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DYNAMICS OF FAST SYNAPTIC EXCITATION IN
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DYNAMICS OF FAST SYNAPTIC EXCITATION IN MYENTERIC NEURONS

By

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ABSTRACT

DYNAMICS OF FAST SYNAPTIC EXCITATION IN MYENTERIC NEURONS

By

Jianhua Jim Ren

The goal of this dissertation is to understand the dynamic properties of fast excitatory synaptic transmission in myenteric neurons in the small intestine. Three specific issues have been addressed: 1) identification of purinergic P2X receptor subtypes expressed in myenteric neurons; 2) characterization of fast excitatory synaptic transmission during bursts of synaptic activity and 3) the cellular and molecular mechanisms by which presynaptic 5-HT_4 receptors mediate facilitation of fast synaptic transmission. Two types of electrophysiological recording methods were used: 1) intracellular microelectrode recordings from single myenteric neurons in acutely isolated longitudinal muscle-myenteric plexus (LMMP) preparations and 2) patch-clamp recordings from single myenteric neurons maintained in primary culture.

Identification of P2X receptor subtypes was conducted in acutely isolated LMMP preparations in *vitro* from the small intestine of guinea pigs and mice which $P2X_2$ or $P2X_3$ gene was knockout. My data suggest that P2X receptors were $P2X_2$ homomers in myenteric S neurons and $P2X_{2/3}$ heteromers in AH neurons in mouse ileum. However, in guinea pig ileum, P2X receptors in myenteric S neurons contained $P2X_3$ subunits. P2X receptors in AH neurons did not contain $P2X_3$ subunits. My data also showed that $P2X_3$ subunit-containing receptors in myenteric S neurons of guinea pig ileum coupled to Ca^{2+}-activated K^+ channels to mediate a hyperpolarization that followed agonist-induced depolarization.

Fast excitatory postsynaptic potentials (fEPSPs) evoked in guinea pig LMMP preparations in *vitro* declined (rundown) during trains of stimulation. The rundown rate was frequency-dependent. Neither desensitization of nAChRs or P2X receptors nor receptor-mediated presynaptic inhibition contributed to rundown of fEPSP. Depletion of transmitter vesicles in the readily releasable pool in presynaptic terminals was proposed as a mechanism to account for synaptic rundown.

5-HT_4 receptors are localized to presynaptic terminals in myenteric neurons. In my study, 5-HT_4 agonists increased fast EPSP amplitude during trains of stimulation and facilitated recovery after rundown. The effects of 5-HT_4 receptor agonists on fEPSPs were mediated through activation of the adenylate cyclase-cAMP-PKA signaling pathway. Detector-patch studies in cultured myenteric neurons suggested that 5-HT_4 agonists facilitated fast synaptic transmission by increasing neurotransmitter release probability from single varicosities.

These results provide novel information on pre- and postsynaptic mechanisms regulating synaptic transmission in the enteric nervous system. Rundown of fEPSPs during bursts may be a presynaptic mechanism to limit the synapses from over-activity. After-hyperpolarization mediated by P2X receptors and facilitation mediated by 5-HT_4 receptors may provide the cellular mechanisms by which neurotransmitters or drugs that can mimic neurotransmitters' action can alter neuronal activity. In the view of practice, the differential expression of P2X subunits in myenteric neurons will help to select drug targets for development of novel treatment of GI motility disorders. Studies of the mechanisms of 5-HT_4 receptor-mediated facilitation of synaptic transmission may provide insights into the neurophysiological basis of GI motility disorders.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my mentor Dr. James J. Galligan for giving me the opportunity working in his laboratory. I have benefited for my scientific career and daily life so much from his patient teaching, his wise advice and the excellent environment he provides. These past few years working with him will be an invaluable experience for rest of my life. I would also like to thank my thesis advisory committee members for their helpful discussion, constructive suggestions and encouragement. They were: Drs. Steven Hedeimann, David Kreulen and Mary Rheuben.

I dedicate this dissertation to my lovely wife, as she has always been a loyal audience for my work.

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INTRODUCTION

THE ENTERIC NERVOUS SYSTEM

Overview

The enteric nervous system (ENS), sympathetic and parasympathetic nervous systems compose the autonomic nervous system (ANS). The ENS is intrinsic to the gastrointestinal (GI) tract. The ENS is the collection of neurons and supporting cells located within the wall of GI tract, including the neurons within the pancreas and gall bladder (Furness and Costa, 1987). The ENS is composed of two major interconnected ganglionic plexuses: the myenteric (Auerbach's) and submucosal (Meissner's) plexuses (Furness and Costa, 1987). Auerbach and Meissner are the two neuroanatomists who first provided detailed descriptions of the arrangement of the neurons and nerve fibers of the plexuses that bear their names. The descriptions of the two groups of neurons as the myenteric and submucosal plexuses are more meaningful in terms of their anatomical arrangement and this terminology is currently used widely.

The ENS is highly autonomous as it can function independent of central nervous system (CNS) control. Although it is innervated by efferent nerve fibers originating in neurons in sympathetic, parasympathetic ganglia and sensory afferent fibers from neurons in the nodose and dorsal root ganglia, the ENS can control most GI functions after these extrinsic nerves have been cut. The ENS is often referred as the "little brain" or "second brain" to denote its functional autonomy (Gershon, 1999). The ENS can operate autonomously because it contains all of the components required for most GI reflexes (Bertrand, 2003; Furness et al., 2004). The original data supporting this conclusion can be tracked back to 1899 when Bayliss and Starling observed a pressure-evoked descending propulsive wave consisting of oral contraction and anal relaxation (Bayliss and Starling, 1899). They removed sympathetic and parasympathetic innervations of the intestine and found no interruption of the pressure-evoked reflex. They proposed the " Law of the Intestine" which stated that *"local stimulation of the gut produces excitation above and inhibition below the excited spot. These effects are dependent on the activity of the local nervous mechanisms."* (Bayliss and Starling, 1899). In 1917 Trendelenburg conducted a further study and observed the same type of reflex in guinea pig gut in *vitro*, which Trendelenburg called peristalsis (Furness and Costa, 1987). These pioneering studies demonstrated that the peristaltic reflex could occur in *vitro* when the bowel is obviously isolated from the CNS and sensory ganglia. They provided indirect evidence that the ENS contains all the elements and complete intrinsic neural circuits that underlie the coordinated movement of GI smooth muscle. Modern experimental techniques have facilitated steady progress in identifying these components and the neural circuits they form (Furness, 2000; Furness et al., 2004; Bertrand 2003. Also see below "classification of myenteric neurons and neural circuits" for details). Briefly, the individual components include sensory neurons that transduce chemical or mechanical stimulation to initiate a reflex; interneurons that relay the inputs and mediate the reflex and; excitatory and inhibitory motor neurons that cause coordinated contraction and relaxation of smooth muscle to accomplish peristalsis (Bertrand, 2003; Furness et al., 2004).

So, when J. N. Langley formally defined the ANS, he cited anatomical and functional evidence that led him classify the ENS as separate from the sympathetic and parasympathetic divisions. In his comprehensive review (Langley, 1921), he pointed out that:

"...we should expect the cells of Auberbach's and Meissner's plexuses to be on the course of the bulbar and sacral nerves, but as there was no clear proof of their central connection, and as their obvious histological characters differed from those of any other peripheral nerve cell, I placed them in a class by themselves as the enteric nervous system..."

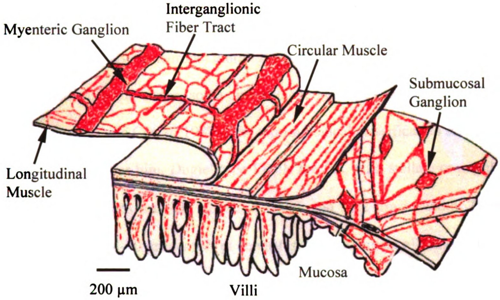
Anatomy of the GI tract and arrangement of the enteric plexuses

The GI tract is a muscular tube extending from the mouth to the anus. In general, the GI tract includes the esophagus, stomach, duodenum, jejunum, ileum and large intestine. The duodenum, jejunum and ileum compose the small intestine. The GI tract has a similar laminar structure in these different segments and consists of two smooth muscle layers, mucosa and the enteric plexuses (Schofield, 1968; Furness and Costa, 1987). There is an inner circular and an outer longitudinal smooth muscle layer. The anatomic arrangement for all these layers is, in order from serosal to lumen side, longitudinal smooth muscle, myenteric plexus, circular smooth muscle, submucosal plexus and mucosal plexus (Figure 1). This kind of arrangement provides an anatomical basis for the function of the two enteric plexuses function. The myenteric plexus controls motility as it exists between two smooth muscle layers. The submucosal plexus regulates secretory and absorptive function and it lies just below the mucosal layer.

Figure 1. The general organization of the enteric nervous system

A schematic diagram adapted from Furness and Costa (1987) illustrates the organization of the enteric nervous system as seen in a whole mount of small intestine. The lumenal mucosa is at the bottom while the serosal surface is at the top. The submucosal plexus is located between the circular muscle and the mucosal layer. The myenteric plexus is located between the longitudinal and circular smooth muscle layers. In guinea pig small intestine, the myenteric plexus is attached tightly to the longitudinal muscle. Therefore, the longitudinal muscle-myenteric plexus (LMMP) preparation is used to study myenteric neurons. Synaptic connections in the myenteric plexus that are present in vivo are preserved in the LMMP preparations.

Figure 1



Interganglionic
Fiber Tract
Myenteric Ganglion
Circular Muscle
Submucosal
Ganglion
Longitudinal
Muscle
200 µm
Villi
Mucosa

Morphology and ultrastructure of enteric neurons

The ENS contains more than 100 million neurons, which are as many as the number of neurons in the spinal cord. Alex Dogiel (1899) provided a complete and accurate description of the morphology of enteric neurons. He used methylene blue as histological stain to reveal neuronal morphology. He demonstrated three groups of enteric neurons based on soma shape, soma size and the number and distribution of neuronal processes (Furness and Costar, 1987). The morphological classification scheme of enteric neurons is named after him. Dogiel type I cells are flat, with stellate or angular shapes. The size of cell bodies was 13- 35 μm in length and 9-22 μm in width. They had one axon (unipolar) and 4-20 or more short dendrites. Because many of these cells sent single axons to the muscle, Dogiel concluded these neurons were motor neurons. Dogiel type II cells were described as angular, star or spindle shaped with multiple long neural processes. The cells had smooth surfaces with long axes of 22-47 um and short axes of 13-22 μm. These cells projected multiple processes to the mucosa and adjoining ganglia and were recognized as sensory neurons. Dogiel type III cells were similar to type I cells. They had a uniopolar shape with 2-10 filamentous dendrites. Type III neurons were suggested to be motor neurons. Later studies have suggested there was an overlap between type I and III neurons in morphology therefore these two cell types were re-classified as type I neurons.

Ultrastructural studies of enteric ganglia reveal important differences from other autonomic ganglia. Enteric ganglia do not have internal capillaries and connective tissue (Cook and Burnstock, 1976) and the enteric neurons in ganglia are packed tightly without satellite cell sheaths (Llewellyn-Smith et al., 1981). Electron microscopy has identified eight types of neurons within myenteric ganglia in guinea pig small intestine based on the size and the intracellular unltrastructure (Cook and Burnstock, 1976). However, the submucosal ganglionic neurons do not differ from each other in their ultrastructural appearance as they all have broad and thin dendrites, axo-somatic and axo-dendritic synapses and similar perikarya of 12-30 μm (Wilson et al., 1981; Komuro et al., 1982). Ultrastructural studies have also revealed that there are multiple types of synaptic vesicles that can be differentiated based on size and vesicle content. These observations have led to the suggestion that enteric neurons use multiple transmitters and some nerve terminals contain and release more than one type of transmitter (Furness and Costa, 1987; Llewellyn-Smith et al., 1989). Myenteric neurons are directly contacted by varicosities. However the pre-and postsynaptic specializations are not different from synapses in other autonomic ganglia or in the CNS (Llewellyn-Smith et al., 1989).

Electrophysiology of myenteric neurons

Electrophysiological studies of the ENS began with extracellular recordings from the myenteric plexus (Wood, 1970). Later, intracellular microelectrode recordings were made from the myenteric plexus (Hirst et al 1974; Nishi and North 1973) and submucosal plexus (Hirst and Mckirdy 1975). Patch-clamp recording techniques have also been used to record from cultured myenteric neurons (Zhou and Galligan, 1996) and submucosal neurons (Barajas-lopez et al., 1998) and in acutely isolated LMMP preparations (Rugiero et al., 2002). Studies in LMMP preparations provide information that represents physiological conditions in *vivo* while studies done in cultured neurons allow investigations of electrophysiological properties in more detail than is possible using the intact plexus preparation. Figure 2 contains schematic diagrams showing the setups for these two types of electrophysiological recordings. These studies have characterized the electrophysiological properties of myenteric neurons and the ion channel basis of these properties (Galligan et al., 1989; Galligan et al., 1990; Galligan, 2002a and b; Rugiero et al., 2002; Furness and Sanger, 2002; Bertrand, 2003).

Most intracellular electrophysiological studies of myenteric neurons have been done in preparations from the guinea pigs. The reason to choose the guinea pigs as animal model for GI studies is because guinea pig gut is readily dissected, reasonably long and mechanically quiescent when studied in *vitro*. There have also been studies done in mouse myenteric plexus (Furukawa et al., 1986). Gene manipulation in mice provides an opportunity to study the electrophysiological properties of specific proteins (Ren et al., 2003; Bian et al., 2003). Most neuronal properties are similar between guinea pigs and mice. The following description is mainly drawn from guinea pigs unless noted.

Two distinct types of myenteric neuron have been identified based on their electrophysiological characteristics. S neurons and AH neurons were described by Hirst et al (1974); while Nishi and North (1973) found neurons with the same properties but called these type I and type II neurons (Figure 3). The nomenclature of Nishi and North is not longer used because of its similarity to the Dogiel classification of cell morphology. In addition, Hirst's nomenclature was based on the properties of the neurons and this is more meaningful. Neurons responding to single focal stimulation of interganglionic nerve fibers with a fast excitatory postsynaptic potential (fEPSP) were termed S neurons; the fEPSP is the predominant mechanism of synaptic excitation in the GI tract. Action

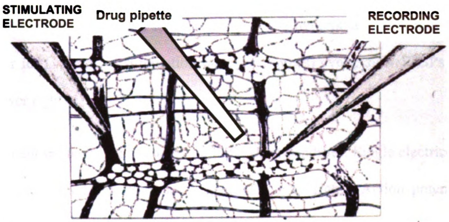
Figure 2. Electrophysiological recording from single myenteric neurons

A. Intracellular electrophysiological recordings are made from single myenteric neurons in LMMP preparations. Myenteric ganglia can be visualized using a microscope with differential interference contrast optics. A sharp microelectrode filled with 2 M KCl and with a tip resistance of ~100 M ohm is used to impale single neurons. Synaptic activity can be evoked by electrical stimulation of interganglionic nerve bundles. Agonists can be applied locally by pressure ejection from a drug pipette positioned close to the impaled neuron.

B. Patch-clamp recordings are made from single myenteric neurons maintained in primary culture. The picture shows the cultured myenteric neurons visualized using a microscope with differential interference contrast optics. Cultured myenteric neurons grow processes that form synapses with other myenteric neurons. Synaptic responses can be elicited by stimulating presynaptic single nerve fibers. Agonists can be applied locally by gravity-driven flow from a drug pipette positioned close to the target neuron.

Figure 2

A


STIMULATING
ELECTRODE
Drug pipette
RECORDING
ELECTRODE

B

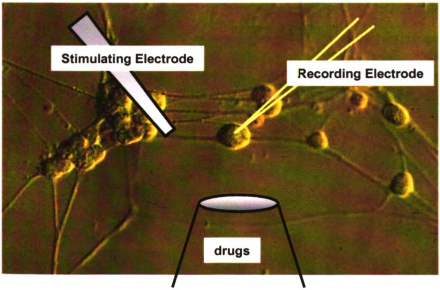
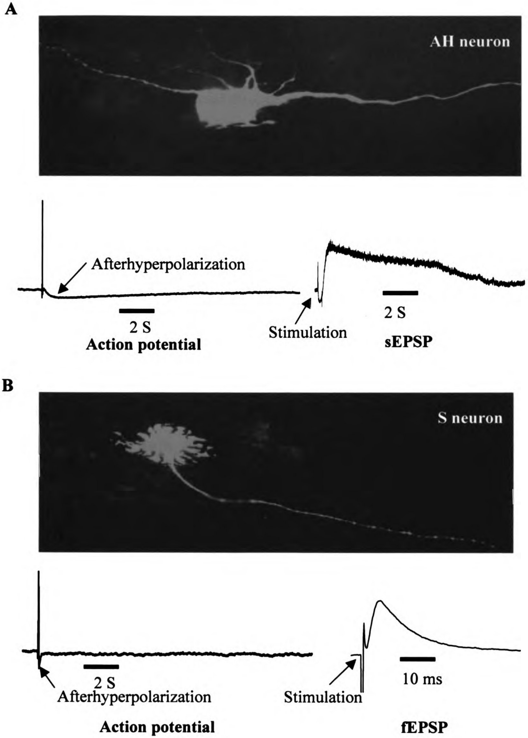

Stimulating Electrode
Recording Electrode
drugs

Figure 3. Myenteric AH and S neurons

A. Photomicrographs of an AH neuron morphology and its electrophysiological properties. AH neurons are bipolar with several dendrites and a smooth cell body. Action potentials in AH neurons have a long-lasting (1-10 s) hyperpolarization pointed by the arrow (lower left). Trains of stimulation (as arrow shows) evoke slow EPSPs from AH neurons (lower right).

B. S neurons have one axon and multiple short dendrites. Single electrical stimuli (as arrow shows) elicit fEPSPs from S neurons (lower right). Action potentials in S neurons have a short afterhyperpolarization as the arrow shows (lower left). For morphological studies, microelectrodes filled with 4% Neurobiotin were used to record from these neurons. Neurobiotin was localized with a Cy3 conjugated avidin.

Figure 3


A
AH neuron
Afterhyperpolarization
2 S
Action potential
Stimulation
2 S
sEPSP
B
S neuron
2 S
Afterhyperpolarization
Action potential
Stimulation
10 ms
fEPSP

potentials recorded from the soma of S neurons were mediated solely by voltage-gated Na^+ channels and they can be blocked by tetrodotoxin (TTX). The action potential in S neurons is followed by an afterhyperpolarizaion of ~20 ms duration (Hirst et al 1974; Bornstein et al., 1994). Most S neurons fire action potentials continuously when depolarized by a current pulse injected through the recording microelectrode (Bornstein et al., 1994). Inward rectifier potassium current (Ik_{ir}) modulates the resting membrane potential of S neurons (Galligan et al., 1989; Rugiero et al 2002). As myenteric S neurons receive mainly excitatory synaptic inputs, the membrane potential is unlikely to go to values more negative than E_k (~ -80 mV) where Kir channels operate more actively and potassium ions leave the cell. Under physiological conditions, IK_{ir} hyperpolarizes the neurons to maintain resting membrane potential.

AH neurons are so named because the action potentials generated in soma are followed by long-lasting (1-10 s) afterhyperpolarization (AHP) (Figure 3) (Nishi and North, 1973; Hirst et al., 1974; Furness et al, 1998). AH neurons cannot fire a train of action potentials because the AHP limits the firing rate. The AHP is mediated by activation of an intermediate conductance calcium-dependent potassium channel (I_{AHP}) (Morita et al., 1982; Hirst et al., 1985; Galligan et al., 1989; Vogalis et al., 2002). The action potential of AH neurons has two components: 1) a TTX-sensitive sodium current and, 2) a calcium current. Calcium action potentials are mediated by N-type calcium channels as N-type calcium channel blocker ω–conotoxin reduces the duration of action potentials and the amplitude of the afterhyperpolarization (Furness et al., 1998; Vogalis et al., 2001; Rugiero et al., 2002). In addition, immunoreactivity for the $\alpha 1B$ (N-type) calcium channel subunits is localized to AH neurons, whereas only weak $\alpha 1A$ (P/Q-type calcium channel subunit) immunoreactivity was observed (Kirchgessner and Liu, 1999). R type calcium channels are only expressed in the nerve fibers (Naidoo, personal communication) therefore it does not seem to contribute to action potential but to contribute to regulation of synaptic transmission. AH neurons contain calbindin immunoreactivity, which is a neurochemical marker to differ AH neurons from S neurons. A cationic current activated by hyperpolarization (I_h) appears to be one major conductance in AH neurons (Galligan et al., 1990; Rugiero et al., 2002). Activation of I_h was suggested to increase excitability in AH neurons by keeping the membrane potential of AH neurons less hyperpolarized in response to activation of I_{AHP} and Ik_{ir}. Some AH neurons receive fast synaptic inputs but slow synaptic transmission is the primary mechanism of synaptic excitation in AH neurons (Galligan et al., 2000; Furness et al., 2004).

Functional classification of myenteric neurons

Classification of myenteric neurons has accomplished using morphological, electrophysiological or neurochemical properties (Furness, 2000; Brookes 2001). A combined classification scheme that includes function would be more significant in a way that can facilitate understanding the functional significance of the myenteric neurons with different properties. This revised scheme requires correlating the electrophysiology, the neurochemistry and the morphology of enteric neurons with their functions. This will help to uncover the mechanisms underlying neural regulation of GI function.

There has been significant progress in defining the types of enteric neurons in the context of their roles in enteric neural circuitry (Bertrand, 2003; Furness et al., 2004). Correlation of electrophysiology, morphology and function has been accomplished by injecting marker dyes into neurons that had been recorded from and subsequently examining cell shape and neurochemical content (Hodgkiss and Lees, 1983; Bornstein et al., 1984; Iyer et al., 1988; Messenger et al., 1994; Clerc et al., 1998; Lomax and Furness, 2000; Tamura et al., 2001; Nurgali et al., 2003). Based on these kinds of studies it has been established that the myenteric plexus contains three major types of neurons. They are motor neurons, interneurons and intrinsic primary afferent neurons (IPANs). IPANs are so termed is to differentiate them from the extrinsic sensory neurons whose cell bodies are in dorsal root and nodose ganglia, which have afferent connections with the ENS (Furness et al., 2004). Motor and interneurons have Dogiel type I morphology and IPANs have Dogiel type II morphology (Figure 3). Five subtypes of motorneurons, one subtype of ascending and four subtypes of descending interneurons have been identified (Furness, 2000; Brookes, 2001; Furness and Sanger, 2002). There are also a small number of intestinofugal neurons (<1% in number) that send afferent fibers to prevertebral ganglion and these connections are part of intestinofugal reflex pathways (Furness, 2002; 2003). Each type of neuron has neurochemical coding and electrophysiological characteristics (Furness, 2000; Furness and Sanger, 2002; Bertrand, 2003). For example, inhibitory longitudinal motor neurons express nitric oxide synthase (NOS), vasoactive intestinal peptide (VIP) and GABA while excitatory longitudinal motor neurons express choline acetyltransferase, calretinin and substance P. IPANs are the only neuron type that expresses calbindin. Electrophysiologically, motor- and interneurons are S neurons while IPANs are AH neurons.

Intrinsic neural circuits in the myenteric plexus

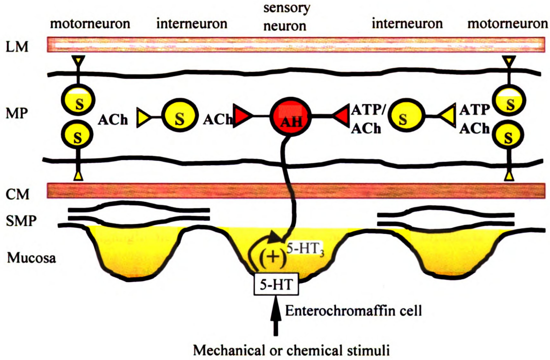
Studies of neuron types have provided the information that helps to understand how myenteric neurons are organized to form a functional network that underlies the peristaltic reflex (Figure 4). Because the peristaltic reflex is based on distention-evoked contraction oral to the point of distention and relaxation anal to the distention point, the organization of the intrinsic neural circuitry must be polarized accordingly. The accumulated data suggest that IPANs are excited indirectly by intraluminal chemical or mechanical stimulation through sensory mediators released from enterochromaffin (EC) cells in mucosa. The identified mediators released from EC cells are 5-HT and ATP (Bertrand, 2003). 5-HT acts at 5-HT_3 receptors and ATP acts at P2X receptors on nerve terminals to activate myenteric IPANs antidromically (Furness et al., 2004). 5-HT_{1P} localized to terminals of submucosal IPANs may also mediate sensory information (Gershon, 2004). IPANs then activate interneurons via cholinergic and purinergic synaptic transmission in the descending pathway and cholinergic synaptic transmission in the ascending pathway (Galligan, 2002b). Interneurons then activate motorneurons, which excite smooth muscle orally and inhibit smooth muscle anally to generate the peristaltic reflex. It is very clear that fast synaptic transmission between these neurons plays an important role in process sensory information (see “fast synaptic transmission” for details). However, the mechanisms by which fast synaptic transmission may encode

Figure 4. Intrinsic neural circuitry in myenteric plexus

This diagram a propose model of the enteric neural circuit underlying the peristaltic reflex. Intrinsic primary afferent neurons (IPANs) are AH neurons while inter- and motorneurons are S neurons. Intraluminal mechanical or chemical stimulation of the mucosa releases 5-HT from enterochromaffin cells. 5-HT acting at 5-HT_3 receptors localized to nerve terminals of myenteric IPANs excites the IPAN antidromically. Activation of IPANs releases transmitters to activate interneurons in orally and anally directed synaptic pathways. Subsequent activation of interneurons stimulates inhibitory or excitatory motor neurons to relax or contract smooth muscle, respectively.

IPAN: intrinsic primary afferent neuron; LM: longitudinal muscle; MP: myenteric plexus; SM: smooth muscle; SMP: submucosal plexus;

Figure 4



sensory neuron
motorneuron
interneuron
interneuron
motorneuron
LM
MP
CM
SMP
Mucosa
S
S
ACh
S
ACh
AH
ATP/ ACh
S
ATP ACh
S
S
(+)
5-HT3
5-HT
Enterochromaffin cell
Mechanical or chemical stimuli

and regulate sensory information causing responses to a variety of physiological stimulation are still unknown.

Extrinsic innervation

Myenteric and submucosal plexuses also receive extrinsic inputs through parasympathetic and sympathetic nerves. The sympathetic preganglionic cell bodies are in thoracic (T) and lumbar (L) segments of the spinal cord. Their axons end on the postganglionic neurons in celiac ganglia, superior and inferior mesenteric ganglia. Sympathetic postganglionic noradrenergic fibers innervate the myenteric plexus, the submucous plexus, and the mucosa, but have little input to the muscle layers (Furness and Costa, 1987; King and Szurszewski, 1989; Llewellyn-smith et al., 1993). The majority of postganglionic sympathetic neurons innervating the ENS are found in celiac-mesenteric ganglia. They project via splanchnic nerves to the stomach and the small intestine. Some sympathetic innervation originates from cervical chain ganglia and projects to the upper small intestine.

The parasympathetic innervation of the GI tract originates in the cervical and sacral portions of the spinal cord and terminates in the esophagus, stomach, pancreas, sigmoid colon, rectum and anus (Kirchgessner and Gershon, 1989). Only few vagal efferent fibers innervate the small intestine. Sympathetic and parasympathetic innervation regulates GI function. Sympathetic postganglionic neurons release norepinephrine (NE) to inhibit enteric excitatory cholinergic neurons through presynaptic mechanism (Hirst and McKirdy, 1974) by acting at $\alpha 2$-adrenergic receptors (Frigo et al., 1984). Activation of sympathetic nervous system results in decreased motility of GI smooth muscle. Parasympathetic nervous system provides only excitatory innervation to GI tract. Stimulation of the parasympathetic nervous systems results in the release of ACh, which causes an increase in GI activity.

There is also afferent innervation that conveys sensory information from the GI tract to the CNS where GI reflex is coordinated and integrated with conscious behavioral responses. The extrinsic sensory innervation originates from enteric plexus and follows two routes to the CNS. The cell bodies of vagal afferent neurons are located in nodose ganglia while the cell bodies of spinal afferent neurons are located in dorsal root ganglia. Pseudo-unipolar sensory neurons in nodose ganglia send their central processes to the nucleus tractus solitarius (NTS) of the medulla in brain stem and their peripheral processes to the GI tract via vagal nerve (Berthoud et al., 2004). Vagal afferent is considered to convey the sensory information from the GI tract that generates the consciousness, for example satiety (Grundy and Scratcherd, 1997). Spinal afferents enters spinal cord to make synapses with the second order neurons that convey GI nociceptive information to the CNS. There is a differential innervation of these afferents throughout the GI tract. Vagal afferents end largely in the upper GI tract including the esophagus and stomach (Powley and Phillips, 2002). For spinal afferents, pelvic afferents are limited in the lower bowel. In contrast, splanchnic afferents innervate the whole GI tract.

Submucosal neurons

The submucosal plexus is a second important component of the ENS. It underlies the regulation of intestinal secretory reflexes (Cooke, 1999). Submucosal neurons receive their intrinsic innervation mainly from myenteric neurons and partly from other submucosal neurons (Bornstein et al., 1987). They also receive major extrinsic innvervation from noradrenergic neurons in the prevertebal sympathetic ganglia (Mihara et al., 1997). In submucosal plexus, four distinct types of submucosal neurons have been identified based on neurochemical properties (Mihara et al., 1997; Furness, 2000; Brookers, 2001). Some submucosal neurons have Dogiel type II morphology. The other submucosal neurons have filamentous morphology (Brookes, 2001). Non-cholinergic secretomotor/vasodilator neurons are immunoreactive for dynophin (DYN), galanin (GAL) and VIP. These neurons account for 45% of submucosal neurons and they use VIP as their secretomotor neurotransmitter. Cholinergic secretmotor neurons account for 30% and they are immunoreactive for ChAT, calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY). Submucosal IPANs (10%) that have immunoreactivity of ChAT, DYN and substance P (SP) and they use Ach as transmitter. Cholinergic interneurons comprise 15% of submucosal neurons and they are immunoreactive for ChAT and DYN.

Electrophysiologically, submucosal neurons can generate three types of synaptic potentials in response to focal nerve stimulation (Mihara, 1993). Fast EPSPs can be recorded from motor and interneurons after single electrical stimuli. Fast EPSPs are mediated by cholinergic inputs from neurons in both myenteric and submucosal plexuses. Slow EPSPs are evoked by trains of stimulation and slow EPSPs are likely mediated by SP, VIP and 5-HT (Mihara et al., 1997). Slow IPSPs can be recoded from non-cholinergic secretmotor neurons: slow IPSPs are mediated by norepinephrine released from extrinsic sympathetic nerves. Norepinephrine acts at α2-adrinergic receptors (Surprenant and North, 1987).

SYNAPTIC TRANSMISSION

Overview

Neurons communicate with each other through synaptic transmission, which occurs at a specialized neuronal structure, the synapse, a milestone-like concept coined by C. Sherrington. Enteric neurons use chemical synaptic transmission to mediate neural signals. Synaptic transmission at chemical synapses is complex in structure, function and modulation mechanisms (Eccles, 1982; Walmsley et al., 1998). Neurotransmitters released from presynaptic nerve terminals act at receptors located at postsynaptic neurons (Ruff, 2003). Transmitter release occurs from nerve terminals after an influx of calcium produced by an action potential, triggers the excytosis of neurotransmitter vesicles clustered at the active zone (Smith and Augustine, 1988; Zucker, 1993; Martin, 2002). Some transmitters are recovered by uptake by specific transporters and some will be degraded in the synaptic cleft by enzymes (McMahan et al., 1978; Amara and Arriza, 1993). Most chemical synaptic transmission is unidirectional. However, the retrograde synaptic transmission may occur in some tissues (Jessel and Kandel, 1993; Levenes et al., 2001; Diana et al., 2002). Synaptic modulation can occur by targeting presynaptic nerve terminals or/and postsynaptic neurons.

There are two broad mechanisms of chemical synaptic transmission based on synaptic delay between a presynaptic action potential and the onset of the postsynaptic change in membrane potential caused by the release neurotransmitter: slow and fast synaptic transmission.

Slow synaptic transmission in myenteric plexus

Slow synaptic transmission occurs through activation of G-protein coupled receptors (GPCRs) by neurotransmitters. GPCR activation alters the membrane potential of postsynaptic neurons through activation or inhibition of a number of second messengers leading to close or open of ion channels. GPCRs are a family of membrane proteins with seven transmembrane domains (Ji et al., 1998). The third intracellular loop is the G-protein binding site (Wess, 1997). G-proteins are trimers composed of α, β and γ subunits (Hamm, 1998). In general, two mechanisms mediate extracellular signals by activation of GPCRs. The first one is a signaling pathway through activated G protein α subunit resulting in an increase or decrease in the activity of a target enzyme (typically a protein kinase) via a second messenger (Gilman, 1987). The protein kinase then modulates the target effectors such as ion channels and gene expression to alter neuronal properties (Hille, 1994; Hamm, 1998). In addition, the G protein β and γ subunit complex can directly act at the ion channels as membrane de-limited factors to modulate ion channel kinetics (Wickman et al., 1994; Huang et al., 1995; Clapham and Neer, 1997). As there are multiple-step processes following activation of GPCRs including agonist binding, the subsequent activation of signaling pathways and the activation of target ion channels, GPCRs mediate slow synaptic transmission with an onset of 50-100 ms and effects on neuronal excitability lasting seconds to minutes. Slow synaptic transmission is an important signaling mechanism in the ENS (Surprenant and North, 1988; Bertrand and Galligan, 1994; Bertrand and Galligan, 1995; Pan et al, 1997; Alex G et al., 2001; Galligan, 2002 a, b). In the ENS, examples of GPCRs include NK-3 receptors activated by substance P (SP) (Holzer and Holzer-Petsche, 2001), 5-HT_{1P} receptors activated by 5-

HT (Pan et al., 1997) and P2Y receptors (Bornstein et al., 2002) activated by ATP. Slow synaptic responses in the ENS are due to a concurrent decrease in potassium conductance and increase in chloride conductance (Bertrand and Galligan, 1994). Slow synaptic transmission occurs predominately in AH neurons (Galligan, 2002a).

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

Ligand-gated ion channel-mediated fast synaptic transmission is the second broad class of synaptic transmission in the ENS (Galligan, 2002a). Ligand-gated ion channels are membrane proteins composed of multiple subunits. The ligand binding sites and the ion channels are localized within the same protein complex (Lester, 1992). There is no second messenger needed to convey the primary signals via ligand-gated channels (Uings and Farrow, 2000; Breitinger, 2001). Therefore, the synaptic delay is less than 1 ms and the duration of transmitter release and response lasts less than 100 ms (Panas et al., 2000). Several ligand-gated ion channels mediate fEPSPs in myenteric neurons. The major receptors include nicotinic cholinergic receptors (nAChRs), purinergic P2X receptors and 5-HT_3 receptors. These receptors have been localized to S neurons and they receive synaptic inputs from AH neurons and other S neurons to mediate reflex activation (Galligan, 2002a).

Nicotinic acetylcholine receptors (nAChRs)

Acetylcholine (ACh), acting at nAChRs, is the predominant contributor to fast synaptic excitatory neurotransmission in the ENS (Nishi and North, 1973; Hirst et al., 1974; Galligan and Bertrand, 1994; Zhou et al, 2002). In myenteric plexus, application of nAChR antagonists inhibits fEPSPs, at least partly, in 92% of S neurons. In submucosal S neurons fEPSPs are exclusively mediated by ACh acting at nAChRs (Hirst and Mckirdy, 1975; Suprenant, 1984). In the myenteric plexus, 25% of S neurons generate fEPSPs that are completely blocked by nAChR antagonists (Galligan et al., 2000); fEPSPs in the remaining neurons have non-cholinergic components (Galligan and Bertrand, 1994; Galligan et al., 2000). Cholinergic fEPSPs play a primary role in ascending reflex pathway in guinea pigs (Johnson et al., 1996; Spencer et al., 2000). Cholinergic fast synaptic transmission also mediates signals in descending reflex pathways in the rat colon and guinea pig ileum (Johnson et al., 1996; Bian et al., 2003).

Neuronal nAChRs are heteromeric pentamers that are composed of different combinations of α and β subunits; each subunit four transmembrane domains. The electrophysiological and pharmacological properties of nAChRs are determined by the specific subunit composition (Albuquerque et al., 1996; Jones et al., 1999; Skok, 2002). Fast EPSPs and ACh-induced responses in myenteric neurons have a reversal potential of 0 mV (Galligan and Bertrand, 1994), which suggests that nAChRs are permeable to cations. Data from immunohistochemical and pharmacological studies done in myenteric neurons maintained in primary culture suggest that S neurons do not express α7 subunit-containing nAChRs in the cell body but most S neurons express nAChRs that contain α3α5β4 subunits (Kirchgessner and Liu, 1998; Zhou et al., 2002). Cholinergic fEPSPs can also be recorded from AH neurons (Furness et al., 1998; Galligan et al., 2000). But the amplitude of fEPSP in AH neurons is smaller than that in S neurons as there may be a lower density of nAChRs expressed in AH neurons (Barajas-Lopez et al., 2001; Galligan, 2002a). Patch-clamp studies performed in cultured myenteric neurons indicate that nAChR-mediated inward currents have a reversal potential of 0 mV and the current-voltage relationship is inward rectified. These results confirm that nAChRs are non-selective cation channels and also suggest that nAChRs operate more actively in depolarizing the neurons to increase excitability. nAChRs desensitize fast within 0.3 second by 80% in response to steady-state application of ACh. Single channel currents have linear relationship with the potentials, which suggests that inward rectification of whole cell currents is due to a decrease in nAChR open probability at positive membrane potential (Zhou et al., 2002; Brown et al, 2003).

In addition to nAChRs localized to somatodendritic region of S neurons, evidence indicates that nAChRs may also be localized to nerve terminals. These nAChRs mediate release of tachykinin peptides in myenteric ganglia and at the motor neuron-circular/longitudinal muscle junction (Galligan, 1999; Schneider and Galligan, 2000; Schneider et al., 2000). The mechanisms and the physiological functions of presynaptic nAChRs remain further studied.

Purinergic P2X receptors

The non-cholinergic component of fEPSPs recorded from 67% of myenteric S neurons is sensitive to the P2X receptor antagonists PPADS or suramin (Galligan and Bertrand, 1994; Galligan 2002a, b). This indicates that ATP acting at P2X receptors is a co-mediator with ACh acting at nAChRs in mediating fast synaptic excitation in myenteric neurons. But the purinergic fEPSPs are not evenly distributed in the whole gastrointestinal tract. Recordings from different segments of the gut indicate that purinergic fEPSPs predominate in ileum compared with that in duodenum, jejunum, proximal and distal colon (LePard et al., 1997). There is also a preferred oral-anal polarity of purinergic projections in myenteric plexus (LePard and Galligan, 1999). In guinea pig ileum, by stroking the mucosal villi and recording synaptic responses, it has been concluded that P2X receptors mediate fast synaptic transmission between interneurons in descending excitatory reflex pathway (Spencer et al., 2000; Monro et al., 2002). P2X receptors are also shown to mediate synaptic transmission from interneurons to inhibitory motor neurons in guinea pig ileum (Bian et al., 2000).

P2X receptors may also mediate activation in AH neurons and contribute to initiation of peristalsis (Bertrand and Bornstein, 2002). In this study, ATP applied to the cell body evoked a large depolarization in most AH neurons. The depolarization was blocked by PPADS. Application of ATP to the mucosal terminals of AH neurons can trigger action potentials that propagate back to the soma. The action potentials were also abolished by PPADS. Although the pharmacology of P2X receptors mediating these responses need to be further studied, P2X receptors obviously play physiological function in AH neurons mediating sensory information. However, the localization of ATP sources remains unclear. Study from vagal neurons has suggested that ATP may be co-transmitter with histamine in mast cells (Kreis et al., 1998). ATP is also localized in the vesicles in EC cells (Tamir and Gershon, 1990). In vivo, ATP released from these sources may serve as stimulator to P2X receptors.

P2X receptors are ligand-gated non-selective cation channels. But the molecular structure is different from nAChRs as P2X receptors may be trimers consisting of combinations of one or more of seven different subunits (Khakh et al., 2001; North, 2002; Galligan, 2002a, b). Each subunit has two membrane-spanning domains (Khakh et al., 2001). Studies of heterogeneous expression of P2X receptor subunits have generated a series of data for the properties of different P2X subunit combinations (North, 2002). P2X receptors can be composed of the same or different subunits to form homomers or heteromers, respectively. All P2X subunits can form homomers except the $P2X_6$, which only combines with other subunits, whereas all P2X subunits can form heteromers except the $P2X_7$, which can only form homomers. The specific composition of the P2X receptor determines the unique pharmacological and physiological properties. Pharmacological data have indicated there are α,β-mATP-sensitive P2X subtypes ($P2X_1$ or $P2X_3$) as well as $P2X_2$ subtype in myenteric S neurons of guinea pig intestine (Zhou and Galligan, 1996; LePard et al., 1997). Immunohistochemical studies have shown that $P2X_2$ and $P2X_3$ subunits are expressed in myenteric neurons (Castelucci et al., 2002; Poole et al., 2002). However, lack of selective drugs has impaired a full pharmacological identification of P2X receptor subtypes in myenteric neurons.

Electrophysiological characterization of P2X receptors made in cultured myenteric neurons has shown similarities and differences to nAChRs. ATP-induced whole-cell currents show inward rectification, which is due to a decrease in the open probability of single channels at more positive membrane potentials as single channel current-voltage relationship is linear (Zhou and Galligan, 1996). The reversal potential of 0 mV indicates that P2X receptors are permeable to cations. A major difference between myenteric P2X receptors and nAChRs is that ATP induces inward currents that desensitize by 80% in 7 seconds and ACh currents desensitize by 80% in less than 1 s (Zhou and Galligan, 1996; Zhou et al., 2002). This difference may contribute to their distinct functions in mediating synaptic signals in enteric neurons.

5-HT$_3$ receptors

5-HT$_3$ receptors are ligand-gated cation channels (Derkach et al., 1989; Fletcher and Barnes, 1998). These receptors belong to a same superfamily with nAChRs in overall structure. 5-HT$_3$ receptors are pentamers with each subunit having four transmembrane domains. In the GI tract, 5-HT$_3$ receptors are found in myenteric sensory nerve endings localized to the mucosal layer where they initiate the motor reflex by responding to 5-HT released from EC cells when mucosal villi are stimulated (Foxx-Orenstein et al., 1996; Bertrand et al., 2000; Galligan, 2002 a, b). In addition to triggering the sensory signal, 5-HT$_3$ receptors also mediate fast synaptic transmission in some S neurons (Zhou and Galligan, 1999). These 5-HT$_3$ receptors are localized to the cell body of myenteric S and AH neurons in small intestine where they mediate rapidly developing and desensitizing depolarization (Zhou and Galligan, 1999; Zhai et al., 1999). Fast EPSPs from about 11% S neurons have components that are not cholinergic or purinergic (Galligan et al., 2000). These fEPSPs are inhibited by ondansetron, a 5-HT$_3$ receptor antagonist. 5-HT$_3$ receptor-mediated fEPSPs are involved in both descending and ascending GI reflex pathway (Monro et al., 2002; Yuan et al., 1994).

The electrophysiological and pharmacological properties of 5-HT$_3$ receptors have been studied in myenteric neurons maintained in primary culture (Zhou and Galligan, 1999; Zhai et al., 1999). More than 80% neurons generate inward currents in response to 5-HT. These currents desensitize with a double exponential time course of 1.1 and 6.9 s. 5-HT$_3$ receptor-mediated currents do not rectify at positive membrane potentials as the nAChRs and P2X receptors do. The current-voltage relationship for 5-HT-induced single channel currents is linear, which suggests that change of potentials does not alter single channel open probability.

Dynamics of neurotransmitter release

A number of chemicals including ATP, ACh, biogenic amines and amino acids have been identified as neurotransmitters in the ENS. A neurotransmitter has to meet the following criteria: 1) it is synthesized in the neuron; 2) it is present in the presynaptic terminal and exerts a defined action on the effectors; 3) it mimics the synaptic response when applied exogenously and 4) there is a mechanism to stop its action (Kandel et al., 2000). In the ENS, identified neurotransmitters mediating fEPSP are ACh, ATP, 5-HT, glutamate (Galligan, 2002a, b). Neurotransmitter release is triggered by calcium influx produced by an action potential leading to exocytosis of neurotransmitter-filled vesicles in quanta units. The exocytosis of transmitter vesicles is via the formation of a complex involving many specific proteins (Hanson et al., 1997). The released vesicles can be recycled by endocytosis, which is one mechanism for generating new synaptic vesicles (Schweizer et al., 1995; Murthy and Stevens, 1998; Sudhof, 2004).

Although many vesicles are contained in a synaptic terminal, the vesicles are organized into functionally different pools (Rizzoli and Betz, 2005); 1) the readily releasable pool, 2) the recycling pool and 3) the reserve pool. A single action potential triggers release of a small percentage (~5%) of vesicles to release (Tong and Jahr, 1994; Stevens and Wang, 1995; Zucker and Regehr, 2002). These vesicles are from the readily releasable pool (RRP) (Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1999; Zucker and Regehr, 2002). Ultrastructural studies have shown that, the vesicles in the

RRP are docked at active zone sites where calcium channels are localized (Schikorski and Stevens, 1997). The RRP can be depleted rapidly by a short train of electrical stimulation at high frequency (Elmqvist and Quastel, 1965, Richards et al., 2003). Most transmitter vesicles (typically 80 - 90%) are contained in the reserve pool, which cannot be released until they move into the active zone to refill RRP, a process called replenishment. Release from the reserve pool occurs only in response to intense stimulation. The recycling pool contains vesicles that are dynamically released and reused under different conditions of neuronal activity (Murthy and Stevens, 1999; Rizzoli and Betz, 2005). Vesicles in the reserve pool can also be released and recycled during periods of prolonged and intense synaptic activity (Murthy and Stevens, 1999). Styryl dyes such as FM1-43 are used to monitor synaptic vesicle cycling and significant progress has been made to elucidate these processes (Cochilla et al., 1999; Sudhof, 2000).

Depletion of transmitter vesicles in the RRP is replenished slowly, which is an explanation for rundown of synaptic transmission (Sudhof, 2000; Zucker and Regehr, 2002). Replenishment of the RRP is also an explanation for synaptic recovery, which is a calcium-dependent process (Dittman and Regehr, 1998; Wang and Kaczmarek, 1998). Synaptic vesicle trafficking between these pools is dependent on a number of proteins (Chi et al., 2003). One of most important proteins is synapsin. Synapsin docks vesicles to the cytoskeleton and is a target protein for phosphorylation by protein kinases (Sudhof et al., 1989; Chi et al., 2003). Synapsin phosphorylation dissociates vesicles from the cytoskeleton and mobilizes them for trafficking from the reserve pool to the RRP (Turner et al., 1999). Activation of 5-HT_4 receptors can stimulate protein kinase A (PKA) in the ENS (see below). Therefore it is a reasonable hypothesis that such receptors can mediate the regulation of neurotransmitter trafficking to affect synaptic activity. In the ENS, the dynamic processes associated with synaptic transmission during trains of stimulation have not been studied.

Recycling of synaptic vesicles

Action potentials induce neurotransmitter release by initiating the processes that lead to fusion of synaptic vesicles with the presynaptic membrane and exocytosis of neurotransmitter. After exocytosis, synaptic vesicles undergo a recycling process including endocytosis and refilling with neurotransmitters so that they are prepared for another cycle of transmitter release in response to the next action potential. This process is an important mechanism that allows synaptic terminals to maintain an active supply of transmitter-containing vesicles.

The pioneering studies of synaptic vesicle recycling were conducted at the frog neuromuscular junction (NMJ) using electrophysiology and electron microscopy (Heuser and Reese, 1973; Ceccarelli et al., 1973). In these studies, NMJ preparations were stimulated in presence of horseradish peroxidase (HRP) or dextran. Exocytosis was assessed by recording the responses of the muscle cells to ACh released from nerve terminals. Endocytosis was measured as the amount of HRP or dextran containing in synaptic vesicles after tissue fixation. The membrane-attached objects with the markers were considered as the endocytosed vesicles. Based on these studies, two models were proposed for synaptic vesicle recycling. Using stimulation at high frequency, Heuser and Reese (1973) suggested "*during stimulation the intracellular compartments of this synapse change shape and take up extracellular protein in a manner which indicates that* *synaptic vesicle membrane added to the surface during exocytosis is retrieved by coated vesicles and recycled into new synaptic vesicles by way of intermediate cisternae".* This model became known as the "slow endocytotic pathway".

Ceccarelli and colleagues, however, proposed a different pathway for synaptic vesicle recycling. They used stimulation at low frequency and found (a), that synaptic vesicles fuse with, and re-form from, the membrane of the nerve terminal during and after stimulation and (b), that the re-formed vesicles can store and release transmitter (Ceccarelli et al., 1973). This suggestion later leads to a "fast endocytotic pathway" model.

Later, extensive studies have been conducted to probe the mechanisms of synaptic vesicle endocytosis. Processes of endocytosis can be directly monitored by measuring capacitance change (Sun et al., 2002; Sun and Wu, 2001) or by optical recording (Gandhi and Stevens, 2003). Recycling can be assessed by monitoring the uptake of fluorescent tracers (Wu and Betz, 1998; Cochilla et al., 1999; Murthy and Stevens, 1999). Based on these studies, three endocytotic pathways have been proposed (Sudhof, 2004): 1) "kiss and stay", 2) "kiss and run", and 3) clathrin-mediated recycling. The "kiss and stay" model proposes that synaptic vesicles recycle directly after closure of the vesicle membrane-plasma membrane fusion pore formed during exocytosis. In this model, vesicles are refilled with neurotransmitters while the vesicles remain docked to the active zone. Vesicle recycling and refilling do not require the formation of a clathrin coat around the vesicle. The "kiss and run" model also proposes that vesicles endocytose without a clathrin-coated intermediate. The difference from the "kiss and stay" model is that the vesicles do not stay in the active zone (Ceccarelli et al., 1973; Richards et al., 2000). After endocytosis, recycled vesicles mix into the reserve pool. "Kiss and stay" and "kiss and run" pathways are mechanisms for fast recycling of synaptic vesicles (Pyle et al., 2000; Sun et al., 2002; Rizzoli et al., 2003). The third pathway, clathrin-mediated endocytosis, is a slow recycling process. Vesicle recycling in this model requires formation of a clathrin-scaffold that organizes several adaptor and accessory proteins (Royle and Lagnado, 2003). The electron-dense coats around vesicles are formed by clathrin (Heuser and Reese, 1973; Cremona and De Camilli, 1997). Clathrin-mediated endocytosis and recycling may occur directly or via endosomes. Both slow and fast endocytosises require GTP hydrolysis (Jockusch et al., 2005; Yamashita et al., 2005).

Rapid recycling including "kiss and stay" and "kiss and run" is a primary pathway for synaptic vesicle retrieval during low frequencies of stimulation (Jockusch et al., 2005). The slow recycling pathway is triggered during high frequency stimulation (Sun et al., 2002; Jockusch et al., 2005). In studies done on synapses in the calyx of Held, capacitance measurements revealed that endocytosis is completed with time constant of 56 ms after a single vesicle exocytotic event. This value increases to 115 ms during stimulation at frequencies < 2 Hz and the time constant increases to 2.3 s and 8.3 s after 10 stimuli at 20 or 333 Hz, respectively (Sun et al., 2002). In retinal bipolar cells, using capacitance measurements of membrane retrieval indicated that clathrin-dependent endocytosis has a slow phase of membrane retrieval with time constant of 10 s while clathrin-independent pathway only takes 1 s (Jockusch et al., 2005).

Although the data suggest that the clathrin-dependent slow recycling pathway is activated selectively upon high-frequency stiumulation and clathrin-independent fast pathway is stimulated by low-frequency stimulation, the mechanisms responsible for these differences remain unclear. The proteins involved in these two recycling pathways have not been fully characterized.

5-HT$_4$ receptors in myenteric neurons

5-HT$_4$ receptors belong to the superfamily of GPCRs (Barnes and Sharp, 1999). 5-HT$_4$ receptors couple to the Gs subtype of G-protein activating adenylate cyclase increasing cAMP leading to activation of PKA. Several 5-HT$_4$ receptor subtypes have been identified as products of alternative splicing in different species (Medhurst et al., 2001). All splice variants of the 5-HT$_4$ receptors possess similar N-terminal sequences up to residue 359, but differ in the length of the C-terminal segment. This segment is an important determinant of constitutive activity and receptor regulation (Blondel et al., 1998). One of the differences is the desensitization rate in which the shorter variants (h5-HT$_{4a}$ and r5-HT$_{4S}$) desensitize slowly whereas the longer variants (h5-HT$_{4b}$ and r5-HT$_{4L}$) desensitize more rapidly (Claeysen et al., 1998). The small intestine expresses predominately 5-HT$_{4b}$ receptors (Blondel et al., 1998). Radioligand binding and functional studies in the intestine suggest that 5-HT$_4$ receptors are present on smooth muscle cells, excitatory cholinergic motor neurons, and terminals of IPANs. More specifically, 5-HT$_4$ receptors are located on the terminals of calcitonin gene-related peptide (CGRP)-containing IPANs. Electrophysiological study indicated 5-HT$_4$ receptor agonists did not alter resting membrane potential of myenteric neurons or and slow synaptic transmission when intracellular recordings were made in cell body (Pan and Galligan, 1995). This result suggested that 5-HT$_4$ receptors were not localized to soma.

Electron microscopic evidence also indicates that 5-HT_4 receptors are localized to synaptic terminals (Liu et al., 2005).

5-HT acting at 5-HT_4 receptors triggers initiation of the peristaltic reflex (Craig et al., 1990; Craig and Clarke, 1991; Foxx-Orenstein et al., 1996; Grider et al., 1996; Grider et al., 1998; Jin et al., 1999). 5-HT_4 receptors modulate fast synaptic transmission (Kibinger and wolf, 1992; Pan and Galligan, 1994), which underlies 5-HT_4 receptor agonist-induced increases in GI motility (Grider et al., 1998). Further studies show that 5-HT_4 receptors couple to facilitation of fast synaptic transmission by enhancing both ACh and ATP release (Pan and Galligan. 1994; LePard et al., 2004). It has been also shown that forskolin mimics 5-HT_4 receptor-mediated facilitation of fast synaptic transmission and PKA selective inhibitors blocked 5-HT_4-mediated synaptic facilitation (Galligan et al., 2003). This 5-HT_4 receptor-mediated facilitation is used as the neurophysiological basis for the prokinetic actions of clinical drugs such as 5-HT_4 receptor agonists, cisapride and tegaserod, which are used to treat GI motility disorders. However, the underlying molecular and cellular mechanisms remain unknown.

RESEARCH GOAL

The goals of the present study were to investigate fast synaptic transmission during trains of nerve stimulation and to identify mechanisms of synaptic modulation and plasticity.

Purinergic P2X receptors in postsynaptic neurons mediate fEPSPs and sensory transduction in the ENS. The subunit compositions that determine P2X receptor properties affect synaptic transmission and sensory transduction. Identification of the P2X receptor subtype expressed by enteric neurons will help to understand the functional significance of fast excitatory synaptic transmission.

Previous studies on fast synaptic transmission have investigated fEPSP induced by a single stimulation. However, fEPSP triggered by physiological stimulation occurs in bursts, which represent the situation in *vivo*. Therefore it is important to investigate fEPSPs elicited by trains of action potentials to understand the physiological functions of fEPSPs.

Presynaptic 5-hydroxytryptamine (5-HT_4) receptors mediate facilitation of fast synaptic transmission in myenteric neurons. This dynamic change mediated by 5-HT_4 receptors is very important as agonists of 5-HT_4 receptors are used as prokinetic drugs to treat certain GI disorders such as irritable bowel syndrome (IBS) and gastroesophageal reflux disease (GERD). Identification of the mechanism underlying 5-HT_4 receptor-mediated facilitation will be useful in understanding the pathology and drug therapy of these GI motility disorders.

Fast synaptic transmission is an important mechanism for enteric neurons to perform their physiological functions. Alteration of fast synaptic transmission by abnormal physiological condition can develop into GI disorders. GI disorders are common problems throughout the world. Although they are rarely life threatening, they negatively impact quality of life (Furness and Sanger, 2002). Therefore study of dynamic change of fast synaptic transmission will, in one way help to understand the neural circuits underlying the ENS regulation of GI physiological functions; in the other way provide information for GI pathology and GI disorder drug therapy.

Specific Aims

1. Identification of P2X receptor subtypes in myenteric neurons in small intestine.

This study was done in tissues from mice and guinea pigs. Although immunohistochemical localization of P2X subunits in guinea pig myenteric neurons has been done (Castelucci et al., 2002; Poole et al., 2002; VanNassauw et al. 2002), the subtypes of P2X receptors mediating fEPSPs in these tissues are not fully identified. Selective agonists and antagonists were used to evaluate eletrophysiological responses to identify P2X receptor subtypes in guinea pig myenteric neurons. In addition to using pharmacological methods, I also took advantage of $P2X_2$ and $P2X_3$ gene knockout mice to investigate the contribution of these two subunits to electrophysiological properties of myenteric neurons in mice.

2. Fast synaptic transmission elicited by trains of electrical stimulation.

This study was conducted in myenteric neurons of guinea pig small intestine in *vitro*. Intracellular electrophysiological recording techniques including single electrode voltage-clamp (SEVC) were used to characterize fast synaptic transmission during trains of electrical stimulation in LMMP preparations in *vitro*. Fast synaptic transmission elicited by single electrical nerve stimulation has been studied extensively. However, fast excitatory postsynaptic potentials (fEPSPs) occur in bursts in response to physiological stimulation. These studies have characterized fast synaptic transmission during trains of stimulation and examined the contributions of ATP and ACh to fEPSPs during a short train of electrical stimulation.

3. Presynaptic 5-HT$_4$ receptor-mediated modulation of fast synaptic transmission.

5-HT$_4$ receptors mediate enhancement of single fEPSPs in myenteric neurons. 5-HT$_4$ receptors may also regulate the dynamic change of synaptic transmission evoked by trains of stimulation. These studies have tested the hypothesis that activation of 5-HT$_4$ receptors reduced the decline in amplitude (rundown) of fEPSPs during trains of stimuli and facilitates the rate of recovery from synaptic run-down. The regulation is mediated by 5-HT agonists activating adenylate cyclase-cyclic 3', 5' adenosine monophosphate (cAMP)-PKA signaling pathway. 5-HT$_4$ receptors mediate facilitation of fEPSPs by increasing transmitter release but the cellular mechanism remains unclear. These studies have examined the hypothesis that activation of 5-HT$_4$ receptors enhances the release probability from single release sites (varicosities). Two experimental methods were used in these studies. Intracellular electrophysiological recordings were made from LMMP preparations from guinea pig tissue in *vitro*. Patch-clamp recordings were made in guinea pig myenteric neurons maintained in primary culture.

METHODS

Animals

All the procedures for handling animals were used in this study were approved by the All University Committee on Animal Use and Care at Michigan State University.

Intact myenteric plexuses were dissected acutely from adult Guinea pigs (Hartley, male, 250 – 350 g) and Mice. Guinea pigs were available from Bioport Company (Lansing, MI). Mice were obtained from Roche Bioscience (Palo Alto, CA). $P2X_2$ or $P2X_3$ gene knockout ($P2X_2^{-/-}$ or $P2X_3^{-/-}$) mice were generated as described (Cockayne *et al.* 2000). All mice used in this study have the genetic background 129Ola X C57BL/6 (Harlan), and were derived from homozygous F2 crosses. The comparison analysis was performed between knockout mouse and its wild type from same family. Genotype confirmation of all animals was carried out by southern blot analysis as previously described (Cockayne *et al.* 2000).

Longitudinal muscle myenteric plexus (LMMP) preparation

Guinea pigs were euthanized by halothane inhalation and by severing the major neck blood vessels. Mice were anesthetized with halothane and then killed by cervical dislocation. The small intestine was removed from mice or guinea pigs and placed in oxygenated (95% O_2 and 5% CO_2) Krebs' solution of the following composition (millimolar): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2;

glucose, 11. The Krebs' solution also contained nifedipine (1 μM) for blocking L-type calcium channels and scopolamine (1 μM) for blocking muscarinic cholinergic receptors to inhibit longitudinal muscle contractions. These drugs do not affect normal myenteric neuronal properties. A 1.5 cm segment of intestine was cut open along the mesenteric border and pinned out flat with the mucosal surface up in a petri dish lined with silastic elastomer. A longitudinal muscle-myenteric plexus (LMMP) preparation was made by peeling away the mucosal, submucosal, and circular muscle layers using fine forceps and scissors. A 5 mm^2 piece of longitudinal muscle myenteric plexus was transferred to a small (2 ml volume) recording chamber lined with silastic elastomer. The preparation was stretched lightly and pinned to the chamber bottom using stainless steel pins (50 μm diameter). The preparation was superfused with 37°C oxygenated Krebs' solution at a flow rate of 4 ml/min. Individual myenteric ganglia were visualized at 200x magnification using an inverted microscope (Olympus CK-2) with differential interference contrast optics.

Myenteric neurons maintained in primary culture

Tissue for primary myenteric cell culture was obtained from newborn guinea pigs (~36 h in age; ~70 g in weight). Newborn guinea pigs were sacrificed by severing the major neck blood vessels and spinal cord after deep halothane anesthesia. The small intestine was placed in cold (4°C) sterile-filtered Krebs' solution of the following composition: 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. The longitudinal muscle myenteric plexus was removed from the entire length of small intestine and cut into 5-mm-long pieces. The dissected tissues were divided into four aliquots and placed in 1 ml of Krebs' solution containing 1600 U of trypsin (Sigma Chemical Co., St. Louis, MO) for 25 to 30 min at 37°C. After trypsin incubation, the tissues were triturated 30 times and then centrifuged at 900*g* for 5 min with a bench-top centrifuge. The supernatant was discarded, and the pellet was then be resuspended in sterile Krebs' solution and incubated (25-30 min, 37°C) in Krebs' solution containing 2000 U crab hepatopancreas collagenase (Calbiochem-Novabiochem, Corp., La Jolla, CA). The suspension was triturated and then centrifuged for 5 min. The pellet was suspended in Eagle's minimum essential medium containing 10% fetal calf serum, gentamicin (10 μg/ml), penicillin (100 U/ml), and streptomycin (50 μg/ml) (all from Sigma). Cells were plated on plastic dishes coated with poly-L-lysine and maintained in an incubator at 37°C in an atmosphere of 5% CO_2 for up to 2 weeks. After 2 days in culture, 10 μM cytosine arabinoside was added to the minimum essential medium to limit smooth muscle and fibroblast proliferation, and the medium was changed twice weekly thereafter.

Intracellular electrophysiological recording from myenteric neurons

Individual myenteric ganglia can be visualized at 200x magnification using an inverted microscope (Olympus CK-2) with differential interference contrast optics. Intracellular recordings were obtained from single neurons using glass microelectrodes filled with 2 M KCl and a tip resistance of 80-120 MΩ. An amplifier with an active bridge circuit (Axoclamp 2A, Axon Instruments, Foster City, CA) was used to record membrane potential. In most experiments, the membrane potential was hyperpolarized using constant DC current to avoid action potentials when evoking fEPSPs. The amplified signals then were filtered at 1 kHz using a four-pole, low-pass Bessel filter (Warner Instruments, Hamden, CT), and digitized at 2 kHz using a Digitdata 1200 analog/digital converter (Axon Instruments). Data were acquired and stored using Axotape 2.02 or Axoscope 8.2 software (Axon Instruments).

All fEPSPs were elicited using a glass pipette (tip diameter 40-60 μM) filled with Krebs' solution as a focal stimulating electrode. The stimulating electrode was positioned closely over an interganglionic nerve strand. Nerve fibers were stimulated electrically using single stimuli of 0.5 msec of duration at a rate of 0.1 Hz. A digital average of eight individual fEPSPs was used as a measurement of fEPSP amplitude in the absence or presence of drug treatments.

Patch-clamp recording from primary cultured neurons

Whole-cell and outside-out patch-clamp recordings were obtained via standard methods. Recordings were carried out at room temperature with patch pipettes with tip resistances of 3 to 5 MΩ for whole-cell and 5 to 10 MΩ for single-channel currents in outside-out patches; seal resistances were >5 GΩ. The tips of pipettes used for single-channel recordings were coated with Sylgard (Dow Corning, Midland, MI). The pipette solution contained the following: 160 mM CsCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 1 mM ATP, and 0.25 mM GTP, the pH and osmolarity were adjusted to 7.4 (with CsOH) and 315 mosmol/kg (with CsCl), respectively. All recordings were made with an Axopatch 200A amplifier. Data were acquired with pClamp 8.0 software. Currents were sampled at 2 kHz and were filtered at 1 kHz (4-pole Bessel filter, Warner Instruments, Hamden, CT) and stored on a computer hard drive for offline analysis.

Immunohistochemistry

After electrophysiological recordings with Neurobiotin-filled microelectrodes, the myenteric plexus-longitudinal muscle preparation was fixed overnight at 4 °C in Zamboni's fixative (2 % (v/v) formaldehyde and 0.2 % (v/v) picric acid in 0.1 M sodium phosphate buffer, pH 7.0). The fixative was removed using three washes of dimethyl sulfoxide at 10 min intervals. Tissues were then washed three times with phosphate-buffered saline (PBS) (0.01 M; pH 7.2) at 10 min intervals. Subsequently the preparation was incubated overnight with a primary antibody against nitric oxide synthase (NOS) at room temperature. After primary antibody incubation, tissues were washed three times at 10 min intervals with PBS. Tissues were then incubated (1.5 h at 23 °C) with goat anti-rabbitt IgG (1:40 dilution in PBS; Jackson Immunoresearch Laboratories, West Grove, PA, USA) conjugated to fluorescein isothiocyanate (FITC) to recognize NOS immunoreactivity and Texas Red to recognize the neurobiotin-filled impaled neurons. Tissues were washed three times with PBS and mounted in buffered glycerol for fluorescence microscopy. Images were obtained using Nikon, Eclipse TE2000-U (Tokyo, Japan).

Drugs and drug application

All drugs used in the studies are listed in Table 1. In general, four different methods were used for drug application.

1. Superfusion: Antagonists were applied by superfusion in a known concentration by addition to the superfusing Krebs' solution. Antagonists were applied for 6-20 minutes prior to measuring the amplitude of evoked responses.

2. Pressure ejection: Local application of agonists was accomplished by ejection from the tip of a micropipette (~20 μm tip diameter) placed within 150 μm of the impaled neuron. Agonists were applied using short pulses of nitrogen gas (3 to 35 ms, 10 psi) using a Picospritzer II (General Valve, Fairfield, NJ).

3. Ionophoresis: ACh or ATP was applied by ionophoresis from an electrode (10 MΩ <tip resistance <20 MΩ) placed directly above the impaled neuron. The concentration of ACh or ATP used for ionophoresis was 1M. When ACh ionophoresis was applied, a retaining current of –6nA was used and a cathodal current pulse of 1-5 msec duration and 100-199 nA intensity were used. When ATP ionophoresis was applied, a retaining current of +8 nA was used and an anodal current pulses of 1-5 msec duration and 100-199 nA intensity was used.

4. Gravity-driven flow: Application of agonists or some antagonists to cultured neurons was accomplished using a linear barrel array of quartz, gravity-fed flow tubes (320 μm i.d. and 450 μm o.d., Poltmicron Technologies, Phoenix, AZ). The precise timing of onset and offset of drug application was controlled by computer-gated solenoid valves (General Valve, Fairfield, NJ).

Table 1. Drugs and ligands used in this dissertation

Ligands	Major Effect
Acetylcholine (ACh)	Endogenous AChR agonist
α,β-methyleneATP (α,β-mATP)	Agonist selective to $P2X_1$ or $P2X_3$ receptors
Apamin	Blocker of K_{Ca} with small conductance (SK)
Adenosine triphosphate (ATP)	Endogenous P2 receptor agonist
Clotrimazole (CLT)	Blocker of K_{Ca} with intermediate conductance (IK)
8-cyclopentyltheophylline (CPT)	Antagonist selective to A_1 Adenosine receptors
Cytisine	nAChR agonist selective for β_4 subunit
DMPP	nAChR agonist selective for β_2 subunit
Forskolin	Adenylate cyclase stimulator
H-89	Protein kinase A blocker
Hexamethonium	nAChR blocker
Iberori toxin (IBTx)	Blocker of K_{Ca} with big conductance (BK)
Idazoxan	α2 Adrenergic receptor antagonist
Mecamylamine	nAChR antagonist
MLA	nAChR antagonist selective for α7 subunits
Nalaxone	Opioid receptor antagonist
NAN-190	$5\text{-}HT_{1A}$ receptor antagonist
Nicotine	nAChR agonist
Nifedipine	L-Calcium channel blocker
PPADS	P2 receptor antagonist
Pertussis toxin (PTX)	Gi/o Protein inhibitor
Prucalopride	$5\text{-}HT_4$ receptor agonist
Renzapride	$5\text{-}HT_4$ receptor agonist
Scoplamine	Muscrinic AChR blocker
Tetraethylammonium (TEA)	BK(<1mM), Kir(10mM)
Tegaserod	$5\text{-}HT_4$ receptor agonist
Tetrodotoxin (TTX)	Voltage-gated Na^+ channel blocker
TNP-ATP	P2X receptor blocker selective for $P2X_1$ and $P2X_3$

Statistics

All data were expressed as the mean ± standard error of the mean (SEM). Data were analyzed for significance using Student's t-test for paired data or analysis of variance. $P < 0.05$ was used to established significant differences between control and treatment groups. The specific methods used to analyze data are explained in the related result parts.

RESULTS

SUBTYPES OF FUNCTIONAL P2X RECEPTORS IN MYENTERIC NEURONS

Pharmacological identification of functional P2X receptor subtypes in myenteric neurons were conducted in tissues from mice (Part A.) and guinea pigs (Part B.).

Part A. P2X Receptor Subtypes In Myenteric Neurons In Mouse Small Intestine

This part of studies used electrohysiological and pharmacological methods in tissues from $P2X_2$ or $P2X_3$ subunit gene deleted or wild type control mice. As these mice were derived from homozygous F2 crosses, each knockout mouse has its corresponding wildtype control. So comparisons were made between each knockout and its corresponding wildtype control.

Characterization of myenteric neurons from $P2X_2^{+/+}$, $P2X_2^{-/-}$, $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice

Intracellular electrophysiological recordings were made from single myenteric neurons in acutely isolated LMMP preparations in small intestine of mice whose genes encoding $P2X_2$ or $P2X_3$ subunits have been deleted. RT-PCR and immunohistochemical analysis confirmed that no $P2X_2$, $P2X_3$ subunit mRNA or protein was expressed in small intestine of $P2X_2^{-/-}$ and $P2X_3^{-/-}$ mouse, respectively (Ren et al., 2003; Bian et al., 2003).

There were no data available that describe the electrophysiological properties of murine small intestinal myenteric neurons. Therefore, I first characterized the electrophysiological properties of myenteric neurons prior to assessing changes that might be associated with $P2X_2$ or $P2X_3$ gene deletion. S neurons were identified by the presence of fEPSPs and action potentials that lacked long lasting afterhyperpolarizations (Hirst et al., 1974). AH neurons were identified by the long- lasting action potential afterhyperpolarization. Recordings were made from 108 S neurons and 12 AH neurons for $P2X_3^{+/+}$ /$P2X_3^{-/-}$ studies and 93 S neurons and 12 AH neurons for $P2X_2^{+/+}$ /$P2X_2^{-/-}$ studies. AH type neurons were infrequently encountered and represented approximately 10 percent of total number of neurons impaled in the study. This observation was similar to previous report in the mouse colon (Furukawa et al., 1986).

Resting membrane potentials (RMP) and input resistance (R_{input}) of AH and S myenteric neurons from $P2X_3^{-/-}$, $P2X_2^{-/-}$ and their corresponding wild type mice were not different (Table 2). Action potentials recorded from S neurons in the different mutants and control mice were similar (Figure 5). The action potentials were blocked by tetrodotoxin (TTX) and followed by a brief afterhyperpolarization. The action potentials had durations at half-amplitude of 1.7 ± 0.1 ms in tissues from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice and 1.6 ± 0.1 ms in tissues from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice.

Action potentials recorded from AH neurons in tissues from these mice were also similar (Figure 6). The action potential durations at half-amplitude were 2.1± 0.1 and 2.3 ± 0.1ms ($P > 0.05$) in tissues from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice, respectively; 2.0 ± 0.2 and 2.4 ± 0.1 ms ($P > 0.05$) in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice, respectively. The peak amplitudes of the action potentials slow afterhyperpolarization were 5.9 ± 1.1 and 5.8 ± 0.7 mV in $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice ($P > 0.05$), respectively. These numbers were 5.2 ± 0.7mV and 5.4 ± 0.6mV in ($P > 0.05$) in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice, respectively. The duration of the

Table 2. Electrical properties of myenteric neurons in mouse small intestine

A. Resting membrane potential (RMP) and input resistance (R_{input}) of AH and S neurons in $P2X_3$ $^{+/+}$ and $P2X_3$ $^{-/-}$ mice.

		AH neurons		S neurons	
		$P2X_3$ $^{+/+}$ (n=5)	$P2X_3$ $^{-/-}$ (n=7)	$P2X_3$ $^{+/+}$ (n=22)	$P2X_3$ $^{-/-}$ (n=22)
RMP (mV)	Mean	63 ± 4	63 ± 2	62 ± 2	59 ± 2
	Range	50 – 71	57 – 73	49 – 73	42 – 72
R_{input} (MΩ)	Mean	172 ± 35	153 ± 20	121 ± 14	127 ± 10
	Range	93 – 285	79 – 212	57 – 277	65 – 204

B. Resting membrane potential (RMP) and input resistance (R_{input}) of AH and S neurons in $P2X_2{}^{+/+}$ and $P2X_2{}^{-/-}$ mice.

		AH neurons		S neurons	
		$P2X_2{}^{+/+}$ (n=3)	$P2X_2{}^{-/-}$ (n=7)	$P2X_2{}^{+/+}$ (n=17)	$P2X_2{}^{-/-}$ (n=21)
RMP (mV)	Mean	69 ± 5	62 ± 4	63 ± 2	58 ± 2
	Range	61 – 80	48 – 81	50 – 74	45 – 80
R_{input} (MΩ)	Mean	136 ± 35	132 ± 21	122 ± 13	112 ± 9
	Range	96 – 207	85 – 221	65 – 241	54 – 167

Figure 5. Action potentials in myenteric S neurons in mouse small intestine

A. Action potentials recorded from myenteric S neurons in tissues from $P2X_2$ knockout mice ($P2X_2^{-/-}$) and wild type mice ($P2X_2^{+/+}$). S neurons have action potentials followed by short duration afterhyperpolarizations. TTX blocks the action potential in S neurons.

B. Action potentials recorded from S neurons in tissues from a $P2X_3^{-/-}$ and $P2X_3^{+/+}$ mice. These action potentials also have a short afterhyperpolarization (as arrows point) and they are blocked by TTX.

Figure 5

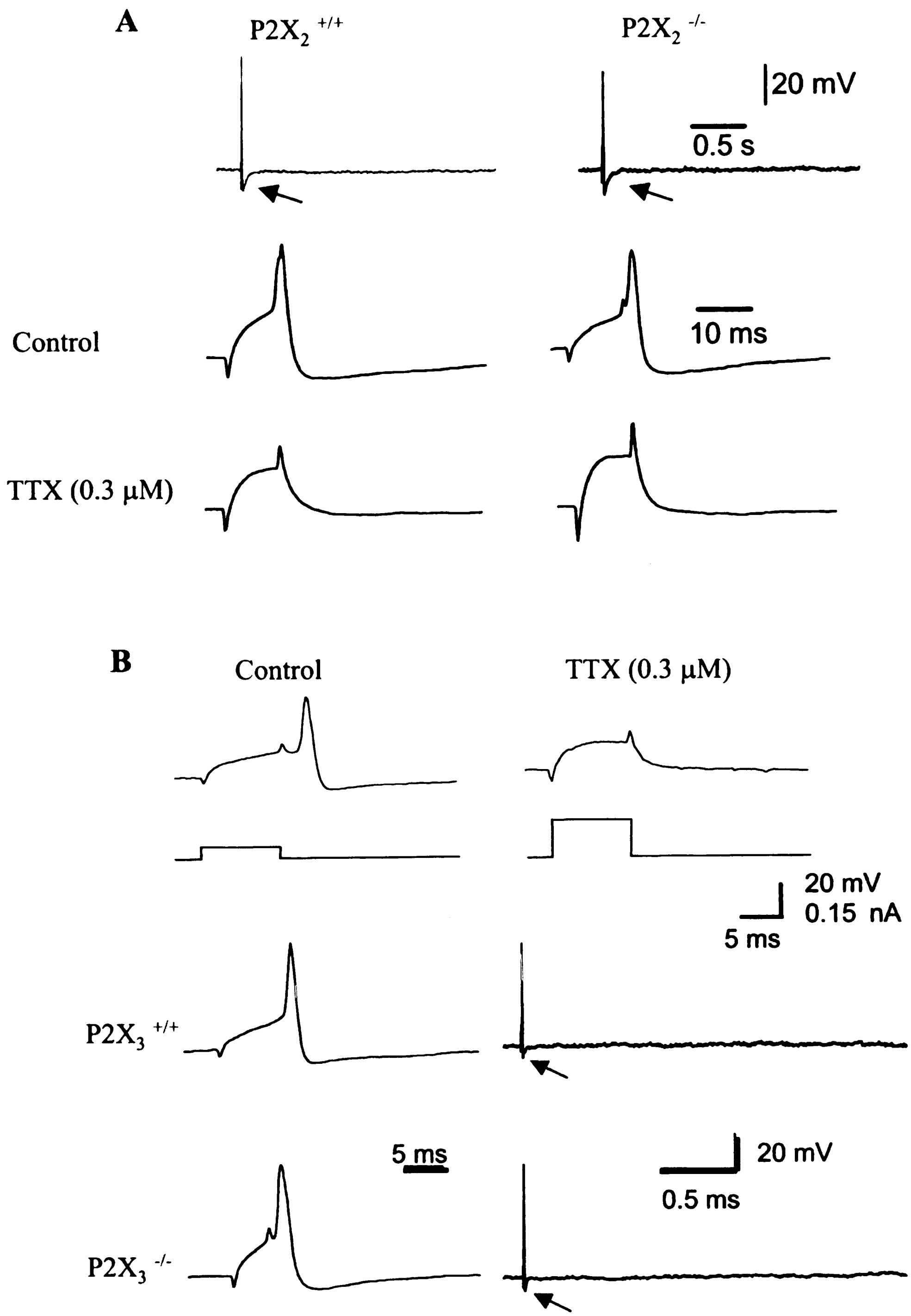
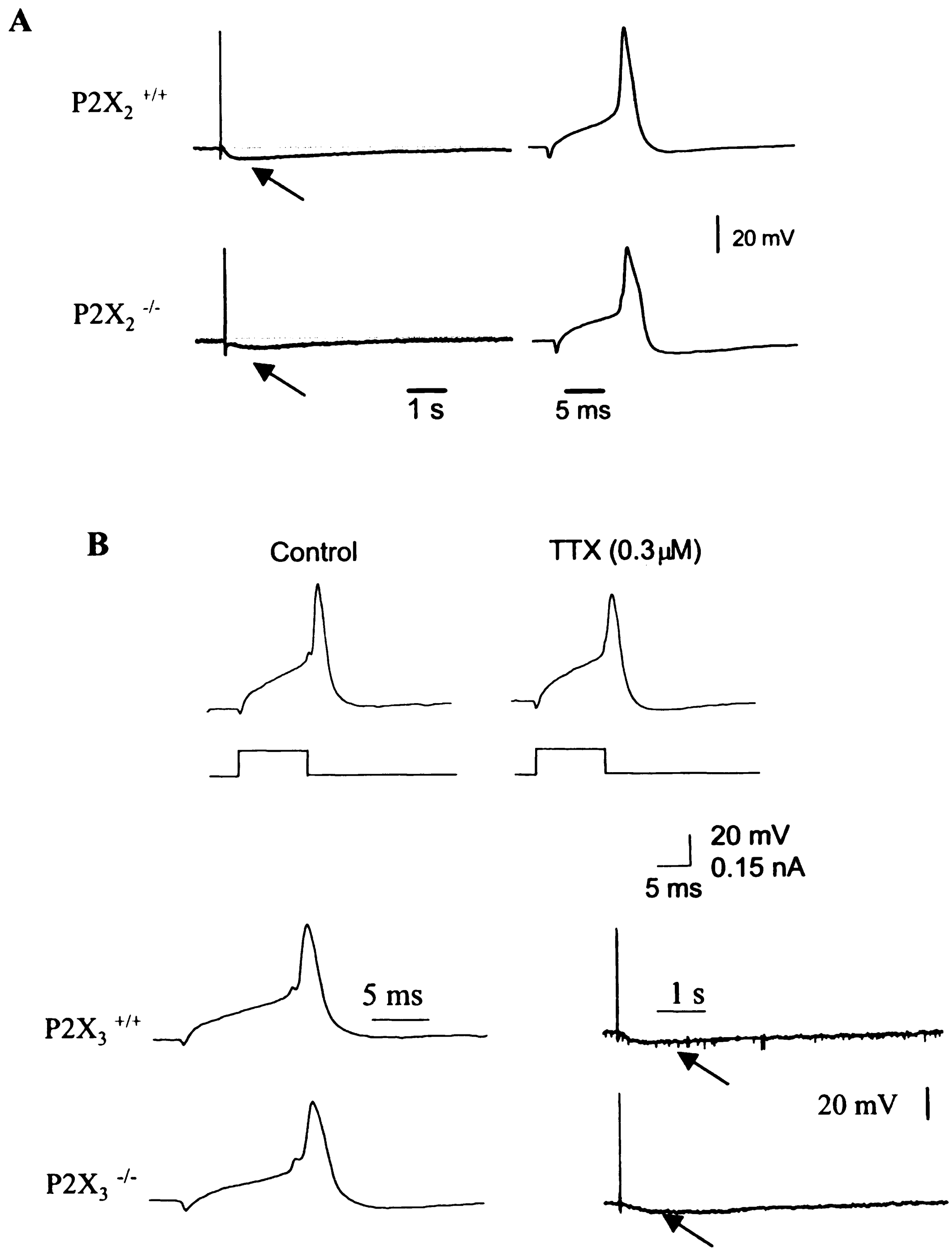

A
$P2X_2$ +/+
$P2X_2$ -/-
20 mV
0.5 s
Control
10 ms
TTX (0.3 μM)
B
Control
TTX (0.3 μM)
20 mV
0.15 nA
5 ms
$P2X_3$ +/+
5 ms
20 mV
0.5 ms
$P2X_3$ -/-

Figure 6. Action potentials in myenteric AH neurons in mouse small intestine

A. Action potentials recorded from AH neurons in $P2X_2^{-/-}$ knockout and $P2X_2^{+/+}$ tissues are similar. They have long-lasting afterhyperpolarization (as arrows show).

B. Similarly, there is no difference between action potentials recorded from AH neurons in $P2X_3^{-/-}$ and $P2X_3^{+/+}$ tissues and they have long-lasting hyperpolarization (arrows). In addition, action potential recorded from AH neurons is not blocked by TTX.

Figure 6


A
P2X2 +/+
P2X2 -/-
20 mV
1 s
5 ms
B
Control
TTX (0.3μM)
20 mV
0.15 nA
5 ms
P2X3 +/+
P2X3 -/-
5 ms
1 s
20 mV

afterhyperpolarization was 6.4 ± 0.9 s in AH neurons from $P2X_2^{+/+}$ mice and 5.7 ± 0.4 s ($P > 0.05$) in AH neurons from $P2X_2^{-/-}$ mice. In $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice, these numbers were 6.3 ± 1.3s and 6.5 ± 0.7 s (*P> 0.05*), respectively.

$P2X_2$ subunit knockout altered P2X receptor-mediated responses in S neurons

Fast EPSPs recorded from S neurons in tissues of $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice were not different in the peak amplitude or time course (Table 2, Figure 7). This result suggests that $P2X_2$ subunits may not contribute to the P2X receptors mediating fEPSPs in S neurons. However, in tissues from $P2X_2^{+/+}$ mice, nearly all fEPSPs were reduced in amplitude by the P2 receptor antagonist pyridoxalphosphate- 6-azophenyl-2,, 4,-disulphonic acid (PPADS, 10 μM) (Figure 7). All fEPSPs were blocked by the combined application of PPADS and the nicotinic acetylcholine receptor antagonist, mecamylamine (10 μM). However, PPADS had no effect on fEPSPs recorded from S neurons in tissues from $P2X_2^{-/-}$ mice (Figure 7). Mecamylamine reduced the amplitude of fEPSPs recorded from S neurons in $P2X_2^{+/+}$ tissues, but blocked fEPSPs recorded from S neurons in tissues from $P2X_2^{-/-}$ mice (Figure 8). The amplitude of fEPSP in presence of mecamylamine recorded from S neurons in $P2X_2^{+/+}$ tissues was larger than that in S neurons of $P2X_2^{-/-}$ tissues (Figure 8). Puff application of nicotine caused a depolarization of S neurons from $P2X_2^{-/-}$ mice and this response was similar in amplitude to that recorded from S neurons in $P2X_2^{+/+}$ tissues (Figure 9). Focal application of ATP, but not α,β-mATP depolarized S neurons in tissues from $P2X_2^{+/+}$ mice (Figure 9). Neither ATP nor α,β-mATP caused a response in S neurons from $P2X_2^{-/-}$ mice (Figure 9).

Figure 7. PPADS inhibits fEPSPs recorded from S neurons in tissues from $P2X_2^{+/+}$ **but not in tissues from** $P2X_2^{-/-}$ mice.

A. Representative fEPSPs recorded from S neurons in $P2X_2^{+/+}$ (left) and $P2X_2^{-/-}$ (right) tissues in the absence or presence of PPADS (10 μM). The fEPSPs recorded from neurons in tissues from $P2X_2^{+/+}$ mice were inhibited by PPADS (left) while fEPSPs recorded from neurons in $P2X_2^{-/-}$ tissues were unaffected by PPADS (right).

B. Pooled data show that the amplitudes of fEPSPs recorded from S neurons in $P2X_2^{+/+}$ (n = 5) and $P2X_2^{-/-}$ (n = 6) tissues were similar. PPADS did not alter fEPSP amplitude recorded from $P2X_2^{-/-}$ S neurons but it inhibited the fEPSP in S neurons from $P2X_2^{+/+}$ tissues. Data are mean ± SEM. "*" Significantly different from $P2X_2^{+/+}$ control ($p<0.05$).

Figure 7

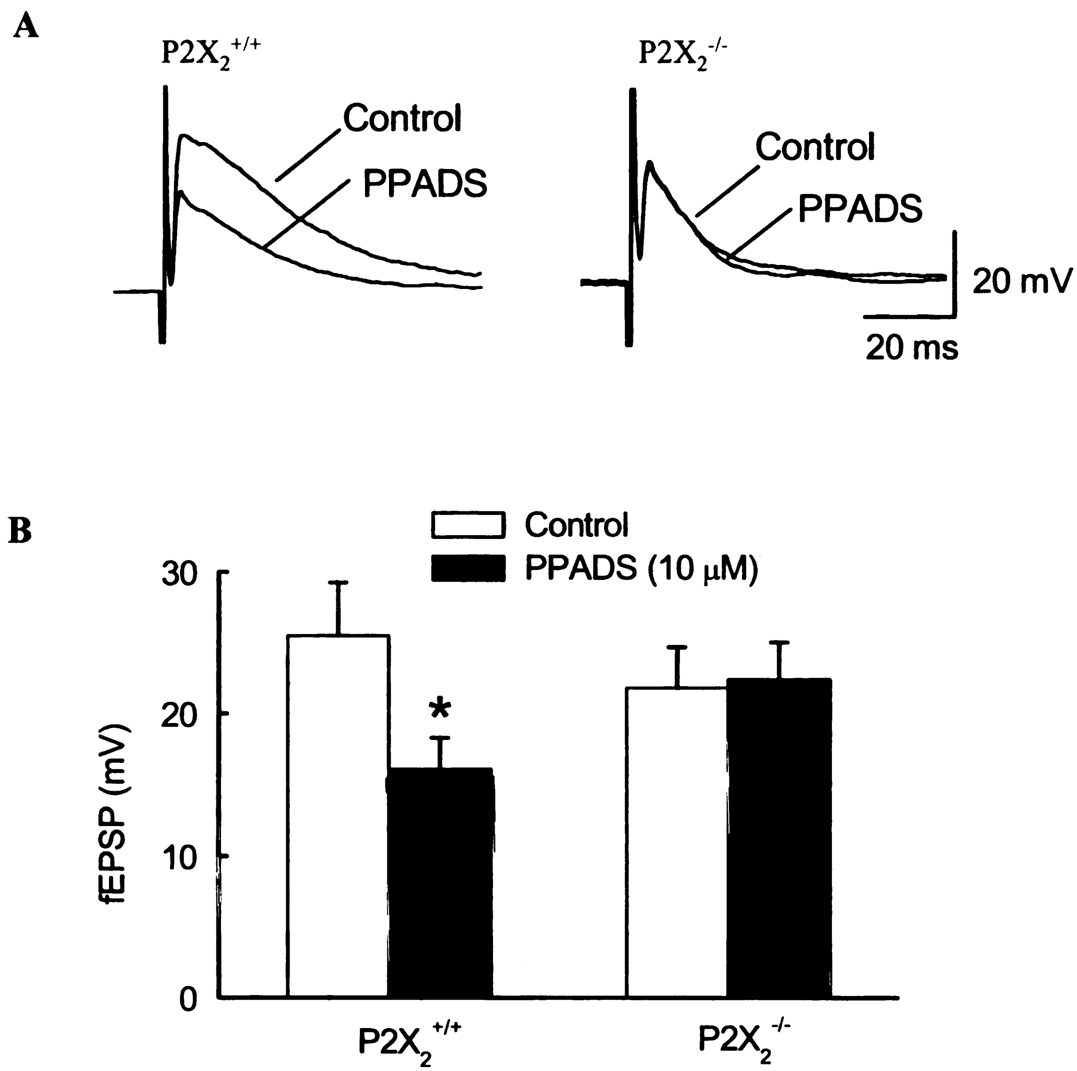

A
P2X2+/+
Control
PPADS
P2X2-/-
Control
PPADS
20 mV
20 ms
B
Control
PPADS (10 μM)
30
20
10
0
fEPSP (mV)
*
P2X2+/+
P2X2-/-

Figure 8. Mecamylamine reduced fEPSPs in S neurons from tissues of $P2X_2^{+/+}$ mice but completely blocked fEPSPs in S neurons from $P2X_2^{-/-}$ mice.

A. Representative fEPSPs recorded from S neurons in $P2X_2^{+/+}$ (left) and $P2X_2^{-/-}$ (right) tissues. The fEPSP in $P2X_2^{+/+}$ neuron is only partly inhibited by mecamylamine (10 μM), but the fEPSP is blocked by mecamylamine in a neuron from a $P2X_2^{-/-}$ mouse.

B. Pooled data show that mecamylamine reduced fEPSP amplitude in $P2X_2^{+/+}$ neurons (n = 5). However, mecamylamine blocked fEPSPs in $P2X_2^{-/-}$ neurons (n = 9). "*" Significantly different from $P2X_2^{+/+}$ or $P2X_2^{-/-}$ control amplitude ($P < 0.05$); "#" Significantly different from the amplitude of the $P2X_2^{+/+}$ fEPSP in the presence of mecamylamine ($P < 0.05$). Data are mean ± SEM.

Figure 8

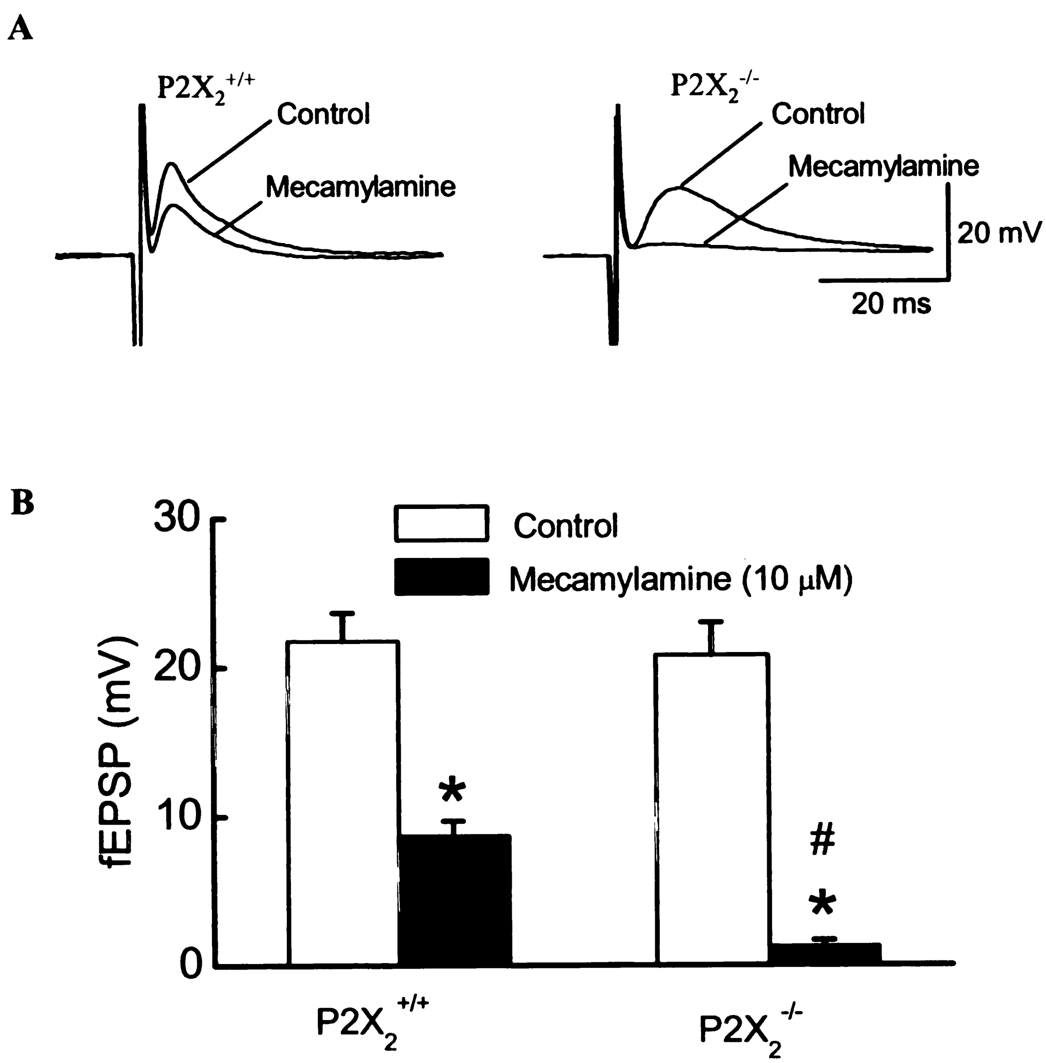
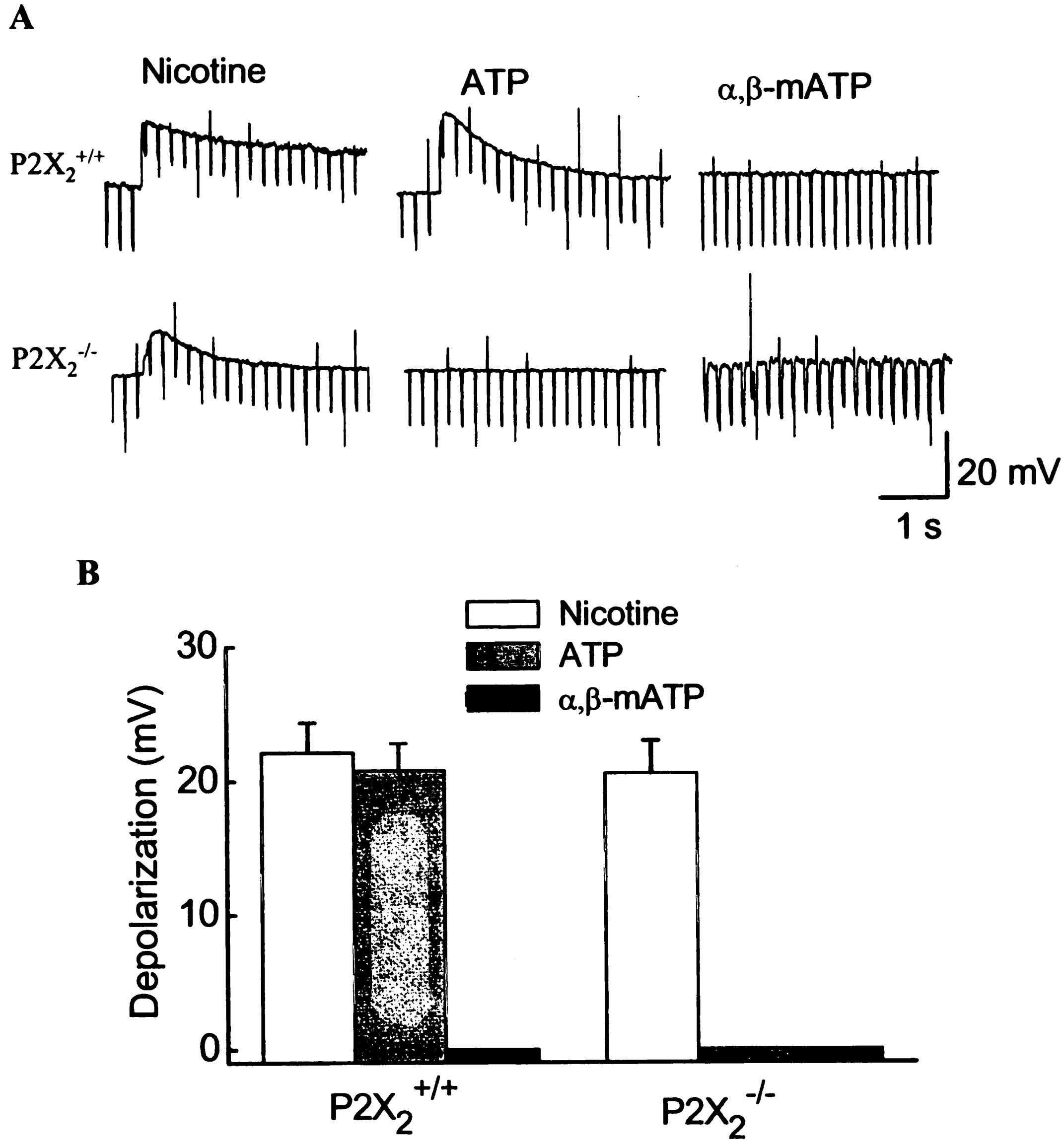

A
P2X2+/+
Control
Mecamylamine
P2X2-/-
Control
Mecamylamine
20 mV
20 ms
B
Control
Mecamylamine (10 μM)
fEPSP (mV)
30
20
10
0
*
#
*
P2X2+/+
P2X2-/-

Figure 9. Responses of murine myenteric S neurons in $P2X_2^{+/+}$ and $P2X_2^{-/-}$ tissues to nicotine, ATP and α, β-mATP

A. Nicotine induced similar amplitude depolarizations of S neurons from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice. ATP depolarized S neurons from $P2X_2^{+/+}$ but not $P2X_2^{-/-}$ mice, while α, β-mATP did not depolarize any S neurons. Drugs were applied (at the arrows) by pressure ejection from a pipette positioned near the impaled neurons. The concentration of each drug in the pipette was 1 mM.

B. Mean data from experiments similar to those shown in A. There was no difference in the amplitude of the nicotine-induced depolarization recorded from S neurons in tissues from $P2X_2^{+/+}$ (n = 15) and $P2X_2^{-/-}$ (n = 12) mice. "*" The ATP-induced depolarization was significantly smaller in $P2X_2^{-/-}$ neurons compared to those in recorded from $P2X_2^{+/+}$ neurons. α, β-mATP did not elicit a response in S neurons from either type of mouse.

Figure 9


A
Nicotine
ATP
α,β-mATP
P2X2+/+
P2X2-/-
20 mV
1 s
B
Nicotine
ATP
α,β-mATP
Depolarization (mV)
30
20
10
0
P2X2+/+
P2X2-/-

Properties of P2X receptors were not affected by $P2X_3$ subunit knockout in S neurons

Single stimuli applied to interganglionic nerve strands evoked fEPSPs in S neurons from tissues of $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice (Figure 10, 11). The mean amplitude of fEPSPs recorded from neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues was not significantly different (Figure 10, 11). The pharmacology of fEPSPs between $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues was similar. All fEPSPs were reduced, but not completely blocked, by the nicotinic receptor antagonist mecamylamine (10 μM) (Figure 10). Amplitudes of fEPSPs in presence of mecamylamine were not different in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. Similarly, fEPSPs in tissues from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice were both partly inhibited by PPADS (Figure 11). Also, fEPSP amplitude in S neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice were not different in presence of mecamylamine. ATP caused a depolarization that was similar in amplitude in S neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice (Figure 12). However, α, β-mATP did not cause depolarization in S neurons in tissues from either type of mice (Figure 12).

α, β-mATP depolarizes AH neurons from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice

Focal application of α,β-mATP (1mM), an agonist selective for $P2X_1$ and $P2X_3$ containing P2X receptors caused a rapidly developing depolarization that was associated with a decrease in membrane input resistance in AH neurons from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice (Figure 13). The α,β-mATP-induced response was mimicked by ATP applied to the same neurons and was blocked by PPADS. Amplitudes of α, β-mATP-induced

Figure 10. Effects of mecamylamine on fEPSPs from murine myenteric S neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues.

A. Representative recordings show mecamylamine (10 μM) reduced the amplitudes of fEPSPs in neurons in both $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues.

B. Pooled data from experiments shown in A. There was no significant difference in the amplitude of fEPSPs recorded from S neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. Mecamylamine reduced the amplitude of the fEPSP in all S neurons but it did not completely block the fEPSP. * Significantly different from fEPSP amplitude recorded in drug free solution.

Figure 10

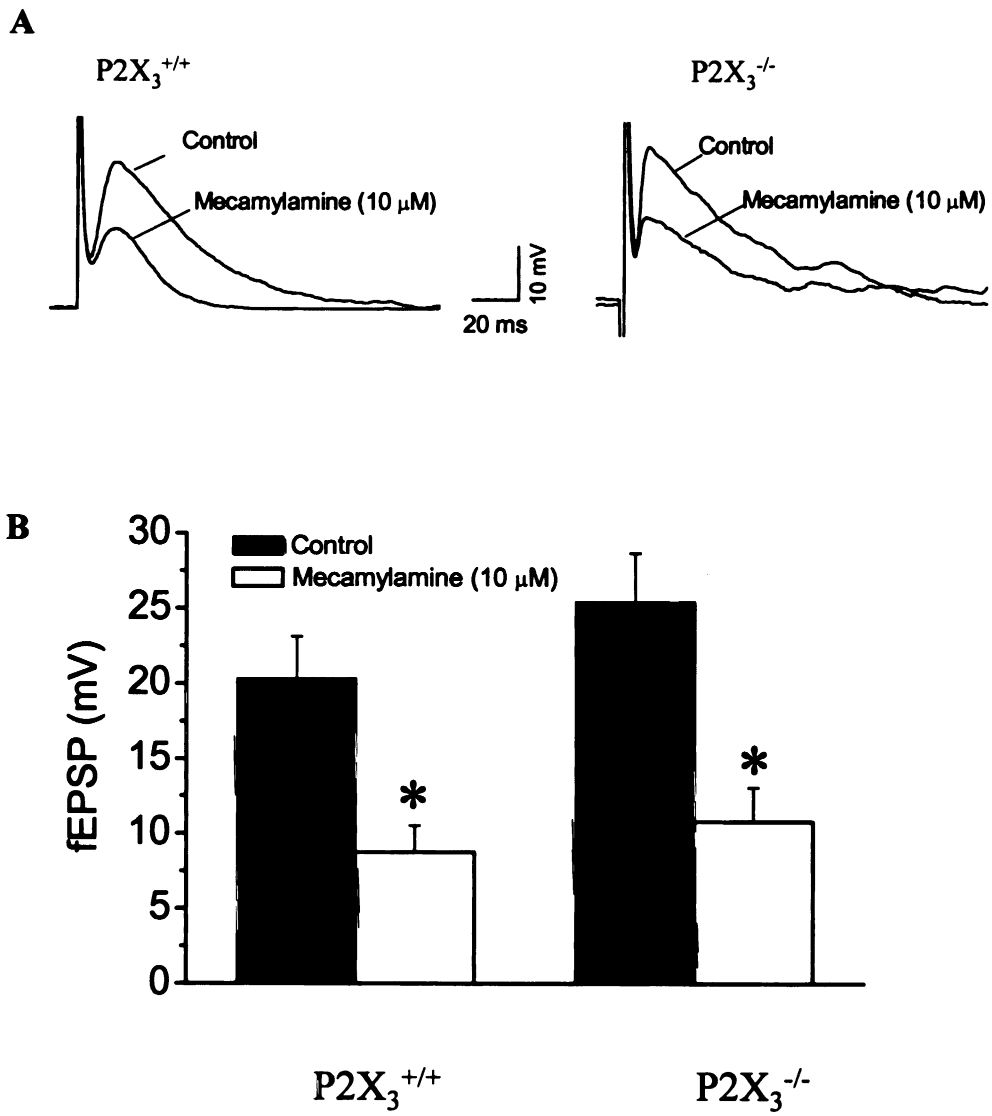

A
$P2X_3^{+/+}$
Control
Mecamylamine (10 μM)
10 mV
20 ms
$P2X_3^{-/-}$
Control
Mecamylamine (10 μM)
B
30
25
20
15
10
5
0
fEPSP (mV)
Control
Mecamylamine (10 μM)
*
*
$P2X_3^{+/+}$
$P2X_3^{-/-}$

Figure 11. Effects of PPADS on fEPSPs from murine myenteric S neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues.

A. Representative fEPSPs recorded from S neurons in tissues from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice are inhibited but not blocked by PPADS.

B. Pooled data from experiment illustrated in A. PPADS produces a significant inhibition of fEPSPs but it does not block the synaptic response. There was no difference in the amplitude of the fEPSPs recorded from S neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ tissues.

Figure 11

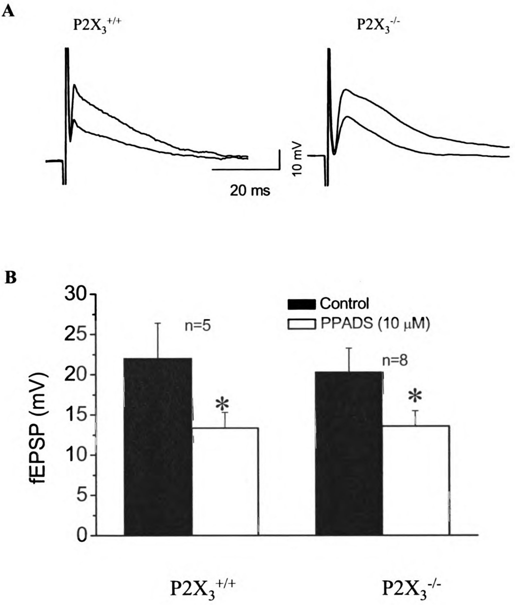

A
P2X3+/+
P2X3-/-
10 mV
20 ms
B
30
25
20
15
10
5
0
fEPSP (mV)
Control
PPADS (10 μM)
n=5
n=8
*
*
P2X3+/+
P2X3-/-

Figure 12. Responses of murine myenteric S neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ **tissues to ATP and α, β-mATP**

A. Representative recordings show that ATP, but not α, β-mATP induced depolarization in S neurons.

B. Pooled data indicate there were no differences in the amplitude of ATP-induced depolarizations in S neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. α, β-mATP did not induce depolarization in any tested S neurons.

Figure 12

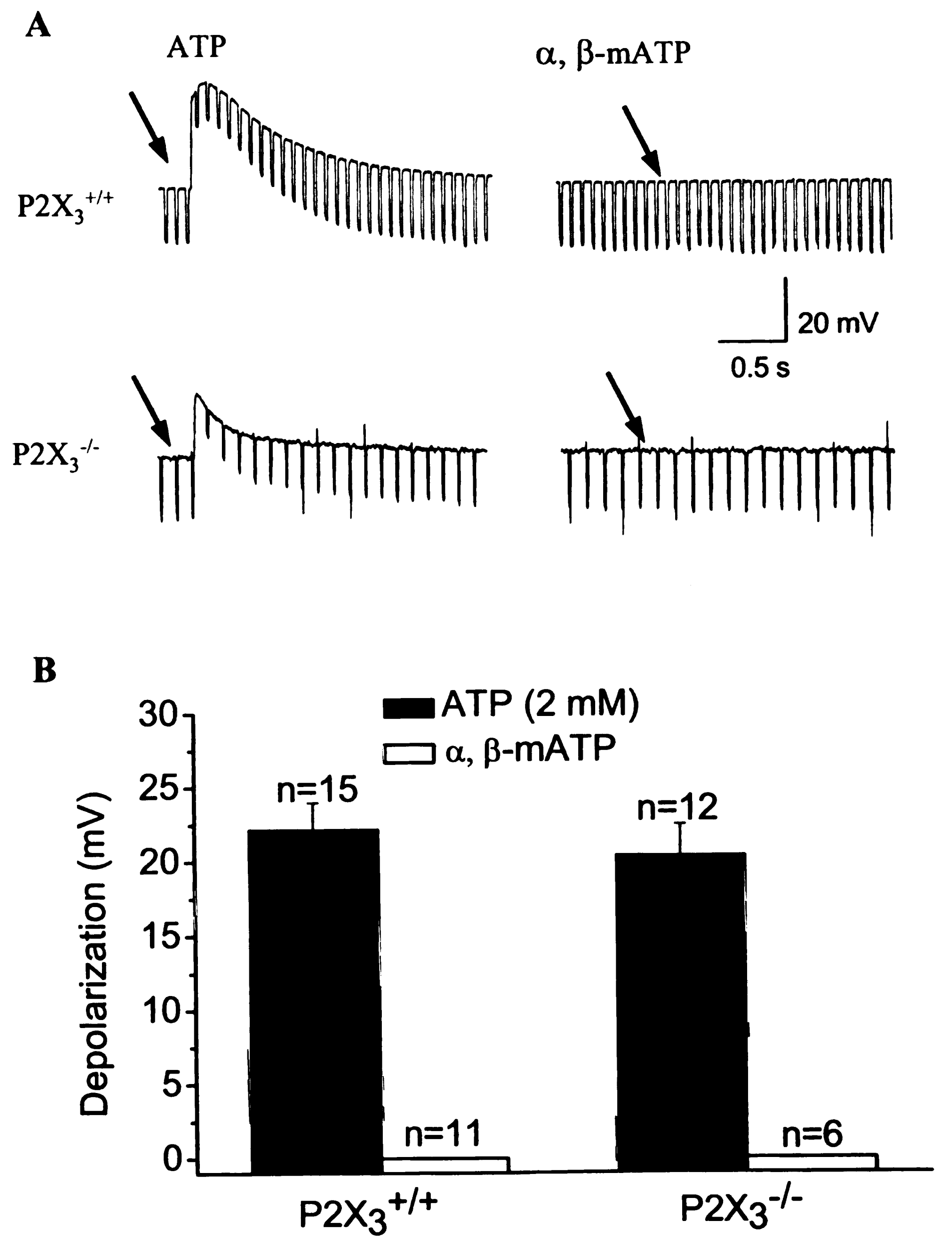
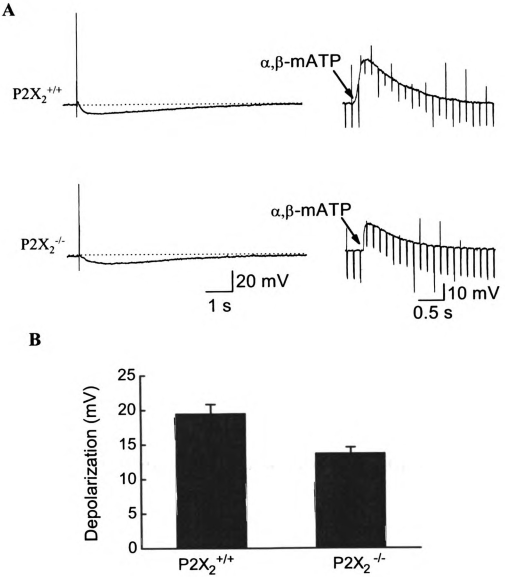

A
ATP
α, β-mATP
P2X3+/+
20 mV
0.5 s
P2X3-/-
B
ATP (2 mM)
α, β-mATP
Depolarization (mV)
30
25
20
15
10
5
0
n=15
n=11
n=12
n=6
P2X3+/+
P2X3-/-

Figure 13. Responses of murine myenteric AH neurons in $P2X_2^{+/+}$ and $P2X_2^{-/-}$ tissues to α, β-mATP

A. Representative recordings of agonist-induced responses from AH neurons in $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mouse tissues. α, β-mATP was applied by pressure ejection (at the arrows) from a pipette positioned near the impaled neuron. The concentration of α, β-mATP in the pipette was 1 mM.

B. Mean data show that the amplitude of the α, β-mATP-induced depolarization was similar in tissues from $P2X_2^{+/+}$ (n = 3) and $P2X_2^{-/-}$ (n = 4) mice.

Figure 13


A
$P2X_2^{+/+}$
α,β-mATP
$P2X_2^{-/-}$
α,β-mATP
20 mV
1 s
10 mV
0.5 s
B
Depolarization (mV)
25
20
15
10
5
0
$P2X_2^{+/+}$
$P2X_2^{-/-}$

depolarizations were similar in tissues from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice (Figure 13).

ATP- and α, β-mATP- induced depolarization in AH neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice

Local application of ATP (2 mM) from a pipette positioned near the neurons caused a depolarization that was similar in amplitude in neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice (Figure 14). The ATP-induced response was blocked by PPADS and was associated with a decrease in membrane input resistance (Figure 14). PPADS blocks some P2X receptors and $P2Y_1$ receptors for ATP so responses by ATP in murine neurons could be mediated by either P2X or $P2Y_1$ receptors. To address this possibility, I tested the effect of α, β-mATP, an agonist of $P2X_1$ and $P2X_3$ subunit-containing P2X receptors. It was found that α, β-mATP caused depolarization in AH neurons in preparations from $P2X_3^{+/+}$ but not $P2X_3^{-/-}$ mice (Figure 14).

B. Pharmacological Identification Of P2X Receptor Subtypes In Guinea Pig Myenteric Neurons

Depolarization induced by ATP and α,β-mATP in AH and S neurons in guinea pigs

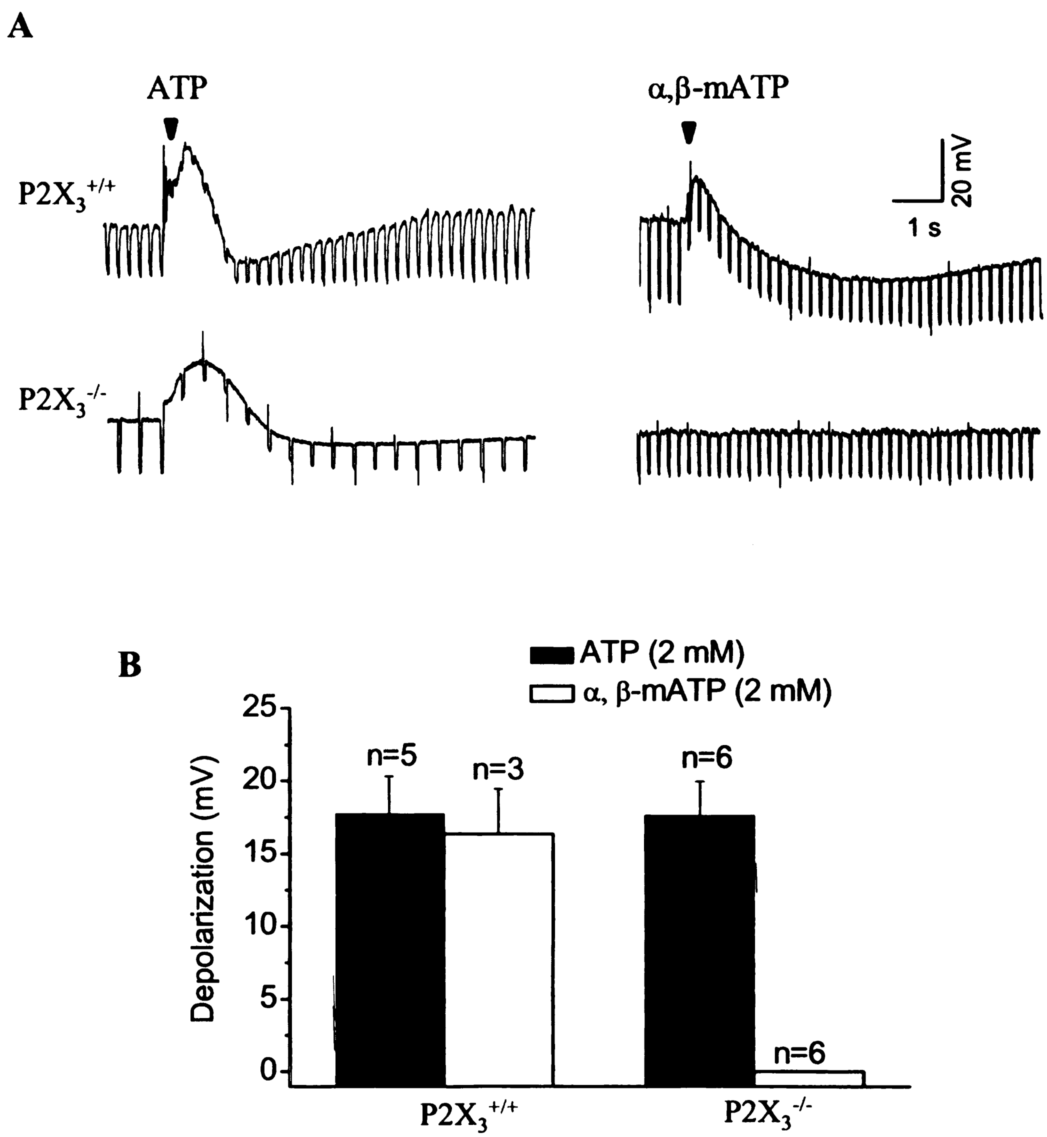
Intracellular electrophysiological recordings were obtained from 112 myenteric S type neurons from guinea pig ileum. 85% of the neurons (95 cells) were depolarized by local application of α, β-mATP, an ATP structure analog identified as an agonist selective

Figure 14. Responses of murine myenteric AH neurons in $P2X_3^{+/+}$ and $P2X_3^{-/-}$ **tissues to ATP and α, β-mATP**

A. Representative responses induced by ATP and α, β-mATP. ATP depolarized AH neurons from both types of mice. α, β-mATP evoked depolarization in AH neurons in tissues from $P2X_3^{+/+}$ but not $P2X_3^{-/-}$ mice.

B. Pooled data from experiments illustrated in A. The amplitude of ATP-induced depolarizations was similar in AH neurons from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice whilst α, β-mATP induced depolarization in AH neurons from $P2X_3^{+/+}$ but not $P2X_3^{-/-}$ mice.

Figure 14


A
ATP
α,β-mATP
$P2X_3^{+/+}$
$P2X_3^{-/-}$
20 mV
1 s
B
ATP (2 mM)
α, β-mATP (2 mM)
Depolarization (mV)
25
20
15
10
5
0
n=5
n=3
n=6
n=6
$P2X_3^{+/+}$
$P2X_3^{-/-}$

for $P2X_1$ and $P2X_3$ subunits containing P2X receptors. The rest 15% of the S neurons (17 cells) were insensitive to ATP or α, β-mATP. They were cholinergic neurons and fast synaptic excitation was mediated solely by ACh acting at nAChRs as mecamylamine (10 μM) completely blocked fEPSPs recorded from these neurons. The amplitudes of ATP and α, β-mATP-induced depolarization were 21.5 mV and 22.5 mV ($P>0.05$) (Figure 15), respectively, which were blocked by PPADS (10 μM).

A total of 28 AH neurons was studied; 17 neurons were tested for ATP sensitivity. 65% of these neurons were depolarized by ATP. All 28 AH type neurons were tested for α, β-mATP sensitivity and only 17% of them were depolarized by local application of α, β-mATP. The amplitudes of depolarization caused by ATP and α, β-mATP were 14.7 mV and 11 mV, respectively (Figure 15).

Effects of TNP-ATP on fEPSPs and ATP-induced depolarization in S neurons

TNP-ATP (10 μM), an ATP analog, is an antagonist selective for $P2X_1$, $P2X_3$ or $P2X_{2/3}$ receptors. It was found that TNP-ATP reversibly reduced depolarizations induced by ATP (Figure 16). Superfusion of TNP-ATP also reversibly reduced amplitude of fEPSPs recorded from S type neurons (Figure 17). These data also suggest that P2X receptors of S neurons contain $P2X_1$, $P2X_3$ or $P2X_{2/3}$ subunits.

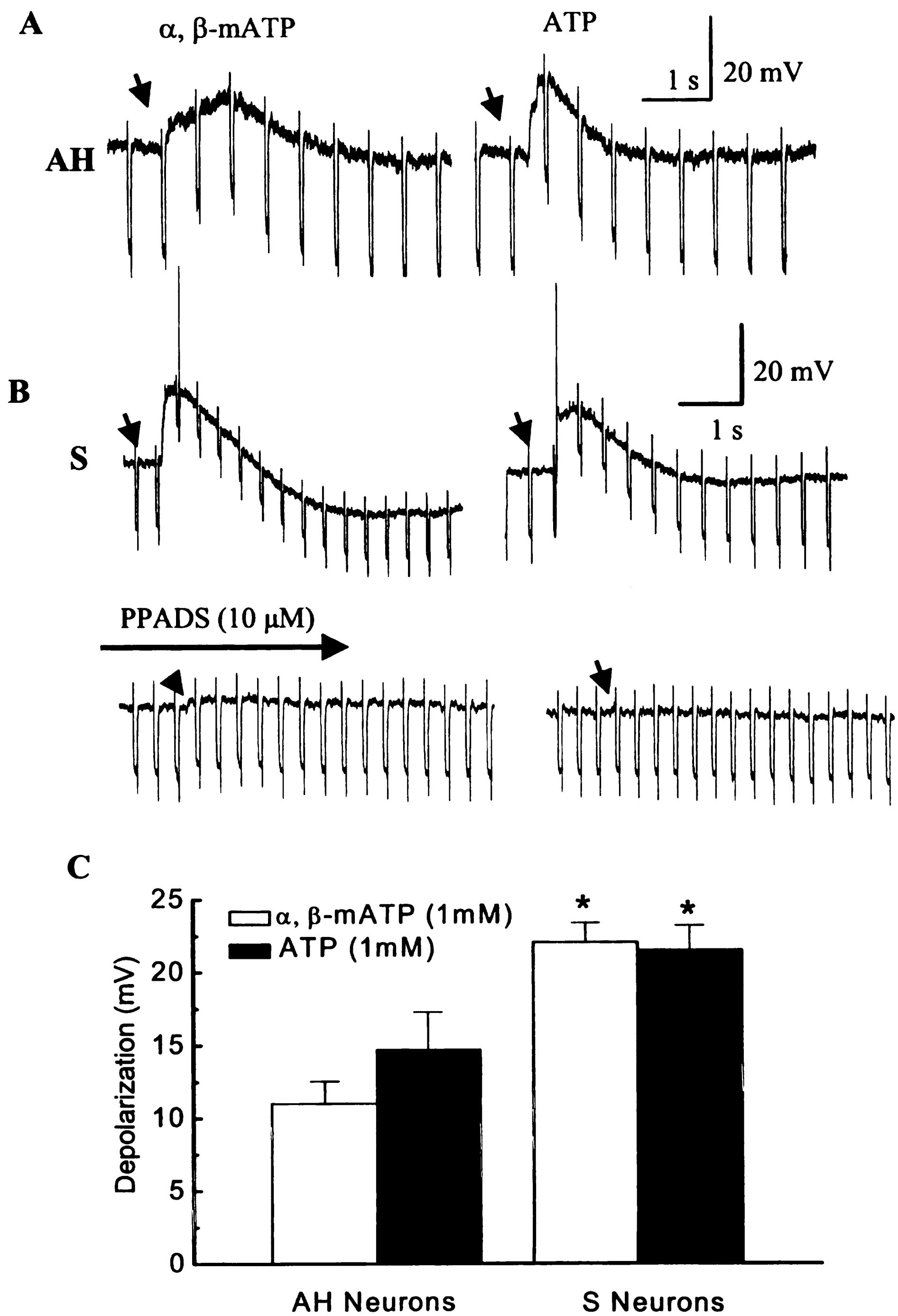
Figure 15. Depolarization induced by ATP (1mM) and α, β-mATP (1 mM) in S and AH myenteric neurons in guinea pig small intestine

A. Representative recordings of depolarizations induced by α,β-mATP and ATP in AH neurons. The arrowheads refer to pressure injection of α,β-mATP or ATP. Only 17% of the tested AH neurons have similar responses to these two agonists.

B. Representative recording traces show depolarization induced by ATP and α,β-mATP in S myenteric neurons. The depolarizations were blocked by PPADS (10 μM). 85% S neurons have similar responses to these two agonists.

C. Pooled data indicated the amplitudes of α, β-mATP and ATP-induced depolarization in S neurons are similar, which are bigger than these in AH neurons. Data are the mean ± SEM obtained from 5 AH neurons and 12 S neurons. "*" indicates a significant difference from responses in AH neurons.

Figure 15



A
α, β-mATP
ATP
1 s
20 mV
AH
B
S
20 mV
1 s
PPADS (10 μM)
C
α, β-mATP (1mM)
ATP (1mM)
Depolarization (mV)
25
20
15
10
5
0
*
*
AH Neurons
S Neurons

Figure 16. Effect of TNP-ATP on depolarizations induced by ATP in S neurons

A. Representative traces show that TNP-ATP reduced amplitude of ATP-induced depolarizations. The effect of TNP-ATP was reversible. Arrowheads indicate pressure ejection of ATP. B. Data are mean ± SEM from experiments (n=7) similar to that shown in A. "*" indicates a significant difference from control and wash groups.

Figure 16

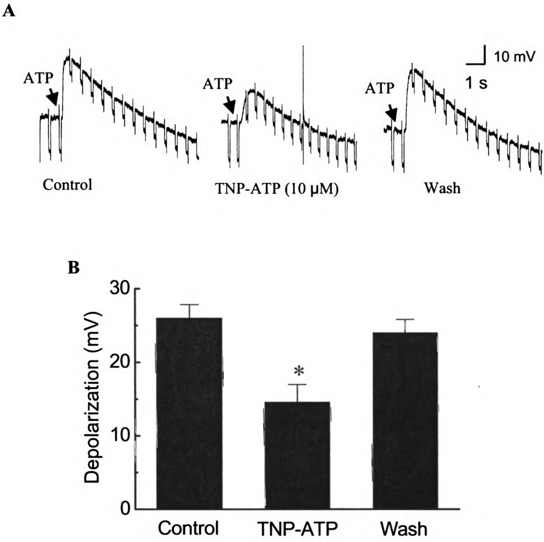
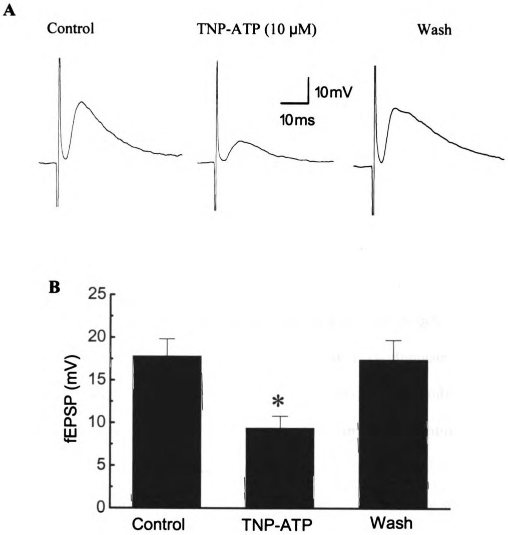

A
ATP
ATP
ATP
10 mV
1 s
Control
TNP-ATP (10 μM)
Wash
B
30
20
10
0
Depolarization (mV)
*
Control
TNP-ATP
Wash

Figure 17. Effect of TNP-ATP on fEPSPs in S neurons

A. Representative recording traces indicate TNP-ATP reduced the amplitude of fEPSPs. The effect of TNP-ATP on fEPSP amplitude was reversible. B. Data are mean ± SEM from experiments (n=4) similar to that shown in A. "*" indicates a significant difference from control and wash groups.

Figure 17


A
Control
TNP-ATP (10 μM)
Wash
10mV
10ms
B
fEPSP (mV)
25
20
15
10
5
0
*
Control
TNP-ATP
Wash

P2X RECEPTORS COUPLE TO CALCIUM-DEPENDENT POTASSIUM CONDUCTANCE IN GUINEA PIG MYENTERIC NEURONS

An afterhyperpolarization is induced by activation of P2X receptors

Activation of P2X receptors by α, β-mATP induced a depolarization in myenteric S neurons of guinea pig small intestine. This resulted from cations flowing through P2X receptors as input resistance (R_{input}) was reduced during depolarization. However, it was observed that, in 52 of 95 (55%) S type neurons, α, β-mATP-induced depolarizations were followed by a hyperpolarization (Figure 18). PPADS abolished both the depolarization and hyperpolarization. The amplitudes of depolarization and hyperpolarization were positively correlated (Figure 18). It was also observed that membrane conductance was increased during hyperpolarization, which suggested that opening of ion channels mediates the after-hyperpolarization. To eliminate the possibility that α, β-mATP-elicited hyperpolarization was due to change of membrane potential, single electrode voltage clamp (SEVC) recordings from similar preparations were made to confirm α, β-mATP-induced depolarization and hyperpolarization. In SEVC recording, inward and outward currents were recorded in S neurons in response to α, β-mATP, which underlie the depolarization and hyperpolarization, respectively (Figure 18). Similarly, the amplitudes of inward and outward currents were also positively correlated and they were blocked by PPADS.

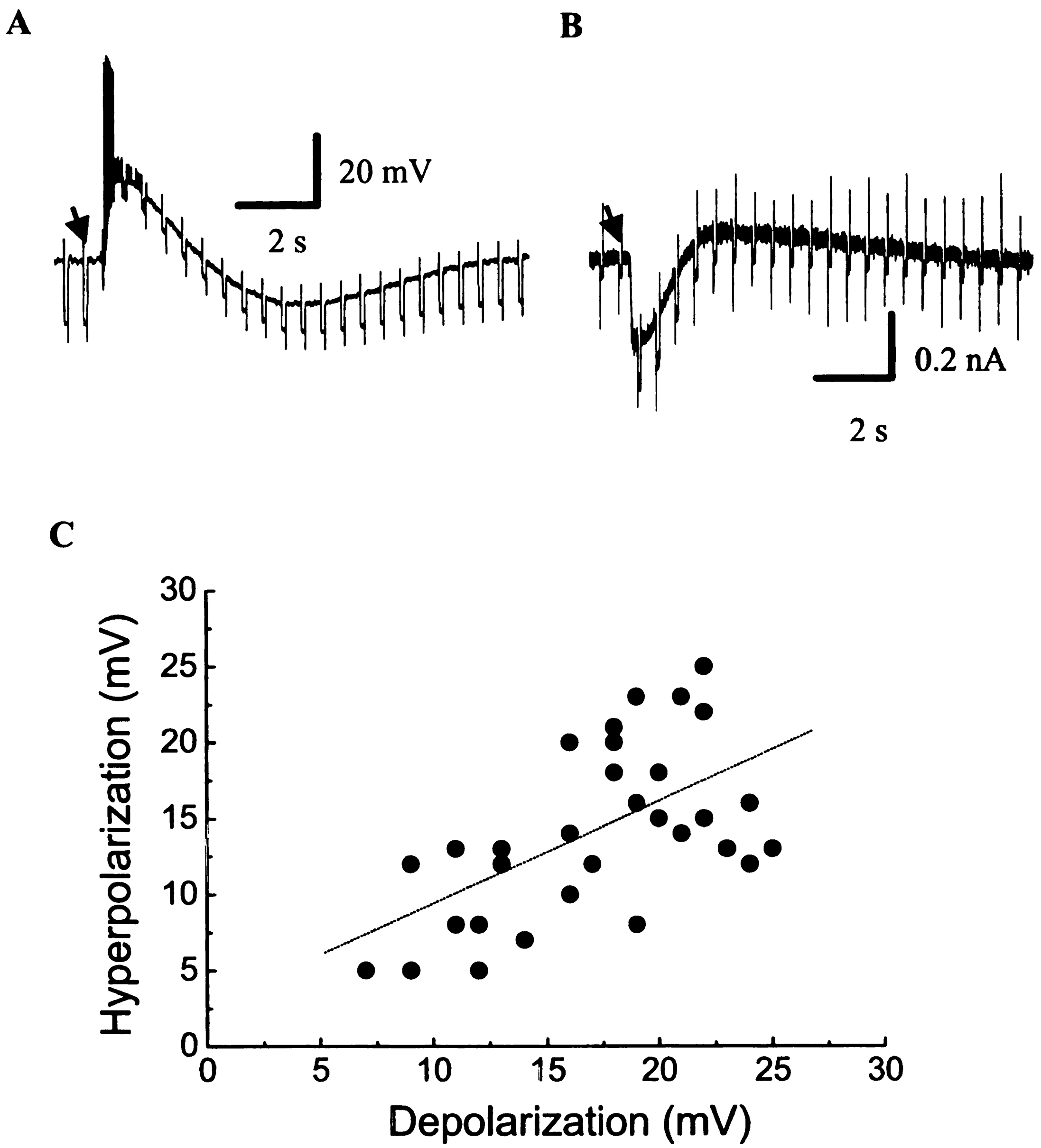
Figure 18. α, β-mATP-induced a biphasic response in S neurons.

A. Representative recording showing that α, β-mATP induced a depolarization followed by a hyperpolarization (resting membrane potential = –50 mV). Downward deflections in the trace are voltage responses caused by hyperpolarizing current pulses. A decrease in the amplitude of these responses during the depolarization and hyperpolarization indicates an increase in membrane conductance. Arrowheads indicate pressure ejection of α,β-mATP.

B. Current recording in SEVC mode shows a similar result as in A. α, β-mATP induced an inward current followed by an outward current at a holding potential of –50 mV. The downward deflections are currents caused by voltage steps. An increase in the amplitude means an increase in membrane conductance. Arrowheads indicate pressure ejection of α, β-mATP.

C. There is a positive correlation between the amplitude of the hyperpolarization and the depolarization.

Figure 18


A
20 mV
2 s
B
0.2 nA
2 s
C
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Hyperpolarization (mV)
Depolarization (mV)

The ionic basis of the afterhyperpolarization

One mechanism for increased conductance in mediating hyperpolarization could be an efflux of K^+ ions. It has been reported that intermediate Ca^{2+}-activited K^+ channels mediate the action potential afterhyperpolarization in AH neurons (Vogalis et al., 2002; Furness et al., 2004; Neylon et al., 2004). In myenteric S neurons, a depolarization induced by nicotine is followed by a hyperpolarization, which is also due to activation of calcium-activated potassium channels (Tokimasa et al., 1983). α, β-mATP-induced hyperpolarizations in S neurons might also be mediated by Ca^{2+}-activated K^+ channels.

Calcium dependency of the α, β-mATP after-hyperpolarization was examined by changing extracellular calcium concentration from normal level of 2.5 mM to 0.25 mM. Lowering extracellular $[Ca^{2+}]$ at 0.25 mM almost abolished fEPSP and reduced α, β-mATP-induced depolarization from 20.6 mV to 17.8 mV (Figure 19). As a consequence, it reversibly and dramatically reduced the hyperpolarization from 14.6 mV to 1.8 mV (Figure 19).

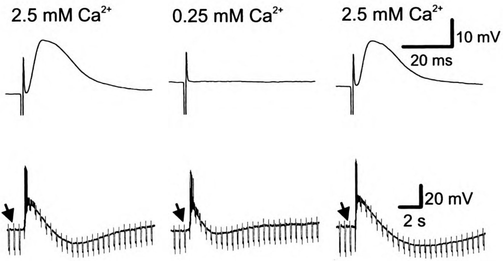
The subsequent experiment was conducted to examine whether the α,β-mATP afterhyperpolarization or outward current was carried by K^+. SEVC recordings were made from LMMP preparations. The inward and outward currents were induced by α, β-mATP. The relationships between holding potential and inward and outward currents were linear (Figure 20). The reversal potentials of inward and outward currents were 3.3 mV and –79 mV, respectively. These data indicate that the inward current underlying depolarization was carried by non-selective cations through P2X receptors as the reversal

Figure 19. α, β-mATP-induced afterhyperpolarization is Ca^{2+} sensitive

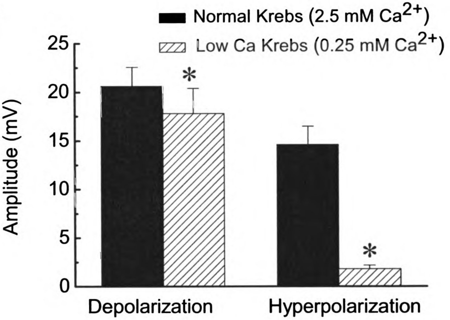
A. Low extracellular Ca^{2+} (0.25 mM) abolished fEPSPs. Restoring extracellular Ca^{2+} to 2.5 mM restored fEPSP amplitude. Ca^{2+} at 0.25 mM reduced the depolarization induced by α, β-mATP. The after-hyperpolarization was also reduced. Restoring Ca^{2+} to 2.5 mM restored the depolarization and hyperpolarization. B. Pooled data (mean ± SEM) obtained from similar experiments (n=7) to that shown in A. "*" indicates a significant difference from 2.5 mM Ca^{2+} conditions.

Figure 19

A


2.5 mM Ca^{2+}
0.25 mM Ca^{2+}
2.5 mM Ca^{2+}
10 mV
20 ms
20 mV
2 s

B


Normal Krebs (2.5 mM Ca^{2+})
Low Ca Krebs (0.25 mM Ca^{2+})
Amplitude (mV)
25
20
15
10
5
0
*
*
Depolarization
Hyperpolarization

Fig

hol

vol

vo

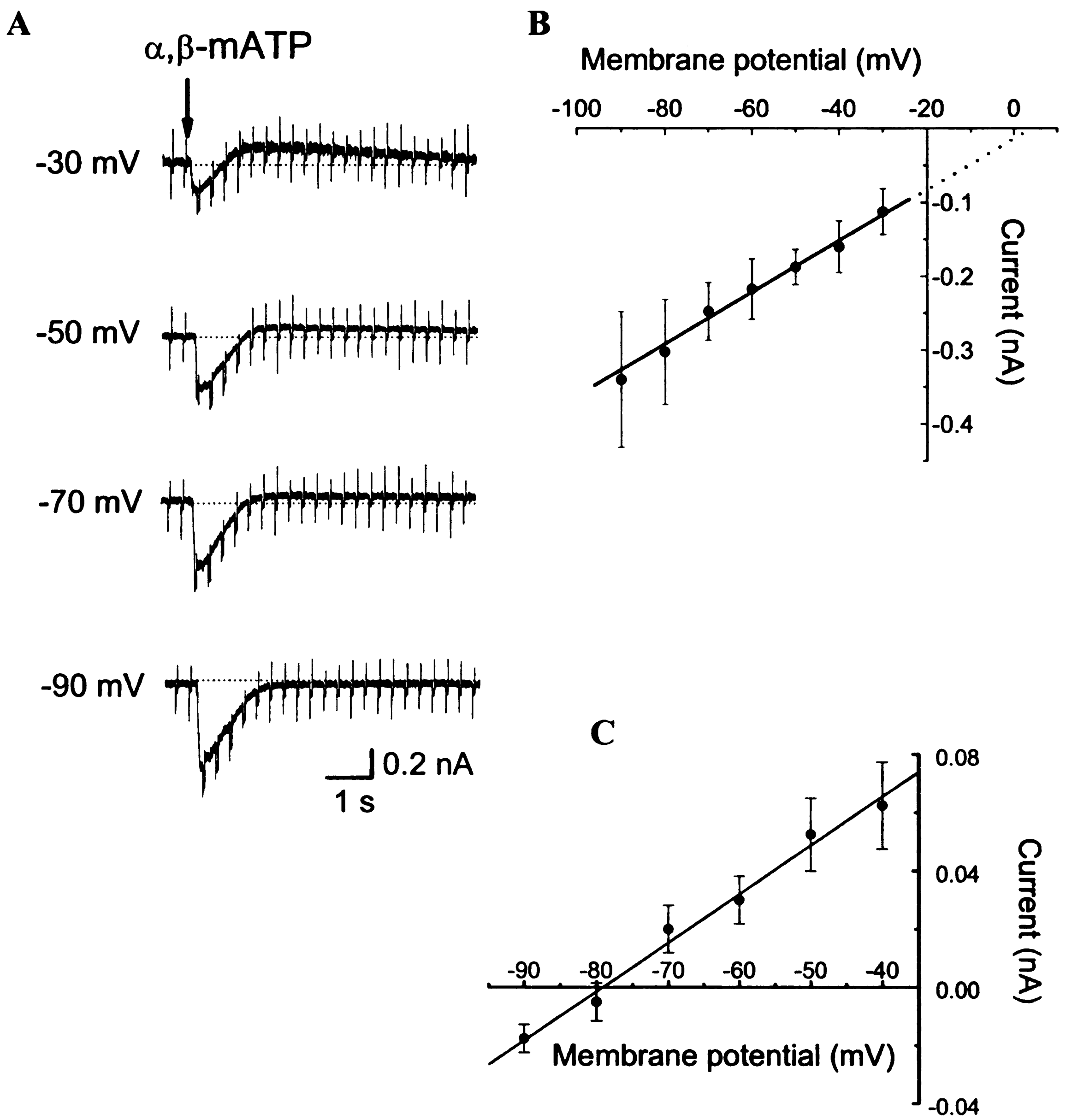
Th

ob

Figure 20. α, β-mATP-induced afterhyperpolarization is K^+ dependent.

A. Representative recordings show inward and outward currents at different holding potentials. B. Current voltage relationship of the inward current. C. Current voltage relationship for outward current induced by α, β-mATP in S-neurons. Current voltage relationships were linear. The reversal potential for the inward current was 3 mV. The reversal potential for the outward current was -79 mV. Data are mean ± SEM obtained from 7 neurons.

Figure 20


A
α,β-mATP
-30 mV
-50 mV
-70 mV
-90 mV
0.2 nA
1 s
B
Membrane potential (mV)
-100
-80
-60
-40
-20
0
-0.1
-0.2
-0.3
-0.4
Current (nA)
C
0.08
0.04
0.00
-0.04
Current (nA)
-90
-80
-70
-60
-50
-40
Membrane potential (mV)

potential is close to 0 mV. The outward current mediating the afterhyperpolarization was carried by K^+ because the reversal potential is approximately same as K^+ equilibrium potential of –80 mV.

I then began to identify the Ca^{2+}-activated K^+ channel (K_{Ca}) subtypes that mediated the afterhyperpolarization or outward currents in myenteric S neurons. Three toxins for major K_{Ca} subtypes were used (Vergara et al., 1998). Iberiotoxin (IBTx) at concentration of 0.1 μM selectively blocks K_{Ca} with large conductance (BK). Apamin at 0.1 μM selectively blocks K_{Ca} with small conductance (SK). Clotrimazole at 10 μM selectively blocks K_{Ca} with intermediate conductance (IK). Application of these antagonists had no effects on AHP induced by α, β-mATP in S neurons (Figure 21).

Immunochemical characterization of S neurons with after-hyperpolarization

$P2X_3$ receptors are expressed primarily in the inhibitory motor neurons ((LePard et al., 1997; Poole et al. 2002; Van Nassauw et al. 2002). As shown above, not in all S type neurons but in an approximately half of them, $P2X_3$ receptors coupled to a K_{Ca} that mediates the after-hyperpolarization. One hypothesis could be this subset S neurons may utilize the $P2X_3$ receptors to accomplish their unique function of those neurons. Here I tested the idea that these S neurons were descending inhibitory motor neurons. The descending inhibitory motor neurons can be labeled by an antibody against NOS. The results indicated that the S neurons with α, β-mATP-induced hyperpolarization are either NOS-ir positive or NOS-ir negative. There was no significant association between this subset of S type neurons and descending inhibitory motor neurons (Figure 22).

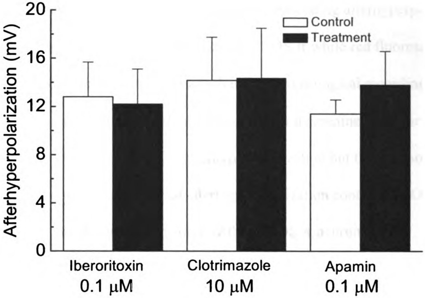
Figure 21. Lack of effect of K_{Ca} channel blockers on the α,β-mATP-induced afterhyperpolarization.

A. IBTx, apamin and clotrimazole, selective blockers of big, small and intermediate conductance calcium-activated potassium channels, respectively, had no effect on the afterhyperpolarization induced by α, β-mATP. Data are mean ± SEM obtained from 6, 4 and 7 neurons in IBTx, apamin and clotrimazole, respectively.

B. Clotrimazole blocked action potential afterhyperpolarization in AH neurons in the same preparations from which S neurons were studied. These data confirmed the effectiveness of application of clotrimazole.

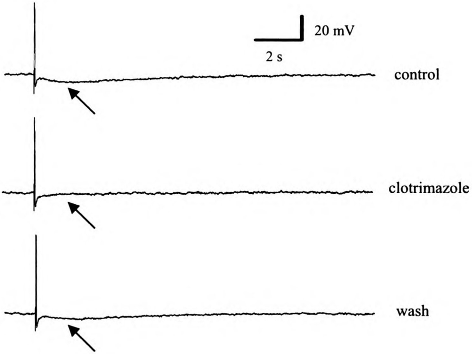
Figure 21

A



Control
Treatment
Afterhyperpolarization (mV)
20
16
12
8
4
0
Iberoritoxin
0.1 μM
Clotrimazole
10 μM
Apamin
0.1 μM

B

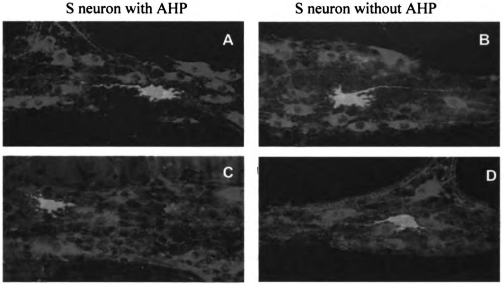


20 mV
2 s
control
clotrimazole
wash

Figure 22. Identification of the neurochemical phenotype of S neurons that generate the α, β-mATP induced afterhyperpolarization

There was no association between S neurons generating afterhyperpolarization and NOS-ir neurons. Green fluorescence indicates NOS-ir while red fluorescence reveals neurobiotin used to mark neurons from which electrophysiological recordings were obtained. A neuron generating the afterhyperpolarization contained NOS-ir (A) while C shows an example of a neuron with an afterhyperpolarization but that did not contain NOS-ir. A neuron that did not show an afterhyperpolarization contained NOS-ir neuron (B) while D shows an example of a neuron without NOS-ir neuron (D).

Figure 22



FAST EXCITATORY SYNAPTIC TRANSMISSION DURING BURSTS OF NEURONAL ACTIVITY

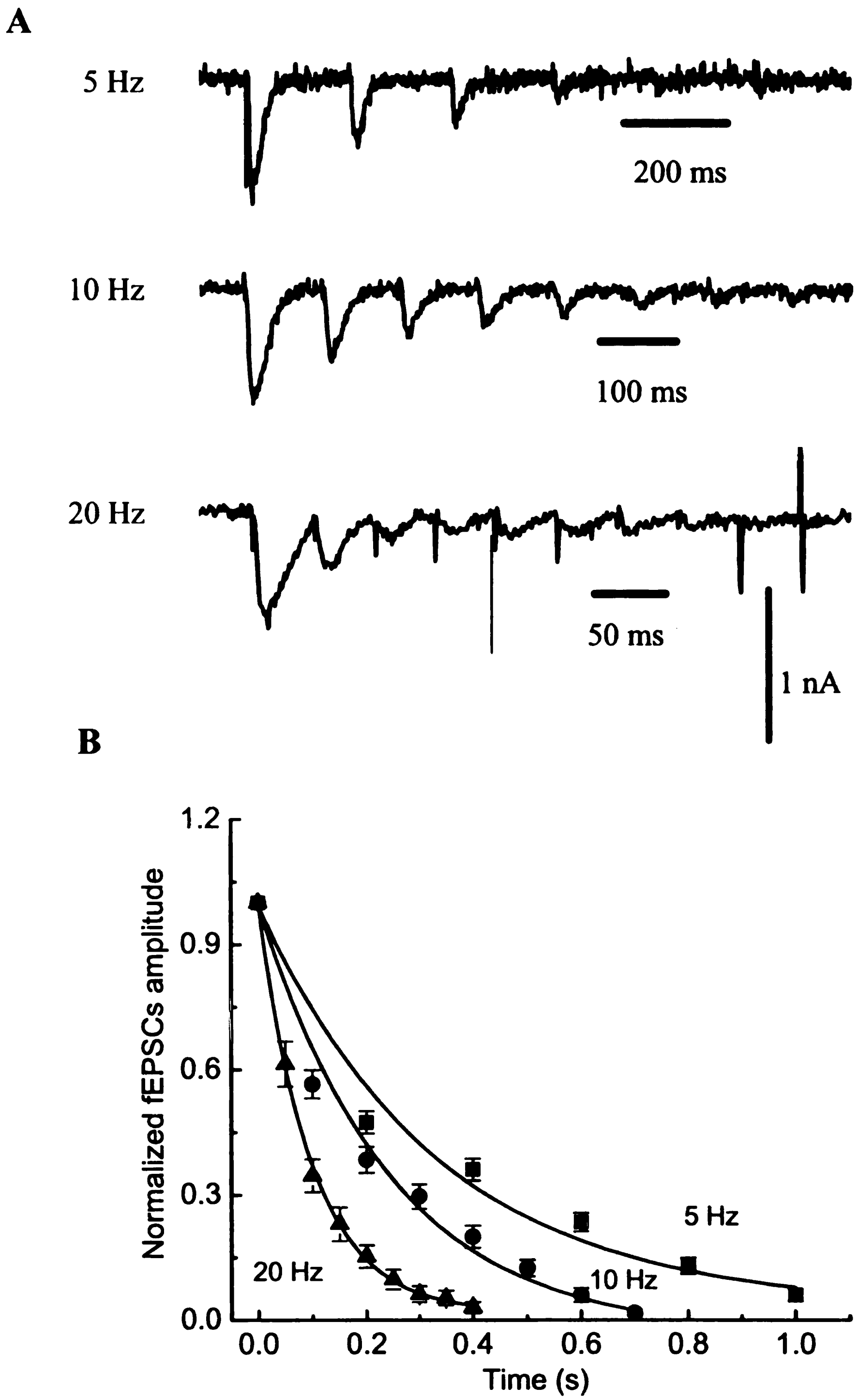
Fast synaptic transmission declined in amplitude during trains of electrical stimulation

First fEPSPs were recorded when a train of electrical stimulation was applied on presynaptic nerve fibers at different frequencies. Fast EPSPs showed a decline in their amplitudes during test stimulus train at 5, 10 and 20 Hz. However, trains of stimulation could also elicit slow excitatory post-synaptic potentials (sEPSPs) in S and AH neurons in the ENS (Grafe et al., 1980; Morita and North, 1985; Wood and Kirchgessner, 2004). The depolarization occurring during sEPSPs would reduce the electrochemical driving force for Na^+ and Ca^{2+} influx through nAChRs and P2X receptors thereby reducing fEPSP amplitude during a stimulus train. Therefore, I used single electrode voltage clamp (SEVC) method to hold the membrane potential at -60 mV in order to record fEPSC amplitude at a constant membrane potential. Holding the membrane potential constant would eliminate changes in driving force as a cause for alterations in the amplitude of fast synaptic responses during trains of stimulation. The SEVC experiments showed the similar results. At 0.5 Hz, the fEPSC declined in amplitude by 50% after the first stimulus but was maintained throughout the remainder of the stimulus train (Figure 23). However, at stimulus frequencies of 5, 10 and 20 Hz, the amplitude of fEPSCs declined to zero and the rate of rundown was frequency-dependent and was fitted by a single exponential decay (Figure 23). The time constants (τ) for rundown of the fEPSCs at 5, 10

Figure 23. Trains of electrical nerve stimulation cause synaptic rundown.

A. Representative recordings of fEPSCs during trains of stimulation at 5, 10 and 20 Hz. B. Pooled data show that the rate of rundown in fEPSC amplitude was frequency-dependent and was fitted by a monoexponential decay (solid lines). Data are the mean ± SEM. obtained from 21 neurons at 5 Hz, 32 neurons at 10 Hz and 19 neurons at 20 Hz.

Figure 23


A
5 Hz
200 ms
10 Hz
100 ms
20 Hz
50 ms
1 nA
B
Normalized fEPSCs amplitude
1.2
0.9
0.6
0.3
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Time (s)
5 Hz
10 Hz
20 Hz

and 20 Hz were 0.35 ± 0.05, 0.2 ± 0.02 and 0.1 ± 0.02 s, respectively. These data indicate that fast excitatory synaptic responses rundown in amplitude during trains of stimulation and that synaptic rundown is not caused by changes in driving force that might occur during sEPSP.

fEPSCs exhibit paired pulse depression

Paired pulse facilitation (PPF) is a property of many synapses in the peripheral and central nervous systems. I tested for the presence of PPF at synapses in the myenteric plexus by measuring the ratio of the amplitude of fEPSCs elicited by pairs of stimuli applied at intervals of 200, 100 and 50 ms (5, 10 and 20 Hz frequencies, respectively) (Figure 24). The second fEPSC in the pair was always smaller than the first indicating that synapses in myenteric plexus exhibit paired pulse depression (PPD) rather than PPF (Figure 24). The second fEPSC evoked at 10 and 20 Hz exhibited less depression than that evoked at 5 Hz (Figure 24).

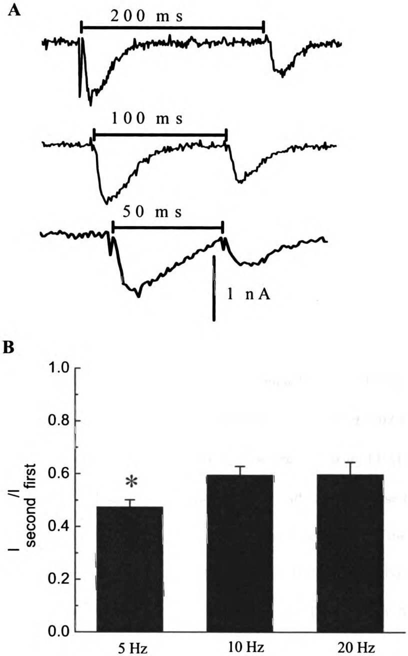
Recovery from synaptic rundown

Synaptic recovery is another important indicator of synaptic properties. It reflects synaptic vesicle trafficking after depletion. I next investigated the time course of recovery from synaptic rundown following a 10 Hz train of stimulation. In these experiments, a train of 20 stimuli at 10 Hz caused fEPSPs to decline in amplitude to 0 mV. Recovery from rundown was tested by waiting for periods of 1 to 12 s before a single stimulation was used to elicit a fEPSP (Vtest); this amplitude was expressed as a

Figure 24. Paired stimuli cause synaptic depression

A. The second fEPSC in a pair evoked by stimuli at 5 (200 ms interval), 10 (100 ms interval) and 20 (50 ms interval) Hz was smaller in amplitude than the first fEPSC. B. Mean data from experiments similar to that shown in A. Data are the ratio of the second fEPSC vs. the first fEPSC (Isecond/Ifirst) in a stimulus pair. * Indicates that synaptic depression at 10 (n=32) and 20 (n=19) Hz is less than that occurring at 5 Hz (n=21). Data are mean ± SEM.

Figure 24


A
200 ms
100 ms
50 ms
1 nA
B
1.0
0.8
0.6
0.4
0.2
0.0
I second /I first
*
5 Hz
10 Hz
20 Hz

fraction of the amplitude of the first EPSP in the preceding stimulus train (V_{first}) (Figure 25). Fully recovery from synaptic rundown occurred in less than 12 s and the recovery time course was fitted by a monoexponential function; the recovery time constant was 7 ± 2 s (Figure 25).

The contribution of nAChRs and P2X receptors to rundown of fEPSPs

As ACh, acting at nAChRs, and ATP, acting at P2X receptors, contribute to most fEPSPs recorded from myenteric neurons in the guinea-pig ileum, I next examined if there was selective rundown of one of these fEPSP components during a 10-Hz train of stimulation. The P2X-mediated component of the fEPSP was isolated by using mecamylamine (10 μM), to block nAChRs. Under these conditions, it was found that the remaining fEPSP declined in amplitude with a time course (τ =0.3 ± 0.07 s, n=6) that was identical to that occurring in the absence of mecamylamine (τ =0.3 ± 0.07 s, n=6) (Figure 26). The cholinergic component of the fEPSP was isolated by using PPADS (10 μM) to block P2X receptors. The cholinergic fEPSP ran down with a time course (τ =0.4 ± 0.08 s, n=6) similar to that measured under control conditions and in the presence of mecamylamine (Figure 26). More than 90% of the fEPSP was abolished by co-application of mecamylamine and PPADS (Figure 26) indicating that the rundown was a mixed cholinergic–purinergic fEPSP.

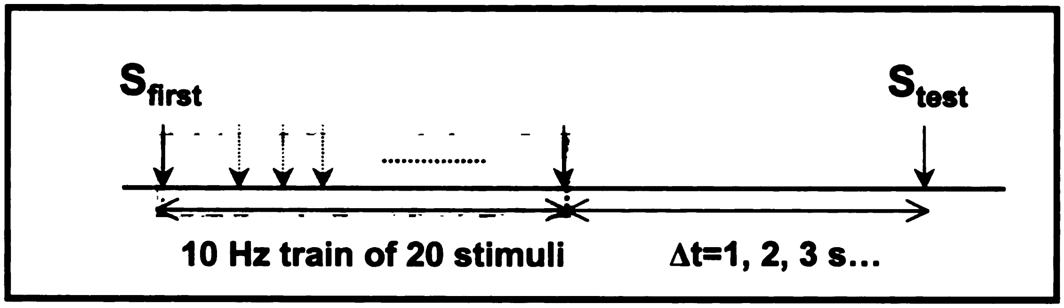
ACh and ATP ionophoresis were utilized to investigate if fEPSP rundown was due to postsynaptic nAChR and/or P2X receptor desensitization. ACh and ATP

Figure 25. Time-dependent recovery of fEPSPs from synaptic rundown

A. Diagram of the protocol used to measure the time course of recovery from synaptic rundown. A conditioning train of 20 stimuli at 10 Hz was used to cause fEPSP rundown. A single test stimulus applied at various intervals after the conditioning train was used to elicit a fEPSP to determine the extent of recovery. Δt is the time interval between the last stimulus in the conditioning train and the test stimulus. Recovery is expressed as the ratio of the amplitude of the fEPSP caused by the test stimulus (V_{test}) vs. the amplitude of the fEPSP caused by the first stimulus (V_{first}) in the conditioning train. B. The time course of recovery was fit by a single exponential function with a $\tau = 7 \pm 2$ s. Data points are the mean ± SEM. of observations made from 14 neurons.

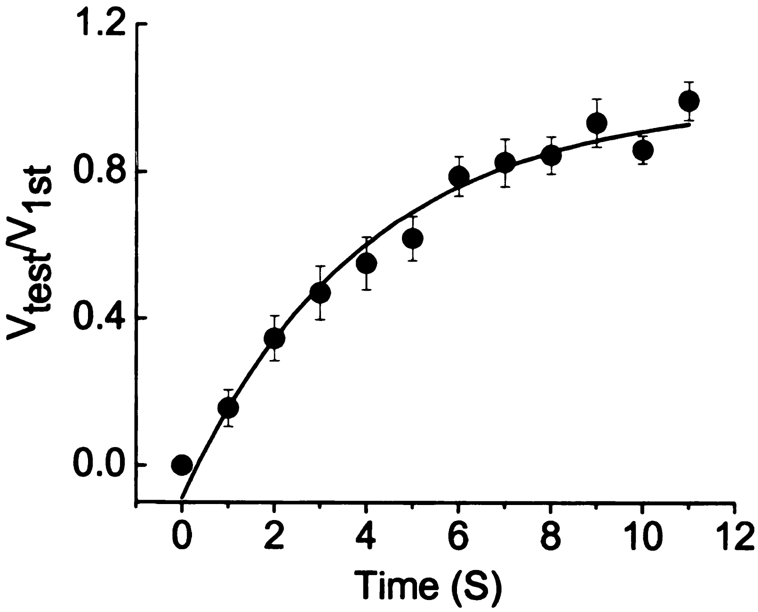
Figure 25

A



S_{first}
S_{test}
10 Hz train of 20 stimuli
Δt=1, 2, 3 s...

B

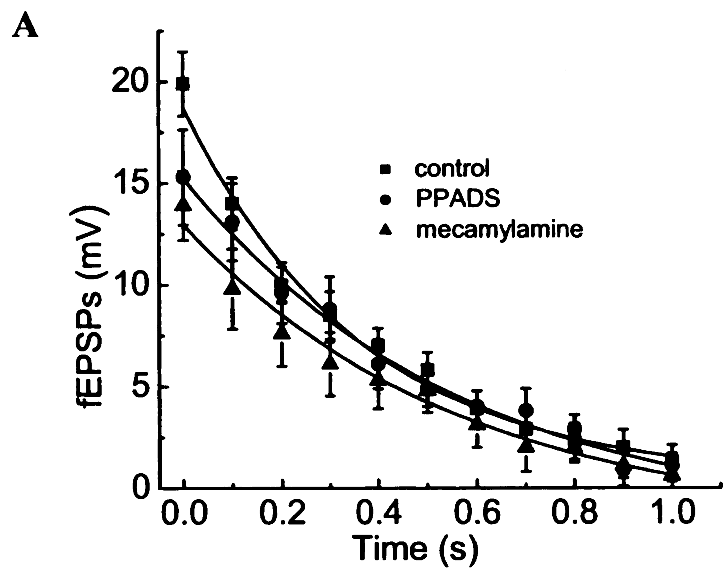


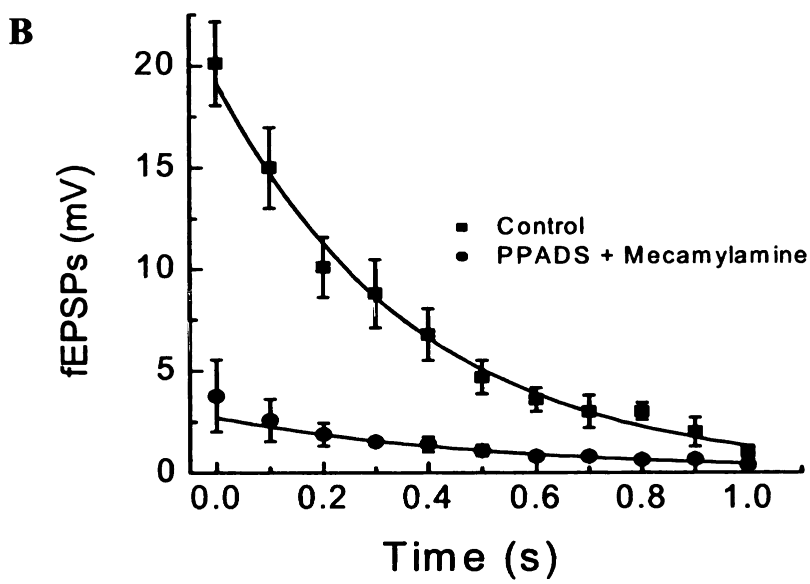
1.2
0.8
0.4
0.0
V_{test}/V_{1st}
0
2
4
6
8
10
12
Time (S)

Figure 26. Mecamylamine or PPADS does not alter fEPSP rundown

A. The amplitude of fEPSPs was reduced by mecamylamine or PPADS (each at 10 μM) but the time course of rundown was not changed by the antagonists. The rate of synaptic rundown was fitted by a monoexponential function with s values of 0.3 ± 0.07, 0.3 ± 0.07 and 0.4 ± 0.08 s under control conditions and in the presence of mecamylamine and PPADS, respectively. Each point is the mean ± SEM. (n=6). B. Co-application of mecamylamine or PPADS inhibited the fEPSP by more than 90%. Data are mean ± SEM (n=6).

Figure 26


A
20
15
10
5
0
fEPSPs (mV)
control
PPADS
mecamylamine
0.0
0.2
0.4
0.6
0.8
1.0
Time (s)


B
20
15
10
5
0
fEPSPs (mV)
Control
PPADS + Mecamylamine
0.0
0.2
0.4
0.6
0.8
1.0
Time (s)

ionophoresis caused depolarizations that were similar in amplitude and time course to fEPSPs recorded from the same neurons, which suggests the same group of receptors were activated by ACh/ATP and by synaptic stimulation (Figure 27). Then ACh and ATP were applied as trains (10 Hz) of ionophoretic pulses and fEPSPs were evoked in the same neurons using a 10 Hz stimulus train. As same as described above, fEPSPs declined in amplitude during the stimulus train. However, in the same neurons, neither ACh nor ATP responses declined in amplitude during a train of agonist application (Figure 27). These data indicate that synaptic rundown is not due to postsynaptic receptor desensitization.

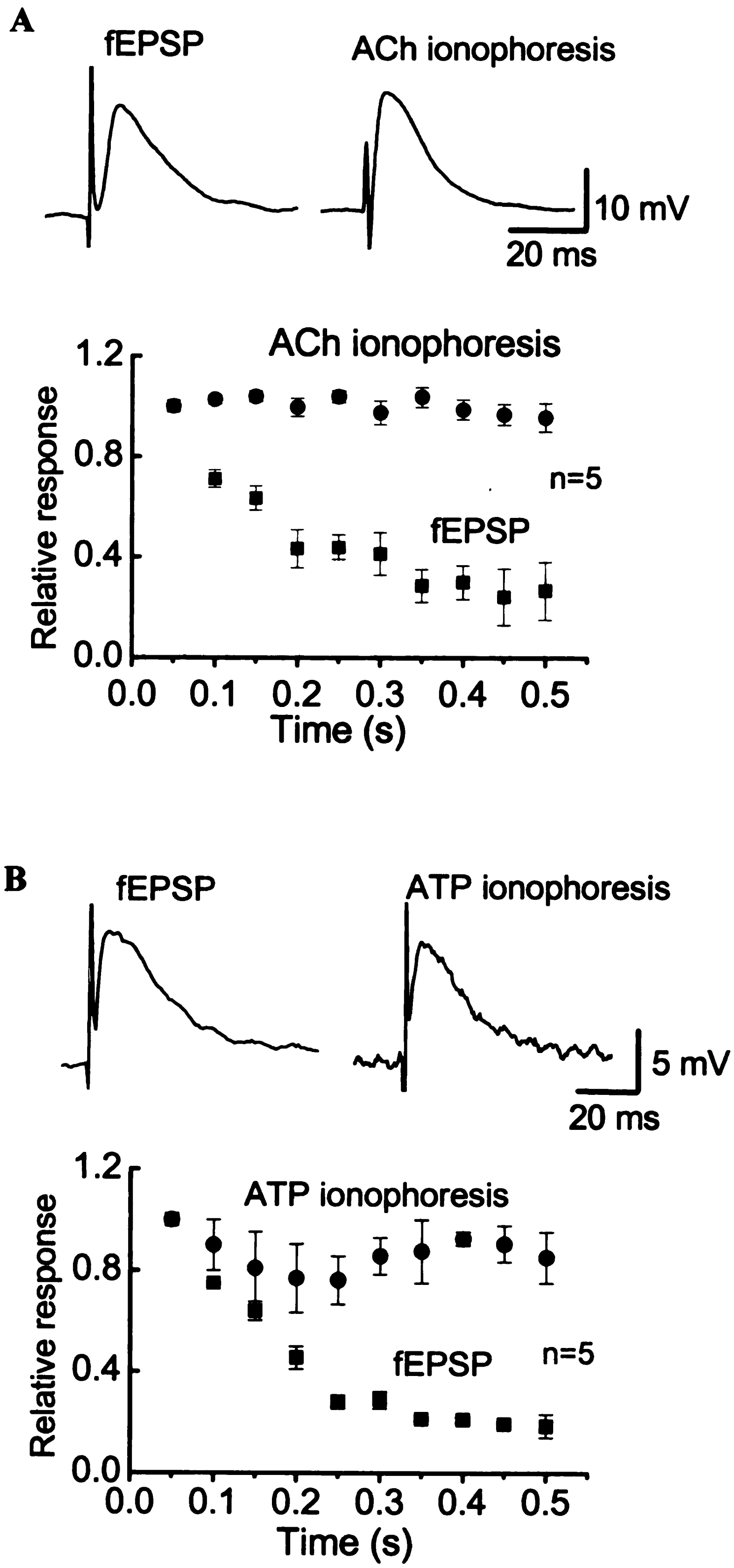
Presynaptic mechanisms

Presynaptic inhibitory receptors. Nerve terminals in the myenteric plexus express a number of receptors that mediate presynaptic inhibition of neurotransmitter release. These receptors include muscarinic M_2 muscarinic receptors, 5-HT_{1A} receptors, opioid receptors, $\alpha 2$ adrenergic receptors and adenosine A1 receptors. It is possible that during trains of stimulation, myenteric nerves release the endogenous agonists for one or more of these receptors and that presynaptic inhibition of ACh and/or ATP is responsible for fEPSP rundown. To test this hypothesis, fEPSP rundown was measured before and after application of antagonists to each of the receptors listed above. The exception being an antagonist for the M_2 muscarinic receptor as scopolamine (1 μM), which blocks all muscarinic receptor subtypes, was present in the Krebs' solution in all studies (see Methods and Materials). I used naloxone (10 μM) to block opioid receptors, NAN-190

Figure 27. Postsynaptic desensitization does not contribute to synaptic rundown

A. ACh applied by ionophoresis caused a depolarization that mimicked the amplitude and time course of the fEPSP. During a 20 Hz stimulation train, the fEPSP ran down while responses caused by a 20 Hz train of ionophoretic pulses of ACh did not. Data are mean ± SEM (n=4). B. ATP applied by ionophoresis caused a depolarization that mimicked the fEPSP. Responses caused by a 20 Hz train of ionophoretic pulses of ATP did not run down while fEPSPs caused by a 20 Hz stimulus train did. Data are Mean ± SEM (n=4).

Figure 27


A
fEPSP
ACh ionophoresis
10 mV
20 ms
1.2
0.8
0.4
0.0
Relative response
ACh ionophoresis
n=5
fEPSP
0.0 0.1 0.2 0.3 0.4 0.5
Time (s)
B
fEPSP
ATP ionophoresis
5 mV
20 ms
1.2
0.8
0.4
0.0
Relative response
ATP ionophoresis
fEPSP
n=5
0.0 0.1 0.2 0.3 0.4 0.5
Time (s)

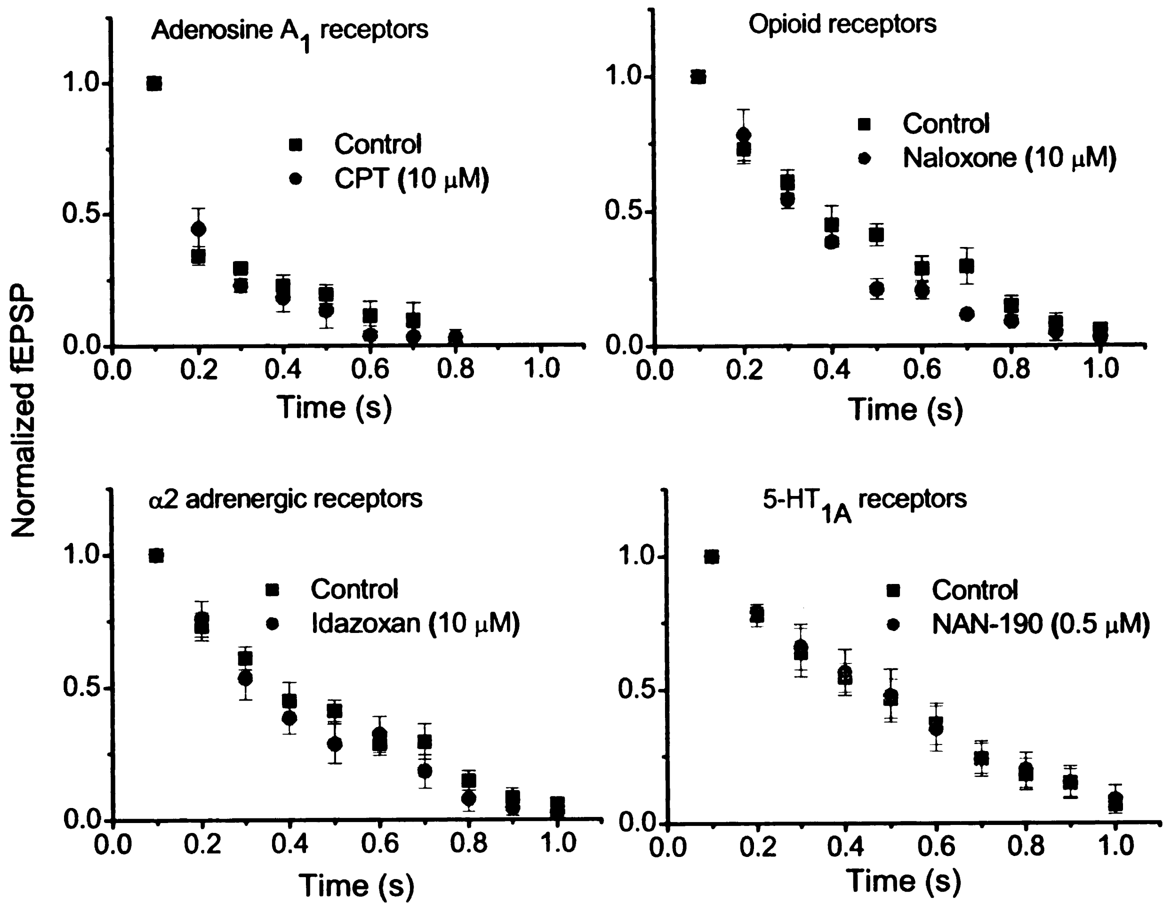
(0.5 mM) to block $5\text{-}HT_{1A}$ receptors, idazoxan (10 μM) to block α2 adrenergic receptors, and 8-cyclopentyl-theophylline (CPT, 10 μM) to block A1 adenosine receptors. None of the antagonists tested altered fEPSP rundown during a 10-Hz stimulus train (Figure 28). It is possible that antagonists tested above have not blocked the receptor(s) responsible for presynaptic inhibition leading to synaptic rundown. In order to comprehensively eliminate presynaptic inhibition as a mechanism of synaptic rundown, I attempted to block the intracellular signaling pathway coupled to presynaptic inhibitory receptors. Most inhibitory presynaptic receptors are G-protein coupled receptors, which link to the pertussis toxin (PTX) sensitive Gi/Go subsets of G-proteins (Zamponi, 2001). So PTX was used to block the signaling pathway to confirm the effects of these receptors on synaptic rundown. Blockade of the inhibitory postsynaptic potential (IPSP) from submucosal S neurons can be an indicator of the effectiveness of the PTX-treatment protocol. Previous work has shown that the IPSP is mediated by norepinephrine acting α2-adrenergic receptors which couple via a PTX-sensitive G-protein to activation of a potassium channel (Surprenant and North, 1988). I showed that IPSPs were blocked in PTX pretreated submucosal preparation while fEPSPs recorded from the same cells were unchanged (Figure 29). The same protocol was used to treat myenteric plexus preparations with PTX. In these tissues, it was found that fEPSPs still exhibited rundown that was similar to that observed under control conditions (Figure 29).

Action potentials. Another possible reason that results in synaptic rundown is that action potentials are not able to follow stimulus train. In order to test the hypothesis that

Figure 28. Antagonists of presynaptic inhibitory receptors do not alter synaptic rundown

Each figure shows the mean ± SEM. of successive fEPSPs normalized to the first fEPSP in the train before and after drug treatment. There were no differences between the control and drug treatment values. Data are mean ± SEM (n = 4–5).

Figure 28

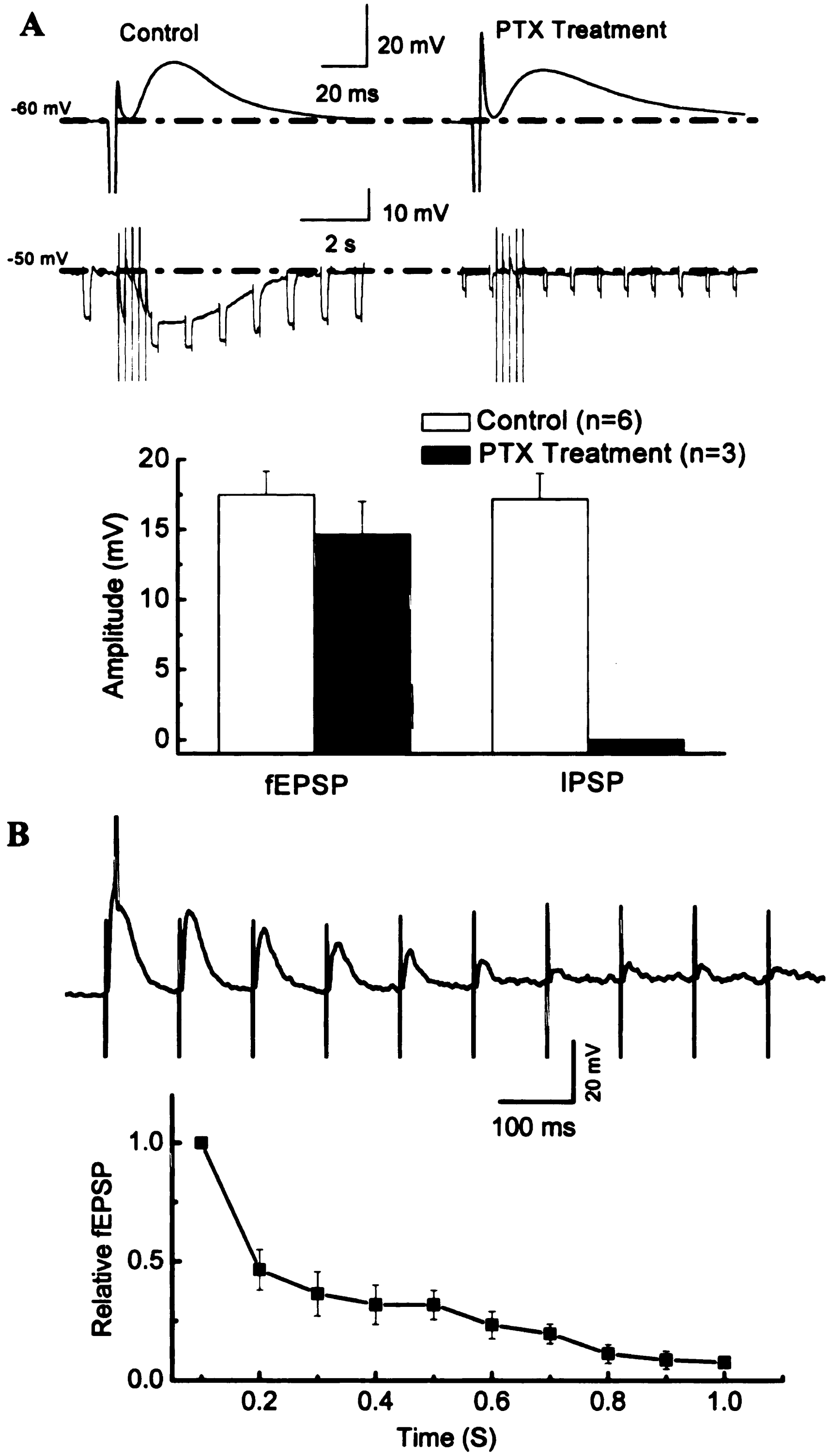


Adenosine A_1 receptors
Control
CPT (10 μM)
Opioid receptors
Control
Naloxone (10 μM)
α2 adrenergic receptors
Control
Idazoxan (10 μM)
5-HT_{1A} receptors
Control
NAN-190 (0.5 μM)
Normalized fEPSP
Time (s)

Figure 29. Pertussis toxin (PTX) does not affect fEPSP rundown

A. PTX pretreatment abolished the IPSP but not the fEPSP in submucosal neurons. Downward deflections in the IPSP traces are voltage responses caused by hyperpolarizing current pulses. A decrease in the amplitude of these responses during the IPSP indicates a decrease in membrane resistance. B. Successive fEPSPs recorded from myenteric neurons with and without PTX pretreatment. There were no differences between values obtained in treated and untreated tissues at any time point. Data are mean ± SEM (n=8).

Figure 29



A
Control
20 mV
20 ms
PTX Treatment
-60 mV
10 mV
2 s
-50 mV
Control (n=6)
PTX Treatment (n=3)
Amplitude (mV)
20
15
10
5
0
fEPSP
IPSP
B
20 mV
100 ms
Relative fEPSP
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1.0
Time (S)

axonal action potential failure is not a mechanism of fEPSP rundown, we made recordings from neurons in which the focal stimulating electrode was positioned on the axon of the neuron from which the fEPSP was recorded. This allowed us to make simultaneous recordings of axonally propagated action potentials and fEPSPs during a 10 Hz stimulus train. These experiments were done in 10 neurons and it was found that antidromic action potentials were maintained throughout the stimulus train while fEPSPs declined in amplitude during the same stimulus train (Figure 30).

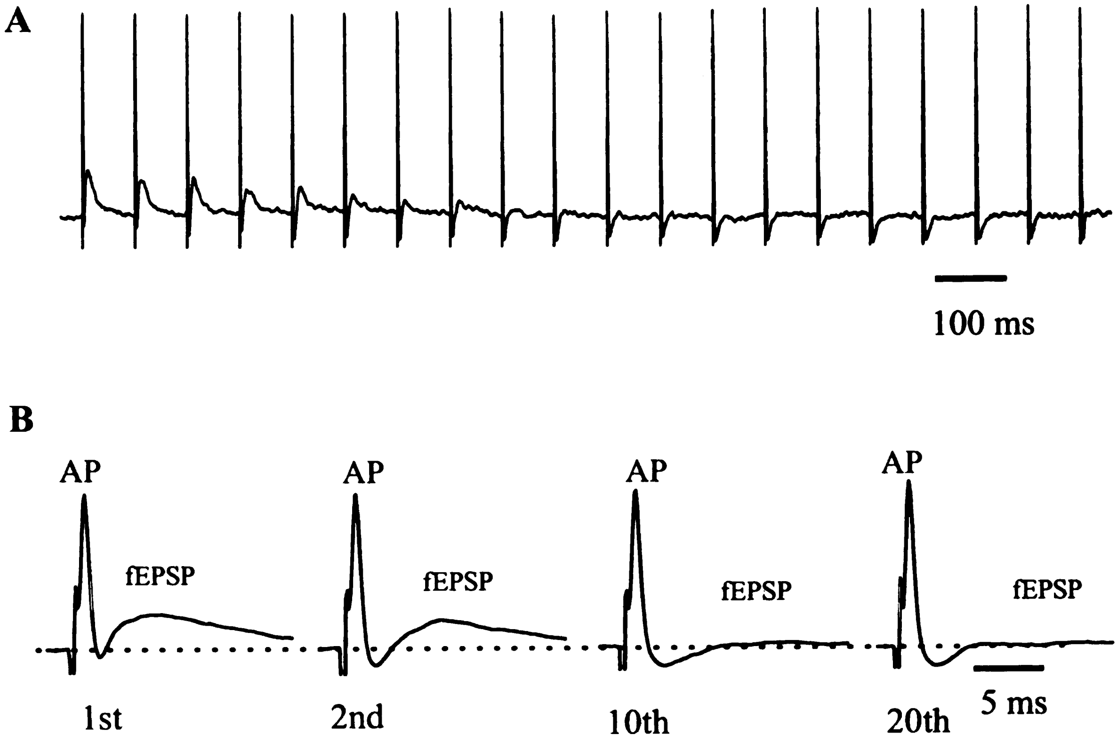
BK channels. The activity of large conductance calcium-activated potassium (BK) channels in nerve terminals can modulate neurotransmitter release (Robitaille and Charlton, 1992). Calcium entering the nerve terminal during the action potential opens BK channels. The opening of BK channels shortens action potential duration and hyperpolarizes the nerve terminal; both effects can reduce transmitter release. I used iberiotoxin (IBTx 100 nM), a selective antagonist of BK channels (Wanner et al., 1999) to determine if activation of these channels contributed to fEPSP rundown. The effect of IBTx on somal action potential duration was studied in 12 S neurons and 5 AH neurons. The duration of somal action potentials triggered by an intracellular current pulse was broadened by IBTx in 8 of 12 S neurons; in these cells, the action potential duration at half amplitude increased from 1.3 ± 0.1 to 2.0 ± 0.2 ms ($P<0.05$) (Figure 31). IBTx did not change action potential duration in AH neurons; the duration at half amplitude was 2.0 ± 0.08 and 2.1 ± 0.07 ms ($P>0.05$) before and after IBTx treatment, respectively (Figure 31). IBTx did not change the resting membrane potential or input resistance in any neuron tested. IBTx did not significantly change the amplitude of the first fEPSP in a

10-Hz train of stimulation but in 8 of 12 neurons IBTx attenuated PPD. In the absence of IBTx, the amplitude of the second fEPSP in a train was 66 ± 7% of the first fEPSP while in the presence of IBTx this value increased to 92 ± 6% ($P<0.05$) (Figure 32). Although IBTx reduced PPD, fEPSP rundown was not altered as the ratio of amplitudes of the first and 10th fEPSP was similar in the absence and presence of IBTx (Figure 32). Similarly, IBTx did not change the time constant of fEPSP rundown. The τ value for rundown at 10 Hz before IBT_X was 0.4 ± 0.1 s and in the presence of IBTx, this value was 0.5 ± 0.1 s ($P>0.05$) (Figure 32).

Figure 30. Action potentials do not fail during the train stimulation at 10 Hz

A. Recordings of antidromic action potentials and fEPSPs elicited by a 10 Hz train of stimulation. The fEPSP declined in amplitude while the antidromic action potential followed each stimulus in the train. B. Examples of the antidromic action potential and fEPSPs were shown on an expanded time scale.

Figure 30



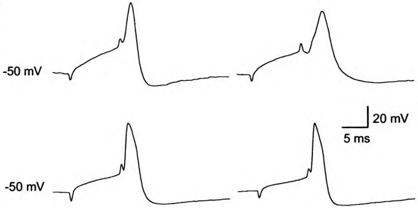
A
100 ms
B
AP
fEPSP
AP
fEPSP
AP
fEPSP
AP
fEPSP
1st
2nd
10th
20th
5 ms

Figure 31. Effects of IBTx on action potentials in S and AH neurons

A. Action potentials triggered by an intracellular depolarizing current pulse were broadened by IBTx in S but not AH neurons. B. Pooled data from experiments similar to that shown in (A) indicate that the action potential duration at half maximum amplitude is lengthened by IBTx in S (n=8) but not AH neurons (n=5). * Indicates a significant difference from control ($P<0.05$). Data are mean ± SEM.

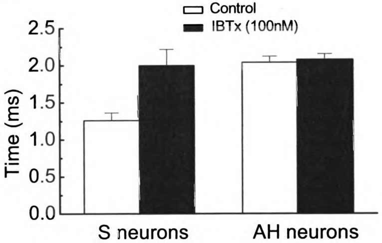
Figure 31

A



-50 mV
20 mV
5 ms
-50 mV

B

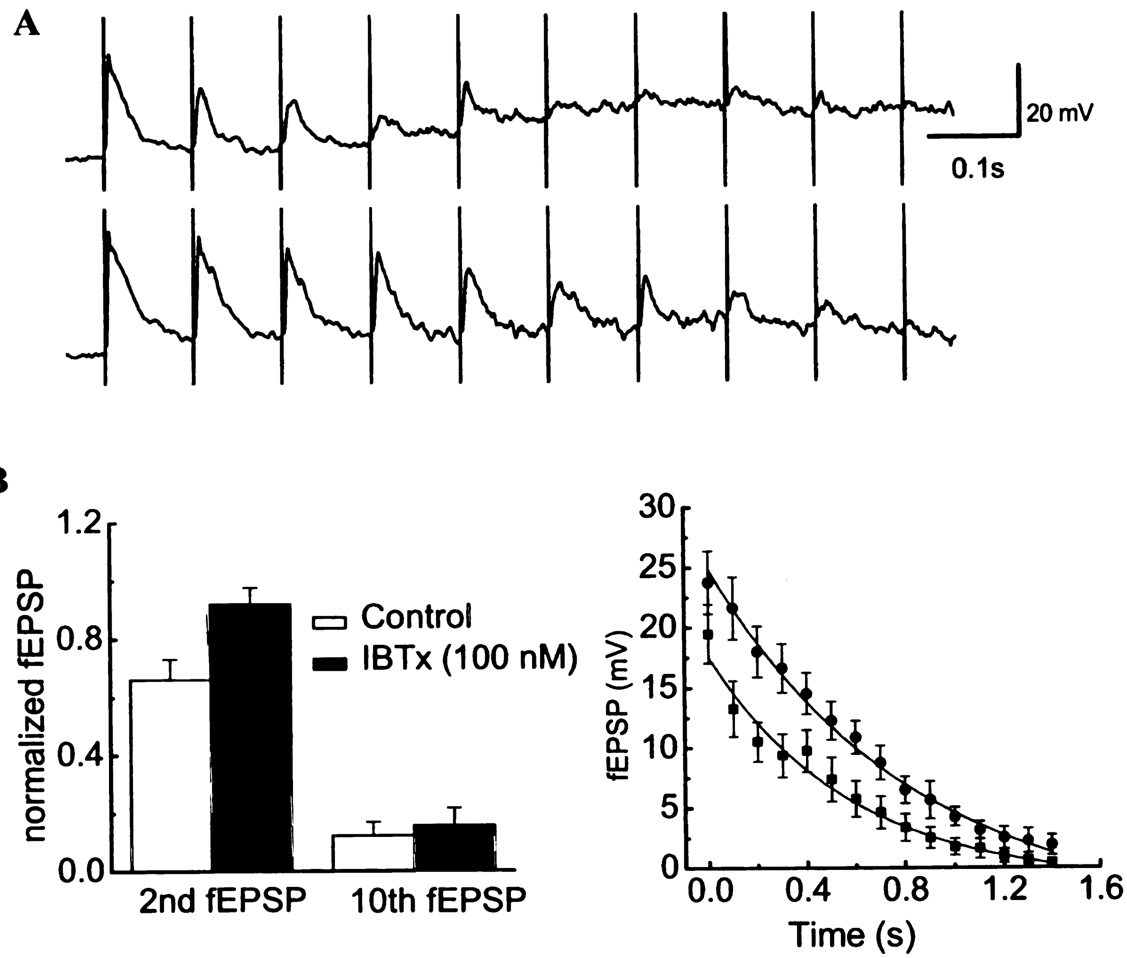


Control
IBTx (100nM)
2.5
2.0
1.5
1.0
0.5
0.0
Time (ms)
S neurons
AH neurons

Figure 32. Effects of IBTx on fEPSPs during a 10 Hz stimulus train

A. Representative fEPSPs before and after IBTx treatment. B. The second fEPSP in the train of fEPSPs is not depressed after IBTx treatment but the 10th fEPSP decreased to the same level occurring in the absence of IBTx. Data are the ratio of the second fEPSP vs. the first fEPSP (V_{second}/V_{first}) in a stimulus pair and the ratio of the 10th fEPSP vs. the first fEPSP in the stimulus train. * Indicates significantly different from control ($P< 0.05$). (C) Frequency-dependent rundown of fEPSPs before and after IBTx treatment. IBTx did not alter the time course of fEPSP rundown. Data are mean ± SEM (n=8).

Figure 32


A
20 mV
0.1s
B
1.2
0.8
0.4
0.0
normalized fEPSP
Control
IBTx (100 nM)
2nd fEPSP
10th fEPSP
30
25
20
15
10
5
0
fEPSP (mV)
0.0
0.4
0.8
1.2
1.6
Time (s)

PRESYNAPTIC 5-HT_4 RECEPTOR-MEDIATED REGULATION OF FAST SYNAPTIC TRANSMISSION

5-HT_4 receptor agonist increases fEPSPs during trains of stimulation

First, I tested the effect of 5-HT_4 receptor agonist on cholinergic and purinergic synaptic transmission induced by single electrical stimulation. Renzapride at 0.1 μM increased both cholinergic and purinergic fEPSPs when presence of purinergic blocker, PPADS and cholinergic blocker, mecamylamine, respectively (Figure 33). Then the effect of renzapride on the fEPSPs elicited by trains of stimulation was examined. Under control condition, fEPSPs declined to zero at about the tenth stimulation during a 20 pulses conditioning stimulation at 10 Hz. The first fEPSP amplitude was 17 ± 2 mV and fEPSP amplitude at the 20th stimulus was 0.7 ± 0.3 mV (n=9). In the presence of renzapride, the amplitude of the first fEPSP was 28 ± 3 mV and this declined to a steady state level at the 20 stimulus of 6 ± 2 mV (n=9). However, the time constants of decline were similar. They were 0.6 ± 0.1 s and 0.6 ± 0.1 s before and after renzapride (Figure 34).

5-HT_4 receptor agonist accelerates recovery after rundown

The effect of renzapride on time of recovery from synaptic rundown following a 10 Hz train of stimulation was also investigated. As described above, in these experiments, a train of 20 stimuli at 10 Hz caused fEPSPs to decline in amplitude to 0 mV at control condition. Recovery from rundown was tested by waiting for periods of 1

Figure 33. Prucalopride increases non-cholinergic and cholinergic fEPSPs recorded from myenteric neurons

A. Representative fEPSP recorded in the absence of drug (control) and in the presence of mecamylamine (10 μM) to isolate the non-cholinergic component of the fEPSP. Subsequent addition of prucalopride (0.1 μM) caused an increase in the amplitude of the non-cholinergic fEPSP. B. The P2 receptor antagonist, PPADS (10 μM), was used to isolate the cholinergic component of the fEPSP in the same neuron. Subsequent addition of prucalopride increased the amplitude of the cholinergic component of the fEPSP.

Figure 33

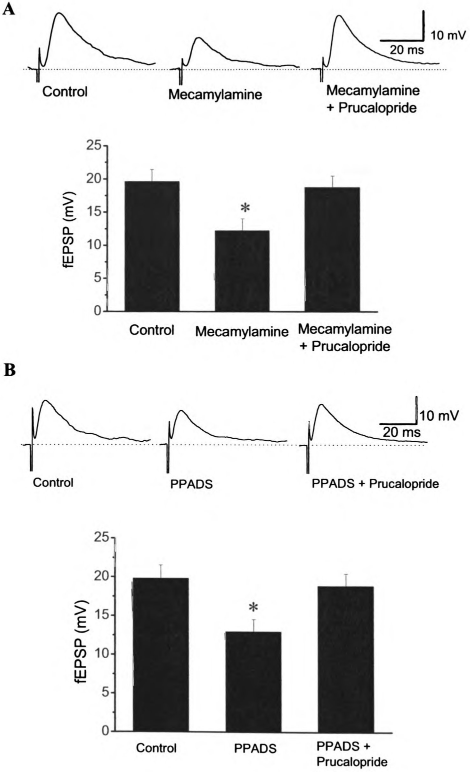
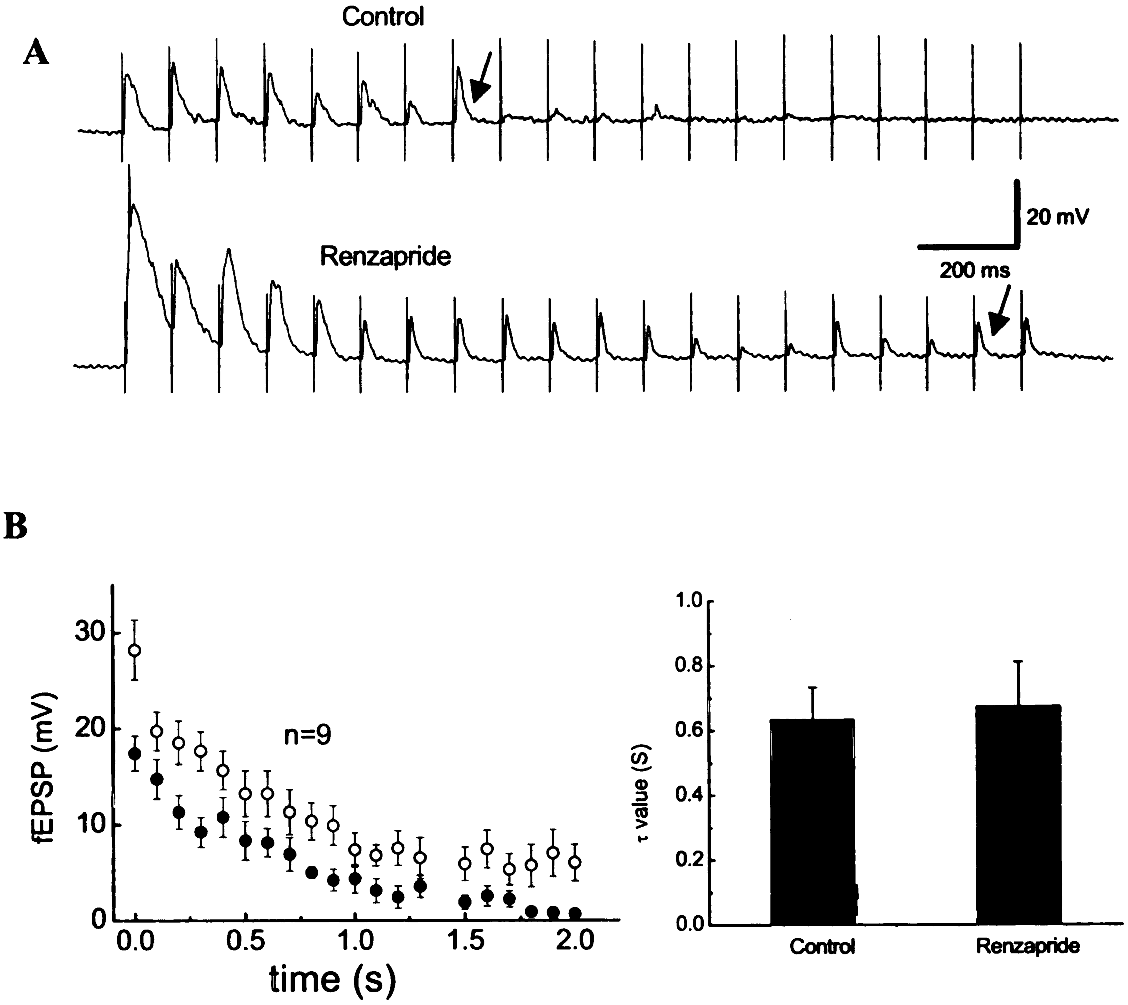

A
10 mV
20 ms
Control
Mecamylamine
Mecamylamine + Prucalopride
fEPSP (mV)
25
20
15
10
5
0
*
Control
Mecamylamine
Mecamylamine + Prucalopride
B
10 mV
20 ms
Control
PPADS
PPADS + Prucalopride
fEPSP (mV)
25
20
15
10
5
0
*
Control
PPADS
PPADS + Prucalopride

Figure 34. Renzapride increases fEPSPs elicited by trains of stimulation

A. Representative recordings of fEPSPs during trains of stimulation. Fast EPSPs declined to zero at the tenth stimulation (arrow) under control conditions. Renzapride increased the amplitude of all fEPSPs during the stimulus train. The fEPSPs did not decline to zero at the last stimulation (arrow). B. Pooled data show that renzapride increased fEPSPs (lower left) but did not alter the time course of synaptic rundown (lower right). Data were fit by a mono-exponential funtion.

Figure 34


A
Control
Renzapride
20 mV
200 ms
B
fEPSP (mV)
time (s)
n=9
τ value (S)
Control
Renzapride

to 12 s before a single stimulus was used to elicit a fEPSP (V_{test}); this amplitude was expressed as a fraction of the amplitude of the first EPSP in the preceding stimulus train (V_{first}) (Figure 35). Renzapride accelerated fEPSP recovery rate from rundown. The recovery time constants as calculated by exponential fittings were 7 ± 2 s under control condition whereas in the presence of renzapride the recovery time constant was reduced to 1.6 ± 0.2 s ($P < 0.05$).

Forskolin mimics 5-HT$_4$ receptor agonist effects

5-HT$_4$ receptors couple to Gs-protein. Activation of 5-HT$_4$ receptors stimulate a cascade including activiting adenylate cyclase, cAMP and PKA. To investigate if 5-HT$_4$ receptor agonist increases fEPSP through stimulating adenylate cyclase, forskolin, an adenylate cyclase stimulator, was superfused into the Krebs' solution. It was found that forskolin mimicked the effects of 5-HT$_4$ receptor agonists. Forskolin increased fEPSPs during 10 Hz train of electrical stimulation (Figure 36). Fast EPSPs still declined with forskolin treatment however the decline curves were parallel before and after forskolin treatment (Figure 36). As 5-HT$_4$ receptor agonists did to fEPSP recovery, forskolin also reduced fEPSP recovery time from rundown (Figure 36). Since 5-HT$_4$ receptors couple to the Gs-protein and forskolin mimics 5-HT$_4$ receptor agonists, effects of 5-HT$_4$ receptors on rundown and recovery must be through activation of adenylate cyclase.

H-89 abolishes the effects of 5-HT$_4$ receptor agonist

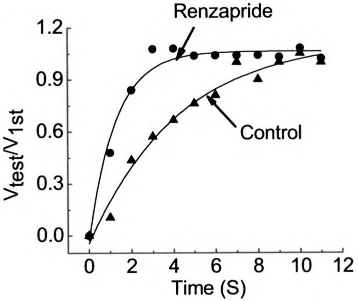
The function of adenylate cyclase is to catalyze coversion of ATP to cyclic AMP (cAMP) which activates PKA. PKA will then phosphorylate a number of targets

Figure 35. Renzapride accelerates recovery from fEPSP rundown

A. Renzapride accelerated recovery rate after fEPSP rundown. V_{first} was the first fEPSP in the train and V_{test} was the fEPSP evoked by a test stimulus at various time intervals after the stimulus train. The recovery time course was fitted by monoexponential function. B. The recovery time constant was reduced by renzapride.

Figure 35

A


Renzapride
1.2
0.9
0.6
0.3
0.0
V_{test}/V_{1st}
Control
0
2
4
6
8
10
12
Time (S)

B

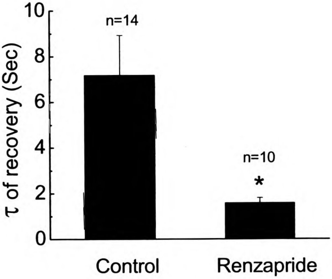
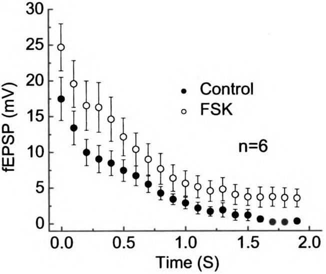

10
8
6
4
2
0
τ of recovery (Sec)
n=14
n=10
*
Control
Renzapride

Figure 36. Forskolin (FSK) mimics the effects of renzapride

A. FSK increased the fEPSP amplitude during a stimulus train. The fEPSPs did not decline to zero at the last stimulation in presence of FSK. B. FSK accelerated recovery rate after fEPSP rundown.

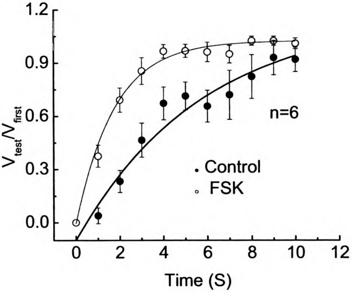
Figure 36

A



30
25
20
15
10
5
0
fEPSP (mV)
Control
FSK
n=6
0.0
0.5
1.0
1.5
2.0
Time (S)

B



1.2
0.9
0.6
0.3
0.0
V_{test}/V_{first}
n=6
Control
FSK
0
2
4
6
8
10
12
Time (S)

such as proteins and ion channels. H-89, a PKA blocker, was used to examined if activation of PKA is needed for 5-HT_4 receptor agonist effects. As shown in Figure 37, renzapride increased fEPSPs and accelerated the recovery rate. Co-application of H-89 and rezapride abolished the renzapride effects (Figure 37). This result suggested that 5-HT_4 receptor-mediated facilitation of synaptic transmission depended on its downstream signaling molecule PKA.

5-HT_4 receptor agonists increase release probability from single varicosities

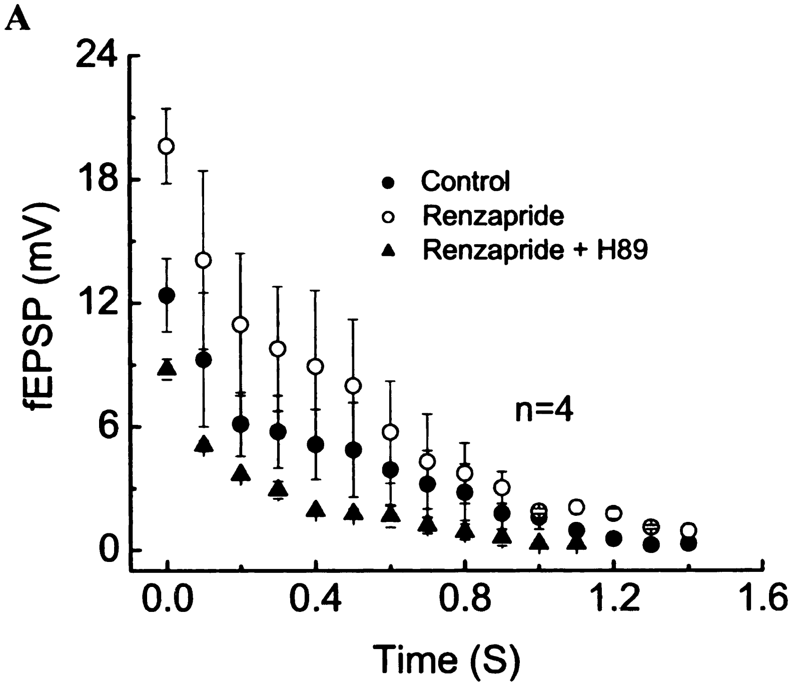
The above studies have shown that activation of 5-HT_4 receptors facilitated synaptic transmission through activating adenylate cyclase and PKA. But how PKA increases fEPSPs was not known. Did it increase release probability from single release site? Those experiments were conducted in acutely isolated LMMP preparations in *vitro*. In these studies, synaptic transmission recorded was triggered by electrical stimulation on a bundle of nerve fibers. However, in the ENS, transmitters are released from varicosities along single nerve fibers. The mechanism that 5-HT_4 receptor agonists increase transmitter release in single varicosities cannot be obtained from such studies. To address this question, the “sniffer” patch (detector patch) technique was employed in cultured myenteric neurons.

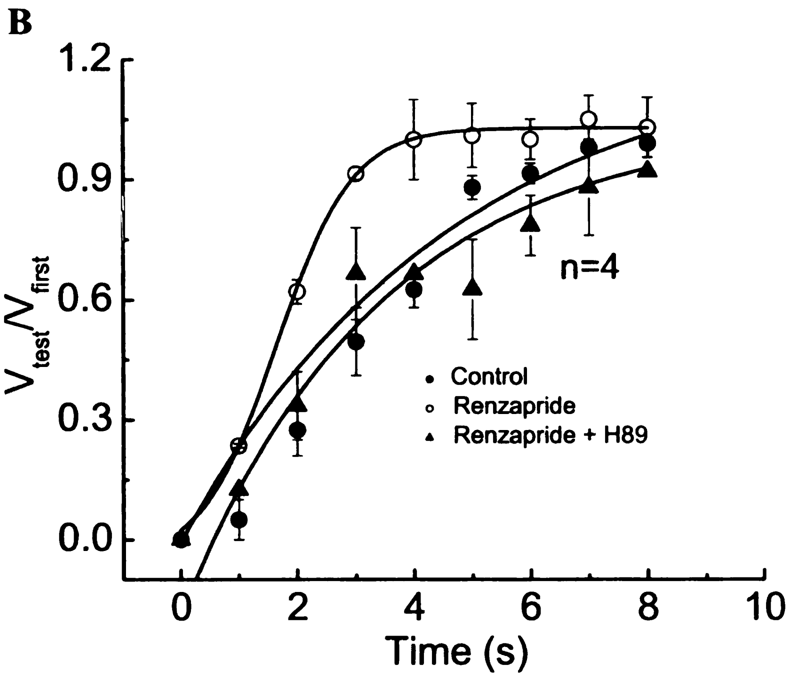
Outside-out patch clamp recordings were made from myenteric neurons maintained in primary culture. ACh and ATP-induced inward currents were recoded to make sure there are nAChRs and/or P2X receptors in the outside-out patches. Then this

Figure 37. H-89 abolishes the effects of renzapride on rundown and recovery rate after fEPSP rundown.

A. Renzapride increased the amplitude of all fEPSPs during stimulus train (10 Hz). Co-application of renzapride and H-89 did not alter fEPSP amplitude. B. Renzapride accelerated recovery rate after fEPSP rundown. The acceleration was abolished by addition of H-89.

Figure 37


A
24
18
12
6
0
fEPSP (mV)
0.0
0.4
0.8
1.2
1.6
Time (S)
Control
Renzapride
Renzapride + H89
n=4


B
1.2
0.9
0.6
0.3
0.0
V_{test}/V_{first}
0
2
4
6
8
10
Time (s)
n=4
Control
Renzapride
Renzapride + H89

patch was used as a detector to measure transmitter released from single varicosities. The transmitter release was triggered by stimulating a single nerve fiber. The currents flow through the detector patch represented the activity of a single release site. Twenty electrical stimuli were given in an interval of eight seconds. Because transmitter release from a given varicosity is intermittent, only some of twenty stimuli can trigger transmitter release to induce currents that can be recorded in the detector patch. Under control conditions, 27% stimulation elicited transmitter release (Figure 38; Table 3). Renzapride increased the ratio to 52% (Figure 38; Table 3). Similarly, another 5-HT_4 receptor agonist teagasrod increased release probability from 27% to 49% (Figure 38; Table 3). This result suggests that activation of 5-HT_4 receptors increases neurotransmitter release probability and this effect leads to facilitation of synaptic transmission.

Figure 38. Activation of presynaptic 5-HT_4 receptors increases release probability from single varicosities

Representative recordings of single channel events evoked by electrical stimulation of a single nerve fiber (as arrows show) before and after 5-HT_4 receptor agonists renzapride (Figure 38a) or tegaserod (Figure 38b). Renzapride or tegaserod increased single channel open probability.

Figure 38a

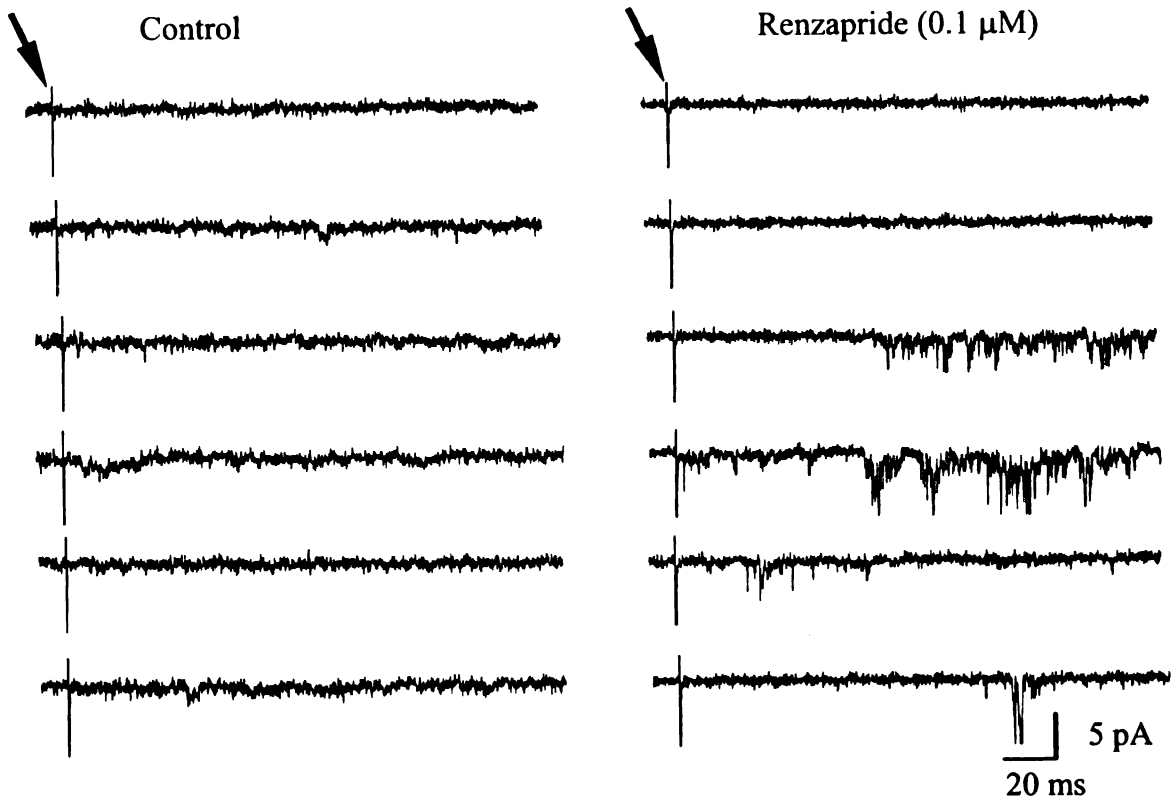


Figure 38b

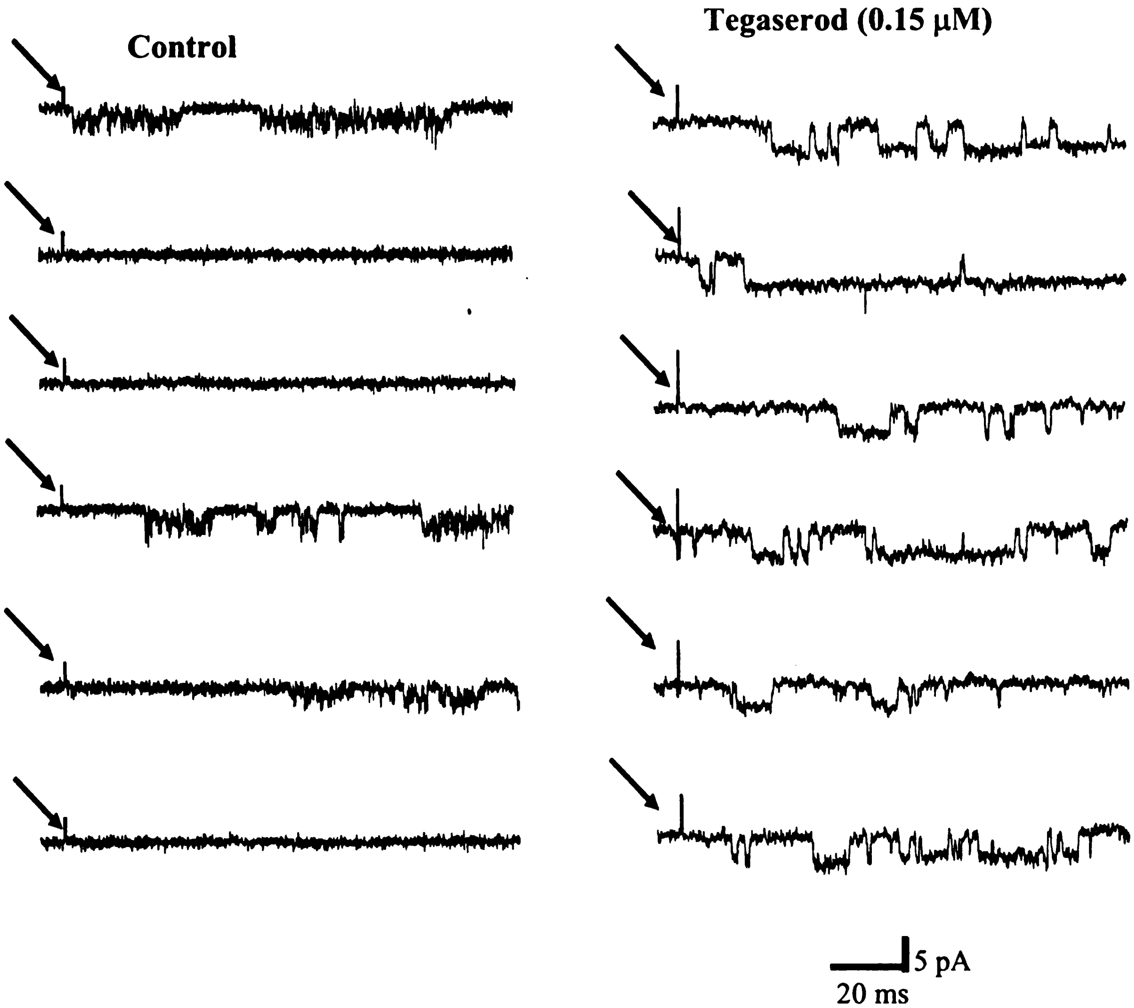


Table 3. Effects of 5-HT_4 receptor agonists on release probability.

Treatment of renzapride (A) or tegaserod (B) increased transmitter release probability evoked by electrical stimulation (see text for details).

A.

	Failure events	Successful events
Control	73	27
Renzapride	48	52*

n=5, $P < 0.001$

B.

	Failure events	Successful events
Control	58	22
Tegaserod	41	39*

n=4; $P < 0.005$

DISCUSSION

P2X RECEPTOR SUBTYPES EXPRESSED IN MYENTERIC NEURONS

In this section, identification of P2X receptor subtypes of myenteric neurons was accomplished in the small intestine of guinea pigs and mice. Guinea pigs have been used as the animal model for studying GI physiology and pharmacology broadly because the gut is readily dissected, reasonably long and mechanically quiescent when studied in *vitro*. Immunohistochemical localization of P2X receptor subtypes has been done in whole mount preparations of guinea pig small intestine (Pools et al., 2002; Castelucci et al., 2002). Pharmacological properties of P2X receptors in myenteric neurons have also been characterized in cultured cells (Zhou and Galligan, 1996; Khakh et al., 2000). However, no data are available about the function of P2X receptors in acutely isolated LMMP preparations. It is important to identify P2X receptor subtypes so that a complete characterization of synaptic mechanisms in neural circuits can be accomplished. I attempted to characterize pharmacological properties of P2X receptors in myenteric neurons in guinea pig tissues using limited selective agents. As a supplement to these pharmacological studies, I also investigated P2X receptor subtypes in tissues from mice in which $P2X_2$ and $P2X_3$ genes have been deleted. The advantage of gene deletion would produce precise information.

My data indicate that, in guinea pig small intestine myenteric S neurons express $P2X_3$ receptors whereas AH neurons express $P2X_2$ receptors. In mice small intestine, myenteric S neurons express $P2X_2$ homomers whereas AH neurons express $P2X_{2/3}$ heteromers. The differences may be due to selective expression of P2X subunits by different species. But I cannot exclude the possibility that lack of specific P2X receptor ligands limited the conclusion made in guinea pigs.

Properties of murine myenteric neurons

Murine myenteric neurons have similar electrophysiological properties to guinea pigs. In the mouse ileum, AH neurons had a broad action potential that was only partly reduced by TTX. The action potential has a prominent calcium component that accounts for the long duration and action potential shoulder in AH neurons from guinea-pig ileum and colon (Furness et al. 1998; Nuragli et al. 2004), rat colon (Browning and Lees, 1996), pig ileum (Cornelissen et al. 2001), mouse colon (Furakawa et al. 1986) and human colon (Brookes et al. 1987). Therefore, the calcium shoulder is a general property of AH neurons in the gastrointestinal tract of a number of species. Although not tested in this study, the long duration and TTX-resistance of mouse ileal myenteric AH neurons suggest that there is also a calcium component to the action potential. The action potential in mouse ileal myenteric AH neurons was followed by an afterhyperpolarization that lasted more than 2 s. This property is also similar to that in AH neurons in the myenteric plexus of the guinea-pig ileum and colon (Hirst et al. 1974; Furness et al. 1998;) rat colon (Browning & Lees, 1996), pig ileum (Cornelissen et al. 2001), mouse colon (Furakawa et al. 1986) and human colon (Brookes et al. 1987). In guinea pig and pig intestine, fEPSPs can be evoked in some AH neurons (Liu et al. 1997; Cornelissen et al. 2001) while sEPSPs can be evoked in nearly all AH neurons in the guinea-pig gastrointestinal tract (Furness et al. 1998). I found that single electrical stimuli did not elicit synaptic responses in mouse ileal AH neurons. As I recorded from only a small number of AH neurons, it is possible that I did not sample those neurons receiving fast synaptic input or that I did not stimulate the interganglionic connectives providing the fast synaptic inputs to the neurons that I did sample. However, I was able to elicit sEPSPs in all AH neurons studied. Slow synaptic excitation is the predominant synaptic mechanism in guinea pig AH neurons (Kunze et al. 1993). Taken together, these data indicate that the properties of AH neurons in the mouse ileal myenteric plexus are similar to those identified in the gastrointestinal tract of other species.

S type enteric neurons have an action potential that is completely blocked by TTX and all S neurons exhibit fEPSPs following single stimuli applied to the interganglionic connectives in the myenteric plexus (Hirst et al. 1974; Bornstein et al. 1994; Galligan et al. 2000). In the mouse myenteric plexus, I identified neurons that received fast excitatory synaptic inputs and the action potential in these neurons was completely blocked by TTX. Therefore, I concluded that these were S neurons. I also found that the fEPSP in S neurons was only partly blocked by mecamylamine, a nicotinic cholinergic receptor antagonist, and that fEPSPs were also inhibited by PPADS. These data indicate that both acetylcholine and ATP are fast synaptic transmitters in the mouse small intestine as they are in the guinea-pig intestine (LePard et al. 1997; Johnson et al. 1999) but there may be additional fast neurotransmitters including 5-HT (Galligan et al. 2000).

Murine myenteric AH neurons were encountered infrequently using a random impalement strategy with intracellular microelectrodes. This observation is similar to that reported previously in a study of myenteric neurons in the mouse colon (Furukawa et al. 1986). If AH neurons are sensory neurons in the mouse intestine, as they are in the guinea pig intestine (Furness et al. 1998), then normal intestinal reflex behaviors may require only a small number of sensory neurons in the mouse. Alternatively, non-AH neurons may serve as sensory neurons in the mouse intestine. A previous study, for example, has shown S neurons in guinea-pig distal colon serve as mechanosensory neurons that are sensitive to stretch (Spencer and Smith, 2004).

P2X receptor-mediated responses in AH neurons

ATP caused a rapidly developing depolarization in all murine myenteric AH neurons. This depolarization was associated with a decrease in input resistance and was blocked by PPADS. These data are consistent with this response being mediated by either a P2X or $P2Y_1$ receptor. α, β-mATP is an agonist at homomeric $P2X_1$, homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ receptors, but it does not activate P2Y receptors (North and Surprenant, 2000). I found that ATP caused a depolarization of AH neurons in $P2X_3^{+/+}$ and in $P2X_3^{-/-}$ tissues, while α, β-mATP depolarized AH neurons in $P2X_3^{+/+}$ but not in $P2X_3^{-/-}$ tissues. These data indicate that $P2X_3$ subunits contribute to the P2X receptor expressed by murine AH neurons. However, sensory neurons and some sympathetic neurons also express $P2X_{2/3}$ heteromeric receptors (Lewis et al. 1995; Zhong et al. 2000), and the loss of response to α, β-mATP in $P2X_3^{-/-}$ mice could result from the loss of $P2X_{2/3}$ heteromeric receptors in murine AH neurons. Other subunits must also be present in order for an ATP-induced depolarization to persist in AH neurons from $P2X_3^{-/-}$ mice. $P2X_2$ subunits remaining in $P2X_3^{-/-}$ mice could account for this ATP sensitivity. α, β-mATP caused similar amplitude depolarizations in AH neurons from $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice. As α, β-mATP activates P2X receptors containing $P2X_1$ or $P2X_3$ subunits (North and Surprenant, 2000), it is possible that AH neurons express P2X receptors composed of

$P2X_1$ or/and $P2X_3$ subunits. However, my work has also shown that α, β-mATP does not depolarize AH neurons in the myenteric plexus of $P2X_3^{-/-}$ mice while ATP depolarized the same neurons. Therefore, the P2X receptors in murine AH neurons are likely to be $P2X_{2/3}$ heteromers. My suggestion that the $P2X_2$ subunits are expressed in AH neurons is also supported by recent immunohistochemical studies in guinea-pig gastrointestinal tissues. These studies showed that $P2X_2$ subunits are localized to neurons that also contain the calcium binding protein, calbindin (Castelucci et al. 2002). Calbindin is a protein marker for most AH neurons in the guinea-pig GI tract (Furness et al. 1998). However, it has also been shown that $P2X_3$ subunits are not found in calbindin-containing neurons in the guinea-pig GI tract but that these subunits are localized to calretinin- and NOS-containing cells (Nassauw et al. 2002; Poole et al. 2002). Calretinin and NOS are contained in S type neurons and are markers for interneurons and motoneurons (Brookes, 2001). It is possible that receptor expression and subunit composition of P2X receptors in subsets of neurons are different in the mouse and guinea pig small intestine. A detailed study of the neurochemical content and receptor expression of subsets of murine enteric neurons is needed to address this issue.

P2X receptors in AH neurons do not mediate fEPSPs as fEPSPs are not recorded from AH neurons. It may be due to no purinergic inputs to AH neurons. The functions of AH neuron somal P2X receptors remain to be addressed.

Fast synaptic transmission and P2X receptor-mediated responses in S neurons

I hypothesized that a P2X receptor contributes to the fEPSP recorded from S neurons in the mouse small intestine. This hypothesis was based on the finding that fEPSPs were only partly inhibited by mecamylamine, indicating that a neurotransmitter in addition to acetylcholine mediates the fEPSP. As fEPSPs were also reduced by PPADS, the additional neurotransmitter in the mouse myenteric plexus is likely to be ATP. It is unlikely that $P2X_3$ receptor subunits contribute to the P2X receptor expressed by S neurons as the properties of fEPSPs in tissues from $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice were identical. Furthermore, S neurons in tissues from $P2X_3^{+/+}$ and $P2X_3^{-/-}$mice were depolarized by ATP but not by α, β-mATP. α, β-mATP insensitivity suggests that the P2X receptor in S neurons is a $P2X_2$ homomeric receptor (Zhou and Galligan, 1996). The fEPSPs recorded from S neurons in tissues from $P2X_2^{+/+}$ mice were partly inhibited by the nicotinic cholinergic receptor antagonist mecamylamine and by the P2 receptor antagonist PPADS. These results indicate that ACh and ATP mediate fast synaptic responses in the murine myenteric plexus as also occurs in the guinea pig intestine (Galligan and Bertand, 1994; LePard et al. 1997; Johnson et al. 1999) and colon (Nurgali et al. 2003). However, the fEPSP in $P2X_2^{-/-}$ mice was blocked by mecamylamine while PPADS had no effect on fEPSPs in $P2X_2^{-/-}$ tissues. These data indicate that $P2X_2$ subunits are a critical component of the P2X receptor expressed by S neurons. Although $P2X_2$ subunits mediate the purinergic component of fEPSPs, the average fEPSP amplitudes recorded from S neurons in $P2X_2^{-/-}$ and $P2X_2^{+/+}$ tissues were not different. This result may be related to the findings from previous studies that showed a functional interaction between P2X and nicotinic acetylcholine receptors (Nakazawa, 1994; Zhou and Galligan, 1998; Searl et al. 1998; Barajas-Lopez et al. 1998; Khakh et al. 2000). In these studies it was found that simultaneous activation of nicotinic and P2X receptors produces a response that is smaller in amplitude than the predicted sum of responses caused by individual activation of each receptor. It was concluded that there is a functional link between P2X and nicotinic receptors that results in cross-inhibition of responses mediated by these receptors (Nakazawa, 1994; Zhou and Galligan, 1998; Searl et al. 1998; Barajas-Lopez et al. 1998; Khakh et al. 2001). Deletion of the $P2X_2$ subunit gene would remove cross-inhibition and synaptic responses mediated by nicotinic receptors in $P2X_2^{-/-}$ mice would be larger in amplitude than those occurring in neurons co-expressing the $P2X_2$ subunit. Lack of $P2X_2$- nicotinic receptor cross-inhibition could account for the maintained fEPSP amplitude in $P2X_2^{-/-}$ tissues.

S neurons in tissues from $P2X_2^{+/+}$ mice were depolarized by ATP but not by α, β-mATP. This result suggests that S neurons express $P2X_2$ homomeric receptors as α,β-mATP does not activate $P2X_2$ homomers, but does activate $P2X_3$ homomeric and $P2X_{2/3}$ heteromeric receptors (Lewis et al. 1995; North and Surprenant, 2000). In addition, ATP failed to elicit a depolarization in S neurons from $P2X_2^{-/-}$ mice. Based on these results, I conclude that the P2X receptor mediating fEPSPs in murine S neurons is a $P2X_2$ homomeric receptor. In the guinea pig intestine, $P2X_2$ subunit immunoreactivity has been localized to NOS-containing neurons (Castelucci et al. 2002). NOS-containing neurons are inhibitory motorneurons and descending interneurons, and both of these classes of neuron would have S-type electrophysiological properties (Brookes, 2001). If the chemical coding of murine myenteric neurons is similar to that in the guinea pig intestine, these data would indicate that inhibitory motorneurons (Johnson et al. 1999) and some descending interneurons (LePard & Galligan, 1999; Bian et al. 2000; Monro et al. 2002) receive fast excitatory synaptic input mediated in part by $P2X_2$ homomeric receptors.

P2X subtypes in myenteric neurons in guinea pig small intestine

Extensive immunohistochemical studies have attempted to identify P2X subunit compositions in guinea pig myenteric neurons (Poole et al., 2002; Castelucci et al., 2002). In these studies, it has been shown that $P2X_2$ subunits are localized to inhibitory motor neurons and AH neurons in myenteric plexus. $P2X_3$ subunits are localized to inhibitory motor neurons and S neurons. They are not found in AH neurons. In inhibitory motor neurons, which are NOS immunoreactive, P2X receptors are $P2X_{2/3}$ heteromers. The pharmacological studies in other paper have found there are α, β-mATP-sensitive P2X receptor subtypes ($P2X_1$ or $P2X_3$) contributing fEPSP in S neurons of guinea pig intestine (LePard et al. 1997). However patch-clamp recordings from myenteric neurons maintained in primary culture indicate that only in a small subset (10 %) of myenteric neurons, α, β-mATP caused a rapidly developing and desensitizing inward current, suggesting that P2X receptors contain $P2X_1$ or $P2X_3$ subunits (Zhou & Galligan, 1996). In present study, all S neurons that were sensitive to ATP were depolarized by α, β-mATP but only 17% AH neurons were sensitive to α, β-mATP. A selective antagonist for $P2X_1$ or $P2X_3$ receptors, TNP-ATP reduced ATP-induced depolarization as well as reducing fEPSP amplitude in S neurons. These results support immunohistochemical identification and studies in acute isolated LMMPs in guinea pigs. However, these results are not consistent with guinea pig myenteric neurons maintained in primary culture and murine myenteric neurons. Most of neurons in primary culture do not desensitize to α, β-mATP may be due to the alteration of $P2X_3$ receptors in cultural condition. The difference between myenteric neurons in guinea pigs and mice may be due to the differentiation expression of P2X receptor between these two species; or myenteric S type neurons in mice and in guinea pigs may play different functions. These data in the present study suggest that, in guinea pig ileum, P2X receptors in myenteric S neurons contained $P2X_3$ subunits while P2X receptors in AH neurons were composed of few $P2X_3$ subunits.

P2X RECEPTOR-MEDIATED AFTERHYPERPOLARIZATION IN GUINEA PIG MYENTERIC S NEURONS

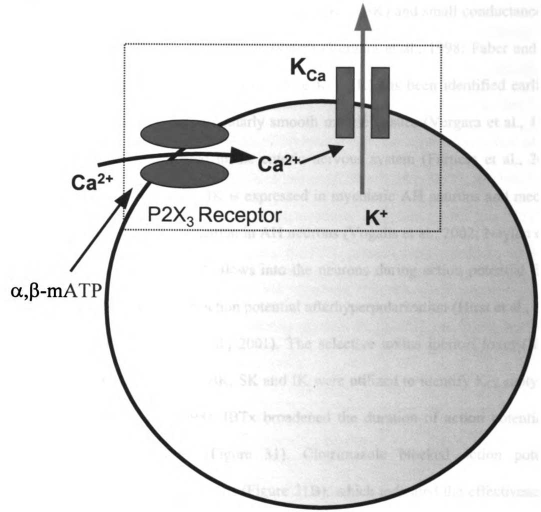
Ionic basis of afterhypolarization

An important finding in the present study was that the depolarization induced by α, β-mATP was followed by a hyperpolarization in 55 percent of tested S neurons. The hyperpolarization was mediated by activation of potassium conductance because the reversal potential of its underlying outward current was equal to K^+ equilibrium potential. The K^+ conductance was gated by calcium because reduction of extracellular Ca^{2+} concentration greatly reduced the hyperpolarization. The influx of Ca^{2+} that activates K^+ conductance was through P2X receptors as PPADS blocked depolarization therefore abolished hyperpolarization consequentially. The proposed model for P2X receptor-mediated hyperpolarization is shown as Figure 39. It has been known that P2X receptors have a predominant Ca^{2+} permeability that equals to or greater than those of the 100 times more Na^+ (Burnashev, 1998). We also noticed that the amplitudes of hyperpolarization and depolarization were positively correlated although the other cations are involved. It may still be arguable that Ca^{2+} could move into the neurons through voltage-gated channels. It is unlikely the case in the present study since we recorded the K_{Ca}-mediated outward currents in single electrode voltage-clamp recordings. The influx of Ca^{2+} can have broad physiological effects by acting at various downstream targets, which are Ca^{2+}-dependent K^+ channels (K_{Ca}) in the present study.

Figure 39. A proposed model for P2X-mediated afterhyperpolarization in S neurons

$P2X_3$ subunit-containing receptors and calcium-dependent potassium channels are expressed in myenteric S neurons in guinea pig small intestine. Specifically, they are located closely within a micro-domain (shown in dashed box). Activation of $P2X_3$ receptors by α,β-mATP induces calcium influx. The calcium ions entry activates K_{Ca} that allows potassium flowing out of the cell, which results in afterhyperpolarization.

Figure 39



I attempted to identify the subtypes of K_{Ca} channels that mediate hyperpolarization in S neurons. Large conductance K_{Ca} (BK) and small conductance K_{Ca} (SK) have been described in the nervous system (Vergara et al., 1998; Faber and Sah, 2003; Stocker, 2004). Intermediate conductance K_{Ca} (IK) has been identified earlier in non-neural peripheral tissues, particularly smooth muscle tissues (Vergara et al., 1998). Recently IK is also documented in the enteric nervous system (Furness et al., 2004). Further studies have shown that IK is expressed in myenteric AH neurons and mediates action potential afterhyperpolarization in AH neurons (Vogalis et al., 2002; Neylon et al., 2004). IK is activated when Ca^{2+} flows into the neurons during action potential firing, which mediates the long lasting action potential afterhyperpolarization (Hirst et al., 1974; Furness et al., 1998; Vogalis et al., 2001). The selective toxins iberiori toxin (IBTx), apamin and clotrimazole against BK, SK and IK were utilized to identify K_{Ca} subtype in S neurons (Vergara et al., 1998). IBTx broadened the duration of action potential by blocking BK in S neurons (Figure 31). Clotrimazole blocked action potential afterhyperpolarization in AH neurons (Figure 21B), which indicated the effectiveness of clotrimazole on blocking IK. However none of these toxins affected P2X receptor-mediated afterhyperpolarization. This result suggests that K_{Ca} in S neurons does not belong to any of these three major types.

Present study provides evidence that K_{Ca} channels are expressed in S neurons. K_{Ca} should mediate an afterhyperpolarization in S neurons as it does in AH neurons. However action potentials are not followed by long lasting hyperpolarization in S neurons. There is no action potential afterhyperpolarization in S type neurons because there is no Ca^{2+} influx during the action potential (Hirst et al., 1974), and not because there are no K_{Ca}

channels in S neurons. Ca^{2+} moves into the neurons during fEPSPs and a hyperpolarization should be observed after fEPSP. However a single purinergic fEPSP evoked by electrical stimulation on presynaptic nerve fibers was not followed by an after-hyperpolarization. One explanation would be that the amount of Ca^{2+} influx through P2X receptors during fEPSP is not sufficient to activate K_{Ca} channels. In addition, fast EPSPs are mediated by $P2X_3$ receptors in most of myenteric S neurons (see discussion above). Ca^{2+} permeability of P2X receptor is dependent on different subtypes (Egan and Khakh, 2004). $P2X_3$ receptors have relative low permeability comparing with the other P2X subtypes. The other possibility would be that the $P2X_3$ receptors expressed at the synaptic area do not couple to K_{Ca} channels. For example, immunohistochemistry staining indicates that only 20% Dogiel type I neurons express IK channel immunoreactivity and it is only seen in cell soma (Neylon et al., 2004).

Functional significance of AHP in myenteric S neurons

Another interesting observation was that only approximate half of tested S neurons displayed the agonist-induced after-hyperpolarization. I proposed that ligand-gated hyperpolarization might be associated with a specific subset of S neurons that have the same function. S type myenteric neurons are motor or interneurons (Furness, 2000). This is based on the studies using combined electrophysiological and immunohistochemical methods (Furness, 2000). Purinergic synaptic transmission contributes to the descending and ascending excitatory nervous reflex pathway as well as inhibitory descending pathway (Spencer et al., 2000; Furness and Sanger, 2002). P2X receptors coupled to K_{Ca} may be a mechanism by which the neuronal firing rate is controlled. I tested if this group of neurons belongs to a specific functional category of purinergic S neurons. I examined the co-localization of these neurons with NOS neurons, which are inhibitory motor neurons. However, my results did not show any association of NOS-ir neurons with the neurons that displayed α,β-mATP-induced ligand-gated hyperpolarization.

A previous study has documented that a depolarization induced by activation of nicotinic AChRs is followed by a hyperpolarization in myenteric S neurons (Tokimasa et al., 1982). The underlying mechanism is similar, which is due to calcium entry and subsequent opening of K_{Ca}. Nicotinic AChRs and P2X receptors are both ligand-gated cation channels, which are permeable to non-selective cations including Ca^{2+}. They are the two primary ligand-gated ion channels in myenteric S neurons and have similar functions in mediating fast synaptic transmission. The similar hyperpolarization they induced may have similar physiological function.

RUNDOWN OF fEPSPS DURING BURSTS OF NEURONAL ACTIVITY

In this section, fast synaptic transmission during trains of electrical stimulation was investigated. I found that fEPSPs declined during stimulus train and there was no contribution of desensitization of nAChRs and P2X receptors. Action potential failure, presynaptic inhibitory receptors or BK channels did not account for synaptic rundown. I proposed that synaptic rundown is due to depletion of vesicles in RRP.

Rundown of fEPSPs

In isolated segments of guinea-pig ileum, it has been shown that mechanical stimulation of mucosal villi generates short bursts of fEPSPs that occur in the frequency range of 15–40 Hz (Bornstein et al., 1991). In similar experiments, it was shown that if the mechanical stimulus is applied at intervals of less than 10 s, the amplitude of fEPSPs declined during repetitive stimulation (Smith et al., 1992). Chemical stimulation (low pH or 5-HT application) of the mucosa also elicits short bursts of fEPSPs (average of 14 fEPSPs per burst) occurring at frequencies between 5 and 20 Hz (Bertrand et al., 1997). I studied the dynamics of fast synaptic excitation during short trains of electrical stimulation of presynaptic nerve fibers. The frequencies of the stimulus trains that I used mimicked the frequencies of fEPSPs evoked by the physiological stimuli described above. In most neurons, these trains of electrical stimulation elicited fEPSPs that declined in amplitude in a frequency dependent manner. The conclusions are based on data obtained in S neurons, as these cells received fast excitatory synaptic input, and the action potentials were not followed a long-lasting (>1 s) action potential afterhyperpolarization.

My data differ from those obtained in studies of fast synaptic input to AH type neurons in the pig intestine where it was found that fEPSPs did not rundown when evoked by trains of stimulation at frequencies up to 20 Hz (Cornelissen et al., 2001). Similarly, in the rat colon, fast synaptic input to most myenteric S neurons is maintained during 10-Hz trains of stimulation (Browning and Lees, 1996). These observations suggest that there are species or tissue differences in the mechanisms regulating the availability and release of stores of fast synaptic transmitters in the myenteric plexus.

Receptor desensitization did not contribute to synaptic rundown

ACh acting at nAChRs and ATP acting at P2X receptors mediate most fEPSPs in myenteric S neurons in the guinea pig ileum (Galligan et al., 2000). Previous work showed that nAChRs expressed by guinea-pig myenteric neurons desensitize faster than P2X receptors expressed by the same cells (Zhou and Galligan, 1998; Brown and Galligan, 2003). Based on this difference in desensitization rate, I proposed that nAChRs and P2X receptors would make a differential contribution to the fEPSPs during trains of stimulation. My hypothesis was that the nicotinic component would desensitize early in a train of stimulation while P2X receptors would maintain synaptic transmission as the stimulus trained continued. However, the data indicate that this does not happen, as selective blockade of nAChRs or P2X receptors did not alter the rate of fEPSP rundown during a 10 Hz train of stimulation. Furthermore, when ACh and ATP were applied by trains of ionophoretic pulses to mimic fEPSPs during same stimulus train, the ionophoretic depolarizations did not decline in amplitude. These data indicate that little desensitization occurs during the brief occupation of nAChRs or P2X receptors by either

synaptically released or ionophoretically applied agonist. It was also found that mecamylamine or PPADS applied individually each reduced fEPSPs by about 35% while coapplication of these two blockers reduced the fEPSP by 90%. This result is consistent with the previous finding that P2X receptors and nAChRs are linked in a mutually inhibitory manner in guinea-pig ileum myenteric neurons (Zhou and Galligan, 1998; Khakh et al., 2000). That is simultaneous activation of the two receptors produces a response that is smaller than the predicted sum of responses caused by individual activation of the two receptors. Blocking either the nAChR or the P2X receptor removes cross-inhibition allowing a larger amplitude response at the unblocked receptor.

Rundown is not caused by presynaptic inhibition

Presynaptic inhibition is a predominant mechanism for modulating synaptic transmission in the ENS. α2-Adrenoceptors are localized to cholinergic neurons and these receptors mediate inhibition of acetylcholine release onto myenteric S neurons (Stebbing et al., 2001; Scheibner et al., 2002). Opioid receptors are expressed on cell bodies and processes of myenteric neurons and activation of opioid receptors inhibits the electrically evoked release of acetylcholine (Sternini, 2001). Muscarinic M_2 receptors also mediate presynaptic inhibition of fast synaptic transmission in the myenteric plexus (North et al., 1985). Adenosine A_1 (Christofi and Wood, 1993) and 5-HT_{1A} (Pan and Galligan, 1994) receptors also couple to presynaptic inhibition of fast synaptic excitation in the ENS. Acetylcholine, norepinephrine, enkephalins and 5-HT-containing nerve fibers are present throughout the myenteric plexus (Costa and Furness, 1984; Costa et al., 1996; Brookes, 2001) and trains of nerve stimulation can release all of the substances.

Adenosine is a breakdown product of neurally released ATP (Westfall et al., 2002) and it will accumulate in the myenteric plexus during trains of nerve stimulation. Therefore, fast synaptic rundown could be due to presynaptic inhibition caused by the accumulation of endogenous agonists for presynaptic inhibitory receptors. However, the data show that fast synaptic rundown persists in the presence of muscarinic, $\alpha 2$ adrenergic, opioid, A1 adenosine and 5-HT_{1A} receptor antagonists. Based on these data, I conclude that presynaptic inhibition mediated by one or more of these receptors are not responsible for fEPSP rundown. However, although I tested the contribution of activation of several presynaptic receptors to fEPSP rundown, there could be many other inhibitory presynaptic receptors localized to myenteric nerve terminals. Inhibitory presynaptic receptors in the ENS belong to the family of G-protein coupled receptors which link to PTX-sensitive mechanisms and inhibition of neurotransmitter release (Zamponi, 2001). In order to eliminate presynaptic inhibition mediated by G-protein coupled receptors, I studied fast synaptic rundown in myenteric plexus preparations that had been treated with PTX to inactivate the Gi/Go class of G-proteins. Fast synaptic rundown was identical in control and in PTX-treated tissues indicating that presynaptic inhibition does not account for fast synaptic rundown. Although PTX-treatment did not alter synaptic rundown, the treatment protocol was effective in blocking the target G-proteins. This conclusion is based on the data showing that the $\alpha 2$-adrenoceptormediated IPSP recorded from submucosal S neurons was blocked in preparations that had been treated using our PTX incubation protocol. IPSPs in submucosal neurons are mediated by $\alpha 2$-adrenoceptors that couple directly to a K^+ channel via a PTX-sensitive G-protein (Surprenant and North, 1988).

Changes in presynaptic action potentials do not contribute synaptic rundown

Frequency-dependent decreases in nerve terminal Ca^{2+} influx could result in synaptic rundown. Decreased nerve terminal Ca^{2+} entry could be due to axonal action potential failure during high frequency nerve stimulation (Cunnane and Stjarne, 1984). I made recordings from neurons in which fEPSPs and antidromically propagated action potentials were recorded simultaneously. These experiments showed that action potentials followed each stimulus in a 10 Hz train while fEPSPs declined in amplitude. These data indicate that action potential propagation into the nerve terminal is maintained throughout a train of stimuli and action potential failure does not contribute to fEPSP rundown. Frequency-dependent shortening of action potential duration could also contribute to synaptic rundown. This could occur either through Ca^{2+} channel inactivation or though a Ca^{2+}-dependent activation of K^{+} channels which would accelerate action potential repolarization (Franciolini et al., 2001). Inactivation of Ca^{2+} channels in myenteric neurons occurs in the time range of 0.1– 1 s (Bian et al., 2004). As an individual action potential duration is 2 ms or less, it is unlikely that Ca^{2+} channel inactivation makes a significant contribution to fEPSP rundown in myenteric neurons. However, in the absence of direct data about calcium channel dynamics in the nerve terminal, I cannot categorically rule out a contribution of Ca^{2+} channel inactivation to fEPSP rundown. BK channels are a class of Ca^{2+}-activated K^{+} channels expressed in nerve terminals and these channels function to limit nerve terminal action potential duration and Ca^{2+} entry (Robitaille and Charlton, 1992). IBTx, a BK channel blocker (Wanner et al., 1999), did not alter the time course of fEPSP rundown indicating that BK channel-dependent shortening of action potential duration do not contribute to fEPSP rundown during trains of stimulation. However, I did find the IBTx inhibited PPD in a subset of neurons as the decline in amplitude of the second fEPSP in the presence of IBTx was less than that occurring in the absence of the toxin. This result suggests that BK channel activation contributes to PPD during the first two fEPSPs in a train of stimulation but that other processes mediate fEPSP rundown as the train duration lengthens. IBTx increased action potential duration in a subset of S neurons. These data indicate that BK channels regulate action potential duration in some S neurons and that Ca^{2+} must enter these neurons during a single action potential. Direct measurements have shown that short trains of action potentials can cause measurable increases in intracellular Ca^{2+} in some myenteric S neurons; N-type Ca^{2+} channels mediate this response (Shuttleworth and Smith, 1999). Although single action potentials did not evoke a measurable Ca^{2+} increase, changes caused by a single spike may be under the detection limits of the assay or there may be local increases in Ca^{2+} that were not detected. These local increases in Ca^{2+} could be restricted to sights near the BK channel as previous studies in hippocampal neurons have shown that there is a close spatial and functional relationship between N-type Ca^{2+} channels and BK channels (Marrion and Tavalin, 1998). A similar relationship may exist in some myenteric S neurons. This relationship is specific for some S neurons as IBTx did not alter the action potential in AH neurons. This result is consistent with previously published work showing that IBTx does not alter action potential duration in AH neurons (Kunze et al., 1994).

Depletion of transmitter accounts for synaptic rundown

When nerve fibers were stimulated at a lower frequency (0.5 Hz), fEPSP amplitude declined by 50% after the first stimulus but was maintained for the remainder of the stimulus train. Higher frequencies of stimulation resulted in complete rundown of the fEPSP. There are two models that could explain these data. Firstly, there may be two separate neurotransmitter pools in myenteric nerve terminals. A readily releasable pool (RRP) is available for exocytosis during the first action potential in a train and at low stimulation frequencies; a reserve pool will partly replenish the RRP prior to subsequent stimuli (Rosenmund and Stevens, 1996; Pyle et al., 2000; Sudhof, 2000; von Gersdorff and Borst, 2002; Zucker and Regehr, 2002). The release probability of the RRP is very high as a single action potential can deplete this pool. In addition, we never observed PPF whose occurrence is most prominent at low probability synapses (Thomson, 2000; Zucker and Regehr, 2002). At stimulation frequencies >5 Hz, fEPSPs decline in amplitude because transmitter release rate exceeds the RRP refilling rate (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Lin et al., 2001). In myenteric nerve terminals, the refilling rate of the RRP is relatively slow (7 s) and the slow filling rate will limit the duration and frequency at which bursts of fEPSPs can occur. The mechanism by which vesicles are replenished after short-term depletion may vary among synapses with different recovery time constants. For example, the τ for recovery is 5 s at synapses between hippocampal CA3 and CA1 pyramidal neurons (Debanne et al., 1996), 3 s at synapses between cerebellar granule and Purkinje cells, 4 s at the calyx of Held in the brain stem (von Gersdorff et al., 1997), and 2.8 s at synapses in the rat superior cervical ganglion (Lin et al., 2001). The recovery rate depends on trafficking between vesicle pools and the mechanisms of vesicle movement at different synapses may be specialized to meet the particular demands of those synapses. Vesicle mobilization from a reserve pool can be a principal mechanism for synaptic recovery after rundown in the myenteric plexus. Mobilization from the reserve pool is relatively slow (Pyle et al., 2000) and this would account for synaptic rundown during trains of stimulation >5 Hz. A second model that would account for our data involves vesicle recycling (Sudhof, 2004). In this scheme, the RRP is released by the first action potential in a train. At low frequencies of stimulation (0.5 Hz), some of these vesicles recycle and are then available for release prior to the next action potential. At stimulation frequencies >5 Hz, the release rate exceeds the recycling rate and the RRP becomes depleted. Rapid vesicle recycling (τ<1 s) contributes to maintained transmission at synapses in the hippocampus where the recycling rate is rapid enough to maintain transmission at a 10 Hz stimulation frequency (Pyle et al., 2000; Sara et al., 2002; Fernandez-Alfonso and Ryan, 2004). If vesicle recycling occurs in the myenteric plexus, it must occur at a much slower rate than in the hippocampus.

To summarize these studies, trains of electrical stimulation evoke fEPSPs that decline in amplitude in a frequency-dependent manner in the guinea-pig ileum myenteric plexus. Rundown of fEPSPs is not due to postsynaptic receptor desensitization, presynaptic inhibition mediated by G-protein coupled receptors or to changes in presynaptic action potentials. The data presented here are consistent with a model in which there are two pools of neurotransmitter in myenteric nerve endings. There is readily releasable pool of transmitter that has a high release probability; this pool is depleted following a single action potential. At a low stimulation frequency (0.5 Hz), fast

synaptic transmission is maintained either by recycling of released synaptic vesicles or by replenishment of the readily releasable pool by a reserve pool of transmitter. At high frequencies of stimulation (20 Hz), fEPSPs decline in amplitude because the stimulation rate exceeds the rate at which the readily releasable pool can be restored either by recycling or by refilling from a reserve pool.

5-HT_4 RECEPTOR-MEDIATED PRESYNAPTIC REGULATION OF FAST SYNAPTIC TRANSMISSION

This section of my dissertation investigated the regulation of fast synaptic transmission by 5-HT_4 receptors. My data show that 5-HT_4 receptor agonists enhance both fast cholinergic and purinergic synaptic transmission. During a short train of nerve stimulation, activation of 5-HT_4 receptors increased amplitudes of each fEPSPs and prevented fEPSP from declining to zero. But the rundown rate was not altered by 5-HT_4 receptor agonists. Stimulation of 5-HT_4 receptors also accelerated the recovery rate of fast synaptic transmission after rundown. Further study in cultured myenteric neurons indicated that 5-HT_4 receptor agonists promote fast synaptic transmission by increasing neurotransmitter release probability in single varicosities. The mechanism of 5-HT_4 receptor-mediated facilitation of fEPSPs can be summarized in Figure 40.

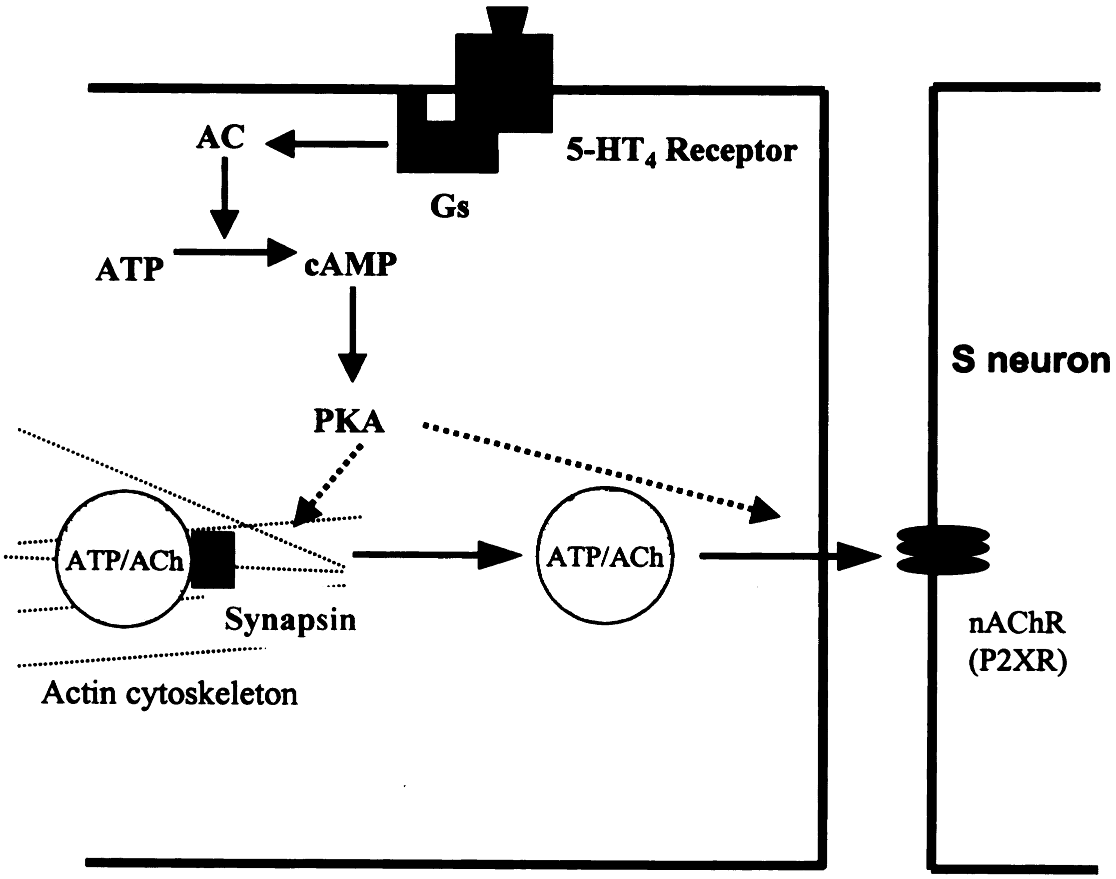
5-HT_4 receptors are localized to nerve terminals of CGRP IPANs (Grider, 2003; Liu et al., 2005). 5-HT_4 receptor stimulation facilitates peristaltic reflexes by increasing release of CGRP and ACh (Grider et al, 1998; Grider, 2003; Gershon, 2004). 5-HT_4 receptors are a target for drug treatment of GI motility disorders. For example, the 5-HT_4 receptor agonist, teagaserod (Zalnorm), is used as prokinetic agent to treat constipation-predominant IBS (IBS-C) and chronic constipation. However, the underlying mechanism is not entirely clear.

As discussed above, fast synaptic transmission occurs in bursts and it declines in response to physiological stimulation. Therefore investigation of the effects of 5-HT_4

Figure 40. A proposed model for 5-HT_4 receptor-mediated presynaptic regulations

Agonists of presynaptic 5-HT_4 receptors activate PKA through stimulating AC and formation of cAMP. PKA phorsphorylates the proteins in the processes of vesicle trafficking and releasing to facilitate neurotransmitter release. The target proteins remain unknown.

Figure 40



AC
5-HT4 Receptor
Gs
ATP
cAMP
S neuron
PKA
ATP/ACh
ATP/ACh
Synapsin
nAChR
(P2XR)
Actin cytoskeleton

receptor agonist on fast synaptic transmission during a train of stimulation could provide more precise information in physiological condition. In addition, I proposed that the rundown of fEPSPs was attributed to depletion of vesicles in readily releasable pool (RRP) and there was no direct proof for it. As 5-HT_4 receptor couples Gs-protein to stimulate adenylate cyclase-cAMP-PKA cascade and synaptic vesicle trafficking and releasing in nerve terminal can be affected by products of this cascade, identification of 5-HT_4 receptor-mediated regulation of fEPSPs rundown and recovery could help to discover more evidence for understanding mechanisms of fEPSP rundown.

Activation of 5-HT_4 receptors attenuates synaptic run-down and accelerates recovery from rundown

Under control conditions, fEPSPs decline to zero amplitude at stimulus trains at 5, 10 and 20 Hz. Renzapride, a selective 5-HT_4 receptor agonist, increases ACh and ATP release elicited by a single shock. Renzapride also increased all fEPSPs elicited by 20 stimuli in a 10 Hz stimulus train. However, the fEPSPs still decline in amplitude. The decline of fEPSPs was also fit by monoexponential equation. This suggests that renzapride acting at 5-HT_4 receptors altered the same process resulting in synaptic rundown. Interestingly, the rundown rates before and after renzapride were similar. As we have discussed above, fEPSP declines during stimulus train and the synaptic rundown is very likely because of the depletion of vesicle pool. Although renzapride increased all fEPSP amplitudes, it did not affect the rate of rundown but maintain fEPSP to a higher non-zero level. In the experiment of examining synaptic recovery, it was observed renzapride extremely reduced the time that takes fEPSP to recover after rundown.

Based on depletion model, synaptic rundown and recovery represent the processes of vesicle trafficking and release (Thakur et al., 2004; Sudhof, 2004; Rizzoli and Betz, 2005). At synaptic terminals of myenteric neurons, the size of readily releasable pool (RRP) is small and/or releasing probability is high since the PPD was observed (Zucker and Regehr, 2002). Activation of 5-HT_4 receptors mobilizes synaptic vesicles in recycling pool and facilitates vesicle trafficking to RRP, which is evidenced by reduced recovery time. The accelerated replenishment of RRP maintains fEPSPs at certain amplitude. However, the time constant of synaptic rundown did not change before and after renzapride treatment. It suggests that activation of 5-HT_4 receptors also stimulates transmitter release or increases releasing probability from a given single releasing site (Schneggenburger et al., 2002).

Postsynaptic current (PSC) is determined by "Npq", where q is postsynaptic quantal size and p is probability of release (Schneggenberger et al., 2002; Zucker and Regehr, 2002). "N" can be the number of vesicles or the number of morphologically defined release sites. In sympathetic neuromuscular junction, transmitters are released from single varicosities and each single varicosity is a synapse with a single active zone (Bennett, 2000). Therefore " N " can be regarded as the number of release sites in the ENS. The increase of transmitter release may be due to increased release probability by enlarged pool size of RRP and/or facilitation of vesicle releasing protein complexes. Because synaptic potentials decline at the similar rate after renzapride treatment, the processes of vesicle trafficking and release should be regulated in an equivalent manner. This result provides an indirect evidence that synaptic rundown is a result of vesicle depletion.

5-HT$_4$ receptor-mediate presynaptic regulation of synaptic transmission is through adenylate cyclase-cAMP-PKA signaling pathway

Significant progress has been made in mechanisms of metabotropic receptor-mediated regulation of transmitter release in the CNS (Varma et al., 2001; Sudhof, 2004; Rizzoli and Betz, 2005) but not many data have been accumulated in the ENS. There are quite a few presynaptic mechanisms to increase synaptic vesicle mobilization and neurotransmitter release (Engelman and MacDermott, 2004). Briefly, two broad models have been proposed: 1) Ca^{2+}-dependent, increase of Ca^{2+} entry presynaptically can promote transmitter release (Brown et al., 2004); and 2) Ca^{2+} independent, the releasing complex including proteins or other molecules can also be regulated to facilitate transmitter release (Blackmer et al., 2001; Takahashi et al., 2001). In both cases, the targets are common: vesicle recycling and vesicle releasing.

My data indicate activation of 5-HT$_4$ receptors stimulates vesicle mobilization and facilitates transmitter release. These regulations were mediated by stimulation of adenylate cyclase and PKA as forskolin mimics renzapride and H-89 abolished effects of renzapride. Because cAMP is a second messenger that activates PKA, cAMP must also be a mediator. PKA can phosphorylate many protein targets, some of which are suggested to regulate vesicle trafficking and release in the CNS (Chavez-Noriega and Stevens, 1994; Weisskopf et al., 1994; Sudhof, 2004). For example, synapsin is a vesicle phosphorprotein in nerve terminals (Chi et al., 2001; Sudhof, 2004). It has been suggested that synapsin functions to bind vesicles to actin cytoskeleton (Sudhof et al., 1989; Chi et al., 2003). Three synapsin subtypes have been identified and all three subtypes have a common N-terminal domain that is a phosphorylation site for PKA.

Phosphorylation of synapsin controls dynamic mobilization of synaptic vesicles to regulate vesicles pool size (Hosaka et al., 1999; Turner et al., 1999). In hippocampal neurons, PKA facilitates transmitter release by acting directly at the proteins of the neurotransmitter releasing machinery (Capogna et al., 1995; Trudeau et al., 1996). SNAP-25 is a target for PKA phosphorylation to regulate transmitter release (Nagy et al., 2004). Another example is Snapin, a SNAP-25 binding protein. Snapin has been shown a target that can be phosphorylated by PKA to increase transmitter release in hippocampal neurons (Thakur et al., 2004). Here my data show that activation of PKA is necessary for 5-HT_4 receptors to facilitate transmitter release and synaptic recover. Although the targets in myenteric nerve terminals for PKA need further identification, 5-HT_4 receptor-activated PKA must affect the processes of both transmitter mobilization and release.

Activation of 5-HT_4 receptors increases probability of transmitter release from single varicosities

The studies of 5-HT_4 receptor-mediated synaptic regulation were carried out in acutely isolated LMMP preparations. Electrical nerve stimulation used to evoke synaptic responses activates multiple nerve fibers instead of a single one. Under such a recording situation, fast synaptic potential or current represents the neurotransmitter released from multi-nerve endings/releasing sites. Different from the CNS, neurotransmitter in the ANS is released from single varicosities. For example, sympathetic nerves end in strings of varicosities, which form individual synapses with smooth muscle cells. In the CNS, each nerve terminal has multi-releasing sites (active zones). The Gaussian distribution of spontaneous release recorded from a nerve terminal in the CNS is actually an indication of transmitter release from multiple active zones. However, in the autonomic nervous system, each varicosity has one active zone (Bennett, 2000). Studies of synaptic potentials from varicosities in the vas deferens indicated that the neurotransmitter release from a single varicosity is positively skewed and transmitter release at different varicosities has a nonuniform probability (Bennett, 1998; Bennett et al., 1995; Brain et al., 2002) and therefore transmitter release from in varicosities is highly intermittent (Brain et al., 2002; Bennett, 1996). This intermittent is not due to intermittent failure of action potential propagation in the nerve terminal (Jackson et al., 2001; Bennett, 1996). To identify how 5-HT_4 receptor stimulation increases transmitter release in the ENS, it is necessary to investigate the transmitter release from one single varicosity.

Varicosities can be visually identified docking along the processes of myenteric neurons maintained in primary culture. Cultured myenteric neurons express P2X and nACh receptors. The synaptic responses are also maintained. "Detector patch" technique can be utilized in the cultured neurons to monitor single varicosity activity. In my experiments, a patch containing P2X or/and nACh receptors was used as a detector. For one given single varicosity, there was no spontaneous current observed in detector patch. This does not necessarily mean there is no spontaneous release. The sensitivity in the experimental setup could limit observation of current induced by spontaneous release. However, electrical stimulation evoked transmitter release and the evoked release probability is 27%. Application of 5-HT_4 receptor agonists, renzapride and tegaserod increased evoked release probability to 50%. The increase of release probability in the given varicosity may be due to an enlarged RRP size or to a direct regulation of proteins in release machinery or both. As discussed above, the increase of release probability is through PKA phosphorylation of proteins in processes of trafficking and releasing.

Studies in neuromuscular junction led to a classic model of quanta release and this model has been widely accepted to be true also in the CNS. The Katz's model defined the number of release site (N), probability of transmitter release (p) and quantal size (q) (del Castillo and Katz, 1954; Dittman et al., 2000).

Considering the equation (Schneggenburger et al., 2002):

PSP=npq; where

PSP: postsynaptic potential;

n: the number of releasing sites (varicosities or active zones);

p: probability of release from one releasing site (varicosity or active zone);

q: postsynaptic quantal size.

Postsynaptic responsive quantal size is independent of release probability and p is the only factor responsible for short-term synaptic plasticity (Biro et al., 2005). In the ANS, a varicosity can be considered as equivalent to an active zone in one nerve terminal since it is one releasing site. Activation of 5-HT_4 receptors increases release probability (p) from the single release sites. All varicosities sitting in the same nerve fiber and in the range that postsynaptic receptors can be activated can be regarded as the different releasing sites in one "terminal". The transmitter release from multi-release sites is heterogeneous upon arrival of an action potential. This release is at low probability.

Renzapride or tegaserod acts at 5-HT_4 receptors to enhance "p" value by activating PKA and therefore increases PSP.

SUMMARY AND RESEARCH SIGNIFICANCE

In this dissertation, I have identified P2X receptor subtypes in myenteric neurons in the small intestine of guinea pigs and mice. I have found a novel calcium-dependent potassium channels that underlie afterhyperpolarization induced by P2X receptor agonists. I have characterized fast synaptic transmission elicited by stimulus train. I have also investigated regulation of fast synaptic transmission mediated by presynaptic 5-HT_4 receptors and identified the underlying mechanism. All these issues were addressed around a central topic: the dynamic properties of fast synaptic transmission.

In guinea pig small intestine, myenteric S neurons express $P2X_3$ containing receptors while AH neurons express $P2X_2$ homomers. However, in mouse small intestine, myenteric S neurons express $P2X_2$ homomers; AH neurons express $P2X_3$ subunit-containing receptors. This difference may be due to the difference in species, which implies the possible different function of these myenteric neurons. $P2X_3$ receptors in half number of guinea pig myenteric neurons mediate an after-hyperpolarization, which is mediated by a novel calcium-dependent potassium conductance. But its physiological function is not clear.

Fast synaptic transmission is depressed during a short stimulus train at 5, 10 and 20 Hz. Synaptic depression is frequency dependent. There is no postsynaptic receptor desensitization, presynaptic inhibition or action potential failure for the depression. Synaptic vesicle depletion is proposed as a mechanism. The synaptic depression can be attenuated by activation of 5-HT_4 receptors. Activation of 5-HT_4 receptors also

accelerates synaptic recovery after depression. PKA has been indicated as a mediator for 5-HT_4 receptor-mediated regulations. The targets for PKA remain unknown. Further study in cultured myenteric neurons indicates that 5-HT4 receptor agonists facilitate transmitter release by increasing release probability from single varicosities.

All these findings are summarized in Figure 41.

The significance of my findings can be discussed from two aspects: neurobiology and gastroenterology.

Neurobiology focuses more on the neural basis of behavior. How the neurons process information to generate a corresponding behavior is a fundamental and also an ultimate question for neurobiologists. The ENS underlies GI behaviors. So one of my goals is to understand the way enteric neurons control GI behaviors. Synaptic encoding is one of mechanisms by which neurons process input signals. For example, synaptic depression has been shown to be a mechanism that regulates synaptic activity in the CNS. My data show there is also synaptic depression in the ENS, which probably has a similar function in limiting synapses from over activity. Vesicle depletion is the mechanism for this regulation. This is also a target for presynaptic modulation such as by presynaptic 5-HT_4 receptors.

In the view of gastroenterology, the pathological change of GI functions draws more concerns. Gastroenterologists would like more to look into the neuropathology of GI and pursue a best therapy for GI disorders. Understanding the fundamental properties of the ENS will provide the basis to identify neurological

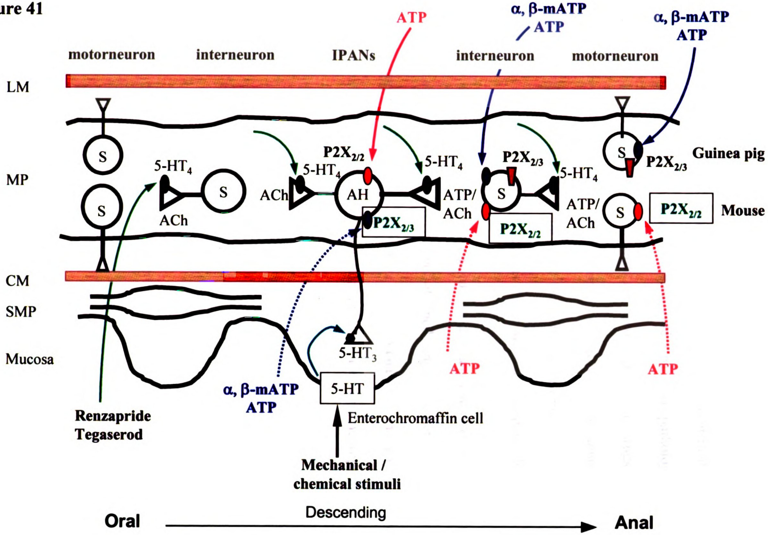
Figure 41. A summary for receptors studied in this dissertation in myenteric neurons

In myenteric plexus, synaptic transmission is polarized as shown in the figure. Synaptic activation is coordinated with oral contraction and anal relaxation, which initiates peristalsis. Receptors are expressed accordingly to accomplish the functions. P2X receptors primarily mediate descending pathway (the anal direction). In guinea pig small intestine, myenteric AH neurons express $P2X_{2/2}$ homomers and S neurons express $P2X_{2/3}$ heteromers. However, in mouse small intestine, myenteric AH neurons express $P2X_{2/3}$ heteromers and S neurons express $P2X_{2/2}$ homomers as pointed by dashed lines. In guinea pig small intestine, fifty percent myenteric S neurons also express calcium-activated potassium channels, which mediate an afterhyperpolarization. 5-HT_4 receptors are expressed in nerve terminals. 5-HT_4 receptor agonists, renzapride or tegaserod increases fEPSP by increasing synaptic transmitter release probability.

5-HT_4 receptors; $P2X_{2/2}$ homomers; $P2X_{2/3}$ heteromers; Calcium-activated potassium channels; The dashed arrows indicate receptors in boxes activated by the agonists in mouse tissue.

LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SMP, submucosal plexus; IPANs, intrinsic primary afferent neurons.

Figure 41


ATP
α, β-mATP
ATP
α, β-mATP
ATP
motorneuron
interneuron
IPANs
interneuron
motorneuron
LM
MP
CM
SMP
Mucosa
5-HT4
ACh
ACh
5-HT4
P2X2/2
AH
P2X2/3
5-HT4
ATP/
ACh
P2X2/3
P2X2/2
5-HT4
ATP/
ACh
P2X2/3
Guinea pig
P2X2/2
Mouse
5-HT3
5-HT
ATP
ATP
α, β-mATP
ATP
Renzapride
Tegaserod
Enterochromaffin cell
Mechanical /
chemical stimuli
Oral
Descending
Anal

disorders and facilitate the treatment. As shown in my data, P2X receptor subtypes are expressed differentially in the different types of myenteric neurons. So P2X receptor subtypes can be selected as potential drug target. 5-HT_4 receptor-mediated dynamic changes of transmitter trafficking and release provide a basis for pathology of some GI motility disorders as 5-HT_4 receptor agonists are used as prokinetic drugs. For example, the patients who suffer from GI motility disorders probably have an abnormal alteration of vesicle trafficking and releasing processes in the myenteric nerve terminals. Meanwhile this also offers more opportunities to find ways to treat these diseases.

Finally, the ENS represents a unique case in the mammalian nervous system. It is a highly isolated system and controls a well-defined range of functions. Therefore the ENS can be considered as a very appropriate model to challenge many questions in the area of fundamental neuroscience and therefore help to understand neuropathology of GI disorders.

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