The role of negatively charged amino acids in channel function, pH potentiation, and zinc modulation of the ATP-gated rat $P2X_2$ receptor

by

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Dedication

This thesis is dedicated with Divine love always to my mother and father (Betty and Gerald Friday), Evan Friday, Dorothy King Cleveland, Melvin Craft, David Friday, Davi and Gambel Oulette, Sam Harris, Celester Anderson, Uncle Jack King, and Carolyn, Houston and Stacy Williams. Also, I would like to express gratitude and appreciation to all of the helpful friends I have met during my odyssey to become a Teacher and Scientist.

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Abstract

P2X receptors are a family of genes encoding seven homologous subunits which form ion channels that are permeable to Na^+, K^+, and Ca^{2+} ions upon activation by ATP. Expressed in excitable and non-excitable cells, the $P2X_2$ receptor has been shown to have broad physiological importance in neuromuscular junction development, gustatory sensation, and the regulation of urinary bladder function. The ATP-evoked responses of $P2X_2$ receptors are allosterically modulated by divalent zinc and protons. We have used site-directed mutagenesis and two-electrode voltage clamp electrophysiology to test whether any of the 34 extracellular aspartate and glutamate residues of the ATP-gated rat $P2X_2$ receptor are important for general channel function, pH potentiation, or zinc modulation. All of the extracellular acidic residues tested produced functional channels with only small changes in ATP potency or maximal response. Seven candidates (D82, E85, E91, E115, D136, D209, and D281) showed significantly reduced zinc potentiation, and one candidate (E84) showed significantly reduced zinc inhibition. Further tests with cysteine versions of the eight identified candidates and thiol-reactive MTSET suggested that only D136 remains a good candidate to directly bind zinc at the potentiation site, and there were no good candidates for zinc inhibition. When the 34 alanine substituted mutants were screened for ability to potentiate to acidic pH, E63

showed significantly reduced pH potentiation as compared with wild type $P2X_2$. Molecular level understanding of the structure-function relationship of allosteric sites of these receptors might allow for development of selective drugs that target allosteric sites, which could lead to safer therapies with fewer side effects than current approaches with neurotransmitter agonists and antagonists.

Chapter One

Introduction

1.0 Receptor-mediated excitation and inhibition in neurons

There are two broad mechanisms of receptor-mediated excitation and inhibition in central and peripheral neurons. One mechanism involves G-protein coupled receptors (GPCRs). GPCRs are the largest family of membrane-bound receptors, and there are five main human GPCR families--Rhodopsin, Secretin, Adhesion, Glutamate and Frizzled/Taste2 (Lagerstrom & Schioth 2008). All GPCRs possess seven hydrophobic membrane-spanning segments (Larhammar *et al.* 1993, Kobilka *et al.* 1988).

In general, GPCRs mediate slowly developing (onset is longer than 50 ms) but long lasting (many seconds to minutes) changes in the excitability of neurons. The slow time course of responses mediated at GPCRs is based on a multistep process that includes agonist binding and subsequent activation of one or more intracellular signaling pathways and possibly some ion channels. Ion channel phosphorylation and resulting changes in channel open probability are important downstream consequences of GPCR activation in neurons (Vial *et al.* 2004).

Ligand-gated ion channels (LGICs), like GPCRs, also mediate changes in neuronal excitability. LGICs are composed of several homologous subunits arranged in a ring to form a central ion-conducting pore, having the ligand binding site and ion channel as part of the same multimeric complex. This broad class of receptors mediates fast synaptic responses. As these receptor/ ion channels do not require generation of intracellular 2nd messenger molecules to convey the primary signal, response latencies (<1 ms) and durations (<100 ms) are very short.

Ion channels are widespread in virtually all organisms where they mediate receptor signaling, membrane potential maintenance, accumulation and transduction of electrochemical energy, and generation and spreading of action potential. Since chemically activated channels are crucial for synaptic transmission in the nervous system, comprehension of the mechanisms involved in the operation of LGICs at the cellular and molecular level is essential for gaining insight into the pathogenesis of many psychiatric and neurological disorders and for efficient development of novel specifically targeted drugs (Krusek *et al.* 2004).

Ion channels can exist in various states e.g. resting, activated, desensitized, and inactivated. Binding of the endogenous ligand (neurotransmitter) to an LGIC causes activation of the channel from the resting (closed) conformational state, and typically stabilizes the channel in an activated (open) state. The binding site for the natural ligand, that activates the receptor, is known as the orthosteric binding site. In addition, the binding of ligands at other

sites on the receptor surface can modify the functioning of the receptor. This concept of modulation of LGIC activity by the binding of a second ligand, or allosteric modulator, is termed, allosteric modulation (Karlin 1967). The binding site for an allosteric modulator is an allosteric binding site and each receptor may have several allosteric binding sites which are selective for different ligands. Activation of these allosteric binding sites results in changes in the affinity for the agonist or efficacy by which the channel is opened (Krusek *et al.* 2004).

Positive allosteric effectors enhance agonist-induced responses whereas negative allosteric effectors reduce receptor function. Allosteric modulators can change the current amplitude (potentiation or inhibition) and/ or time course (desensitization rate) of agonist-evoked responses. In addition to modulating LGICs, allosteric modulation can affect a wide range of cellular proteins including enzymes and metabotropic membrane receptors (Soudijn *et al.* 2002, Christopoulos 2002).

In mammals, one way to classify LGICs is according to the number of transmembrane (TM) segments present in the subunits that form the channels. By this criterion there are three families, with two, three and four transmembrane segments respectively. The simplest LGIC family includes the adenosine 5'-triphosphate (ATP)-activated P2X receptors and acid-sensing ion channels (ASICs); each subunit of these cationic channels contains two TM segments separated by a large extracellular loop (Khakh & North 2006). These are made up of three homologous subunits. It remains unclear whether these two classes

of channels (ASICS and P2X) share a common evolutionary origin, as there is no sequence similarity.

A second family is the ionotropic glutamate-activated cationic receptors, which include N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid (AMPA) and kainate receptors. Members of this family have subunits with three TM segments with a 'P-loop' separating the first and second TM segments. These are made up of four homologous subunits. These channels clearly are distantly related to the voltage gated sodium, potassium and calcium channel family.

The third and largest LGIC family is the 'Cys-loop receptor superfamily', so called because of a conserved cysteine loop in their extracellular domain. This family includes both anionic glycine (GlyR) and γ-aminobutyric acid type A and type C ($GABA_aR$, $GABA_cR$) channels, and cationic nicotinic acetylcholine receptors (nAChRs), serotonin receptors ($5\text{-}HT_3$), histamine receptors and recently identified zinc-activated channels (Davies *et al.* 2003). The subunits of Cys-loop receptors have four TM segments, with a large intracellular domain between TM segments 3 and 4. Functional receptors of this family are made up of five homologous subunits.

In all three families of channels the subunit composition can be homomeric or heteromeric and, as a result they display a great diversity of physiological and pharmacological properties (Hogg et al. 2005).

AMPA and NMDA receptors mediate most of the fast excitatory synaptic transmission in the brain and spinal cord, although their excessive activation

causes glutamate-induced neurodegeneration e.g. Alzheimer dementia (Henneberry 1992). They are permeable to monovalent cations. All NMDA receptors and some AMPA receptors are also highly permeable to calcium. Glutamate is the sole neurotransmitter activating AMPA receptors, but NMDA receptors are gated by two different agonists, glutamate and glycine. Small ions such as protons, Zn^{2+}, Ca^{2+}, and Cu^{2+} act through allosteric sites to affect the efficacy of channel opening, and thereby affect excitatory synaptic transmission mediated by AMPA and NMDA receptors and other LGICs.

In contrast, the $GABA_A$ receptor represents the main fast inhibitory LGIC in the brain. This chloride ion conducting channel is activated by γ-amino butyric acid and allosterically modulated by benzodiazepines and other compounds such as the antihelmintic avermectin drugs (Krusek et al. 2004).

1.1 Adenosine 5'-Triphosphate (ATP)

Adenosine 5'-triphosphate (ATP) is widely recognized for its fundamental metabolic role as the energy currency of the cell. Expenditure of ATP provides the input of free energy necessary for cellular work, biosynthesis, catabolism, and active transport. In addition to this indispensible role in all living things, ATP has further importance in multicellular animals: ATP acts as an intercellular signaling molecule which regulates various processes such as the cell cycle, programmed cell death, membrane and cytoskeletal remodeling, and modulation of excitability in neurons.

ATP and other nucleotides can be released from cells through regulated pathways, or following the loss of plasma membrane integrity (Brake & Julius 1996). Extracellular ATP activates cell-surface metabotropic and ionotropic nucleotide (P2) receptors in vascular, neural, connective, and immune tissues. P2 receptors mediate a wealth of physiological processes, including nitric oxide-dependent vasodilation of vascular smooth muscle (Ralevic & Burnstock 1991) and fast excitatory neurotransmission in sensory afferents (Lustig et al. 1993). Thus, ATP has multiple critical functions as the source of chemical energy for intracellular housekeeping, while also serving as an autocrine or paracrine signal of homeostasis, pathogenesis, and injury at the cellular and tissue level (Gerasimovskaya *et al.* 2002).

ATP is known to be a co-transmitter at neuroneuronal and neuroeffector synapses. ATP may act at pre- and postsynaptic sites and therefore, it may participate in the phenomena of long-term potentiation and long-term depression of excitatory synaptic transmission. The regulated release of ATP into the extracellular space, e.g., by exocytosis, membrane transporters, and connexin hemichannels, is a widespread physiological process (Franke *et al.* 2006).

Criteria for a neurotransmitter include regulated release, the presence of specific receptors, and a mechanism for clearance (Finger et al. 2005). ATP is released by vesicular, anion channel, and hemichannel mechanisms, activates P2 receptors, and is cleared from the extracellular space by ectonucleotidases (Burnstock 2007b). Therefore, ATP meets all criteria for being a major neurotransmitter.

Metabotropic pyrimidine and purine nucleotide receptors (P2YRs) belong to the GPCR superfamily. They are distinguishable from adenosine receptors (P1) as they bind adenine and/or uracil nucleotide triphosphates or diphosphates depending on the subtype. Over the past decade, P2Y receptors have been cloned from a variety of tissues and species, and as many as eight functional subtypes have been characterized (Schoneberg *et al.* 2007). However, the focus of this thesis is the ionotropic $P2X_2$ receptor.

1.2 Ionotropic P2X Purinoceptors

ATP-gated ion channels were first cloned from smooth muscle of rat vas deferens ($rP2X_1$) and rat Phaeochromocytoma (PC12) cells ($rP2X_2$). So far, seven different mammalian genes have been found to encode P2X receptor subunits ($P2X_1$ – $P2X_7$), ranging from 379 ($P2X_6$) to 595 ($P2X_7$) amino acid residues in length. The products of this gene family are 40 to 50% identical in amino acid sequence (Girdler & Khakh 2004). There is no report of homology of sequence between P2X receptors and other proteins, although a similarity has been suggested to class II aminoacyl-tRNA synthetases (North 2002).

No P2X-related gene has been identified in the whole genomes of the model invertebrates *Drosophila* and *C. elegans*. However, there are now several reports of homologous sequences from invertebrate species: including the parasitic flatworm *Schistosoma mansoni* (Raouf *et al.* 2005, Agboh *et al.* 2004), the social amoeba *Dictyostelium discoideum* (Fountain *et al.* 2007), and

Ostreococcus tauri, a primitive green algae of Prasinophyceae, that is close to the evolutionary origins of photosynthetic plants (Fountain *et al.* 2008).

In vertebrates, P2X receptors are expressed in the central and peripheral nervous systems, as well as in non-neuronal cell types in vascular, connective, and immune tissues (North 2002). These receptors have been characterized on smooth muscle cells and autonomic and sensory neurons, where they mediate membrane depolarization and in some cases, Ca^{2+} entry. P2XRs allow released extracellular ATP to act as a signaling molecule in autocrine and paracrine signaling processes (Stojilkovic & Koshimizu 2001, Gerasimovskaya *et al.* 2002, Placido *et al.* 2006) and neurotransmission (Burnstock 2007a).

P2X receptors functionally resemble glutamate-gated, acetylcholine-gated and serotonin-gated channels with respect to cationic selectivity and fast activating kinetics of channel gating, but as noted above they belong to completely different gene familes and make proteins with very different structures. Thus, the P2X receptor provides a striking example of convergent evolution, whereby proteins have been fashioned with similar functional properties from subunits having very different structural characteristics (Brake et al. 1994). Ionotropic P2X receptors are also structurally distinct from the G-protein coupled metabotropic P2Y ATP receptors and other LGICs.

P2X receptors show considerable differences in their sensitivity to naturally occurring agonists, P2 receptor antagonists, allosteric modulators and furthermore show differences in kinetics of receptor activation and inactivation. Such diversity in the operational profiles of ATP-gated ion channels may be

attributable to the subunit composition of native P2XRs because other classes of ionotropic receptors show differing phenotypes that depend on subunit composition (King et al. 2000).

1.3 P2XR distribution, topology, and expression

P2X receptors are abundantly distributed, and functional responses are seen in neurons, glia, epithelia, endothelia, bone, muscle, and hemopoietic tissues (North 2002).

Investigation of transmembrane topology was initially done by Torres and others, where they expressed $P2X_2$ subunits in tandem revealing that the N and C-termini are on the same side of the plasma membrane. Immunofluorescence further resolved this to both termini being intracellular (Fig. 1.1). Studies with N-glycosylation reporters showed that the central loop between the two transmembrane domains is extracellular (Torres et al. 1998). The N-terminus is quite short and has a strictly conserved protein kinase C (PKC) phosphorylation site at Threonine-18 ($P2X_2$ numbering), which is important for desensitization. The C-terminus is variable in length among P2XRs, but is uniformly larger than the N-terminus (Girdler & Khakh 2004). While not all P2X receptors have as many as 400 amino acids, the $P2X_2$ receptor is phosphorylated by protein kinase A at Serine-431 on the intracellular C-terminal domain which regulates the amplitude but not the kinetics of ATP-evoked responses in HEK293 cells (Chow & Wang 1998).

Some cells express more than one type of P2X receptor subunit. Six of the seven cloned P2X receptors are believed to form functional homotrimeric assemblies (Barrera *et al.* 2005). However, the $P2X_6$ receptor does not form stable homotrimers, and when expressed alone is retained in the endoplasmic reticulum in monomeric form (Ormond *et al.* 2006).

P2XRs can also coassemble with other P2X subunits to form heteromeric P2X assemblies of three protein subunits per ATP-gated ion channel. Examples of functional heteromeric P2X receptors include $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$, and $P2X_{1/5}$ (King et al. 2000). The $rP2X_{2/6}$ receptor is a functionally modified $P2X_2$-like receptor with a distinct pattern of pH modulation of ATP activation. Although homomeric $P2X_6$ receptors function poorly, the $P2X_6$ subunit can contribute to functional heteromeric P2X channels and may influence the phenotype of native P2X receptors in those cells in which it is expressed (King et al. 2000). $P2X_5$ can coassemble with all other P2XRs except $P2X_7$. The prevailing view until recently was that $P2X_7$ subunits only form homomeric channels. There is now a report of biochemical and electrophysiological evidence for the existence of $P2X_{4/7}$ heteromeric receptors (Dubyak 2007).

Channels composed of splice variants of a P2X subunit (e.g. $rP2X_{2a}$ and $rP2X_{2b}$) can also generate different phenotypic forms of P2X receptors (King et al. 2000). ATP-activated currents mediated by heterologously expressed $P2X_{2a}$ and $P2X_{2b}$ receptors showed significant differences in desensitization time constants and steady-state currents in the continuous presence of ATP (Brandle *et al.* 1997). These results imply functional differences between cells

differentially expressing these $P2X_2$ isoforms. Further differences in function may possibly be generated by heteromeric combinations of isoforms of a given subunit type.

1.4 *In vivo* studies of P2X receptors using genetically altered mice

Genetically altered mice are an extremely powerful tool for understanding the *in vivo* function of proteins and provide useful models of human genetic disorders. Many different manipulations are possible, including gene knock out, knock in replacement and transgenic insertion at a different chromosomal location. Many of the uses of genetically altered mice in physiology have been recently reviewed. (Picciotto & Wickman 1998).

Currently, there around 50 reports of *in vivo* studies that used genetically modified mouse models to study the biological function of members of the P2X receptor family. None of the reports describe embryonic lethality owing to P2XR knockout, so the receptors do not appear to be critical for regulation of prenatal developmental events. The majority of studies have been done on $P2X_7$ knockout mice, and there are no reports of transgenic or knockout rodent models of $P2X_5$ or $P2X_6$ receptor function. Further, knock in studies are needed to test whether P2XR mutations identified to be candidates for functional importance by chimerization, mutagenesis, and biochemical studies in heterologous expression and *in vitro* systems are in fact relevant in the whole intact organism.

Nonetheless, the available studies have provided a wealth of insight into the broad physiological relevance of P2XRs, while also identifying molecular

targets for design of therapeutics. The following section is intended to illustrate, using representative examples from the literature, some of the ways in which transgenic or knockout mice have been used to analyze the physiological relevance of the P2X receptor family of ATP-gated cation channels in a variety of tissues and organ systems.

$P2X_1$

Knock-out studies using mice have shown that the $P2X_1$ receptor has a direct physiological role in normal male reproductive function (Mulryan et al. 2000), cytokine release from peritoneal macrophages (Sim *et al.* 2007), platelet aggregation (Hechler *et al.* 2003), and autoregulation of preglomerular vasoconstriction (Inscho *et al.* 2004).

$P2X_2$

No deficits in a wide variety of central nervous system behavioral tests were observed in $P2X_2$-/- mice (Cockayne *et al.* 2005). However, mice lacking the $P2X_2$ nucleotide receptor showed abnormalities in neuromuscular junction structure and skeletal muscle function (Ryten *et al.* 2007), urinary voiding (Wang *et al.* 2005), and peristalsis of the small intestine (Ren *et al.* 2003). Additional studies have demonstrated that ATP-evoked currents measured in rat otic neurons are due to the expression of $P2X_2$ receptors (Ma *et al.* 2004), and that $P2X_2$ plays a pivotal role in carotid body function and in mediating ventilatory responses to hypoxia (Rong *et al.* 2003).

$P2X_1$ / $P2X_2$

$P2X_1$ knock out studies have shown that heteromeric channels incorporating the properties of $P2X_1$ and $P2X_2$ receptors are present in sympathetic postganglionic neurons from the superior cervical ganglia. Thus, the $P2X_1$ receptor can contribute to the properties of heteromeric P2X receptors, and it is likely that regulation of the properties of P2X receptors by this subunit occurs in other $P2X_1$ expressing neurons (Calvert & Evans 2004).

$P2X_3$

Mice lacking the $P2X_3$ ATP receptor exhibit attenuated responses to bladder distension (Vlaskovska *et al.* 2001), impaired peristalsis in the intestine (Bian *et al.* 2003), persistent nociceptive signaling in models of acute and chronic pain (Jarvis 2003), altered coding of peripheral warm stimuli (Shimizu *et al.* 2005), and abnormalities in long-term depression of hippocampal neurons (Wang *et al.* 2006).

$P2X_2$ / $P2X_3$

P2X receptors on sensory neurons involve almost exclusively $P2X_2$ and $P2X_3$ subunits. $P2X_2$-/-, $P2X_3$-/-, and $P2X_2$/$P2X_3$(Dbl-/-) mice had reduced urinary bladder reflexes and decreased pelvic afferent nerve activity in response to bladder distension, revealing an important contribution of heteromeric $P2X_{2/3}$

receptors to nociceptive responses and mechanosensory transduction within the urinary bladder (Cockayne et al. 2005).

Mice lacking both $P2X_2$ and $P2X_3$ receptors have enlarged spleens and this is correlated with an increase in the number of immune cells, perhaps as a consequence of a compromised immune system and chronic infection (Coutinho-Silva *et al.* 2005).

Genetic elimination of $P2X_2$ and $P2X_3$ eliminates taste responses in the gustatory nerves, although the nerves remain responsive to touch, temperature, and menthol. Similarly, P2X-knockout mice show greatly reduced behavioral responses to sweeteners, glutamate, and bitter substances. Loss of either $P2X_2$ or $P2X_3$ alone resulted in only a moderate change in taste-mediated behaviors in contrast to profound deficit seen in $P2X_2$/ $P2X_3$ Dbl -/- (KO) animals. Thus neither homomeric $P2X_2$ or homomeric $P2X_3$ suffice for normal function in the peripheral taste system (Finger et al. 2005).

$P2X_4$

Long-term potentiation at Schaffer collateral synapses is reduced in mice lacking the $P2X_4$ ATP receptor relative to that in wild-type mice (Sim *et al.* 2006). Knockout studies have also shown $P2X_4$ currents are functionally expressed in recruited peritoneal macrophages of WT mice and that the $P2X_4$-like current is absent in $P2X_4$ (-/-) mice (Brone *et al.* 2007).

$P2X_5$

Although no studies of $P2X_5$ knock out mice have been reported, it has been reported that the most common human allele for $P2X_5$ produces a non-functional protein (Bo *et al.* 2003), so it appears that many humans are homozygous for this null mutation. It therefore appears that this subunit does not carry out an essential function in our species.

$P2X_7$

Of the seven P2XR subunits ($P2X_{1-7}$), the first six are expressed throughout the peripheral and central nervous systems. The $P2X_7$ purinoceptor is expressed predominantly by cells of hematopoietic origin (Chessell *et al.* 2005) and its expression has been demonstrated in microglial cells, granulocytes, monocytes/macrophages, and B and T lymphocytes. $P2X_7$ receptor protein is strongly and reliably detected in the submandibular gland and lung of wild-type mice but not in either of the $P2X_7$-/- mice (Sim *et al.* 2004).

Upon sustained activation, the $P2X_7$ receptor forms large pores in the plasma membrane. $P2X_7$ was termed the cytolytic non-neuronal P2X receptor because it had not been detected in neurons until recently immunolocalized to several brain regions, particularly the hippocampus, where it might be involved in presynaptic modulation of transmitter release (Sim *et al.* 2004).

In cells of hematopoietic origin, $P2X_7$ receptors act not only as cationic channels but uniquely couple with multiple downstream events including rapid release of proinflammatory cytokines such as IL-1 beta, IL-6 and TNFalpha,

cytoskeletal rearrangements, cell permeabilization, and apoptosis or necrotic cell death. $P2X_7$ receptors are rapidly upregulated and activated in immune cells as a result of inflammatory stimuli, and have been involved in several cellular mechanisms including those related to inflammation and immunological response (Sim et al. 2004).

Indeed mouse transgenic studies have established the $P2X_7$ receptor as an important component of *in vivo* inflammatory, immunological, and nociceptive responses (Chessell et al. 2005). Direct evidence for $P2X_7$ function in inflammatory response has been reported on the effects on leukocyte function and macrophage infiltration (Labasi *et al.* 2002), and apoptosis in response to ureteral obstruction (Goncalves *et al.* 2006). $P2X_7$ receptors play a key role in the release of brain cytokine IL-1 beta in response to immune stimuli (Mingam *et al.* 2008) and have been shown to inhibit intracellular infection by chlamydiae and mycobacteria in macrophages (Darville *et al.* 2007). ATP stimulation of cell surface $P2X_7$ receptors results in cytolysis and cell death of macrophages (Le Feuvre *et al.* 2003).

Knockout studies of $P2X_7$ have provided direct evidence for a role of this receptor in the non-neuronal physiology of skeletal mechanotransduction (Li *et al.* 2005) and osteogenesis during skeletal development (Panupinthu *et al.* 2007), and the generation of multinucleated giant cells, polykaryons, and osteoclasts (Gartland *et al.* 2003). The established regulatory roles in bone formation and resorption make the $P2X_7$ receptor a novel therapeutic target for the management of skeletal disorders such as osteoporosis (Ke *et al.* 2003).

In parallel, $P2X_7$ knockout studies of have provided some evidence for a role of this receptor in neuronal physiology as well. Functional $P2X_7$ receptors have been demonstrated to be present in synaptic terminals from midbrain of wild type mice (Marin-Garcia *et al.* 2008). Further, $P2X_7$ receptor -/- mice exhibited lack of ATP-evoked GABA and glutamate release in mouse hippocampal slices (Papp *et al.* 2004). However, Sim and others failed to find evidence for $P2X_7$ receptor protein in hippocampal neurons or their input-output projections (Sim et al. 2004).

There is clearly an abundance of *in vivo* evidence to substantiate the importance of P2X receptors in the modulation of a broad range of physiological processes in excitable and nonexcitable cells. It will be interesting to see if transgenic mouse models can aid in uncovering the physiological significance of heteromers involving $P2X_5$ or $P2X_6$ in cells where they are coexpressed with other P2XRs. Also, knock in studies aimed at assessing the validity of functional attributes assigned to residues from conclusions reached *in vitro* are anxiously awaited.

1.5 P2XR cross talk with other Ligand-Gated Ion Channels

When LGICs of different families are coexpressed in cells, activation can result in one channel modulating the function of the other, and vice-versa. This phenomenon is known as receptor cross-talk, and is usually manifest as non-additivity of the respective individual responses. That is, peak currents evoked by the respective agonists for each receptor are less than when only one of the

agonists is present. Interactions between P2XRs and other LGICs coexpressed in nerve terminals could play a regulatory role by buffering the paracrine effects of ATP and other neurotransmitters on reflex actions.

$P2X_2$ and $\alpha3\beta4$ nicotinic channels influence each other when coactivated in *Xenopus* oocytes and synaptically coupled myenteric neurons. Coactivation results in non-additive responses owing to inhibition of both channel types. Thus, ATP occludes synaptically activated nicotinic channels, and this may be relevant to the effects of ATP spillover from purinergic synapses, ATP released during local tissue damage, and ATP released during inflammation (Khakh et al. 2000). The functional cross-inhibition between ATP-gated channels and other excitatory transmitter-gated channels of the nicotinic receptor family may regulate neuronal excitability and synaptic plasticity by limiting both the level of depolarization and the flow of calcium ions through calcium-permeable P2X receptors. Alternatively, in pathological conditions of neuronal hyperactivity, interchannel cross-talk may also play a protective role by preventing overexcitation and calcium-dependent excitotoxicity (Boue-Grabot et al. 2003).

Concurrent activation of $P2X_2$ ATP-gated channels and $5\text{-}HT_3$ serotonin-gated channels leads to functional interaction and nonadditive currents in mammalian myenteric neurons as well as in *Xenopus* oocytes or transfected HEK293 cell heterologous systems. These two cation channels were also shown to coimmunoprecipitate constitutively and to be associated in clusters (Boue-Grabot et al. 2003).

Epithelial sodium channels (ENaC) coexist with P2X receptors in the renal collecting duct. Coexpression studies in *Xenopus* oocytes showed that ENaC was downregulated by the activation of $P2X_2$, $P2X_{2/6}$, $P2X_4$, and $P2X_{4/6}$ receptors. Conversely, ENaC increased the plasma membrane expression of $P2X_2$, $P2X_{2/6}$, and $P2X_5$ receptors (Wildman *et al.* 2005).

Finally, activation of synaptically coupled neurons of the lateral hypothalamus elicits reliable and concurrent release of ATP with GABA (Jo & Role 2002). Nonadditivity of P2X and $GABA_a$-mediated currents has been observed in rat dorsal root ganglion neuron receptors, providing more evidence for nonindependence of activity between P2X and several members of the nicotinic receptor superfamily (Sokolova et al. 2001).

P2X receptors, notably $P2X_7$, form large pores that can mediate apoptotic cell death. These megapores are non-selective cation channels that are inwardly permeable to large cations such as ethidium bromide and $NMDG^+$. Two alternate views to explain this variability in selectivity and magnitude of responses have been proposed. The first holds that the megapore state is due to a change in the intrinsic channel gating properties, whereas the other invokes recruitment of accessory proteins as the mechanism underlying the dramatic changes in receptor characteristics. Pannexin1 appears to be the molecular substrate for this permeabilization pore and dye-uptake pathway recruited into the $P2X_7$ signaling complex in *Xenopus* oocytes and macrophages (Locovei *et al.* 2007, Pelegrin & Surprenant 2006). It remains to be demonstrated whether

pannexins or other hemichannels are required for megapore conductance in other P2X receptors.

1.6 Heterologous expression in *Xenopus* oocytes and HEK293 cells

Experiments by King and colleagues (2000) confirmed that there are difficulties associated with $P2X_6$ receptor expression in *Xenopus* oocytes and in all probability, in other cell systems. It is possible that *Xenopus* oocytes and most HEK293 cells fail to produce an essential protein necessary to insert $P2X_6$ subunits into the cell membrane. One plausible candidate for this protein is another P2X subunit, perhaps the $P2X_2$ or $P2X_4$ subunits. Functional heteromeric $P2X_{2/6}$ are indeed formed and inserted into the membrane of *Xenopus* oocytes (King et al. 2000).

2.0 Metals needed by living organisms

There are fourteen metals currently known to be essential for plants and animals. Four of these, sodium, potassium, magnesium and calcium, are present in large quantities and are known as bulk metals. Nine of the remaining ten are d-block elements: vanadium, chromium, molybdenum, manganese, iron, cobalt, nickel, copper, and zinc. Also of high significance is the non-metal selenium, which is in the same family of elements as oxygen (Alexander 2007). These ten species are present in small quantities and are known as the trace metals. The bulk metals form 1-2% of the human body weight, whereas the trace elements represent less than 0.01%. Concentrations of metals in cells are

strictly regulated at their respective optimum levels: too much or too little is often harmful and may even be lethal to the organism (Wilson *et al.* 2004).

Most of the trace metals are found as obligatory constituents of a variety of proteins and vitamins. More than 30% of all proteins in cells exploit one or more metals to perform their specific functions. Metal-binding proteins fold in a cellular environment where their cognate cofactors are present, either free in the cytoplasm or bound to delivery proteins. The bound metal causes energetic and structural effects on the folded protein, which in turn has consequences on function. Thus, metals generally stabilize the folded forms of metalloproteins (Wilson et al. 2004).

The amino acids that regularly act as metal ligands in proteins are thiolates of cysteines, imidazoles of histidines, and carboxylates of glutamic and aspartic acids (Maret 2006). Each metal favors different sets of protein ligands. Due to the unique chemical properties of each metal, different metals are apt for different types of biological functions, although there is overlap in some cases (Wilson et al. 2004). Several reports have shown that transition metals such as zinc and copper can modulate the ATP-evoked currents in P2X receptors.

2.1 Biological roles of the bulk metal, calcium

Calcium ions (Ca^{2+}) impact nearly every aspect of cellular life (Clapham 2007). Calcium is the most abundant mineral in the human body where it plays a critical role in determining the strength and hardness of teeth and bone. More than 99% of the total body calcium is stored in the bones and teeth where it

functions to support their structure. The remaining 1% is found throughout the body in blood, muscle, and the extracellular fluid (Shils 1999).

Non-structural Ca^{2+} localized outside of the hard tissues has multiple dynamic roles in cellular processes involving both rapid and sustained cellular events. Calcium is a critical regulating factor in a broad range of functions including cellular homeostasis (Wolfe & Pearce 2006), membrane excitability (Smith *et al.* 2003), cell cycle control (Munaron *et al.* 2004), cytoskeletal dynamics (Zheng & Poo 2007), and gene expression (Carrasco *et al.* 2004). Calcium is needed for contractility of smooth, skeletal, and cardiac muscle (Isojima & Bozler 1963, Frank 1964, Nayler 1966), blood vessel tone (Akata 2007), regulated exocytosis (Penner & Neher 1988), and sending messages throughout the nervous system.

A constant level of Ca^{2+} is maintained in body fluid and tissues so that these vital body processes function efficiently (Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. 1997). When Ca^{2+} intake is low or Ca^{2+} is poorly absorbed, bone breakdown occurs because the body must use the Ca^{2+} stored in bones to maintain normal biological functions such as nerve and muscle function. Disease processes associated with calcium deficiency, or hypocalcemia, include: hypertension, cancer, kidney stones, obesity and related adverse health outcomes (Shils 1999).

Ca^{2+} movements across the cell membrane and between various intracellular compartments play a major role in the regulation of neuronal

excitability and neurotransmitter release (Smith et al. 2003). Activation of P2XRs produces fast excitatory responses which can support membrane depolarization (Bertrand & Bornstein 2002) and influx of extracellular calcium in sensory and autonomic neurons (Surprenant 1996) and other cell types expressing P2XRs (Egan & Khakh 2004). For example, in cultured rat myenteric neurons, ATP mainly activates ionotropic $P2X_2$ receptors, resulting in an increase in intracellular calcium dependent on external calcium rather than internal stores (Ohta *et al.* 2005). P2X receptor activation causes the accumulation of calcium ions in the cytoplasm, which is responsible for activating numerous signaling molecules (Erb *et al.* 2006). Thus, P2X receptors have the potential to modulate numerous cellular events that are regulated by transient or sustained changes in intracellular Ca^{2+} in cells expressing these ATP-gated channels.

2.2 Biological roles of the trace metal, zinc

The essential metal zinc (Zn^{2+}) is the second most abundant trace metal in the body after iron, and is found in almost every cell. Zinc is physiologically important for all organisms because it is a catalytic and/ or structural cofactor for numerous zinc-dependent enzymes and proteins. Zinc supports a healthy immune system (Solomons 1998), helps maintain one's sense of taste and smell (Heyneman 1996), is needed for wound healing and for nucleic acid synthesis (Barceloux 1999). Zinc also supports normal growth and development during pregnancy, childhood, and adolescence (Fabris & Mocchegiani 1995, Simmer & Thompson 1985). Zinc is not only an important cofactor of numerous enzymes

and transcription factors, but it also acts as an intracellular mediator, similarly to calcium. As a result of efficient homeostatic control, zinc does not accumulate in excess (Zatta et al. 2003).

Zinc has particular biological roles in some specialized cells. Examples of biological roles for Zn^{2+} include the regulation of gene expression and cellular signal transmission (Zatta et al. 2003). Zinc is stored in pre-synaptic terminals and released upon nerve stimulation (Palmiter *et al.* 1996) where it acts as a paracrine signaling molecule and regulator in pancreatic cells and neurons by a mechanism of vesicle-mediated metal excretion and uptake (Chimienti et al. 2003).

Zinc has been shown to act as an endogenous modulator of excitatory and inhibitory ligand-gated ion channels, and some members of the voltage-gated-like ion channel superfamily (Mathie *et al.* 2006). Zinc can be stored with either glutamate or GABA, respectively the main excitatory and inhibitory transmitters in the brain (Beaulieu *et al.* 1992). Although Zn^{2+} affects most LGICs, to date putative zinc-binding sites have been identified in the subunits of NMDA, $GABA_a$ and $GABA_c$ receptors, GlyRα1, ASICs, P2XRs, and nAChRs as well as P2XRs.

GlyR activated currents are potentiated by zinc at low concentrations, and are inhibited by zinc at higher concentrations. Aspartate-80 has been implicated in the potentiating zinc binding site, because the D80A mutation abolished potentiation, while inhibition was unimpaired (Lynch et al. 1998). The E110A mutation reduced sensitivity to zinc inhibition by 16-fold, indicating it could be a

coordinating residue for zinc either directly or through a water molecule. Although zinc bound within proteins is normally coordinated by at least 3 side chains, coordination by only 2 residues may be sufficient to explain the relatively low binding affinity of the inhibitory zinc-binding site (Nevin et al. 2003).

A knock in study was successfully accomplished for the glycine receptor (GlyR) alpha1 subunit gene (Glra1), to test the *in vivo* relevance of Zn^{2+} neuromodulation by producing knock in mice carrying the mutation D80A in Glra1. Homozygous Glra1(D80A) mice developed a severe neuromotor phenotype, hyperekplexia (startle disease). In spinal neurons and brainstem slices, GlyR expression, synaptic localization, and basal glycinergic transmission were normal; however, potentiation of spontaneous glycinergic currents by Zn^{2+} was significantly impaired (Hirzel *et al.* 2006).

Several lines of evidence indicate that zinc and other divalent cations coexist with ATP or P2X purinoceptors in central and peripheral neuronal regions. For example, zinc is abundant in hippocampal nerve endings, and RNA from $P2X_4$ is highly expressed in this region. Zn^{2+} potentiates ATP-evoked responses in rat PC12 cells, and in some mammalian peripheral neurons, apparently by increasing the sensitivity to ATP, but not by increasing the maximal responses (Nakazawa & Ohno 1997). The $P2X_2$ receptor is modulated by zinc in a reversible fashion that is dependent on both the ATP and zinc concentrations and is voltage independent (Lustig et al. 1993, Wildman *et al.* 1998).

Extracellular zinc reaches concentrations in the high micromolar range during neuronal activity and thus may physiologically regulate ATP-P2X receptor

interactions in the hypothalamus (Vorobjev *et al.* 2003). The proximity of synaptic zinc to P2X receptors and the coincident timing of release with endogenous agonist ATP suggests that zinc could affect ATP-evoked responses *in vivo*.

In contrast to the redox-active metals iron and copper that participate in electron transfer reactions of photosynthesis and/ or respiration, redox-inactive zinc serves as a superacid center in several metalloenzymes, promoting hydrolysis or cleavage of a variety of chemical bonds. Representative proteins that use catalytic zinc ions are carboxypeptidases, carbonic anhydrase, and alcohol dehydrogenase. In addition, zinc ions play structural roles in zinc-finger (ZF) motifs and proteins such as superoxide dismutase. Most ZFs serve as DNA-binding domains in proteins that regulate gene expression. However, recent work has demonstrated that ZFs can also mediate protein-protein and protein-lipid interaction (Wilson et al. 2004).

The classic ZF motif is a peptide comprised of ~30 residues that is folded around a divalent zinc cation. Cysteine and/ or histidine residues provide the side chains that coordinate Zn^{2+}, most often in a tetrahedral geometry. Raman spectroscopy experiments have shown that when both histidine and cysteine are present, cysteine residues bind to the Zn^{2+} prior to coordination of the histidines. Conserved hydrophobic residues as well as the spacing between the zinc ligands further contribute to the ZF's metal binding characteristics. The zinc binds tightly; the dissociation constant for a typical ZF-Zn^{2+} complex is ~10 pM. Nuclear magnetic resonance and circular dichroism experiments have demonstrated that

modification or elimination of the zinc ligands, or the conserved hydrophobic residues, in ZF peptides strongly affect metal affinity (Wilson et al. 2004). None of the known consensus sequences for ZF or metalloenzymes are present in the P2X receptors (Ennion et al. 2001).

2.3 Pathologies of Zn^{2+} dysregulation

Excess zinc can be cytotoxic, and zinc transporters as well as metallothioneins serve as zinc detoxification systems (Chimienti et al. 2003). There has as yet been no evidence that zinc is directly involved in the etiology of neurodegenerative diseases in humans. However, the concentration of free zinc and copper either in the synaptic cleft or neuronal cytoplasm may contribute to the etiology of certain disease states such as Alzheimer's disease (AD) and epilepsy. Hence, zinc appears to be a contributing factor in the progression of senile plaque formation in the brains of patients with AD (Mathie et al. 2006), and epileptic brain damage caused by neuronal injury induced by the excessive release of endogenous Zn^{2+} at central glutamatergic synapses (Koh & Choi 1994).

The amyloid β (Aβ) peptide is the major constituent of senile plaques. Deposition in the brain of insoluble aggregates of Aβ causes neurodegeneration. Several studies have shown that Cu^{2+} and Zn^{2+} promote aggregation of Aβ *in vitro*. *In vivo,* high concentrations of Cu^{2+} (0.4 mM) and Zn^{2+} (1 mM) have been reported in aggregates isolated from diseased brains. Zn^{2+} ions induce Aβ aggregation at pH >6, whereas Cu^{2+} is only effective between pH 6 and 7.

Raman spectroscopy and NMR have demonstrated that three histidines situated in the N-terminal hydrophilic region constitute the primary metal-binding sites. Upon Zn^{2+} binding to one histidine, peptides aggregate through intermolecular His-Zn^{2+}-His bridges. The same mechanism is proposed for Cu^{2+} induced Aβ aggregation (Wilson et al. 2004).

Conformational changes of metallopeptides play an important role in a range of human diseases. Further understanding of metallopeptide and metalloprotein folding may also aid in curing of diseases related to metal metabolism, such as Menkes syndrome and Wilson's disease, which both involve erroneous cofactor-protein interactions (Wilson et al. 2004).

2.4 Residues necessary for Zn^{2+} and pH modulation of $rP2X_2$ receptors

Previous reports have shown that extracellular pH and divalent zinc are two endogenous modulators that differentially affect the seven P2X receptor subtypes that have been cloned from rat (Stoop *et al.* 1997, Wildman *et al.* 1998). For example, extracellular zinc (<100 μM) potentiates the responses of homomeric $P2X_2$, $P2X_3$, $P2X_4$, and heteromeric $P2X_{2/6}$, and $P2X_{4/6}$ receptors. In contrast, the responses of $P2X_1$ (Wildman *et al.* 1999b) and $P2X_7$ (Virginio *et al.* 1997) are inhibited in the same range of zinc concentration. Since P2X receptors on sensory neurons include $P2X_2$ subunits, the attendant acidosis and ATP-release associated with tissue injury may play a role in sensitizing sensory nerve fibers (Wildman *et al.* 1997). Proton and zinc modulation have both been suggested to occur by allosteric regulation of the P2X receptors rather than by an

effect on ATP, because changes in the species of ATP present do not correlate with changes in receptor modulation properties (Clyne et al. 2002a).

Histidine residues are prominent in the zinc-binding domains of many structural proteins, transcription factors, and enzymes. Also, histidines are essential for the zinc sensitivity of GABA, NMDA, and Glycine receptors. Our lab showed that mutating histidine-319 of $rP2X_2$ greatly reduced pH potentiation with no effect on zinc modulation (Clyne et al. 2002a). In contrast, mutating either one of two histidines (H120 or H213) to an alanine nearly eliminated zinc potentiation without altering pH modulation. Mutations at H120 and H213 do not dramatically alter the response to ATP, suggesting that these mutations do not dramatically disrupt receptor gating. Further, it was shown that zinc potentiation of $P2X_2$ responses to 10 μM ATP attenuates at zinc concentrations greater than 100 μM. Zinc inhibition was maintained in H120A and H213A suggesting that there are independent, low and high-affinity sites that mediate inhibition and potentiation, respectively.

In most zinc-binding proteins, zinc complexes with either three or four residues (Maret 2006). Loss of one or more of the zinc-coordinating side chains likely reduces the affinity of the binding site. Thus, with respect to potentiation, zinc is probably coordinated by one or two residues in addition to H120 and H213.

It has been shown by Nagaya et al. (2005) that these coordinating histidines originate from adjacent $P2X_2$ subunits, instead of from the same subunit. Assuming the intersubunit binding site is tetrahedral, the coordinating residues

might consist of two H120 and two H213 residues, or consist of H120 and H213 residues from different subunits, and one or two as yet unidentified residues contributed from one subunit or adjacent subunits. The first possibility is unlikely because that would predict that only one zinc binding site for potentiation is present for each trimer of subunits. Concatemeric constructs have been used to show that there are up to three intersubunit sites for the high-affinity coordination of zinc, and that sequential removal of each one results in progressive decreases in potentiation to zinc (Nagaya *et al.* 2005).

In studies parallel to those of Clyne and colleagues (2002a), complete metal concentration curves were used by Lorca and others (2005), to investigate the role of extracellular histidines in metal modulation of $rP2X_2$. To assess the role of residues in zinc modulation, assays were performed to compare fold potentiation vs. ascending concentrations of zinc while holding ATP concentration at the EC_{10}. The results of their screen confirmed the original observations of Clyne et al. (2002a), that H120 and H213 are required for the modulator action of zinc at the high-affinity metal coordination site. However, unlike the results reported in the original histidine scan by Clyne et al. (2002a), Lorca and colleagues claim that residues H192 and H245 may also participate in the modulator action of zinc. While WT exhibited 6-7 fold potentiation under the conditions used, H192A and H245A exhibited potentiation of approximately two-fold. As not all mutants resulted in an equal phenotype for zinc or other trace metals, they proposed that these histidines form part of a metal-binding pocket, where each metal coordinates differentially and with varying affinity to these and/or to additional

amino acid residues. Because of these phenotypic differences, the authors propose a model where Zn^{2+} is coordinated in a complex involving H120, H213, H245, and further stabilized in an auxiliary role by the more distant H192.

Lorca and colleagues used 10 μM ATP, which is the EC_{10} value they report for the WT receptor expressed in *Xenopus laevis* oocytes. For the H245A mutant, they used 1 μM ATP because the ATP concentration-response curve they obtained in characterization of this mutant was left-shifted compared to WT. Our lab has not been able to replicate the report that the H245A mutant is left-shifted. Our data show that both H192A and H245A are slightly right-shifted relative to WT. Further, our lab has not been able to reproduce results that support the claim that H245 and H192 are involved in Zn^{2+}-potentiation.

3.0 Site-directed mutagenesis studies of P2X receptors

There are predictions that one can make regarding the functional roles of conserved and unconserved residues. On the one hand conservation of a residue at a given position in a family of related subunits likely indicates that the residue has a structural or functional role. On the other hand, properties that vary among the family members such as sensitivity to protons and Zn^{2+} are likely due to unconserved amino acids.

Site-directed mutagenesis has been used to identify residues of general importance to channel function. Briefly, phosphorylatable intracellular residues have been shown to regulate channel gating and kinetics, transmembrane residues have been shown to affect ionic permeation; and extracellular residues

have been shown to be important for ligand binding, glycosylation and trafficking, gating, desensitization and allosteric modulation (Evans 2008).

3.1 Charged extracellular residues

There are eleven aspartate (D) and glutamate (E) residues in $hP2X_1$ that are conserved among the seven family members of hP2XRs. Ennion et al. (2001) examined the role of the conserved negatively charged D and E residues within the ECD of human $P2X_1$ by alanine-scanning mutagenesis. Of the eleven residues tested for effects on ATP potency and cell surface localization, no major shifts in EC_{50} values were observed. Their results showed that individual conserved negatively charged amino acids are not essential for ATP recognition by $hP2X_1$, and coordinated binding of the positive charge on Mg^{2+} complexed ATP by negatively charged amino acids is not required. The most striking change observed in $P2X_1$ receptor phenotype was a pronounced (~90%) decrease in current amplitude for the D86A and D264A mutants. Possible explanations for these results are 1) altered gating capability, or 2) decreased cell surface expression of the mutant channels. Evidence supporting the latter hypothesis was obtained in biotinylation experiments, verifying that decreased receptor expression accounts for the lack of functional receptors in cells transfected with mutants E85A and D261A. Conservative mutation of charge and shape of amino acid side chain at these positions is able to compensate for an aspartate residue at positions 86 and 264 (Ennion et al. 2001).

Little or no change in ATP potency was seen at individual point mutations to replace negatively charged glutamate or aspartate residues at $P2X_1$ receptors; a similar pattern was seen on a $P2X_2$ receptor background with the exception of an ~100-fold decrease in ATP potency at the D259A mutant and no responses were detected for E85A and D261A mutant $P2X_2$ receptors. Interaction of conserved and non-conserved amino acids may play a role in regulation or fine-tuning of channel properties (Roberts et al. 2006).

3.2 Uncharged extracellular residues

Uncharged polar amino acids can also contribute to ligand action at receptors either by hydrogen bonding with the agonist or stabilizing structural changes. Non-polar aromatic amino acids have also been shown to be involved in ATP binding at a range of proteins. Extracellular $P2X_1$ receptor residues F185 T186 and N290 F291 R292 could be involved in coordinating the binding of the adenine ring of ATP (Roberts et al. 2006). Tyrosine residues are candidates for zinc coordination because of the known ability of the Π faces of aromatic rings to bind positive charges (Lynch et al. 1998).

Studies on the inhibitory zinc binding site of the GlyRα1 subunit have shown that in addition to histidines 107 and 109, threonine-112 appears to have a significant structural role in the zinc binding site but may have an additional direct role in zinc coordination.

3.3 Non-polar extracellular residues

Conserved proline and glycine residues are also of interest, as these may give some structural restraint to the receptor through the introduction of kinks/tight turns in the protein or introduction of flexibility, respectively. Proline imino amino acids can introduce particular structural features to proteins. Generally, peptide bonds between two amino acids in proteins are planar and trans. However, proline residues can form cis peptide bonds. Cis/ trans isomerization can therefore lead to reversible conformation changes and prolines can act as hinges in transmembrane α-helices. The lack of a hydrogen bond donor makes proline a classical breaker of α-helical structure. Proline can also promote folding and is frequently associated with β-turns. Thus, proline residues in the extracellular loop may be essential for structural integrity or involved in ATP action or allosteric modulation. Early reports suggested that conserved proline and glycine residues in the extracellular loop do not appear to play an essential role in ATP action at the $P2X_1$ receptor (Roberts et al. 2006).

The extracellular domain of $hP2X_2$ receptors contains four prolines that are totally conserved across the P2X receptor family (P93, P166, P228, and P272) and three less conserved residues (P196, P174, P225) and P330 ($hP2X_1$) conserved in all rat and human isoforms except for the $P2X_7$ and $hP2X_5$ receptor. Amino acid substitution at P272 indicated that this residue contributes to ATP action. Replacement with either alanine, aspartate, or lysine abolished channel function. In contrast, isoleucine substitution at P272 had essentially no effect whereas replacement with glycine or phenylalanine increased ATP potency. This

region of the receptor is very sensitive to amino acid substitution, indicating that it may play an important structural role in channel function. The effects of mutation at P272 were dependent on the amino acid substitution indicating that the conformation at this region of the receptor contributes to the ATP action at the $P2X_1$ receptor (Roberts & Evans 2005).

Extracellular glycine residues are also important for the function of some P2X receptors. When expressed in *Xenopus* oocytes, $P2X_1$ serine substitution at glycine 250 produced receptors that gave functional responses to ATP with no effect on ATP sensitivity but a reduction in peak amplitude; in contrast, functional responses were not recorded when glycine 250 was replaced by the amino acids alanine, cysteine, aspartate, phenylalanine, isoleucine, lysine, proline or asparagine. These results suggest that glycine 250 plays an important role in determining the function of P2X receptors.

In the cloned $P2X_2$ channel, responsiveness to ATP was lost when Gly247 was replaced by alanine. The sensitivity to ATP was reduced when Gly248 was replaced by alanine, and the responsiveness to ATP was lost when Gly248 was replaced by valine. The results suggest that the neighboring glycine residues (G247-G250) are essential for $P2X_2$ receptor function (Nakazawa & Ohno 1999).

3.4 Extracellular cysteine residues

Cysteines form essential components of metal ion binding sites in many proteins (e.g. zinc finger motifs and metalloenzymes). There are ten conserved cysteines in the extracellular loop of P2X receptors. The role of conserved

extracellular cysteine residues in the function of $P2X_2$ and the modulation of its responses to ATP by pH and zinc has been investigated previously (Clyne *et al.* 2002). Although mutation of many of these conserved cysteines resulted in changes in zinc modulation, it was inferred that the action was indirect. Rather than playing a role in binding zinc, the cysteine residues form disulfide bonds (DSBs), and breaking these bonds results in the structural changes that might account for the reduced effects of zinc. Disulfide bonds are known to play an important role in the formation and maintenance of ion channel structure.

Until recently, it was widely believed that all ten cysteine residues in the $rP2X_2$ ECD are disulfide bonded because the receptor fails to label in the presence of MTS reagents. Now there is evidence that a cysteine residue of the $P2X_4$ receptor is critical for the modulator role of zinc. This suggests that the cysteine residue in question does not form a disulfide bridge with other cysteines as has been suggested for other P2X subtypes (Coddou *et al.* 2007), while also asserting that a second extracellular cysteine remains unpaired.

To gain insight into the folding/ structural organization of the extracellular loop, Ennion and Evans sought to determine effects on ligand binding, surface expression, and ATP potency, and existence of free cysteines in the $P2X_1$ extracellular domain (Ennion & Evans 2002). Disruption of two bonds (C261 - C270 and C217 – C227) results in >90% decrease in peak current, but little to no effect on ATP potency and time-course of response. Notably, C261A and C270A mutations resulted in inefficient trafficking of $P2X_1$ receptors to the cell surface,

suggesting that these bonds confer important tertiary structure (Ennion & Evans 2002).

Although known to stabilize individual subunits, DSBs can also form between subunits, supporting oligomerization. Cysteine residues can also contribute to stabilization of quaternary structure when liganding zinc between different polypeptide chains termed, protein interface zinc sites (Messerschmidt 2001). However, multiple investigators have produced evidence that there are no intersubunit disulfide bonds in wild type P2X receptors.

4.0 The role of negatively charged extracellular residues in allosteric modulation by pH and zinc of the rat $P2X_2$ receptor

4.1 Specific Aim 1

We used site-directed alanine-scanning mutagenesis and two-electrode voltage clamp recording of mutant channels heterologously expressed in *Xenopus* oocytes to test if any of the 34 aspartate (D) and glutamate (E) residues of the $P2X_2$ extracellular domain were individually required for channel function and potentiation or inhibition of channel activity by zinc. The rationale for this approach was that given the residues that had already been tested, they were the most likely remaining candidates. Characterizing these mutants also provided the opportunity to test if they play a more general role in channel function. Negatively charged residues might coordinate Mg^{2+}ATP, or they might interact with the positively charged residues in the adenine moiety of ATP, and hence facilitate channel function.

4.2 Specific Aim 2

$P2X_2$ receptors can be modulated by protons (Wildman *et al.* 1997). Prior studies in our lab have shown that $P2X_2$ histidine 319 is required for wild type-like pH potentiation. None of the other extracellular histidine or cysteine residues are required for potentiation by protons, so some mechanism to account for the residual pH potentiation in the H319A mutants remains to be discovered.

Because acidic residues have been reported to bind protons in other ligand-gated ion channels such as cyclic nucleotide-gated channels and the VR1 capsaicin receptor, it is plausible that they contribute to pH potentiation of $P2X_2$. Therefore, I tested the hypothesis that negatively charged residues of the $P2X_2$ extracellular domain act to coordinate protons at sites for pH potentiation by studying the same 34 mutations created for the zinc study.

Figure 1.1

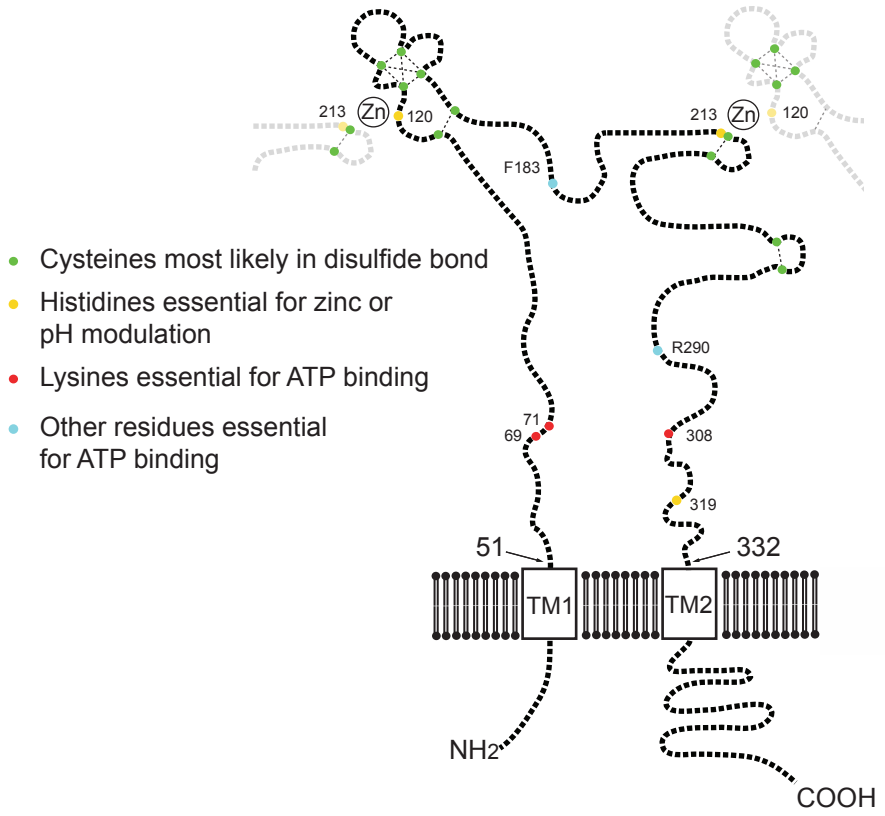


Figure 1.1 Schematic diagram of the membrane topology and key residues of rat $P2X_2$ receptors.

$P2X_2$ receptors function as homotrimers. The full length of one of the subunits (black) is shown with pieces of the two neighboring subunits (gray) that contribute to the intersubunit coordination of zinc at the potentiating site.

Chapter Two

Contribution of extracellular negatively charged residues to ATP action and zinc modulation of rat $P2X_2$ receptors

Abstract

Two histidines are known to be essential for zinc potentiation of rat $P2X_2$ receptors, but the chemistry of zinc coordination would suggest that other residues also participate in this zinc binding site. There is also a second lower affinity zinc binding site in $P2X_2$ receptors whose constituents are unknown. To assess whether extracellular acidic residues of the $P2X_2$ receptor contribute to zinc potentiation or inhibition, site-directed mutagenesis was used to produce alanine substitutions at each extracellular glutamate or aspartate. Two electrode voltage clamp recordings from *Xenopus* oocytes indicated that 7 of the 34 mutants (D82A, E85A, E91A, E115A, D136A, D209A, and D281A) were deficient in zinc potentiation and one mutant (E84A) was deficient in zinc inhibition. Additional tests on cysteine mutants at these 8 positions indicated that D136 is the only residue that is a strong candidate to be at the potentiating zinc binding site, and that E84 is unlikely to be at the inhibitory zinc binding site.

Introduction

The divalent cation zinc is found in the mammalian central nervous system either tightly bound to proteins or as a smaller pool of free, chelatable zinc (Cuajungco & Lees 1997, Zatta et al. 2003). Genetic deletion of the zinc transporter ZnT3 in mice results in a dramatic decrease in the amount of zinc that can be detected in synaptic terminals but produces no obvious change in the behavior of mice (Palmiter et al. 1996, Cole *et al.* 1999, Salazar *et al.* 2005). Despite this observation, there is considerable evidence in support of the idea that synaptically released zinc plays a role as a regulator of synaptic transmission in the brain (Li *et al.* 2003). The most direct *in vivo* evidence for zinc as a physiological modulator in the brain is the observation that mice that express a mutant form of the glycine receptor α1 subunit gene (*Glra1*) that is zinc insensitive, but fully responsive to glycine, show a dramatic startle-prone phenotype, hyperekplexia (Hirzel et al. 2006).

In addition to modulating the activity of glycine receptors *in vivo*, *in vitro* studies indicate that extracellular zinc can potentiate or inhibit the current responses of nicotinic acetylcholine receptors (Hsiao *et al.* 2001, Hsiao *et al.* 2006); NMDA receptors (Forsythe *et al.* 1988, Rassendren *et al.* 1990); and GABA type A receptors (Gibbs *et al.* 2000). It has also been shown that zinc potentiates ATP-activated currents in many cell types including rat sympathetic neurons (Cloues *et al.* 1993), rat nodose ganglion neurons (Wright & Li 1995), and acutely isolated rat hypothalamic neurons (Vorobjev et al. 2003). ATP released from cells can function as a synaptic neurotransmitter, an autocrine

signal, or a paracrine signal in a wide range of mammalian tissues (Ralevic & Burnstock 1998, Stojilkovic & Koshimizu 2001). This signaling is mediated by ionotropic P2X receptors (P2XRs) and G-protein-coupled P2Y receptors. Because ATP and zinc are both released from some neurons in an activity-dependent manner (Wright & Li 1995), it is plausible that neurotransmission at P2X utilizing synapses is modulated *in vivo* by zinc.

The P2X receptor family consists of seven genes ($P2X_1$-X_7) and the proteins they encode function as homomeric or heteromeric oligomers of three subunits (North 2002). Each P2X subunit has a short intracellular amino terminus, a first transmembrane domain, a large extracellular domain, a second transmembrane domain and a carboxy terminal domain of diverse length among subunits (Fig. 1). With respect to $P2X_2$ receptors, zinc exerts a concentration-dependent biphasic modulation that is thought to result from an increase in ATP affinity at low concentrations of zinc, and a voltage-independent inhibition that reduces the efficacy of channel opening at high concentrations of zinc (Wildman et al. 1998, Clyne et al. 2002a, Vorobjev et al. 2003). It has been shown previously by alanine-scanning mutagenesis that two extracellular histidines, H120 and H213, are required for zinc potentiation of $P2X_2$ (Clyne et al. 2002a). Furthermore, the coordination of zinc by $P2X_2$ has been shown to occur at the interface between adjacent subunits (Nagaya et al. 2005). Prior studies on zinc finger transcription factors and metalloenzymes have shown that zinc is typically coordinated in a tetrahedral configuration that requires nitrogen, oxygen, and sulfur ligands from the side chains of histidine, aspartate, glutamate, or cysteine

residues (Maret 2005). All of the extracellular histidines (Clyne et al. 2002a) and cysteines (Clyne et al. 2002) of $P2X_2$ have already been tested for their potential role in zinc potentiation. Therefore, in the current study we tested whether negatively charged residues of the $P2X_2$ extracellular domain are structural determinants of the excitatory and/ or inhibitory zinc binding sites.

Experimental Procedures:

Mutagenesis

Rat $P2X_2$ cDNA (encoding a 472-amino acid protein) in pcDNA1 was obtained from Dr. D. Julius (University of California, San Francisco, CA). The mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of mutant subunits were confirmed by DNA sequencing (University of Michigan DNA Sequencing Core). Each mutant is referred to by the original amino acid (one-letter code) followed by the residue number and the substituted amino acid (one-letter code).

Expression of $P2X_2$ Receptors

$P2X_2$ receptors were expressed in defolliculated stage V-VI *Xenopus laevis* oocytes. Oocytes were harvested using procedures approved by the University of Michigan Committee on the Use and Care of Vertebrate Animals and have been described in detail previously (Zhou & Hume 1998). RNAs encoding wild type and mutant $P2X_2$ receptors were synthesized using the mMessage mMachine T7 kit

(Ambion, Austin, TX). Each oocyte was injected with 50 nl of RNA at 100–150 ng/µl, concentrations that produce a maximal response in wild type $P2X_2$ (Clyne *et al.* 2003).

Electrophysiological Recordings

Two-electrode voltage clamp experiments were performed 2–5 days after RNA injection. All of the recordings were made at a holding potential of -50 mV. Recording electrodes were pulled from thin-walled borosilicate glass on a model P-87 Flaming Brown puller (Sutter Instrument Company, Novato, CA) and had resistances of 0.5–1 MΩ. The currents were recorded with a Turbo TEC-03 voltage clamp amplifier (npi electronic GmBH, Tamm, Germany). Data acquisition was performed using a Digidata 1322A interface controlled by pCLAMP 9 (Molecular Devices, Union City, CA).

Materials

[2-(trimethylammonium) ethyl] methanethiosulfonate bromide (MTSET) and sodium (2-sulfonatoethyl) methane thiosulfonate (MTSES) were obtained from Toronto Research Chemicals. All other chemicals were obtained from Sigma.

Solutions

The external recording solution contained (in mM): 90 NaCl, 1 KCl, 1.3 $MgCl_2$, and 10 HEPES, pH 7.5. Electrodes were filled with an internal solution of 3 M KCl. Disodium ATP was prepared as a 100 mM stock in double-distilled H_2O

and stored at -20 °C. For recording, ATP solutions were made by diluting the stock in external recording solution. ATP solutions with concentrations of 200 μM and above were supplemented with $MgCl_2$ to account for chelation of Mg^{2+} by ATP. The amount to add to each concentration of ATP was calculated using Bound and Determined (Brooks & Storey 1992) so that the free magnesium concentration was 1 mM. The ATP concentrations of recording solutions were verified by spectroscopic measurement at 259 nm (using an extinction coefficient of $15.4*10^3$ $M^{-1}cm^{-1}$). Zinc chloride was prepared as a 100 mM stock in double-distilled H_2O that was acidified with 0.01 M HCl to prevent precipitation. The pH of ATP solutions with and without zinc was adjusted to 7.5 prior to recording. For experiments assaying pH potentiation, the pH of ATP solutions to be tested was adjusted to 6.5 prior to recording. All ATP recording solutions were used within 48 hours.

The sulfhydryl-reactive reagents MTSET and MTSES were prepared as 1 M stocks in dimethyl sulfoxide and stored in 10-μl aliquots at -20 °C. For recording, the stocks were diluted in the standard HEPES-buffered external recording solution to a working concentration of 1 or 10 mM. For each incubation, 50 μl of the working solution was added to the recording chamber with flow through the chamber stopped. After incubation for 1-2 minutes, the oocytes were washed in external recording solution for one minute, and then assayed with the same protocol used before treatment. This was repeated until there was no further change in response.

Quantification of Zinc Modulation and Selection of Zinc and ATP Concentrations

The response of $P2X_2$ receptors to zinc is biphasic; at low levels, zinc causes potentiation, but at high levels it results in inhibition of current (Wildman et al. 1998). In a previous study, we used the non-potentiating mutants H120A and H213A to demonstrate that these two effects of zinc are separate processes. At a zinc concentration of 20 μM or below, the inhibition is negligible, whereas at all higher zinc concentrations both processes are occurring (Clyne et al. 2002a). We therefore used 20 μM zinc in all experiments testing only zinc potentiation, even though 50 and 100 μM zinc produce greater potentiation.

To compare the magnitude of zinc potentiation between groups of oocytes, it is essential that all of the oocytes be studied at similar points on the ATP concentration response relation, because as the concentration of ATP increases, potentiation decreases so that there is no zinc potentiation when a saturating concentration of ATP is present (Wildman et al. 1998). Furthermore, the EC_{50} for ATP of different oocytes expressing the same construct can vary significantly (Clyne et al. 2003). We dealt with these complications by testing each oocyte with both a low concentration of ATP which we expected would be close to the EC_{10} based on the average concentration response relation for each construct, and with 1,000 μM ATP, a maximal concentration for all constructs used in this study. Only data from oocytes for which it was verified that the low ATP concentration used was between the EC_5 and the EC_{15} (so that at least a 6-fold increase in

current was possible) were analyzed. All currents were measured after the responses had come to a steady state.

To test for zinc potentiation, oocytes were given low ATP followed by low ATP plus 20 μM zinc. We defined potentiation to 20 μM zinc as [(current in low ATP plus 20 μM zinc/ current in low ATP) -1]. Thus, a cell that showed no increase in current in response to zinc had zinc potentiation equal to 0, and a cell inhibited by 20 μM zinc had negative zinc potentiation.

We followed a similar approach to test for zinc inhibition phenotypes. Oocytes were given low ATP plus 20 μM zinc followed by low ATP plus 1,000 μM zinc. Zinc inhibition was defined as [1-(current in low ATP with 1000 μM zinc/ current in low ATP with 20 μM zinc)] %. Therefore, a construct unresponsive to high zinc would have 0% inhibition, a construct in which 1,000 μM zinc potentiated more than 20 μM zinc would have negative inhibition, and a construct in which high zinc completely inhibited the response would have an inhibition index of 100%.

Data Analysis

Data were analyzed using Clampfit and Microsoft Excel. Error bars shown in all figures are the standard error of the mean (SEM). The significance of differences between experimental conditions was tested using the student's *t* test function of Excel. When the same oocyte was tested before and after some treatment, the paired *t* test was used, while when groups of oocytes were compared with each other the unpaired *t* test was used. To minimize the

possibility that with so many mutants compared to the wild type apparent significance might arise based on chance alone, significance was taken to be $p<0.01$.

Concentration response relations for ATP were fit to the three parameter Hill equation using the nonlinear curve fitting program of Sigmaplot 9.0 (Systat Software Inc., San Jose, CA). For displaying average concentration response data, the points from each oocyte were normalized to between 0 and 100% based on the maximum value of the fitted curve. The scaled data were then averaged and plotted with error bars indicating the standard error of the mean. The lines fit to the data indicate the average parameters of the individual fits.

Results

When voltage clamped at -50 mV, oocytes expressing all 34 of the $P2X_2$ mutants tested (Fig. 2.1) responded to 100 μM ATP with readily detectable inward currents. Concentration response relations were assessed by giving an ascending series of ATP concentrations without intervening washes (Fig. 2.2A, B), and the data were fit to the Hill equation (Fig. 2.2C). All shifts in the EC_{50} were less than 10-fold (Table 1). The mutant receptor E85A showed the greatest rightward shift in EC_{50} (about 5-fold), and the mutant receptor D315A showed the greatest leftward shift in EC_{50} (about 6.5-fold, Fig. 2.2B). In addition to changes in EC_{50}, some mutants showed Hill coefficients that were somewhat larger or smaller than wild type. We did not explore the mechanisms that caused these changes because there was no apparent correlation between having an altered Hill

coefficient or EC_{50} and having a change in the ability to respond to zinc (Table 1). To determine the maximum response that each construct could produce when saturating levels of RNA were injected, we used 1,000 μM ATP, which is maximal for all mutants studied. All mutants had maximal currents of at least -3 μA (Table 1), indicating that several million functional receptors were successfully delivered to the cell surface.

Zinc potentiation was assayed (Fig. 2.3A) and quantified as described in Methods. Wild type $P2X_2$ exhibited mean zinc potentiation of about 7 while the zinc insensitive mutations previously studied (H120A and H213A) gave potentiation near 0. All 34 mutants tested in this study gave detectable zinc potentiation (Fig. 2.3B). None of the mutants showed a significant increase in zinc potentiation. Five mutants (D82A, E85A, E91A, D136A, and D209A), all with an average zinc potentiation below 3, potentiated significantly less ($p<0.01$) than wild type $P2X_2$. Two other mutants (E115A and D281A) had error bars that extended below 3 and just missed passing our test of significance (with $p<0.015$ but not <0.01). We focused the rest of our studies of zinc potentiation on these seven mutants.

In wild type $P2X_2$, the potentiation to zinc declines as zinc concentration rises above 100 μM. This is due to the action of a low affinity inhibitory site with an estimated IC_{50} of about 120 μM (Clyne et al. 2002a). As a screen for whether any of these mutations might have altered the inhibitory zinc binding site, we compared the amplitude of responses to the EC_{10} concentration of ATP alone, the EC_{10} concentration of ATP plus 20 μM zinc, the EC_{10} concentration of ATP plus

1,000 μM zinc, and 1,000 μM ATP (a saturating concentration) alone (Fig. 2.4A). The quantitative index of zinc inhibition (Fig. 2.4B) was calculated as defined in Methods. For wild type $P2X_2$, the current declined about 5-fold as zinc was increased from 20 μM to 1,000 μM, such that the amplitude of responses to low ATP alone and low ATP plus 1,000 μM zinc were similar. Of the 34 alanine mutants assayed, 25 exhibited no significant difference from the ~75% inhibition by 1,000 μM zinc measured in wild type $P2X_2$. Eight mutants, (E85A, E91A, E115A, D127A, D136A, E167A, D172A and D261) showed significantly enhanced zinc inhibition (Fig 2.4B; $p<0.01$), and one mutant (E84A) showed significantly decreased zinc inhibition. An increase in zinc inhibition is opposite to the result expected if these residues were part of the inhibitory zinc binding site. It should be noted that when the combination of low ATP and high zinc was applied or removed, currents of wild type $P2X_2$ receptors showed transients at both the onset and the offset (see Fig 2.4A). These transients are a manifestation of the slower on-kinetics and faster off-kinetics of the low affinity zinc inhibition as compared to the higher affinity zinc potentiation process (Clyne et al. 2002a). The on and off transients were much larger in the currents of the E84A mutant (Fig. 2.4A).

The decrease in either potentiation to low zinc (20 μM) or inhibition to high zinc (1,000 μM) might reflect an impeded ability to bind zinc at the modulatory sites, a diminution of communication between the occupied zinc binding site and the gating machinery, enhanced opposing modulation, or a general defect in channel gating. In the latter case, these candidate mutants might have been expected to also be deficient in potentiation to protons, an allosteric modulator

(Nakazawa *et al.* 1997b) that acts at a different site (Clyne et al. 2002a). In wild type receptors, application of an ATP concentration that produced an EC_{10} response at pH 7.5 produced a large increase in current when the pH was dropped to 6.5. Each of the mutants that were highly deficient in zinc potentiation or inhibition had pH potentiation that was indistinguishable from wild type $P2X_2$ (wild type 6.3±1.1, D82A 6.2±0.7, E84A 8.4±1.3, D85A 7.0±0.8, E91A 6.1±0.5, E115A 6.2±0.8, D136A 5.4±1.0, D209A 6.9±0.7, D281A 7.2±1.5), so none of these mutations produced a general defect in channel gating.

Cysteines support greater zinc potentiation than alanines at several candidate sites

To test the role of amino acid residues identified in the alanine screen, we generated individual cysteine substitutions at the candidate positions and determined their effects on zinc potentiation and inhibition. If a candidate residue participates in zinc binding, it would be expected that the response to zinc would be enhanced in the “C” version over the “A” version, because it is known that cysteine residues perform better than non-polar alanine residues as ligands for zinc (Maret 2005). Responses to ATP were recorded from all the cysteine substituted candidates. Cysteine substitution increased zinc potentiation over alanine substitution at positions 82, 136, and 209. There was no significant difference between zinc potentiation for A and C mutants at positions 84, 85, 91 and 281, and a cysteine at position 115 virtually eliminated zinc potentiation (Fig. 2.5A).

To test whether any of the cysteine substituted zinc modulation candidates were accessible to the positively charged, thiol-reactive compound MTSET (Javitch *et al.* 1994), oocytes expressing the cysteine substituted candidates were assayed for zinc potentiation or inhibition before and after treatment with MTSET. For a positive control to verify that the MTSET had not been degraded, we used H213C, which had previously been shown to be accessible to MTSET. For H213C, MTSET treatment produced a significant decrease in potentiation as expected. Of the cysteine substituted potentiation candidates, the zinc potentiation before and after MTSET treatment was significantly different for only D136C (Fig. 2.5B, C). The other cysteine substituted mutants showed potentiation ratios similar to levels observed before MTSET treatment (Fig. 2.5C). These results show that position 136 is accessible to MTSET, and therefore meets an essential criterion for participation in a site coordinating extracellular zinc.

Because oocytes expressing the E115A mutant showed modest zinc potentiation, the failure to see any zinc potentiation in oocytes expressing E115C was unexpected. One possible explanation for this result is a cysteine at this site is unable to engage in zinc binding because it has been trapped in an unnatural disulfide bond with one of the 10 endogenous cysteines of the extracellular domain of $P2X_2$. To test this possibility, we treated oocytes expressing E115C with the reducing agent dithiothreitol (DTT), and then retested for zinc potentiation. It had previously been demonstrated that DTT has no effect on zinc potentiation in wild type oocytes, but that receptors that carry cysteines replacing

both of the histidines required for zinc potentiation (H120C/H213C) form disulfide bonds that make them zinc resistant until after DTT treatment (Nagaya et al. 2005). Treatment of oocytes expressing E115C with DTT (10 mM for 5 minutes) caused no significant increase in potentiation to 20 μM zinc (before -0.6 ± 0.1, after -0.4 ± 0.1; N=3), while DTT caused a robust and highly significant increase in the positive control oocytes expressing the H120C/H213C double mutant (before 0.1 ± 0.1, after 2.4 ± 0.4; N=3; $p<0.001$). As the failure of E115C to allow zinc potentiation did not appear to be caused by trapping the cysteine in a disulfide bond, we considered the possibility that the absence of a negative charge at position 115 forced the receptor into a new conformation that either indirectly eliminated the zinc binding site, or prevented bound zinc from modifying channel gating. To test these possibilities, we treated oocytes expressing E115C with MTSES, a negatively charged cysteine reactive reagent (Fig. 2.6). In contrast to the slight zinc inhibition shown before treatment (which gave negative zinc potentiation of -0.4 ± 0.1), MTSES treated oocytes expressing E115C showed pronounced potentiation to 20 μM zinc (4.2 ± 0.5; N=6; $p<0.0001$). The zinc potentiation after MTSES treatment was a consequence of both a significant decrease in the response to low ATP alone (to 18% ± 3% of its previous amplitude) and an increase in the response to ATP plus 20 μM zinc (to 166% ± 28% of its previous amplitude) with relatively little change in the response to a high concentration (2 mM) of ATP (85% ± 3%; N=6 for each). The effect on E115C was specific for a negatively charged reagent, as treatment with positively charged MTSET had no effect on zinc potentiation on oocytes expressing E115C

(potentiation before = -0.5 ± 0.1; potentiation after = -0.2 ± 0.3). However, MTSET treatment did decrease the amplitude of currents in response to low ATP (to 19% ± 5%) and the currents in response to 2 mM ATP (to 85% ± 8%; N=5), which were very similar to the effects of MTSES treatment on these parameters. There was no significant effect of MTSES treatment on the zinc potentiation of oocytes expressing wild type $P2X_2$.

The currents from oocytes expressing the E84C mutant showed several unexpected features (Fig. 2.7A). Like wild type and the E84A mutant (Fig. 2.5A), the response of E84C to an EC_{10} concentration of ATP plus 20 μM zinc resulted in substantial zinc potentiation. However, a large component of this potentiation was only transient, while in wild type and E84A it was sustained. Furthermore, the steady state responses to low ATP plus 20 μM zinc or 1,000 μM zinc were quite similar in E84C, while in wild type and E84A the currents were much smaller in high zinc. MTSET treatment of individual oocytes expressing E84C resulted in little or no change in the responses to low ATP alone or to low ATP plus 20 μM zinc (Fig. 2.7A), but the steady state inhibition by 1,000 μM zinc was substantially enhanced (Fig. 2.7A, B). MTSET treatment of oocytes expressing E84C also attenuated the current evoked by 1,000 μM ATP alone (from 9,900 nA ± 1,100 to 6,800 nA ± 700, N=5).

Discussion

The goal of this study was to test whether any of the negatively charged residues in the extracellular domain of $P2X_2$ might directly contribute to zinc binding. As an initial test of this idea, we made alanine mutations at all 34 extracellular positions at which either a glutamate or an aspartate was present, characterized the concentration response relation to ATP of each mutant and then carried out assays for zinc potentiation and zinc inhibition.

General effects of mutation of negatively charged residues of $P2X_2$

When the sequences of the seven rat P2X receptors are compared, 7 of the 34 positions we tested have negatively charged residues only in $P2X_2$. Of the remaining positions tested, 16 have a negatively charged residue in two to four subunits, 3 have a negatively charged residue in five or six subunits, and 8 have a negatively charged residue in all seven subunits (Table 1). When alanine was substituted at each of the 23 negatively charged positions that are not highly conserved, there were only modest changes in the concentration response relations for ATP. Similarly, although conservation between multiple members of a gene family often indicates that a residue plays an essential role, we found only modest changes in the concentration response relations for ATP when the 11 most highly conserved residues were replaced with alanines. This agrees with previous work on all of the homologous residues in human $P2X_1$ (Ennion et al. 2001) and many of the same residues in rat $P2X_2$ (Jiang *et al.* 2000). Similar to the previous work, the mutants with the most distinctive phenotypes in our study

were E85A and D315A (which are equivalent to D89A and D316A in human $P2X_1$). In oocytes, both the $P2X_1$ D89A and the $P2X_2$ E85A mutants produce a modest rightward shift in ATP potency. A previous attempt to express $P2X_2$ E85A and D261A in HEK293 cells resulted in no detectable current (Jiang et al. 2000). Perhaps the ability to express very high levels of protein in oocytes accounted for our ability to observe currents from these mutants. In all three studies, the most left-shifted mutant of the 11 conserved extracellular acidic residues was produced by placing an alanine at the position equivalent to D315 of rat $P2X_2$. All changes at this position are not equivalent, as mutation to valine resulted in a 60-fold decrease in ATP potency (Nakazawa *et al.* 1998). In summary, neither the conserved nor the unconserved negative residues of the extracellular domain could be demonstrated to play an essential direct role in allowing $P2X_2$ receptors to respond to ATP. This stands in stark contrast to the dramatic effect on the EC_{50} caused by mutating some of the conserved positive residues of P2X receptors (Ennion *et al.* 2000, Jiang et al. 2000).

Are any negatively charged resides involved in binding zinc?

If a residue is required for binding zinc to the potentiating site, our expectation was that when the residue was replaced with an alanine the enhancement of ATP-evoked currents by 20 μM zinc would be substantially attenuated. Of the 34 positions tested in our alanine scan, seven (D82, E85, E91, E115, D136, D209, and D281) showed substantial attenuation of zinc potentiation, although all showed normal pH potentiation. Similarly, if a residue was required

for binding to the inhibitory site, then the attenuation of ATP-evoked currents in the presence of 1,000 μM zinc would be significantly lessened. Only one mutant, E84A, passed this preliminary test. Each of these 8 candidate residues was subjected to a secondary screen which involved producing mutants that carried a cysteine at each site under study and then testing the effect of MTSET on zinc modulation. The rationale for this approach was that if the candidate residue is part of the zinc binding site, modification with a bulky compound like MTSET should occlude the site, and weaken zinc binding.

The candidate for participation in the inhibitory zinc binding site, E84, can be ruled out as a participant in zinc binding, because MTSET treatment of oocytes expressing E84C resulted in greater zinc inhibition. If position 84 contributed directly to zinc binding at the inhibitory site, less zinc inhibition would be the expected result. It was also noted that zinc inhibition was significantly enhanced when alanine was placed in 8 positions. Because the zinc concentration response relations for potentiation and inhibition overlap (Clyne et al. 2002a), it was not a surprise that some mutations that depressed zinc potentiation (E85A, E91A, E115A, and D136A) showed enhanced inhibition, as no change to the inhibitory process would be needed to obtain this result. It is unclear how mutations D127A, E167A, D172A and D261A enhanced inhibition while producing no significant reduction of potentiation. It was not surprising that the E84A mutation, which had a decreased ability to produce inhibition to high zinc did not result in enhanced potentiation to 20 μM zinc, because very little inhibition is produced in wild type oocytes by 20 μM zinc (Clyne et al. 2002a).

Of the seven candidates for participation in the potentiating site, only D136C showed significant attenuation of zinc potentiation following MTSET treatment. However, even when the MTSET effect had reached saturation (as determined by a failure to change when the duration of MTSET exposure was doubled), substantial zinc potentiation remained after MTSET treatment. Like the modification of D136C we report here, MTSET modification of H120C and H213C, residues known to be in the zinc binding site, produces only attenuation and not elimination of zinc potentiation. One potential explanation is that although $P2X_2$ receptors are homotrimers with three zinc binding sites producing potentiation, it may not be possible to simultaneously modify all three zinc binding sites with MTSET. If this is so, residual zinc potentiation would be expected after MTSET treatment, because it was previously demonstrated that even a single zinc binding site is sufficient to produce substantial potentiation (Nagaya et al. 2005).

Of the other six candidates for involvement in zinc binding to the excitatory site, the cysteine mutant E115C did not potentiate to zinc. Additional experiments demonstrated that it is unlikely that E115 is in the potentiating zinc binding site, as MTSES treatment restored the ability to respond to zinc, while attaching such a large molecule to a cysteine in the zinc binding site would have been expected to occlude zinc binding. There are two possible explanations why the cysteine mutants of the other five candidates did not have altered responses to zinc following MTSET treatment. A simple one is that MTSET bound to the cysteine, but had no effect because the candidate residue was not near the zinc binding site. An alternative is that one or more of these residues are part of the excitatory

zinc binding site, but MTSET was not able to access the cysteine replacing the endogenous residue. This second explanation seems unlikely because we know that MTSET can access cysteines replacing the two histidines (H120 or H213) that are known to be part of the zinc binding site (Nagaya et al. 2005). If these six candidates are not direct participants in zinc binding, what else might have caused alanine mutations at these sites to attenuate zinc potentiation? A plausible idea is that these negative charges are essential for the normal execution of the conformational changes that follow zinc binding, but not for opening the channel in response to ATP alone, or to potentiation by acidic pH, both of which are relatively normal in these mutants.

To summarize these results, none of the extracellular acidic residues is required for $P2X_2$ function and the evidence presented here best supports D136 as the most likely of the 34 residues tested to directly bind zinc at the potentiation site, while none of the tested residues is likely to directly coordinate zinc at the inhibitory site. If D136 proves to be part of the excitatory zinc binding site, then one additional residue likely remains to be discovered, as most zinc sites are tetrahedral, and two histidines had previously been identified as participating in zinc binding. It is potentially of interest that the position equivalent to D136 is also negatively charged in $P2X_1$, $P2X_4$, and $P2X_7$. $P2X_4$ is able to potentiate in response to zinc (Wildman *et al.* 1999a, Xiong *et al.* 1999), while $P2X_1$ and $P2X_7$ are inhibited by zinc (Virginio et al. 1997, Wildman et al. 1999b). None of these subunits contain histidines at positions equivalent to H120 or H213 of $P2X_2$, so if the aspartate equivalent to D136 plays a role in zinc potentiation in $P2X_4$, one

would have to look elsewhere in the sequence of this subunit for the rest of the excitatory binding site. Similarly, if E84 proves to be involved in binding zinc at the inhibitory site of $P2X_2$, one would have to seek a different site to explain zinc inhibition of $P2X_1$ and $P2X_7$, as a negative residue is not found at the equivalent position in these subunits (nor in any subunit other than $P2X_2$).

Table 1. Characteristics of rat $P2X_2$ receptors and mutant receptors with an alanine substitution at one negatively charged extracellular residue. Values reported are the mean and the SEM for each construct tested. N indicates the number of oocytes tested to determine the concentration response relation. Data from additional oocytes were sometimes included in estimates of the maximal current. The seven mutants with significantly decreased zinc potentiation (five with $p<0.01$ plus two additional mutants that differed from wild type at $p<0.015$) are indicated with light gray shading, and the one mutant with significantly decreased zinc inhibition is indicated with dark gray shading. Asterisks indicate EC_{50}s or Hill coefficients that were significantly different from wild type $P2X_2$ ($p<0.01$, two tailed t test assuming equal variance).

Mutant	EC_{50} (μM)		Hill Coefficient		Peak inward current at -50 mV (μA)	N	Number of rat P2X receptors with a negative residue at this position
wild type	15 ± 1.3		1.9 ± 0.05		12.8 ± 1.6	20	
D57A	43 ± 9.9 *		1.3 ± 0.21 *		12.0 ± 1.4	6	4
E59A	10 ± 0.6		1.9 ± 0.08		9.8 ± 1.5	4	6
E63A	20 ± 6.9		1.0 ± 0.08 *		3.1 ± 0.3	9	2
E77A	22 ± 1.0		1.7 ± 0.03		6.3 ± 1.4	5	1
D78A	33 ± 5.5 *		1.6 ± 0.12		6.1 ± 1.0	5	2
D82A	55 ± 8.0 *		1.1 ± 0.07 *		9.6 ± 1.2	11	7
E84A	3 ± 0.2 *		2.1 ± 0.09		16.7 ± 1.4	5	1
E85A	66 ± 5.2 *		1.8 ± 0.09		7.3 ± 1.9	8	7
E91A	48 ± 6.6 *		1.4 ± 0.03 *		7.3 ± 0.4	5	1
E103A	7 ± 1.0 *		1.9 ± 0.09		12.5 ± 1.5	6	1
E115A	42 ± 2.7 *		1.5 ± 0.04		8.0 ± 0.7	5	7
D127A	12 ± 1.4		3.0 ± 0.55 *		25.5 ± 5.3	9	7
D128A	35 ± 2.9		1.3 ± 0.06 *		18.1 ± 1.4	6	3
D129A	20 ± 1.5		1.9 ± 0.07		23.0 ± 4.6	5	4
D136A	17 ± 3.6		2.0 ± 0.10		9.3 ± 1.5	6	4
D154A	9 ± 0.6		3.2 ± 0.64 *		16.3 ± 1.6	5	1
E159A	9 ± 0.3 *		4.1 ± 0.52 *		16.1 ± 1.7	8	7
E167A	7 ± 0.3 *		1.3 ± 0.16 *		16.0 ± 3.2	5	7
D168A	20 ± 2.5		1.5 ± 0.11		24.8 ± 2.8	5	4
D172A	11 ± 0.6		1.7 ± 0.04		17.1 ± 2.4	5	2
D209A	43 ± 4.5 *		1.5 ± 0.05 *		9.1 ± 0.5	6	2
D217A	10 ± 0.5		2.1 ± 0.09		18.6 ± 2.2	5	2
D219A	10 ± 0.3		1.8 ± 0.06		15.1 ± 1.0	5	2
D221A	5 ± 0.7 *		2.3 ± 0.10 *		16.4 ± 3.2	6	2
E234A	15 ± 1.0		1.8 ± 0.13		15.3 ± 0.8	6	1
E238A	12 ± 1.4		1.5 ± 0.04 *		20.8 ± 2.8	6	2
E242A	2 ± 0.2 *		1.9 ± 0.05		18.5 ± 2.4	5	5
D259A	19 ± 3.0		1.7 ± 0.17		11.0 ± 1.8	5	4
D261A	9 ± 0.5 *		2.0 ± 0.09		7.7 ± 0.8	8	7
E264A	8 ± 0.4 *		2.3 ± 0.20 *		17.9 ± 3.7	7	1
E266A	7 ± 0.4 *		1.7 ± 0.09		18.2 ± 2.1	5	2
D277A	22 ± 4.2		1.2 ± 0.10 *		11.6 ± 2.2	5	6
D281A	4 ± 0.3 *		1.9 ± 0.04		12.7 ± 2.0	6	2
D315A	2 ± 0.4 *		2.5 ± 0.45		26.6 ± 8.1	6	7

Figure legends

Figure 2.1. Residues of the extracellular domain of $P2X_2$ tested in this study.

$P2X_2$ receptors function as homotrimers. The full length of one of the subunits (black) is shown with pieces of the two neighboring subunits (gray) that contribute to the intersubunit coordination of zinc at the potentiating site. All of the negative residues in the extracellular region are numbered in accord with the rat $P2X_2$ sequence. Within the extracellular domain aspartate residues are shown in cyan, and glutamate residues are shown in red. D or E residues with black outlines are conserved in at least 6 of the 7 rat P2X receptors. The two extracellular histidines (H120 and H213) known to coordinate zinc between subunits at the site for potentiation are shown in yellow. The seven other extracellular histidines are not shown. With the exception of the histidines that bind zinc and the 10 conserved extracellular cysteines that are believed to form 5 disulfide bonds (dashed lines), the relative positions of residues is unknown, so residues quite far apart in this schematic diagram might be physically quite close.

Figure 2.2. Method for obtaining ATP concentration response relations.

A and B. Representative traces illustrating concentration response relationship of wild type $P2X_2$ receptors and the mutant D315A (the most left-shifted mutant studied). The numbers in the boxes above each trace indicate the ATP concentration in μM and the length of the boxes indicates the duration of each application. All traces were obtained from *Xenopus* oocytes voltage clamped at -50 mV. **C.** Concentration response relationships of oocytes

expressing wild type $P2X_2$ and D315A. The error bars represent the standard error of the mean (SEM).

Figure 2.3. Potentiation of wild type and mutant $P2X_2$ receptors by 20 μM zinc.

A. Representative traces from wild type $P2X_2$ and each mutant receptor that was deficient in zinc potentiation. Each construct was first tested with an amount of ATP that produced approximately 10% of the maximal response (EC_{10} ATP), then with EC_{10} ATP plus 20 μM zinc, and finally with 1,000 μM ATP. All traces were scaled so that the response to 1,000 μM ATP was of equal height. The current in response to this concentration of ATP produced a maximal response for all constructs. The dotted line across all the traces represents the predicted amplitude of the current if zinc potentiation was unchanged. The dashed line intersecting each trace indicates the actual amount of zinc potentiation. **B.** Potentiation in response to 20 μM zinc for all mutants studied. Zinc potentiation was calculated as defined in Methods. The dashed line indicates a potentiation ratio of 3.0. For each construct, data from 5-10 oocytes were averaged. Error bars represent the SEM. Black bars indicate mutants that showed significantly reduced zinc potentiation ($p<0.01$) compared to wild type $P2X_2$. Two additional mutants, E115A and D281A differed from wild type at $p<0.015$ and are indicated with diagonal lines.

Figure 2.4. Inhibition of wild type and mutant $P2X_2$ receptors by 1,000 μM zinc.

A. Examples of inhibition by high zinc in wild type $P2X_2$ and in mutants that showed less inhibition (E84A) and enhanced inhibition (D172A). Each trace is the response as the bathing solution was switched from external solution alone to the indicated solutions for the duration indicated by the bar. Five minutes of recovery was allowed between adjacent traces. Zinc inhibition was determined based on the amplitude of the current with EC_{10} ATP + 1000 μM zinc (dashed line) and the current with EC_{10} ATP + 20 μM zinc (solid line). **B.** Inhibition in response to 1,000 μM zinc for all mutants studied. Zinc inhibition was calculated as defined in Methods, with 100% representing complete inhibition, and 0% representing no inhibition. The dashed line indicates 50% inhibition. For each construct, data from 3-10 oocytes were averaged. Error bars represent the SEM. The black bar indicates the one mutant that showed significantly reduced zinc inhibition ($p<0.01$) compared to wild type $P2X_2$. Asterisks indicate mutants that showed significantly increased zinc inhibition ($p<0.01$) compared to wild type $P2X_2$.

Figure 2.5. Zinc potentiation in mutants with cysteines at candidate zinc binding site locations.

A. Potentiation to 20 μM zinc when an alanine or a cysteine was substituted at each indicated position. Each bar represents the average zinc potentiation (as defined in Methods) of 4-9 oocytes for each construct assayed. White bars represent alanine mutants, gray bars represent cysteine mutants, and

error bars represent SEM. The dashed line represents the mean potentiation for wild type. The data for the alanine mutants are the same as shown in Figure 3. Asterisks indicate when the A and C mutants at the same position were significantly different ($p<0.01$). **B.** Representative traces for wild type, H213C, and D136C expressing oocytes before and after MTSET treatment. Zinc potentiation was present in the untreated H213C mutant, but was greatly reduced following a 1 minute exposure to 1 mM MTSET. The dashed lines illustrate the expected amplitude of the current if MTSET did not interfere with zinc responses. **C.** Results from the MTSET experiments done on potentiation candidates shown as the mean potentiation before MTSET treatment (gray bars) and the mean potentiation after treatment (white bars). Only the potentiated responses of oocytes expressing D136C and the H213C positive controls were significantly ($p<0.01$) reduced by MTSET treatment (asterisks). Error bars represent SEM for 3-7 oocytes per construct.

Figure 2.6. Effect of 20 μM zinc on oocytes expressing E115C before and after treatment with disulfide reactive reagents.

Following the initial test for responses to ATP and zinc, each oocyte was incubated for 2 minutes in 10 mM of the indicated reagent and then washed for one minute (arrow) before being retested. **A.** MTSES restored zinc potentiation. **B.** MTSET did not restore zinc potentiation.

Figure 2.7. MTSET sensitivity of zinc inhibition in oocytes expressing E84C.

A. Representative traces for E84C before and after treatment with 1 mM MTSET. Shown are responses to low ATP, low ATP with 20 μM zinc, low ATP with 1,000 μM zinc, and 1,000 μM ATP. **B.** Histogram representing the average percent inhibition of responses of E84A (N = 4), and E84C before and after treatment with MTSET (N = 6). Error bars represent the SEM. The dashed line represents the average percent inhibition for wild type $P2X_2$ (76% ± 3%, N=6) stimulated with low ATP containing 1,000 μM zinc.

Figure 2.1

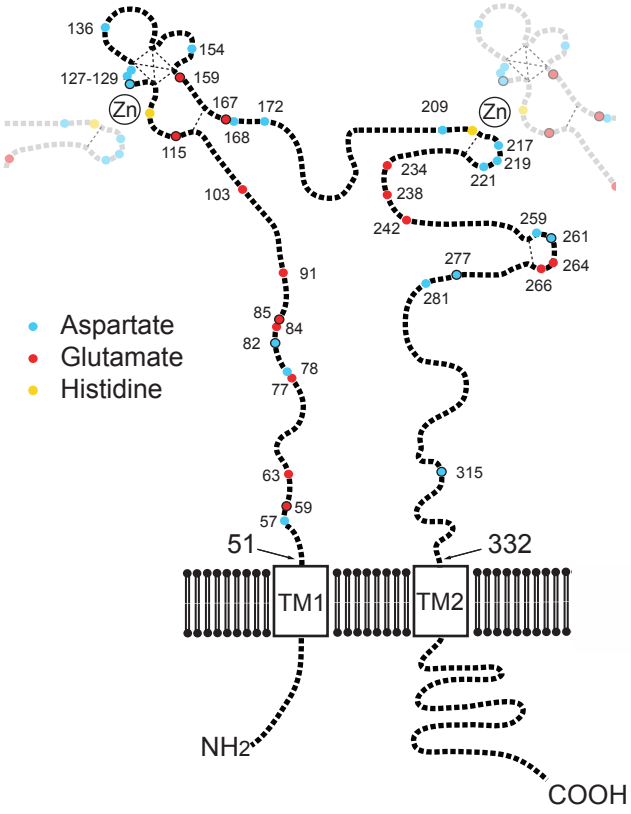

136
154
127-129
159
Zn
167
172
168
115
103
209
Zn
217
219
234
221
238
242
259
261
277
264
266
281
91
85
84
82
78
77
63
59
57
315
51
332
TM1
TM2
Aspartate
Glutamate
Histidine
NH2
COOH

Figure 2.2

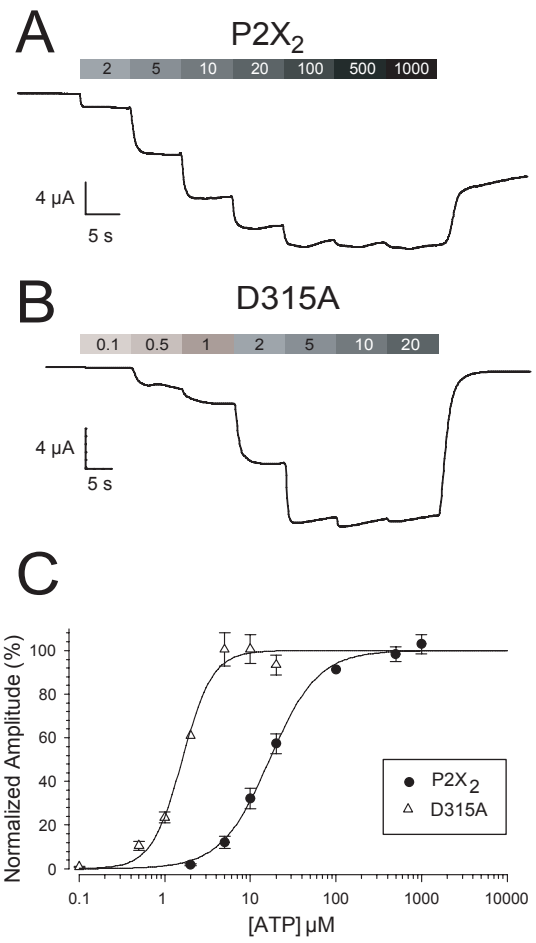
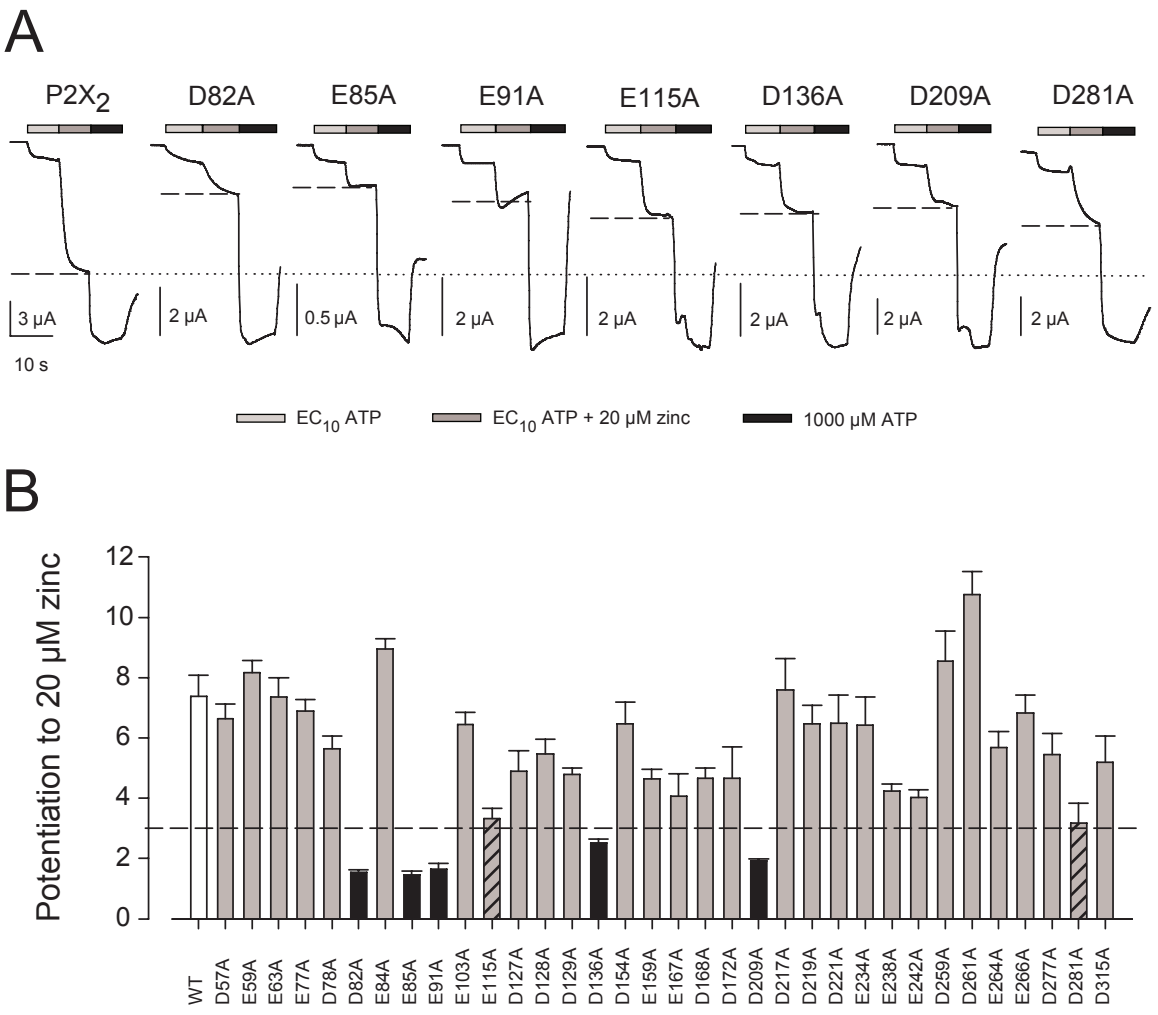

A
P2X2
2 5 10 20 100 500 1000
4 µA
5 s
B
D315A
0.1 0.5 1 2 5 10 20
4 µA
5 s
C
Normalized Amplitude (%)
0 20 40 60 80 100
0.1 1 10 100 1000 10000
[ATP] µM
P2X2
D315A

Figure 2.3


A
P2X2
D82A
E85A
E91A
E115A
D136A
D209A
D281A
3 µA
10 s
2 µA
0.5 µA
2 µA
2 µA
2 µA
2 µA
2 µA
EC10 ATP
EC10 ATP + 20 µM zinc
1000 µM ATP
B
Potentiation to 20 µM zinc
0
2
4
6
8
10
12
WT
D57A
E59A
E63A
E77A
D78A
D82A
E84A
E85A
E91A
E103A
E115A
D127A
D128A
D129A
D136A
D154A
E159A
E167A
D168A
D172A
D209A
D217A
D219A
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E266A
D277A
D281A
D315A

Figure 2.4

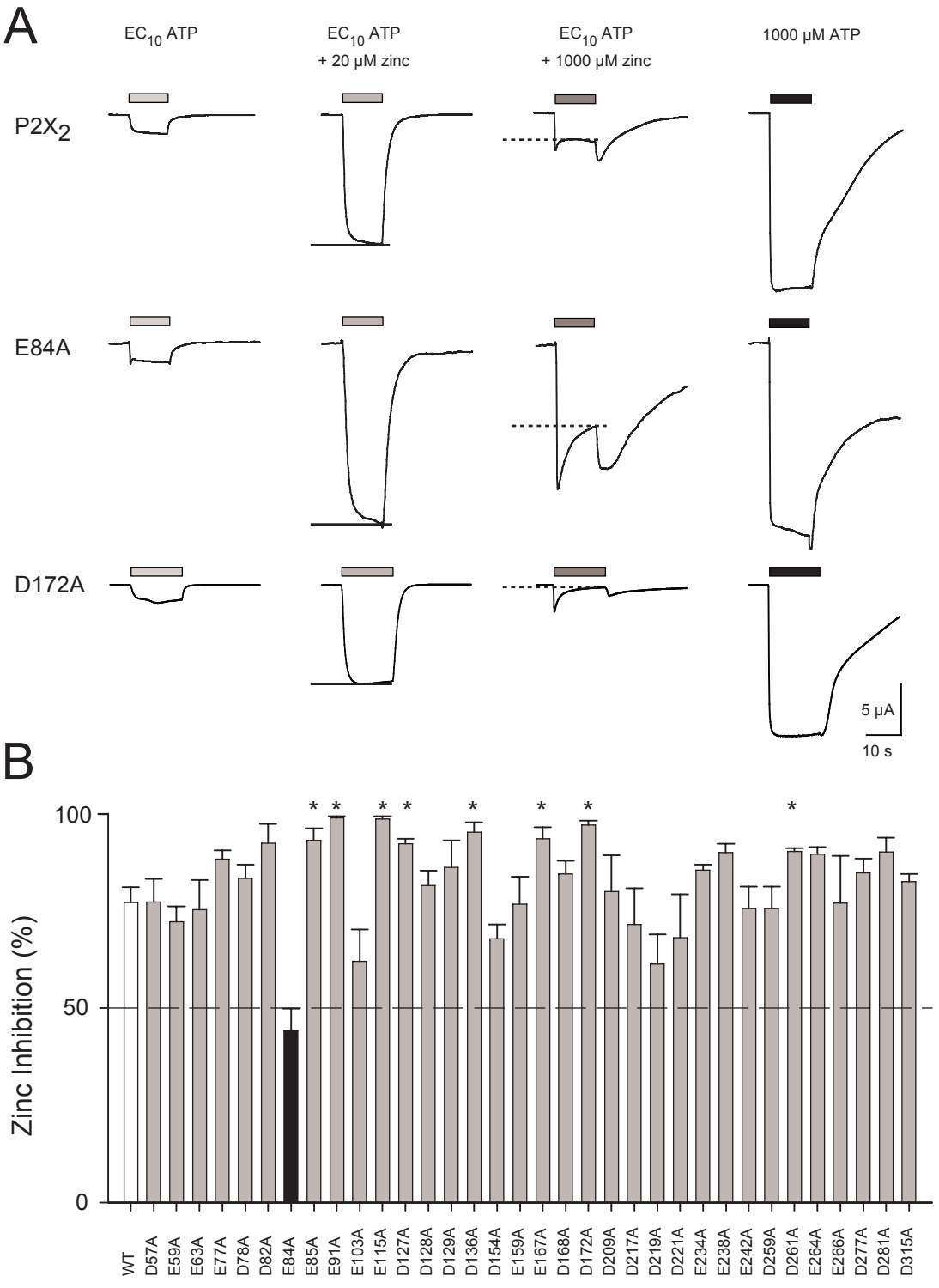

A
EC10 ATP
EC10 ATP
+ 20 μM zinc
EC10 ATP
+ 1000 μM zinc
1000 μM ATP
P2X2
E84A
D172A
5 μA
10 s
B
Zinc Inhibition (%)
100
50
0
WT
D57A
E59A
E63A
E77A
D78A
D82A
E84A
E85A
E91A
E103A
E115A
D127A
D128A
D129A
D136A
D154A
E159A
E167A
D168A
D172A
D209A
D217A
D219A
D221A
E234A
E238A
E242A
D259A
D261A
E264A
E266A
D277A
D281A
D315A

Figure 2.5

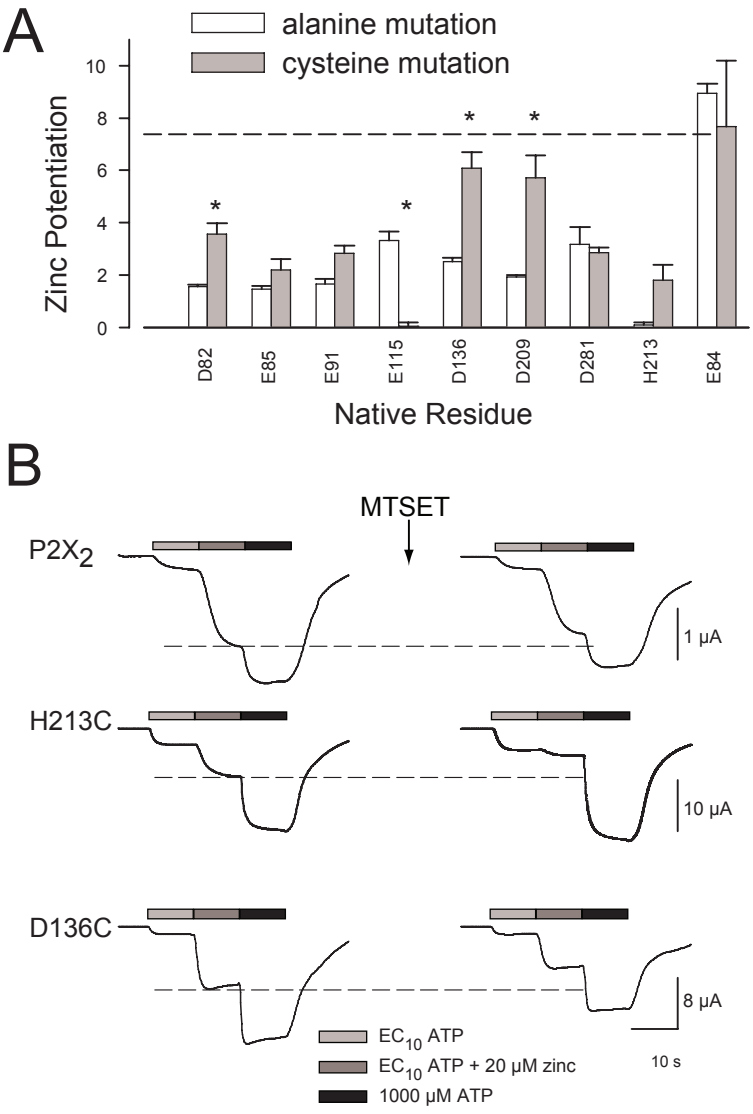

A
alanine mutation
cysteine mutation
Zinc Potentiation
0
2
4
6
8
10
*
*
*
*
D82
E85
E91
E115
D136
D209
D281
H213
E84
Native Residue
B
MTSET
P2X2
1 µA
H213C
10 µA
D136C
8 µA
EC10 ATP
EC10 ATP + 20 µM zinc
1000 µM ATP
10 s
C
Before MTSET
After MTSET
Zinc Potentiation
0
2
4
6
*
*
D82C
E85C
E91C
E115C
D136C
D209C
D281C
H213C

Figure 2.6

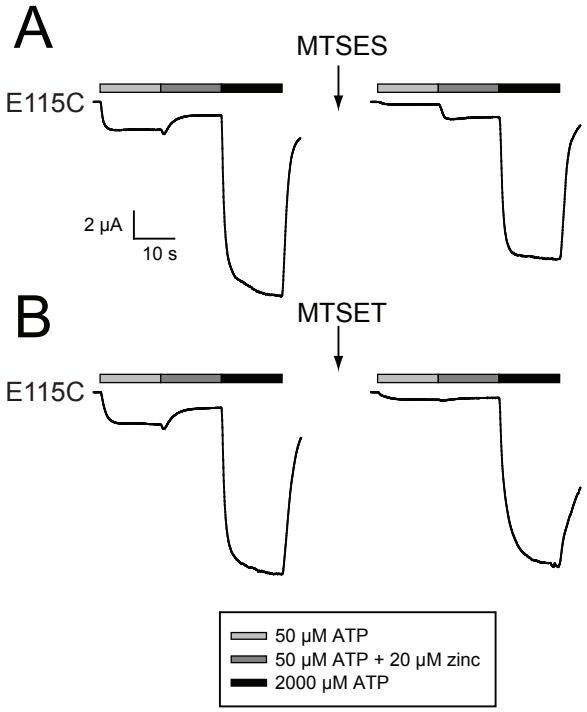
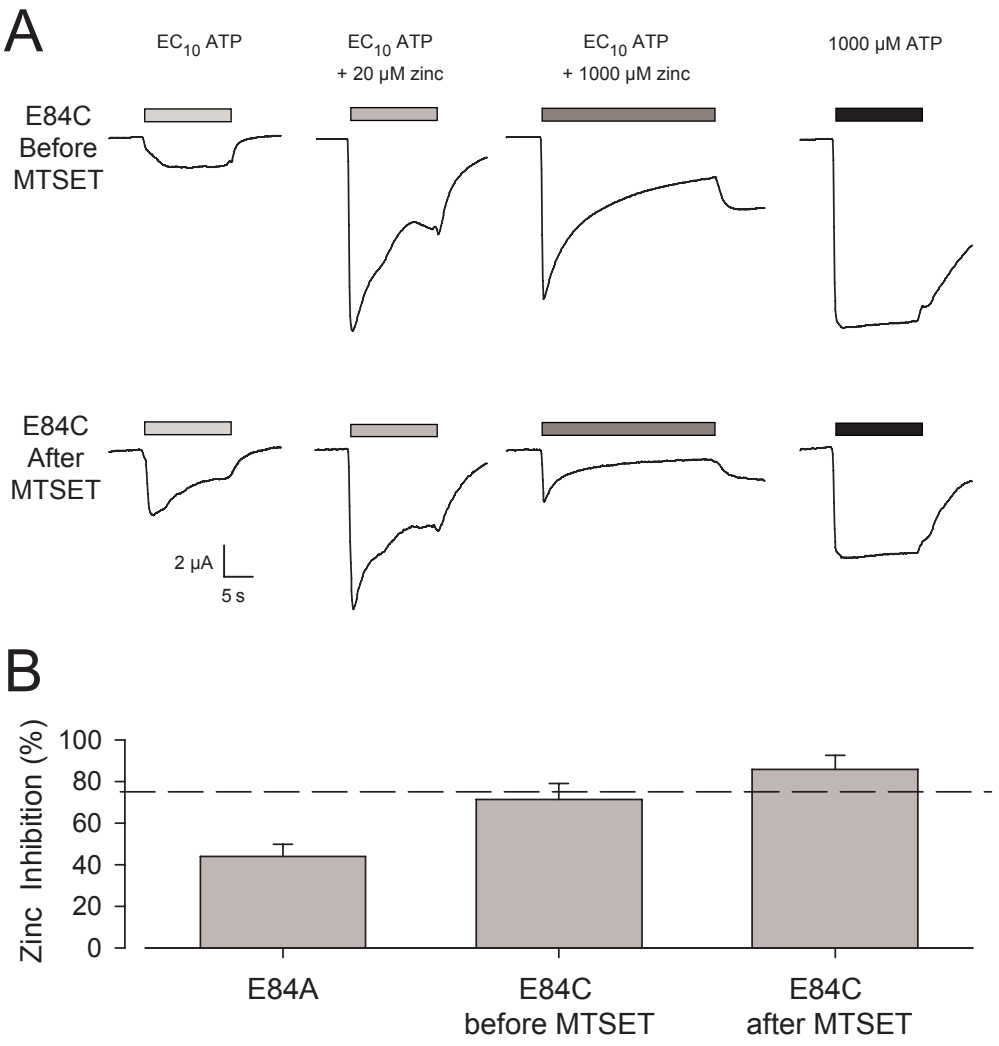

A
MTSES
E115C
2 µA
10 s
B
MTSET
E115C
50 µM ATP
50 µM ATP + 20 µM zinc
2000 µM ATP

Figure 2.7


A
EC10 ATP
EC10 ATP
+ 20 μM zinc
EC10 ATP
+ 1000 μM zinc
1000 μM ATP
E84C
Before
MTSET
E84C
After
MTSET
2 μA
5 s
B
Zinc Inhibition (%)
100
80
60
40
20
0
E84A
E84C
before MTSET
E84C
after MTSET

Chapter Three

Extracellular acidic residues as pH sensors at $rP2X_2$ receptors

Introduction:

Arthritis, infarction, tumor growth, and other forms of injury can cause levels of extracellular protons to increase from the normal physiological concentration (which produce a pH of ~7.6) to that typically associated with tissue acidosis (pH 7.0–6.0) (Reeh & Steen 1996). Local acidosis is primarily sensed as pain. Hydrogen ions are able to excite sensory afferents such as bullfrog dorsal root ganglion (DRG) neurons via the activation and/or modulation of inward cation-selective currents, including the acid-sensing ion channels (ASICs), the transient receptor potential vanilloid receptor 1 (TRPV1), and P2X receptors (Gerevich *et al.* 2007).

ATP is the endogenous ligand that activates P2X receptors. ATP is stored in neurosecretory vesicles, often together with other neurotransmitters and is released from neural cells and synaptic terminals via regulated exocytosis. ATP is also released from non-neuronal cells including astrocytes and astrocyte-like cell lines (Lazarowski 2006). In addition to vesicular release mechanisms, connexin hemichannels have been shown to mediate regulated release of ATP.

For example, hemichannels contribute to ATP release on mechanical stimulation in bovine corneal endothelial cells (Gomes *et al.* 2005). In vascular endothelium, vesicular exocytosis, volume-regulated anion channels, and connexin hemichannels have been reported as candidates for ATP release pathways (Oike *et al.* 2004). Data from physiological and pharmacological experiments suggests that ATP is released from taste cells via specific channels, likely to be connexin or pannexin hemichannels. Tastant-responsive taste cells release the neurotransmitter ATP via a non-exocytotic mechanism dependent upon the generation of an action potential (Romanov *et al.* 2007).

In peripheral tissues, large quantities of ATP may leave the intracellular space in response to tissue trauma, tumors, inflammation, migraine, or visceral distension. The resulting P2X receptor activation and the subsequent depolarization of the sensory cell membrane initiate action potentials that are perceived centrally as pain. Although sensory neurons express all known P2X subunits, the homomeric $P2X_3$ and the heteromeric $P2X_{2/3}$ receptors occur in these cells at the highest density (Gerevich *et al.* 2007).

Many receptors have allosteric binding sites that modulate the activity of the receptor, contributing an additional level of complexity in the cell signaling process (Hogg *et al.* 2005). Protons are capable of modulating the activity of a number of receptors and ion channels expressed by primary afferent nociceptors, including vanilloid receptors (Caterina *et al.* 1997), acid-sensitive channels of the degenerin family (Waldmann & Lazdunski 1998), and ATP gated channels (Stoop *et al.* 1997). Protons have been shown to modulate ion channels through

effects on affinity of ligand binding (Varnum *et al.* 1995, Gordon *et al.* 1996), efficacy of channel gating (Jordt & Jentsch 1997), ion permeation ratio (Root & MacKinnon 1994), or altering the rate of receptor desensitization (Gerevich et al. 2007).

$P2X_2$ receptors can be modulated by protons (Wildman et al. 1997), zinc (Cloues et al. 1993), copper (Xiong et al. 1999), neuroamines (Nakazawa & Ohno 1997), and some types of ion channels (Khakh *et al.* 2000, Wildman *et al.* 2005). The molecular basis of the sites of regulation by protons has been investigated in a number of mutagenesis studies on P2X receptor subtypes. $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$ have each been shown to have sensitivity to pH, with a non-conserved extracellular histidine residue serving as a proton sensor. Acidification inhibits $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ receptor currents by increasing the EC_{50} value for ATP without a concomitant change in the maximum ATP effect. The response of $P2X_2$ receptors to protons is biphasic: acidic pH supports potentiation, while alkaline pH inhibits current (King *et al.* 1997). Protons potentiate both recombinant and native $P2X_2$ receptor currents by reducing the EC_{50} value without altering the maximum effect of ATP (Acuna-Castillo *et al.* 2007, Gerevich et al. 2007). Prior studies have shown that histidine 319 of $P2X_2$ is necessary for wild type-like potentiation by low pH and that none of the other extracellular histidine (Clyne et al. 2002a) residues contributed to pH potentiation. It remains unclear if other extracellular residues contribute to $P2X_2$ potentiation by protons. The acidic residues glutamate and aspartate are electrostatically suitable to coordinate protons directly, but are titrated poorly at

physiological pH because of their low pKa's of ~4.39 and ~4.04, respectively (Tollinger *et al.* 2002). However, pKa-elevating carboxyl-carboxylate pairs can coordinate protons at more physiologically relevant pH (Morrill & MacKinnon 1999).

Glutamate residues have been shown to be important for acid sensing for the capsaicin receptor VR1. VR1 is homologous to members of the transient receptor potential family of ion channels first identified in the *Drosophila* phototransduction pathway (Caterina et al. 1997, Montell & Rubin 1989). Cells expressing wild-type VR1 receptors show especially dynamic modulation of heat-evoked currents between pH 8 and 6, a sensitivity range that matches the extent of local acidosis attained during most forms of tissue injury (Reeh & Steen 1996). Extracellular protons are believed to modulate the capsaicin receptor primarily by increasing the probability of channel opening (Tominaga *et al.* 1998). VR1 mutants bearing other amino acids at the E600 site, E600D or E600H, do not exhibit the same robust and dynamic increase in heat-evoked currents over this pH range, highlighting the physiological significance of having glutamate at this position. For E600Q or other mutants having non-titratable amino acids at this position, the magnitude of heat-evoked currents was largely independent of extracellular pH (Jordt et al. 2000). These investigators speculated that E600 formed a pair with an unidentified residue. Another example where carboxyl-carboxylate pairs serve as proton binding sites of ion channels are four glutamate residues that probably lie within the channel pore of cyclic nucleotide-gated (CNG) channels. These CNG glutamate residues are thought to mediate the

proton-dependent transitions between different conductance states with a pKa ~7.6 (Root & MacKinnon 1994, Wildman et al. 2005). Similar pairings of pore glutamates confer proton-dependent block in L-type calcium channels with an even higher pKa of ~8.5, nearly four pH units greater than the pKa of an individual glutamate residue (Chen *et al.* 1996, Chen & Tsien 1997).

The mechanism of proton sensing involving carboxyl-carboxylate pairs of acidic residues is best understood for Acid-sensing Ion Channels (ASICs). ASICs are a distinct family of extracellular proton-gated cation channels with some similarity in topology (Jasti *et al.* 2007) and distribution of expression (Masin et al. 2006) to P2XRs. ASIC2b, a splice variant of ASIC2a, is acid-insensitive. Five extracellular sites occupied by protonatable residues in ASIC2a but not in ASIC2b may be involved in activation of ASIC2a by protons (Smith et al. 2007). Mutation of ASIC2a Histidine 72 to an alanine abolishes the pH sensitivity of ASIC2a, however it is not the unique determinant of its pH sensitivity (Baron et al. 2001).

Evidence from a report describing a low-pH crystal structure of chicken ASIC1 at 1.9 Å resolution demonstrated that acidic residues of the extracellular domain formed at least one proton-bearing carboxyl-carboxylate pair. Asp 238–Asp 350 and Glu 239–Asp 346 of chicken ASIC1 define two pairs of unusually close (2.8 Å) carboxyl-carboxylate interactions between the side chains of aspartate or glutamate residues. Electrophysiological studies confirmed that the candidate aspartate residues are part of proton-sensing carboxyl-carboxylate pairs. Neutralization of Asp 346 and Asp 350 had profound effects on either pH_{50}

or apparent Hill coefficient, or both. These results confirmed identification of two key residues involved in proton sensing, yet they also suggest that the pH sensor is distributed over multiple residues (Jasti *et al.* 2007).

Because of this extensive body of work on other channel classes, we sought to test the hypothesis that negatively charged residues of the $P2X_2$ extracellular domain might act to coordinate protons at the allosteric site for pH potentiation.

Experimental Procedures:

All methods were as detailed in Chapter 2 with the following additions:

Solutions—For assaying pH potentiation, the pH of ATP solutions was adjusted to either 7.5 or 6.5 prior to recording. All ATP recording solutions were used within 48 hours.

Selection of ATP Concentrations and Quantification of pH Potentiation —

ATP-evoked steady-state responses were measured with two-electrode voltage clamp recording 48 hours after injection of *Xenopus* oocytes with 50 nl RNA (100 ng/ µl) encoding $P2X_2$ or mutants bearing single alanine substitutions at extracellular aspartate and glutamate residues. To compare the magnitude of pH potentiation between groups of oocytes, it is essential that all of the oocytes be studied at similar points on the ATP concentration-response relation, because as the concentration of ATP increases, potentiation decreases so that there is no pH potentiation when a saturating concentration of ATP is present (Wildman *et al.*

1998). Furthermore, the EC_{50} for ATP of different oocytes expressing the same construct can vary significantly (Clyne *et al.* 2003). We dealt with these complications by testing each oocyte first with a low concentration of ATP at pH 7.5, which we expected would be close to the EC_{10} based on the average concentration-response relation for each construct, and then with 1,000 μM ATP at pH 7.5, which was a maximal concentration for all constructs used in this study. We set a criterion of responses to low ATP at pH 7.5 between 8-12% of the maximal response evoked by 1000 μM ATP (pH 7.5), which was a refinement from our previous screen for zinc potentiation and inhibition phenotypes. We used a protocol that consisted of the following sequential applications without intervening washes: low ATP (pH 7.5), low ATP (pH 6.5), and 1000 μM ATP (pH 7.5).

Data Analysis—Data were analyzed using Clampfit and Microsoft Excel. The F test was used to determine if the variance of the data was equal or unequal ($p<0.01$), and the significance of differences between groups was then tested using the two-tailed, unpaired *t* test function of Excel, with significance taken to be $p<0.0014$ based on a Bonferroni correction for $p< 0.05$ and 35 subjects.

Results:

To test whether mutation of negatively charged extracellular residues altered the responses of rat $P2X_2$ receptors to a shift from the normal pH to a more acidic level, we used a three pulse protocol (Fig. 3.1A). We first applied a concentration of ATP at pH 7.5 that produced approximately 10% of the maximal response to ATP, then switched to the same concentration of ATP at pH 6.5, and finally switched to 1,000 μM ATP at pH 7.5, a concentration that produced maximal responses to ATP at both pH 7.5 and 6.5. Because potentiation is an allosteric effect, the fold potentiation diminishes as the ATP concentration rises. Therefore, data analysis was restricted to those cells for which the initial response was no less than 5% and no more than 15% of the final response to high ATP, and that the average for the population of cells was between 8 and 12% of maximum (Fig. 3.1B). We therefore refer to the first test pulse as low ATP.

When care was taken to study cells close to the EC_{10}, wild type $P2X_2$ receptors had approximately an 8 fold increase in current on being shifted from pH 7.5 to 6.5. To quantify pH potentiation (Fig. 3.1C), we calculated a pH potentiation index (PPI):

PPI = [current in low ATP (pH 6.5)/ current in low ATP (pH 7.5)]-1.

With this index, an absence of potentiation produces a ratio of 0 and the maximum possible potentiation from an EC_{10} concentration of ATP is 9.0. Thus, a PPI of 7 corresponds to ATP activation to 80% of maximal.

For wild type $rP2X_2$, a concentration of ATP eliciting ~10% of the maximal response at pH 7.5 exhibited a PPI of 6.77 ± 0.69 when the pH of the external solution was lowered to 6.5. For 33 of the 34 mutants tested, pH potentiation was not significantly different from wild type (Fig. 3.1C). However, the mutant E63A showed significantly reduced potentiation (2.28 ± 0.25) compared with wild type $rP2X_2$ ($p < 0.0014$, 2 tail t test; Fig. 3.1A, C).

Discussion:

Titration of free glutamate residues by protons usually occurs with a pKa ~4.45 (Marti 2005). However, carboxyl-carboxylate pairs can raise the pKa values above that of the respective single acidic residues of the binding site and into the normal physiological range (Morrill & MacKinnon 1999). In the present study, we tested whether any of the 34 extracellular acidic residues were necessary for wild type-like pH potentiation of the $rP2X_2$ receptor. These alanine substituted mutants were previously characterized for channel function in a companion study that focused on the role of the extracellular aspartate and glutamate residues in modulation of the channel by divalent zinc (Friday & Hume 2008). None of these mutations produced non-functional channels or significantly affected ATP potency or channel gating, but 7 candidates were identified that exhibited deficient zinc potentiation. All 7 of these zinc modulation candidates showed normal potentiation to acidic pH. In the current work we found 26 additional sites at which substituting an alanine for a glutamate or

aspartate also did not significantly alter pH potentiation, and found a single site, E63A, that is substantially deficient in pH potentiation.

As none of the extracellular histidines of $rP2X_2$ are required for pH potentiation (Clyne et al, 2002a) (although potentiation is greatly attenuated in H319A), it was therefore tempting to speculate that the local microenvironment elevates the pKa of E63 so that it can be titrated at pH 6.5. However, our results do not support the interpretation that the defect in pH potentiation observed for E63A was from disruption of a carboxyl-carboxylate pair mechanism for sensing protons. The prediction if this were the case was that negatively charged residues important for pH potentiation should be revealed not singly but in pairs, with mutations of either negatively charged residue of a proton-sensing carboxyl-carboxylate pair producing similar changes in pH potentiation. As none of the other 33 alanine substituted negatively charged extracellular positions exhibited potentiation defects at pH 6.5, the pH potentiation defect observed for E63A seems unlikely to represent disruption of a carboxyl-carboxylate pair. To resolve this definitively, one could test double mutants of E63A and the remaining 33 extracellular alanine-substituted acidic residues for exacerbation of the pH potentiation defect, or altogether abolition of potentiation by protons. It also might be of interest to pair E63A with H319A, the only other residue known to show a deficit in pH potentiation.

Figure Legend:

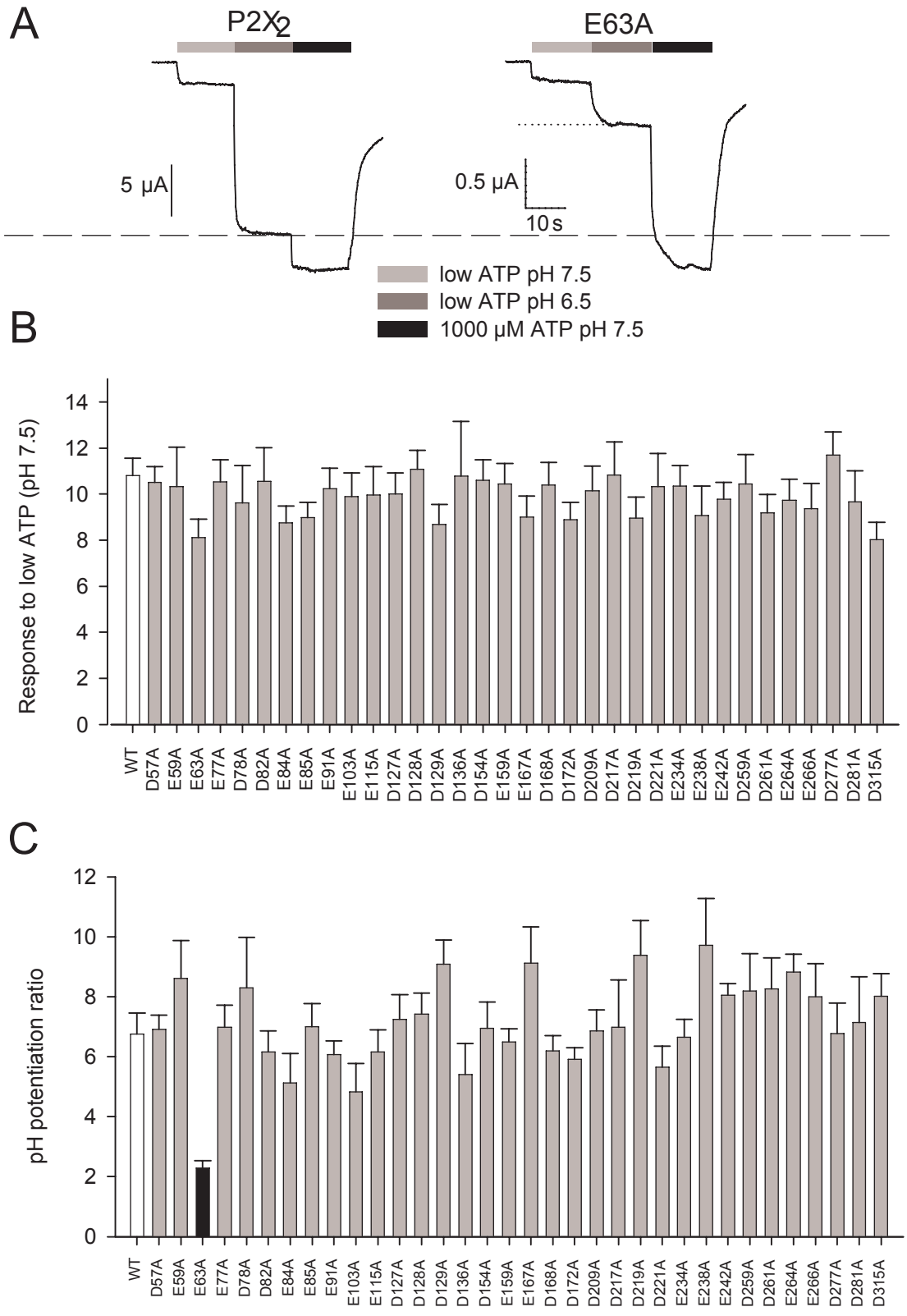
Figure 3.1: Effects of changes in the extracellular pH on currents through rat $P2X_2$ receptors expressed in *Xenopus* oocytes.

A. Representative traces of pH potentiation assays performed on *Xenopus* oocytes expressing saturating amounts of RNA encoding $rP2X_2$ and E63A. Horizontal bars represent the duration of application of low ATP (EC_{8-12}, pH 7.5), low ATP (EC_{8-12}, pH 6.5), and 1000 μM ATP (pH 7.5). The dotted line represents the maximal pH potentiation evoked by low ATP (pH 6.5) for oocytes expressing the E63A mutation. The dashed line represents the magnitude of proton-potentiated current expected if pH potentiation was unaffected by the alanine substitutions. The traces were re-scaled so that the maximal responses to 1000 μM ATP were similar. The time scale applies to each trace.

B. Histogram demonstrating that all mutants were tested at close to the EC_{10} for ATP at pH 7.5. Error bars represent standard error of the mean (N= 3–19).

C. Histogram summarizing the results of experiments as in A for $rP2X_2$ (white bar) and 34 mutants in which a single negatively charged amino acid was mutated to alanine. Data are presented as the pH potentiation index (PPI) described in Results. Only the E63A mutation exhibited a significantly reduced pH potentiation ratio ($p<0.0014$). Error bars represent standard error of the mean (sem).

Figure 3.1


A
P2X2
E63A
5 μA
0.5 μA
10 s
low ATP pH 7.5
low ATP pH 6.5
1000 μM ATP pH 7.5
B
Response to low ATP (pH 7.5)
0
2
4
6
8
10
12
14
WT
D57A
E59A
E63A
E77A
D78A
D82A
E84A
E85A
E91A
E103A
E115A
D127A
D128A
D129A
D136A
D154A
E159A
E167A
D168A
D172A
D209A
D217A
D219A
D221A
E234A
E238A
E242A
D259A
D261A
E264A
E266A
D277A
D281A
D315A
C
pH potentiation ratio
0
2
4
6
8
10
12
WT
D57A
E59A
E63A
E77A
D78A
D82A
E84A
E85A
E91A
E103A
E115A
D127A
D128A
D129A
D136A
D154A
E159A
E167A
D168A
D172A
D209A
D217A
D219A
D221A
E234A
E238A
E242A
D259A
D261A
E264A
E266A
D277A
D281A
D315A

Chapter Four

General Discussion

In this thesis I set out to test whether any of the extracellular glutamates or aspartates of $P2X_2$ might play an essential role in three processes, high affinity zinc binding that leads to potentiation of ATP responses, low affinity zinc binding that leads to inhibition of ATP responses and proton binding that leads to potentiation of ATP responses. The focus was on these negatively charged residues because the phenotypes of mutations previously made to other potential candidates for involvement in these modulations (histidines and cysteines) seemed insufficient to fully account for these phenomena. I identified a single residue (D136) that is a very strong candidate for involvement in the zinc potentiating site, a single residue that remains a candidate for involvement in a pH potentiating site (E63A) and no good candidates for involvement in the zinc inhibitory site, although I did find one mutation (E84A) that can significantly attenuate zinc inhibition. I begin this section with consideration of the next steps that should be taken to understand each of these three binding sites, and end with a general consideration of the potential uses that might follow from understanding any of these allosteric sites in detail.

Potential future experiments to explore the zinc potentiation site

To gain further understanding of the mechanism by which D136A leads to attenuation of zinc potentiation, it would be of great interest to know if this residue is in close physical proximity to the known zinc coordinating residues H120 and H213. To test whether this candidate acidic residue is in close enough proximity to directly participate in zinc binding with H120 and H213, we have initiated two parallel approaches.

Electrophysiologically, when a double mutant of H120C/H213C H213C is expressed in *Xenopus* oocytes, there is a significant rightward shift in the ATP dose response relationship, and zinc potentiation is absent. Treatment with the reducing agent DTT, shifts the ATP potency back toward that of wild type controls, and partially restores zinc potentiation. Furthermore, for some concentrations of ATP there is a very large increase in zinc potentiation after DTT treatment.

Results from electrophysiology experiments performed so far have shown that the ATP dose response relationships for oocytes expressing the single mutants H120C, H213C, and D136C are similar to wild type, and responses to 100 μM ATP before and after DTT are no different. When the D136/H120C double mutant was tested, the ATP potency was shifted slightly to the left and the response to 100 μM ATP was unchanged by DTT treatment. However, the D136C/H213C double mutant was right shifted similar to the H120C/H213C double mutant. After DTT treatment, the responses evoked by 100 μM ATP significantly increased for the control H120C/H213C and the test D136C/H213C

combinations. While these changes in receptor function are suggestive of an intersubunit disulfide occurring at the zinc binding site, an alternate possibility is that flexibility between subunits in this region of the ectodomain is sufficient to allow ectopic disulfides to form non-specifically with residues not at the binding site. To test this possibility, electrophysiological recordings will be performed on the remaining 6 candidates as double mutants with either H120C or H213C.

With respect to zinc potentiation, the control H120C/H213C showed a significant increase in zinc potentiation ratio after DTT treatment. However, the results are not as clear for the test combinations D136C/H120C and D136C/H213C because it has been difficult to identify the concentration of ATP to evoke 10% of the maximal response, so most of the data generated so far are not within our established criteria for low ATP. However, it is clear that the zinc potentiation ratios of the control H120C/H213C and D136C/H120C, D136C/H213C test combinations are lower than those of wild type and singly expressed H120C, H213C, and D136C mutations. Larger sample sizes are needed to draw conclusions about whether the lower zinc potentiation can be restored by DTT treatment.

Biochemically, under non-reducing SDS/PAGE conditions, $P2X_2$ proteins from oocytes coexpressing the H120C and H213C constructs migrate as a mix of monomers and dimers and oocytes expressing the H120C/H213C double mutant migrate as trimers. Under reducing conditions, both coexpressed proteins and proteins from double mutants migrate as monomers, consistent with formation of reductant-sensitive ectopic disulfides. A prediction is that when D136C is in a

double mutant with either H120C or H213C, the channel function phenotypes resulting from either combination would not be equivalent, because one or the other should be an intersubunit ectopic disulfide, which might constrain global channel flexibility more than an intrasubunit ectopic disulfide. The strategy for the biochemical approach has been to make double mutant constructs encoding H120C or H213C with the cysteine version of each of the seven identified zinc potentiation candidates, D82C, E85C, E91C, E115C, D136C, D209C, and D281C. Protein extracted from HEK293 cells or *Xenopus* oocytes expressing these combinations will be resolved by denaturing SDS/PAGE under reducing and non-reducing conditions. These studies are not yet complete, as only several of the possible combinations have been tested so far. Efforts are currently underway to optimize western blotting protocols to reduce detection of spurious high molecular weight bands that confound interpretation.

Regardless of whether D136 is proved to be part of the zinc binding site, we began with the assumption that the site would be tetrahedral, which if correct would imply that one residue remains to be identified (or two if D136 is subsequently ruled out).

To look for the remaining zinc binding residues that mediate potentiation or inhibition at $P2X_2$ receptors, one could move further down the list of likely candidates. While histidine, cysteine, aspartate, and glutamate are the most common residues known to bind zinc, there are other candidates. Lysine, a positively charged residue, has been shown by site-directed mutagenesis to be involved in high-affinity Zn^{2+} modulation of NMDA channels (Fayyazuddin *et al.*

2000) and ASIC1a (Chu *et al.* 2004). Also, two polar, uncharged threonine residues of the GlyRα1 subunit, T112 and T133, have been shown by site-directed mutagenesis to be important determinants of Zn^{2+}-mediated inhibition of the glycine receptor (Laube *et al.* 2000, Miller *et al.* 2005). Therefore, it might be reasonable to investigate the extracellular lysine and threonine residues for involvement in zinc modulation of $P2X_2$ receptors.

Potential future experiments to explore mechanism(s) of pH potentiation

With respect to the mechanism by which E63 contributes to pH potentiation, there are several hypotheses that might explain these results that were discussed in detail in Chapter 3. Briefly, the steric properties of glutamate 63 may be a critical determinant of structural aspects of the proton binding site, in which case a glutamine substitution would be predicted to have a less dramatic effect on pH potentiation than an aspartate substitution. Alternatively, the electrostatic properties of E63 may be the critical chemical feature enabling wild type-like pH potentiation, in which case an aspartate substitution would be predicted to be less dramatic than the effects of a glutamine substitution on pH potentiation.

Mechanism of zinc inhibition

The same classes of residues that were candidates for participation in the zinc potentiating binding site were candidates for participation in the inhibitory site. The question of whether inhibition of current in response to high

concentrations of zinc (>100 μM) in the alanine substituted conserved extracellular cysteines was previously addressed by our lab. It was noted that zinc inhibition was operative for each of the 10 mutants, although the magnitude of the inhibition was not quantitatively characterized. As there is good evidence that all of these residues are in disulfide bonds, they do not seem to be promising candidates, although if the suggestion that one pair is not disulfide bonded (Coddou *et al.* 2007) were to be verified for $P2X_2$, these residues should be tested. Similarly, in the study of Clyne et al (2002a) the nine extracellular histidines of $P2X_2$ were tested with a careful quantitative assay for participation in the potentiating zinc binding site, but it was simply noted that inhibition was present in all 9 alanine mutants without quantitation. Therefore I believe that the role of the histidines in zinc inhibition should be looked at more carefully.

To definitively assess whether the histidine residues are necessary for zinc inhibition, two approaches might be taken. The first approach would be to rescreen the 9 alanine substituted histidine mutants with a more quantitative protocol like the one used here in the zinc inhibition screen of the aspartate and glutamate mutants. The better, but more time consuming approach would be to make double mutants of each of the remaining seven histidines with H120A or H213A. Because these mutants do not potentiate to zinc, but are inhibited by high concentrations of zinc, mutants that fail to exhibit inhibition would likely be more easily identified in this background.

If a rescreen of the histidines (and possibility some cysteines) does not reveal the members of the zinc inhibitory site, the same approaches I mentioned

for the excitatory site would be relevant, although I would consider this to be of lower priority because the affinity of this site in rats is so low. However, it should be noted that recent studies by Rachel Tittle in our lab have demonstrated that human $P2X_2$ has no zinc potentiation and a much higher affinity inhibitory zinc binding site, so a higher priority than continuing to study the rat receptor should be to understand the nature of zinc inhibition on the human receptor.

Additional modulatory actions on $P2X_2$ that could be studied

The focus of the work presented here has been to identify amino acids that play a role in the allosteric modulation of $P2X_2$ by zinc and pH. There are at least two other modulatory systems acting on $P2X_2$, and there might be more. Previous work from our lab (Zhou & Hume 1998) demonstrated that $P2X_2$ receptors exhibit inward rectification and voltage-dependence of gating, which can affect receptor function. Like the allosteric modulators zinc and pH, the effect of negative membrane potential is to decrease the concentrtation of ATp needed to achieve and EC50 response, with little change in the Hill coefficient or response to a maximal concentration of ATP. Work of others has shown that copper can potentiate $P2X_2$ responses by acting at a site that probably has some residues in common with the high affinity zinc site, but is not identical to it (Lorca *et al.* 2005).

I am nearing completion of a study testing whether any of the negative charges thought to be in the extracellular loop can alter the voltage dependence of $P2X_2$ gating. To date, I have completed tests on approximately one-fourth of

the 34 mutants, and I have found that none alter the amount of inward rectification in the current-voltage relation, which is an indicator of the amount of allosteric regulation by membrane potential. Furthermore, for the following reason the likelihood that we will find anything is low. Although charged amino acids are usually part of voltage sensing mechanisms for voltage-gated ion channels (Bezanilla 2008), in order to be part of a voltage sensor the charges have to be within the membrane voltage field, and in our current model of the $P2X_2$ receptor, all of these residues are in the extracellular space. However, because re-entrant loops into the membrane are a feature of some ion channels (Zhorov & Tikhonov 2004), and it has been recently proposed that $P2X_2$ might have a reentrant loop in addition to the two transmembrane domains (Cao *et al.* 2007) it seems worthwhile to complete this study. It should be noted that human $P2X_2$ shows voltage dependent modulation similar to rat $P2X_2$ (Rachel Tittle, personal communication).

The role of $P2X_2$ modulatory mechanisms in normal physiology

While my dissertation studies focused on only two allosteric ligands for $P2X_2$ receptors, they nonetheless illustrate that the function of these channels can potentially be greatly influenced by molecules in the immediate milieu, adding a potential for complex regulation of channel activity. An absolutely critical question for future studies is whether there are actually changes in the levels of zinc or pH (or copper) to make any of these binding sites physiologically meaningful. Clearly the membrane potential does change enough to act upon

the voltage modulatory system. In order to test the role of these modulatory systems *in vivo*, (probably through the use of knock in mice) two requirements must be met. There must be mutants identified that significantly decrease, (or ideally eliminate) the modulatory process without affecting other receptor properties. The H120A mutant seems ideal for testing the role of zinc potentiation, but the mutants that attenuate pH modulation (H319A and E63A), zinc inhibition (E84A), and voltage modulation (D315A) (Clyne et al. 2002a, Friday & Hume 2008, Zhou 1998), are not particularly potent or have other actions on receptor function and so it seems premature to consider building mice with these mutants. However, there is a more serious problem, which is that the phenotype of mice with a complete knock out of $P2X_2$ is quite mild (Cockayne *et al.* 2005) and so it is unclear how a mouse that was lacking one of these modulatory sites would be different from a wild type mouse.

Because $P2X_2$ knockout mice have been reported to show abnormalities in neuromuscular junction formation and ventilitory responses to hypoxia, knock in mice carrying the H120A mutation might allow one to determine whether normal zinc potentiation is necessary for $P2X_2$ receptor function in those physiological roles.

Potential uses that might arise from a better understanding of the allosteric binding sites of P2X receptors

Positive and negative allosteric effectors are intriguing as potential therapeutic targets for different LGIC families, and may provide an additional and perhaps safer strategy for affecting the function of LGICs than agonists and

competitive or non-competitive inhibitors (Hogg et al. 2005). Orthosteric agonists (those that act at the binding site for the primary ligand) can evoke sustained activation of the receptors, which can have a cytotoxic effect is the dosage is too high. Alternatively, sustained exposure can cause receptor desensitization that may result in the opposite of the desired effects. In contrast, the effects of an allosteric effector are limited by the nature of the receptor modulation, such that presence of higher concentrations of the modulator will saturate the effector sites but not result in effects beyond maximal modulation. This ceiling effect sometimes offers a much larger safety margin in drug administration and compliance (Hogg *et al.* 2005).

Specific examples of the utility of this approach are the use of benzodiazepenes such as valium as allosteric modulators of some types of GABA A receptors (Barbaccia *et al.* 1990), and the use of UCI-30002 [N-(1,2,3,4-tetrahydro-1-naphthyl)-4-nitroaniline], a novel, negative allosteric modulator with selectivity for the major neuronal nAChR subtypes over muscle-type nAChRs (Yoshimura *et al.* 2007). These therapeutics represents validation of the concept that allosteric modulators may have significant benefits as a strategy for treating some neurological disorders and smoking cessation.

If allosteric modulation of $P2X_2$ is indeed physiologically relevant, receptor specific positive allosteric therapeutics might be beneficial where there is an underlying condition of zinc deficiency or impaired zinc metabolism, while negative allosteric therapeutics might be indicated to attenuate activation of endogenous agonist or elevated amounts of allosteric modulator.

Bibliography

Acuna-Castillo, C., Coddou, C., Bull, P., Brito, J. and Huidobro-Toro, J. P. (2007) Differential role of extracellular histidines in copper, zinc, magnesium and proton modulation of the P2X7 purinergic receptor. *J Neurochem,* **101,** 17-26.

Agboh, K. C., Webb, T. E., Evans, R. J. and Ennion, S. J. (2004) Functional characterization of a P2X receptor from Schistosoma mansoni. *J Biol Chem,* **279,** 41650-41657.

Akata, T. (2007) Cellular and molecular mechanisms regulating vascular tone. Part 1: basic mechanisms controlling cytosolic Ca2+ concentration and the Ca2+-dependent regulation of vascular tone. *J Anesth,* **21,** 220-231.

Alexander, J. (2007) Selenium. *Novartis Found Symp,* **282,** 143-149; discussion 149-153, 212-148.

Barbaccia, M. L., Berkovich, A., Guarneri, P. and Slobodyansky, E. (1990) DBI (diazepam binding inhibitor): the precursor of a family of endogenous modulators of GABAA receptor function. History, perspectives, and clinical implications. *Neurochem Res,* **15,** 161-168.

Barceloux, D. G. (1999) Zinc. *J Toxicol Clin Toxicol,* **37,** 279-292.

Baron, A., Schaefer, L., Lingueglia, E., Champigny, G. and Lazdunski, M. (2001) Zn2+ and H+ are coactivators of acid-sensing ion channels. *The Journal of biological chemistry,* **276,** 35361-35367.

Barrera, N. P., Ormond, S. J., Henderson, R. M., Murrell-Lagnado, R. D. and Edwardson, J. M. (2005) Atomic force microscopy imaging demonstrates that P2X2 receptors are trimers but that P2X6 receptor subunits do not oligomerize. *J Biol Chem,* **280,** 10759-10765.

Beaulieu, C., Dyck, R. and Cynader, M. (1992) Enrichment of glutamate in zinc-containing terminals of the cat visual cortex. *Neuroreport,* **3,** 861-864.

Bertrand, P. P. 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.

Bezanilla, F. (2008) How membrane proteins sense voltage. *Nat Rev Mol Cell Biol,* **9,** 323-332.

Bian, X., Ren, J., DeVries, M., Schnegelsberg, B., Cockayne, D. A., Ford, A. P. and Galligan, J. J. (2003) Peristalsis is impaired in the small intestine of mice lacking the P2X3 subunit. *J Physiol,* **551,** 309-322.

Bo, X., Jiang, L. H., Wilson, H. L., Kim, M., Burnstock, G., Surprenant, A. and North, R. A. (2003) Pharmacological and biophysical properties of the human P2X5 receptor. *Mol Pharmacol,* **63,** 1407-1416.

Boue-Grabot, E., Barajas-Lopez, C., Chakfe, Y., Blais, D., Belanger, D., Emerit, M. B. and Seguela, P. (2003) Intracellular cross talk and physical interaction between two classes of neurotransmitter-gated channels. *J Neurosci,* **23,** 1246-1253.

Brake, A. J. and Julius, D. (1996) Signaling by extracellular nucleotides. *Annual review of cell and developmental biology,* **12,** 519-541.

Brake, A. J., 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. et al. (1997) Desensitization of the P2X(2) receptor controlled by alternative splicing. *FEBS Lett,* **404,** 294-298.

Brone, B., Moechars, D., Marrannes, R., Mercken, M. and Meert, T. (2007) P2X currents in peritoneal macrophages of wild type and P2X4 -/- mice. *Immunol Lett,* **113,** 83-89.

Brooks, S. P. and Storey, K. B. (1992) Bound and determined: a computer program for making buffers of defined ion concentrations. *Analytical biochemistry,* **201,** 119-126.

Burnstock, G. (2007a) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev,* **87,** 659-797.

Burnstock, G. (2007b) Purine and pyrimidine receptors. *Cell Mol Life Sci,* **64,** 1471-1483.

Calvert, J. A. and Evans, R. J. (2004) Heterogeneity of P2X receptors in sympathetic neurons: contribution of neuronal P2X1 receptors revealed using knockout mice. *Mol Pharmacol,* **65,** 139-148.

Cao, L., Young, M. T., Broomhead, H. E., Fountain, S. J. and North, R. A. (2007) Thr339-to-serine substitution in rat P2X2 receptor second transmembrane domain causes constitutive opening and indicates a gating role for Lys308. *J Neurosci,* **27,** 12916-12923.

Carrasco, M. A., Jaimovich, E., Kemmerling, U. and Hidalgo, C. (2004) Signal transduction and gene expression regulated by calcium release from internal stores in excitable cells. *Biol Res,* **37,** 701-712.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D. and Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* **389,** 816-824.

Chen, X. H., Bezprozvanny, I. and Tsien, R. W. (1996) Molecular basis of proton block of L-type Ca2+ channels. *J Gen Physiol,* **108,** 363-374.

Chen, X. H. and Tsien, R. W. (1997) Aspartate substitutions establish the concerted action of P-region glutamates in repeats I and III in forming the protonation site of L-type Ca2+ channels. *J Biol Chem,* **272,** 30002-30008.

Chessell, I. P., Hatcher, J. P., Bountra, C. et al. (2005) Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain,* **114,** 386-396.

Chimienti, F., Aouffen, M., Favier, A. and Seve, M. (2003) Zinc homeostasis-regulating proteins: new drug targets for triggering cell fate. *Curr Drug Targets,* **4,** 323-338.

Chow, Y. W. and Wang, H. L. (1998) Functional modulation of P2X2 receptors by cyclic AMP-dependent protein kinase. *J Neurochem,* **70,** 2606-2612.

Christopoulos, A. (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov,* **1,** 198-210.

Chu, X. P., Wemmie, J. A., Wang, W. Z., Zhu, X. M., Saugstad, J. A., Price, M. P., Simon, R. P. and Xiong, Z. G. (2004) Subunit-dependent high-affinity zinc inhibition of acid-sensing ion channels. *J Neurosci,* **24,** 8678-8689.

Clapham, D. E. (2007) Calcium signaling. *Cell,* **131,** 1047-1058.

Cloues, R., Jones, S. and Brown, D. A. (1993) Zn2+ potentiates ATP-activated currents in rat sympathetic neurons. *Pflugers Arch,* **424,** 152-158.

Clyne, J. D., Brown, T. C. and Hume, R. I. (2003) Expression level dependent changes in the properties of P2X2 receptors. *Neuropharmacology,* **44,** 403-412.

Clyne, J. D., LaPointe, L. D. and Hume, R. I. (2002a) The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH. *J Physiol,* **539,** 347-359.

Clyne, J. D., Wang, L. F. and Hume, R. I. (2002) Mutational analysis of the conserved cysteines of the rat P2X2 purinoceptor. *J Neurosci,* **22,** 3873-3880.

Cockayne, D. A., Dunn, P. M., Zhong, Y. et al. (2005) P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor

subunit in mediating multiple sensory effects of ATP. *J Physiol,* **567,** 621-639.

Coddou, C., Acuna-Castillo, C., Bull, P. and Huidobro-Toro, J. P. (2007) Dissecting the facilitator and inhibitor allosteric metal sites of the P2X4 receptor channel: critical roles of CYS132 for zinc potentiation and ASP138 for copper inhibition. *J Biol Chem,* **282,** 36879-36886.

Cole, T. B., Wenzel, H. J., Kafer, K. E., Schwartzkroin, P. A. and Palmiter, R. D. (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. *Proc Natl Acad Sci U S A,* **96,** 1716-1721.

Coutinho-Silva, R., Knight, G. E. and Burnstock, G. (2005) Impairment of the splenic immune system in P2X(2)/P2X(3) knockout mice. *Immunobiology,* **209,** 661-668.

Cuajungco, M. P. and Lees, G. J. (1997) Zinc metabolism in the brain: relevance to human neurodegenerative disorders. *Neurobiology of disease,* **4,** 137-169.

Darville, T., Welter-Stahl, L., Cruz, C., Sater, A. A., Andrews, C. W., Jr. and Ojcius, D. M. (2007) Effect of the purinergic receptor P2X7 on Chlamydia infection in cervical epithelial cells and vaginally infected mice. *J Immunol,* **179,** 3707-3714.

Davies, P. A., Wang, W., Hales, T. G. and Kirkness, E. F. (2003) A novel class of ligand-gated ion channel is activated by Zn2+. *J Biol Chem,* **278,** 712-717.

Dubyak, G. R. (2007) Go it alone no more--P2X7 joins the society of heteromeric ATP-gated receptor channels. *Mol Pharmacol,* **72,** 1402-1405.

Egan, T. M. and Khakh, B. S. (2004) Contribution of calcium ions to P2X channel responses. *J Neurosci,* **24,** 3413-3420.

Ennion, S., Hagan, S. and Evans, R. J. (2000) The role of positively charged amino acids in ATP recognition by human P2X1 receptors. *J Biol Chem,* **275,** 35656.

Ennion, S. J. and Evans, R. J. (2002) Conserved cysteine residues in the extracellular loop of the human P2X(1) receptor form disulfide bonds and are involved in receptor trafficking to the cell surface. *Mol Pharmacol,* **61,** 303-311.

Ennion, S. J., Ritson, J. and Evans, R. J. (2001) Conserved negatively charged residues are not required for ATP action at P2X(1) receptors. *Biochemical and biophysical research communications,* **289,** 700-704.

Erb, L., Liao, Z., Seye, C. I. and Weisman, G. A. (2006) P2 receptors: intracellular signaling. *Pflugers Arch,* **452,** 552-562.

Evans, R. J. (2008) Orthosteric and allosteric binding sites of P2X receptors. *Eur Biophys J*.

Fabris, N. and Mocchegiani, E. (1995) Zinc, human diseases and aging. *Aging (Milano),* **7,** 77-93.

Fayyazuddin, A., Villarroel, A., Le Goff, A., Lerma, J. and Neyton, J. (2000) Four residues of the extracellular N-terminal domain of the NR2A subunit control high-affinity Zn2+ binding to NMDA receptors. *Neuron,* **25,** 683-694.

Finger, T. E., 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.

Forsythe, I. D., Westbrook, G. L. and Mayer, M. L. (1988) Modulation of excitatory synaptic transmission by glycine and zinc in cultures of mouse hippocampal neurons. *J Neurosci,* **8,** 3733-3741.

Fountain, S. J., Cao, L., Young, M. T. and North, R. A. (2008) Permeation properties of a P2X receptor in the green algae ostreococcus tauri. *J Biol Chem*.

Fountain, S. J., Parkinson, K., Young, M. T., Cao, L., Thompson, C. R. and North, R. A. (2007) An intracellular P2X receptor required for osmoregulation in Dictyostelium discoideum. *Nature,* **448,** 200-203.

Frank, G. B. (1964) Calcium and the Initiation of Contraction. *Circ Res,* **15,** SUPPL 2:54-63.

Franke, H., Krugel, U. and Illes, P. (2006) P2 receptors and neuronal injury. *Pflugers Arch,* **452,** 622-644.

Friday, S. C. and Hume, R. I. (2008) Contribution of extracellular negatively charged residues to ATP action and zinc modulation of rat P2X2 receptors. *J Neurochem,* **105,** 1264-1275.

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. (2003) Multinucleated osteoclast formation *in vivo* and *in vitro* by P2X7 receptor-deficient mice. *Crit Rev Eukaryot Gene Expr,* **13,** 243-253.

Gerasimovskaya, E. V., Ahmad, S., White, C. W., Jones, P. L., Carpenter, T. C. and Stenmark, K. R. (2002) Extracellular ATP is an autocrine/paracrine

regulator of hypoxia-induced adventitial fibroblast growth. Signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor. *J Biol Chem,* **277,** 44638-44650.

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.

Gibbs, J. W., 3rd, Zhang, Y. F., Shumate, M. D. and Coulter, D. A. (2000) Regionally selective blockade of GABAergic inhibition by zinc in the thalamocortical system: functional significance. *Journal of neurophysiology,* **83,** 1510-1521.

Girdler, G. and Khakh, B. S. (2004) ATP-gated P2X channels. *Curr Biol,* **14,** R6.

Gomes, P., Srinivas, S. P., Van Driessche, W., Vereecke, J. and Himpens, B. (2005) ATP release through connexin hemichannels in corneal endothelial cells. *Invest Ophthalmol Vis Sci,* **46,** 1208-1218.

Goncalves, R. G., Gabrich, L., Rosario, A., Jr., Takiya, C. M., Ferreira, M. L., Chiarini, L. B., Persechini, P. M., Coutinho-Silva, R. and Leite, M., Jr. (2006) The role of purinergic P2X7 receptors in the inflammation and fibrosis of unilateral ureteral obstruction in mice. *Kidney Int,* **70,** 1599-1606.

Gordon, S. E., Oakley, J. C., Varnum, M. D. and Zagotta, W. N. (1996) Altered ligand specificity by protonation in the ligand binding domain of cyclic nucleotide-gated channels. *Biochemistry,* **35,** 3994-4001.

Hechler, B., Lenain, N., Marchese, P. et al. (2003) A role of the fast ATP-gated P2X1 cation channel in thrombosis of small arteries *in vivo*. *J Exp Med,* **198,** 661-667.

Henneberry, R. C. (1992) Cloning of the genes for excitatory amino acid receptors. *Bioessays,* **14,** 465-471.

Heyneman, C. A. (1996) Zinc deficiency and taste disorders. *Ann Pharmacother,* **30,** 186-187.

Hirzel, K., Muller, U., Latal, A. T., Hulsmann, S., Grudzinska, J., Seeliger, M. W., Betz, H. and Laube, B. (2006) Hyperekplexia phenotype of glycine receptor alpha1 subunit mutant mice identifies Zn(2+) as an essential endogenous modulator of glycinergic neurotransmission. *Neuron,* **52,** 679-690.

Hogg, R. C., Buisson, B. and Bertrand, D. (2005) Allosteric modulation of ligand-gated ion channels. *Biochem Pharmacol,* **70,** 1267-1276.

Hsiao, B., Dweck, D. and Luetje, C. W. (2001) Subunit-dependent modulation of neuronal nicotinic receptors by zinc. *J Neurosci,* **21,** 1848-1856.

Hsiao, B., Mihalak, K. B., Repicky, S. E., Everhart, D., Mederos, A. H., Malhotra, A. and Luetje, C. W. (2006) Determinants of zinc potentiation on the alpha4 subunit of neuronal nicotinic receptors. *Mol Pharmacol,* **69,** 27-36.

Inscho, E. W., Cook, A. K., Imig, J. D., Vial, C. and Evans, R. J. (2004) Renal autoregulation in P2X1 knockout mice. *Acta Physiol Scand,* **181,** 445-453.

Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. (1997) *Dietary reference intakes : for calcium, phosphorus, magnesium, vitamin D, and fluoride*. National Academy Press, Washington, D.C.

Isojima, C. and Bozler, E. (1963) Role of Calcium in Initiation of Contraction in Smooth Muscle. *Am J Physiol,* **205,** 681-685.

Jarvis, M. F. (2003) Contributions of P2X3 homomeric and heteromeric channels to acute and chronic pain. *Expert Opin Ther Targets,* **7,** 513-522.

Jasti, J., Furukawa, H., Gonzales, E. B. and Gouaux, E. (2007) Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. *Nature,* **449,** 316-323.

Javitch, J. A., Li, X., Kaback, J. and Karlin, A. (1994) A cysteine residue in the third membrane-spanning segment of the human D2 dopamine receptor is exposed in the binding-site crevice. *Proc Natl Acad Sci U S A,* **91,** 10355-10359.

Jiang, L. H., Rassendren, F., Surprenant, A. and North, R. A. (2000) Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. *J Biol Chem,* **275,** 34190-34196.

Jo, Y. H. and Role, L. W. (2002) Coordinate release of ATP and GABA at *in vitro* synapses of lateral hypothalamic neurons. *J Neurosci,* **22,** 4794-4804.

Jordt, S. E. and Jentsch, T. J. (1997) Molecular dissection of gating in the ClC-2 chloride channel. *EMBO J,* **16,** 1582-1592.

Jordt, S. E., Tominaga, M. and Julius, D. (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci U S A,* **97,** 8134-8139.

Karlin, A. (1967) On the application of "a plausible model" of allosteric proteins to the receptor for acetylcholine. *J Theor Biol,* **16,** 306-320.

Ke, H. Z., Qi, H., Weidema, A. F. et al. (2003) Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. *Mol Endocrinol,* **17,** 1356-1367.

Khakh, B. S. and North, R. A. (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature,* **442,** 527-532.

Khakh, B. S., Zhou, X., Sydes, J., Galligan, J. J. and Lester, H. A. (2000) State-dependent cross-inhibition between transmitter-gated cation channels. *Nature,* **406,** 405-410.

King, B. F., Townsend-Nicholson, A., Wildman, S. S., Thomas, T., Spyer, K. M. and Burnstock, G. (2000) Coexpression of rat P2X2 and P2X6 subunits in Xenopus oocytes. *J Neurosci,* **20,** 4871-4877.

King, B. F., Wildman, S. S., Ziganshina, L. E., Pintor, J. and Burnstock, G. (1997) Effects of extracellular pH on agonism and antagonism at a recombinant P2X2 receptor. *Br J Pharmacol,* **121,** 1445-1453.

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. and Lefkowitz, R. J. (1988) Chimeric alpha 2-,beta 2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science,* **240,** 1310-1316.

Koh, J. Y. and Choi, D. W. (1994) Zinc toxicity on cultured cortical neurons: involvement of N-methyl-D-aspartate receptors. *Neuroscience,* **60,** 1049-1057.

Krusek, J., Dittert, I., Hendrych, T. et al. (2004) Activation and modulation of ligand-gated ion channels. *Physiol Res,* **53 Suppl 1,** S103-113.

Labasi, J. M., Petrushova, N., Donovan, C. et al. (2002) Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol,* **168,** 6436-6445.

Lagerstrom, M. C. and Schioth, H. B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov,* **7,** 339-357.

Larhammar, D., Blomqvist, A. G. and Wahlestedt, C. (1993) The receptor revolution--multiplicity of G-protein-coupled receptors. *Drug Des Discov,* **9,** 179-188.

Laube, B., Kuhse, J. and Betz, H. (2000) Kinetic and mutational analysis of Zn2+ modulation of recombinant human inhibitory glycine receptors. *J Physiol,* **522 Pt 2,** 215-230.

Lazarowski, E. (2006) Regulated release of nucleotides and UDP sugars from astrocytoma cells. *Novartis Found Symp,* **276,** 73-84; discussion 84-90, 107-112, 275-181.

Le Feuvre, R. A., Brough, D., Touzani, O. and Rothwell, N. J. (2003) Role of P2X7 receptors in ischemic and excitotoxic brain injury *in vivo*. *J Cereb Blood Flow Metab,* **23,** 381-384.

Li, J., Liu, D., Ke, H. Z., Duncan, R. L. and Turner, C. H. (2005) The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem,* **280,** 42952-42959.

Li, Y. V., Hough, C. J. and Sarvey, J. M. (2003) Do we need zinc to think? *Sci STKE,* **2003,** pe19.

Locovei, S., Scemes, E., Qiu, F., Spray, D. C. and Dahl, G. (2007) Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. *FEBS Lett,* **581,** 483-488.

Lorca, R. A., Coddou, C., Gazitua, M. C., Bull, P., Arredondo, C. and Huidobro-Toro, J. P. (2005) Extracellular histidine residues identify common structural determinants in the copper/zinc P2X2 receptor modulation. *J Neurochem,* **95,** 499-512.

Lustig, K. D., Shiau, A. K., Brake, A. J. and Julius, D. (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc Natl Acad Sci U S A,* **90,** 5113-5117.

Lynch, J. W., Jacques, P., Pierce, K. D. and Schofield, P. R. (1998) Zinc potentiation of the glycine receptor chloride channel is mediated by allosteric pathways. *J Neurochem,* **71,** 2159-2168.

Ma, B., Ruan, H. Z., Cockayne, D. A., Ford, A. P., 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.

Maret, W. (2005) Zinc coordination environments in proteins determine zinc functions. *J Trace Elem Med Biol,* **19,** 7-12.

Maret, W. (2006) Zinc coordination environments in proteins as redox sensors and signal transducers. *Antioxid Redox Signal,* **8,** 1419-1441.

Marin-Garcia, P., Sanchez-Nogueiro, J., Gomez-Villafuertes, R., Leon, D. and Miras-Portugal, M. T. (2008) Synaptic terminals from mice midbrain exhibit functional P2X7 receptor. *Neuroscience,* **151,** 361-373.

Marti, D. N. (2005) Apparent pKa shifts of titratable residues at high denaturant concentration and the impact on protein stability. *Biophys Chem,* **118,** 88-92.

Masin, M., Kerschensteiner, D., Dumke, K., Rubio, M. E. and Soto, F. (2006) Fe65 interacts with P2X2 subunits at excitatory synapses and modulates receptor function. *The Journal of biological chemistry,* **281,** 4100-4108.

Mathie, A., Sutton, G. L., Clarke, C. E. and Veale, E. L. (2006) Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. *Pharmacol Ther,* **111,** 567-583.

Messerschmidt, A. (2001) *Handbook of metalloproteins*. Wiley, Chichester ; New York.

Miller, P. S., Beato, M., Harvey, R. J. and Smart, T. G. (2005) Molecular determinants of glycine receptor alphabeta subunit sensitivities to Zn2+-mediated inhibition. *J Physiol,* **566,** 657-670.

Mingam, R., De Smedt, V., Amedee, T., Bluthe, R. M., Kelley, K. W., Dantzer, R. and Laye, S. (2008) *In vitro* and *in vivo* evidence for a role of the P2X7 receptor in the release of IL-1 beta in the murine brain. *Brain Behav Immun,* **22,** 234-244.

Montell, C. and Rubin, G. M. (1989) Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron,* **2,** 1313-1323.

Morrill, J. A. and MacKinnon, R. (1999) Isolation of a single carboxyl-carboxylate proton binding site in the pore of a cyclic nucleotide-gated channel. *The Journal of general physiology,* **114,** 71-83.

Mulryan, K., Gitterman, D. P., Lewis, C. J. et al. (2000) Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. *Nature,* **403,** 86-89.

Munaron, L., Antoniotti, S. and Lovisolo, D. (2004) Intracellular calcium signals and control of cell proliferation: how many mechanisms? *J Cell Mol Med,* **8,** 161-168.

Nagaya, N., Tittle, R. K., Saar, N., Dellal, S. S. and Hume, R. I. (2005) An intersubunit zinc binding site in rat P2X2 receptors. *J Biol Chem,* **280,** 25982-25993.

Nakazawa, K., Liu, M., Inoue, K. and Ohno, Y. (1997) pH dependence of facilitation by neurotransmitters and divalent cations of P2X2 purinoceptor/channels. *Eur J Pharmacol,* **337,** 309-314.

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.

Nakazawa, K. and Ohno, Y. (1999) Neighboring glycine residues are essential for P2X2 receptor/channel function. *Eur J Pharmacol,* **370,** R5-6.

Nakazawa, K., Ohno, Y. and Inoue, K. (1998) An aspartic acid residue near the second transmembrane segment of ATP receptor/channel regulates agonist sensitivity. *Biochemical and biophysical research communications,* **244,** 599-603.

Nayler, W. G. (1966) Influx and efflux of calcium in the physiology of muscle contraction. *Clin Orthop Relat Res,* **46,** 157-182.

Nevin, S. T., Cromer, B. A., Haddrill, J. L., Morton, C. J., Parker, M. W. and Lynch, J. W. (2003) Insights into the structural basis for zinc inhibition of the glycine receptor. *J Biol Chem,* **278,** 28985-28992.

North, R. A. (2002) Molecular physiology of P2X receptors. *Physiological reviews,* **82,** 1013-1067.

Ohta, T., Kubota, A., Murakami, M., Otsuguro, K. and Ito, S. (2005) P2X2 receptors are essential for [Ca2+]i increases in response to ATP in cultured rat myenteric neurons. *Am J Physiol Gastrointest Liver Physiol,* **289,** G935-948.

Oike, M., Droogmans, G. and Ito, Y. (2004) [ATP release pathways in vascular endothelial cells]. *Nippon Yakurigaku Zasshi,* **123,** 403-411.

Ormond, S. J., Barrera, N. P., Qureshi, O. S., Henderson, R. M., Edwardson, J. M. and Murrell-Lagnado, R. D. (2006) An uncharged region within the N terminus of the P2X6 receptor inhibits its assembly and exit from the endoplasmic reticulum. *Mol Pharmacol,* **69,** 1692-1700.

Palmiter, R. D., Cole, T. B., Quaife, C. J. and Findley, S. D. (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. *Proc Natl Acad Sci U S A,* **93,** 14934-14939.

Panupinthu, N., Zhao, L., Possmayer, F., Ke, H. Z., Sims, S. M. and Dixon, S. J. (2007) P2X7 nucleotide receptors mediate blebbing in osteoblasts through a pathway involving lysophosphatidic acid. *J Biol Chem,* **282,** 3403-3412.

Papp, L., Vizi, E. S. and Sperlagh, B. (2004) Lack of ATP-evoked GABA and glutamate release in the hippocampus of P2X7 receptor-/- mice. *Neuroreport,* **15,** 2387-2391.

Pelegrin, P. and Surprenant, A. (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J,* **25,** 5071-5082.

Penner, R. and Neher, E. (1988) The role of calcium in stimulus-secretion coupling in excitable and non-excitable cells. *J Exp Biol,* **139,** 329-345.

Picciotto, M. R. and Wickman, K. (1998) Using knockout and transgenic mice to study neurophysiology and behavior. *Physiol Rev,* **78,** 1131-1163.

Placido, R., Auricchio, G., Falzoni, S., Battistini, L., Colizzi, V., Brunetti, E., Di Virgilio, F. and Mancino, G. (2006) P2X(7) purinergic receptors and extracellular ATP mediate apoptosis of human monocytes/macrophages infected with Mycobacterium tuberculosis reducing the intracellular bacterial viability. *Cell Immunol,* **244,** 10-18.

Ralevic, V. and Burnstock, G. (1991) Roles of P2-purinoceptors in the cardiovascular system. *Circulation,* **84,** 1-14.

Ralevic, V. and Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacological reviews,* **50,** 413-492.

Raouf, R., Blais, D. and Seguela, P. (2005) High zinc sensitivity and pore formation in an invertebrate P2X receptor. *Biochim Biophys Acta,* **1669,** 135-141.

Rassendren, F. A., Lory, P., Pin, J. P. and Nargeot, J. (1990) Zinc has opposite effects on NMDA and non-NMDA receptors expressed in Xenopus oocytes. *Neuron,* **4,** 733-740.

Reeh, P. W. and Steen, K. H. (1996) Tissue acidosis in nociception and pain. *Prog Brain Res,* **113,** 143-151.

Ren, J., Bian, X., DeVries, M., Schnegelsberg, B., Cockayne, D. A., Ford, A. P. and Galligan, J. J. (2003) P2X2 subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. *J Physiol,* **552,** 809-821.

Roberts, J. A. and Evans, R. J. (2005) Mutagenesis studies of conserved proline residues of human P2X receptors for ATP indicate that proline 272 contributes to channel function. *J Neurochem,* **92,** 1256-1264.

Roberts, J. A., Vial, C., Digby, H. R., Agboh, K. C., Wen, H., Atterbury-Thomas, A. and Evans, R. J. (2006) Molecular properties of P2X receptors. *Pflugers Arch,* **452,** 486-500.

Romanov, R. A., Rogachevskaja, O. A., Bystrova, M. F., Jiang, P., Margolskee, R. F. and Kolesnikov, S. S. (2007) Afferent neurotransmission mediated by hemichannels in mammalian taste cells. *EMBO J,* **26,** 657-667.

Rong, W., Gourine, A. V., Cockayne, D. A., Xiang, Z., Ford, A. P., Spyer, K. M. and Burnstock, G. (2003) Pivotal role of nucleotide P2X2 receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. *J Neurosci,* **23,** 11315-11321.

Root, M. J. and MacKinnon, R. (1994) Two identical noninteracting sites in an ion channel revealed by proton transfer. *Science,* **265,** 1852-1856.

Ryten, M., Koshi, R., Knight, G. E., Turmaine, M., Dunn, P., Cockayne, D. A., Ford, A. P. and Burnstock, G. (2007) Abnormalities in neuromuscular junction structure and skeletal muscle function in mice lacking the P2X2 nucleotide receptor. *Neuroscience,* **148,** 700-711.

Salazar, G., Craige, B., Love, R., Kalman, D. and Faundez, V. (2005) Vglut1 and ZnT3 co-targeting mechanisms regulate vesicular zinc stores in PC12 cells. *Journal of cell science,* **118,** 1911-1921.

Schoneberg, T., Hermsdorf, T., Engemaier, E., Engel, K., Liebscher, I., Thor, D., Zierau, K., Rompler, H. and Schulz, A. (2007) Structural and functional evolution of the P2Y(12)-like receptor group. *Purinergic Signal,* **3,** 255-268.

Shils, M. E. (1999) *Modern nutrition in health and disease*. Williams & Wilkins, Baltimore.

Shimizu, I., Iida, T., Guan, Y., Zhao, C., Raja, S. N., Jarvis, M. F., Cockayne, D. A. and Caterina, M. J. (2005) Enhanced thermal avoidance in mice lacking the ATP receptor P2X3. *Pain,* **116,** 96-108.

Sim, J. A., Chaumont, S., Jo, J., Ulmann, L., Young, M. T., Cho, K., Buell, G., North, R. A. and Rassendren, F. (2006) Altered hippocampal synaptic potentiation in P2X4 knock-out mice. *J Neurosci,* **26,** 9006-9009.

Sim, J. A., Park, C. K., Oh, S. B., Evans, R. J. and North, R. A. (2007) P2X1 and P2X4 receptor currents in mouse macrophages. *Br J Pharmacol,* **152,** 1283-1290.

Sim, J. A., Young, M. T., Sung, H. Y., North, R. A. and Surprenant, A. (2004) Reanalysis of P2X7 receptor expression in rodent brain. *J Neurosci,* **24,** 6307-6314.

Simmer, K. and Thompson, R. P. (1985) Zinc in the fetus and newborn. *Acta Paediatr Scand Suppl,* **319,** 158-163.

Smith, E. S., Zhang, X., Cadiou, H. and McNaughton, P. A. (2007) Proton binding sites involved in the activation of acid-sensing ion channel ASIC2a. *Neuroscience letters,* **426,** 12-17.

Smith, T. K., Kang, S. H. and Vanden Berghe, P. (2003) Calcium channels in enteric neurons. *Curr Opin Pharmacol,* **3,** 588-593.

Sokolova, E., Nistri, A. and Giniatullin, R. (2001) Negative cross talk between anionic GABAA and cationic P2X ionotropic receptors of rat dorsal root ganglion neurons. *J Neurosci,* **21,** 4958-4968.

Solomons, N. W. (1998) Mild human zinc deficiency produces an imbalance between cell-mediated and humoral immunity. *Nutr Rev,* **56,** 27-28.

Soudijn, W., van Wijngaarden, I. and AP, I. J. (2002) Allosteric modulation of G protein-coupled receptors. *Curr Opin Drug Discov Devel,* **5,** 749-755.

Stojilkovic, S. S. and Koshimizu, T. (2001) Signaling by extracellular nucleotides in anterior pituitary cells. *Trends Endocrinol Metab,* **12,** 218-225.

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,** 1837-1840.

Surprenant, A. (1996) Functional properties of native and cloned P2X receptors. *Ciba Found Symp,* **198,** 208-219; discussion 219-222.

Tollinger, M., Forman-Kay, J. D. and Kay, L. E. (2002) Measurement of side-chain carboxyl pK(a) values of glutamate and aspartate residues in an unfolded protein by multinuclear NMR spectroscopy. *J Am Chem Soc,* **124,** 5714-5717.

Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I. and Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron,* **21,** 531-543.

Torres, G. E., Egan, T. M. and Voigt, M. M. (1998) Topological analysis of the ATP-gated ionotropic [correction of ionotrophic] P2X2 receptor subunit. *FEBS Lett,* **425,** 19-23.

Varnum, M. D., Black, K. D. and Zagotta, W. N. (1995) Molecular mechanism for ligand discrimination of cyclic nucleotide-gated channels. *Neuron,* **15,** 619-625.

Vial, C., Tobin, A. B. and Evans, R. J. (2004) G-protein-coupled receptor regulation of P2X1 receptors does not involve direct channel phosphorylation. *Biochem J,* **382,** 101-110.

Virginio, C., Church, D., North, R. A. and Surprenant, A. (1997) Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. *Neuropharmacology,* **36,** 1285-1294.

Vlaskovska, M., Kasakov, L., Rong, W., Bodin, P., Bardini, M., Cockayne, D. A., Ford, A. P. and Burnstock, G. (2001) P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci,* **21,** 5670-5677.

Vorobjev, V. S., Sharonova, I. N., Sergeeva, O. A. and Haas, H. L. (2003) Modulation of ATP-induced currents by zinc in acutely isolated hypothalamic neurons of the rat. *Br J Pharmacol,* **139,** 919-926.

Waldmann, R. and Lazdunski, M. (1998) H(+)-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. *Curr Opin Neurobiol,* **8,** 418-424.

Wang, E. C., 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 Invest,* **115,** 2412-2422.

Wang, Y., Mackes, J., Chan, S., Haughey, N. J., Guo, Z., Ouyang, X., Furukawa, K., Ingram, D. K. and Mattson, M. P. (2006) Impaired long-term depression in P2X3 deficient mice is not associated with a spatial learning deficit. *J Neurochem,* **99,** 1425-1434.

Wildman, S. S., King, B. F. and Burnstock, G. (1997) Potentiation of ATP-responses at a recombinant P2x2 receptor by neurotransmitters and related substances. *British journal of pharmacology,* **120,** 221-224.

Wildman, S. S., King, B. F. and Burnstock, G. (1998) Zn2+ modulation of ATP-responses at recombinant P2X2 receptors and its dependence on extracellular pH. *Br J Pharmacol,* **123,** 1214-1220.

Wildman, S. S., King, B. F. and Burnstock, G. (1999a) Modulation of ATP-responses at recombinant rP2X4 receptors by extracellular pH and zinc. *Br J Pharmacol,* **126,** 762-768.

Wildman, S. S., King, B. F. and Burnstock, G. (1999b) Modulatory activity of extracellular H+ and Zn2+ on ATP-responses at rP2X1 and rP2X3 receptors. *Br J Pharmacol,* **128,** 486-492.

Wildman, S. S., Marks, J., Churchill, L. J., Peppiatt, C. M., Chraibi, A., Shirley, D. G., Horisberger, J. D., King, B. F. and Unwin, R. J. (2005) Regulatory interdependence of cloned epithelial Na+ channels and P2X receptors. *J Am Soc Nephrol,* **16,** 2586-2597.

Wilson, C. J., Apiyo, D. and Wittung-Stafshede, P. (2004) Role of cofactors in metalloprotein folding. *Q Rev Biophys,* **37,** 285-314.

Wolfe, D. M. and Pearce, D. A. (2006) Channeling studies in yeast: yeast as a model for channelopathies? *Neuromolecular Med,* **8,** 279-306.

Wright, J. M. and Li, C. (1995) Zn2+ potentiates steady-state ATP activated currents in rat nodose ganglion neurons by increasing the burst duration of a 35 pS channel. *Neuroscience letters,* **193,** 177-180.

Xiong, K., Peoples, R. W., Montgomery, J. P., Chiang, Y., Stewart, R. R., Weight, F. F. and Li, C. (1999) Differential modulation by copper and zinc of P2X2 and P2X4 receptor function. *Journal of neurophysiology,* **81,** 2088-2094.

Yoshimura, R. F., Hogenkamp, D. J., Li, W. Y., Tran, M. B., Belluzzi, J. D., Whittemore, E. R., Leslie, F. M. and Gee, K. W. (2007) Negative allosteric modulation of nicotinic acetylcholine receptors blocks nicotine self-administration in rats. *J Pharmacol Exp Ther,* **323,** 907-915.

Zatta, P., Lucchini, R., van Rensburg, S. J. and Taylor, A. (2003) The role of metals in neurodegenerative processes: aluminum, manganese, and zinc. *Brain Res Bull,* **62,** 15-28.

Zheng, J. Q. and Poo, M. M. (2007) Calcium signaling in neuronal motility. *Annu Rev Cell Dev Biol,* **23,** 375-404.

Zhorov, B. S. and Tikhonov, D. B. (2004) Potassium, sodium, calcium and glutamate-gated channels: pore architecture and ligand action. *J Neurochem,* **88,** 782-799.

Zhou, Z. (1998) *Molecular determinants of rectification and desensitization in the P2X b2 s-class of ATP-gated channels*.

Zhou, Z. and Hume, R. I. (1998) Two mechanisms for inward rectification of current flow through the purinoceptor P2X2 class of ATP-gated channels. *J Physiol,* **507 (Pt 2),** 353-364.