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A novel calcium-sensitive potassium conductance is coupled to P2X₃ subunit containing receptors in myenteric neurons of guinea pig ileum

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Abstract

This study characterized P2X receptors in guinea pig ileum myenteric S neurons (n=112) *in vitro* using electrophysiological methods. ATP or α,β -methylene ATP (α,β -mATP), an agonist at P2X₁ and P2X₃ subunit containing receptors depolarized 95 neurons (85%). PPADS (10 μ M) blocked ATP- and α,β -mATP-induced depolarizations. ATP-induced depolarizations and fast excitatory postsynaptic potentials (fEPSPs) were reduced by TNP-ATP (10 μ M), an antagonist that can block P2X₃ receptors. Ivermectin (10 μ M), a modulator of P2X₄ and P2X_{4/6} receptors, had no effect on α,β -mATP-induced depolarizations. In 58% of neurons, the α,β -mATP induced-depolarization was followed by an afterhyperpolarization (P2X-afterhyperpolarization). Under voltage clamp, α,β -mATP induced an inward current followed by an outward current which reversed polarity at 0 and –80 mV, respectively. The P2X-afterhyperpolarization was reduced in low extracellular Ca²⁺ solutions. Blockers of large, intermediate and small conductance Ca²⁺-activated K⁺ channels or voltage-gated K⁺ channels did not inhibit the P2X-afterhyperpolarization. Half of the neurons exhibiting the P2X-afterhyperpolarization contained nitric oxide synthase (NOS)-immunoreactivity (ir). In summary, NOS-ir S neurons express P2X₃ subunit containing P2X receptors. P2X receptors couple to activation of a Ca²⁺-activated K⁺ conductance that mediates an afterhyperpolarization. As P2X receptors contribute to fEPSPs, the P2X-afterhyperpolarization may modulate S neuron excitability during purinergic synaptic transmission.

Keywords

Enteric nervous system; electrophysiology; ion channels

P2X receptors are ligand-gated cation channels activated by ATP (1) and they are composed of combinations of one or more of seven subunits (P2X_{1–7})(2,3,4). The subunit composition determines the electrophysiological and pharmacological properties of the P2X receptor. α,β -Methylene ATP (α,β -mATP) selectively activates P2X₁, P2X₃ or P2X_{2/3} receptors; while trinitrophenyl-ATP (TNP-ATP) is an antagonist of P2X₁, P2X₃ or P2X_{2/3} receptors. Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) is an antagonist that blocks P2X receptors that contain, P2X₁, P2X₂, P2X₃ and P2X₅ subunits. P2X₁ and P2X₃ receptors desensitize quickly while P2X₂ containing receptors desensitize slowly (2,5). All P2X receptor subtypes are Ca²⁺ permeable to some extent (6,7).

Myenteric neurons are classified as AH or S neurons based on their electrophysiological properties (8,9). In longitudinal muscle, myenteric plexus preparations (LMMP), electrical stimulation of nerve fibers elicits fast excitatory post-synaptic potentials (fEPSP) from S neurons (8,10). P2X receptors contribute to fast synaptic excitation in most S neurons (11, 12,13,14). Action potentials in S neurons are blocked completely by the voltage-gated sodium channel antagonist, tetrodotoxin, while action potentials in AH neurons have a calcium-mediated component and an afterhyperpolarization (AHP) that lasts 1–10 seconds (8,15). Somatic action potentials in AH neurons are only partly reduced by TTX. The long lasting action potential AHP in AH neurons is mediated by an intermediate conductance Ca^{2+} -activated K^{+} channel (15,16) and the AHP limits the firing rate of AH neurons. AH neurons express P2X receptors in the soma and nerve terminals. Nerve terminal localization of P2X receptors is supported by data from *in vitro* studies that showed that application of ATP to mucosal villi elicits antidromic action potentials in AH type neurons. These results led to the conclusion that P2X receptors might contribute to detection of mucosal stimuli by myenteric AH neurons (17,18).

Data from patch clamp recordings from guinea pig small intestinal myenteric neurons maintained in primary culture indicate that most of these neurons express homomeric P2X₂ receptors while a small fraction of neurons also express P2X₃ subunit containing receptors (19). Pharmacological studies in acutely dissected LMMP preparations of guinea pig ileum indicate that α,β -mATP-sensitive P2X receptor subtypes (P2X₁ or P2X₃) as well as P2X₂ subunit containing receptors are expressed in S neurons (12). Finally, immunohistochemical data from LMMP preparations from the rat (20) and guinea pig ileum (21,22,23) show that P2X₂ and P2X₃ subunits are expressed by some myenteric neurons. Despite these data, there is no clear evidence to indicate the specific subunit composition of P2X receptors that contribute to synaptic transmission in the adult guinea pig ileum myenteric plexus. Therefore, the goal of this study was to use a combined electrophysiological and pharmacological approach to characterize functional P2X receptors in myenteric neurons in the acutely isolated LMMP preparation. This preparation preserves most synaptic connections present in the intact animal and the properties of the neurons in this tissue are likely to be similar to those present *in vivo*.

METHODS

Tissue preparation

Animal protocols were approved by the Institutional Animal Use and Care Committee at Michigan State University. Male guinea pigs weighing 250–350 g were anesthetized *via* halothane inhalation, stunned and exsanguinated by severing the major neck blood vessels. An ileal segment was harvested 15–20 cm proximal to the ileocecal junction 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 contained nifedipine (1 μM) to block L-type calcium channels and scopolamine (1 μM) to block muscarinic cholinergic receptors and to inhibit longitudinal muscle contractions. A longitudinal muscle-myenteric plexus (LMMP) preparation was made using standard dissection procedures. The LMMP preparation was pinned to the bottom of a recording chamber 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.

Intracellular electrophysiological recording

Individual myenteric ganglia were visualized at 200 $\times$ magnification using an inverted microscope (Olympus CK-2) with differential interface contrast optics (Hoffman Modulation Contrast). Intracellular recordings were using glass microelectrodes filled with 2 M KCl (tip

resistance, ~100 M Ω). In some experiments, electrodes were filled with Neurobiotin tracer (N-(2-aminoethyl)biotinamide hydrochloride, Vector Laboratories, Burlingame, CA) at 30 mg/ml in 2 M KCl. Cells were filled with tracer by passing repeated 200 ms - 0.5 nA current pulses through the recording microelectrode after pharmacological and physiological studies were completed in that neuron. Preparations containing Neurobiotin-filled neurons were processed for immunohistochemistry in order to identify the neurochemical phenotype of filled neurons (see below). An amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA) was used to record membrane potentials in bridge mode and to inject current. Changes in membrane resistance occurring during drug responses were determined by measuring the amplitude of voltage changes caused by 0.1 nA, 200 ms duration hyperpolarizing current pulses. When recording fEPSPs, the membrane potential was adjusted to -70 mV using constant DC current to avoid generating action potentials. A digital average of eight fEPSPs was used to measure the amplitude of responses before and after drug treatments. Synaptic responses were evoked using a glass pipette (tip diameter 40–60 μ m) filled with Krebs' solution as a focal stimulating electrode. The stimulating electrode was positioned over an interganglionic nerve strand supplying the ganglion containing the impaled neuron. Signals were filtered at 1 kHz using a four-pole, low-pass Bessel filter (Warner Instruments, Hamden, CT), and digitized at 5 kHz using Digitdata 1320 analog/digital converter (Axon Instruments, Foster City, CA). Data were acquired and analyzed using Axoscope 8.2 software (Axon Instruments) and a desktop computer. In some experiments, single electrode voltage clamp (SEVC) techniques were used to record currents caused by local application of α , β -mATP when the membrane potential of impaled neurons was clamped at different holding potentials. In SEVC studies, the switching rate was adjusted to 3 kHz and the gain setting was 3–5. Drug-induced changes in membrane conductance during SEVC experiments were determined by measuring the amplitude of current responses caused by 10 mV, 200 ms duration hyperpolarizing voltage steps.

Immunohistochemistry

LMMP preparations were 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 dimethylsulfoxide 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. Preparations were incubated overnight with a primary antibody against nitric oxide synthase (NOS) at room temperature. Tissues were washed three times at 10 min intervals with PBS and then incubated (1.5 h at 23°C) with goat anti-rabbit IgG (1:40 in PBS; Jackson Immunoresearch Laboratories, West Grove, PA, USA) conjugated to fluorescein isothiocyanate (FITC) to reveal NOS immunoreactivity (ir) and Texas Red-avidin (1–200 dilution) to reveal Neurobiotin-filled neurons. Tissues were washed three times with PBS and mounted in buffered glycerol for fluorescence microscopy. Images were obtained using Nikon Eclipse TE2000-U microscope (Tokyo, Japan) a 40 X objective, a Cool Snap ES camera (Photometrics) and processed using Metaimaging Series 6.1 and Adobe Photoshop 8.0 software.

Drug application

Antagonists were applied by addition to the superfusing Krebs' solution for 5–20 minutes prior to measuring the amplitude of evoked responses. ATP or α , β -mATP (each at 1 mM) were applied by ejection from a micropipette (~20 μ m tip diameter) placed within 150 μ m of the impaled neuron. Agonists were applied using short pulses of nitrogen gas (3–35 ms, 10 p.s.i.) applied by a Picospritzer II (General Valve, Fairfield, NJ, USA).

Drugs

All drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO). All reagents and drugs were diluted in deionized water except for nifedipine and clotrimazole, which were dissolved in 95% ethanol to make a 10 mM concentrated stock solution. The final working concentration of all drugs was made daily by diluting concentrated solutions in Krebs' solution.

Statistics

All data are mean $\pm$ SEM and "n" values indicate the number of neurons from which the data were obtained. Student's t-test for paired data or analysis of variance were used to establish significant differences between control and treatment groups. A P value < 0.05 was considered statistically significant.

RESULTS

ATP and α,β -mATP depolarize myenteric neurons

Data were obtained from 124 S neurons; 103 of these neurons (83%) were depolarized by either ATP or α,β -mATP, an ATP analog that is an agonist at P2X₁ and P2X₃ subunit containing receptors (Fig. 1A). The remaining 21 neurons (17%) were insensitive to ATP or α,β -mATP. In this latter group of neurons, fEPSPs were cholinergic as mecamylamine (10 μ M), a nicotinic cholinergic receptor antagonist, completely blocked the fEPSP. The mean amplitude of the ATP and α,β -mATP-induced depolarization in the same neurons (n = 12) were similar (Fig. 1B) and these responses were blocked by PPADS (10 μ M)(Fig. 1C).

Pharmacological identification of P2X receptors in myenteric S neurons

As S neurons were excited by α,β -mATP it is likely that these cells express receptors that contain either P2X₁ or P2X₃ subunits. To confirm the presence of P2X₁ or P2X₃ subunits, TNP-ATP (10 μ M), an antagonist that can block P2X₁, P2X₃ or P2X_{2/3} receptors (5), was added to the recording bath solution to test the sensitivity of fEPSPs and agonist-induced depolarizations to this antagonist. TNP-ATP reversibly reduced the amplitude of fEPSPs recorded from S neurons (Fig. 2). TNP-ATP also reversibly reduced the amplitude of α,β -mATP-induced depolarizations (Fig. 3). These data provide pharmacological evidence that S neurons express P2X₁ and/or P2X₃ subunits. Additional data that support this conclusion come from studies using ivermectin which potentiates responses mediated by P2X₄ subunit containing receptors (24). It was found that ivermectin (10 μ M) did not affect α,β -mATP-induced depolarizations in any S neuron tested. The control depolarization was 12.7 ± 3.9 mV while the depolarization in the presence of ivermectin was 13.5 ± 2.7 mV (n = 5, P > 0.05).

α,β -mATP-induced depolarization is followed by a hyperpolarization in S neurons

An unexpected finding was that in 60 of 103 (58%) S neurons tested, the ATP or α,β -mATP-induced depolarization was followed by an afterhyperpolarization (Fig. 4A); this response will be called the P2X-afterhyperpolarization. Changes in membrane resistance were assessed during the P2X-mediated responses by measuring the amplitude of voltage responses caused by brief intracellular hyperpolarizing current injections passed through the recording microelectrode. Membrane resistance decreased during both the depolarization and the P2X-afterhyperpolarization as indicated by a decline in the amplitude of these voltage transients (Fig. 4A). These data suggest that ion channels were opening during both the depolarization and P2X-afterhyperpolarization. The amplitudes of the depolarization and P2X-afterhyperpolarization were positively correlated (Fig. 4B).

The ionic mechanism underlying P2X-afterhyperpolarization in S neurons

We next determined if the P2X-afterhyperpolarization was mediated by activation of a K^+ channel. This was accomplished by using single electrode voltage clamp to measure inward and outward currents caused by P2X receptor activation (Fig. 4C). Membrane conductance increased during both responses as indicated by an increase in the amplitude of current responses caused by hyperpolarizing voltage commands applied before and during α,β -mATP-induced responses (Fig. 4C). This result is consistent with ion channels opening during these responses. However, unlike the data obtained under current clamp conditions, voltage clamp measurements showed that the amplitudes of the inward and outward currents caused by α,β ATP were not correlated (Fig. 4D). Measurements of inward and outward currents caused by α,β -mATP showed that their current-voltage relationships were linear in the range of membrane potentials studied (Fig. 5). The inward and outward currents had reversal potentials of 0.5 ± 2.5 mV and -80 ± 1.3 mV ($n = 6$), respectively. These values suggest that the inward current was carried by cations, including Ca^{2+} , through P2X receptors while the outward current was mediated by a K^+ channel as the reversal potential is near the K^+ equilibrium potential (-85 mV).

The depolarization caused by α,β -mATP could activate voltage-sensitive Ca^{2+} channels with subsequent activation of a Ca^{2+} sensitive channel which mediates the P2X-afterhyperpolarization. However, the voltage clamp data described above indicate that activation of voltage-gated Ca^{2+} channels is not required for the biphasic response as α,β -mATP caused an inward current followed by an outward current which would underlie the depolarization and hyperpolarization, respectively (Fig. 4C). P2X receptors are Ca^{2+} permeable ion channels and Ca^{2+} can enter neurons directly through these channels under voltage clamp conditions. In order to test for a Ca^{2+} -dependence of the P2X-afterhyperpolarization, we reduced the extracellular Ca^{2+} concentration from 2.5 mM to 0.25 mM. This treatment blocked fEPSPs (Fig. 6A) recorded from the same S neurons and slightly reduced the amplitude of the α,β -mATP-induced depolarization (Fig. 6B,C). However, a 10-fold reduction in extracellular Ca^{2+} blocked the P2X-afterhyperpolarization (Fig. 6B,C).

The data summarized above suggest that the P2X-afterhyperpolarization could be mediated by a Ca^{2+} -activated K^+ channel. Three types of Ca^{2+} -activated K^+ channel have been identified: large conductance (BK), intermediate conductance (IK) and small conductance (SK) Ca^{2+} -activated K^+ channels. Iberitoxin (IBTx, 0.1 μ M) selectively blocks BK channels, clotrimazole (10 μ M) blocks IK channels while apamin (0.1 μ M) blocks SK channels (24, 25). None of these blockers affected the P2X-afterhyperpolarization (Fig. 7).

The data described above indicate that the P2X-afterhyperpolarization was mediated by activation of a K^+ channel but blockers of known Ca^{2+} -activated K^+ channels did not alter this response. The next experiments were designed to determine if voltage-gated K^+ channels contributed to the P2X-afterhyperpolarization. In these studies, tetraethylammonium (TEA, 10 mM) and 4-aminopyridine (4-AP, 1 mM), two antagonists which block a number of voltage-gated K^+ channels, were used. Neither 4-AP (Fig. 8A,B) or TEA (Fig. 8C) changed significantly the depolarization or P2X-afterhyperpolarization caused by local application of ATP.

Immunochemical characterization of S neurons with the P2X-afterhyperpolarization

We tested the hypothesis that nitric oxide synthase (NOS) immunoreactivity (ir) would be a marker of neurons exhibiting the P2X-afterhyperpolarization. Recordings were obtained from 15 neurons using Neurobiotin-filled microelectrodes and tissues were processed for NOS-ir after electrophysiological studies. Three neurons did not respond to α,β -mATP; these neurons did not contain NOS-ir. Five neurons exhibited the P2X-afterhyperpolarization; 3 contained

NOS-ir and 2 did not. Seven neurons were depolarized by α,β -mATP without the P2X-afterhyperpolarization; 4 of these neurons contained NOS-ir and 3 did not (Fig. 9). These data indicate that there is not a specific association between NOS-ir and expression of the ion channel mediating the P2X-afterhyperpolarization. However, the data also indicate that about half of NOS-ir neurons will express the P2X-afterhyperpolarization.

Discussion

P2X receptor subtypes in myenteric neurons

Immunohistochemical studies have identified P2X receptor subunits expressed by rat (20) and guinea pig myenteric neurons (21,22,23) and P2X receptors contribute to fEPSPs in descending pathways in the guinea pig small intestine (27,28,29) and these fEPSPs are mediated α,β -mATP-sensitive P2X receptors (P2X₁ or P2X₃)(27). Data from the present study show that most S-type neurons are excited α,β -mATP and some fEPSPs are inhibited by TNP-ATP, an antagonist that blocks P2X₃ subunit containing receptors (5). Taken together, these data indicate that many S neurons in the guinea-pig ileum myenteric express P2X₃ subunit-containing receptors.

NOS-ir is a marker for descending interneurons and inhibitory motoneurons in the guinea pig intestine myenteric plexus (30). We used combined intracellular dye-labeling and immunohistochemical methods to determine if neurons excited by α,β -mATP contained NOS-ir. It was found that 7 of 12 neurons excited by α,β -mATP were NOS-ir. These neurons would be either descending interneurons or inhibitory motoneurons. Previous immunohistochemical studies have shown that P2X₂ and P2X₃ subunits are localized together in NOS-ir inhibitory motoneurons (21,22). Therefore, the NOS-ir neurons excited by α,β -mATP identified in our study are probably inhibitory motoneurons which express heteromeric P2X₂/P2X₃ receptors (22). There were neurons (5 of 12) that were excited by α,β -mATP but that did not contain NOS-ir. P2X₃ subunits have been localized to NOS-negative ascending interneurons and excitatory motoneurons in the guinea pig ileum myenteric plexus (22) and to neurons that contain the calcium binding proteins, calretinin and calbindin in the rat ileum myenteric plexus (20). As α,β -mATP sensitivity is a property of P2X₃ subunit containing receptors (5), the NOS-negative neurons that were excited by α,β -mATP are likely to be ascending interneurons or excitatory motoneurons. These neurons express P2X₃ but not P2X₂ subunits, so the receptors in ascending interneurons or excitatory motoneurons are P2X₃ homomeric receptors. This conclusion is based only on immunohistochemical data as there are no drugs which can reliably discriminate among responses mediated by P2X₃ homomeric from P2X₂/P2X₃ heteromeric receptors.

The pharmacological and immunohistochemical data discussed above indicate that P2X₃ subunits contribute to P2X receptors in rat and guinea pig ileum myenteric neurons. However, previous data from whole-cell patch-clamp recordings obtained from guinea pig small intestinal myenteric neurons maintained in primary culture revealed that while ATP caused an inward current in more than 80% of neurons studied, α,β -mATP caused an inward current in only a small subset (10 %) of neurons (19). These data led to the conclusion that most guinea pig intestinal myenteric neurons expressed P2X₂ homomeric receptors. Data from electrophysiological and calcium imaging studies of myenteric neurons from neonatal rats also indicate that these neurons express homomeric P2X₂ receptors (31). The patch clamp studies were done on neurons obtained from newborn guinea pigs or rats, while data obtained in the present study were obtained in adult myenteric neurons in the acutely isolated LMMP preparation. It is possible that there are developmental changes in P2X receptor subunit composition where P2X₂ homomeric receptors predominate in neonates while P2X₂ homomeric and/or P2X₂/P2X₃ heteromeric receptors predominate in adult guinea pigs. However, developmental studies of P2X₃ receptor expression in the myenteric plexus of the

rat stomach have shown that there is a postnatal decline in P2X₃ subunit expression. In these studies 45% of myenteric neurons express P2X₃ subunits at time up to postnatal day 14 but this percentage decline to about 11% two months after birth (32). It is also possible that tissue culture conditions may lead to a change in P2X receptor subunit composition in myenteric neurons.

There are also species differences in the subunit composition of P2X receptors expressed by myenteric neurons. Studies done in acutely isolated LMMP preparations obtained from P2X₂ subunit gene knockout mice showed that all fEPSPs were blocked completely by mecamylamine, a nicotinic cholinergic receptor antagonist. However, fEPSPs recorded from neurons in preparations from wild type mice were blocked only by combined application of mecamylamine and PPADS (33). When the same studies were done in preparations from P2X₃ subunit knockout mice it was found that fEPSPs were unaffected by P2X₃ subunit gene deletion (34). Therefore, murine small intestinal myenteric neurons express P2X₂ homomeric receptors (33,34).

Hyperpolarization induced by P2X receptors in myenteric S type neurons

An important finding from the present study was that the depolarization induced by ATP or α,β -mATP was followed by a hyperpolarization in more than half of S neurons studied. Both responses required P2X receptor activation as they were activated by α,β -mATP and blocked by PPADS. The hyperpolarization was mediated by activation of a K⁺ conductance because the reversal potential of its underlying outward current was near the K⁺ equilibrium potential. We conclude that the K⁺ conductance is gated by Ca²⁺ for two reasons. Firstly, a 10-fold reduction of the extracellular Ca²⁺ concentration blocked the P2X-afterhyperpolarization while having only a modest effect on the depolarization. Secondly, P2X receptors are Ca²⁺ permeable (6,7,31) and we used voltage clamp methods to record α,β m-ATP-induced inward and outward currents that correspond to the depolarization and subsequent hyperpolarization. Using SEVC, contributions of voltage-gated Ca²⁺ channels would be eliminated so Ca²⁺ would only enter neurons directly through the P2X receptor in order to activate the K⁺ channel. While we did not find a correlation between the amplitudes of inward and outward currents, the amplitudes of hyperpolarization and depolarization recorded under current clamp conditions were correlated positively. The positive correlation of the depolarization and P2X-afterhyperpolarization is based on the following proposed scheme. During the depolarization caused by P2X receptor activation, Ca²⁺ enters the neurons directly through the P2X receptors and also through voltage-gated Ca²⁺ channels. Larger depolarizations will activate more voltage-gated Ca²⁺ channels allowing more Ca²⁺ to enter the neuron. The higher intracellular Ca²⁺ concentrations that result from large depolarizations will open more Ca²⁺ activated K⁺ channels leading to a larger P2X- afterhyperpolarization.

We then attempted to identify the subtype of Ca²⁺-activated K⁺ channel that mediates the P2X-afterhyperpolarization in S type neurons. Large conductance (BK) and small conductance (SK) Ca²⁺-activated K⁺ channels are expressed throughout the nervous system (25,35,36). Intermediate conductance (IK) Ca²⁺-activated K⁺ channels are expressed by smooth muscle cells (25) and by enteric neurons (15,16) as IK channels mediated the action potential AHP in myenteric AH neurons (15,16,37). We used antagonists of BK (iberiotoxin), SK (apamin) and IK (clotrimazole) channels at concentrations that are effective in blocking the target channels (26). None of the toxins inhibited the P2X-afterhyperpolarization suggesting that a novel Ca²⁺-activated K⁺ channel mediates this response.

A previous study showed that a depolarization induced by activation of nicotinic acetylcholine receptors (nAChRs) is followed by a hyperpolarization in myenteric S neurons (38). These authors also concluded that the acetylcholine-gated afterhyperpolarization was due to activation of an unidentified Ca²⁺-activated K⁺ channel. Action potentials in S neurons are not

associated with long-lasting afterhyperpolarizations and even trains of action potentials, which cause a substantial rise in intracellular Ca^{2+} , elicit only small afterhyperpolarizations (39). This suggests that there may be a spatial relationship between P2X, nAChRs and the unidentified Ca^{2+} -activated K^{+} channel such that Ca^{2+} entering some S neurons through P2X receptors and/or nAChRs has access to the K^{+} channel while action potential dependent Ca^{2+} entry does not. Support for this proposed arrangement comes from studies showing that P2X and nAChRs may be closely clustered in some neurons, including myenteric neurons (40,41). It is unclear if P2X receptors and nAChRs form a multi-receptor cluster alone or if other proteins contribute to this complex. Close coupling of a Ca^{2+} -activated K^{+} channel to the proposed P2X-nAChR cluster would account for the selective activation of the K^{+} channel by Ca^{2+} entering the neuron through P2X receptors or nAChRs. There is precedent for selective close spatial coupling of Ca^{2+} permeable channels with specific Ca^{2+} -activated K^{+} channels in neurons. For example, L-type Ca^{2+} channels cluster with SK channels while N-type calcium channels cluster with BK channels in hippocampal neurons (42). This leads to selective activation of the K^{+} channel only by Ca^{2+} entering the neuron through the closely clustered Ca^{2+} channel.

Functional significance of the P2X-afterhyperpolarization in myenteric S neurons

Ca^{2+} -activated K^{+} channels play an important role in synaptic integration, pacemaking and bursting behaviors throughout the nervous system (43). It is possible that the P2X- (and nAChR)-afterhyperpolarization or its underlying conductance provides a mechanism for limiting the extent of postsynaptic excitation during bursts of synaptic activity. Aftercurrents have not been detected following trains of fast synaptic currents in myenteric neurons (44). However, the increase in conductance associated with activation of the Ca^{2+} -activated K^{+} channel may act as an electrical shunt to limit action potential firing during bursts of synaptic input mediated by P2X and nAChRs. Detailed studies of the function of the P2X-afterhyperpolarization will require identification of the specific Ca^{2+} -activated K^{+} channel mediating this response and the use of drugs which can modify selectively its function.

Summary and Conclusion

Most myenteric S neurons in guinea pig small intestine have P2X receptors that contain P2X_3 subunits while half of these S neurons contained NOS-ir. As nitric oxide is an inhibitor neurotransmitter to the muscle layers in the intestine, some P2X_3 subunit containing receptors are localized to inhibitory motoneurons. Activation of P2X_3 containing receptors in about half of the S neurons studied caused a depolarization followed by an afterhyperpolarization. The P2X-afterhyperpolarization is mediated by an as yet unidentified Ca^{2+} -activated K^{+} channel. The P2X-afterhyperpolarization may be a mechanism by which S neurons control their excitability during purinergic synaptic excitation.

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Figure 1.

Depolarization induced by α , β -mATP and ATP in S neurons. A, Depolarizations caused by local application of ATP (1 mM) and α , β -mATP (1 mM) were followed by an afterhyperpolarization. Arrow heads indicate α , β -mATP and ATP application. B, Depolarization caused by α , β -mATP and ATP in the same S neurons ($n = 12$) were similar in amplitude. Data are mean $\pm$ SEM. C, Responses caused by blocked by α , β -mATP and ATP were completely blocked by PPADS (10 μ M).

Figure 2. Effect of TNP-ATP on fEPSP in S neurons. A, Recordings show that TNP-ATP (10 μ M) reversibly reduced fEPSP amplitude. B, Mean data $\pm$ SEM from experiments ($n = 4$) similar to that shown in A. *indicates significantly different from control ($P < 0.05$).

Figure 3. Effect of TNP-ATP (10 μ M) on depolarizations caused by ATP (1 mM) in S neurons. A, Recordings show that TNP-ATP reversibly inhibits ATP-induced depolarizations. Arrow heads indicate ATP application. B, Data are mean $\pm$ SEM from experiments (n=7) similar to that shown in A. *indicates the significant different from control and after wash groups.

Figure 4.

α, β -mATP caused by biphasic response in S neurons. A, α, β -mATP induced a depolarization followed by a hyperpolarization. Downward deflections are voltage responses caused by hyperpolarizing current pulses (0.1 nA, 200 ms). A decrease in the amplitude of these responses during the depolarization and hyperpolarization indicates a decrease in membrane resistance (resting membrane potential = -50 mV). B, There was a positive correlation between the amplitude of the depolarization and hyperpolarization caused by α, β -mATP in the same neurons. Points are measurements made in individual neurons. C, SEVC recording of inward and outward currents caused by α, β -mATP in an S neuron. Downward deflections are currents caused by a 10 mV, 200 ms hyperpolarizing voltage step. An increase in the amplitude indicates an increase in membrane conductance (holding potential = -50 mV). Arrow heads indicate α, β -mATP application.

Figure 5.

Current-voltage relationship for inward and outward currents caused by α, β -mATP in S neurons. A, Recordings show the amplitude of inward and outward currents at the indicated holding potentials. B, Mean current-voltage relationship for the inward current caused by α, β -mATP, the extrapolated reversal potential was near 0 mV. C, Current voltage relationship for the outward current caused by α, β -mATP. The reversal potential was near -80 mV. Data are mean $\pm$ SEM obtained from 7 neurons.

Figure 6.

The P2X-afterhyperpolarization was Ca^{2+} -dependent. A, Reducing extracellular Ca^{2+} to 0.25 mM blocked fEPSPs in a reversible manner. B, Lowering extracellular Ca^{2+} to 0.25 mM reversibly reduced the amplitude of the depolarization and hyperpolarization caused by α, β -mATP. C, Mean $\pm$ SEM of data from experiments ($n=7$) shown in B. *indicates a significantly different from 2.5 mM Ca^{2+} conditions.

Figure 7. Antagonists of Ca^{2+} -activated K^+ channels do not inhibit the P2X-afterhyperpolarization. Iberitoxin (IBTx), apamin and clotrimazole (CLT) the selective blockers of large, small and intermediate calcium-activated K^+ channel subtypes, respectively, did not affect the P2X-afterhyperpolarization. Data are mean $\pm$ SEM obtained from 6, 4 and 7 neurons in IBTx, apamin and clotrimazole, respectively.

Figure 8.

4-aminopyridine (4-AP) and tetraethylammonium (TEA) do not inhibit the P2X₂-afterhyperpolarization. A, Representative recordings of ATP-induced depolarization and afterhyperpolarization in an S neuron. 4-AP, at a concentration (1 mM) that blocks some voltage-gated K⁺ channels, did not alter either the depolarization or afterhyperpolarization. B, Data from experiments shown in “A” (mean ± SEM, n = 4). C, Mean data from experiments similar to that shown in “A” except TEA (10 mM) was used in an effort to block the P2X₂-afterhyperpolarization. TEA did not alter either the depolarization or the afterhyperpolarization. Data are mean ± SEM (n = 4).

Figure 9.

There was no association between NOS-ir and the P2X-afterhyperpolarization. Neurons with green fluorescence are NOS-ir neurons. Red fluorescence is Neurobiotin labeled with Texas Red to identify neurons from which electrophysiological recordings were obtained. A,B show neurons in which NOS-ir and Texas Red fluorescence overlapped (indicated by yellow labeling in the neurons (arrow)). The neuron in "A" exhibited the P2X-afterhyperpolarization, the neuron in B did not. C,D, Texas Red labeled neurons that did not contain NOS-ir. The neuron in "C" exhibited the P2X-afterhyperpolarization while the neuron in "D" did not.