) ATP Is Released From Guinea Pig Ureter Epithelium on Distension. *Am J Physiol Renal Physiol* **282**:F281-F288.

Knight GE and Burnstock G (2004) The Effect of Pregnancy and the Oestrus Cycle on Purinergic and Cholinergic Responses of the Rat Urinary Bladder. *Neuropharmacology* **46**:1049-1056.

Koshimizu T, Tomic M, Koshimizu M and Stojilkovic S S (1998a) Identification of Amino Acid Residues Contributing to Desensitization of the $P2X_2$ Receptor Channel. *J Biol Chem* **273**:12853-12857.

Koshimizu T, Tomic M, Van Goor F and Stojilkovic S S (1998b) Functional Role of Alternative Splicing in Pituitary $P2X_2$ Receptor-Channel Activation and Desensitization. *Mol Endocrinol* **12**:901-913.

Krause RM, Buisson B, Bertrand S, Corringer P J, Galzi J L, Changeux J P and Bertrand D (1998) Ivermectin: a Positive Allosteric Effector of the A7 Neuronal Nicotinic Acetylcholine Receptor. *Mol Pharmacol* **53**:283-294.

Krusek J and Zemkova H (1994) Effect of Ivermectin on Gamma-Aminobutyric Acid-Induced Chloride Currents in Mouse Hippocampal Embryonic Neurones. *Eur J Pharmacol* **259**:121-128.

Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette M M, Brissette W, Wicks J R, Audoly L and Gabel C A (2002) Absence of the $P2X_7$ Receptor Alters Leukocyte Function and Attenuates an Inflammatory Response. *J Immunol* **168**:6436-6445.

Lachnit WG, Oglesby I B, Gever J R, Gever M, Huang C C, Li X C, Jin H, McGivern J G and Ford A P D W (2000) Regulated Expression of the Rat Recombinant P2X3 Receptor in Stably Transfected CHO-K1 TTA Cells. *Journal of the Autonomic Nervous System* **81**:75-81.

Lai HH, Smith C P, Munoz A, Boone T B, Szigeti G P and Somogyi G T (2008) Activation of Cholinergic Receptors Blocks Non-Adrenergic Non-Cholinergic Contractions in the Rat Urinary Bladder. *Brain Research Bulletin* **77**:420-426.

Lalo U, Pankratov Y, Wichert S P, Rossner M J, North R A, Kirchhoff F and Verkhratsky A (2008) P2X1 and P2X5 Subunits Form the Functional P2X Receptor in Mouse Cortical Astrocytes. *J Neurosci* **28**:5473-5480.

Lambrecht G, Friebe T, Grimm U, Windscheif U, Bungardt E, Hildebrandt C, Baumert H G, Spatz-Kumbel G and Mutschler E (1992) PPADS, a Novel Functionally Selective Antagonist of P2 Purinoceptor-Mediated Responses. *Eur J Pharmacol* **217**:217-219.

Lambrecht G, Rettinger J, Baumert H G, Czeche S, Damer S, Ganso M, Hildebrandt C, Niebel B, Spatz-Kumbel G, Schmalzing G and Mutschler E (2000) The Novel Pyridoxal-5'-Phosphate Derivative PPNDS Potently Antagonizes Activation of $P2X_1$ Receptors. *Eur J Pharmacol* **387**:R19-R21.

Lamont C, Vial C, Evans R J and Wier W G (2006) P2X1 Receptors Mediate Sympathetic Postjunctional Ca2+ Transients in Mesenteric Small Arteries. *Am J Physiol Heart Circ Physiol* **291**:H3106-H3113.

Langer SZ and Pinto J E (1976) Possible Involvement of a Transmitter Different From Norepinephrine in the Residual Responses to Nerve Stimulation of the Cat Nictitating Membrane After Pretreatment With Reserpine. *J Pharmacol Exp Ther* **196**:697-713.

Lazzeri, M. The physiological function of the urothelium - more than a simple barrier. Urol.Int. 76, 289-295. 2006.

Le Feuvre R, Brough D and Rothwell N (2002) Extracellular ATP and P2X7 Receptors in Neurodegeneration. *Eur J Pharmacol* **447**:261-269.

Le Feuvre RA, Brough D, Touzani O and Rothwell N J (2003) Role of $P2X_7$ Receptors in Ischemic and Excitotoxic Brain Injury in Vivo. *J Cereb Blood Flow Metab* **23**:381-384.

Le KT, Babinski K and Seguela P (1998) Central $P2X_4$ and $P2X_6$ Channel Subunits Coassemble into a Novel Heteromeric ATP Receptor. *J Neurosci* **18**:7152-7159.

Le KT, Boue-Grabot E, Archambault V and Seguela P (1999) Functional and Biochemical Evidence for Heteromeric ATP-Gated Channels Composed of $P2X_1$ and $P2X_5$ Subunits. *J Biol Chem* **274**:15415-15419.

Le KT, Paquet M, Nouel D, Babinski K and Seguela P (1997) Primary Structure and Expression of a Naturally Truncated Human P2X ATP Receptor Subunit From Brain and Immune System. *FEBS Lett* **418**:195-199.

Lee GA, Joshi BV, Chen W, Jeong LS, Moon HR, Jacobson KA and Kim YC (2008) Synthesis and Structure-Activity Relationship Studies of Tyrosine-Based Antagonists at the Human $P2X_7$ Receptor. *Bioorg Med Chem Lett* **18**:571-575.

Lee GA, Lee HS, Lee SD, Kim JH, Kim WK, Kim YC (2009) Synthesis and Structure-Activity Relationships of Novel, Substituted 5,6-Dihydrodibenzo[*a,g*]quinolizinium $P2X_7$ Antagonists. *Bioorg Med Chem Lett* **19**:954-958.

Lee HY, Bradini M and Burnstock G (2000) Distribution of P2X Receptors in the Urinary Bladder and the Ureter of the Rat. *J Urol* **163**:2002-2007.

Lewis C, Neidhart S, Holy C, North R A, Buell G and Surprenant A (1995) Coexpression of $P2X_2$ and $P2X_3$ Receptor Subunits Can Account for ATP-Gated Currents in Sensory Neurons. *Nature* **377**:432-435.

Lewis CJ and Evans R J (2001) P2X Receptor Immunoreactivity in Different Arteries From the Femoral, Pulmonary, Cerebral, Coronary and Renal Circulations. *J Vasc Res* **38**:332-340.

Lewis, S. A. and Lewis, J. R. Kinetics of urothelial ATP release. AJP - Renal Physiology 291, F332-F340. 2006.

Li C, Peoples R W, Li Z and Weight F F (1993) Zn^{2+} Potentiates Excitatory Action of ATP on Mammalian Neurons. *Proc Natl Acad Sci U S A* **90**:8264-8267.

Li C, Peoples R W and Weight F F (1996a) Acid PH Augments Excitatory Action of ATP on a Dissociated Mammalian Sensory Neuron. *Neuroreport* **7**:2151-2154.

Li C, Peoples R W and Weight F F (1996b) Proton Potentiation of ATP-Gated Ion Channel Responses to ATP and Zn^{2+} in Rat Nodose Ganglion Neurons. *J Neurophysiol* **76**:3048-3058.

Li J, Liu D, Ke H Z, Duncan R L and Turner C H (2005) The $P2X_7$ Nucleotide Receptor Mediates Skeletal Mechanotransduction. *J Biol Chem* **280**:42952-42959.

Liang L and Schwiebert E M (2005) Large Pore Formation Uniquely Associated With $P2X_7$ Purinergic Receptor Channels. Focus on "Are Second Messengers Crucial for Opening the Pore Associated With $P2X_7$ Receptor?". *Am J Physiol Cell Physiol* **288**:C240-C242.

Liu M, King B F, Dunn P M, Rong W, Townsend-Nicholson A and Burnstock G (2001) Coexpression of $P2X_3$ and $P2X_2$ Receptor Subunits in Varying Amounts Generates Heterogeneous Populations of P2X Receptors That Evoke a Spectrum of Agonist Responses Comparable to That Seen in Sensory Neurons. *J Pharmacol Exp Ther* **296**:1043-1050.

Liu X, Surprenant A, Mao H J, Roger S, Xia R, Bradley H and Jiang L H (2008) Identification of Key Residues Coordinating Functional Inhibition of P2X7 Receptors by Zinc and Copper. *Mol Pharmacol* **73**:252-259.

Lundstrom, K., Mills, A., Buell, G., Allet, E., Adami, N., and Liljestrom, P. High-level expression of the human neurokinin-1 receptor in mammalian cell lines using the Semliki Forest virus expression system. Eur J Biochem 224, 917-921. 1994.

Lynch KJ, Touma E, Niforatos W, Kage K L, Burgard E C, Van Biesen T, Kowaluk E A and Jarvis M F (1999) Molecular and Functional Characterization of Human $P2X_2$ Receptors. *Mol Pharmacol* **56**:1171-1181.

Ma B, Ruan H Z, Burnstock G and Dunn P M (2005) Differential Expression of P2X Receptors on Neurons From Different Parasympathetic Ganglia. *Neuropharmacology* **48**:766-777.

Ma B, Ruan H Z, Cockayne D A, Ford A P D W, Burnstock G and Dunn P M (2004) Identification of P2X Receptors in Cultured Mouse and Rat Parasympathetic Otic Ganglion Neurones Including P2X Knockout Studies. *Neuropharmacology* **46**:1039-1048.

MacKenzie AB, Mahaut-Smith M P and Sage S O (1996) Activation of Receptor-Operated Cation Channels Via P_{2X1} Not P_{2T} Purinoceptors in Human Platelets. *J Biol Chem* **271**:2879-2881.

Maggi, C. A. Prostanoids as local modulators of reflex micturition. Pharmacol Res 25, 13-20. 1992.

Mahaut-Smith MP, Ennion S J, Rolf M G and Evans R J (2000) ADP Is Not an Agonist at $P2X_1$ Receptors: Evidence for Separate Receptors Stimulated by ATP and ADP on Human Platelets. *Br J Pharmacol* **131**:108-114.

Mason HS, Bourke S and Kemp P J (2004) Selective Modulation of Ligand-Gated P2X Purinoceptor Channels by Acute Hypoxia Is Mediated by Reactive Oxygen Species. *Mol Pharmacol* **66**:1525-1535.

McCoy DE, Taylor A L, Kudlow B A, Karlson K, Slattery M J, Schwiebert L M, Schwiebert E M and Stanton B A (1999) Nucleotides Regulate NaCl Transport in MIMCD-K2 Cells Via P2X and P2Y Purinergic Receptors. *Am J Physiol Renal Physiol* **277**:F552-F559.

McGaraughty S, Chu K L, Namovic M T, Donnelly-Roberts D L, Harris R R, Zhang X F, Shieh C C, Wismer C T, Zhu C Z, Gauvin D M, Fabiyi A C, Honore P, Gregg R J, Kort M E, Nelson D W, Carroll W A, Marsh K, Faltynek C R and Jarvis M F (2007) P2X7-Related Modulation of Pathological Nociception in Rats. *Neuroscience* **In Press, Corrected Proof**.

McGaraughty S, Wismer C T, Zhu C Z, Mikusa J, Honore P, Chu K L, Lee C H, Faltynek C R and Jarvis M F (2003) Effects of A-317491, a Novel and Selective $P2X_3$/$P2X_{2/3}$ Receptor Antagonist, on Neuropathic, Inflammatory and Chemogenic Nociception Following Intrathecal and Intraplantar Administration. *Br J Pharmacol* **140**:1381-1388.

Mehta VB, Hart J and Wewers M D (2001) ATP-Stimulated Release of Interleukin (IL)-1β and IL-18 Requires Priming by Lipopolysaccharide and Is Independent of Caspase-1 Cleavage. *J Biol Chem* **276**:3820-3826.

Merriman GH, Ma L, Shum P, McGarry D, Volz F, Sabol J S, Gross A, Zhao Z C, Rampe D, Wang L, Wirtz-Brugger F, Harris B A and Macdonald D (2005) Synthesis and SAR of Novel 4,5-Diarylimidazolines As Potent $P2X_7$ Receptor Antagonists. *Bioorg Med Chem Lett* **15**:435-438.

Meyer MP, Groschel-Stewart U, Robson T and Burnstock G (1999) Expression of Two ATP-Gated Ion Channels, $P2X_5$ and $P2X_6$, in Developing Chick Skeletal Muscle. *Dev Dyn* **216**:442-449.

Michel AD, Chessell I P and Humphrey P P A (1999) Ionic Effects on Human Recombinant $P2X_7$ Receptor Function. *Naunyn-Schmiedebergs Arch Pharmacol* **359**:102-109.

Michel AD, Grahames C B A and Humphrey P P A (1996a) Functional Characterisation of P2 Purinoceptors in PC12 Cells by Measurement of Radiolabelled Calcium Influx. *Naunyn-Schmiedebergs Arch Pharmacol* **354**:562-571.

Michel, A. D. and Humphrey, P. P. High affinity P2X-purinoceptor binding sites for [35S]-adenosine 5'-O-[3-thiotriphosphate] in rat vas deferens membranes. British Journal of Pharmacology 117, 63-70. 1996.

Michel AD, Kaur R, Chessell I P and Humphrey P P A (2000) Antagonist Effects on Human $P2X_7$ Receptor-Mediated Cellular Accumulation of YO-PRO-1. *Br J Pharmacol* **130**:513-520.

Michel AD, Lundstrom K, Buell G N, Surprenant A, Valera S and Humphrey P P (1996b) The Binding Characteristics of a Human Bladder Recombinant P2X Purinoceptor, Labelled With 3H -Alpha Beta MeATP, 35S -ATP Gamma S or 33P -ATP. *Brit J Pharmacol* **117**:1254-60.

Michel AD, Miller K J, Lundstrom K, Buell G N and Humphrey P P A (1997) Radiolabeling of the Rat P2X4 Purinoceptor: Evidence for Allosteric Interactions of Purinoceptor Antagonists and Monovalent Cations With P2X Purinoceptors. *Molecular Pharmacology* **51**:524-532.

Miller KJ, Michel A D, Chessell I P and Humphrey P P (1998) Cibacron Blue Allosterically Modulates the Rat $P2X_4$ Receptor. *Neuropharmacology* **37**:1579-1586.

Mills, E. and Smith, P. G. Functional development of the cervical sympathetic pathway in the neonatal rat. Fed Proc 42, 1639-1642. 1983.

Mio K, Kubo Y, Ogura T, Yamamoto T and Sato C (2005) Visualization of the Trimeric $P2X_2$ Receptor With a Crown-Capped Extracellular Domain. *Biochem Biophys Res Commun* **337**:998-1005.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C., and Sakmann, B. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406-411. 1986.

Mok MH, Knight G E, Andrews P L, Hoyle C H and Burnstock G (2000) The Effects of Cyclophosphamide on Neurotransmission in the Urinary Bladder of Suncus Murinus, the House Musk Shrew. *J Auton Nerv Syst* **80**:130-136.

Moore KH, Ray F R and Barden J A (2001) Loss of Purinergic $P2X_3$ and $P2X_5$ Receptor Innervation in Human Detrusor From Adults With Urge Incontinence. *J Neurosci* **21**:RC166-2401.

Moore SF and MacKenzie A B (2008) Species and Agonist Dependent Zinc Modulation of Endogenous and Recombinant ATP-Gated P2X7 Receptors. *Biochemical Pharmacology* **76**:1740-1747.

Morytko MJ, Betschmann P, Woller K, Ericsson A, Chen H, Donnelly-Roberts DL, Namovic MT, Jarvis MF, Carroll WA and Rafferty P (2008) Synthesis and In Vitro Activity of *N'*-Cyano-4-(2-phenylacetyl)-*N-o*-tolylpiperazine-1-carboximidamide $P2X_7$ Antagonists. *Bioorg Med Chem Lett* **18**:2093-2096.

Mulryan K, Gitterman D P, Lewis C J, Vial C, Leckie B J, Cobb A L, Brown J E, Conley E C, Buell G, Pritchard C A and Evans R J (2000) Reduced Vas Deferens Contraction and Male Infertility in Mice Lacking $P2X_1$ Receptors. *Nature* **403**:86-89.

Murgia M, Hanau S, Pizzo P, Rippa M and Di Virgilio F (1993) Oxidized ATP. An Irreversible Inhibitor of the Macrophage Purinergic P2Z Receptor. *J Biol Chem* **268**:8199-8203.

Naemsch LN, Weidema A F, Sims S M, Underhill T M and Dixon S J (1999) $P2X_4$ Purinoceptors Mediate an ATP-Activated, Non-Selective Cation Current in Rabbit Osteoclasts. *J Cell Sci* **112 (Pt 23)**:4425-4435.

Naemsch LN, Dixon S J and Sims S M (2001) Activity-Dependent Development of $P2X_7$ Current and Ca^{2+} Entry in Rabbit Osteoclasts. *J Biol Chem* **276**:39107-39114.

Nagaya N, Tittle R K, Saar N, Dellal S S and Hume R I (2005) An Intersubunit Zinc Binding Site in Rat $P2X_2$ Receptors. *J Biol Chem* **280**:25982-25993.

Nakatsuka T and Gu J G (2001) ATP P2X Receptor-Mediated Enhancement of Glutamate Release and Evoked EPSCs in Dorsal Horn Neurons of the Rat Spinal Cord. *J Neurosci* **21**:6522-6531.

Nakatsuka T, Tsuzuki K, Ling J X, Sonobe H and Gu J G (2003) Distinct Roles of P2X Receptors in Modulating Glutamate Release at Different Primary Sensory Synapses in Rat Spinal Cord. *J Neurophysiol* **89**:3243-3252.

Nakazawa K and Ohno Y (1997) Effects of Neuroamines and Divalent Cations on Cloned and Mutated ATP-Gated Channels. *Eur J Pharmacol* **325**:101-108.

Namasivayam S, Eardley I and Morrison J F B (1999) Purinergic Sensory Neurotransmission in the Urinary Bladder: an in Vitro Study in the Rat. *Br J Urol Int* **84**:854-860.

Neelands TR, Burgard E C, Uchic M E, McDonald H A, Niforatos W, Faltynek C R, Lynch K J and Jarvis M F (2003) 2', 3'-O-(2,4,6,Trinitrophenyl)-ATP and A-317491 Are Competitive Antagonists at a Slowly Desensitizing Chimeric Human $P2X_3$ Receptor. *Br J Pharmacol* **140**:202-210.

Nelson DW, Gregg R J, Kort M E, Perez-Medrano A, Voight E A, Wang Y, Grayson G, Namovic M T, Donnelly-Roberts D L, Niforatos W, Honore P, Jarvis M F, Faltynek C R and Carroll W A (2006) Structure-Activity Relationship Studies on a Series of Novel, Substituted 1-Benzyl-5-Phenyltetrazole P2X7 Antagonists. *Journal of Medicinal Chemistry* **49**:3659-3666.

Nelson DW, Sarris K, Kalvin D M, Namovic M T, Grayson G, Donnelly-Roberts D L, Harris R, Honore P, Jarvis M F, Faltynek C R and Carroll W A (2008) Structure-Activity Relationship Studies on *N*'-Aryl Carbohydrazide $P2X_7$ Antagonists. *Journal of Medicinal Chemistry* **51**:3030-3034.

Newbolt A, Stoop R, Virginio C, Surprenant A, North R A, Buell G and Rassendren F (1998) Membrane Topology of an ATP-Gated Ion Channel (P2X Receptor). *J Biol Chem* **273**:15177-15182.

Nicke A (2008) Homotrimeric Complexes Are the Dominant Assembly State of Native P2X7 Subunits. *Biochemical and Biophysical Research Communications* **377**:803-808.

Nicke A, Baeumert H.G., Rettinger J, Eichele A, Lambrecht G, Mutschler E and Schmalzing G (1998) $P2X_1$ and $P2X_3$ Receptors Form Stable Trimers: a Novel Structural Motif of Ligand-Gated Ion Channels. *EMBO J* **17**:3016-3028.

Nicke A, Kerschensteiner D and Soto F (2005) Biochemical and Functional Evidence for Heteromeric Assembly of $P2X_1$ and $P2X_4$ Subunits. *J Neurochem* **92**:925-933.

Norenberg W and Illes P (2000) Neuronal P2X Receptors: Localisation and Functional Properties. *Naunyn Schmiedebergs Arch Pharmacol* **362**:324-339.

Nori S, Fumagalli L, Bo X, Bogdanov Y and Burnstock G (1998) Coexpression of MRNAs for $P2X_1$, $P2X_2$ and $P2X_4$ Receptors in Rat Vascular Smooth Muscle: an in Situ Hybridization and RT-PCR Study. *J Vasc Res* **35**:179-185.

North, R. A. Families of ion channels with two hydrophobic segments. Curr Opin Cell Biol 8, 474-483. 1996.

North RA (2002) Molecular Physiology of P2X Receptors. *Physiol Rev* **82**:1013-1067.

North RA and Surprenant A (2000) Pharmacology of Cloned P2X Receptors. *Annu Rev Pharmacol Toxicol* **40**:563-580.

Novakovic SD, Kassotakis L C, Oglesby I B, Smith J A, Eglen R M, Ford A P and Hunter J C (1999) Immunocytochemical Localization of P2X3 Purinoceptors in Sensory Neurons in Naive Rats and Following Neuropathic Injury. *Pain* **80**:273-82.

O'Connor SE, Wood B E and Leff P (1990) Characterization of P2x-Receptors in Rabbit Isolated Ear Artery. *Br J Pharmacol* **101**:640-644.

O'Reilly BA, Kosaka A H, Chang T K, Ford A P D W, Popert R, Rymer J M and McMahon S B (2001) A Quantitative Analysis of Purinoceptor Expression in Human Fetal and Adult Bladders. *J Urol* **165**:1730-1734.

O'Reilly BA, Kosaka A H, Knight G F, Chang T K, Ford P D W, Rymer J M, Popert R, Burnstock G and McMahon S B (2002) P2X Receptors and Their Role in Female Idiopathic Detrusor Instability. *J Urol* **167**:157-164.

Obara K, Lepor H and Walden P D (1998) Localization of P2Y1 Purinoceptor Transcripts in the Rat Penis and Urinary Bladder. *The Journal of Urology* **160**:587-591.

Oglesby IB, Lachnit W G, Burnstock G and Ford A P D W (1999) Subunit Specificity of Polyclonal Antisera to the Carboxy Terminal Regions of P2X Receptors, P2X1 Through P2X7. *Drug Development Research* **47**:189-195.

Ohta T, Kubota A, Murakami M, Otsuguro K and Ito S (2005) $P2X_2$ Receptors Are Essential for $[Ca^{2+}]_i$ Increases in Response to ATP in Cultured Rat Myenteric Neurons. *Am J Physiol Gastrointest Liver Physiol* **289**:G935-G948.

Oury C, Kuijpers M J E, Toth-Zsamboki E, Bonnefoy A, Danloy S, Vreys I, Feijge M A H, De Vos R, Vermylen J, Heemskerk J W M and Hoylaerts M F (2003) Overexpression of the

Platelet $P2X_1$ Ion Channel in Transgenic Mice Generates a Novel Prothrombotic Phenotype. *Blood* **101**:3969-3976.

Palea S, Pietra C, Trist D G, Artibani W, Calpista A and Corsi M (1995) Evidence for the Presence of Both Pre- and Postjunctional P2-Purinoceptor Subtypes in Human Isolated Urinary Bladder. *Br J Pharmacol* **114**:35-40.

Pandita RK and Andersson K-E (2002) Intravesical Adenosine Triphosphate Stimulates the Micturition Reflex in Awake, Freely Moving Rats. *J Urol* **168**:1230-1234.

Pankratov Y, Castro E, Miras-Portugal M T and Krishtal O (1998) A Purinergic Component of the Excitatory Postsynaptic Current Mediated by P2X Receptors in the CA1 Neurons of the Rat Hippocampus. *Eur J Neurosci* **10**:3898-3902.

Pankratov Y, Lalo U, Krishtal O and Verkhratsky A (2003) P2X Receptor-Mediated Excitatory Synaptic Currents in Somatosensory Cortex. *Mol Cell Neurosci* **24**:842-849.

Pankratov Y, Lalo U, Verkhratsky A and North R (2006) Vesicular Release of ATP at Central Synapses. *Pflugers Archiv European Journal of Physiology* **452**:589-597.

Pankratov Y, Lalo U, Krishtal A and Verkhratsky A (2009) P2X Receptors and Synaptic Plasticity. *Neuroscience* **158**:137-148.

Parvathenani LK, Tertyshnikova S, Greco C R, Roberts S B, Robertson B and Posmantur R (2003) $P2X_7$ Mediates Superoxide Production in Primary Microglia and Is Up-Regulated in a Transgenic Mouse Model of Alzheimer's Disease. *J Biol Chem* **278**:13309-13317.

Pelegrin P and Surprenant A (2006) Pannexin-1 Mediates Large Pore Formation and Interleukin-1β Release by the ATP-Gated P2X7 Receptor. *EMBO J* **25**:5071-5082.

Pelegrin P and Surprenant A (2009) The $P2X_7$ Receptor-Pannexin Connection to Dye Uptake and Il-1β Release. *Purinergic Signalling* **5**:129-137.

Pelegrin P, Barroso-Gutierrez C and Surprenant A (2008) $P2X_7$ Receptor Differentially Couples to Distinct Release Pathways for Il-1β in Mouse Macrophage. *J Immunol* **180**:7147-7157.

Perregaux DG, McNiff P, Laliberte R, Conklyn M and Gabel C A (2000) ATP Acts As an Agonist to Promote Stimulus-Induced Secretion of IL-1β and IL-18 in Human Blood. *J Immunol* **165**:4615-4623.

Pintor J, King B F, Miras-Portugal M T and Burnstock G (1996) Selectivity and Activity of Adenine Dinucleotides at Recombinant $P2X_2$ and $P2Y_1$ Purinoceptors. *Br J Pharmacol* **119**:1006-1012.

Poole DP, Castelucci P, Robbins H L, Chiocchetti R and Furness J B (2002) The Distribution of $P2X_3$ Purine Receptor Subunits in the Guinea Pig Enteric Nervous System. *Auton Neurosci* **101**:39-47.

Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I., and West, A. B. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. AJP - Renal Physiology 277, C183-C201. 1999.

Prasad M, Fearon I M, Zhang M, Laing M, Vollmer C and Nurse C A (2001) Expression of P2X2 and P2X3 Receptor Subunits in Rat Carotid Body Afferent Neurones: Role in Chemosensory Signalling. *J Physiol* **537**:667-677.

Pratt EB, Brink T S, Bergson P, Voigt M M and Cook S P (2005) Use-Dependent Inhibition of $P2X_3$ Receptors by Nanomolar Agonist. *J Neurosci* **25**:7359-7365.

Priel A and Silberberg S D (2004) Mechanism of Ivermectin Facilitation of Human $P2X_4$ Receptor Channels. *J Gen Physiol* **123**:281-293.

Qu Y, Franchi L, Nunez G and Dubyak GR (2007) Nonclassical IL-1β Secretion Stimulated by P2X7 Receptors is Dependent on Inflammasome Activation and Correlated with Exosome Release in Murine Macrophages. *J Immunol* **179**:1913-1925.

Queiroz G, Talaia C and Goncalves J (2003) ATP Modulates Noradrenaline Release by Activation of Inhibitory P2Y Receptors and Facilitatory P2X Receptors in the Rat Vas Deferens. *J Pharmacol Exp Ther* **307**:809-815.

Radford KM, Virginio C, Surprenant A, North R A and Kawashima E (1997) Baculovirus Expression Provides Direct Evidence for Heteromeric Assembly of $P2X_2$ and $P2X_3$ Receptors. *J Neurosci* **17**:6529-6533.

Rae MG, Rowan E G and Kennedy C (1998) Pharmacological Properties of P2X3-Receptors Present in Neurones of the Rat Dorsal Root Ganglia. *Br J Pharmacol* **124**:176-180.

Ralevic, V. and Burnstock, G. Postjunctional synergism of noradrenaline and adenosine 5'-triphosphate in the mesenteric arterial bed of the rat. Eur.J.Pharmacol. 175[3], 291-299. 1990.

Ralevic V and Burnstock G (1998) Receptors for Purines and Pyrimidines. *Pharmacol Rev* **50**:413-492.

Rampe D, Wang L and Ringheim G E (2004) P2X7 Receptor Modulation of B-Amyloid- and LPS-Induced Cytokine Secretion From Human Macrophages and Microglia. *J Neuroimmunol* **147**:56-61.

Rassendren F, Buell G N, Virginio C, Collo G, North R A and Surprenant A (1997) The Permeabilizing ATP Receptor, $P2X_7$. Cloning and Expression of a Human CDNA. *J Biol Chem* **272**:5482-5486.

Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne D A, Ford A P D W and Galligan J J (2003) P2X2 Subunits Contribute to Fast Synaptic Excitation in Myenteric Neurons of the Mouse Small Intestine. *The Journal of Physiology* **552**:809-821.

Rettinger J, Braun K, Hochmann H, Kassack M U, Ullmann H, Nickel P, Schmalzing G and Lambrecht G (2005) Profiling at Recombinant Homomeric and Heteromeric Rat P2X Receptors Identifies the Suramin Analogue NF449 As a Highly Potent P2X1 Receptor Antagonist. *Neuropharmacology* **48**:461-468.

Rettinger J and Schmalzing G (2004) Desensitization Masks Nanomolar Potency of ATP for the $P2X_1$ Receptor. *J Biol Chem* **279**:6426-6433.

Rettinger J, Schmalzing G, Damer S, Muller G, Nickel P and Lambrecht G (2000) The Suramin Analogue NF279 Is a Novel and Potent Antagonist Selective for the $P2X_1$ Receptor. *Neuropharmacology* **39**:2044-2053.

Roberts JA and Evans R J (2004) ATP Binding at Human $P2X_1$ Receptors. Contribution of Aromatic and Basic Amino Acids Revealed Using Mutagenesis and Partial Agonists. *J Biol Chem* **279**:9043-9055.

Robertson SJ, Rae M G, Rowan E G and Kennedy C (1996) Characterization of a P2X-Purinoceptor in Cultured Neurones of the Rat Dorsal Root Ganglia. *Br J Pharmacol* **118**:951-956.

Rolf MG, Brearley C A and Mahaut-Smith M P (2001) Platelet Shape Change Evoked by Selective Activation of $P2X_1$ Purinoceptors With Alpha,Beta-Methylene ATP. *Thrombosis and Hemostasis* **85**:303-308.

Rong W and Burnstock G (2004) Activation of Ureter Nociceptors by Exogenous and Endogenous ATP in Guinea Pig. *Neuropharmacology* **47**:1093-1101.

Rong W, Gourine A V, Cockayne D A, Xiang Z, Ford A P D W, Spyer K M and Burnstock G (2003) Pivotal Role of Nucleotide $P2X_2$ Receptor Subunit of the ATP-Gated Ion Channel Mediating Ventilatory Responses to Hypoxia. *J Neurosci* **23**:11315-11321.

Rong W, Spyer K M and Burnstock G (2002) Activation and Sensitisation of Low and High Threshold Afferent Fibres Mediated by P2X Receptors in the Mouse Urinary Bladder. *J Physiol* **541**:591-600.

Rubin RP (2007) A Brief History of Great Discoveries in Pharmacology: In Celebration of the Centennial Anniversary of the Founding of the American Society of Pharmacology and Experimental Therapeutics. *Pharmacol Rev* **59**:289-359.

Rubio ME and Soto F (2001) Distinct Localization of P2X Receptors at Excitatory Postsynaptic Specializations. *J Neurosci* **21**:641-653.

Ruggieri MR (2006) Mechanisms of Disease: Role of Purinergic Signaling in the Pathophysiology of Bladder Dysfunction. *Nat Clin Pract Urol* **3**:206-215.

Ruppelt A, Ma W, Borchardt K, Silberberg S D and Soto F (2001) Genomic Structure, Developmental Distribution and Functional Properties of the Chicken $P2X_5$ Receptor. *J Neurochem* **77**:1256-1265.

Ryten M, Dunn P M, Neary J T and Burnstock G (2002) ATP Regulates the Differentiation of Mammalian Skeletal Muscle by Activation of a $P2X_5$ Receptor on Satellite Cells. *J Cell Biol* **158**:345-355.

Ryten M, Hoebertz A and Burnstock G (2001) Sequential Expression of Three Receptor Subtypes for Extracellular ATP in Developing Rat Skeletal Muscle. *Dev Dyn* **221**:331-341.

Sak K and Illes P (2005) Neuronal and Glial Cell Lines As Model Systems for Studying P2Y Receptor Pharmacology. *Neurochem Int* **47**:401-412.

Satchell DG and Maguire M H (1975) Inhibitory Effects of Adenine Nucleotide Analogs on the Isolated Guinea-Pig Taenia Coli. *J Pharmacol Exp Ther* **195**:540-548.

Schadlich, H., Wirkner, K., Franke, H., Bauer, S., Grosche, J., Burnstock, G., Reichenbach, A., Illes, P., and Allgaier, C. P2X2, P2X2-2 and P2X5 receptor subunit expression and function in rat thoracolumbar sympathetic neurons. J Neurochem 79, 997-1003. 2001.

Scheibler P, Pesic M, Franke H, Reinhardt R, Wirkner K, Illes P and Norenberg W (2004) $P2X_2$ and $P2Y_1$ Immunofluorescence in Rat Neostriatal Medium-Spiny Projection Neurones and Cholinergic Interneurones Is Not Linked to Respective Purinergic Receptor Function. *Br J Pharmacol* **143**:119-131.

Schwab JM, Guo L and Schluesener H J (2005) Spinal Cord Injury Induces Early and Persistent Lesional $P2X_4$ Receptor Expression. *J Neuroimmunol* **163**:185-189.

Seabrook, G. R., Fieber, L. A., and Adams, D. J. Neurotransmission in neonatal rat cardiac ganglion in situ. Am J Physiol 259, H997-H1005. 1990.

Seguela P, Haghighi A, Soghomonian J J and Cooper E (1996) A Novel Neuronal P2x ATP Receptor Ion Channel With Widespread Distribution in the Brain. *J Neurosci* **16**:448-55.

Sharp CJ, Reeve A J, Collins S D, Martindale J C, Summerfield S G, Sargent B S, Bate S T and Chessell I P (2006) Investigation into the Role of P2X3//P2X2//3 Receptors in Neuropathic Pain Following Chronic Constriction Injury in the Rat: an Electrophysiological Study. *Br J Pharmacol*.

Shigematsu Y, Shimoda Y and Kaneda M (2007) Distribution of Immunoreactivity for P2X3, P2X5, and P2X6-Purinoceptors in Mouse Retina. *Journal of Molecular Histology* **38**:369-371.

Shigetomi E, Bowser DN, Sofroniew MV and Khakh BS (2008) Two Forms of Astrocyte Calcium Excitability Have Distinct Effects on NMDA Receptor-Mediated Slow Inward Currents in Pyramidal Neurons. *J Neurosci* **28**:6659-6663.

Sigel E (2002) Mapping of the Benzodiazepine Recognition Site on GABA(A) Receptors. *Curr Top Med Chem* **2**:833-839.

Silva, A. M., Rodrigues, R. J., Tome, A. R., Cunha, R. A., Misler, S., Rosario, L. M., and Santos, R. M. Electrophysiological and immunocytochemical evidence for P2X purinergic receptors in panceatic beta cells. Pancreas 36[3], 279-283. 2008.

Sim JA, Young M T, Sung H Y, North R A and Surprenant A (2004) Reanalysis of $P2X_7$ Receptor Expression in Rodent Brain. *J Neurosci* **24**:6307-6314.

Simon J, Kidd E J, Smith F M, Chessell I P, Murrell-Lagnado R, Humphrey P P and Barnard E A (1997) Localization and Functional Expression of Splice Variants of the $P2X_2$ Receptor. *Mol Pharmacol* **52**:237-248.

Skaper SD, Facci L, Culbert A, Chessell I, Davis J B and Richardson J C (2005) P2X7 Receptors on Microglial Cells Mediate Toxicity to Cortical Neurons in Vitro. *Abstract Viewer/Itinerary Planner Washington, DC: Society for Neuroscience* **2005.Online**:Program No. 937.7.

Sluyter R, Barden J A and Wiley J S (2001) Detection of P2X Purinergic Receptors on Human B Lymphocytes. *Cell Tissue Res* **304**:231-236.

Smith AB, Hansen M A, Liu D-M and Adams D J (2001) Pre- and Postsynaptic Actions of ATP on Neurotransmission in Rat Submandibular Ganglia. *Neuroscience* **107**:283-291.

Smith FM, Humphrey P P and Murrell-Lagnado R D (1999) Identification of Amino Acids Within the $P2X_2$ Receptor C-Terminus That Regulate Desensitization. *J Physiol* **520**:91-99.

Smolen, A. and Raisman, G. Synapse formation in the rat superior cervical ganglion during normal development and after neonatal deafferentation. Brain Res 181, 315-323. 1980.

Sneddon, P. and Burnstock, G. ATP as a co-transmitter in rat tail artery. Eur.J.Pharmacol. 106[1], 149-152. 1984.

Solini A, Chiozzi P, Morelli A, Fellin R and Virgilio F D (1999) Human Primary Fibroblasts in Vitro Express a Purinergic $P2X_7$ Receptor Coupled to Ion Fluxes, Microvesicle Formation and IL-6 Release. *J Cell Sci* **112**:297-305.

Solle M, Labasi J, Perregaux D G, Stam E, Petrushova N, Koller B H, Griffiths R J and Gabel C A (2001) Altered Cytokine Production in Mice Lacking P2X7 Receptors. *J Biol Chem* **276**:125-132.

Soto F, Garcia-Guzman M, Gomez-Hernandez J M, Hollmann M, Karschin C and Stuhmer W (1996) $P2X_4$: an ATP-Activated Ionotropic Receptor Cloned From Rat Brain. *Proc Natl Acad Sci U S A* **93**:3684-3688.

Soto F, Lambrecht G, Nickel P, Stuehmer W and Busch A E (1999) Antagonistic Properties of the Suramin Analogue NF023 at Heterologously Expressed P2X Receptors. *Neuropharmacology* **38**:141-149.

Souslova V, Cesare P, Ding Y, Akopian A N, Stanfa L, Suzuki R, Carpenter K, Dickenson A, Boyce S, Hill R, Nebenius-Oosthuizen D, Smith A J H, Kidd E J and Wood J N (2000) Warm-Coding Deficits and Aberrant Inflammatory Pain in Mice Lacking $P2X_3$ Receptors. *Nature* **407**:1015-1017.

Spehr J, Spehr M, Hatt H and Wetzel C H (2004) Subunit-Specific P2X-Receptor Expression Defines Chemosensory Properties of Trigeminal Neurons. *Eur J Neurosci* **19**:2497-2510.

Spelta V, Jiang L H, Surprenant A and North R A (2002) Kinetics of Antagonist Actions at Rat $P2X_{2/3}$ Heteromeric Receptors. *Br J Pharmacol* **135**:1524-1530.

Sperlagh B, Erdelyi F, Szabo G and Vizi E S (2000) Local Regulation of [3H]-Noradrenaline Release From the Isolated Guinea-Pig Right Atrium by P2X-Receptors Located on Axon Terminals. *Br J Pharmacol* **131**:1775-1783.

Stokes L, Jiang LH, Alcaraz L, Bent J, Bowers K, Fagura M, Furbur M, Mortimore M, Lawson M, Theaker J, Laurent C, Braddock M and Surprenant A (2006) Characterization of a Selective and Potent Antagonist of Human $P2X_7$ receptors, AZ11645373. *Br J Pharmacol* **149**:880-887.

Stoop R, Surprenant A and North R A (1997) Different Sensitivities to PH of ATP-Induced Currents at Four Cloned P2X Receptors. *J Neurophysiol* **78**:1837-40.

Stucky, C. L., Medler, K. A., and Molliver, D. C. The P2Y agonist UTP activates cutaneous afferent fibers. Pain 109, 36-44. 2004.

Studeny S, Torabi A and Vizzard M A (2005) $P2X_2$ and $P2X_3$ Receptor Expression in Postnatal and Adult Rat Urinary Bladder and Lumbosacral Spinal Cord. *Am J Phypsiol Regul Integ Comp Physiol* **289**:R1155-R1168.

Sugiyama T, Kawamura H, Yamanishi S, Kobayashi M, Katsumura K and Puro D G (2005) Regulation of $P2X_7$-Induced Pore Formation and Cell Death in Pericyte-Containing Retinal Microvessels. *Am J Physiol Cell Physiol* **288**:C568-C576.

Sui, G. P., Wu, C., and Fry, C. H. Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts. BJU Int 97, 1327-1331. 2006.

Sun Y and Chai T C (2002) Effects of Dimethyl Sulphoxide and Heparin on Stretch-Activated ATP Release by Bladder Urothelial Cells From Patients With Interstitial Cystitis. *BJU Int* **90**:381-385.

Sun, Y. and Chai, T. C. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. Am J Physiol Cell Physiol 290, C27-C34. 2006.

Sun, Y., Keay, S., DeDeyne, P., and Chai, T. Stretch-activated release of adenosine triphosphate by bladder uroepithelia is augmented in interstitial cystitis. Urology 57, 131. 2001.

Surprenant A, Rassendren F, Kawashima E, North R A and Buell G (1996) The Cytolytic P2Z Receptor for Extracellular ATP Identified As a P2X Receptor (P2X7). *Science* **272**:735-738.

Surprenant A, Schneider D A, Wilson H L, Galligan J J and North R A (2000) Functional Properties of Heteromeric $P2X_{1/5}$ Receptors Expressed in HEK Cells and Excitatory Junction Potentials in Guinea-Pig Submucosal Arterioles. *J Auton Nerv Syst* **81**:249-263.

Suzuki-Kerr H, Vlajkovic S, Donaldson P J and Lim J (2008) Molecular Identification and Localization of P2X Receptors in the Rat Lens. *Experimental Eye Research* **86**:844-855.

Tempest HV, Dixon A K, Turner W H, Elneil S, Sellers L A and Ferguson D R (2004) $P2X_2$ and $P2X_3$ Receptor Expression in Human Bladder Urothelium and Changes in Interstitial Cystitis. *BJU Int* **93**:1344-1348.

Tenneti L, Gibbons S J and Talamo B R (1998) Expression and Trans-Synaptic Regulation of P2x4 and P2z Receptors for Extracellular ATP in Parotid Acinar Cells. Effects of Parasympathetic Denervation. *J Biol Chem* **273**:26799-26808.

Theobald RJ, Jr. (1996) The Effect of NG-Monomethyl-L-Arginine on Bladder Function. *Eur J Pharmacol* **311**:73-78.

Thomas S, Virginio C, North R A and Surprenant A (1998) The Antagonist Trinitrophenyl-ATP Reveals Co-Existence of Distinct P2X Receptor Channels in Rat Nodose Neurones. *J Physiol* **509**:411-417.

Tittle RK and Hume R I (2008) Opposite Effects of Zinc on Human and Rat P2X2 Receptors. *J Neurosci* **28**:11131-11140.

Torres GE, Egan T M and Voigt M M (1998a) Topological Analysis of the ATP-Gated Ionotropic [Correction of Ionotrophic] $P2X_2$ Receptor Subunit. *FEBS Lett* **425**:19-23.

Torres GE, Egan T M and Voigt M M (1999) Hetero-Oligomeric Assembly of P2X Receptor Subunits. Specificities Exist With Regard to Possible Partners. *J Biol Chem* **274**:6653-6659.

Torres GE, Haines W R, Egan T M and Voigt M M (1998b) Co-Expression of $P2X_1$ and $P2X_5$ Receptor Subunits Reveals a Novel ATP- Gated Ion Channel. *Mol Pharmacol* **54**:989-993.

Townsend-Nicholson A, King B F, Wildman S S and Burnstock G (1999) Molecular Cloning, Functional Characterization and Possible Cooperativity Between the Murine $P2X_4$ and $P2X_{4a}$ Receptors. *Mol Brain Res* **64**:246-254.

Trezise DJ, Michel A D, Grahames C, Khakh B S, Surprenant A and Humphrey P P (1995) The Selective P_{2X} Purinoceptor Agonist, B,γ-Methylene-L-Adenosine 5'-Triphosphate, Discriminates Between Smooth Muscle and Neuronal P_{2X} Purinoceptors. *Naunyn-Schmiedebergs Arch Pharmacol* **351**:603-609.

Truschel ST, Ruiz WG, Shulman T, Pilewski J, Sun TT, Zeidel ML and Apodaca G (1999) Primary Uroepithelial Cultures. A Model System to Analyze Umbrella Cell Barrier Function. *J Biol Chem* **274**:15020-15029.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter M W and Inoue K (2003) $P2X_4$ Receptors Induced in Spinal Microglia Gate Tactile Allodynia After Nerve Injury. *Nature* **424**:778-783.

Tsuda, M., Toyomitsu, E., Komatsu, T., Masuda, T., Kunifusa, E., Nasu-Tada, K., Koizumi, S., Yamamoto, K., Ando, J., and Inoue, K. Fibronectin/integrin system is involved in P2X4 receptor upregulation in the spinal cord and neuropathic pain after nerve injury. *Glia* 56, 579-585. 2008.

Tsuda M, Ueno S and Inoue K (1999a) Evidence for the Involvement of Spinal Endogenous ATP and P2X Receptors in Nociceptive Responses Caused by Formalin and Capsaicin in Mice. *Br J Pharmacol* **128**:1497-1504.

Tsuda M, Ueno S and Inoue K (1999b) In Vivo Pathway of Thermal Hyperalgesia by Intrathecal Administration of α,β-Methylene ATP in Mouse Spinal Cord: Involvement of the Glutamate-NMDA Receptor System. *Br J Pharmacol* **127**:449-456.

Ueno S, Moriyama T, Honda K, Kamiya H, Sakurada T and Katsuragi T (2003) Involvement of $P2X_2$ and $P2X_3$ Receptors in Neuropathic Pain in a Mouse Model of Chronic Constriction Injury. *Drug Dev Res* **59**:104-111.

Ulmann L, Hatcher J P, Hughes J P, Chaumont S, Green P J, Conquet F, Buell G N, Reeve A J, Chessell I P and Rassendren F (2008) Up-Regulation of P2X4 Receptors in Spinal Microglia After Peripheral Nerve Injury Mediates BDNF Release and Neuropathic Pain. *J Neurosci* **28**:11263-11268.

Urbanek E, Nickel P and Schlicker E (1990) Antagonistic Properties of Four Suramin-Related Compounds at Vascular Purine P2X Receptors in the Pithed Rat. *Eur J Pharmacol* **175**:207-210.

Valera S, Hussy N, Evans R J, Adami N, North R A, Surprenant A and Buell G (1994) A New Class of Ligand-Gated Ion Channel Defined by P2X Receptor for Extracellular ATP. *Nature* **371**:516-519.

Valera S, Talabot F, Evans R J, Gos A, Antonarakis S E, Morris M A and Buell G N (1995) Characterization and Chromosomal Localization of a Human P2X Receptor From the Urinary Bladder. *Receptors & Channels* **3**:283-9.

Van Nassauw L, Brouns I, Adriaensen D, Burnstock G and Timmermans J P (2002) Neurochemical Identification of Enteric Neurons Expressing $P2X_3$ Receptors in the Guinea-Pig Ileum. *Histochem Cell Biol* **118**:193-203.

Varani K, De Mattei M, Vincenzi F, Tosi A, Gessi S, Merighi S, Pellati A, Masieri F, Ongaro A and Borea P A (2008) Pharmacological Characterization of P2X1 and P2X3 Purinergic Receptors in Bovine Chondrocytes. *Osteoarthritis and Cartilage* **16**:1421-1429.

Vial C and Evans R J (2000) P2X Receptor Expression in Mouse Urinary Bladder and the Requirement of $P2X_1$ Receptors for Functional P2X Receptor Responses in the Mouse Urinary Bladder Smooth Muscle. *Br J Pharmacol* **131**:1489-1495.

Vial C and Evans R J (2001) Smooth Muscle Does Not Have a Common P_{2X} Receptor Phenotype: Expression, Ontogeny and Function of P_{2X1} Receptors in Mouse Ileum, Bladder and Reproductive Systems. *Auton Neurosci Basic Clin* **92**:56-64.

Vial C and Evans R J (2002) $P2X_1$ Receptor-Deficient Mice Establish the Native P2X Receptor and a $P2Y_6$-Like Receptor in Arteries. *Mol Pharmacol* **62**:1438-1445.

Vial C, Roberts J A and Evans R J (2004) Molecular Properties of ATP-Gated P2X Receptor Ion Channels. *Trends Pharmacol Sci* **25**:487-493.

Virginio C, Church D, North R A and Surprenant A (1997) Effects of Divalent Cations, Protons and Calmidazolium at the Rat $P2X_7$ Receptor. *Neuropharmacology* **36**:1285-1294.

Virginio C, MacKenzie A, Rassendren F A, North R A and Surprenant A (1999) Pore Dilation of Neuronal P2X Receptor Channels. *Nature Neurosci* **2**:315-321.

Virginio C, North R A and Surprenant A (1998a) Calcium Permeability and Block at Homomeric and Heteromeric $P2X_2$ and $P2X_3$ Receptors, and P2X Receptors in Rat Nodose Neurones. *J Physiol* **510**:27-35.

Virginio C, Robertson G, Surprenant A and North R A (1998b) Trinitrophenyl-Substituted Nucleotides Are Potent Antagonists Selective for $P2X_1$, $P2X_3$, and Heteromeric $P2X_{2/3}$ Receptors. *Mol Pharmacol* **53**:969-973.

Vizi ES, Liang S-D, Sperlagh B, Kittel A and Juranyi Z (1997) Studies on the Release and Extracellular Metabolism of Endogenous ATP in Rat Superior Cervical Ganglion: Support for Neurotransmitter Role of ATP. *Neuroscience* **79**:893-903.

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne D A, Ford A P and Burnstock G (2001) $P2X_3$ Knock-Out Mice Reveal a Major Sensory Role for Urothelially Released ATP. *J Neurosci* **21**:5670-5677.

Volonte, C., Amadiio, S., Cavaliere, F., D'Ambrosi, N., Vacca, F., and Bernardi, G. Extracellular ATP and neurodegeneration. Curr Drug Targets CNS Neurol Disord 2, 403-412. 2003.

Volonte C, Amadio S, D'Ambrosi N, Colpi M and Burnstock G (2006) P2 Receptor Web: Complexity and Fine-Tuning. *Pharmacology & Therapeutics* **112**:264-280.

von Kugelgen I, Krumme B, Schaible U, Schollmeyer P J and Rump L C (1995) Vasoconstrictor Responses to the P_{2x}-Purinoceptor Agonist B,γ-Methylene-L-ATP in Human Cutaneous and Renal Blood Vessels. *Br J Pharmacol* **116**:1932-1936.

Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North R A and Elde R (1996) Differential Distribution of Two ATP-Gated Channels (P2X Receptors) Determined by Immunocytochemistry. *Proc Natl Acad Sci U S A* **93**:8063-8067.

Vulchanova L, Riedl M S, Shuster S J, Buell G, Surprenant A, North R A and Elde R (1997) Immunohistochemical Study of the $P2X_2$ and $P2X_3$ Receptor Subunits in Rat and Monkey Sensory Neurons and Their Central Terminals. *Neuropharmacology* **36**:1229-42.

Vulchanova L, Riedl M S, Shuster S J, Stone L S, Hargreaves K M, Buell G, Surprenant A, North R A and Elde R (1998) $P2X_3$ Is Expressed by DRG Neurons That Terminate in Inner Lamina II. *Eur J Neurosci* **10**:3470-8.

Wang CZ, Namba N, Gonoi T, Inagaki N and Seino S (1996) Cloning and Pharmacological Characterization of a Fourth P2X Receptor Subtype Widely Expressed in Brain and Peripheral Tissues Including Various Endocrine Tissues. *Biochem Biophys Res Commun* **220**:196-202.

Wang ECY, Lee J M, Ruiz W G, Balestreire E M, von Bodungen M, Barrick S, Cockayne D A, Birder L A and Apodaca G (2005) ATP and Purinergic Receptor-Dependent Membrane Traffic in Bladder Umbrella Cells. *J Clin Inves* **115**:2412-2422.

Wang X, Arcuino G, Takano T, Lin J, Peng W G, Wan P, Li P, Xu Q, Liu Q S, Goldman S A and Nedergaard M (2004) $P2X_7$ Receptor Inhibition Improves Recovery After Spinal Cord Injury. *Nat Med* **10**:821-827.

Watters JJ, Sommer J A, Pfeiffer Z A, Aga M, Prabhu U and Guerra A N (2001) $P2X_7$ Nucleotide Receptor: Modulation of LPS-Induced Macrophage Signaling and Mediator Production. *Drug Dev Res* **53**:91-104.

Wein, A. J. The urothelium in overactive bladder: passive bystander or active participant. J Urol 173, 2199-2200. 2005.

Wells, D. G., Zawisa, M. J., and Hume, R. I. Changes in responsiveness to extracellular ATP in chick skeletal muscle during development and upon denervation. Dev Biol 1722, 585-590. 1995.

Werner P, Seward E P, Buell G N and North R A (1996) Domains of P2X Receptors Involved in Desensitization. *Proceedings of the National Academy of Sciences of the United States of America* **93**:15485-15490.

Wewers MD and Sarkar A (2009) $P2X_7$ Receptor and Macrophage Function. *Purinergic Signalling* **5**:189-195.

Wildman SS, Brown S G, King B F and Burnstock G (1999a) Selectivity of Diadenosine Polyphosphates for Rat P2X Receptor Subunits. *Eur J Pharmacol* **367**:119-123.

Wildman SS, Brown S G, Rahman M, Noel C A, Churchill L, Burnstock G, Unwin R J and King B F (2002) Sensitization by Extracellular Ca^{2+} of Rat $P2X_5$ Receptor and Its Pharmacological Properties Compared With Rat $P2X_1$. *Mol Pharmacol* **62**:957-966.

Wildman SS, King B F and Burnstock G (1998) Zn^{2+} Modulation of ATP-Responses at Recombinant $P2X_2$ Receptors and Its Dependence on Extracellular PH. *Br J Pharmacol* **123**:1214-1220.

Wildman SS, King B F and Burnstock G (1999b) Modulatory Activity of Extracellular H^+ and Zn^{2+} on ATP-Responses at $RP2X_1$ and $RP2X_3$ Receptors. *Br J Pharmacol* **128**:486-492.

Wildman SS, Unwin R J and King B F (2003) Extended Pharmacological Profiles of Rat P2Y2 and Rat P2Y4 Receptors and Their Sensitivity to Extracellular H+ and Zn2+ Ions. *Br J Pharmacol* **140**:1177-1186.

Wiley JS, Chen J R, Snook M B and Jamieson G P (1994) The P2Z-Purinoceptor of Human Lymphocytes: Actions of Nucleotide Agonists and Irreversible Inhibition by Oxidized ATP. *Br J Pharmacol* **112**:946-950.

Wilson HL, Wilson SA, Surprenant A and North RA (2002) Epithelial Membrane Proteins Induce Membrane Blebbing and Interact with the $P2X_7$ Receptor C Terminus. *J Biol Chem***277**:34017-34023

Wirkner K, Sperlagh B and Illes P (2007) P2X3 Receptor Involvement in Pain States. *Molecular Neurobiology* **36**:165-183.

Wirkner, K., Stanchev, D., Milius, D., Hartmann, L., Kato, E., Zadori, Z. S., Mager, P. P., Rubini, P., Norenberg, W., and Illes, P. Regulation of the pH sensitivity of human $P2X_3$ receptors by N-linked glycosylation. Journal of Neurochemistry 107, 1216-1224. 2008.

Wiseman, O. J., Fowler, C. J., and Landon, D. N. The role of the human bladder lamina propria myofibroblast. *BJU Int* 91, 89-93. 2003.

Wong AY, Burnstock G and Gibb A J (2000) Single Channel Properties of P2X ATP Receptors in Outside-Out Patches From Rat Hippocampal Granule Cells. *J Physiol* **527**:529-547.

Worthington RA, Dutton J L, Poronnik P, Bennett M R and Barden J A (1999) Localisation of P2X Receptors in Human Salivary Gland Epithelial Cells and Human Embryonic Kidney Cells by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis/Western Blotting and Immunofluorescence. *Electrophoresis* **20**:2065-2070.

Wu G, Whiteside G T, Lee G, Nolan S, Niosi M, Pearson M S and Ilyin V I (2004) A-317491, a Selective $P2X_3$/$P2X_{2/3}$ Receptor Antagonist, Reverses Inflammatory Mechanical Hyperalgesia Through Action at Peripheral Receptors in Rats. *Eur J Pharmacol* **504**:45-53.

Wynn G, Ma B, Ruan H Z and Burnstock G (2004) Purinergic Component of Mechanosensory Transduction Is Increased in a Rat Model of Colitis. *Am J Physiol Gastrointest Liver Physiol* **287**:G647-G657.

Wynn G, Rong W, Xiang Z and Burnstock G (2003) Purinergic Mechanisms Contribute to Mechanosensory Transduction in the Rat Colorectum. *Gastroenterology* **125**:1398-1409.

Xiang Z, Bo X and Burnstock G (1998) Localization of ATP-Gated P2X Receptor Immunoreactivity in Rat Sensory and Sympathetic Ganglia. *Neurosci Lett* **256**:105-108.

Xiang Z and Burnstock G (2005) Changes in Expression of P2X Purinoceptors in Rat Cerebellum During Postnatal Development. *Dev Brain Res* **156**:147-157.

Xiong K, Hu X Q, Stewart R R, Weight F F and Li C (2005) The Mechanism by Which Ethanol Inhibits Rat $P2X_4$ Receptors Is Altered by Mutation of Histidine 241. *Br J Pharmacol* **145**:576-586.

Yamamoto K, Korenaga R, Kamiya A, Qi Z, Sokabe M and Ando J (2000) P2X4 Receptors Mediate ATP-Induced Calcium Influx in Human Vascular Endothelial Cells. *Am J Physiol Heart Circ Physiol* **279**:H285-H292.

Yamamoto K, Sokabe T, Matsumoto T, Yoshimura K, Shibata M, Ohura N, Fukuda T, Sato T, Sekine K, Kato S, Isshiki M, Fujita T, Kobayashi M, Kawamura K, Masuda H, Kamiya A and Ando J (2006) Impaired Flow-Dependent Control of Vascular Tone and Remodeling in P2X4-Deficient Mice. *Nat Med* **12**:133-137.

Yan Z, Li S, Liang Z, Tomic M and Stojilkovic S S (2008) The P2X7 Receptor Channel Pore Dilates Under Physiological Ion Conditions. *J Gen Physiol* **132**:563-573.

Yiangou Y, Facer P, Baecker P A, Ford A P, Knowles C H, Chan C L, Williams N S and Anand P (2001a) ATP-Gated Ion Channel $P2X_3$ Is Increased in Human Inflammatory Bowel Disease. *Neurogastroenterol Motil* **13**:365-369.

Yiangou Y, Facer P, Ford A, Brady C, Wiseman O, Fowler C J and Anand P (2001b) Capsaicin Receptor VR1 and ATP-Gated Ion Channel $P2X_3$ in Human Urinary Bladder. *BJU Int* **87**:774-779.

Yin DX, Zhu L and Schimke R T (1996) Tetracycline-Controlled Gene Expression System Achieves High-Level and Quantitative Control of Gene Expression. *Analytical Biochemistry* **235**:195-201.

Young MT, Fisher J A, Fountain S J, Ford R C, North R A and Khakh B S (2008) Molecular Shape, Architecture, and Size of P2X4 Receptors Determined Using Fluorescence Resonance Energy Transfer and Electron Microscopy. *J Biol Chem* **283**:26241-26251.

Yu, W., Zacharia, L. C., Jackson, E. K., and Apodaca, G. Adenosine receptor expression and function in bladder uroepithelium. AJP - Renal Physiology 291, C254-C265. 2006.

Zhang M, Zhong H, Vollmer C and Nurse C A (2000) Co-Release of ATP and ACh Mediates Hypoxic Signalling at Rat Carotid Body Chemoreceptors. *J Physiol* **525**:143-158.

Zhao Q, Logothetis D and S+¬gu+¬la P (2007) Regulation of ATP-Gated P2X Receptors by Phosphoinositides. *Pfl++gers Archiv European Journal of Physiology* **455**:181-185.

Zhong Y, Dunn P M and Burnstock G (2000a) Guinea-Pig Sympathetic Neurons Express Varying Proportions of Two Distinct P2X Receptors. *J Physiol* **523**:391-402.

Zhong Y, Dunn P M and Burnstock G (2000b) Pharmacological Comparison of P2X Receptors on Rat Coeliac, Mouse Coeliac and Mouse Pelvic Ganglion Neurons. *Neuropharmacology* **39**:172-180.

Zhong Y, Dunn P M and Burnstock G (2001) Multiple P2X Receptors on Guinea-Pig Pelvic Ganglion Neurons Exhibit Novel Pharmacological Properties. *Br J Pharmacol* **132**:221-233.

Zhong Y, Dunn P M, Xiang Z, Bo X and Burnstock G (1998) Pharmacological and Molecular Characterization of P2X Receptors in Rat Pelvic Ganglion Neurons. *Br J Pharmacol* **125**:771-81.

Zhou X and Galligan J J (1996) P2X Purinoceptors in Cultured Myenteric Neurons of Guinea-Pig Small Intestine. *J Physiol* **496**:719-729.

Zimmermann H (2008) ATP and Acetylcholine, Equal Brethren. *Neurochemistry International* **52**:634-648.

Ziyal R, Ziganshin A U, Nickel P, Ardanuy U, Mutschler E, Lambrecht G and Burnstock G (1997) Vasoconstrictor Responses Via P2X-Receptors Are Selectively Antagonized by NF023 in Rabbit Isolated Aorta and Saphenous Artery. *Br J Pharmacol* **120**:954-960.