P2X2 receptors are essential for [Ca\(^{2+}\)]\text{\textsubscript{i}} increases in response to ATP in cultured rat myenteric neurons

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Ohta, Toshio, Akane Kubota, Matsuka Murakami, Ken-ichi Otsuguro, and Shigeo Ito. P2X2 receptors are essential for [Ca\(^{2+}\)]\text{\textsubscript{i}}, increases in response to ATP in cultured rat myenteric neurons. Am J Physiol Gastrointest Liver Physiol 289: G935–G948, 2005. First published May 19, 2005; doi:10.1152/ajpgi.00017.2005.—We characterized ATP-induced changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) and membrane current in cultured rat myenteric neurons using ratiometric Ca\(^{2+}\) imaging with fura-2 and the whole cell patch-clamp technique, respectively. Neuronal cells were functionally identified by [Ca\(^{2+}\)]\textsubscript{o}, responses to high K\textsuperscript{+} and nicotine, which occurred only in cells positive for neuron-specific protein gene product 9.5 immunoreactivity. ATP evoked a dose-dependent increase of [Ca\(^{2+}\)]\textsubscript{i}, that was greatly decreased by the removal of extracellular Ca\(^{2+}\) channel blockers. In [Ca\(^{2+}\)]\textsubscript{o}-free solution, ATP produced a small transient rise in [Ca\(^{2+}\)]\textsubscript{i}, similar to that induced by P2Y agonists. At −60 mV, ATP evoked a slowly inactivating inward current that was suppressed by the removal of extracellular Na\textsuperscript{+} concentration. The current-voltage relation for ATP showed an inward rectification with the reversal potential of about 0 mV. The apparent rank order of potency for the purinoceptor agonist-induced increases of [Ca\(^{2+}\)]\textsubscript{i} was ATP ≥ adenosine 5’-O-3-triphosphate ≥ CTP ≥ 2-methylthio-ATP > benzoylbenzoyl-ATP. A similar potency order was observed with current responses to these agonists. P2 antagonists inhibited inward currents induced by ATP, Ca\(^{2+}\) and Mg\(^{2+}\) suppressed the ATP-induced current, and Zn\(^{2+}\), Cu\(^{2+}\), and protons potentiated it. RT-PCR and immunocytochemical studies showed the expression of P2X2 receptors in cultured rat myenteric neurons. These results suggest that ATP mainly activates ionotropic P2X2 receptors, resulting in a [Ca\(^{2+}\)]\textsubscript{i} increase dependent on [Ca\(^{2+}\)]\textsubscript{o} in rat myenteric neurons. A small part of the ATP-induced [Ca\(^{2+}\)]\textsubscript{i} increase may be also mediated via a P2Y receptor-related mechanism.

enteric neuron; fura-2; patch clamp; purinoceptor

THE ENTERIC NERVOUS SYSTEM (ENS) plays a key role in controlling various gastrointestinal functions, including motor activity, secretion, absorption, and local circulation (60). The ENS is composed of functionally different neurons that contain a variety of potential neurotransmitters and express receptors for these substances (20). Accumulation of ATP in the extracellular space evokes various responses in neurons, immune/inflammatory cells, smooth muscle, and glandular epithelium by activating different P2 purinoceptor subtypes (10, 49). In the gastrointestinal tract, ATP was first recognized as an inhibitory neurotransmitter released from myenteric motor neurons to relax smooth muscles (16). In enteric neurons of the guinea pig, a part of the fast excitatory postsynaptic potentials (fEPSPs) is suppressed by a nicotinic receptor antagonist, and the remaining fEPSPs are further reduced by P2 purinoceptor antagonists (22), indicating the presence of purinergic excitatory neurotransmission in the ENS (45). An experiment using mice lacking the P2X receptor subtype suggested that ATP was involved in fEPSPs (51). Moreover, it has recently been reported that ATP functions as a putative sensory mediator from epithelial sources to the intrinsic sensory nerve terminals (3). Therefore, purinergic signaling in the ENS is considered to be important in the regulation of gastrointestinal functions (17).

P2 purinoceptors are identified as ionotropic and metabotropic receptors based on their pharmacological properties, mechanisms of signal transudation, and deduced amino acid sequences (32). Ionotropic P2 purinoceptors, P2X, are ligand-gated nonselective cation channels that are permeable for Na\textsuperscript{+} and Ca\(^{2+}\) (44). Seven P2X receptors, identified as P2X1–P2X7, have been cloned. In the ENS, P2X2 (11, 58), P2X1 (48), P2X2 and P2X3 (59, 61), and P2X7 (30) have been detected immunohistochemically. It has been reported that P2X2, P2X5 (64), and P2X2 (30) are involved in the generation of fEPSPs in the ENS. A recent study demonstrated that in P2X1 knockout mice, P2X2 was not involved in fEPSP but in the detection of distention or the intraluminal pressure increase and in the initiation of reflex contraction (4). The same group reported that the P2X2 receptor subtype contributed to fEPSP in neural pathways underlying peristalsis using P2X2 knockout mice (51). Unusual subtypes of purinoceptors sharing some properties with P2X4 and P2X6 are reported to be involved in ATP-induced currents in myenteric neurons of the guinea pig (1). Furthermore, inconsistent results have been reported with respect to the effect of suramin, a P2 antagonist, i.e., it antagonizes (21), has no effect (24), and potentiates (1) responses to ATP in guinea pig myenteric neurons. It has also been suggested that there are some inter- and intraspecies variances in P2X receptor subtypes (63). Because most electrophysiological and cytochemical studies have been performed using guinea pig myenteric neurons, P2X receptors in the ENS of other mammalian species, including the rat, remain to be characterized.

Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) is important in the control of cell excitability in neurons because it triggers neurotransmitter release and regulates protein synthesis and gene expression (2, 33). Myenteric neurons in culture display [Ca\(^{2+}\)]\textsubscript{i} changes when stimulated by various neuroligands (35). In guinea pig myenteric neurons, it has been reported that ATP

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produces an increase of \([\text{Ca}^{2+}]\), through activation of P2 purinoceptors (14, 36), but the related subtype has not been fully determined. Moreover, \([\text{Ca}^{2+}]\) sources involved in the ATP-induced increase of \([\text{Ca}^{2+}]\) have not been investigated in myenteric neurons. Because myenteric neurons in culture form new networks and consist of several morphological and functional classes of neurons, reflecting the diversity of the myenteric neurons in situ (28, 42), a cultured myenteric neuronal system would be a valid model for the study of ENS function in vitro. It is quite difficult to evaluate these cellular functions systematically and individually by the electrophysiological approach. P2X receptors are well known to be capable of passing cations, including \(\text{Na}^+, \text{K}^+,\) and \([\text{Ca}^{2+}]\) (19, 44). In the present study, we first established a primary culture of myenteric neurons isolated from neonatal rats. For examining \([\text{Ca}^{2+}]\) responses in single myenteric neurons, we used a \([\text{Ca}^{2+}]\) imaging technique with the fluorescent dye fura-2. We then investigated \([\text{Ca}^{2+}]\), and current responses to purinergic agonists to assess the purinoceptor subtypes responsible for ATP in rat myenteric neurons by pharmacological, molecular, and immunocytochemical techniques.

**MATERIALS AND METHODS**

**Isolation and culture of myenteric neurons.** All protocols for the use of animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Neonatal Wistar rats (1–4 days old) of either sex were deeply anesthetized with ether and then killed by bloodletting from the abdominal aorta. The small intestine was removed and placed in cold DMEM (Sigma) containing 100 U/ml penicillin G (Meiji-Seika) and 100 \(\mu\text{g}/\text{ml}\) streptomycin (Banyu). The tissue was cut into pieces of 2–3 mm in length, and the serosa was stripped away under optical control using fine forceps. The muscle layer containing the myenteric plexus was dissociated by incubation at 37°C in collagenase (1 mg/ml, type II, Worthington)- and DNase (1 mg/ml, Roche)-containing DMEM for 30 min to isolate myenteric neurons as reported (54). After enzyme digestion, tissues were gently triturated with a fire-polished Pasteur pipette and stood on ice for several minutes. The pellet was again subjected to enzyme digestion for a further 30 min and triturated again. The cell suspension was then centrifuged (800 rpm, 5 min, 4°C), and the pellet containing cells was suspended with medium 199 supplemented with 10% fetal bovine serum (Sigma), 100 \(\mu\text{g}/\text{ml}\) penicillin G, and 100 \(\mu\text{g}/\text{ml}\) streptomycin (Banyu). The tissue was cut into pieces of 2–3 mm in length, and the serosa was stripped away under optical control using fine forceps. The muscle layer containing the myenteric plexus was dissociated by incubation at 37°C in collagenase (1 mg/ml, type II, Worthington)- and DNase (1 mg/ml, Roche)-containing DMEM for 30 min to isolate myenteric neurons as reported (54). After enzyme digestion, tissues were gently triturated with a fire-polished Pasteur pipette and stood on ice for several minutes. The pellet was again subjected to enzyme digestion for a further 30 min and triturated again. The cell suspension was then centrifuged (800 rpm, 5 min, 4°C), and the pellet containing cells was suspended with medium 199 supplemented with 10% fetal bovine serum (Sigma), 100 \(\mu\text{g}/\text{ml}\) penicillin G, and 100 \(\mu\text{g}/\text{ml}\) streptomycin (Banyu). Aliquots were placed onto glass coverslips coated with poly-n-lysine (Sigma) and cultured in a humidified atmosphere of 95% air-5% CO\(_2\) at 37°C. Nerve growth factor (10 ng/ml, 7 s, GIBCO), cytosine \(\beta\)-n- arabinofuranoside (10 \(\mu\text{M}\), Sigma), and 5-fluoro-2-deoxyuridine (25 \(\mu\text{M}\), Sigma) were added to the culture medium. The culture medium was renewed every day. In the present study, cells cultured for 2–3 days were used because relatively large increases of \([\text{Ca}^{2+}]\), in response to high \(\text{K}^+\) (80 mM) were obtained during this period (data not shown).

**Measurement of \([\text{Ca}^{2+}]\).** \([\text{Ca}^{2+}]\), in single cells was measured with a fluorescent \([\text{Ca}^{2+}]\) indicator, fura-2 (25), by dual excitation using a fluorescent imaging system controlling illumination and acquisition with software (Aqua Cosmos, Hamamatsu Photonics) as described previously (41). To load fura-2, cells were incubated for 1.5 h at room temperature with 10 \(\mu\text{M}\) fura-2 AM in normal external solution containing (in mM) 134 NaCl, 6 KCl, 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), 10 glucose, and 10 HEPES (pH 7.4 with NaOH). A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Diaphot 300, Nikon). Cells were illuminated every 2.5 s with lights at 340 and 380 nm, and the respective fluorescence signals (F\(_{340}\) and F\(_{380}\)) were detected. The fluorescence emitted was projected onto a charge-coupled device camera, and the ratio of fluorescent signals (F\(_{340}/F_{380}\)) was stored on the hard disk of a computer (Pro-600L, EPSON). Calibration of fura-2 was performed with a \([\text{Ca}^{2+}]\) calibration buffer solution (Molecular Probes) containing 5 \(\mu\text{M}\) fura-2. Cells were continuously superfused with the external solution at a flow rate of 1 ml/min through a multibarreled puffer pipette placed close to the cells (46). The composition of high-\(\text{K}^+\) solution was (in mM) 60 NaCl, 80 KCl, 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), 10 glucose, and 10 HEPES (pH 7.4 with NaOH). Drugs were applied through different tubes of the puffer pipettes. All experiments were carried out at room temperature (20–24°C).

**Whole cell current recording.** Membrane currents were recorded using the conventional whole cell configuration of the patch-clamp technique (26, 46). Whole cell recordings were made with 4- to 5-M\(\Omega\) electrodes mounted on the head stage of a patch-clamp amplifier (Axopatch200B, Axon). Current data were filtered at 1 kHz and sampled at 2–5 kHz by an analog-to-digital converter (PowerLab System, AD Instruments) in conjunction with a personal computer (MacintoshG3, Apple) and stored on the hard disk of the personal computer. Cell capacitance of myenteric neurons was 1.5 – 3.2 pF. After cells were voltage-clamped at a holding potential of 80 mV, a step depolarization to 0 mV lasting 50 ms was applied to activate voltage-dependent \(\text{Na}^+\) channels. We identified as neural cells those that showed a fast inward current through voltage-dependent \(\text{Na}^+\) channels. The standard pipette solution contained (in mM) 120 CsCl, 20 tetraethylammonium chloride, 1.2 MgCl\(_2\), 2 ATPNa\(_2\), 0.2 GTPNa\(_3\), 10 HEPES, and 10 EGTA (pH 7.2 with CsOH). In some experiments, current responses to ATP were measured using a pipette solution without EGTA. A multibarreled puffer system similar to that used for \([\text{Ca}^{2+}]\), measurement was used for drug application and external perfusion.

**RT-PCR.** The design of the oligonucleotides used for the specific amplification of rat P2X receptor cDNA was based on sequences registered in GenBank for each of the receptors in the rat. The nucleotide sequence and the length of the expected PCR products for each primer pair are shown in Table 1. Total RNA from rat myenteric plexus cells cultured for 3 days or rat spinal cord with dorsal root ganglion was extracted with TRZol reagent (Iogen, Nippogen) and then treated with DNase I (Promega). First-strand cDNA was synthesized from oligo (dT)-primed total RNA with Superscript II reverse transcriptase (GIBCO). The reaction mixture was then subjected to PCR amplification with the use of Taq DNA polymerase (Promega). Samples were heated to 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. To exclude the possibility of genomic DNA giving a false positive result in the PCR, a reverse transcriptase-free (negative) control was run in parallel with each primer pair. The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining followed by ultraviolet transillumination. To identify the amplified products as the respective subtypes of P2X receptors detected in the rat ENS, they were extracted from the gel with a gel extraction kit (Qiagen), subcloned with pGEM-T-easy vector (Promega), and sequenced (CEQ8000, Beckman).

**Immunocytochemistry.** After the measurement of \([\text{Ca}^{2+}]\), cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature and subsequently rinsed with 0.01 M PBS with 0.3% Triton X-100 (Triton-PBS). Nonspecific binding sites were blocked with 10% normal goat serum in Triton-PBS for 1 h. Cells were then incubated with a rabbit antiserum for protein gene product 9.5 (PGP9.5; diluted 1:5,000, Chemicon) or P2X\(_2\) (diluted 1:200, Alomone) for 1 h at room temperature. Double labeling was achieved using antisera with neuronal nitric oxide synthase (nNOS) mouse IgG (diluted 1:500, Sigma) in combination with PGP9.5. For immunocytochemical classification of myenteric neurons, cells were stained with antibodies against mouse monoclonal anti-calbindin (diluted 1:1,000, Sigma) or rabbit anti-calretinin (diluted 1:1,000, Sigma). For double staining with calretinin and PGP9.5, mouse monoclonal anti-PGP9.5 (diluted 1:20, Abcam) was used. After sev—

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eral rinses with Triton-PBS, the antibody was visualized by incubation with Alexa-labeled goat anti-rabbit IgG (10 μg/ml, Molecular Probes) or Alexa-labeled goat anti-mouse IgG (10 μg/ml, Molecular Probes) in Triton-PBS for 1 h. For nuclear staining, Hoechst 33752 (10 μM, Molecular Probes) was included with the second antibody-containing solution. Finally, cells were extensively rinsed with Triton-PBS and then with distilled water. Preparations were examined with a laser confocal microscope (FV500, Olympus) using software (Fluoview). A negative control was prepared by the omission of primary antibodies, resulting in no staining.

Chemicals. The following drugs were used: adenosine, ADP, adenosine 5'-O-3-triphosphate (ATP-s), ATP, 2',3'-O-(2,4,6-trinitrophényl)ATP (TNP-ATP), α,β-methylene ATP (α,β-MeATP), AMP, 2',3'-O-(4-benzoylbenzoyl)ATP (BzATP), CTP, nicotine bitartrate, thapsigargin, HEPES, UDP, and UTP, all from Sigma. Pyridoxal phosphate-6-azo-(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS) and suramin were from Tocris. Acetylcholine chloride (ACh) was from Daiichi. Nifedipine and tetrodotoxin were from Wako. EGTA and fura-2 AM were obtained from Dojindo. ω-Agatoxin IVA (ω-ATX) and ω-conotoxin GVIA (ω-CTX) were from Peptide. All other drugs used were of analytic grade.

Data analysis. Data are presented as means ± SE, where n is the number of observations. Comparisons were made by the unpaired Student’s t-test or by one-way ANOVA followed by the Tukey-Kramer test when more than two groups were compared (StatView 5.0, SAS Institute). Differences with a P value of <0.05 were considered significant.

RESULTS

Functional identification of myenteric neural cells. Cultured myenteric plexus cells on coverslips contained neurons, fibroblasts, and glial cells. The differences in morphological appearance allowed us to distinguish between neurons and fibroblasts, because fibroblasts had flat cell bodies, sticking close to the coverslip surface. However, it was not always easy to distinguish some glial cells from neurons. It has been reported that voltage-dependent Ca2⁺ channels (VDCCs) are primarily expressed in neurons (27). However, some glial cells also express VDCCs (40). Nicotinic ACh receptors are reported to be expressed in myenteric neurons (38, 65). First, we attempted to identify myenteric neurons from other cells. For this purpose, [Ca2⁺]i responses to high K⁺, nicotine, and ACh were observed, and these cells were then subjected to immunocytochemical staining with an antibody for neuron-specific protein, PGP9.5 (53). We examined the relation between the [Ca2⁺]i responses and PGP9.5 immunoreactivities (IR). Fura-2-loaded cells were sequentially stimulated by high K⁺ (80 mM), nicotine (50 μM), and ACh (50 μM) for 30 s at 5-min intervals. Figure 1, A and B, shows cells responding to these stimulants in the presence and absence of PGP9.5-IR. Only PGP9.5-IR cells exhibited increases of [Ca2⁺]i, induced by both high K⁺ and nicotine. On the other hand, ACh elicited a [Ca2⁺]i increase in some cells without PGP9.5-IR (cell 4; Fig. 1, A and B). Changes in [Ca2⁺]i in response to nicotine, high K⁺, and ACh were summarized in cells with positive and negative PGP9.5-IR (Fig. 1C). These results clearly indicated that only cells positive for PGP9.5-IR, probably myenteric neurons, showed [Ca2⁺]i increases induced by both high K⁺ and nicotine. Thus, by examining [Ca2⁺]i responses to these stimuli, we differentiﬁed neural cells from other cells in the following experiments.

ATP-induced [Ca2⁺]i increase in single myenteric neurons. The baseline [Ca2⁺]i level of myenteric neurons was 41.8 ± 0.5 nM (n = 237). ATP (30 μM) evoked [Ca2⁺]i increases in most myenteric neurons (96.6%, n = 229) in a dose-dependent manner (Fig. 2A). At higher concentrations, the rate of rise in [Ca2⁺]i became rapid, and the level quickly peaked. When applied at the maximal concentration of ATP (300 μM), a [Ca2⁺]i transient was followed by a slowly declining increase of [Ca2⁺]i. The peak [Ca2⁺]i increase induced by 300 μM ATP was 221.6 ± 11.3 nM, and the time to peak and half-decay time from the peak were 10.2 ± 0.2 s and 44.8 ± 1.1 s, respectively (n = 110). The EC50 for the ATP-induced [Ca2⁺]i increase was 25.9 μM, estimated from the concentration-response curve shown in Fig. 2B. A similar concentration-response curve was obtained for nicotine, with an EC50 of 9.6 μM (Fig. 2, C and D). Neither ATP-induced nor nicotine-induced [Ca2⁺]i responses were affected by tetrodotoxin (0.2 μM). For neurochemical classiﬁcation, after measurement of the [Ca2⁺]i response to ATP, neurons were subjected to immunocytochemical staining with nNOS, calbindin, and calretinin. Figure 3 shows a double-staining image with antibodies against nNOS together with PGP9.5 and representative [Ca2⁺]i responses to high K⁺, nicotine, and ATP in two neurons. Neuronal NOS-IR cells were observed 19.5% (66 of 338 cells

Table 1. P2X receptor and β-actin primers for RT-PCR

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<th>Gene Accession No.</th>
<th>Primer Sequence</th>
<th>Predicted Length, bp</th>
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<td>P2X1 X80447 Sense 5'-GAAGTGTGATCTCGACTGAGCCT-3'</td>
<td>452</td>
<td></td>
</tr>
<tr>
<td>P2X2 U14414 Antisense 5'-GGTCAAGACTGGAATGGAGCCT-3'</td>
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<td>354</td>
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<tr>
<td>β-Actin J00691 Sense 5'-AGCACTGTAGTGAAGGATGTCA-3'</td>
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Fig. 1. Effects of high K⁺, nicotine (Nic), and acetylcholine (ACh) on intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in cultured rat myenteric cells. A: morphological features of isolated myenteric cells. a, Image under transmission light; b, nuclear staining with Hoechst 33752; c, protein gene product 9.5 (PGP9.5) immunoreactivity (IR). B: [Ca²⁺]ᵢ responses to high K⁺ (80 mM), Nic (50 μM), and ACh (50 μM) in PGP9.5-IR-positive cells [PGP(+), cells 1 and 2] and PGP9.5-negative cells [PGP(-), cells 3 and 4]. Numbers (1–4) correspond to cells shown in A. These stimulants were applied for 30 s at 5-min intervals. C: summarized effects of [Ca²⁺]ᵢ responses to high K⁺, Nic, and ACh in PGP9.5-positive (n = 89) and PGP9.5-negative cells (n = 45). The increase of [Ca²⁺]ᵢ was estimated by the subtraction of basal [Ca²⁺]ᵢ before agonist application from each peak [Ca²⁺]ᵢ.
positive for PGP9.5-IR). ATP increased \([\text{Ca}^{2+}]_i\), in both nNOS-IR-positive and -negative cells (Fig. 2E). For the immunoreactivity for calbindin and calretinin, calbindin-IR neurons were only 5.7% (15 of 261 cells positive for PGP9.5-IR), but most of the cells (90.6%) showed positive for calretinin-IR (331 of 365 cells positive for PGP9.5-IR). ATP elicited a \([\text{Ca}^{2+}]_i\) increase in myenteric neurons regardless of positive or negative immunoreactivity for calbindin and calretinin.

ATP-induced inward currents. At a holding potential of \(-60\) mV, the application of ATP (30 \(\mu\)M) evoked a rapid inward current followed by a slight reduction of its amplitude in the presence of ATP. The inward current induced by ATP (112.1 \(\pm\) 26.6 pA, \(n = 54\)) with a standard internal solution containing 10 mM EGTA was not different from that with an internal solution without EGTA (130.3 \(\pm\) 25.4 pA, \(n = 40\)). The ATP-induced current only slightly declined during its presence (86.9 \(\pm\) 1.9% of the peak value at 20 s). As shown in Fig. 4A, the ATP-induced inward current was greatly reduced by the removal of external Na\(^+\), which was replaced by N-methyl-D-glucamine\(^+\). In the presence of tetrodotoxin (0.2 \(\mu\)M) and Cd\(^{2+}\) (0.1 mM), ramp voltages from +50 to \(-90\) mV for 200 ms were applied before and during the application of ATP. In the current-voltage relation, the ATP-induced current showed inward rectification with a reversal potential of +2.3 \(\pm\) 0.4 mV (\(n = 12\)).

To examine the steady-state current-voltage relation for ATP, ATP (30 \(\mu\)M) was repetitively applied for 5 s at various holding potentials (Fig. 4B). In this experiment, the current-voltage relation for nicotine was also examined in the same cell (Fig. 4C). The ATP-induced current showed larger and slower inactivation than the nicotine-induced one at all holding potentials tested. The peak current-voltage relation for ATP was similar to that of nicotine. These results suggest that ATP activates cation-permeable channels with slow inactivation in rat myenteric neurons.

Extracellular \(\text{Ca}^{2+}\) dependency of the ATP-induced \([\text{Ca}^{2+}]_i\) increase. To study the \(\text{Ca}^{2+}\) source for the ATP-induced \([\text{Ca}^{2+}]_i\) increase in rat myenteric neurons, the effects of external \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}]_o\)] removal on ATP-induced \([\text{Ca}^{2+}]_i\) responses were investigated. One minute after the removal of [\(\text{Ca}^{2+}]_o\), cells were stimulated with ATP in the absence of [\(\text{Ca}^{2+}]_o\). Only a transient [\(\text{Ca}^{2+}]_i\) increase occurred (Fig. 5B) compared with the presence of [\(\text{Ca}^{2+}]_o\] (Fig. 5A). The transient [\(\text{Ca}^{2+}]_i\), increase in the absence of [\(\text{Ca}^{2+}]_o\] was eliminated by treatment with thapsigargin (1 \(\mu\)M), a \(\text{Ca}^{2+}\)-pump inhibitor of the sarco(endo)plasmic reticulum (data not shown). A similar increase of [\(\text{Ca}^{2+}\)] was induced by the P2Y receptor agonists, UTP or UDP (Fig. 5, C and D). These results suggest that the major \(\text{Ca}^{2+}\) source for [\(\text{Ca}^{2+}]_i\)] responses to ATP is extracellular \(\text{Ca}^{2+}\) influx and that a small part is mediated by \(\text{Ca}^{2+}\) release from intracellular \(\text{Ca}^{2+}\) stores.

Because ATP activates cation channels in rat myenteric neurons (Fig. 4), it is possible that VDCCs are activated due to membrane depolarization by ATP. To determine the involvement of VDCCs in the ATP-induced \([\text{Ca}^{2+}]_i\) increase, we examined the effects of VDCC blockers on the [\(\text{Ca}^{2+}]_i\), increase induced by ATP. High K\(^+\) (80 mM) and ATP (100 \(\mu\)M) were applied twice with an interval of 20 min. A second stimulation was carried out in the presence of a mixture of VDCC blockers (3 \(\mu\)M nifedipine, 1 \(\mu\)M \(\omega\)-CTX and 0.1 \(\mu\)M \(\omega\)-ATX). Typical [\(\text{Ca}^{2+}\)], responses to high K\(^+\) and ATP before and after blockade of VDCCs are shown in Fig. 5E. The [\(\text{Ca}^{2+}\)], increase induced by high K\(^+\) was greatly suppressed by these blockers, but that caused by ATP was reduced by half. Summarized data are shown in Fig. 5F. These results indicate that about half of [\(\text{Ca}^{2+}\)], responses to ATP are mediated by \(\text{Ca}^{2+}\) entry through VDCCs.

[\(\text{Ca}^{2+}\), responses to P2 purinoceptor agonists. Because P2X receptor subtypes are characterized by the potency of purinoceptor agonists, we examined the effects of P2X agonists on [\(\text{Ca}^{2+}\)] in rat myenteric neurons. As shown in Fig. 6A, ATP, ATP\(_\gamma\)S, BzATP, CTP, 2-methylthio-ATP (2MeSATP), and ADP elicited concentration-dependent increases of [\(\text{Ca}^{2+}\)]. Adenosine, AMP, and \(\alpha\)-\(\beta\)-MeATP hardly evoked increases of [\(\text{Ca}^{2+}\)] even at the highest concentrations used (1 mM). For CTP, 2MeSATP, and BzATP, maximal responses

\[\text{Ca}^{2+}\_i \text{ increase} \]

\[A \text{ and } C: \text{ representative traces of } [\text{Ca}^{2+}]_i \text{ responses to ATP (A) and Nic (C) at various concentrations for 30 s. B and D: concentration-response relation for the peak } [\text{Ca}^{2+}]_i \text{: led induced by ATP (B) and nicotine (D) (n = 102-237).}\]
were not obtained at the concentrations used. To determine the potency order of these agonists, the amplitude at 30 μM of each agonist was compared for convenience. The apparent potency order for evoking the [Ca^{2+}]_{i} response was ATP > ATP_S > CTP > 2MeSATP > BzATP.

We examined the effects of sequential applications of 30 μM ATP, BzATP, ATP_S, 2MeSATP, and CTP to the same cells and compared their amplitudes of [Ca^{2+}]_{i} increase. Similar studies were carried out for inward current in voltage-clamped cells at a holding potential of −60 mV. The representative [Ca^{2+}]_{i} and current responses to these agonists are summarized in Fig. 6, B and C. For both responses, a similar order of amplitude by these agonists (ATP ≥ ATP_S ≥ CTP ≥ 2MeSATP > BzATP) was obtained.

Inhibitory effects of P2 antagonists. The effects of P2 purinoceptor antagonists on ATP-induced current were examined. ATP (30 μM) was applied to voltage-clamped cells at −60 mV for 15 s at 5-min intervals in the presence of the P2 antagonists suramin, TNP-ATP, and PPADS. Figure 7A shows the actual traces showing the inhibitory effects of suramin on ATP-induced current. Suramin dose dependently reduced the amplitude of inward currents evoked by ATP, and the inhibitory effect completely disappeared after its washout. The other two P2 antagonists also inhibited ATP-induced currents in a dose-dependent manner with similar sensitivities. The estimated IC_{50} was 3.6 μM for PPADS, 4.5 μM for TNP-ATP, and 9.8 μM for suramin. Like suramin, the inhibitory effect of TNP-ATP also quickly vanished after its washout, but
PPADS produced a long-lasting inhibition of ATP-induced currents.

**Effects of external cations on ATP-induced currents.** It is known that P2X receptors receive subtype-specific modulation by external cations (43, 44). To identify the P2X subtype related to the current responses to ATP in rat myenteric neurons, we examined the effects of external cations on ATP-induced currents. ATP (30 μM) was applied to voltage-clamped cells at a holding potential of −60 mV for 15 s at 5-min intervals in the presence of various cations. Figure 8A shows the typical effects of removal of external Ca^{2+} and/or Mg^{2+}. The inward current evoked by ATP in normal external solution (containing 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+}) was increased by the removal of either external Ca^{2+} or Mg^{2+}. Further augmentation occurred with the removal of both. These two divalent cations produced concentration-dependent inhibition of ATP-induced currents with IC_{50} values of 0.45 mM for Mg^{2+} and 0.72 mM for Ca^{2+} (Fig. 8B).

Next, we examined the effects of some divalent and trivalent metal ions on ATP-induced currents. Various concentrations of metal ions were applied together with ATP (30 μM) for 15 s at 5-min intervals at −60 mV. In this series of experiments, metal ions were simply added to the normal external solution. Figure 8C shows the typical effects of increasing concentrations of Zn^{2+} on ATP-induced currents. At up to 200 μM Zn^{2+}, the inward current evoked by ATP was potentiated in a concentration-dependent manner but was completely suppressed by 2 mM Zn^{2+}. Concentration-response relations for other metal ions in ATP-induced currents are shown in Fig. 8D. Similar to the effect of Zn^{2+}, ATP-induced currents were concentration dependently increased by Cu^{2+}, Cd^{2+}, and Co^{2+} at up to 200 μM. Maximal potentiating effects were observed around 20–200 μM. Among these cations, Cu^{2+} and Zn^{2+} had similar effects on ATP-induced currents, i.e., potentiation at low concentrations and blockade in the millimolar range. No remarkable effects on the current response to ATP occurred with the addition of Ni^{2+}, Sr^{2+}, or Ba^{2+}, but La^{3+} and Mn^{2+} produced suppression in a concentration-dependent manner (>20 μM).

Protons showed distinctive effects on ATP-induced currents (Fig. 8E). At low pH for the external solution (pH 6.8), the ATP-induced current was pronouncedly enlarged. On the other hand, at high pH (pH 8.0) an opposite effect was observed. Figure 8F shows a concentration-dependent curve for protons in ATP-induced currents. Considering these biophysical properties, especially taking the effects of Cu^{2+}, Zn^{2+}, and protons into consideration, it is suggested that ATP produces inward
current through the activation of P2X2 receptors in rat myenteric neurons.

RNA expression of myenteric neurons in culture. To identify the subtypes of P2X receptors expressed in the rat ENS, RT-PCR using seven pairs of primers for P2X receptors was performed. The major PCR products detected in rat myenteric plexus in culture were P2X2, P2X4, and P2X5 (Fig. 9A). These PCR products were subcloned, sequenced, and confirmed to be identical to the known sequences of mRNAs for each rat P2X subtype. As reported by Chen et al. (12), mRNAs for all P2X receptor subtypes were expressed in the spinal cord with the dorsal root ganglion (Fig. 9B).

P2X2 immunoreactivity in myenteric neurons. As expected from the pharmacological, biophysical, and molecular characteristics, P2X2 receptors seemed to be the main P2X subtype mediating ATP-induced responses in rat myenteric neurons. To obtain further evidence, we conducted immunocytochemistry against P2X2 receptors. Cells after measurement of [Ca2+]i responses to various agonists (ATP, ATPγS, BzATP, CTP, and 2MeSATP) were stained with an anti-P2X2 antibody. As
shown in Fig. 9C, P2X2-IR was detected in cells exhibiting a $[\text{Ca}^{2+}]_i$ increase in response to these purinergic agonists (Fig. 9D) with the same magnitude of order as shown in Fig. 6.

**DISCUSSION**

In the present study, we established a primary culture of myenteric neurons isolated from the neonatal rat intestine to characterize $[\text{Ca}^{2+}]_i$ and current responses at the single cell level. $[\text{Ca}^{2+}]_i$ and current responses to ATP, a putative neurotransmitter underlying fEPSPs in the ENS, were analyzed in functionally identified neural cells. ATP evoked a $[\text{Ca}^{2+}]_i$ increase in most of the neurons, of which the response was mainly derived from extracellular $\text{Ca}^{2+}$ influx. A small part of the $[\text{Ca}^{2+}]_i$ response was mediated by $\text{Ca}^{2+}$ release from internal stores through P2Y receptor activation. From the pharmacological characteristics, biophysical properties of the current responses, and analysis of RT-PCR and immunocytochemistry, it was suggested that P2X2 mainly contributed to the $[\text{Ca}^{2+}]_i$ response to ATP in rat myenteric neurons.

In cultured myenteric neurons from neonatal rat, results of double staining of nNOS, calbindin, and calretinin together with PGP 9.5 showed that 19.5% of neurons were nNOS positive, 90.6% were positive for calretinin, and only 5.7% were positive for calbindin. nNOS-IR has been observed in a subpopulation of descending inhibitory neurons (15, 18). It has been reported that calbindin-IR is correlated with afterhyperpolarization (AH)-type neurons, which are thought to be a sensory neurons (39), and calretinin is likely to be excitatory in motor neurons to the longitudinal muscle or ascending interneurons (7, 8). Therefore, these immunocytochemical data suggest that the major classes of myenteric neurons are motor and interneurons and a small population of them are AH-type sensory neurons. Similar to the present results, it has been reported that there are few AH-type neurons in cultured rat myenteric neurons by patch-clamp study (29).

In the myenteric neuron culture used in the present study, it was not possible to completely discriminate glial cells and fibroblasts from neurons. The $[\text{Ca}^{2+}]_i$ increase induced by high...
K\textsuperscript+ is generated through VDCCs, because glial cells and fibroblasts usually lack VDCCs (27). The high-K\textsuperscript+ -induced \([\text{Ca}^{2+}]_i\) response has been used to discriminate neurons from other cells in guinea pig myenteric neurons (14, 56). However, it has been reported that some glial cells possess VDCCs (40). Nicotinic ACh receptors are known to be expressed in myenteric neurons (38, 65). In the present experiment, therefore, to identify myenteric neurons, \([\text{Ca}^{2+}]_i\) responses to nicotine were also observed in addition to those to high K\textsuperscript+. This method for identification of neurons was supported by the immunocytochemical study with PGP9.5, a neuronal marker protein (53), because only cells that responded to both high K\textsuperscript+ and nicotine possessed immunoreactivity to PGP9.5.

Because the ATP-induced increase in \([\text{Ca}^{2+}]_i\) in myenteric neurons was greatly decreased by the removal of \([\text{Ca}^{2+}]_o\), the major source for the increase of \([\text{Ca}^{2+}]_i\) appeared to be extracellular Ca\textsuperscript+ influx. In addition, in the absence of \([\text{Ca}^{2+}]_o\), a small transient \([\text{Ca}^{2+}]_i\) increase remained. This \([\text{Ca}^{2+}]_i\) transient was abolished by pretreatment with thapsigargin. A similar small \([\text{Ca}^{2+}]_i\) increase was elicited by UTP or UDP, P2Y receptor agonists, under external Ca\textsuperscript+ -free conditions. It is known that the activation of P2Y receptors produces inositol 1,4,5-trisphosphate, resulting in Ca\textsuperscript+ release from internal stores (52, 57). These results, therefore, suggest that a small part of the \([\text{Ca}^{2+}]_i\) increase induced by ATP is mediated by the Ca\textsuperscript+ release from internal stores through the activation of P2Y receptors. Extracellular ATP evokes a \([\text{Ca}^{2+}]_i\) increase via a phospholipase C-dependent mechanism in guinea pig myenteric neurons (36). Moreover, the existence of functional Ca\textsuperscript+ stores has been reported in guinea pig myenteric neurons (37), and P2Y\textsubscript{1} is reported to mediate slow excitatory postsynaptic potentials in the guinea pig ENS (31).

P2X receptors are well known as ligand-gated ion channels, passing cations including Ca\textsuperscript+ (19, 49). To examine P2X
receptor channel activity by purinoceptor agonists, we measured whole cell currents. Under voltage-clamped conditions at negative holding potentials, ATP produced inward currents with slow inactivation. The current-voltage relation for ATP-induced currents showed pronounced inward rectification with a reversal potential of about 0 mV, which was similar to the nicotinic current obtained in the same cells. ATP-induced inward currents were greatly suppressed by the removal of external Na$^{+}$. These results suggest that ATP acts on ionotropic cation-permeable P2X receptors in rat myenteric neurons as reported in guinea pig myenteric neurons (64). It is reasonable that increased cation conductance by ATP leads to membrane depolarization, resulting in opening of VDCCs. This was the case in rat myenteric neurons. Under current-clamped conditions, ATP evoked sustained depolarization similar to nicotine (data not shown). Whereas the [Ca$^{2+}$], response to high K$^+$ was almost abolished in the presence of VDCC blockers, the ATP-induced [Ca$^{2+}$] increase was reduced by half. These results indicate that VDCCs are involved in the [Ca$^{2+}$] increase in response to ATP in rat myenteric neurons.

In the present study, ATP, ATP$\gamma$S, CTP, 2MeSATP, and BzATP produced [Ca$^{2+}$] increases in a concentration-dependent manner, but there was almost no response to α,β-MeATP, AMP, or adenosine. When agonists were compared at 100 μM, the order of magnitude of responses was BzATP ≥ ATP ≥ CTP > ATP$\gamma$S = 2MeSATP > ADP. However, low concentrations (<10 μM) of BzATP evoked only a slight [Ca$^{2+}$], response. Although BzATP is known to be a selective agonist for the P2X$_7$ receptor (50), at higher concentrations it also acts as agonist at P2X$_1$, P2X$_2$, and P2X$_7$ (34). Moreover, recombinant P2X$_7$ receptors are weakly activated by ATP with 100 μM range of EC$_{50}$ (34). RT-PCR analysis showed the absence of mRNA of P2X$\gamma$, but the presence of mRNAs of P2X$_2$, P2X$_4$, and P2X$_5$ in rat myenteric culture. Therefore, BzATP may act on subtypes other than P2X$\gamma$ in rat myenteric neurons, as reported for heterogeneous expression in human astrocytoma cells (5). In guinea pig myenteric neurons, however, BzATP produces large inward currents, and the P2X$\gamma$ receptor is suggested to function as an ATP-mediating fEPSP (30). We estimated that agonist sensitivity to [Ca$^{2+}$], increased at 30
μM, and the apparent rank order of the potency was ATP ≅ ATPγS ≅ CTP ≅ 2MeSATP > BzATP. Current responses to these agonists were well correlated with [Ca²⁺]i responses, supporting the finding that major Ca²⁺ influx pathways were P2X channels and VDCCs activated via membrane depolarization. It has been reported that the pharmacological profile determined by measuring Ca²⁺ influx is correlated with the respective electrophysiological properties in a heterologous expression study (5). This is supported by the fact that the Ca²⁺ permeability ratio to Na⁺ is large in P2X receptors expressed in the Chinese hamster ovary (19). In the present experiment, αβ- meATP had no effect on [Ca²⁺]i. Only P2X₁ and P2X₃ subtypes and heteromeric P2X₂/3, P2X₁/5, and P2X₄/₆ subtypes are sensitive to αβ-meATP (44). From these agonist sensitivities and the PCR results, we could dismiss the possibility that P2X₁, P2X₃, P2X₄, and P2X₇ were involved in ATP-induced responses in rat myenteric neurons.

It has been reported that there are distinctive biophysical properties dependent on P2X subtypes. The inward current evoked by ATP showed markedly slow inactivation compared with the nicotinic current observed in the same cells. It is reported that the inactivation rate is slow in heterogeneously expressed P2X₂, P2X₄, and P2X₇ but not other types (43). Among slow inactivation channel receptors, P2X₅ is characterized by smaller conductance of the channel than others. In this experiment, external Ca²⁺ influx is correlated with external cations such as containing neurotransmitters and neural communication. Their IC₅₀ values were equivalent to those reported for heterogeneously expressed P2X₁, P2X₂, P2X₃, and P2X₇ (43). Unlike the report on guinea pig myenteric neurons (1), no potentiation was induced by suramin in rat myenteric neurons in the present experiment. It is reported that interspecies differences are present, with differences in pharmacological properties among P2X receptor orthologues. Recombinant human P2X₄ receptors display notably higher sensitivity to suramin and PPADS than their rat homologs, and a single-point mutation on rat P2X₄ is sufficient to account for this increase in suramin sensitivity (23). Similarly, a lysine residue on rat P2X₉ and P2X₇ seems to be crucial for the potency and kinetics of the antagonism by PPADS (9). In light of these results, it is possible that the difference in amino acids between different species may confer distinct pharmacological phenotypes. Similar species differences are reported between pelvic ganglion neurons in the mouse and rat (63). Although the neurons investigated in this study suggest a pharmacological and biophysical profile of the P2X₂ phenotype, it is still possible that P2X receptors are heteromultimers, with P2X₂ being the dominant component. The presence of alternatively spliced variants of P2X₂ receptor subunits may add additional complexity to the characterization of endogenous P2X₂ receptors (6, 47, 55).

The myenteric neurons in the ENS are functionally and morphologically classified into sensory, motor, and interneurons (20). The present results indicated that, because most of the neurons responded to ATP regardless of positive or negative for immunoreactivity against nNOS, calbindin, and calretinin, although the magnitude was different from cell to cell, ATP mainly plays an excitatory role in myenteric neurons of the rat. Functional phenotypes after the activation of purinoceptor in neurons may be dependent on each neural function, such as containing neurotransmitters and neural communication.

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