This is to certify that the
dissertation entitled

CROSS-INHIBITION BETWEEN P2X AND NACHR LIGAND-GATED ION CHANNELS IN THE ENTERIC NERVOUS SYSTEM

presented by

Dima Alkawwas Decker

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Biochemistry

Major Professor's Signature

5-11-09

Date

CROSS-INHIBITION BETWEEN P2X AND NACHR LIGAND-GATED ION CHANNELS IN THE ENTERIC NERVOUS SYSTEM

By

Dima Alkawwas Decker

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

DOCTOR of PHILOSOPHY

Biochemistry and Molecular Biology

2009

ABSTRACT

CROSS-INHIBITION BETWEEN P2X AND NACHR LIGAND-GATED ION CHANNELS IN THE ENTERIC NERVOUS SYSTEM

By

Dima Alkawwas Decker

The enteric nervous system (ENS) resides in the gut wall and contains neurons that can control gastrointestinal function independently of the central nervous system. The ENS contains two main types of ligand-gated ion channels that are important mediators of fast excitatory synaptic transmission; P2X and the nicotinic acetylcholine receptors (nAChRs). Although these channels are structurally distinct and have been assumed to function independently, recent studies have shown that these receptors have a mutually inhibitory interaction. Whole-cell patch clamp techniques were used to assess the function of $P2X_2$ and $\alpha_3\beta_4$ nAChRs in cultured myenteric neurons and P2X and nAChRs expressed separately and together in human embryonic kidney (HEK-293) cells, a heterologous system that permits molecular manipulations. These molecular aspects included a detailed look at interactions between these receptors in three different states; open, desensitized, and closed. This work provided evidence that $P2X_2$ and nAChRs cross-inhibition occurs in all three of these states, including constitutive inhibition between these receptors in the closed state. The specificity of this interaction was investigated among the different subtypes of the P2X receptors. Cross-inhibition occurred between $\alpha_3\beta_4$ nAChRs and the $P2X_3$ and $P2X_4$ receptor subtypes in open and desensitized states.

More specific molecular manipulations included the determination of the direct molecular mechanisms responsible for this interaction via the use of a truncated $P2X_2$ receptor with a stop codon to shorten the C-terminal tail. Studies with this truncated receptor provided evidence that the C-terminal tail of the $P2X_2$ receptor is important in mediating cross inhibition of $\alpha_3\beta_4$ nAChRs in the open and closed states. Furthermore, with the use of a purified protein construct corresponding to the C-terminal tail of the $P2X_2$ receptor, competitive experiments were performed that provide evidence for a physical interaction between the $P2X_2$ receptor and the $\alpha_3\beta_4$ nAChR. A better understanding of receptor interactions in enteric neurons would likely lead to novel drug targets that would be useful in the treatment of common gastrointestinal motility disorders such as irritable bowel syndrome.

To my mother, may she be proud of me.

ACKNOWLEDGMENTS

This dissertation is culmination of 5 years of work, and would not have been possible without the support of many people.

To my advisor, Jim Galligan, I thank you for your continued encouragement, guidance, support and patience. The ideal environment that you provided was crucial in my journey to becoming an independent investigator.

My committee members: Dr. John Lapres, Dr. Dave DeWitt, Dr. Eric Hegg, and Dr. Tim Zhacharewski for your time and effort.

I would like to thank my colleagues and friends in the Galligan lab for their help and for creating an amiable work environment.

I also need to thank my 'biochem' collegues. The Kodrofske lab for many thought provoking discussions. Dr. John Wang for his advice and generosity. And Patty Voss for her help with experiments, friendship and always finding the exact thing to say to make me laugh my face off.

Without the support of my family, this would have not been possible. I want to especially thank my husband Marc.

TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

Chapter 1: Introduction 1

1. **The enteric nervous system** 2
   - Overview 2
   - Anatomy of the enteric nervous system 3
   - Reflex circuitry of the enteric nervous system 5
2. **Synaptic transmission** 11
   - Overview 11
   - Slow synaptic transmission 12
   - Fast synaptic transmission 13
3. **Purinergic signaling** 16
   - ATP as an intercellular signaling molecule 16
   - Purinergic receptors 16
   - P2X receptors 17
   - Multimerization 18
   - Electrophysiological and pharmacological characteristic of heterologously expressed P2X receptors 18
   - P2X receptor subunit structure 20
4. **Cholinergic receptors** 25
   - Muscarinic cholinergic receptors 25
   - Nicotinic acetylcholine receptors (nAChRs) 26
   - Multimerization 27
   - Electrophysiological and pharmacological characteristic of heterologously expressed nACRs 28
   - nAChR subunit structure 39
5. **Cross-inhibition between nAChRs and P2X receptors** 34
6. **Research Goal** 35
7. **References** 37

Chapter 2: Hypothesis and Specific Aims 42

Chapter 3: Cross-inhibtion between nicotinic acetylcholine receptors and P2X receptors in myenteric neurons and HEK-293 cells 46

- Abstract 47
- Introduction 48
- Materials and Methods 51
- Results 55
- Discussion 72
- References 79

Chapter 4: Molecular mechanisms responsible for cross-inhibition **83**
Abstract 84
Introduction 86
Materials and Methods 88
Results 93
Discussion 105
References 111

Chapter 5: Concluding remarks and future perspectives **115**

LIST OF TABLES

Chapter 1.

Table 1.1: Neuron classification criteria 6

LIST OF FIGURES

Images in this thesis/dissertation are presented in color.

Chapter 1:

Figure 1.1 The anatomy of the enteric nervous system 4

Figure 1.2 Reflex circuitry of the enteric nervous system 8

Figure 1.3 Myenteric AH and S neurons 10

Figure 1.4 Fast excitatory synaptic transmission in myenteric neurons 15

Figure 1.5 Membrane topology of the $P2X_2$ receptor 22

Figure 1.6 C-terminal tail length of P2X subtypes 24

Figure 1.7 Membrane topology of nicotinic acetylcholine receptors 31

Chapter 3:

Figure 3.1 Cross- inhibition between P2X receptors and nAChRs in guinea pig ileum cultured myenteric neurons 56

Figure 3.2 Immunocytochemistry of HEK-293 cells 57

Figure 3.3 Comparison of pharmacological and functional properties of $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed by cultured myenteric neurons and HEK-293 cells. 59

Figure 3.4 Cross-inhibition between $P2X_2$ stable receptors and $\alpha_3\beta_4$ nAChRs expressed by HEK-293 cells 61

Figure 3.5 Cross-inhibition between $\alpha_3\beta_4$ nAChRs stable and $P2X_2$ receptors expressed by HEK-293 cells 62

Figure 3.6 Cross-inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed by HEK-293 cells is maintained when receptors were activated by agonist EC_{50} concentrations 64

Figure 3.7 ACh and ATP do not directly modulate $P2X_2$ receptor or $\alpha_3\beta_4$ nAChR function. 65

Figure 3.8 Cartoon illustrating P2X subunit C-terminal tail amino acid length differences among $P2X_2$, $P2X_3$ and $P2X_4$ subunits 66

Figure 3.9 P2X subunit C-terminal tail length does not alter cross-inhibition of $\alpha_3\beta_4$ nAChR function 68

Figure 3.10 Cross inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs is maintained during receptor desensitization 69

Figure 3.11 Cross inhibition between $P2X_3$ or $P2X_4$ receptors and $\alpha_3\beta_4$ nAChRs is maintained during receptor desensitization 71

Chapter 4:

Figure 4.1 Truncation of the $P2X_2$ C-terminal tail stops cross-inhibition between $P2X_2$ and nAChRs 94

Figure 4.2 Purification of GST-fusion construct 96

Figure 4.3 Cross-inhibition between nAChRs and $P2X_2$ receptors is mediated by the $P2X_2$ receptor C-terminal tail 98

Figure 4.4 Cross-inhibition between nAChRs and $P2X_2$ receptors in myenteric neurons is mediated by the $P2X_2$ receptor C-terminal tail 100

Figure 4.5 Constitutive inhibition of nAChRs 101

Figure 4.6 PPADS does not alter $P2X_2$ receptor mediated constitutive inhibition of $\alpha_3\beta_4$ nAChRs 103

Figure 4.7 nAChRs and $P2X_2$ receptors constitutively inhibit each other 104

LIST OF ABBREVIATIONS

ENS: Enteric nervous system

CNS: Central nervous system

ANS: Autonomic nervous system

GPCRs: G-protein coupled receptors

nAChRs: Nicotinic acetylcholine receptors

P2X: Purinergic 2X receptor

ACh: Acetylcholine

ATP: Adenosine triphosphate

fEPSPs: Fast excitatory post-synaptic potentials

sEPSPs: Slow excitatory post-synaptic potentials

HEK-293: Human embryonic kidney cells

DMEM: Dulbecco's modified Eagle's medium

EC_{50}: Half-maximal effective concentration

FRET: Fluorescence Resonance Energy Transfer

5-HT: 5-hydroxy tryptamine

GABA: gamma-Aminobutyric acid

GST: glutatione S-transferase

CHAPTER 1

Introduction

1. THE ENTERIC NERVOUS SYSTEM

Overview

The enteric nervous system (ENS) is a division of the autonomic nervous system (ANS) that resides in the intestinal wall and controls gastrointestinal function (22). The ANS is a system of neurons that control effectors that are not under voluntary direction and include all neurally regulated tissues and organs (23). The ANS innervates visceral organs of the thoracic, abdominal, and pelvic cavities including but not limited to the lungs, heart, digestive organs, kidneys, and the urinary bladder (23). Although parasympathetic and sympathetic nerves supply the intestine, the ENS can still function if these connections are severed. Therefore, the ENS can function independently of central nervous system (CNS) control because it contains all the network elements needed for reflex control of gastrointestinal function (29, 31). These elements include motor neurons, inter-neurons, and sensory neurons that respond to mechanical and chemical stimuli provided by intraluminal content (21).

The ANS is divided into three branches: the parasympathetic branch, the sympathetic branch, and the enteric nervous system. Langley (1903, 1921) provided three main features of the ENS branch. The ENS is a system of autonomic ganglia and nerve fibers that reside within the walls of digestive organs. First, enteric neurons are histologically different from other neurons of the ANS. Second, the autonomic pathways from the CNS that make connections with enteric ganglia are different from those made to other autonomic ganglion. Connections of the ANS are organized in a manner that CNS connections are directly proportional to peripheral connections; one presynaptic

neuron connects to one postsynaptic neuron in the ganglia. However, ENS connections differ in that CNS projections are not proportional to the connections in the ENS. More specifically, there are many fewer extrinsic nerve fibers that make connections to the ENS than there are enteric neurons. Lastly, there are complete reflex pathways that are capable of operating without any connections to the CNS which reside within the enteric division (38, 39). The ENS is the only substantial group of neurons that form circuits capable of complete autonomous reflex activity outside of the CNS. This reflex arc includes sensory, inter-neurons and motor neurons (21, 22).

Anatomy of the enteric nervous system

The digestive organs referred to as the tubular digestive tract include the esophagus, stomach, and the small and large intestines. The ENS in these organs is composed of an interconnected network of neurons and glial cells. These networks of nerve cells are found in two sets of ganglionated plexuses: the myenteric plexus and the submucosal plexus. Axons from these plexuses project to and innervate the two muscle layers of the ENS (longitudinal and circular) and the mucosa (22, 20).

The anatomic arrangement of these layers is in the following order from the serosal to the lumen side: longitudinal smooth muscle, myenteric plexus, circular smooth muscle, submucosal plexus, and mucosal layer (Figure 1.1). In the myenteric plexus, individual ganglia contain up to 100 neurons. Interganglionic connections contain axons which connect the ganglia together. Because the myenteric plexus is sandwiched between the

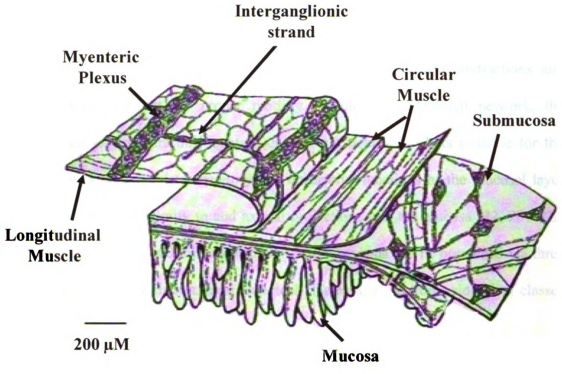


Figure 1.1: The anatomy of the enteric nervous system.

A diagram adapted from Furness and Costa 1987 illustrates the organization of the enteric nervous system in whole mounts of the intestine. From top to bottom: the serosal surface starts with the longitudinal muscle layer, followed by the myenteric plexus, circular muscle, submucosal plexus and the mucosal layer.

muscle layers with axonal projections, there is an anatomical basis for the myenteric plexus to have full control of both muscle layers. It is through its interganglionic connective strands that the myenteric plexus is able to coordinate contractions and relaxations along the gut for proper motility. In the other neuronal network, the submucosal plexus, ganglia only contain up to eight neurons. It is possible for the submucosal plexus to regulate secretory and absorptive functions of the mucosal layer due to its very close proximity to and axonal projections into the mucosa (22, 20). The ENS contains more than 100 million neurons. These neurons can be organized via three main criteria: functional classes, morphological classes and electrophysiological classes (Table 1.1).

Reflex circuitry of the enteric nervous system and functional classifications

Myenteric neurons are organized into a functional network that regulates and initiates the peristaltic reflex also known as motility. This reflex is activated via distension. A bolus of food in the intestine causes distension and activates the peristaltic reflex to relay information for coordinated contractions and relaxations. A bolus of food can only be propagated along the intestine due to a distension-evoked contraction at the oral side of the bolus followed by a relaxation at the anal side of the bolus. Repeated cycles of this process are what mediate propulsion of food from the beginning of the intestine down through the colon (22).

As mentioned earlier, the ENS can function without external nerve supply because it contains three functional classes of neurons needed for a complete reflex circuit: sensory neurons, interneurons, and motor neurons. When a bolus of food enters

Table 1.1: Neuron classification criteria

Functional definition	Morphology	Electrophysiological Properties
Sensory neuron (IPAN)	Dogiel type II	AH neuron
Interneuron	Dogiel type I	S neuron
Motor neuron	Dogiel type I	S neuron

The enteric nervous system contains over 100 million neurons. These neurons are organized via three main criteria: functional classes, morphological classes, and electrophysiological classes. The table lists the neuron types found in the guinea-pig small intestine.

the intestine, there is a distention in the intestinal wall. This distension activates mechanoreceptors that indirectly activate intrinsic primary afferent neurons (IPANS) at their nerve terminal (they can also be activated by chemical stimuli). IPANS can propagate the resulting action potential back to the cell body and relay the excitatory cascade by releasing neurotransmitters onto a connecting interneuron. It is from there that this cascade propagates to more interneurons, or activates motor neurons to cause contractions or relaxations by releasing excitatory or inhibitory neurotransmitters, respectively, onto the muscle (Figure 1.2; 22).

Morphological classifications

It is generally true that neurons in different species that have the same function also have the same shape; this is also true for neurons in the ENS. By using morphology as an indicator of equivalence of neurons of different species, ENS networks among difference species can be studied and compared. It is important to note, however, that neurons from different species do not have exactly the same shape according to function. Neurons in larger animals have more elaborate dendritic trees compared to smaller animals which contain less elaborate dendrites. This makes it less difficult to classify neurons in smaller animals such as guinea-pigs compared to humans (22).

The first classification of enteric neurons by their morphology was studied by Dogiel via methylene blue staining of neurons in the ENS. He provided a comprehensive description of neurons of the myenteric and submucosal plexes of the intestine from humans, guinea-pig, rabbit, rat, dog, and cat. These descriptions also included illustrations of guinea-pig and human samples. Dogiel demonstrated two main groups of enteric neurons based on soma shape, soma size and the number and distributions of

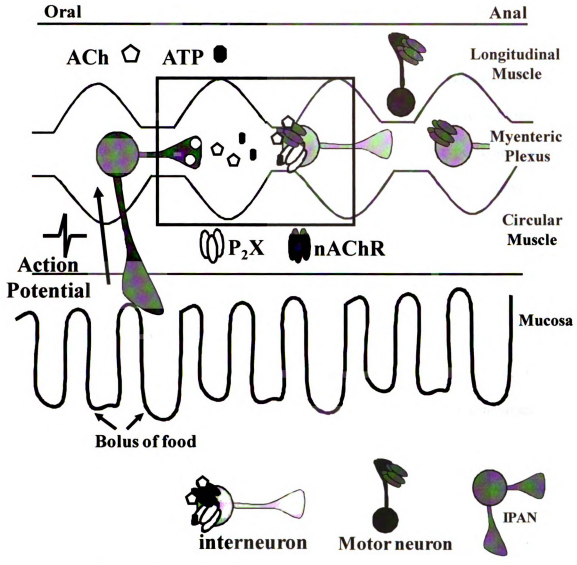


Figure 1.2: Reflex circuitry of the enteric nervous system.

A diagram of the proposed model of the reflex circuitry of the enteric nervous system responsible for mediating the peristaltic reflex. Intraluminal mechanical or chemical stimulation indirectly activates intrinsic primary afferent neurons (IPANs , dark grey) nerve terminals. This results in an antidromal action potential that results in the release of neurotransmitters onto connecting interneurons. Interneurons (light grey) activation results in the subsequent stimulation of motor neurons (black) to either contract or relax the muscle. IPANS are AH neurons, while interneurons and motor neurons are S neurons.

dendrites. Therefore, the morphological classification scheme of enteric neurons is named after him. Dogiel type I neurons are flat with stellate or angular shapes with cell bodies ranging between 13-35 μm in length and 9-22 μm in width (Figure 1.3A; 58). They have one axon (unipolar) and contain 4-20 short dendrites. These neurons have been concluded to be motor and interneurons of the ENS. Dogiel type II neurons are angular, star or spindle shaped with multiple axonal (multiaxonal) projections to the mucosa and adjoining neurons. With long axes of 22-47 μm and short axes of 13-22 μm, these neurons are recognized as sensory neurons (Figure 1.3B; 22, 58).

Electrophysiological classifications

Due to their electrophysiological characteristics, neurons in the reflex arc can be divided into two types: AH neurons and S neurons. AH neurons are multi-axonal neurons (also referred to as Dogiel type II neurons; Figure 1.3B) in which a distinctive component of the action potential is mediated by Ca^{2+}. AH neuron action potentials are immediately followed by a characteristic delayed and prolonged afterhyperpolarizing (AHP) potential that lasts 1-20s (27, 58). As a result of the Ca^{2+} mediated shoulder, AH neuron action potentials are only partially blocked by the sodium channel blocker tetrodotoxin (TTX). Although most AH neurons only receive slow synaptic input, some AH neurons receive fast synaptic input (see synaptic transmission). Therefore, trains of stimuli elicit slow excitatory potentials (sEPSPs) from all AH neurons while single electrical stimuli fail to elicit a synaptic response in most AH neurons. All sensory neurons (IPANS) of the reflex arc are AH neurons (22, 21, 7, 10, 27, 25).

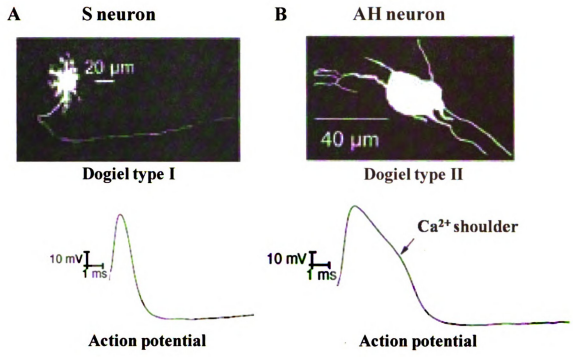


Figure 1.3: Myenteric AH and S neurons

A. Representative characteristics of S neuron morphology and electrophysiological properties. S neurons are uniaxonal with multiple short dendrites. Single stimuli elicit action potentials in S neurons that are tetrodotoxin sensitive. *B.* Representative characteristics of AH neuron morphology and electrophysiological properties. AH neurons are multiaxonal. Action potentials in AH neurons have a calcium shoulder making them insensitive to tetrodotoxin, and a long-lasting afterhyperpolarization.

S neurons (also referred to as Dogiel type I neurons; Figure 1.3A) are interneurons and motor neurons in this reflex arc. These neurons are uniaxonal, and lack the Ca^{2+} and AHP components of the action potential, and they are therefore completely blocked by TTX. Moreover, S neurons receive fast excitatory postsynaptic potentials (fEPSPs) that are generally not observed in AH neurons. Single electrical stimuli elicit fEPSPs in S neurons, while only some sEPSPs are elicited due to short trains of electrical stimuli. Sensory processing of information in the intestine is mostly due to fast synaptic transmission between these neurons (22, 21, 7, 10, 25, 27).

2. SYNAPTIC TRANSMISSION

Overview

Neurons communicate with each other through synaptic transmission, a process mediated via specialized neuronal structures called synapses. A synapse is composed of a presynaptic cell that is separated from a postsynaptic cell by a narrow cleft filled with extracellular fluid preventing direct electrical communication between the cells (32, 34, 56, 42).

Pre-synaptic actions. Cells communicate via neurotransmitters, these are small hydrophilic molecules that are synthesized and stored in vesicles by neurons (51). Release occurs due to the propagation of an action potential resulting in depolarization of the nerve terminal. This activates calcium channels resulting in an influx of calcium, and triggers the exocytosis of the content of neurotransmitter-containing vesicles clustered near the nerve terminal membrane. The molecules of neurotransmitter diffuse across the

synaptic cleft and can bind to post-synaptic receptors. Some neurotransmitter molecules are recovered via uptake through specialized transporters, while other molecules will be degraded via enzymes located in the synaptic cleft (32, 34, 56, 42). The **post-synaptic actions** of chemical synaptic transmission are classified into two main types: slow and fast synaptic transmission.

Slow synaptic transmission

Slow synaptic transmission occurs via neurotransmitters acting at G-protein coupled receptors (GPCRs). GPCR signaling is mediated by activating excitatory or inhibitory interactions with intracellular heteromeric G-proteins (Bridges & Lindsley 2008). This activation alters the membrane potential of the post-synaptic cell. GPCR family members are membrane proteins with an extracellular N-terminus, seven transmembrane domains, and an intracellular C-terminus (35). The large third intracellular loop of the receptor binds G-proteins which are trimeric containing α, β, and γ subunits. Agonist binding induces conformational changes which are thought to involve at minimum the rearrangement of membrane helices 6 and 3. The activated receptor now has a greater binding affinity for the G-protein and serves as a guanine-nucleotide exchange factor to promote GDP dissociation, and GTP binding and activation. The activated G-protein complex dissociates into Gα and Gβγ subunits. These subunits can now bind their respective downstream effectors including kinases, phosphatases, small GTPases, and ion channels. Gα-proteins include: G_s coupled to stimulation of adenylyl cyclase; G_i coupled to inhibition of adenylyl cyclase; G_q coupled to the activation of phospholipase C; and G_o which activates K^+ and Ca^{2+} channels. Gβγ

subunits can also directly activate channels and kinases. Hydrolysis of GTP to GDP by the regulator of G-protein signaling proteins leads to the re-association of the heterotrimer and termination of the activation cycle (13, 9, 50, 33).

GPCRs mediate slowly developing responses (latency 50 ms - 2s) of long duration (many seconds to minutes). GPCRs have latency in response because they initiate a multi-step process that begins with agonist binding and depends on intracellular signaling pathways. GPCRs mediate slow excitatory transmission, an important signaling mechanism of the ENS (27). Some examples of slow synaptic transmission in the ENS are acetylcholine acting at muscarinic M_1 receptors, substance P acting at $NK\text{-}_3$ receptors, 5-HT acting at $5\text{-}HT_{1P}$ receptors, and ATP acting at P2Y receptors. Activation of these receptors results in a decrease in potassium conductance and an increase in chloride conductance in myenteric neurons (25).

Fast synaptic transmission and ligand-gated ion channels in myenteric neurons

The second class of synaptic transmission in the ENS is ligand-gated ion channel mediated fast synaptic transmission (25). Ligand-gated ion channels are membrane proteins composed of multiple subunits in which the ligand binding site and the ion channel are part of the same complex, forming an ionophore. Fast synaptic transmission in the ENS is always excitatory. Since second messenger molecules are not needed, such channels mediate a fast response (latency < 1 ms) with a short duration (< 100 ms). Ligand-gated ion channels are localized to the somatodendritic region of the neuron where they mediate fast excitatory synaptic transmission, and also to nerve endings where they couple to the release of other excitatory neurotransmitters (25, 26, 27).

Several ligand-gated ion channels mediate fEPSPs in myenteric neurons, including cholinergic nicotinic acetylcholine receptors (nAChRs), purinergic P2X receptors, and serotinergic 5-HT_3 receptors (26). These channels are localized to S type neurons and receive synaptic inputs from AH neurons and other S neurons for reflex activation (see Figure 1.2). Moreover, the predominant mechanisms of excitatory fast synaptic transmission of myenteric neurons of the myenteric plexus are mediated by nAChRs and P2X receptors (22).

Studies performed by Galligan and Bertrand (24) using intracellular electrophysiology methods to record from isolated preparations of guinea pig ileum myenteric neurons maintained *in vitro* have shown that fast excitatory post-synaptic potentials (fEPSPs) recorded from 25% of neurons are fully blocked by hexamethonium (a nAChR antagonist). However, at 67% of neurons, hexamethonium only partially blocked fEPSP responses, and complete inhibition was only established after the addition of pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), a P2X receptor blocker. This suggests that ATP and ACh are co-released by enteric neurons, and nAChR and P2X are both located at the same synapse (24, 26; Figure 1.4).

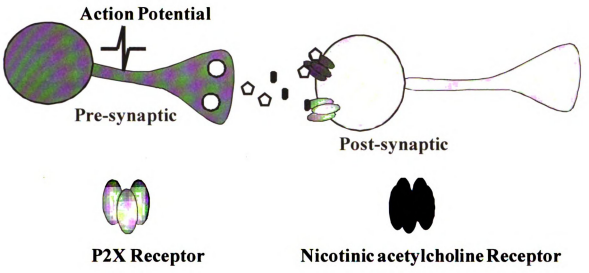


Figure 1.4: Fast excitatory synaptic transmission in myenteric neurons.

A depiction of fast synaptic transmission synapse in the myenteric plexus. In 67% of myenteric neurons, fast excitatory post-synaptic potentials (fEPSPs) are mediated by P2X and nicotinic acetylcholine receptors (nAChRs). This suggests that these receptors are co-expressed by S type neurons of the myenteric plexus. In this type of synaptic transmission, neurotransmitter would be released from the pre-synaptic cells to act on their corresponding receptors of P2X and nAChR on the post-synaptic cell membrane.

3. PURINERGIC SIGNALING

ATP as an intercellular signaling molecule

The first concept of ATP as a neurotransmitter, and not just as an energy carrier for cellular metabolism, was proposed in 1972 by Burnstock after studying non-adrenergic (norepinephrine) and non-cholinergic (acetylcholine) nerve mediated transmission in the gut and urinary bladder (30). There are many possible sources of extracellular ATP. ATP can be released from injured cells as might occur under hypoxia or inflammation (17, 6). ATP in millimolar concentrations is also stored inside vesicles and granules of many neuronal and non-neuronal cells (62). These cells provide large amounts of extracellular ATP from exocytotic release (16, 18). Once ATP is released into the extracellular space, it is hydrolyzed into ADP by the membrane-bound enzyme ecto-ATPase. Another enzyme ecto-diphosphohydrolase (ecto-apyrase) is capable of hydrolyzing both ATP and ADP further down the cascade to AMP. AMP is then hydrolyzed by 5'-nucleotidase to adenosine. Both AMP and adenosine are ligands for P1 receptors as mentioned above (62, 63). ATP acts at all P2 receptors; however, this research will focus on ionotoropic P2X receptors as mediators of fast synaptic transmission in the ENS.

Purinergic receptors

In 1978 purinergic receptors were divided into two pharmacological categories by Burnstock (11, 12). P1 for adenosine gated receptors and P2 for ATP/ADP gated receptors (11, 1). All P1 adenosine receptors are metabotropic GPCRs that have been divided into four cloned subtypes, A_1, A_{2A}, A_{2B}, and A_3 (4). It was not until 1985 that

there was a proposal distinguishing two types of P2 receptors: P2X and P2Y. There have been eight P2Y receptor subtypes identified to date, all of which belong to the GPCRs family. The P2X receptor family are all ligand-gated ion channels of which seven subtypes have been cloned so far (11, 1, 12).

P2X receptors

ATP-gated P2X receptors are a family of receptors that can mediate fast-synaptic transmission in the nervous system. P2X receptors can be composed of seven ($P2X_1$-$P2X_7$) different subunits activated by the neurotransmitter ATP (55, 18). The different P2X subunits have physiologically distinct distributions. The rat $P2X_1$ receptor was isolated by expression cloning of the vas deferens smooth muscle, with abundant expression in smooth muscle preparations. $P2X_2$ receptors are widely expressed in the nervous system, including but not limited to myenteric neurons, mouse and rat celiac neurons, and dorsal horn neurons. $P2X_3$ receptors have been observed in sensory neurons and myenteric neurons, and they are believed to mediate pain sensations. $P2X_4$ receptors are extensively found in the CNS. $P2X_5$ receptors are present in some parts of the brain, heart spinal cord, adrenal medulla, and lymphocytes. $P2X_6$ receptors cannot form homomers and are believed to exist as heteromers with other subtypes. $P2X_7$ receptors are localized to immune cells and rat brain (37, 48). These subunits range in size between 379 ($P2X_6$) and 595 ($P2X_7$) amino acids in length and present between 30 to 50% pair-wise identity in sequence (49, 37).

Multimerization

The stoichiometry of P2X receptors involves three subunits, thus the channel could be a homo- or heterotrimer. Homomer association of the P2X subtypes lead, in most of the receptors, to a functional ion channel and thus is the preferred state to study the pharmacological properties of the receptors. For when the receptors form as heteromers, some P2X subtypes dominate the pharmacological properties of the other subtypes. All P2X subtypes can form homomers, except $P2X_6$ which can only complex with other subtypes to form a functional channel. Conversely, $P2X_7$ receptors can only form functional receptors as homomers, and do not mix with other subtypes (48, 49, 6).

Pharmacological studies have provided evidence of heteromeric receptors including: $P2X_{2/3}$ receptors in sensory neurons and ganglion cells (49, 28), $P2X_{4/6}$ receptors in CNS neurons (40), $P2X_{1/5}$ receptors, and $P2X_{2/6}$ receptors (54, 49). P2X subunits have different pharmacologic characteristics. For example, ATP activates all the P2X subtypes, but α,β-methylene ATP is a more potent agonist at $P2X_1$ and $P2X_3$ receptors. These pharmacological differences coupled with the different biophysical properties of these channels are characteristics used to identify the composition of these receptors in native and heterologous tissues (54, 6).

Electrophysiological and pharmacological characteristics of heterologously expressed P2X receptors.

P2X receptors are non-selective cation channels with equal permeability for Na^+, K^+, and even greater permeability for Ca^{2+}. ATP binding activates P2X receptors within

a few milliseconds, with important kinetic differences among the different subtypes (48). The continuous presence of agonist results in desensitization. The kinetics of desensitization are contingent upon the time duration of agonist application. Heterologously expressed $P2X_1$ and $P2X_3$ receptors desensitize in the presence of ATP with time constants in the 100-300 ms range, whereas $P2X_2$ and $P2X_4$ receptors show sustained currents with a very slow desensitization (time constant of seconds to tens of seconds), occurring more rapidly in $P2X_4$ subunits. $P2X_7$ receptors show no desensitization during application lasting for many seconds (48, 37, 49).

All P2X receptors are non-selective cation channels with equal permeability for Na^+, K^+, and even greater permeability for Ca^{2+}. However, different subtypes display variable permeability properties. Homomeric $P2X_1$ and $P2X_4$ receptors present a high Ca^{2+} permeability (P_{Ca} / P_{Na} ~ 4), while permeability through $P2X_2$ receptors is lower (P_{Ca}/P_{Na} 2.5), and lowest at $P2X_3$ receptors with P_{Ca}/P_{Na} 1.2 (37, 49). At the whole cell level, the currents induced by ATP show an inward rectification; this is due to a decrease in the open probability of channels at more positive membrane potentials. This current-voltage relationship also has a reversal potential of 0 mV, which is indicative of non-selective cation permeability (49).

Agonist and antagonist pharmacology of P2X receptors. All cloned P2X receptors are activated by ATP. Receptor subtype half maximal effective concentrations (EC_{50}) vary from 1 μM for $P2X_1$ and $P2X_3$ to ~10 μM for $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$. The EC_{50} for $P2X_7$ receptors is 100 μM (48).

There are other P2X agonists that are ATP analogs, α,β-methylene-ATP (α,β-meATP), 2-methylthio-ATP (2MeSATP), and 2',3'-(4-benzoyl)-benzoyl ATP (BzATP). α,β-meATP is a selective agonist for $P2X_1$ and $P2X_3$ subunit containing receptors, while BzATP is a selective agonist for $P2X_7$ receptors (37, 48, 49). The two main antagonists for P2X receptors are suramin and PPADS. There are different sensitivities among the subtypes to both antagonists. $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_5$ are blocked by suramin and PPADS, while $P2X_4$ and $P2X_7$ are not (59, 37, 48, 49).

P2X receptor subunit structure

Each subunit of P2X receptors has two transmembrane domains (TM1 and TM2) with intracellular N and C-terminal tails and a large extracellular loop (49). This topology was determined by hydrophobicity plots and was confirmed by glycosylation site determination and antibody identification towards epitope tags at the N- and C-terminus that were only accessible due to permealization of the membrane (49, 37). Since the main focus of this research is on myenteric neurons which mainly express $P2X_2$ receptors, this work will mainly discuss the molecular physiology of $P2X_2$ receptors (Figure 1.5).

The amino-terminal tail. The N-terminal domains of P2X subunits are short (ranging between 20 – 30 amino acids in length) relative to their C-terminal domains. A consensus protein kinase C (PKC; Thr-X-Lys/Arg) site is present in the amino-terminus of all P2X subunits. This site is especially important for $P2X_2$ subunit function; mutation of the Thr18 amino acid prevents phosphorylation by PKC and results in rapidly desensitizing kinetics. This disrupts its typical slow desensitizing characteristics (as

explained above; 37, 8). Mutations of residues Asp15, Pro19, Val23, and Val24 in the amino-terminal via cystiene-scanning mutagenesis indicated that methenethiosulfonates binding to free cysteine recidues resulted in 60% inhibition of current, while mutations at Tyr-16 resulted in a non-functional channel. These results indicate the importance of the *intracellular* N-terminals in P2X channel conductance and function (49).

The extracellular loop. This domain is the bulk of P2X receptors at 280 amino acids in length (amino acid 50-330 of the rat $P2X_2$ receptor), and it presents a high degree of homology among the seven different mammalian P2X subtypes. It contains 10 conserved cysteines and 6 conserved lysines (two of which compose the binding site for ATP see below). Also, depending on the P2X subtype, there are two to six putative N-linked glycosylation sites (three sites for rat $P2X_2$ at asparagines 182, 239 and 298) that help channel function and proper insertion into the membrane (49, 37). The extracellular loop domain is believed to contain the binding site for ATP and antagonists. The lysine residue at position 246 in $P2X_2$ receptors has been determined to be crucial for proper binding of PPADS, while alanine scanning mutagenesis techniques indicate that lysine residues at positions 69 and 71 are critical for ATP binding (37).

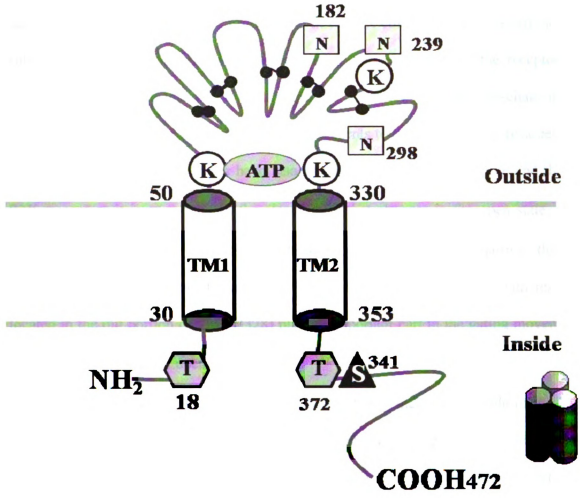


Figure 1.5: Membrane topology of the $P2X_2$ receptor.

Each subunit of P2X receptors has two transmembrane domains (TM1 and TM2) with intracellular N and C-terminal tails and a large extracellular loop. The N-terminal domain contains a protein kinase C site at Thr18 (●) that mediates channel kenetics. The extracellular loop contains 10 conserved cystiene residues (●) and lysine residues (○) that mediate the binding site of ATP and the anagonist PPADs, along with three glycosylation (□) sites. The C-terminal of the P2X2 receptor contains protein kinase A (▲) and protein kinase C (●) sites that mediate channel kenetics. P2X interactions with other proteins are believed to be mediated by the C-terminal tail.

The pore. The channel pore is composed of two transmembrane domains (TM1 and TM2) which span the plasma membrane in α-helix conformation as determined by substituted cysteine accessibility. This method is where residues of the receptor are mutated to cysteine residues individually and the accessibility of the side-chain in the expressed channels are probed with modifying reagents to determine which residues line the pore of the receptor. Studies have also shown that TM2 lines the pore and that in $P2X_2$ receptors it is an α-helix in the closed state that changes in the open state. The narrowest part of the pore is at position 342 at a glycine residue about halfway through the TM2 (37). Also, Val48 located in the outer edge of TM1 together with residues located in the out portion of TM2 (I328) play an important part in the gating movements of $P2X_2$ receptor channel opening (49).

The carboxy-terminal tail. The C-terminal tail is the most variable of the P2X structure among different subtypes. It varies in amino acid composition and length with the shortest for the $P2X_4$ receptor (30 amino acids) and the longest for the $P2X_7$ receptor (240 amino acids; Figure1.6). The C-terminal tail of $P2X_2$ receptors contains a consensus phosphorylation site for cyclic AMP (cAMP)-dependent protein kinase A (PKA, S431), phosphorylation results in inhibition of current by increasing desensitization of the channel. There is also another possible PKC site at T372 (37, 49).

These sites seem to play a stabilizing role on the slow phenotype of the $P2X_2$ receptor (8). For truncations, deletions, mutations, and splicing of the C-terminal domain greatly affect the kinetics, permeation and desensitization of the channel (37). In addition, it seems that the C-terminal tail of the P2X receptors is responsible for its interactions with other proteins (37).

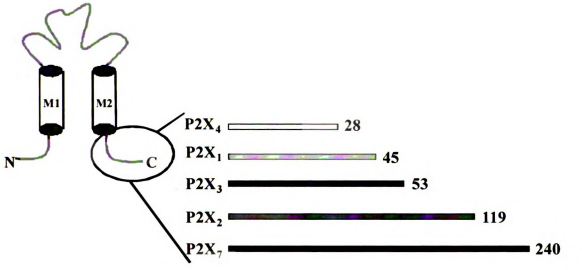


Figure 1.6: C-terminal tail length of P2X subtypes.

The C-terminal tail is the most variable of the P2X structure among different subtypes. It varies in amino acid composition and length with the shortest for $P2X_4$ (30 amino acids) and the longest for the $P2X_7$ receptor (240 amino acids).

4. CHOLINERGIC RECEPTORS

Cholinergic signaling is mediated by the neurotransmitter acetylcholine (ACh). Acetylcholine is synthesized in nerve terminals from acetyl coenzyme A (acetyl CoA) and choline in a reaction catalyzed by choline acetyltransferase (ChAT), an enzyme which exist in soluble form in the nerve terminal cytoplasm (64, 51). Choline is transported into cholinergic neurons by a high affinity transporter (this is the rate limiting step of the reaction), while acetyl CoA is produced by mitochondria. After synthesis, ACh is packaged into vesicles at the synapse via a vesicular ACh transporter (VAChT) due to the proton gradient established by the proton pumping activity of a synaptic vesicle-located vacuolar ATPase (64). Once released into the synapse, ACh is hydrolyzed via acetylcholinesterase into acetate and choline; choline is then transported back into the terminal for more ACh synthesis (51).

Adolf von Beayer was the first to discover choline, and he prepared ACh in 1867. It was not until 1914 that ACh was discovered in ergot-containing plant material by Dale and Ewins, and deduced to be a possible neurotransmitter. Many years later, in 1926, Loewi provided strong evidence that ACh was the transmitter mediating parasympathetic transmission in the frog heart (64). ACh acts at both muscarinic acetylcholine receptors and nicotinic acetylcholine receptors (nAChRs). However, this work will focus on nAChRs and their role in fast synaptic transmission.

Muscarinic cholinergic receptors

ACh produces its diverse physiological actions by binding and activating two structurally and functionally distinct families of receptors. Muscarinic receptors

(mAChR) are GPCRs and encompass the metabotropic side of ACh mediated transmission. Cloning studies have revealed five distinct mammalian subtypes M_1-M_5 (57). These five subtypes are divided into two major functional classes according to their G-protein coupling affinities. M_1, M_3, and M_5 receptors are selective for the G_q/G_{11} G-protein which couples to activation of phospholipase C (PLC) and generation of inositol triphosphate and diacylglycerol (DAG). DAG activates PKC which can phosphorylate a variety of intracellular targets, including ion channels. The M_2 and M_4 receptor subtypes activate G_i G-protiens which are coupled to inhibition of adenylate cyclase activity. Slow synaptic excitatory transmission in the ENS is mediated by M_1 receptors (57, 58, 25). Fast excitatory synaptic transmission in the ENS is mediated by ACh acting at nicotinic acetylcholine receptors (nAChRs; 25, 27,28).

Nicotinic acetylcholine receptors (nAChRs)

nAChRs do not only bind acetylcholine, but are also activated by nicotine (an alkaloid extracted from tobacco plants, *Nicotiana tabacum)* and is antagonized by curare. Curare is a mixture of plant toxins from *Chondodendron tomentosum* adopted by South American Indians who used it as an arrowhead poison to immobilize their quarry (51). Using these resources, it was Langley's work in 1901 that first revealed the idea of the nAChR. He reported that nicotine stimulated sympathetic nerve cells by 'direct action upon them'. In 1905 Langley reported that curare acts on the same 'receptive substance' as nicotine, and antagonizes its effects; what we now simply call a receptor (5). The cloned subunit of the nAChR was the ACh binding α-subunit from the electric organ of *Torpedo*, this electric ray produces and electric pulse to stun its prey. This electric organ

was a great source for receptor purification since nAChRs comprised 40% of the total protein of this organ (3). This was the first primary structure of any vertebrate ion channel protein to be purified (64).

nAChRs belong to the cys-loop superfamily of ligand-gated ion channels. This family comprises both excitatory cation conducting (nAChR and 5-hydroxytryptamine receptors 5-HT_3) and inhibitory Cl^- conducting channels (γ-aminobutyric acid receptor $GABA_A$ and the glycine receptor). Cys-loop family members are composed of five subunits (pentameric), each of which has four transmembrane domains and *extracellular* N and C-terminal tails (44). Nicotinic acetylcholine receptors can be subdivided into muscle and neuronal types.

Multimerization

Muscle types of nAChRs consist of five subunits, two α_1 subunits, one β_1 subunit, one γ subunit and either one δ subunit, or one ε subunit. Muscle receptors are usually expressed postsynaptically (3, 64). This work will only focus on neuronal types of this receptor. Neuronal nAChRs are heteromeric pentamers composed of two α and three β subunits. There are eight different neuronal α (α_2- α_9) and three different neuronal β (β_2- β_4) subunits (43). In the brain, neuronal nAChR receptors have a presynaptic and modulatory function by regulating the release of both ACh and other neurotransmitters as autoreceptors via increasing the influx of Na^+ and especially Ca^{2+} ions (16; 64). CNS expressed subtypes include $\alpha_4\beta_2$, $\alpha_6\beta_3$, α_7, α_9, α_5, and α_2 (14).

In the peripheral nervous system, including the ENS, the prominent subtype of expression is $\alpha_3\beta_4$ and these receptors mediate fast excitatory transmission (64, 14). This

subtype will be the focus of this research. The electrophysiological and pharmacological properties of nAChRs are determined by their specific subunit composition (64, 14, 3).

Electrophysiolocial and pharmacological characteristics of heterologously expressed nAChRs

The predominant mechanism of excitatory neurotransmission in the ENS is via nerve released ACh acting at nAChRs (25, 27, 28). In fEPSPs recorded from myenteric plexus S neurons, 25% of neurons were purely cholinergic, and 67% of neurons were a mix of purinergic and cholinergic signaling (26). ACh activates $\alpha_3\beta_4$ nAChRs with a very short latency of 2 ms, with fastly desensitizing characteristics, within 4 seconds the response is desensitized by 80% due to steady-state application of ACh (25, 61). ACh-induced responses in myenteric neurons have an inwardly rectifying current-voltage relationship with a reversal potential near 0 mV. This is a characteristic of non-selective cation channels; nAChRs are permeable to Na^+, K^+, and Ca^{2+} with a P_{Ca}/P_{Na} of 1 (61, 27, 19).

Data from immunocytochemical and pharmacological studies done in cultured myenteric neurons suggest that S neurons do not express α_7 subunits, but instead express $\alpha_3\beta_4$ subunits. Although fEPSPs can be recorded from AH neurons, the amplitude of nAChR mediated currents where smaller in amplitude from AH neurons compared to current amplitudes recorded from S neurons. This suggests that AH neurons express fewer nAChRs than S neurons (27, 26)

Agonist and antagonist pharmacology of nAChRs. All nAChRs are activated by ACh application; however, there are other agonists for nAChR, such as nicotine,

DMPP, and cytisine. Cytisine is only a full agonist at nAChRs containing β_4 subunits. Concentration response curves in cultured myenteric neurons revealed that DMPP is the most potent at $\alpha_3\beta_4$ receptors with an EC_{50} of 44 μM, while ACh and nicotine where equipotent at 200 μM, and the least potent agonist was cytisine at 400 μM. These pharmacological studies provided evidence that myenteric neurons contain β_4 subunits (61).

Different nAChR subtypes also have different antagonist. α-bungarotoxin (a southeast Asia snake *Bungarus multicinctus* venom that binds receptors in the neuromuscular junction causing paralysis and death) only binds α_7 subtypes. α-bungarotoxin has no effect on nAChRs in the ENS, suggesting that α_7 subunits are not expressed there (27, 3). Other antagonists include hexamethonium, mecamylamine, and DHβE, with mecamylamine inducing the most potent antagonism at β_4 subunits, and DHβE for β_2 subunits. Half-maximal inhibition concentration values (IC_{50}) of antagonists in myenteric neurons were in the following rank order potency: mecamylamine > hexamethonium > DHβE (27, 61). These data suggest that $\alpha_3\beta_4$ nAChRs are the most prominently expressed subtype in the ENS.

nAChR subunit structure

The membrane topology of nAChRs (a member of the cys-loop superfamily) differs from that of P2X receptors. Cys-loop family members are composed of five subunits (pentameric), each of which has four transmembrane domains and extracellular N and C-terminal tails (44; Figure 1.7). They are referred to as cys-loop due to the characteristic loop which contains a conserved sequence of 13 residues flanked by a

disulfide bond between two cysteine (Cys) residues that is important for complete nAChR assembly (65, 15).

Since 2001, structural studies have described the cys-loop members in general with an emphasis on nAChR structure. The first step was the elucidation of the X-ray crystal structure of the *molluscan Lymnaea stagnalis* ACh-binding protein AChBP at 2.7 Å resolution. This protein aligns with the extracellular domains of nAChR at 24%, and consists of a soluble homopentamer of 210 amino acid subunit. In the *molluscan* synaptic cleft, AChBP is secreted from perisynaptic glial cells to the cleft under conditions of active neurotransmission, and captures ACh molecules, therefore diminishing or terminating synaptic transmission (65). The AChBP structure along with a subsequent 4 Å resolution electron microscopy study of the *Torpedo* nAChR were used to create the refined 4 Å model of the whole receptor in its closed state. In this model, the receptor was shown to have a length of 160 Å, a diameter of 80 Å, and a vestibule 20 Å in diameter (65, 15).

The extracellular domains. The amino terminal, an extracellular linker between M2 and M3 (transmembrane domains) and the caboxy terminal of this receptor form the extracellular domains. These extracellular domains of the receptor contain the ligand binding site, glycosylation sites, and the 15 residue Cys-loop. The extracellular domain is organized with 10 β-sheet structures connected via the Cys-loop and an amino α- helix. This region contains many loop regions important for receptor function and assembly. The ligand binding site is mainly mediated by the α subunits, but has been shown to be formed at the interface between two subunits, with a "principle component" from α subunits, and a "complementary component" via a non-α subunits (2, 65, 41, 15). The

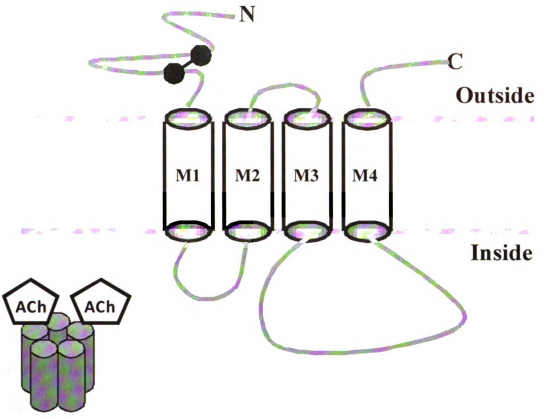


Figure 1.7: Membrane topology of nicotinic acetylcholine receptors.

Nicotinic acetylcholine receptors belong to the Cys-loop superfamily of receptors. Cys-loop family members are composed of five subunits (pentameric), each of which has four transmembrane domains and extracellular N- and C-terminal tails. They are referred to as Cys-loop due to the characteristic loop with contains a conserved sequence of 13 residues flanked by a disulfide bond between two cysteine (Cys; ●) residues that are important for complete nAChR assembly.

binding of agonist is mediated by five conserved aromatic side chains referred to as the 'aromatic box' that serves to capture the agonist (41).

Dose-response relationships for agonist activity have a Hill coefficient approaching two, indicating that the channel is activated by two agonist molecules; one for each α subunit (41, 15). Ligand binding causes a rotation of part of the extracellular binding region around an axis that passes the cys-loop via a series of interactions. The Cys-loop makes contact with a β1-β2 loop and the M2-M3 transmembrane domain linker that projects above the membrane. The β1-β2 also interacts with residues near the M2 C-terminal domain. All these events result in the M2-M3 linker causing the M2 region to move opening the channel (41, 65).

The pore. Each subunit is formed by 4 highly hydrophobic α- helix segments termed M1, M2, M3 and M4. The M2 transmembrane α-helix contributes to the pore, while M1, M3 and M4 are in contact with the lipid membrane (2). The outer portion of the vestibule that is exposed to the extracellular portion of the membrane is lined with negatively charged residues on the α subunit (residues Glu13, Asp44, Glu51, Asp71) and β subunits (residues Asp84, Asp 89, Asp 97, Glu 45, Glu82, Glu100) promoting a cation-stabalizing environment and explains the vestibules selectivity for cations (65). The pore is maximally constricted in the middle of the membrane (~6 Å) due to side chain interactions between hydrophobic residues of neighboring helices, forming a hydrophobic girdle or gate with major contributions by Leu 251 and Val 255 of the M2 transmembrane domain (2, 65) .

Intracellular loop. This main hydrophilic portion of the receptor consists of a large cytoplasmic loop between M3 and M4. This loop varies in length between subunits

(110-270 amino acids) and contains a curved α- helix. There are many phosphorylation sites contained within this loop which modulate receptor function via desensitization, and expression. Moreover, this portion of the receptor is involved in governing channel kinetics and mediates protein-protein interactions (2, 65). These interactions include binding with the cytoskeleton to induce receptor clustering. Also, it has been demonstrated that residues in the large cytoplasmic loop near the intracellular end of M4 affect the assembly of subunits and electrophysiological properties of nAChRs (65).

5. CROSS-INHIBITION BETWEEN NICOTINIC ACETYLCHOLINE AND P2X RECEPTORS.

P2X receptors and nAChRs are co-expressed by guinea-pig coeliac ganglion neurons (53, 52), rat pheochromocytoma PC12 cells (45), myenteric neurons, (60) and rat superior cervical ganglion neurons (46). P2X receptors and nAChRs are very structurally distinct (Figure 1.5 and Figure 1.7), and they have therefore been assumed to function independently. However, studies have shown a functional interaction between these channels. Interactions between these receptors were first observed by Nakazawa et al in 1991. ATP activated currents and nicotine induced currents were studied in NGF differentiated rat pheochromcytoma-12 cells isolated from the adrenal medulla (45, 47). Nakazawa observed that ATP and nicotine mediated currents were not additive. When simultaneously activated, these receptors mediate an inhibitory interaction. Simultaneous activation of both channels produces inward currents that are smaller than the additive sum of individual currents (predicted current) that would occur if the channels functioned independently.

P2X/nAChR interactions were later observed in guinea-pig celiac ganglion neurons (52, 53), and superior cervical sympathetic neurons (46). These studies suggested that $P2X_2$-nAChR cross-inhibition is a result of "channel overlap" (45, 46). This hypothesis is based on the idea that ATP and ACh activate a common channel with different agonist binding sites (45, 52, 53). Another theory to explain this occlusion of current was that P2X receptors have an allosteric inhibitory binding site for ACh, and that nAChRs have a similar binding site for ATP (47, 52). These hypotheses are difficult to test in these model systems where both P2X and nAChRs are endogenously expressed.

Further work included P2X and nAChRs co-expressed in cultured native peripheral neurons, and cultured myenteric neurons that have shown this occlusion of response in which the combined activation of receptors resulted in only 74% of the predicted response (60). This non-additivity has also been reproduced in *Xenopus* oocytes where P2X receptors and nAChRs were expressed heterologously (36). Unlike G-coupled protein receptors, P2X receptors and nAChRs mediate quick responses (latency, < 1 ms); hence, it is unclear how interactions take place in a short period of time.

6. REASEARCH GOAL

The ENS is responsible for regulating motility, immune, and inflammatory processes of the gastrointestinal tract. Disorders of this system result in motor, secretory and circulatory dysfunctions (31). Details of the neuronal circuitry, receptor localization and signaling responsible for regulating excitatory synaptic transmission in the ENS are only partly understood. In particular, specific receptor sites responsible for regulation would be excellent targets for drugs to facilitate neurotransmission and enhance or decrease gut motility. Such drugs would help to better treat GI motility disorders such as irritable bowel syndrome (29).

The goals of the present study were to investigate the interactions between nAChRs and P2X receptors. This interesting interaction has not yet been studied thoroughly. Since the ENS is the only part of the nervous system where ACh and ATP have been shown to be co-transmitters at functionally defined synapses (26), it is an ideal

system to study this novel mechanism of synaptic integration. In this study, I will investigate the interactions between P2X and nAChR during fast synaptic excitation.

A majority of previous work has been limited in its study of this unique ion channel interaction because the systems utilized endogenously expressed both receptors. This makes it difficult to verify such hypothesis as the "channel overlap" theory, or allosteric binding sites. A heterologous system in which these receptors are not endogenously expressed is a crucial tool in answering these scientific questions. Although this interaction has been touched upon in *Xenepos oocytes*, our studies focus on a mammalian system that has the same processing and trafficking of proteins as the native environment of myenteric neurons, compared to an amphibian system.

REFERENCES

1. **Abbracchiio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight G, Fumagalli M, Gachet C, Jacobson KA, Weisman GA.** International Union of Pharmacology LVIII: Update on the P2Y G Protein-Coupled Nucleotide Receptors: From Molecular Mechanisms and Pathophysiology to Therapy. *Pharmacol Rev,* **58:** 281–341, 2006.

2. **Arias HR, Bhumireddy P, Bouzat C.** Molecular mechanisms and binding site locations for noncompetitive antagonists of nicotinic acetylcholine receptors. *The International Journal of Biochemistry & Cell Biology,* **38:** 1254–1276, 2006.

3. **Albuquerque EX, Pereira EFR, Alkondon M, Rogers, SW.** Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol Rev* **89:** 73–120, 2009.

4. **Benarroch, EE.** Adenosine and its receptors. *Neurology,* **70:** 231-236, 2008.

5. **Bennett MR.** The concept of transmitter receptors: 100 years on. *Neuropharmacology* **39:** 523–546, 2000.

6. **Bertrand PP.** ATP and Sensory Transduction in the Enteric Nervous System. *Neuroscientist,* **9(4):** 243–260, 2003.

7. **Bornstein JC, Costa M, Grider JR.** Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol. Motil.* **16** (suppl 1): 34-38, 2004.

8. **Boue-Grabot E, Archambault V, Seguela P.** A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of $P2X_2$ ATP-gated channels. *J. Biol. Chem.* **275**(14): 10190-5, 2000.

9. **Bridges TM, Lindsley CW.** G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS Chem. Biol.* **3 (9):** 530-541, 2008.

10. **Brookes SJH.** Classes of enteric nerve cells in the guinea-pig small intestine. *The anatomical record,* **262:** 58-70, 2001.

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

12. **Burnstock G.** Purinergic signalling: past, present and future. *Braz J Med Biol Res* **42(1):** 2-8, 2009.

13. **Clapham DE , Neer EJ.** G-protein βγ subunits. *Annu. Rev. Pharmacol. Toxicol* **37:**167–203, 1997.

14. **Cordero-Erausquin M, Merubio LM, Klink R, Changeux JP.** Nicotinic receptor function: new perspectives from knockout mice. *TiPS* **21:** 211-217, 2000.

15. **Costa V, Nistri A, Cavalli A, Carloni P.** A structural model of agonist binding to the alpha3beta4 neuronal nicotinic receptor. *Br J Pharmacol.* **140 (5):** 921-31, 2003.

16. **Dajas-Bailador F, Wonnacott S.** Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci.* **25(6):**317-24, 2004.

17. **Dubyak GR, el-Moatassim C.** Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol-Cell.* **265(3 Pt 1):** C577-606, 1993.

18. **Edwards FA, Gibb AJ.** ATP- a fast neurotransmitter. *FEBS* **325:** 86-89, 1993.

19. **Fieber LA, Adams DJ.** Acetylcholine-evoked currents in cultured neurons dissociated from rat parasympathetic cardiac ganglia. *J of Physiology* **434**, 215-237, 1991.

20. **Furness JB, Costa M.** The enteric nervous system. pp 1-290, New York: Churchill Livingstone. 1987.

21. **Furness JB, Sanger GJ.** Intrinsic nerve circuits of the gastrointestinal tract; identification of drug targets. *Current opinion in Pharmacology*, **2**: 612- 622, 2002.

22. **Furness JB.** The Enteric Nervous System. Blackwell Publishing, Massachusetts, USA. p 1-274, 2006a.

23. **Furness JB.** The organisation of the autonomic nervous system: peripheral connections. *Auton Neurosci.* **30**: 1-5. 2006b.

24. **Gallgian and Bertrand PP.** ATP mediates fast synaptic potentials in enteric neurons. *J Neurosci* **14:** 7563-7571,1994.

25. **Galligan JJ.** Mechanisms of excitatory synaptic transmissionin the enteric nervous system. *Tokia J Exp Clin Med.* **23 (5):** 129-136, 1998.

26. **Gallligan JJ, LePard KJ, Schneider DA, Zhou X.** Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* **81:** 97-103, 2000.

27. **Galligan JJ.** Pharmacology of synaptic transmission in the enteric nervous system. *Current opinion in pharmacology*, **2:** 623-629, 2002.

28. **Galligan JJ, North RA.** Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil,* **16 (suppl 1)** 64-70, 2004.

29. **Gershon MD.** Review article: serotonin receptros and transporters – roles in normal and abnormal gastrointestinal motility. *Ailment pharmacol Ther.* **20** (suppl. 7), 3-14, 2004.

30. **Gourine AV, Wood JD, Burnstock G.** Purinergic signalling in autonomic control. *Trends Neuro* , April 7. [Epub ahead of print]

31. **Goyal RK, Hirano I.** The enteric nervous system. *The New England Journal of Medicine* **334**, 1106-1115,1996.

32. **Hall ZW.** Release of neurotransmitters and their interaction with receptors. *Annu Rev Biochem.* **41:**925-52, 1972.

33. **Hamm HE.** The many faces of G protein signaling. *J of Biol Chem,* **273(2):** 669–672, 1998.

34. **Hubbard JI.** Mechanism of transmitter release. *Prog Biophys Mol Biol.* **21:**33-124, 1970.

35. **Ji TH, Grossmann M, Ji I.** G protein-coupled receptors. *J Biol Chem.* **273:** 17299-302, 1998.

36. **Khakh BS, Zhou X, Sydes J, Galligan JJ, Lester HA.** State-dependent cross-inhibition between transmitter-gated cation channels. *Nature* **406:** 405-10, 2000.

37. **Khakh BS.** Molecular physiology of P2X receptors and ATP signaling at synapses. *Nature Reviews* **2:** 165-174, 2001.

38. **Langley JN.** The autonomic nervous system. *Brain* **26:** 1-26, 1903.

39. **Langley JN.** The autonomic nervous system. Heffer, Cambridge. p 80, 1921.

40. **Le KT, Babinski K, Seguela P.** Central $P2X_4$ and $P2X_6$ channel subunits coassemble into a novel heteromeric ATP receptor. *J. Neurosci.* **18** (18): 7152-9, 1998.

41. **Lester HA, Dibas MI, Hahan DS, Leite JF, Dougherty DA.** Cys-loop receptors: new twists and turns. *Trends Neurosci* **27(6):** 329-36, 2004.

42. **Maio VD.** Regulation of information passing by synaptic transmission: A short review. *Brain Research* **1225:** 26– 38, 2008.

43. **McGehee DS, Role LW.** Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol.* **57**,521-46, 1995.

44. **Miyazawa A, Fujiyoshi Y, Unwin N.** Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423:** 949-955, 2003.

45. **Nakazawa K, Fujimori K, Takanaka A, Inoue K.** Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. *J Physiol* **434**: 647-60, 1991.

46. **Nakazawa K.** ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. *J Neurosci* **14:**740-750, 1994.

47. **Nakazawa K.** Reduction of acetylcholine-activated current by low concentrations of extracellular adenosine-triphosphate. *Life Sciences* **57:** 351-356, 1995.

48. **North RA , Surprenant A.** Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol,* **40:** 563-8, 2000.

49. **North RA.** Molecular physiology of P2X receptors. *Physiol Rev* **82**: 1013-1067, 2002.

50. **Pierce KL, Permont RT, Lefkowitz RJ.** Seven-transmemebrane receptors. *Nature Rev, molecular cell biology.* **3:** 639-650, 2002.

51. **Purves D, Augustine G J, Fitzpatrick D, Katz LC, LaMantia AS, McNamara JO, Williams SM.** Neuroscience Second edition. Sunderland (MA): Sinauer Associates, Inc., 2001.

52. **Searl TJ, Redman RS, Silinsky EM.** Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J Physiol* **510:** 783-791, 1998.

53. **Silinsky EM, Gerzanich V.** On the excitatory effects of ATP and its role as a neurotransmitter in coeliac neurons of the guinea-pig. *J Physiol* **464:**197-212, 1993.

54. **Torres G E, Egan TM.** Voigt MM. Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. *J. Biol. Chem.* **274**(10): 6653-9, 1999.

55. **Vial C, Roberts JA, Evans RJ.** Molecular properties of ATP-gated P2X receptor ion channels. *TRENDS in Pharmacological Sciences.* **25**, 487-493, 2004.

56. **Walmsley B, Alvarez FJ, Fyffe RE.** Diversity of structure and function at mammalian central synapses. *Trends Neurosci* **21:** 81-8, 1998.

57. **Wess J, Eglen RM, Gautam D.** Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. N*ature reviews: drug discovery*, **6**: 72 , 2007.

58. **Wood JD, Kirchgessner A.** Slow excitatory metabotropic signal transmission in the enteric nervous system. *Neurogastroenterol Motil.* **16 (Suppl. 1):**71–80, 2004.

59. **Zhou X, Galligan JJ.** P2X purinoceptors in cultured myenteric neurons of the guinea-pig small intestine. *J Physiol* **496**: 719-729, 1996.

60. **Zhou X, Galligan JJ.** Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *J Physiol* **513**: 685-697, 1998.

61. **Zhou X, Ren J, Brown E, Schneider D, Caraballo-Lopez Y, Galligan JJ.** Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther* **302**: 889-897, 2002.

62. **Zimmermann H.** Signalling via ATP in the nervous system. *TINS* **17(10):** 420-426, 1994.

63. **Zimmerman H.** Extracellular Purine metabolism. *Drug Development Res.* **39:** 337-352, 1996.

64. **Zimmermann H.** ATP and acetylcholine, equal brethren. *Neurochemistry International*, **52 :** 634–648, 2008.

65. **Zouridakis M, Zisimopoulou P, Poulas K, Tzartos SJ.** Recent advances in understanding the structure of nicotinic acetylcholine receptors. *IUBMB Life*, **61(4):** 407–423, 2009.

CHAPTER 2

Hypothesis and Specific Aims

OVERALL HYPOTHESIS

My hypothesis is that: **nAChRs and P2X receptors form a heterodimer that regulates fast synaptic excitation in the ENS.** An alternative explanation for their fast mutual inhibition would be that their interaction is charge-mediated. The first concern regarding this interaction would be that it occurs as a result of Na^+ or Ca^{2+} dependence, especially since Ca^{2+} is a known mediator of downstream signaling events (both receptors are Ca^{2+} permeable). However, studies in Ca^{2+} free solutions have not altered the non-additive response (Zhou and Galligan 1998). These studies also provided evidence, that this interaction is not Na^+ dependent (Zhou and Galligan 1998). These results rejected the idea that there are chemical interactions that can account for this occlusion of response.

If these interactions are not ion dependent, further reflection would suggest that this receptor-receptor interaction is associated with the formation of a heterodimer; a hypothesis that is supported by the following evidence. First, the cross-inhibitory response of these receptors is dependent on receptor density, that is a larger amplitude in response to ATP or a greater number of receptors expressed correlate with a more prominent occlusion. Second, this interaction is still observed in membrane patches, suggesting no need for second messengers (Zhou and Galligan, 1998; Khakh et al., 2000). Third, Fluorescence Resonance Energy Transfer (FRET) analysis of a $\alpha_4\beta_2$ nAChR and $P2X_2$, which are subtypes expressed in the brain, revealed localization of these receptors in a close proximity of ~80Å apart (Khakh et al., 2005). This is evidence

of an Angstrom scale interaction between full-length $P2X_2$ and $\alpha_4\beta_2$ nAChR in the plasma membrane of living HEK-293 cells. The FRET data imply that $P2X_2$ interact more strongly with the β subunits than with α subunit.

My hypothesis of heterodimer formation will be tested by two specific aims. The goal of the first specific aim is to reproduce the inhibitory interaction between nAChR and P2X in cultured myenteric neurons. The second will be to determine whether a functional interaction occurs between nicotinic acetylcholine receptors (nAChRs) and P2X receptors in a heterologous system expressing the specific receptor subtypes found at enteric neuronal synapses. These receptor subtypes are the $\alpha_3\beta_4$ nAChR subtype and the $P2X_2$ homotrimeric receptor. The rationale is to utilize the advantage of the human embryonic kidney (HEK-293) heterologous expression system to determine the specificities of this interaction.

Specific Aims:

Specific aim #1: Reproduce nAChR and P2X inhibitory interaction in cultured myenteric neurons. The overall aim of this goal is to verify that this interaction can be reproduced.

Specific aim #2: Express $\alpha_3\beta_4$ nAChR and $P2X_2$ in HEK-293 cells.

Specific aim 2a: Determine the function of $\alpha_3\beta_4$ nAChR and $P2X_2$ in this heterologous system and how it compares to receptors in cultured myenteric neurons.

Specific aim 2b: Reproduce the functional interaction between $P2X_2$ and $\alpha_3\beta_4$ nAChR.

Specific aim 2c: Determine the specificity of the mutually inhibitory interaction between nAChR and P2X.

Specific aim 2d: Determine if desensitization of individual receptors affect the mutually inhibitory interaction.

Specific aim 2e: Determine the role of the C-terminal tail of the $P2X_2$ receptor.

Specific aim 2f: Determine basal level of cross-inhibition between receptors.

CHAPTER 3

Cross-inhibition Between Nicotinic Acetylcholine Receptors and P2X Receptors in Myenteric Neurons and HEK-293 cells[1]

[1] Data contained in this chapter have been published.
Decker, DA, Galligan, JJ. (April 2, 2009) American Journal of Physiology GI and Liver [Epub ahead of print]

ABSTRACT

The enteric nervous system (ENS) controls gut function. P2X and nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that mediate fast synaptic excitation in the ENS. Close molecular coupling in enteric neuronal membranes contributes to a mutually inhibitory interaction between these receptors; this effect is called cross-inhibition. We studied the molecular mechanisms responsible for cross-inhibition. Whole-cell patch clamp techniques were used to measure P2X- and nAChR-mediated currents in cultured enteric neurons and HEK-293 cells. In cultured myenteric neurons, ACh (3 mM) and ATP (1 mM) co-application evoked an inward current that was only 57 ± 6% ($P < 0.05$) of the predicted current that would have occurred if the two populations of channels were activated independently. In HEK-293 cells co-expressing $\alpha_3\beta_4$ nAChR/$P2X_2$ receptors, co-application of ATP and ACh caused a current that was 58 ± 7% of the predicted current ($P < 0.05$). To test the importance of P2X subunit C-terminal tail length on cross-inhibition, $P2X_3$ and $P2X_4$ subunits, which have shorter C-terminal tails were studied. Cross-inhibition with $\alpha_3\beta_4$ nAChRs and $P2X_3$ or $P2X_4$ subunits was similar to that occurring with $P2X_2$ subunits. P2X receptor or $\alpha_3\beta_4$ nAChR desensitization did not prevent receptor cross-inhibition. These data indicate that the $\alpha_3\beta_4$-P2X receptor interaction is not restricted to $P2X_2$ subunits. In addition, active and desensitized conformations of the P2X receptor inhibit nAChR function. These molecular interactions may modulate the function of synapses that use ATP and ACh as fast synaptic transmitters in the ENS.

INTRODUCTION

The enteric nervous system (ENS) is the division of the autonomic nervous system (ANS) that controls gastrointestinal function (8). Although parasympathetic, sympathetic and sensory nerves supply the intestine, the ENS can control gut function independently from CNS control. This is possible because the ENS contains all the neural elements needed for reflex control of gastrointestinal function (10,12). These elements include motor neurons, inter-neurons and sensory neurons that respond to mechanical and chemical stimuli provided by intraluminal content (9).

There are two classes of ligand-gated ion channel receptors that mediate fast synaptic excitation of enteric neurons; the cys-loop receptors and P2X receptors. Cys-loop receptors include nicotinic acetylcholine receptors (nAChRs), 5-HT_3 receptors and $GABA_A$ receptors. Cys-loop receptors are pentameric and each subunit has four transmembrane domains and *extracellular* N and C-terminal tails (17,18). Neuronal nAChRs are composed of two α and three β subunits; there are eight neuronal α (α_2-α_9) and three neuronal β (β_2-β_4) nAChR subunits (17). In the ENS, the predominant nAChR subtype is composed of α_3 and β_4 subunits (39) and acetylcholine acting at nAChRs is the primary fast excitatory neurotransmitter in the ENS (10). P2X receptors are trimeric and each subunit has two transmembrane domains and *intracellular* N and C-terminal tails. P2X receptors are homomeric or heteromeric receptors composed of one or more of seven ($P2X_1$-$P2X_7$) subunits (7, 14, 26, 27, 31, 35). Enteric neurons express $P2X_2$ and $P2X_3$ subunits which can form homomeric and heteromeric $P2X_2/P2X_3$ receptors (6, 29, 38). ATP acting at P2X receptors contributes to fast synaptic excitation in subsets of

enteric neurons and ATP and ACh are co-transmitters at many synapses in the ENS (10, 16, 20, 28, 30).

P2X receptors and nAChRs are structurally different and it would be expected that they would function independently. However, P2X receptors and nAChRs co-expressed by rat pheochromcytoma-12 cells (23, 25) and superior cervical sympathetic neurons (24) are functionally linked. Simultaneous activation of both channels produces inward currents that are smaller than the additive sum of individual currents (predicted current) that would occur if the channels functioned independently. The functional interaction between nAChRs and P2X receptors was subsequently shown to occur in guinea pig celiac ganglion neurons (32) and cultured myenteric neurons from the guinea-pig ileum (38). This latter result is particularly important because ATP and ACh are co-transmitters at functionally identified synapses in the guinea pig ileum myenteric plexus (1, 16, 27) and ATP modulates the function of nAChRs activated by synaptically-released ACh (13). The functional interaction between nAChRs and P2X receptors is proportional to receptor density and the interaction occurs in cell-free patches obtained from cultured myenteric neurons (38). These data suggest that intracellular signaling mechanisms are not required for the functional interaction and that there may be direct molecular interactions between the two types of receptors. A similar functional interaction occurs between P2X receptors and other cys-loop receptors including 5-HT_3 receptors in guinea pig submucosal neurons (2) and $GABA_A$ receptors in dorsal root ganglion neurons (34). Studies of heterologously expressed $P2X_2$ and 5-HT_3 receptors revealed that a portion of the cytoplasmic C-terminal tail of the $P2X_2$ receptor and a portion of the second cytoplasmic loop of the 5-HT_{3A} subunit are responsible for not only a functional

interaction but also may mediate a physical interaction between these receptors (3). Similarly, the C-terminal tail is required for interaction of $P2X_3$ receptors with $GABA_A$ receptors expressed in *Xenopus* oocytes or dorsal root ganglion neurons (34). These studies suggest that the P2X receptor C-terminal tail might contribute to interactions with cys-loop receptors, a hypothesis that we test by studying interactions between nAChRs and P2X subunits with varying C-terminal tail lengths.

Although a functional interaction between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs occurs when these receptors are expressed in *Xenopus* oocytes (13), the molecular mechanism responsible for the interaction between $P2X_2$ and $\alpha_3\beta_4$ nAChRs has not been studied in detail. The present study was designed to test the hypothesis that the functional interaction between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs could be reproduced in a heterologous expression system (HEK-293 cells) and that this system could be used to probe the molecular mechanisms responsible for interactions between these receptors.

MATERIALS AND METHODS

Tissue Culture

Cultured myenteric neurons. Neurons were cultured as described previously (34,35). Two newborn guinea-pigs (<36 hrs old) were killed by severing the major neck blood vessel following halothane anesthesia. The small intestine was removed from the animals and placed in cold (4 °C) Krebs solution of the following composition (millimolar): 117 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 $MgCl_2$; 1.2 NaH_2PO_4; 25 $NaHCO_3$ and 11 glucose. The longitudinal muscle myenteric plexus was stripped free using a cotton swab and cut into 5 mm pieces. Tissues were digested with 1600 U of trypsin, followed by trituration with a fire-polished Pasteur pipette. After incubation with 2000 U crab hepatopancreas collagenase, the tissues were triturated again. The neurons were resuspended in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum, gentamicin (10 $\mu g\ ml^{-1}$), penicillin (100 units ml^{-1}) and streptomycin (100 units ml^{-1}) and plated onto sterile, poly-L-lysine coated 35 mm plastic dishes and maintained in an incubator at 37 °C in a 5% CO_2 atmosphere for up to 3 weeks.

Human embryonic kidney (HEK-293) cells. Cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) F-12 containing 10% fetal bovine serum, 10% GluMax (Invitrogen, Carlsbad, CA), and 100 units ml^{-1} penicillin and streptomycin, except for the rat $\alpha_3\beta_4$ nAChR stable HEK-293 cell line which also contained 0.5 mg/ml geneticin (GIBCO, G418). Cells were maintained at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged once every 3 days when they reached 90% confluence. Transient transfection

of cells was accomplished using electroporation. A 0.4 cm Gene Pulser Cuvette (BIO-RAD) was used with 2 μg of total plasmid protein along with 0.2 μg of GFP to help identify transfected cells. The electroporation machine used was Gene Pulser Xcell™ Electroporation System (BIORAD). This resulted in 80% transfection efficiency. Afterwards, cells were plated on 35 mm coverslips and maintained in the incubator for 24 hrs before use in electrophysiology experiments. A rat $\alpha_3\beta_4$ nAChR stable HEK-293 cell line was provided by Dr. Yingxian Xiao from Georgetown University (36). Plasmids containing the coding sequences for rat $P2X_2$, $P2X_3$ and $P2X_4$ receptor subunits were a gift from Dr. Alan North (University of Manchester, Manchester, UK). Plasmids containing the coding sequences for the murine α_3 and murine β_4 nAChR subunits were provided by Dr. Jerry A. Stitzel (University of Colorado, Denver Colorado).

Whole cell recording: Whole-cell voltage-clamp measurements were obtained at room temperature using standard methods. Coverslips containing cells were placed on a stage of an inverted microscope (Nikon Diaphot, Mager Scientific, Inc. Dexter, MI) using phase-contrast optics. For identifying co-transfected cells, a mercury bulb with excitation wavelength of 495 nm was used with a filter for 520 nm emission. The pipette solution contained (millimolar): 122.5 K-aspartate; 20 KCl; 1 $MgCl_2$; 10 EGTA; 5 HEPES; and 2 ATP. The pH was adjusted to 7.3 with KOH. The extracellular solution was a HEPES-based buffer composed of (millimolar): 155 NaCl; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 HEPES; and 12 glucose, pH was adjusted to 7.4 with NaOH. All recordings were made using an Axopatch 200B amplifier (Molecular Devices, Inc. Sunnyvale, CA). Data was acquired using pCLAMP 9.1 software (Molecular Devices). Whole-cell recordings were carried out using patch pipettes with tip resistances of 3-5 MΩ; seal resistances were greater than

1 GΩ. Steady state current-voltage relationships for agonist-induced whole-cell currents were obtained using step depolarization that changed the holding potential from an initial value of −70 to 50 mV in 20 mV increments. There was a two minute interval between all successive agonist applications at each holding potential, -70 mV for HEK-293 cells and -60 mV for myenteric neurons.

Drug application. Agonists were applied onto individual neurons/cells by gravity flow from linear array quartz tubes placed near the cell. The distance from the mouth of the tubes to the cells was ~200 μm with flow controlled manually using a micromanipulator. Computer-controlled solenoid valves (General Valve, Fairfield, NJ) were used to gate solution flow through the tubes. Agonist-induced currents were measured as the peak current amplitude. The difference between the predicted sum of the individual peak currents was compared with the current amplitude caused by co-application of two agonists.

Statistics. Data are expressed as the mean ± S.E.M. and "*n*" refers to the number of cells from which the data were obtained. The predicted current amplitude that would occur during agonist co-application was calculated by measuring the peak currents caused by previous individual agonist applications in the same cell and then summing these values. These are repeated measurements within the same cell; therefore, there is no concern for error within a single measurement because it cancels itself out. These are directly measured data that were used for statistical comparisons of actual and predicted currents. Traces illustrating predicted currents were constructed using a simple math function in Origin 7 (OriginLab Corp., Northhampton, MA). Student's *t* test or analysis of variance were used to establish significant differences between control and treatment

groups. The significance level was $P< 0.05$. EC_{50} is the half-maximal effective concentration and n_H is the slope. Y_{min} and Y_{max} are the minimum and maximum respectively. Agonist concentration-response curves were fit to the following logistic function using Origin:

$$y = \frac{(Y_{min} - Y_{max})}{1 + (x/EC_{50})^{nH}} + Y_{max}$$

Immunocytochemistry (ICC): After transfection, cells were washed three times with 0.1 M phosphate buffered solution (PBS) and then fixed for 20 minutes with Zamboni's fixative (2% formaldehyde and 0.2% picric acid in 0.1 M PBS), pH 7.0. The cells were washed again with PBS and incubated with 0.1% Triton for 30 minutes prior to addition of primary antibodies. Primary antibodies in PBS with 5% serum were applied and incubated at 37°C for 2 hr. The cells were then washed with PBS, followed by incubation with secondary antibody for 1hr at 37°C. Cells were washed again with PBS before mounting on slides with anti-fade solution (Zhou *et al.* 2002). Primary antibodies were at 1:200 dilution: rabbit anti-$P2X_2$ (Alomone, Jerusalem), goat anti-alpha 3 (SC-1711, Santa Cruz Biotechnology), mouse anti-EE (Covance Research Products). Secondary antibodies were FITC at (1:40) and Cy3 (1:200) IgG (Jackson Immunoresearch Laboratories). Cells were viewed and analyzed using a Leica Microsystems confocal microscope and image acquisition and analysis software.

RESULTS

Inhibitory interaction between nAChRs and P2X receptors in cultured myenteric neurons. ACh (3 mM) caused an inward current in 98% of the myenteric neurons tested, while ATP (1 mM) caused an inward current in 80% of the neurons tested. The mean current amplitudes caused by individual application of ACh (3 mM) and ATP (1 mM) were not statistically different (Fig. 3.1). However, when the two agonists were applied simultaneously to the same neurons the peak inward current was only 57 ± 6% of the predicted current that would have occurred had the two channels functioned in an independent manner ($n = 8$, $P < 0.05$, Fig. 3.1); these data confirm previously published work (35). In the remainder of the paper, we will call the inhibitory interaction between nAChRs and P2X receptors "cross-inhibition".

Properties of $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed in HEK-293 cells. We wanted to verify expression of these receptors in this heterologous system. First we utilized a HEK-293 $P2X_2$ stable cell line to visualize $P2X_2$ receptors near the plasma membrane (Fig. 3.2A). Wild-type HEK-293 cells were also transiently transfected with $\alpha_3\beta_4$ nAChR and GFP (Fig. 3.2B). $P2X_2$ stable HEK-293 cells transiently transfected with $\alpha_3\beta_4$ nAChR subunits displayed co-expression in the plasma membrane (Fig. 3.2C-E). Therefore, we have succeeded in attaining HEK-293 cells that express both receptors, singly and together. We verified that the pharmacological and functional properties of heterologously expressed $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs were similar to those seen in cultured myenteric neurons. In HEK-293 cells stably expressing $P2X_2$ receptors, ATP (1 mM) evoked a rapidly developing current that was largely sustained throughout the

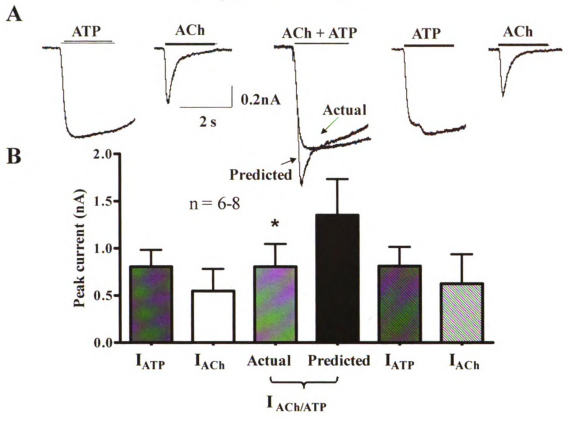


Figure. 3.1: Cross- inhibition between P2X receptors and nAChRs in guinea pig ileum cultured myenteric neurons.

A: Inward currents caused by ATP (1 mM), an agonist for P2X receptors and ACh (3 mM) an agonist for nAChRs in the same neuron. Co-application of ACh and ATP caused an inward current that was smaller than the predicted current that would have occurred if the two channels functioned independently ($P < 0.05$). Individual re-application of ATP and ACh caused inward currents that were not statistically different from the original responses ($P > 0.05$). *B:* Pooled data from experiments shown in "A". "n" indicates the number of neurons. *Indicates significantly different from the predicted current.

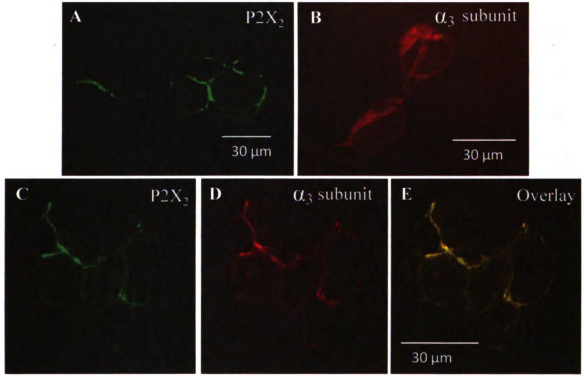


Figure 3.2: Immunocytochemistry of HEK-293 cells.

A. In a $P2X_2$ stable HEK-293 cell line, immunofluorescence for an antibody for the $P2X_2$ receptor. *B.* In $\alpha_3\beta_4$ nAChR HEK-293 cells, immunofluorescence for an antibody against the α_3 subunit of the nAChR. *C-E.* In $P2X_2$ stable HEK-293 cells transiently transfected with $\alpha_3\beta_4$ nAChR, immunofluorescence for $P2X_2$ (*C*), α_3 subunit of nAChR (*D*), and an overlay (*E*) of *C* and *D*.

period of ATP application. This response was similar to that caused by ATP in cultured myenteric neurons (Fig. 3.3A). ACh (3 mM) evoked a rapidly developing and desensitizing current in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs and this response was similar to that measured in cultured myenteric neurons (Fig. 3.3B). Concentration-response curves and current-voltage relationships were determined for ATP in HEK-293 cells stably expressing $P2X_2$ receptors and for ACh in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs. ATP caused a concentration-dependent increase in current amplitude with a half-maximal effective concentration (EC_{50}) of 20 ± 10 μM; the maximum current occurred at 300 μM (Fig. 3.3C). The Hill slope was 0.9 ± 0.3 (n = 6). These values were similar to those reported previously for ATP evoked inward currents in cultured myenteric neurons (38).

ACh caused a concentration-dependent inward current in HEK-293 cells with an EC_{50} of 130 ± 20 μM and a Hill slope of 2.0 ± 0.6 (Fig. 3.3C). These values were similar to those reported previously for ACh-induced activation of nAChRs expressed by cultured myenteric neurons (36). Current-voltage relationships for ACh- or ATP-induced currents were studied in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs or $P2X_2$ receptors, respectively. ACh-induced currents exhibited an inwardly rectifying current voltage relationship with a reversal potential of 8.0 ± 4.5 (n = 5, Fig. 3.3D). The slope conductance at -50 was 33 ± 6 nS while at 50 mV this value was 7 ± 4 nS. ATP-induced currents in $P2X_2$ stable cells also exhibited inward rectification and the reversal potential was 15 ± 6 (n = 4-5). At -50 mV the slope conductance was 48 ± 5 nS while at 50 mV

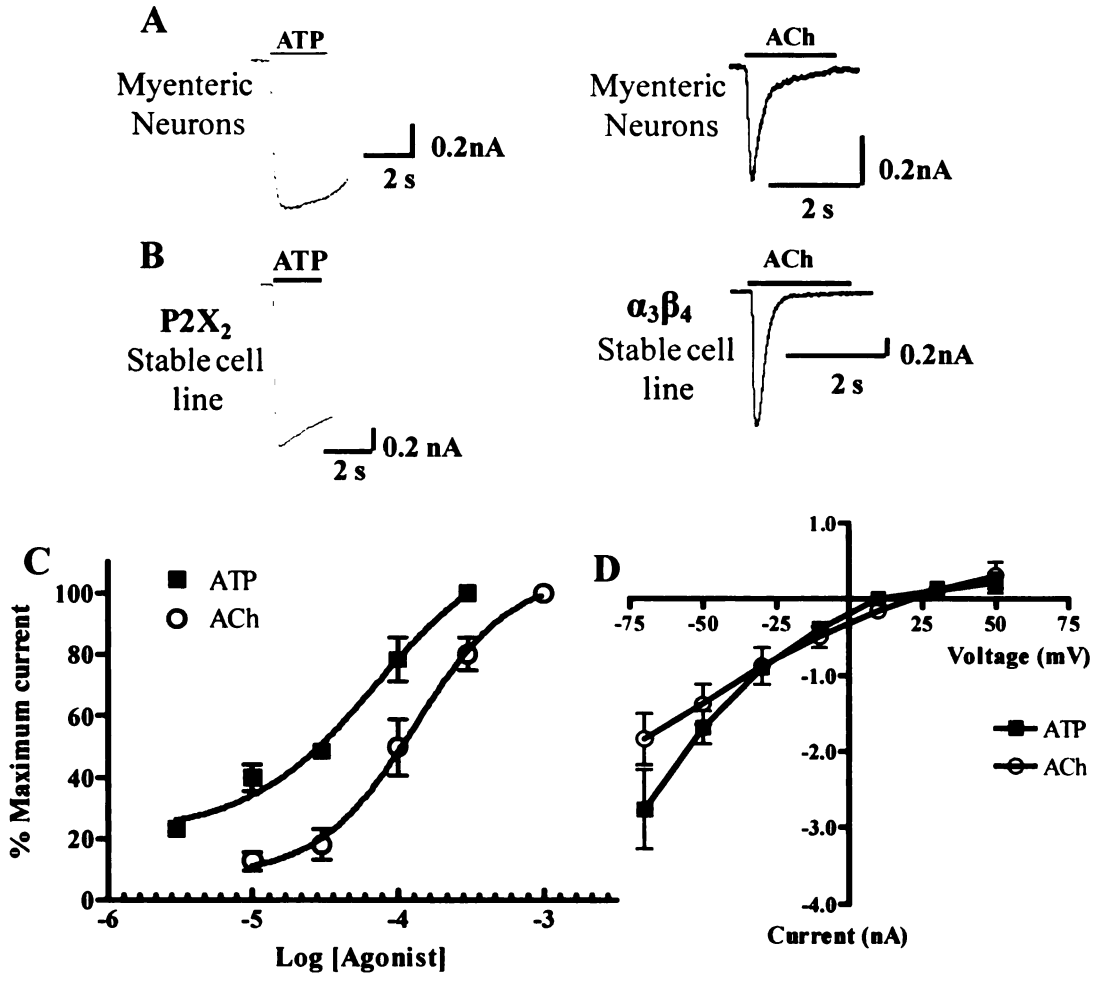


Figure 3.3: Comparison of pharmacological and functional properties of $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed by cultured myenteric neurons and HEK-293 cells.

A: Inward currents caused by ATP (1 mM, left) and ACh (3 mM, right) in a cultured myenteric neuron. The ATP response desensitized slowly while the ACh response desensitized completely during ACh application. *B*: Inward currents caused by ATP (1 mM) in an HEK-293 cell line stably expressing $P2X_2$ receptors (left) and by ACh (3 mM) in an HEK-293 cell line stably expressing $\alpha_3\beta_4$ nAChRs (right). Although larger in amplitude, the properties of the currents in cultured myenteric neurons and HEK-293 cells were similar. *C*: Concentration-response curves for ATP and ACh in HEK-293 cells stably expressing $P2X_2$ and $\alpha_3\beta_4$ nAChRs. *D:* Current-voltage relationship for ATP- (1 mM) and ACh- (3 mM) induced currents in HEK-293 cells stably expressing $P2X_2$ and $\alpha_3\beta_4$ nAChRs.

this value was 6 ± 3 nS. These properties are very similar to those reported previously for nAChRs and P2X receptors expressed by cultured myenteric neurons (37, 38, 39). All subsequent experiments use saturating concentrations of ATP (1 mM) and ACh (3 mM) unless specified otherwise.

Cross-inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs in HEK-293 cells. The data described above indicate that the functional and pharmacological properties of heterologously expressed nAChRs and P2X receptors are similar to those of the receptors expressed by cultured myenteric neurons. Therefore, we used HEK-293 cells that stably expressed rat $P2X_2$ receptors and that were also transiently transfected with murine α_3 and β_4 nAChR subunits to study cross-inhibition between these receptors. Due to receptor overexpression, the mean current amplitudes caused by individual application of ACh and ATP were larger than those recorded from cultured myenteric neurons (Fig. 3.4). However, as was observed in cultured myenteric neurons, simultaneous application of maximum concentrations of ACh and ATP caused an inward current that was only 61 ± 3 % ($P < 0.05$, n=9) of the predicted current that would have occurred had the heterologously expressed $P2X_2$ receptors $\alpha_3\beta_4$ nAChRs functioned independently. Responses caused by individual application of ACh and ATP obtained after agonist co-application were not statistically different from the original current amplitudes ($P > 0.05$; n = 9, Fig. 3.4). Furthermore, when the predicted current value was calculated using individual agonist-evoked currents obtained after agonist co-application, the actual current was significantly smaller than this predicted value ($P < 0.05$; n=6). In the next experiments, we used a cell line that stably expressed $\alpha_3\beta_4$ nAChRs and that was

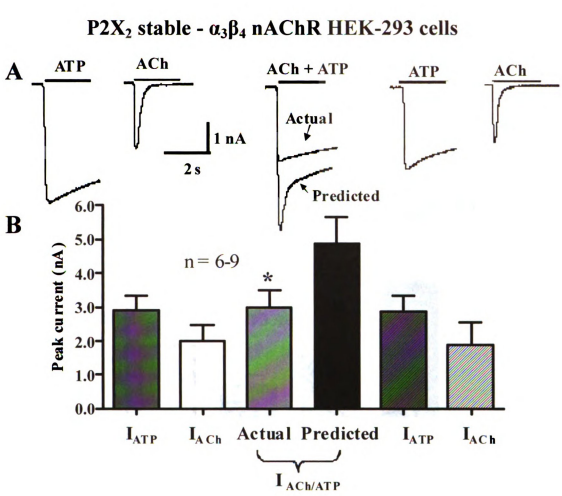


Figure 3.4: Cross-inhibition between $P2X_2$ stable receptors and $\alpha_3\beta_4$ nAChRs expressed by HEK-293 cells.

A: ATP- (1 mM) and ACh- (3 mM) induced inward currents recorded from a HEK-293 cell stably expressing $P2X_2$ receptors and transiently transfected with $\alpha_3\beta_4$ nAChRs. Currents caused by agonist co-application exhibited cross-inhibition. Individual re-application of ATP and ACh caused inward currents that were not statistically different from the original responses ($P > 0.05$). *B*: Pooled data from experiments shown in "A". "n" indicates the number of cells. *Indicates significantly different from the predicted current.

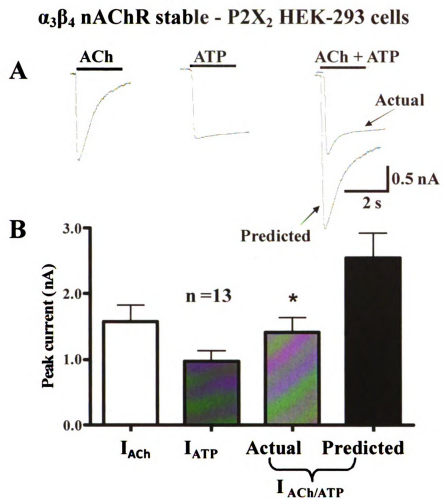


Figure 3.5: Cross-inhibition between $\alpha_3\beta_4$ nAChRs stable and $P2X_2$ receptors expressed by HEK-293 cells.

A: ATP- (1 mM) and ACh- (3 mM) induced inward currents recorded from a HEK-293 cell stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2$ receptors. Currents caused by agonist co-application exhibited cross-inhibition. *B*: Pooled data from experiments shown in "A". "n' indicates the number of cells. *Indicates significantly different from the predicted sum of currents ($P < 0.05$).

transiently transfected with $P2X_2$ receptors. In these cells, currents caused by simultaneous application of ACh and ATP were only 58 ± 5% of the predicted current (Fig. 3.5, $P < 0.05$, $n = 13$) that would have occurred if the receptors functioned independently. Subsequent individual re-application of ACh and ATP caused currents that were not statistically different from those elicited before agonist co-application ($P > 0.05$, $n = 13$, data not shown). Furthermore, when the predicted current value was calculated using individual agonist-evoked currents obtained after agonist co-application, the actual current was significantly smaller than this predicted value ($P<0.05$, $n = 13$).

As mentioned above, receptor over-expression resulted in current amplitudes in HEK-293 cells that were larger than those in cultured myenteric neurons. It is possible that nAChR and P2X receptor activation caused by co-application of maximum agonist concentrations produced local Na^+ or Ca^{2+} depletion reducing the driving force required for inward current. Another concern would be poor voltage control of large amplitude currents evoked by co-application of maximum agonist concentrations. In order to rule out these possibilities, EC_{50} concentrations of ACh (100 μM) and ATP (30 μM) were used to activate nAChRs and P2X receptors, respectively. Because submaximal agonist concentrations were used, neither receptor desensitized (Fig. 3.6). However, the inward current caused by simultaneous activation of $\alpha_3\beta_4$ nAChRs and $P2X_2$ receptors was only 58 ± 7 % of predicted response ($P < 0.05$, $n = 7$).

ACh may interact directly with P2X receptors to modulate their function or ATP may directly interact with nAChRs to modulate their function. Cultured myenteric neurons cannot be used to evaluate this possibility as nearly all neurons express both

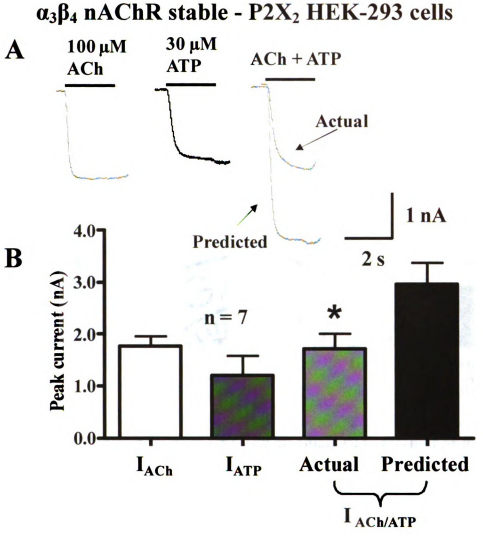


Figure 3.6: Cross-inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed by HEK-293 cells is maintained when receptors were activated by agonist EC_{50} concentrations.

A: ATP- (30 μM) and ACh- (100 μM) induced inward currents recorded from a HEK-293 cell stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2$ receptors. ACh current activated by the EC_{50} concentration does not desensitize. Currents caused by agonist co-application exhibited cross-inhibition. *B*: Pooled data from experiments shown in "A" "n' indicates the number of cells. *Indicates significantly different from the predicted current ($P < 0.05$).

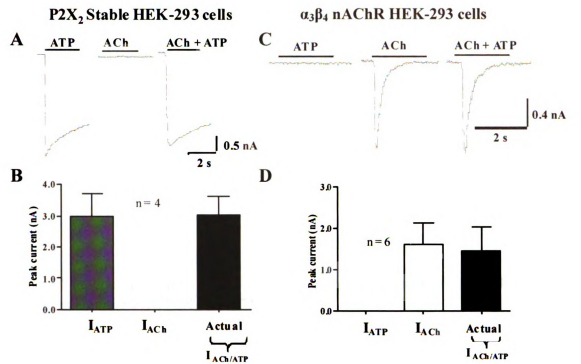


Figure 3.7: ACh and ATP do not directly modulate $P2X_2$ receptor or $\alpha_3\beta_4$ nAChR function.

A: ATP (1 mM) caused an inward current in a HEK-293 cell stably expressing only the $P2X_2$ receptor ACh (3 mM) did not cause an inward current in these cells. ATP and ACh co-application caused an inward current that did not differ from the response caused by ATP alone. *B*: Pooled data from experiments shown in "A". In cells expressing only the $P2X_2$ receptor, co-application of ATP and ACh caused an inward current that is not different from the response caused by application of ATP alone ($P > 0.05$). *C*: ACh (3 mM) caused an inward current in a HEK-293 cell stably expressing only the $\alpha_3\beta_4$ nAChR. ATP (1 mM) did not cause an inward current in these cells. ATP and ACh co-application caused an inward current that did not differ from the response caused by ACh alone. *C*: Pooled data from experiments shown in "B". In cells expressing only the $\alpha_3\beta_4$ nAChR, co-application of ATP and ACh caused an inward current that was not different from the response caused by application of ACh alone ($P > 0.05$).

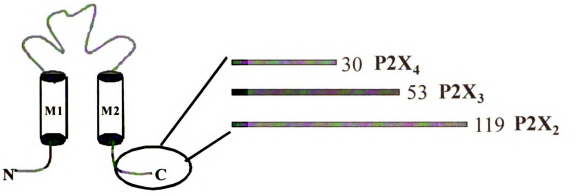


Figure 3.8: Cartoon illustrating P2X subunit C-terminal tail amino acid length differences among $P2X_2$, $P2X_3$ and $P2X_4$ subunits.

receptors. Therefore, we used HEK-293 cells expressing only $P2X_2$ or $\alpha_3\beta_4$ nAChRs to test for the direct agonist modulation mechanism of cross inhibition. ACh (3 mM) did not cause an inward current in HEK-293 cells expressing only $P2X_2$ receptors and ACh did not alter currents caused by ATP in these cells ($P > 0.05$, $n = 4$, Fig. 3.7A). Likewise, ATP (1 mM) did not cause an inward current in cells expressing only $\alpha_3\beta_4$ nAChRs and ATP did not alter currents caused by ACh in these cells ($P > 0.05$, $n= 7$, Fig. 3.7B).

$P2X_3$ and $P2X_4$ subunits interact with nAChRs. The specificity of the P2X and nAChR receptor interaction was determined by transiently expressing $P2X_3$ or $P2X_4$ subunits in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs. $P2X_3$ and $P2X_4$ subunits differ from $P2X_2$ subunits in the length of the intracellular C-terminal tail (Fig. 3.8). In cells co-expressing $\alpha_3\beta_4$ nAChR/$P2X_3$ receptors co-application of ACh and ATP caused an inward current that was only 53 ± 5% of the predicted current (Fig. 3.9A,B, $n = 25$, $P < 0.05$). In cells co-expressing $\alpha_3\beta_4$ nAChR/$P2X_4$ receptors, agonist co-application caused an inward current that was only 58 ± 5% of the predicted current ($P < 0.05$, $n = 21$, Fig. 3.9C,D).

Receptor desensitization. We next investigated if the desensitized state of nAChRs could maintain cross-inhibition of $P2X_2$ receptors, individual activation currents were 1.9 ± 0.3 nA and 2.5 ± 0.3 nA for ACh and ATP respectively. ACh was continuously applied toHEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2$ receptors. This treatment caused nAChR desensitization and then ATP and ACh were co-applied. Because the nAChR was desensitized, the current should only be due to activation of $P2X_2$ receptors. The ATP-induced current was only 71 ± 7%

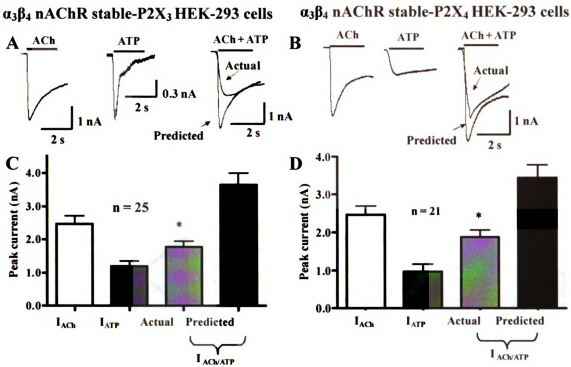


Figure 3.9: P2X subunit C-terminal tail length does not alter cross-inhibition of $\alpha_3\beta_4$ nAChR function.

A: $P2X_2$, $P2X_3$ and $P2X_4$ subunits differ mainly in their C-terminal tail amino acid length. $P2X_4$ subunits have the shortest C-terminal tail (30 amino acids). *B*: Inward currents caused by ACh (3 mM) and ATP (1 mM) in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_3$ receptors. Inward currents carried by $P2X_3$ receptors desensitize rapidly. The inward currents caused by co-application of ACh and ATP were smaller than the predicted current that would occur if the receptors functioned independently. Histograms show pooled data from experiments shown in the upper traces. *C*: Inward currents caused by ACh and ATP in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_4$ receptors. The inward currents caused by co-application of ACh and ATP were smaller than the predicted current that would occur if the receptors functioned independently. Histograms show pooled data from experiments shown in the upper traces. *Indicates significantly different from the predicted current ($P < 0.05$) in both sets of experiments.

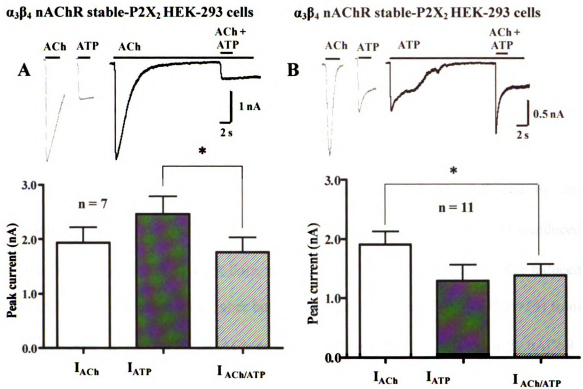


Figure 3.10: Cross inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs is maintained during receptor desensitization.

A: Inward currents caused by ACh (3 mM) and ATP (1 mM) in HEK-293 cells stably transfected with $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2$ receptors. Prolonged ACh application desensitized the $\alpha_3\beta_4$ nAChR but the inward current caused by ATP in the continued presence of ACh was smaller than the current caused by ATP prior to nAChR desensization. Histograms show pooled data from experiments shown in the upper traces. *B*: Similar results were obtained in the same cells when the $P2X_2$ receptor was first desensitized by prolonged application of ATP. The inward current caused by ACh during $P2X_2$ receptor desensitization was smaller than the ACh current recorded before $P2X_2$ receptor desensitization. Histograms show pooled data from experiments shown in the upper traces. *Indicates significantly different from the current recorded prior to ($P < 0.05$) in both sets of experiments.

of the initial current obtained prior to nAChR desensitization ($P < 0.05$, $n = 7$, Fig. 3.10A). The current recorded during simultaneous agonist application in the presence of nAChR desensitization was 71% of the predicted current; this value is higher but not statistically different ($58 \pm 7\%$, $n=13$; $P > 0.05$) than that obtained during ATP and ACh application when both the $P2X_2$ and $\alpha_3\beta_4$ nAChRs were fully function (i.e., not desensitized). The same experiment was repeated, except this time the $P2X_2$ receptors were desensitized using prolonged ATP application immediately followed by co-application of ACh and ATP. After $P2X_2$ receptor desensitization, the ACh-induced current was only 72 ± 6 % ($P < 0.05$, $n = 11$, Fig. 3.10B) of the initial ACh induced current. Again, this value was higher but not statistically different ($P > 0.05$, $n=13$) from that measured during ATP and ACh co-application when the $P2X_2$ and $\alpha_3\beta_4$ nAChRs were fully functional.

To determine the specificity of this interaction, similar experiments as above were performed with the $P2X_3$ and the $P2X_4$ subtypes of the receptor. For nAChR/$P2X_3$ cells, ACh-induced currents after $P2X_3$ receptor desensitization were $74 \pm 9\%$ of the initial ACh current ($n = 9$, $P < 0.05$, Fig. 3.11A). This value was significantly different from that occurring during simultaneous $P2X_3$ receptor and nAChR activation when these receptors were fully functional (non-desensitized) (see above, $53 \pm 5\%$; $P < 0.05$). For nAChR/$P2X_4$ cells, ACh-induced currents after $P2X_4$ receptor desensitization were only $75 \pm 6\%$ of initial activation ($P < 0.05$, $n = 9$, Fig. 3.11B). This value was significantly different from that occurring during simultaneous $P2X_4$ receptor and nAChR activation when these receptors were fully functional (see above, $58 \pm 5\%$; $P < 0.05$).

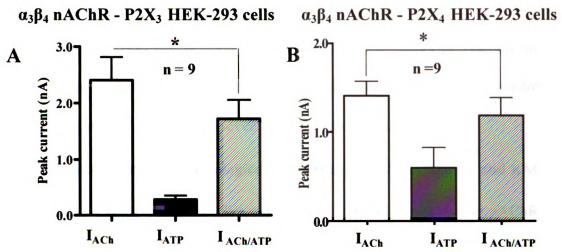


Figure 3.11: Cross inhibition between $P2X_3$ or $P2X_4$ receptors and $\alpha_3\beta_4$ nAChRs is maintained during receptor desensitization.

A: Histograms showing the amplitude of ACh (3 mM) and ATP (1 mM) currents before $P2X_3$ receptor desensitization. Then ATP was applied continuously to desensitize the $P2X_3$ receptors and ACh was re-applied in the continued presence of ATP (see Figure 8 for protocol). The ACh current was significantly smaller than that recorded prior to $P2X_3$ receptor desensitization. *B*: Histograms showing results of experiments similar to those in "A" but using cells co-expressing $P2X_4$ receptors and $\alpha_3\beta_4$ nAChRs. The ACh current recorded during $P2X_4$ receptor desensitization was smaller than that recorded prior to the desensitization protocol. *Indicates significantly different from the current recorded prior to desensitization ($P < 0.05$) in both sets of experiments.

DISCUSSION

ACh and ATP are co-transmitters in the ENS (10, 28, 30) and interactions between their target receptors has been shown in guinea-pig celiac ganglion neurons (32, 33), rat PC-12 cells (23, 25), superior cervical sympathetic neurons (24), cultured myenteric neurons (38) and in heterologously expressed receptors in *Xenopus* oocytes (13). In the present study, we have reproduced this receptor interaction in mammalian cells expressing $\alpha_3\beta_4$ nAChRs and several P2X receptor subunits.

Functional and pharmacological properties of $P2X_2$ receptors and nAChRs are retained in HEK-293 cells. We confirmed that $P2X_2$ receptors and nAChRs are functionally linked in guinea pig cultured myenteric neurons (38) and show that this interaction can be reproduced in a heterologous expression system. Heterologous expression of these receptors did not alter their localization and pharmacological function. When the $P2X_2$ and $\alpha_3\beta_4$ nAChRs are heterologously expressed in HEK-293 cells individually, they are localized to the cell membrane. In cells that express both $P2X_2$ and $\alpha_3\beta_4$ nAChRs, both receptors are co-localized to the membrane as observed by immunecytochemistry. This displays a spatial proximity of these receptors in the cell membrane. $P2X_2$ receptors desensitize slowly while responses mediated by enteric $\alpha_3\beta_4$ nAChRs desensitize in less than 2 s; these properties were retained in HEK-293 cells expressing $\alpha_3\beta_4$ nAChRs or $P2X_2$ receptors. Concentration-response curves for ACh- and ATP-induced inward currents were also similar in cultured myenteric neurons and HEK-293 cells expressing $\alpha_3\beta_4$ nAChRs or $P2X_2$ receptors. The current voltage relationship for currents carried by $P2X_2$ receptors $\alpha_3\beta_4$ nAChRs exhibit inward

rectification (37, 38, 39) and this property was also retained in HEK-293 cells. Therefore, HEK-293 cells are an appropriate model system to study the details of the molecular interactions between P2X and nAChRs.

Cross- inhibition between nAChRs and P2X receptors expressed in HEK-293 cells. We used HEK-293 cells that stably expressed $P2X_2$ receptors and transiently expressed $\alpha_3\beta_4$ nAChRs or HEK-293 cells that stably expressed $\alpha_3\beta_4$ nAChRs and transiently expressed $P2X_2$ receptors to study receptor cross inhibition. Similar data were obtained with both types of receptor combinations. As occurs with cultured myenteric neurons, when $P2X_2$ receptors and nAChRs are co-activated in HEK-293 cells expressing both receptors, the resulting inward current is substantially smaller than the current that would have occurred if the two receptors functioned independently. If P2X receptors and nAChRs function independently, co-activation of receptors would cause fully additive currents. The response amplitude would be predicted by summing the currents evoked by individual agonist application; this is the predicted current. The data obtained in HEK-293 cells indicate that cross-inhibition is not a neuronal specific response and it does not require neuron specific proteins or other molecules to maintain the receptor interaction. We have also ruled out receptor rundown or desensitization as a mechanism responsible for the proposed cross-inhibition as we were able to show that the currents caused by individual application of ACh or ATP were stable in amplitude through the time course of our studies.

It is possible that cross-inhibition occurs because P2X receptors have an allosteric inhibitory binding site for ACh, or that nAChRs have a similar binding site for ATP (25, 31). Another possibility is that ACh activates muscarinic receptors or ATP activates P2Y

receptors which are both endogenously expressed by HEK-293 cells (22). These receptors link to protein kinase C activation which could then alter the function of nAChRs or P2X receptors. These hypotheses would be difficult to test in cultured myenteric neurons, as nearly every neuron co-expresses $P2X_2$ receptors and nAChRs (37, 38) and many myenteric neurons also express muscarinic and P2Y receptors (11, 21). However, using HEK-293 cells it is possible to control receptor expression. We showed that ACh does not activate $P2X_2$ receptors, and more importantly, ACh does not modify activation of the $P2X_2$ receptor by ATP in the absence of nAChR expression. Similarly, ATP does not modify nAChR function in the absence of $P2X_2$ receptor expression. These data indicate that ACh or ATP do not directly modulate the function of $P2X_2$ receptors or nAChRs and do not indirectly modify their function via activation of muscarinic- or P2Y-receptor linked signaling pathways.

We used saturating agonist concentrations in the studies of cross inhibition. It is possible that cross inhibition is not due to direct receptor interaction but could be due to local depletion of Na^+ or Ca^{2+} ions near the channel during maximal activation. Inadequate voltage control during maximal receptor activation could also account for non-additivity of agonist responses. To address these concerns, we studied receptor cross inhibition using EC_{50} concentrations of ACh and ATP. We found that even during submaximal receptor activation, agonist co-application produced responses that were smaller in amplitude than the predicted additive response. These data indicate non-additivity between $P2X_2$ and nAChRs is not attributable to local ion depletion or poor voltage clamp of large amplitude currents.

Cross inhibition of $\alpha_3\beta_4$ nAChR function is not specific for $P2X_2$ receptors. We next determined if inhibition of $\alpha_3\beta_4$ nAChR function was specific for $P2X_2$ subunits. P2X receptor subunits differ largely in the length and composition of their C-terminal intracellular tail. The $P2X_2$ subunit has a C-terminal tail length of 119 amino acids (26). We focused on $P2X_3$ subunits, with a C-terminal tail length of 53 amino acids, as these are expressed by myenteric neurons (29) and the $P2X_4$ subunit because it has the shortest C-terminal tail at 30 amino acids (26). By narrowing down the region of the C-terminal tail that might be responsible for this interaction, a better understanding of the sequence regions or motif that mediate this interaction can be elucidated. We detected cross-inhibition between $\alpha_3\beta_4$ nAChRs and both $P2X_3$ and $P2X_4$ receptors suggesting that $\alpha_3\beta_4$ nAChRs can interact with multiple P2X receptors subunits. Although $P2X_2$ and $P2X_4$ C-terminal tails do not have identical sequences (24), the 30 amino acid C-terminal tail length of the $P2X_4$ subunit is sufficient to produce the proposed allosteric linking to the $\alpha_3\beta_4$nAChR. Others have shown that a segment of the C-terminal tail of the $P2X_2$ subunit (amino acids 22-119 from the end of the second transmembrane domain) interacts with the cys-loop 5-HT_{3A} and $GABA_A$ receptors (3, 4).

Desensitized states of P2X and nAChRs can mediate cross-inhibition. Previous work (21) showed that cross inhibition between neuronal nAChRs and P2X receptors requires the open state of the channels and little cross inhibition occurred during desensitization of one of the receptors. However, we found cross-inhibition of $\alpha_3\beta_4$ nAChR function when $P2X_2$, $P2X_3$ or $P2X_4$ receptors co-expressed by the same cells were desensitized. Similarly, $P2X_2$ receptor function could be inhibited by

desensitized $\alpha_3\beta_4$ nAChRs in cells expressing both receptors. Although cross-inhibition was maintained during receptor desensitization the amount of cross- inhibition was not equivalent to that occurring when fully active, non-desensitized receptors were studied. This difference was most prominent when nAChRs were co-expressed with $P2X_3$ or $P2X_4$ subunits. This suggests that the desensitized state of one receptor (either the P2X or $\alpha_3\beta_4$ nAChR) is less efficient at impairing current flow through the linked receptor. Therefore, differences in our data from those of Nakazawa (24) are based on the magnitude of cross-inhibition caused by desensitized receptors rather than complete inability of the desensitized receptor to cause cross inhibition. Khakh and co-workers (13) studied the effects of receptor desensitization on interactions between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs expressed in *Xenopus* oocytes. They used a mutant $P2X_2$ receptor (T18A) which desensitizes more than 10-fold faster than wild type $P2X_2$ receptors. These studies showed that currents carried by $\alpha_3\beta_4$ nAChRs were inhibited when the T18A $P2X_2$ receptor was fully activated but that nAChR currents partly recovered as the T18A $P2X_2$ receptor desensitized. These data are again consistent with our data showing that the desensitized state of one receptor in the interacting pair ($\alpha_3\beta_4$ nAChR-$P2X_2$) inhibits current flow through the coupled receptor, but the inhibition is reduced compared to that occurring when the channels are in the fully open state.

Conclusions. Cross-inhibition between Cys-loop type receptors and P2X receptors maybe a mechanism of receptor modulation in the enteric nervous system (2, 38). This interaction does not require intracellular signaling mechanisms and occurs in cell free patches of cultured neurons suggesting close coupling of the two receptors in the

neuronal membrane (38). Functional interaction between nAChRs and $P2X_2$ receptors also occurs when these receptors are expressed heterologously in HEK-293 cells suggesting that the receptor interaction does not require nervous system specific proteins. Conformational spread could be a mechanism for cross-inhibition between $\alpha_3\beta_4$ nAChRs and P2X receptors (13, 14) as may also occur with other receptors and channels (5). Conformational spread would require that nAChRs and P2X receptors are so closely associated in the membrane that opening of one channel impairs opening of the second channel. Fluorescence resonance energy transfer analysis of $\alpha_4\beta_2$ nAChRs (brain specific receptors) and $P2X_2$, receptors expressed in HEK-293 cells revealed that the receptors were localized within 100 nm of each other in the plasma membrane. This close association would make conformational spread during receptor activation possible perhaps through heterodimer formation (15). Conformational spread would also be consistent with our desensitization data. P2X receptors and nAChRs adopt different conformations in the closed, open and desensitized states (12, 19). Conformation spread from the open state readily impair current flow in the adjacent channel, while conformation spread from the desensitized state of one receptor impairs current flow in the adjacent channel less effectively.

Acknowledgements

We are grateful to Dr. Xiao (Georgetown University, Washington D.C.) for generously donating the $\alpha_3\beta_4$ nAChR stable HEK-293 cell line, and Dr. Alan North (University of Manchester, Manchester, UK) for the $P2X_2$, $P2X_3$ and $P2X_4$ plasmids. We

would also like to thank Dr. Jerry A. Stitzel (University of Colorado, Boulder, CO) for providing the mouse α_3 and β_4 plasmids.

REFERENCES

1. **Bian X, Bertrand PP, Bornstein JC.** Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. *J Physiol* 528: 551-560, 2000.

2. **Barajas-López C, Montaño LM, Espinosa-Luna R.** Inhibitory interactions between 5-HT_3 and P2X channels in submucosal neurons. *Am J Physiol* 283: G1238-1248, 2002.

3. **Boué-Grabot E, Barajas-López C, Chakfe Y, Blais D, Bélanger D, Emerit MB, Séguéla P.** Intracellular cross talk and physical interaction between two classes of neurotransmitter-gated channels. *J Neurosci* 23: 1246-1253, 2003.

4. **Boué-Grabot E, Toulmé E, Emerit MB, Garret M.** Subunit-specific coupling between gamma-aminobutyric acid type A and $P2X_2$ receptor channels. *J Biol Chem* 279: 52517-52525, 2004.

5. **Bray D, Duke T.** Conformational spread: the propagation of allosteric states in large multiprotein complexes. *Annu Rev Biophys Biomol Struct* 33:53-73, 2004.

6. **Castelucci P, Robbins HL, Poole DP, Furness JB.** The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system. Histochem Cell Biol 117: 415-422, 2002.

7. **Edwards FA, Gibb AJ.** ATP - a fast neurotransmitter. *FEBS* 325: 86-89, 1993.

8. **Furness, JB, Sanger GJ.** Intrinsic nerve circuites of the gastrointestinal tract: identification of drug targets. *Curr Opin Pharmacol* 2: 612-622, 2002.

9. **Furness JB.** The organisation of the autonomic nervous system: peripheral connections. *Auton Neurosci* 30: 1-5, 2006.

10. **Gallligan JJ, LePard KJ, Schneider DA, Zhou X.** Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* 81: 97-103, 2000.

11. **Gwynne RM, Bornstein JC.** Synaptic transmission at functionally identified synapses in the enteric nervous system: roles for both ionotropic and metabotropic receptors. *Current Neuropharmacology* **5**: 1-17, 2005.

12. **He ML, Koshimizu TA, Tomić M, Stojilkovic SS.** Purinergic $P2X_2$ receptor desensitization depends on coupling between ectodomain and C-terminal domain. M*ol Pharmacol* 62: 1187-1197, 2002.

13. **Khakh BS, Zhou X, Sydes J, Galligan JJ, Lester HA.** State-dependent cross-inhibition between transmitter-gated cation channels. *Nature* 406: 405-10, 2000.

14. **Khakh BS.** Molecular physiology of P2X receptors and ATP signaling at synapses. *Nature Reviews* 2:165-174, 2001.

15. **Khakh BS, Fisher JA, Nashmi R, Bowser DN, Lester HA.** An Angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with fluorescence resonance energy transfer and total internal reflection fluorescence microscopy. *J Neurosci* 25: 6911-6920, 2005.

16. **Lepard KJ, Galligan JJ.** Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. *Am J Physiol* 276: G529-G538, 1999.

17. **McGehee DS, Role LW.** Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Ann Rev Physiol* **57**:521-546, 1995.

18. **Miyazawa A, Fujiyoshi Y, Unwin N.** Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423**: 949-955, 2003.

19. **Mourot A, Rodrigo J, Kotzyba-Hibert F, Bertrand S, Bertrand D, Goeldner M.** Probing the reorganization of the nicotinic acetylcholine receptor during desensitization by time-resolved covalent labeling using [^{3}H]AC5, a photoactivatable agonist. *Mol Pharmacol* **69**: 452-461, 2006.

20. **Monro RL, Bertrand PP, Bornstein JC.** ATP participates in three excitatory postsynaptic potentials in the submucous plexus of the guinea pig ileum. *J Physiol* **556**: 571-584, 2004.

21. **Morno RL, Bornstein JC, Bertrand PP.** Synaptic transmission from the submucosal plexus to the myenteric plexus in Guinea-pig ileum. *Neurogastroenterol Motil* **20**: 1165–1173, 2008.

22. **Mundell SJ, Benovic JL.** Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem* **275** (17): 12900-12908, 2000.

23. **Nakazawa K, Fujimori K, Takanaka A, Inoue K.** Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. *J Physiol* **434**: 647-60, 1991.

24. **Nakazawa K.** ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. *J Neurosci* **14:**740-750, 1994.

25. **Nakazawa K.** Reduction of acetylcholine-activated current by low concentrations of extracellular adenosine-triphosphate. *Life Sciences* 57: 351-356, 1995.

26. **North RA, Surprenant A.** Pharmacology of cloned P2X receptors. *Ann Rev Pharmacol Toxicol* 40: 563-80, 2000.

27. **North RA.** Molecular physiology of P2X receptors. *Physiol Rev* 82: 1013-1067, 2002.

28. **Nurgali K, Furness JB ,Stebbing MJ.** Analysis of purinergic and cholinergic fast synaptic transmission to identified myenteric neurons. *Neuroscience* 116: 335-347, 2003.

29. **Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB.** The distribution of $P2X_3$ purine receptor subunits in the guinea pig enteric nervous system. *Auton Neurosci* 101: 39-47, 2002.

30. **Ren J, Bertrand PP.** Purinergic receptors and synaptic transmission in enteric neurons. *Purinergic Signal* 4: 255-266, 2008.

31. **Robertson SJ, Ennion SJ, Evans RJ, Edwards FA.** Synaptic P2X receptors. *Curr Opin Neurobiol* 11: 378-386, 2001.

32. **Searl TJ, Redman RS, Silinsky EM.** Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J Physiol* 510: 783-791, 1998

33. **Silinsky EM, Gerzanich V.** On the excitatory effects of ATP and its role as a neurotransmitter in coeliac neurons of the guinea-pig. *J Physiol* 464:197-212, 1993.

34. **Toulmé E, Blais D, Léger C, Landry M, Garret M, Séguéla P, Boué-Grabot E.** An intracellular motif of $P2X_3$ receptors is required for functional cross-talk with $GABA_A$ receptors in nociceptive DRG neurons. *J Neurochem* 102: 1357-1368, 2007.

35. **Vial C, Roberts JA, Evans RJ.** Molecular properties of ATP-gated P2X receptor ion channels. *Trends Pharmacol Sci* 25: 487-493, 2004.

36. **Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ.** Rat α_3/β_4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. *Mol Pharmacol* **54**: 322-333, 1998.

37. **Zhou X, Galligan JJ.** P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. *J Physiol* **496**: 719-729, 1996.

38. **Zhou X, Galligan JJ.** Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *J Physiol* **513**: 685-697, 1998.

39. **Zhou X, Ren J, Brown E, Schneider D, Caraballo-Lopez Y, Galligan JJ.** Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther* **302**: 889-897, 2002.

CHAPTER 4

Molecular Mechanisms of Cross-inhibition Between Nicotinic Acetylcholine Receptors and P2X Receptors in Myenteric Neurons and HEK-293 cells

ABSTRACT

$P2X_2$ and nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast synaptic excitation in the enteric nervous system (ENS). P2X receptors and nAChRs are functionally linked. This study examined the mechanisms responsible for interactions between $P2X_2$ and $\alpha_3\beta_4$ subunit-containing nAChRs in HEK-293 cells and cultured myenteric neurons from the guinea pig ileum. Whole-cell patch clamp techniques were used to measure P2X and nAChR currents caused by acetylcholine and ATP. In HEK-293 cells expressing $\alpha_3\beta_4$ nAChRs and $P2X_2$ receptors, co-application of ATP and ACh caused an inward current that was 56 ± 7% of the current that should occur if these channels functioned independently ($P < 0.05$, $n = 9$); we call this interaction cross-inhibition. Cross-inhibition did not occur in HEK-293 cells expressing $\alpha_3\beta_4$ nAChRs and transfected with a C-terminal tail truncated $P2X_2$ receptor ($P2X_2TR$; $P > 0.05$, $n = 8$). Intracellular application of the C-terminal tail of the $P2X_2$ receptor blocked nAChR-P2X receptor cross-inhibition in HEK-293 cells and myenteric neurons. In the absence of ATP, $P2X_2$ receptors constitutively inhibited nAChR currents. Constitutive inhibition did not occur in cells expressing $\alpha_3\beta_4$ nAChRs transfected with $P2X_2TR$. ATP-induced currents in cells stably expressing $P2X_2$ receptors were unaffected by co-expression with $\alpha_3\beta_4$ nAChRs in the absence of ACh. These data indicate that a portion of the C-terminal tail of $P2X_2$ receptors mediates cross-inhibition between $\alpha_3\beta_4$ nAChR-$P2X_2$ receptors. The closed state of the $P2X_2$ receptor

inhibits nAChR function. These molecular interactions may modulate transmission at enteric synapses that use ATP and acetylcholine as neurotransmitters.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) and P2X receptors are ligand-gated cation channels that mediate cholinergic and purinergic fast synaptic excitation of enteric neurons (9, 10, 11). NAChRs are a member of the Cys-loop receptor family which also includes 5-HT_3 receptors and $GABA_A$ receptors. Cys-loop receptors are composed of 5 subunits and each subunit has four transmembrane domains and extracellular N and C-terminal tails (21, 22). Neuronal nAChRs are composed of two α and three β subunits; there are eight neuronal α (α_2-α_9) and three neuronal β (β_2-β_4) nAChR subunits (21). In the ENS, the predominant nAChR subtype is composed of α_3 and β_4 subunits, and acetylcholine (ACh) acting at nAChRs is the primary fast excitatory neurotransmitter in the ENS (9, 42). P2X receptors belong to a different family of ligand-gated cation channels. P2X receptors are composed of three subunits; each subunit has two transmembrane domains and intracellular N and C-terminal tails. P2X receptors are homomeric or heteromeric receptors composed of one or combinations seven ($P2X_1$-$P2X_7$) subunits (7, 16, 28, 29, 34, 38). Enteric neurons express $P2X_2$ and $P2X_3$ subunits (5, 31, 41). ATP acting at P2X receptors contributes to fast synaptic excitation in subsets of enteric neurons and ATP and ACh are co-transmitters at many synapses in the ENS (9, 19, 30, 33).

Because P2X receptors and nAChRs are so differently structured, they have been assumed to function independently. However, studies have shown a functional interaction between these channels. P2X and nAChR co-expressed in cultured myenteric neurons and in human embryonic kidney cells (HEK-293), when simultaneously

activated, mediate a mutually inhibitory interaction. That is, activation of one channel inhibits responses mediated by the other channel, resulting in fewer channels opening than expected, also referred to as cross-inhibition (41, 15, 6). This functional interaction has also been shown to occur between nAChRs and different subtypes of the P2X receptor including $P2X_2$, $P2X_3$ and $P2X_4$ (20). It seems that P2X receptors interact promiscuously with different Cys-loop family members; studies have shown interactions between P2X receptors and nAChRs, $5\text{-}HT_3$ and $GABA_A$ receptors (41, 15, 6, 3, 4). More detailed studies have also observed that the functional interactions between these receptors occur not only in the active, but also in the desensitized states of these receptors in HEK-293 cells (6).

This study examines in more detail the molecular mechanisms that underlie the functional relationship between these receptors and its dependence on a concurrent physical association in HEK-293 cells and cultured myenteric neurons. Because HEK-293 cells do no endogenously express any of these receptors, they are a unique mammalian model to investigate the specific interactions that involve specific domains of the receptors which may attribute to the functional coupling between them.

MATERIALS AND METHODS

Human embryonic kidney (HEK-293) cells

Cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) F-12 containing 10% fetal bovine serum, 10% GluMax (Invitrogen, Carlsbad, CA), and 100 units ml^{-1} penicillin and streptomycin, except for the rat $\alpha_3\beta_4$ nAChR stable HEK-293 cell line which also contained 0.5 mg/ml geneticin (GIBCO, G418). Cells were maintained at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. Cells were passaged once every 3 days when they reached 90% confluence. Transient transfection of cells was accomplished using electroporation. A 0.4 cm Gene Pulser Cuvette (BIO-RAD) was used with 2 μg of total plasmid protein along with 0.2 μg of GFP to help identify transfected cells. The electroporation machine used was Gene Pulser Xcell™ Electroporation System (BIORAD). This resulted in 80% transfection efficiency. Afterwards, cells were plated on 35 mm coverslips and maintained in the incubator for 24 hrs before use in electrophysiology experiments. A rat $\alpha_3\beta_4$ nAChR stable HEK-293 cell line was provided by Dr. Yingxian Xiao from Georgetown University (40). Plasmids containing the coding sequences for rat $P2X_2$ receptor subunit was provided by Dr. Alan North (University of Manchester, Manchester, UK). Plasmids containing the coding sequence for the rat $P2X_2TR$ where a generous gift from Dr. Boué-Grabot (CNRS Université Victor Segalen Bordeaux, France). Plasmids containing the coding sequences for the murine α_3 and murine β_4 nAChR subunits were provided by Dr. Jerry A. Stitzel (University of Colorado, Denver Colorado).

Myenteric neurons

Neurons were cultured as described previously (41, 6). Two newborn guinea-pigs (<36 hrs old) were killed by severing the major neck blood vessel following halothane anesthesia. The small intestine was removed from the animals and placed in cold (4 °C) Krebs solution of the following composition (millimolar): 117 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 $MgCl_2$; 1.2 NaH_2PO_4; 25 $NaHCO_3$ and 11 glucose. The longitudinal muscle myenteric plexus was stripped free using a cotton swab and cut into 5 mm pieces. Tissues were digested with 1600 U of trypsin, followed by trituration with a fire-polished Pasteur pipette. After incubation with 2000 U crab hepatopancreas collagenase, the tissues were triturated again. The neurons were resuspended in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum, gentamicin (10 μg ml^{-1}), penicillin (100 units ml^{-1}), and streptomycin (100 units ml^{-1}) and plated onto sterile, poly-L-lysine coated 35 mm plastic dishes and maintained in an incubator at 37 °C in a 5% CO_2 atmosphere for up to 3 weeks.

Whole cell patch clamp recording

Whole-cell voltage-clamp measurements were obtained at room temperature using standard methods. Coverslips containing cells were placed on a stage of an inverted microscope (Nikon Diaphot, Mager Scientific, Inc. Dexter, MI) using phase-contrast optics. For identifying co-transfected cells, a mercury bulb with excitation wavelength of 495 nm was used with a filter for 520 nm emission. The pipette solution contained (millimolar): 122.5 K-aspartate; 20 KCl; 1 $MgCl_2$; 10 EGTA; 5 HEPES; and 2 ATP. The

pH was adjusted to 7.3 with KOH. The extracellular solution was a HEPES-based buffer composed of (millimolar): 155 NaCl; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 HEPES; and 12 glucose, pH was adjusted to 7.4 with NaOH. All recordings were made using an Axopatch 200B amplifier (Molecular Devices, Inc. Sunnyvale, CA). Data was acquired using pCLAMP 9.1 software (Molecular Devices). Whole-cell recordings were carried out using patch pipettes with tip resistances of 3-5 MΩ; seal resistances were greater than 1 GΩ. There was a two minute interval between all successive agonist applications at each holding potential, -70 mV for HEK-293 cells and -60 mV for myenteric neurons.

Protein construct expression and purification

pGEX vectors with a GST gene Fusion System were generously provided by Dr. Boué-Grabot from the CNRS Université Victor Segalen Bordeaux, France. We were provided with A pGEX with GST (pGEX-GST) as a control vector along with another vector that contains the C-terminal domain of the $P2X_2$ receptor ($P2X_2$-CT, corresponding to amino acids 365-469). The vectors were heat-shock transformed into $BL21D3^+$ cells and grown on Cam (34ug/ml) and Amp (100ug/ml) LB plates. Transformed clones were first grown in a 10 ml culture (LB media containing Cam and Amp) at 30 °C to an A_{600} of 0.5 and then this was expanded into a 500 ml culture containing Cam/Amp and 20% Glucose. These bacterial cells where grown until A_{600} of 0.6 at which point 0.1 mM isopropylβ-D -thiogalactoside (IPTG) was added. Cultures were induced for 3 hours at 30 °C and cells were finally collected by centrifugation, snap frozen and stored at -80 °C.

Cell-free extracts (soluble protein fractions of cells disrupted by sonication) from induced cultures were affinity purified on immobilized Glutathione agarose beads (Pierce), 10 fractions were collected. Fractions were checked by SDS-Phage that was silver stained. The two fractions that contained the most protein were pooled and dialyzed overnight at 4 °C into the intracellular solution buffer. Bradford assay was performed to determine protein concentration and proteins were also visualized with SDS-Page silver-staining and Western blotting analysis.

Western blot analysis

Samples were run on a 12.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing Tween 20 (10mM Tris, 0.5% Tween 20, 0.5M NaCl, T-TBS) overnight at room temperature with gentle agitation. Membranes were then incubated with the appropriate primary antibody diluted in 1% milk-T-TBS. Primary antibodies are rabbit anti-GST (generously provided by Dr. John Wang's lab (39); Michigan State University) or rabbit anti-$P2X_2$ (1:1,200) form Alamone Labs, Jerusalem. This was incubated for 1 hour at room temperature. After washing four times, 15 minutes each in T-TBS, the secondary HRP-conjugated Goat anti-Rabbit (1:10,000; Jackson Laboratories) were added in 1%-milk-T-TBS for 1 h at room temperature. Membranes were washed T-TBS as above, and proteins where visualized using Western Lightning Chemiluminescence System (Perkin Elmer Life Sciences).

Drug application

Agonists were applied onto individual neurons/cells by gravity flow from linear array quartz tubes placed near the cell. The distance from the mouth of the tubes to the

cells was ~200 μm with flow controlled manually using a micromanipulator. Computer-controlled solenoid valves (General Valve, Fairfield, NJ) were used to gate solution flow through the tubes. Agonist-induced currents were measured as the peak current amplitude. The difference between the predicted sum of the individual peak currents was compared with the current amplitude caused by co-application of two agonists.

Statistics. Data are expressed as the mean ± s.e.m. and "*n*" refers to the number of cells from which the data were obtained. The predicted current amplitude that would occur during agonist co-application was calculated by measuring the peak currents caused by previous individual agonist applications and then summing these values. . These are repeated measurements within the same cell; therefore, there is no concern for error within a single measurement because it cancels itself out. These are directly measured data that were used for statistical comparisons of actual and predicted currents. Traces illustrating predicted currents were constructed using a simple math function in Origin 8 (OriginLab Corp., Northhampton, MA). Student's *t* test was used to establish significant differences between control and treatment groups. The significance level was $P < 0.05$. EC_{50} is the half-maximal effective concentration and n_H is the slope. Y_{min} and Y_{max} are the minimum and maximum respectively. Agonist concentration-response curves were fit to the following logistic function using Origin:

$$y = \frac{(Y_{min} - Y_{max})}{1 + (x/EC_{50})^{nH}} + Y_{max}$$

RESULTS

Cross-inhibition between $P2X_2$ receptors and $\alpha_3\beta_4$ nAChRs in HEK-293 cells

We used HEK-293 cells stably expressed rat $\alpha_3\beta_4$ nAChR receptors and transiently transfected with rat $P2X_2$ receptors to study cross-inhibition. Ach (3 mM) and ATP (1 mM) were first applied individually and peak inward currents were measured. Then the agonists were applied simultaneously and the resulting inward current was only 56 ± 7 % (Fig. 4.1A and 4.1B, $P < 0.05$, $n = 9$) of the predicted current that would have occurred had the $P2X_2$ receptors $\alpha_3\beta_4$ nAChRs functioned independently. The predicted current value was calculated by summing the amplitudes of the individual agonist-evoked currents. Subsequent individual re-application of ACh and ATP caused currents that were not statistically different from those elicited before agonist co-application ($P > 0.05$, data not shown).

The $P2X_2$ C-terminal tail mediates cross-inhibition

In the next experiments, we used HEK-293 cells that stably expressed $\alpha_3\beta_4$ nAChRs and that were transiently transfected with a C-terminal tail truncated $P2X_2$ receptor ($P2X_2TR$). This receptor has a stop codon at amino acid 374, truncating a majority of the $P2X_2$ receptor cytoplasmic C-terminal domain. The truncated subunit can assemble into a functional receptor (2). In these cells, currents caused by simultaneous application of ACh and ATP caused an inward current that was not statistically different from the predicted current value (82 ± 6%, $P > 0.05$, $n = 8$, Fig. 4.1C).

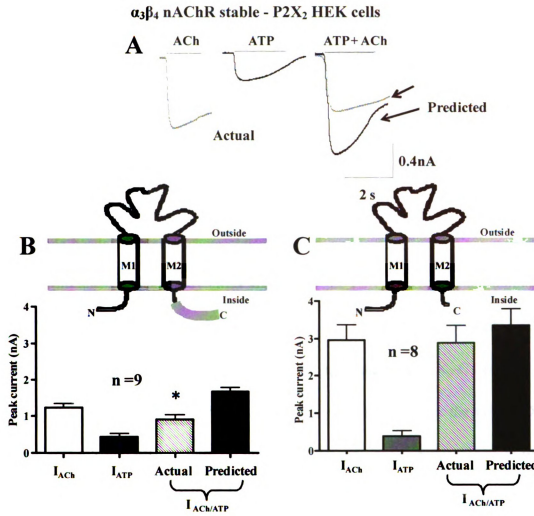


Figure 4.1: Truncation of the $P2X_2$ C-terminal tail stops cross-inhibition between $P2X_2$ and nAChRs.

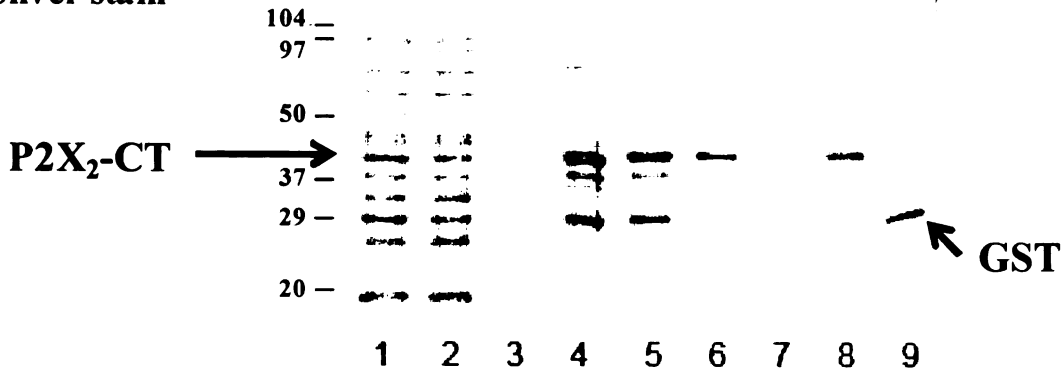
A. Representative traces ATP- (1 mM) and ACh- (3 mM) induced inward currents recorded from $\alpha_3\beta_4$ nAChRs stable HEK-293 cell line transiently transfected with $P2X_2$ receptors. *B*. Pooled data from experiments shown in "A". In cells expressing only the $\alpha_3\beta_4$ nAChR and $P2X_2$ receptors, co-application of ATP and ACh exhibited cross-inhibition. *C*. $\alpha_3\beta_4$ nAChRs stable HEK-293 cell line transiently with a truncated form of the $P2X_2$ receptor ($P2X_2TR$), cross-inhibition was abolished. 'n' indicates the number of cells. *Indicates significantly different from the predicted sum of currents ($P < 0.05$).

These data described above suggest that the C-terminal domain of the $P2X_2$ receptor is a critical determinant of cross inhibition. To test this hypothesis further, we used the C-terminal tail peptide as a competitive antagonist to block cross inhibition. The peptide is a soluble form of the C-terminal tail of the $P2X_2$ receptor (GST-$P2X_2$-CT for amino acids 365-469). A control peptide containing just the GST construct was also tested. These constructs where expressed and purified via affinity chromatography on glutathione beads. Proteins where eluted, pooled, and verified with silver staining and blotted with anti-$P2X_2$ antibody (with affinity for the $P2X_2$ C-terminal tail amino acids 457-472) and anti-GST (Fig. 4.2). Using a rat $\alpha_3\beta_4$ nAChR HEK-293 stable cell line transiently transfected with rat $P2X_2$ receptors, 50 μg of the GST-$P2X_2$-CT peptide or the control GST protein were added to the recording electrode. After the GST-$P2X_2$-CT peptide was allowed diffuse into the interior of the cell, individual application of ACh (1.15 ± 0.2 nA) and ATP (0.62 ± 0.2) were followed by co-application in which the actual at 1.6 ± 0.3 nA was not statistically different from predicted at 1.8 ± 0.3 nA (Fig. 4.3B, $P > 0.05$, $n = 6$). To verify that the interaction was in fact disrupted by the $P2X_2$-CT coding region, and not the GST-peptide itself, similar experiments with the GST-control peptide were performed. After the GST-control peptide diffused into the cell, co-application of agonists still resulted in cross-inhibition of receptors in which actual (0.9 ± 0.3 nA) was significantly less that predicted (1.5 ± 0.3 nA, Fig 4.3A, $n = 6$, $P< 0.05$).

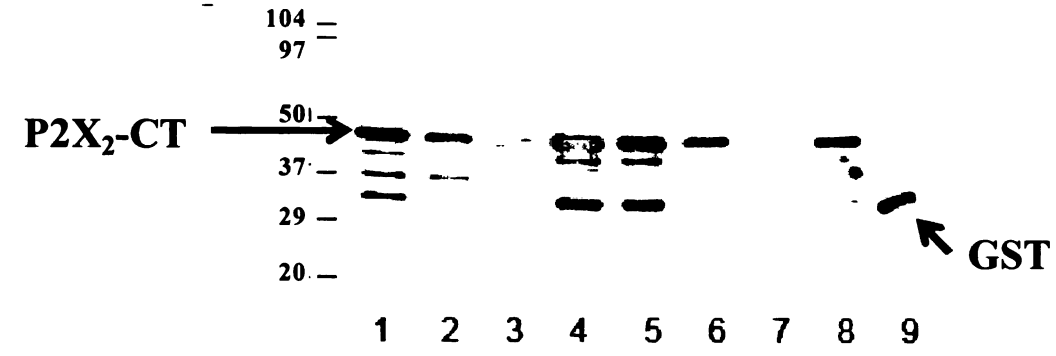
Figure 4. 2: Purification of GST-fusion construct.

Lanes in A, B and C are in the following order from left to right (lanes 1-8 for GST-$P2X_2$-CT and lane 9 for GST): Lane , 1 lysate; lane 2, unbound fraction; lane 3, elution fraction #2; lane 3, elution fraction #3; lane 5, elution fraction #4; lane 6, elution fraction #5; lane 7, elution fraction #6; lane 8, pooled fractions #3 and #4; lane 9, pooled GST fraction. *A.* Silver staining. *B.* Immunoblot with anti-GST. *C.* Immunoblot with anti-$P2X_2$.

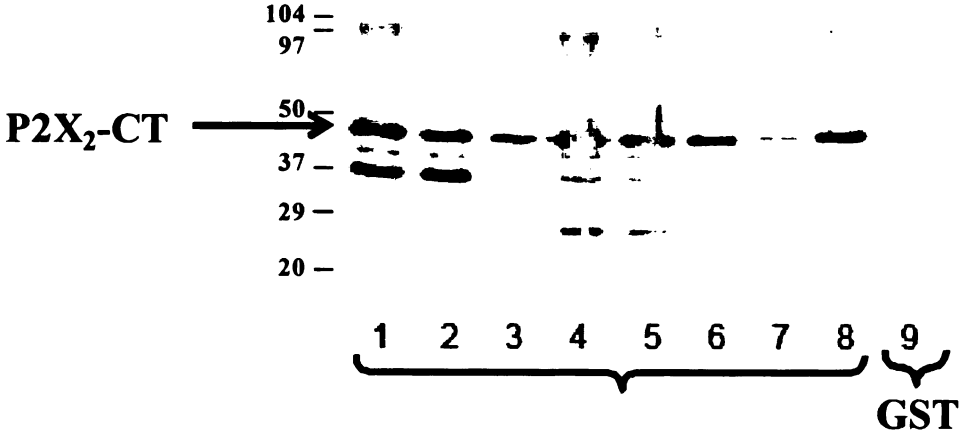
A. Silver-stain



B. a-GST-Blotting



C. a-P2X$_2$-Blotting



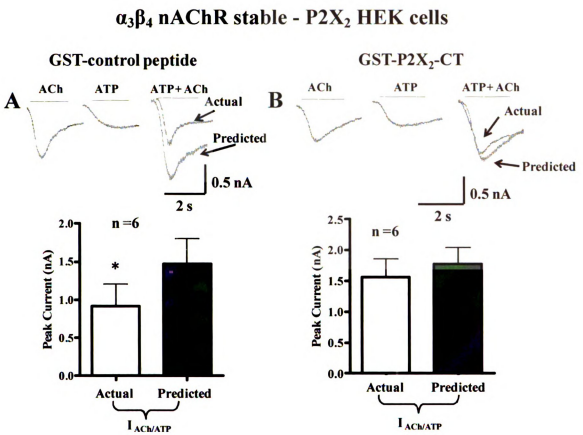


Figure 4.3: Cross-inhibition between nAChRs and $P2X_2$ receptors is mediated by the $P2X_2$ receptor C-terminal tail.

A. A GST-control peptide was added into the intracellular electrode and allowed to diffuse into the cell. This did not alter cross-inhibition between nAChRs and $P2X_2$ receptors. *B.* The addition of GST-$P2X_2$-CT peptide construct corresponding to amino acids 365-469 of the $P2X_2$ C-terminal tail abolishes cross-inhibition. 'n' indicates the number of cells. *Indicates significantly different from the predicted sum of currents ($P < 0.05$).

The $P2X_2$ C-terminal tail mediates cross-inhibition in myenteric neurons

Whole cell recordings were obtained from myenteric neurons using pipette solutions that contained the GST-control peptide. ACh (3 mM) and ATP (1mM) were individually applied to myenteric neurons caused inward currents of 0.16 ± 0.02 nA and 0.25 + 0.03 nA, respectively. Agonist co-application caused an actual response of 0.26 ± 0.04 nA that was statistically different from the predicted (0.4 ± 0.05, Fig 4.4A, $P < 0.05$, $n = 6$). After allowing sufficient time for the GST-$P2X_2$-CT peptide to diffuse into the neurons, cross-inhibition was abolished with no difference between actual and predicted responses (Fig. 4.4B), 0.20 ± 0.05 nA and 0.25 ± 0.05 respectively ($P > 0.05$, $n = 6$).

Constitutive inhibition of nAChR function by $P2X_2$ receptors

We found that ACh currents in HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs alone (2.7 ± 0.2 nA, $n = 10$) were significantly larger than ACh current recorded from HEK-293 cells stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2$ receptors (Fig. 4.5A). ACh currents were significantly smaller 1.7 ± 0.2 nA (Fig 4.5A, $P < 0.05$, $n = 10$). Constitutive inhibition did not occur in cells stably expressing $\alpha_3\beta_4$ nAChRs and transiently transfected with $P2X_2TR$ (Fig 4.5B, $P > 0.05$, $n = 16$). In $P2X_2$ stable cells, ATP currents were 1.9 ± 0.3 nA; when these cells were transiently transfected with $\alpha_3\beta_4$ nAChRs, ATP currents (2.0 ± 0.3 nA, Fig 4.5C, $P > 0.05$, $n = 12$) were not different from those measured in cells expressing only $P2X_2$ receptors. These data suggest that $P2X_2$ receptors in the closed state inhibit $\alpha_3\beta_4$ nAChRs. It is possible

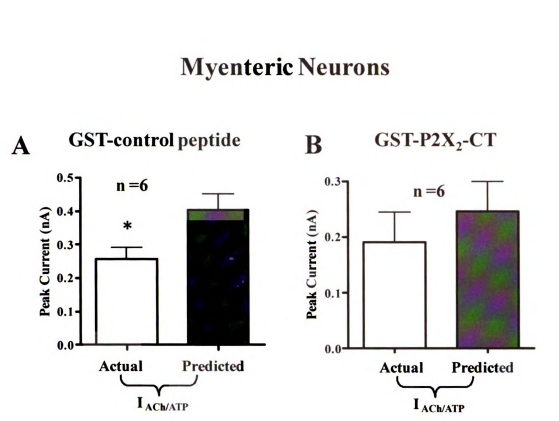


Figure 4.4: Cross-inhibition between nAChRs and $P2X_2$ receptors in myenteric neurons is mediated by the $P2X_2$ receptor C-terminal tail.

A. Experiments were performed in cultured guinea pig ileum myenteric neurons. When the GST-control peptide diffused into cell, cross-inhibition between nAChRs and $P2X_2$ receptors was still present. *B.* Upon addition of the GST-$P2X_2$-CT peptide construct to the myenteric neurons, inhibitory interactions between the receptors where abolished. 'n' indicates the number of cells. *Indicates significantly different from the predicted sum of currents ($P < 0.05$).

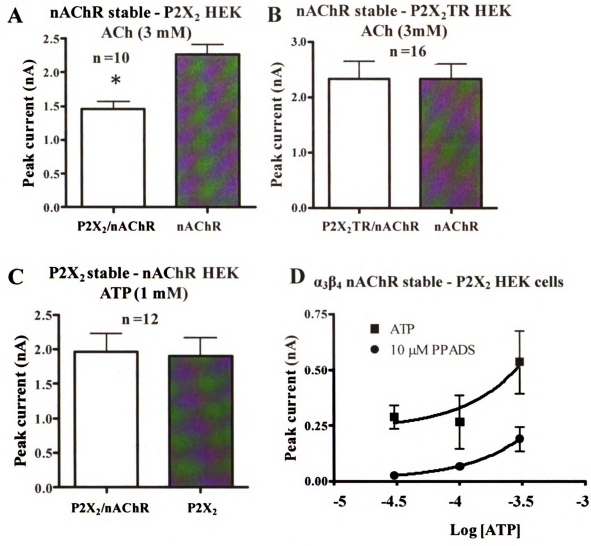


Figure 4.5: Constitutive inhibition of nAChRs.

A. ACh (3mM) induced currents in cells expressing $\alpha_3\beta_4$ nAChRs alone, currents were much larger than when the $P2X_2$ receptor is co-express. *B.* When $\alpha_3\beta_4$ nAChRs are co-expressed with a truncated form of the $P2X_2$ receptor ($P2X_2TR$), ACh induced currents are no longer suppressed compared to co-expression of $\alpha_3\beta_4$ nAChRs with wild-type $P2X_2$ receptors. *C.* ATP (1mM) induced currents from $P2X_2$ receptors alone are not altered by co-expression with $\alpha_3\beta_4$ nAChRs. *D.* PPADS (10uM) inhibits ATP induced currents via the $P2X_2$ receptor. 'n' indicates the number of cells. *****Indicates statistically different from control currents ($P < 0.05$).

that ATP released from surrounding cells might activate $P2X_2$ receptors causing cross-inhibition of nAChRs. Therefore recordings were obtained in the presence of the P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS 10 μM) to block $P2X_2$ receptors. At this concentration, PPADS blocked inward currents caused by exogenous applied ATP (Fig. 4.5D). In HEK-293 cells only expressing $\alpha_3\beta_4$ nAChR, PPADS did not alter ACh induced currents ($P > 0.05$, n= 8, Fig. 4.6). However, when these cells were transfected with $P2X_2$ receptors, the ACh current was reduced (Fig. 4.6, $P<0.05$, n=21). This effect was not altered by PPADS which blocks $P2X_2$ activation ($P < 0.05$ compared to initial ACh application, n = 10, Fig. 4.6), suggesting that $P2X_2$ receptors constitutively inhibit nAChRs.

To determine if there is allosteric inhibition of the nAChR by the $P2X_2$ receptor, concentration response curves with ACh were performed in cells only expressing nAChR where the EC_{50} was $1.2x\ 10^{-4} \pm 30$ μM, and hill slope of 1.4 (n = 4-8). In cells with nAChR/$P2X_2$ co-expression the EC_{50} was $4.4x\ 10^{-4} \pm 90$ μM and a hill slope of 0.6 (Fig 4.7A, n = 5-8). There is significant divergence of the nAChR/$P2X_2$ curve from the nAChR curve at 100 μM to 1 mM ACh concentrations ($P < 0.05$), and significant shift of the curve to the right. When similar experiments are performed, but this time for the ATP concentration response curve, here the EC_{50} is $3.4 \times 10^{-5} \pm 7$ μM, and a hill slope of 1.4 for only $P2X_2$ receptors (n = 5-6, and upon co-expression with nAChR the EC_{50} is $7.1 \times 10^{-5} \pm 7$ μM with a hill slope of 1.9 with statistical significance ATP concentrations of 3 – 30 μM and a significant rightward shift of the curve (Fig 4.7B, n = 4).

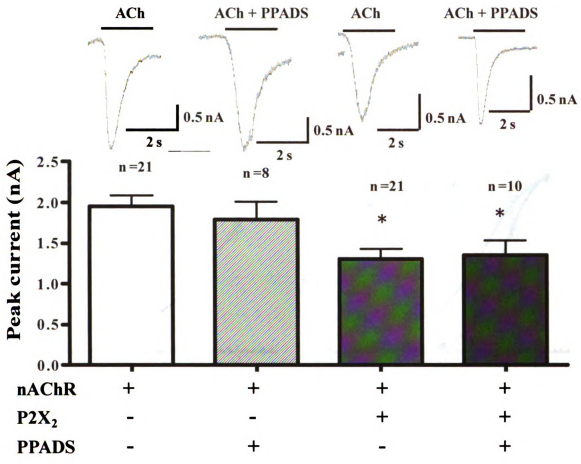


Figure 4.6: PPADS does not alter $P2X_2$ receptor mediated constitutive inhibition of $\alpha_3\beta_4$ nAChRs.

ACh (3mM) induced currents of $\alpha_3\beta_4$ nAChRs expressed individually are not altered by PPADS. However, these currents are inhibited upon co-expression of $\alpha_3\beta_4$ nAChRs with the $P2X_2$ receptor. The addition of PPADS does not alter the constitutive inhibition. 'n' indicates the number of cells. *Indicates significantly different from control currents of $\alpha_3\beta_4$ nAChRs expressed alone ($P < 0.05$).

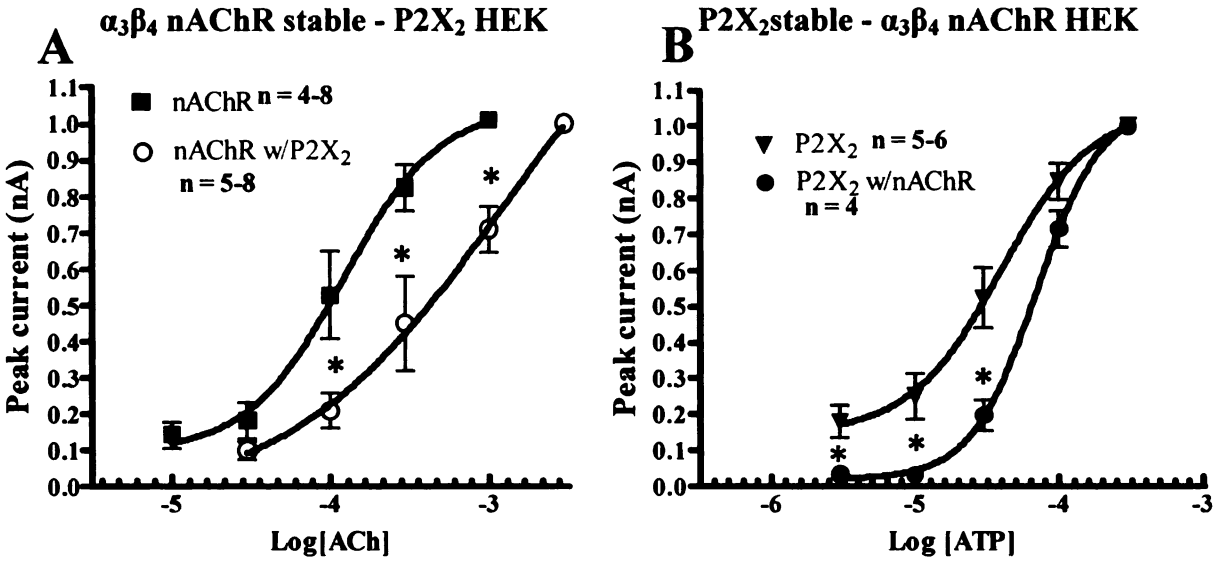


Figure 4.7: nAChRs and $P2X_2$ receptors constitutively inhibit each other.

A. Concentration response curves to ACh in cells expressing $\alpha_3\beta_4$ nAChRs alone, and in cells co-expressing $\alpha_3\beta_4$ nAChRs and $P2X_2$ receptors. *B.* Concentration response curves to ATP in cells expressing $P2X_2$ receptors alone, and in cells co-expressing $\alpha_3\beta_4$ nAChRs and $P2X_2$ receptors. 'n' indicates the number of cells. *Indicates significantly different between concentration response curves expressing individual receptors and both receptors ($P < 0.05$).

DISCUSSION

I present evidence that two structurally distinct receptors, $P2X_2$ and nAChR, which mediate fast synaptic transmission in the ENS do not only interact functionally as previously established, but in fact have a physical association. Many studies have looked at the functional aspects of this interaction in guinea-pig celiac ganglion neurons (35, 36), rat PC-12 cells (25,27), superior cervical sympathetic neurons (26), cultured myenteric neurons (41) and in heterologously expressed receptors in *Xenopus* oocytes (15) and human embryonic kidney (HEK-293) cells (6). This study establishes the physical aspects of the relationship between $P2X_2$ and nAChR and how it affects receptor function.

The $P2X_2$ C-terminal tail mediates cross-inhibition between receptors in HEK-293 cells. Upon co-application of agonist to co-activate the receptors there is a receptor cross-inhibition resulting in a mean current value that is significantly smaller than the additive predicted current had the receptors functioned independently. Cross-inhibition occurs in cells with low $P2X_2$ receptor expression. However, when the C-terminal tail of the $P2X_2$ receptor is truncated from 119 to 21 amino acids, functional interaction with nAChRs is eliminated. This suggests that the C-terminal tail of the $P2X_2$ receptor contributes to cross-inhibition of nAChR function. Although similar experiments have been previously performed in *Xenopus oocytes* (3), this is the first time that they have been performed in a mammalian system.

The importance of the $P2X_2$ receptor C-terminal tail in its interaction with other cys-loop receptors such as $5HT_3$ (3) and $GABA_A$ (4) receptors has been established using

the $P2X_2TR$. It seems that it is a general mechanism of regulation in which $P2X_2$ receptors interact with cys-loop family members via their C-terminal tail. To better understand the role of the $P2X_2$ C-terminal tail, a GST-$P2X_2$-CT construct (corresponding to amino acids 365-469) was expressed in bacteria and purified. After purity was verified, this peptide was utilized to compete for the binding sites between $P2X_2$ and nAChRs. The addition of this construct to cells expressing wild type $P2X_2$ receptors and nAChRs resulted in a dominant negative effect in which cross-inhibition between receptors was abolished. Loss of the functional interaction is not an artifact of peptide addition into the recording electrode, as the GST-control peptide did not alter cross-inhibition. These data demonstrate the role of the cytoplasmic sequence of $P2X_2$ receptors in its functional and physical interaction with nAChRs in the heterologous expression system of HEK-293 cells and in native cultured myenteric neurons. The GST-$P2X_2$-CT construct has also previously been utilized to abolish interactions between $P2X_2$ and other cys-loop members ($5HT_3$ and $GABA_A$ 3, 4). And although the GST-$P2X_2$-CT has been used previously to disrupt the interaction between $P2X_2$ and nAChRs in *Xenopus oocytes* (3), it has never before been attempted in the mammalian systems of HEK-293 cells and cultured myenteric neurons as this study has shown.

The direct correlation of loss of cross-inhibition due to $P2X_2$ receptor truncation or competitive binding with GST-$P2X_2$-CT minimizes the possibility that cross-inhibition is due to second messenger molecules generated by endogenous G-protein-coupled receptors such as P2Y or muscarinic receptors that are expressed by both HEK-293 cells

and myenteric neurons (24, 13, 23). This suggest that although it has been shown that metabotropic receptors like dopamine (D_5) receptors can modulate ion channels such $GABA_A$ receptors, that is not the case here (20,1).

$P2X_2$ receptors in the closed state produce constitutive inhibition of nAChRs. Our data suggests a physical association between these receptors that mediates a cross-inhibition upon activation. Moreover, that there is a constitutive inhibition present in the closed states of the receptors as ACh currents mediated by nAChRs are reduced when co-expressed with $P2X_2$ receptors. This constitutive inhibition of nAChR function is lost when the $P2X_2$ truncated receptor is co-expressed. It seems that even in its closed state, the $P2X_2$ receptor can lead to inhibition of nAChRs via its C-terminal tail. To exclude the possibility that ATP is being release from surrounding HEK-293 cells to activate the $P2X_2$ receptor, PPADS was used to block $P2X_2$ receptors. Even in the presence of maximum PPADS concentrations constitutive inhibition of nAChRs persisted. This was not due to PPADS altering nAChR function. To better test constitutive inhibition, concentration response curves (CRC) of ACh with nAChRs alone and nAChRs/$P2X_2$ receptors were constructed. Upon co-expression of receptors, there was a shift to the right of the ACh CRC suggesting that there is allosteric inhibition of nAChRs by $P2X_2$ receptors. These data suggest that $P2X_2$ receptors result in a shift of agonist sensitivity for nAChRs and that functional cross-inhibition does not only occur when both receptors are activated. Previous studies performed by Ren *et al.* (32) support this hypothesis. ACh and ATP mediate fast synaptic responses in murine myenteric plexus as it occurs in

guinea pig intestine, suggesting a role for both $P2X_2$ receptors and nAChRs (8, 18, 14). In studies by Ren e*t al*, $P2X_2$ subunit contribution to fast synaptic excitation in mouse myenteric neurons was studied using wild type and $P2X_2$ knockout mice. Ren *et al.* where able to show that fast excitatory post-synaptic potentials (fEPSPs) in wild type mice were the same in amplitude, rise time and time to half-decay as knockout $P2X_2$ mice. If $P2X_2$ receptors contributed to fEPSPs in wild type mice (as shown by a decrease in amplitude of fEPSPs by the addition of PPADS), how can $P2X_2$ knockout mice show the same amplitude fEPSPs? The answer is that in the presence of $P2X_2$ receptors, there is a constitutive inhibition of nAChRs. When the $P2X_2$ receptors are deleted, this constitutive inhibition is lifted, allowing full activation of the nAChRs. This results in fEPSPs that are the same in amplitude as in wild type mice. These data support our hypothesis that $P2X_2$ receptors can constitutively inhibit nAChRs even in the closed state.

However, the same was not true for the reverse scenario; experiments showed that nAChRs did not constitutively inhibit $P2X_2$ receptors, at least not as effectively as the inhibition of $P2X_2$ receptors. At maximal concentrations, ATP (1mM) mediated current was not altered by co-expression with nAChRs. In the ATP CRC there is a small change at lower concentrations of $P2X_2$ receptor mediated currents upon co-expression with nAChRs. Our studies indicate that there is constitutive inhibition between these receptors that is not equal in intensity, that $P2X_2$ receptors more potently inhibit nAChRs than nAChR inhibition of $P2X_2$ receptors. In *Xenopus oocytes,* nAChR occlusion by

$P2X_2$ receptors was stronger than the occlusion of $P2X_2$ receptors by nAChRs (15). A similar disproportion of inhibition between P2X and $GABA_A$ receptors in dorsal root ganglion neurons has also been previously reported, in which one receptor is more effective at inhibiting than the other (37).

Conclusions. ACh and ATP are co-transmitters in the ENS (9, 30, 33). The interactions between $P2X_2$ and nAChR could play a regulatory role in excitatory synaptic transmission. This cross-inhibition may regulate neuronal excitability and plasticity to limit the level of calcium influx and depolarization of neurons, a mechanism that might be missing in pathological conditions of hyperactivity such as irritable bowel syndrome (IBS). IBS is a functional bowel disorder in which there is a change in motility and in the function of sensory innervations (12). Fluorescence resonance energy transfer analysis of $\alpha_4\beta_2$ nAChRs (brain specific receptors) and $P2X_2$, receptors expressed in HEK-293 cells revealed that the receptors were localized within 100 nm of each other in the plasma membrane (17). This supports our finding that $P2X_2$ and nAChRs physically associated via the $P2X_2$ C-terminal tail, and that this physical association mediates cross-inhibition of these receptors. We also conclude that cross-inhibition is not only mediated in the active states, but also in the closed states of the receptors. A constitutive inhibition that is not proportional with $P2X_2$ receptors more effectively inhibiting nAChRs. An inhibition state that was supported by experiments in myenteric neuron fEPSPs in $P2X_2$ knockout mice (32). This receptor-receptor mediated interaction which is isolated to the intracellular portion of the membrane is a very direct method of regulation that might be

more prominent in the nervous system than our current knowledge base can attest to; something that more exploration of receptor mediated interactions would elucidate.

Acknowledgements

We are grateful to Dr. Boue-Garabot (CNRS Université Victor Segalen Bordeaux, France) for providing the $P2X_2TR$ plasmid along with the GST-control peptide and GST-$P2X_2$-CT constructs. Dr. Xiao (Georgetown University, Washington D.C.) for donating the $\alpha_3\beta_4$ nAChR stable HEK-293 cell line, and Dr. Alan North (University of Manchester, Manchester, UK) for the $P2X_2$ plasmids. And most importantly, we are very grateful for the Wang lab for its guidance and aid in expressing and purifying the GST peptide constructs.

REFERENCES

1. **Agnati LF, Ferre S , Lluis C, Franco R, Fuxe K.** Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. *Pharmacol Rev* 2003; **55**:509–550.

2. **Boue-Grabot E, Archambault V, Seguela P.** A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of $P2X_2$ ATP-gated channels. *J. Biol. Chem.* 2000; **275**(14): 10190-5.

3. **Boué-Grabot E, Barajas-López C, Chakfe Y, Blais D, Bélanger D, Emerit MB, Séguéla P.** Intracellular cross talk and physical interaction between two classes of neurotransmitter-gated channels. *J Neurosci* 2003; **23**: 1246-1253.

4. **Boué-Grabot E, Toulmé E, Emerit MB, Garret M.** Subunit-specific coupling between gamma-aminobutyric acid type A and $P2X_2$ receptor channels. *J Biol Chem* 2004; **279**: 52517-52525.

5. **Castelucci P, Robbins HL, Poole DP, Furness JB.** The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system. Histochem Cell Biol 2002; **117:** 415-422.

6. **Decker DA, Galligan JJ.** Cross-inhibition Between Nicotinic Acetylcholine Receptors and P2X Receptors in Myenteric Neurons and HEK-293 cells. *Am J Physiol Gastrointest Liver Physiol.* 2009; Apr 2. [Epub ahead of print].

7. **Edwards FA, Gibb AJ.** ATP - a fast neurotransmitter. *FEBS* 1993; **325**: 86-89.

8. **Galligan JJ, Bertrand PP.** ATP mediates fast synaptic transmission in enteric nerves. *J Neurosci* 1994; **14**: 7563-7571.

9. **Gallligan JJ, LePard KJ, Schneider DA, Zhou X.** Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* 2000; **81:** 97-103.

10. **Galligan JJ.** Pharmacology of synaptic transmission in the enteric nervous system. *Current opinion in pharmacology* 2002; **2:** 623-629.

11. **Galligan JJ, North RA.** Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil,* 2004; **16 (suppl 1)** 64-70.

12. **Galligan JJ.** Enteric P2X receptors as potential targets for drug treatment of the irritable bowel syndrome. *Br J of Pharm* 2004; **141**: 1294-1302.

13. **Gwynne RM, Bornstein JC.** Synaptic transmission at functionally identified synapses in the enteric nervous system: roles for both ionotropic and metabotropic receptors. *Current Neuropharmacology* 2005; **5**: 1-17.

14. **Johnson PJ, Shum OR, Thornton PD, Bornstein JC**. Evidence that inhibitory motor neurons of the guinea pig small intestine exhibit fast excitatory postsynaptic potentials mediated via P2X receptors. *Neurosci Lett* 1999; **266**: 169-172.

15. **Khakh BS, Zhou X, Sydes J, Galligan JJ and Lester HA.** State-dependent cross-inhibition between transmitter-gated cation channels. *Nature* 2000; **406**:405-10.

16. **Khakh BS.** Molecular physiology of P2X receptors and ATP signaling at synapses. *Nature Reviews* 2001; **2**:165-174.

17. **Khakh BS, Fisher JA, Nashmi R, Bowser DN, Lester HA**. An Angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with fluorescence resonance energy transfer and total internal reflection fluorescence microscopy. *J Neurosci* 2005; **25**: 6911-6920.

18. **LePard KJ, Messori E, Galligan JJ.** Purinergic (P2X) fast excitatory postsynaptic potentials in myenteric neurons of guinea pig; distribution and pharmacology. Gastroenterology 1997; **113**: 1522-1534.

19. **Lepard KJ, Galligan JJ.** Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. *Am J Physiol* 1999; **276**: G529-G538.

20. **Liu F, Wan Q, Pristupa ZB, Yu X-M, Wang YT, Niznik HB.** Direct protein - protein coupling enables cross-talk between dopamine D_5 and g-aminobutyric acid A receptors. *Nature* 2000; **403**: 274 – 280.

21. **McGehee DS, Role LW.** Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Ann Rev Physiol* 1995; **57**:521-546.

22. **Miyazawa A, Fujiyoshi Y, Unwin N.** Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 2003; **423**: 949-955.

23. **Morno RL, Bornstein JC, Bertrand PP.** Synaptic transmission from the submucosal plexus to the myenteric plexus in Guinea-pig ileum. *Neurogastroenterol Motil* 2008; **20**: 1165–1173.

24. **Mundell SJ, Benovic JL.** Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem* 2000; **275** (17): 12900-12908.

25. **Nakazawa K, Fujimori K, Takanaka A, Inoue K.** Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. *J Physiol* 1991; **434**: 647-60.

26. **Nakazawa K.** ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. *J Neurosci* 1994; **14:**740-750.

27. **Nakazawa K.** Reduction of acetylcholine-activated current by low concentrations of extracellular adenosine-triphosphate. *Life Sciences* 1995; **57**: 351-356.

28. **North RA, Surprenant A.** Pharmacology of cloned P2X receptors. *Ann Rev Pharmacol Toxicol* 2000; **40**: 563-80.

29. **North RA.** Molecular physiology of P2X receptors. *Physiol Rev* 2002; **82**: 1013-1067.

30. **Nurgali K, Furness JB ,Stebbing MJ.** Analysis of purinergic and cholinergic fast synaptic transmission to identified myenteric neurons. *Neuroscience* 2003; **116**: 335-347.

31. **Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB.** The distribution of $P2X_3$ purine receptor subunits in the guinea pig enteric nervous system. *Auton Neurosci* 2002; **101**: 39-47.

32. **Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne, DA, Ford AP, and Galligan JJ.** $P2X_2$ subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. *J Physiol* 2003; **522.3**: 809-821.

33. **Ren J, Bertrand PP.** Purinergic receptors and synaptic transmission in enteric neurons. P*urinergic Signal* **4**: 2008;255-266.

34. **Robertson SJ, Ennion SJ, Evans RJ, Edwards FA.** Synaptic P2X receptors. *Curr Opin Neurobiol* 2001; **11:** 378-386.

35. **Searl TJ, Redman RS, Silinsky EM.** Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. *J Physiol* 1998; **510**: 783-791.

36. **Silinsky EM, Gerzanich V.** On the excitatory effects of ATP and its role as a neurotransmitter in coeliac neurons of the guinea-pig. *J Physiol* 1993; **464**:197-212.

37. **Sokolova E, Nistri A, Giniatullin R.** Negative cross talk between anionic $GABA_A$ and cationic P2X ionotropic receptors of rat dorsal root ganglion neurons. *J Neurosci* 2001; *21*(14): 4958-4968.

38. **Vial C, Roberts JA, Evans RJ.** Molecular properties of ATP-gated P2X receptor ion channels. *Trends Pharmacol Sci* 2004; **25**: 487-493.

39. **Voss PG, Gray RM, Dickey SW, Wang W, Park JW, Kasai KI, Hirabayashi J, Patterson RJ, Wang JL.** Dissociation of the carbohydrate-binding and splicing activities of galectin-1. Arch of Biochem and Biophys 2008; **478(1):** 18–25.

40. **Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J, Kellar KJ.** Rat α_3/β_4 subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: pharmacology of ligand binding and function. *Mol Pharmacol* 1998; **54**: 322-333.

41. **Zhou X, Galligan JJ.** Non-additive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. *J Physiol* 1998; **513**: 685-697.

42. **Zhou X, Ren J, Brown E, Schneider D, Caraballo-Lopez Y, Galligan JJ.** Pharmacological properties of nicotinic acetylcholine receptors expressed by guinea pig small intestinal myenteric neurons. *J Pharmacol Exp Ther* 2002; **302**: 889-897.

CHAPTER 5
Concluding remarks and future perspectives

Previous work to investigate the interactions between $P2X_2$ and nAChRs in model systems such as guinea-pig coeliac ganglion neurons, rat pheochromocytoma PC12 cells, myenteric neurons and rat superior cervical ganglion neurons has provided a barrier for detailed probing into this interaction. For all of these systems express both $P2X_2$ and nAChRs, a draw back that my work has circumvented.

My studies have utilized the *mammalian* heterologous system of HEK-293 cells; these cells do not endogenously express $P2X_2$ and nAChRs, making it possible to test previous hypotheses such as the "channel overlap" theory, and the presence of agonist allosteric binding sites on these receptors. Another major concern in previous work has been that other receptors (muscarinic for ACh, P2Y for ATP) whose activation can lead to desensitization of $P2X_2$ and nAChRs results in a cross-inhibition artifact. Being able to express $P2X_2$ and nAChRs individually, as in our system, has provided a powerful tool to test hypotheses that have been difficult to test in previous model systems that express both receptors. In cells that express only nAChRs, ACh induced currents were not altered by co-application of ATP. The same was true for P2X receptors expressed individually. Our studies have provided evidence that the 'channel overlap' theory, the presence of allosteric binding sites, and a role for desensitization of P2X and nAChR by muscarinic or P2Y receptors mediated hypotheses are not accurate.

The work presented in this thesis furthers our understanding of interactions between P2X and nAChRs in three significant ways: this is the first comprehensive study

of interactions between these receptors in the open, desensitized and closed states. Second, these studies have provided evidence that interactions of the nAChR with the P2X receptor are not specific to the $P2X_2$ subtype, but also occur with the $P2X_3$ and $P2X_4$ subtypes in the open and desensitized states. And third, the molecular mechanisms responsible for these interactions were determined to be mediated by a physical interaction of the $P2X_2$ C-terminal tail with the $\alpha_3\beta_4$ nAChR in HEK-293 cells and myenteric neurons. This verifies results suggesting that a physical interaction between these receptors are not an artifact of expression in a heterologous system, but in fact due to the specific interaction between the $P2X_2$ receptor and the $\alpha_3\beta_4$ nAChR.

Future studies to investigate the specificity of this physical interaction would be fruitful. A more direct physical association between these receptors can be studied with a co-immunoprecipitation of these receptors. Preliminary data was collected from experiments in which an antibody against the $\alpha_3\beta_4$ nAChR was cross-linked to protein A beads. I observed that when incubated with an $\alpha_3\beta_4$ nAChR stable cell line transfected with $P2X_2$ receptors, the $\alpha_3\beta_4$ nAChR can be immunoprecipitated and blotted, but you cannot blot of the $P2X_2$ receptor. If the converse cell line was incubated with the same cross-linked beads (a $P2X_2$ stable cell line transiently transfected with $\alpha_3\beta_4$ nAChR) the $P2X_2$ receptor can be blotted for (the opposite of protein of the antibody) while there would be no signal for the immunoprecipitation or blot of the $\alpha_3\beta_4$ nAChR. Similar experiments were repeated with beads that where cross-linked to the $P2X_2$ receptor antibody. It seems that for a proper signal to be detected on the western, the protein has

to be stably expressed, for if it is not, it seems that there is an insufficient amount of protein to be detected.

These preliminary results are positive, because it shows that even if you immunoprecipitate for the $\alpha_3\beta_4$ nAChR, you can still blot for the $P2X_2$ receptor, and the reciprocal was also true. However, it is disconcerting how the signal for the transfected proteins on the immunoblot cannot be detected. It does not make sense that there are enough proteins there to co-immunoprecipitate the opposite protein, and that they can be activated via whole cell voltage clamp, but not enough for the immunoblot. One concern would be that the stably expressed proteins are being randomly enriched by the beads, and that is the signal that was observed. However, I performed experiments with a 'dummy' antibody, and there were no such result.

To solve these problems in the following attempts, the transfected protein concentration can be increased, or the total amount of protein (I added 1mg) added to the beads can be increased to a higher concentration. Also, this might be due to not enough antibody population to pick up all of the stably transfected protein, so more antibody can be cross-linked to beads (I used 2 μg, it can be increased to 4 μg or 6 μg). Furthermore, there are other controls that have to be performed to verify these previous results. Controls in which: cells are only stably expressing each of the individual proteins, and show that the converse protein cannot be immunoblotted for both the $\alpha_3\beta_4$ nAChR and the $P2X_2$ receptor. Controls of 'pre-immune' antibody, or of that is not available, a sample of rabbit antibodies that have been affinity purified in the same manner as the

antibodies used in the previous experiments against a protein that is known to not interact with either the $\alpha_3\beta_4$ nAChR or the $P2X_2$ receptor also have to be performed.

Another method to determine direct physical interactions is with site direct mutagenesis which can be implemented to determine more specifically which amino acid residues or sequence motifs might mediate these protein-protein interactions. There is evidence in the literature that $P2X_2$ receptors also interact with other cys-loop family members such as the $GABA_A$ receptor and the 5-HT_3 receptor. Work by Boue-Garabot *et al.* has shown that interactions between the $P2X_2$ receptor and the 5-HT_3 receptor are mediated via the $P2X_2$ C-terminal tail and the second large intracellular loop of the 5-HT_3 receptor. Sequence homology for the second large intracellular loop of the $5HT_3$ receptor and the nAChR is 26% for the α_3 subunit and 30% for the β_4 subunit. I believe that interactions between the $P2X_2$ receptor and the $\alpha_3\beta_4$ nAChR occur in a similar manner. A peptide constructs of the large intracellular loop for the α_3 subunit and β_4 subunit of the nAChR could be utilized in a similar manner as the GST-$P2X_2$-CT to determine if the large intracellular loop of the nAChR is in fact responsible for interactions between the $P2X_2$ and the $\alpha_3\beta_4$ nAChR, and more specifically, if both α_3 and β_4 subunits mediate interactions with the $P2X_2$ receptor, or if only one of them does.

Future work to investigate the trafficking of these receptors would also be of interest. This study has provided evidence of cross-inhibition in the desensitized states of the receptors. It would be interesting to determine if even upon desensitization and

internalization of the receptors co-localize would still be observed. A stable association would be suggested if desensitization and internalization of nAChR results in subsequent internalization of the $P2X_2$ receptor. The same would be true for the opposite, if desensitization and internalization of the $P2X_2$ receptor also internalizes the nAChR. Ratios of the $P2X_2$ receptor and nAChR in the endocytosed vesicles can be determined using confocal microscopy.

The foundation for this unique protein-protein interaction between P2X receptors and nAChRs has been laid by this thesis. In the future, the detailed physical mechanism between these receptors can be investigated. For this unique mechanism of regulation during fast excitatory synaptic transmission plays an important role in gastrointestinal motility. Gastrointestinal propulsion of food down the intestine is based on neuron activity. If myenteric plexus neurons in the reflex arc are under-stimulated then there are fewer contractions and relaxations of the muscle. This results in a low level of motility in the intestine leading to constipation. If the same neurons are over-stimulated, there is an increase in propulsion resulting in diarrhea. The level and relay of neuronal stimulation is regulated via fast synaptic transmission. The accurate amount of stimulation results in proper propulsion.

$P2X_2$ receptors and nAChRs are located on descending neurons in the reflex arc of the ENS; more specifically on inter-neurons and motor neurons that release nitric oxide onto the muscle. The descending pathway of neuronal stimulation mediates relaxation of the muscle. Relaxation of the muscle is a crucial response for motility, for without it food could not be propelled down the intestine. I believe that $P2X_2$ receptors

and nAChRs are co-localized in the descending pathway as a safety mechanism. Even though both receptors are cation permeable, both are present as a guarantee that there will be at least one functional receptor to mediate relaxation. Even if one receptor desensitizes or no longer functions, there is a backup receptor to pick up the slack. If both receptors are present and fully functional, cross-inhibition occurs between the $P2X_2$ and nAChR receptors to prevent a large infux of Ca^{2+} into the cell (high intracellular Ca^{2+} can by cytotoxic).

In most gastrointestinal motility disorders, there is an imbalance of neuronal communication that results in hypo-contractility (constipation) or hyper-contractility (diarrhea). By better understanding the mechanisms that regulate the relay of information between neurons in the myenteric plexus (synaptic transmission), we can better understand what goes wrong in disease states. It is a possibility that in disease states of hyper-contractility; this unique mechanism of regulation between P2X receptors and nAChRs is no longer present. That lack of cross-inhibition between these receptors results in an increase in synaptic transmission compared to non-diseased states. By better understanding this protein complex, new drugs can be discovered and implemented to treat many gastrointestinal motility disorders in which this mechanism of regulation might be lacking.