Full Paper

Participation of ATP in Nonadrenergic, Noncholinergic Relaxation of Longitudinal Muscle of Wistar Rat Jejunum

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Abstract. A role of ATP in nonadrenergic, noncholinergic (NANC) relaxations was examined in the Wistar rat jejunum. Electrical field stimulation (EFS) induced NANC relaxation of longitudinal muscle of the jejunal segments in a frequency-dependent manner. A purinoceptor antagonist, adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS, 100 μ M) inhibited the relaxation: relaxations induced by EFS at lower or higher frequencies were either completely or partially inhibited, respectively. After the jejunal segments had been desensitized to ATP, the relaxations were decreased to the same extent as those inhibited by A3P5PS. An inhibitor of small conductance Ca^{2+} -activated K^{+} channels (SK channels), apamin (100 nM), completely inhibited EFS-induced relaxations. Treatment of the segments with an inhibitor of sarcoplasmic reticulum Ca^{2+} -ATPase, thapsigargin (1 μ M), significantly inhibited the relaxations. The exogenous ATP-induced relaxation of longitudinal muscle occurred with a concomitant decrease in intracellular Ca^{2+} levels. Apamin and thapsigargin abolished these ATP-induced responses. A3P5PS significantly inhibited the inhibitory junction potentials which were induced in the longitudinal muscle cells. In addition, apamin significantly inhibited the hyperpolarization that was induced by exogenous ATP in the cells. These findings in the Wistar rat jejunum suggest that ATP participates in the NANC relaxation via activation of SK channels induced by Ca^{2+} ions that are released from the thapsigargin-sensitive store site.

Keywords: ATP; rat jejunum; nonadrenergic, noncholinergic relaxation; SK channel; sarcoplasmic reticulum

Introduction

The role of ATP as the mediator of nonadrenergic, noncholinergic (NANC) relaxation was first suggested from the evidence that ATP was released from the guinea pig and toad stomachs on stimulation of vagus neurons (1). A similar role of ATP was suggested in the circular muscle of the human colon from indirect evidence: ATP mimicked the relaxation induced by electrical field stimulation (EFS), and EFS-induced or exogenous ATP-induced relaxations were slightly or significantly inhibited by apamin, respectively (2). A subsequent study reported that EFS-induced NANC

relaxation of the longitudinal muscle with myenteric plexus preparations of the rat ileum was partially inhibited after desensitization of the preparation to ATP (3). EFS-induced relaxations of the internal anal sphincter (4) and tenia coli (5) of the guinea pig were partially inhibited by a selective P2 purinoceptor antagonist. Neurogenic relaxation induced in the rat distal colon was also partly inhibited by a P2Y purinoceptor antagonist (6, 7). Recent studies reported that EFS-induced relaxation of the gastric fundus of the rat was inhibited by the P2Y purinoceptor antagonist and after the desensitization to ATP (8), and that the relaxation of circular muscle strips of the mouse jejunum, which persisted after inhibition of nitric oxide synthesis, was almost abolished after the desensitization

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to ATP (9). More recently, involvement of purinergic neurons in the relaxant response and interaction of the neurons with nitrergic neurons were suggested in the rat distal colon (10). On the other hand, exogenous ATP-induced relaxation was shown to be apamin-sensitive (2). EFS-induced fast inhibitory junction potentials (i.j.ps) were suggested to be mediated by ATP and to be apamin-sensitive in the circular muscle of the human jejunum (11), the guinea pig ileum (12), the rat colon (13), the guinea pig colon (14), and the mouse distal colon (15). Although these results suggest a role of ATP in NANC relaxation, several reports present contradictory results: no role for ATP was suggested in the rat ileum (16), proximal colon (17), and caecum (18) and the guinea pig gastric fundus (19). This discrepancy may be due to the regional, species, and strain differences in the mediator of NANC relaxation (20, 21).

The mediator of NANC relaxation has been extensively studied in the Wistar rat small intestine. Specifically, it was studied in the duodenum where partial participation of nitric oxide in the relaxation was suggested (22). Studies in the jejunum suggested partial participation of neurotensin (23), but not nitric oxide (24), vasoactive intestinal peptide (VIP), or pituitary adenylate cyclase-activating polypeptide (PACAP) (21), and in the ileum, it was suggested that there was partial participation of nitric oxide, but not VIP or PACAP (24). However, all the possible mediators that exist were not studied in every region of the small intestine of the Wistar rat. In the present study, we further examined another mediator of NANC relaxation of longitudinal muscle of the Wistar rat small intestine, mainly in the jejunum, and found partial participation of ATP in the relaxation. The mechanism of relaxation is discussed in relation to small conductance Ca^{2+} -activated K^{+} (SK) channels.

Materials and Methods

Six to eight-week-old male Wistar rats obtained from JCL (Osaka) were used in the studies described in this report. Rats were lightly anaesthetized with diethyl ether and then stunned by a blow to the head and bled via the carotid arteries. The animal experiments were performed using procedures approved by the institutional Animal Use and Care Committee at the Osaka Prefecture University. Segments of each small intestinal region were removed and placed in Tyrode solution consisting 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.1 mM MgCl_2 , 0.42 mM NaH_2PO_4 , 11.9 mM NaHCO_3 and 5.6 mM glucose. The contents of the excised segments were gently flushed out with Tyrode solution. Whole segments of the duodenum were used. The jejunal

segments were obtained from the part which was defined as the part (2.5–3.0 cm in length) 1-cm-apart from the duodenum. The distal jejunal part was defined as the part adjacent to the proximal jejunum as defined above. The ileal segments were from the mid part of the ileum.

Recording of responses of longitudinal muscle to EFS

Intestinal segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO_2 and 95% O_2 and maintained at 37°C. Since the segments were suspended in a Magnus apparatus in the direction of the longitudinal axis, contractile responses of longitudinal muscle were mainly recorded. The longitudinal muscle of each segment was subjected to a load of 0.5 g to obtain the most reproducible responses and a stable resting tone. Atropine (1 μM) and guanethidine (5 μM) were added throughout the experiment to record NANC responses. After an equilibration period of 30 min, responses of the longitudinal muscle to EFS for 10 s with trains of 1–100 pulses of 0.5-ms width at 30 V, 0.1–10-Hz frequencies were recorded isotonicallly with a 10-min interval between tests. The extent of relaxation was expressed as the area under the line of resting tone that was drawn on the bottom of resting spontaneous contractile activity (broken lines in Fig. 1) as described elsewhere (25).

Recording of membrane potentials in longitudinal muscle of jejunum

The segments of the proximal jejunum were mounted in a 1.5-ml organ bath maintained at 30°C and perfused continuously with Tyrode solution at a rate of 3 ml min^{-1} . This temperature allowed stable recording of the membrane potentials, since the spontaneous and evoked mechanical responses were reduced at this temperature. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing solution throughout the experiment. Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 M Ω . Inhibitory junction potentials were elicited by EFS to intramural nerves within the segment with square-wave pulses of 0.5-ms duration at an appropriate intensity (10–30 V). The electrode impalement was made into the longitudinal muscle cells of the superficial layer from the serosal side (26). The stimulus pulses were delivered with a pair of Ag-AgCl wire electrodes, one on the serosal surface 1–2-mm away from the impaled glass microelectrode and the other in the solution. The distance between the two electrodes was about 20 mm.

Measurement of intracellular Ca^{2+} level of longitudinal muscle cells of proximal jejunum

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) level was measured by using fura-2 AM as described previously (27). Briefly, small strips (about 2.0×7.0 mm) of proximal jejunal longitudinal muscle were incubated in Tyrode solution containing $5 \mu\text{M}$ fura-2 acetoxymethyl ester (fura-2 AM) and 0.02% cremophor EL for 5–6 h at 25°C under 5% CO_2 in O_2 and for about 18 h at 4°C . Contractile responses were recorded isometrically by a strain gauge transducer (U gauge, UL-2GR; Minebea, Tokyo). Changes in $[\text{Ca}^{2+}]_i$ were measured simultaneously in a CAF-100 spectrofluorometer (Nihonbunko, Tokyo). A load of 0.25 g was applied as a resting tension. The relaxation and changes in $[\text{Ca}^{2+}]_i$ were examined after the contraction induced by $1 \mu\text{M}$ carbachol reached a constant level.

Drugs

Apamin, thapsigargin, adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS), and ATP were purchased from Sigma Chemical Co., ST. Louis, MO, USA. The neurotensin antagonist SR48692 was a kind gift from Sanofi Recherche, Toulouse, France. 1-[6-[(17 β -3-Methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122), atropine sulfate, guanethidine, and papaverine hydrochloride were from Wako Pure Chemical Industries, Ltd., Osaka. All other chemicals were of analytical grade. Drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. The volume of vehicle of the drugs, redistilled water, did not affect either the spontaneous contractile activity or the muscle tone. SR48692 and thapsigargin were dissolved in dimethyl sulphoxide as stock solutions. The final dimethyl sulphoxide concentration was 0.1%, which did not have any effect on the preparations.

Statistical analyses

Results were analyzed statistically by Student's *t*-test and a *P* value of <0.05 was regarded as significant.

Results

Effects of ATP-desensitization and an ATP-receptor antagonist on EFS-induced NANC relaxation in proximal jejunal segments prepared from Wistar rats

EFS of the proximal jejunal segment induced relaxation of longitudinal muscle in the presence of $1 \mu\text{M}$ atropine and $5 \mu\text{M}$ guanethidine in a frequency-dependent manner. Although EFS at 0.1 Hz, a single pulse, induced only a transient relaxation, the relaxation became larger with increasing the frequency of EFS and

Fig. 1. Responses of segments of the proximal jejunum of Wistar rats to electrical field stimulation with various frequencies. **A:** Responses of longitudinal muscle of jejunal segments were induced by EFS at the various frequencies indicated. Bars indicate the duration of EFS for 10 s. EFS at 0.1 Hz means a single pulse. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the relaxant response clear. **B:** Summary of EFS-induced NANC relaxation. Relaxations at frequencies indicated are expressed as a percentage of relaxation induced at 10 Hz. Note that contraction began midway through relaxation at 10 Hz. The extent of relaxation was expressed as the area under the line of resting tone that was drawn on the bottom of resting spontaneous contractile activity (broken lines). Values are means $\pm$ S.E.M. for 6 experiments.

was followed by a contraction (Fig. 1). However, relaxation induced at 10 Hz was smaller than that at 3 Hz, since contraction began halfway through relaxation at 10 Hz (Fig. 1). Relaxation induced by EFS at 3 Hz was $18.0 \pm 2.3\%$ ($n = 5$) of the maximal relaxation which was induced by $30 \mu\text{M}$ papaverine. Exogenously added ATP at a concentration of $100 \mu\text{M}$ caused a transient relaxation of the jejunal segments. The extent of the relaxation induced by $100 \mu\text{M}$ ATP was $24.8 \pm 4.3\%$ ($n = 6$) of the maximal relaxation induced by $30 \mu\text{M}$ papaverine. Repetitive application of $100 \mu\text{M}$ ATP, every 5 min without washing, markedly decreased the sensitivity of the segments to ATP and significant desensitization was consistently achieved after 2 or 3 consecutive applications of ATP. EFS-induced NANC

Fig. 2. Inhibitory effects of desensitization of the preparations to ATP and an ATP receptor antagonist on relaxations induced by EFS at various frequencies in the proximal jejunum. A: Relaxations were induced by EFS at 0.3 or 3 Hz before and after desensitization to ATP ($100 \mu\text{M}$). ATP ($100 \mu\text{M}$) was added at the times indicated by triangles. The lines indicate the presence of ATP. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the relaxant response clear. B: Summary of the effects of the desensitization and A3P5PS ($100 \mu\text{M}$) on relaxation at various frequencies. Inhibitions were expressed as a percentage of the corresponding control before the desensitization or addition of the antagonist. Values are means $\pm$ S.E.M. for 3 or 4 experiments. All values are significantly different from the value of the corresponding control, $P < 0.05$.

relaxations at 0.1–1.0 Hz were completely inhibited in the desensitized preparation and those at 3 or 10 Hz were significantly inhibited (Fig. 2).

In another series of experiments, we examined the effect of A3P5PS, a purinoceptor antagonist, on the relaxation induced by EFS at different frequencies. A3P5PS at a concentration of $100 \mu\text{M}$ had a transient inhibitory effect on the basal tone but no effect on spontaneous contractile activity of the longitudinal muscle of the jejunum. A3P5PS significantly inhibited NANC relaxation. The magnitude of the inhibition of relaxation induced by EFS at every frequency examined

Fig. 3. Effects of A3P5PS and SR48692 on relaxations induced by EFS at 1–10 Hz in the proximal jejunum. A: Relaxations were induced by EFS at 10 Hz in the absence or presence of $1 \mu\text{M}$ SR48692 without or with $100 \mu\text{M}$ A3P5PS. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the relaxant response clear. B: Summary of the effects of SR48692, A3P5PS, and a combination of both antagonists. Inhibitions were expressed as a percentage of the relaxation before the treatment. Values are mean $\pm$ S.E.M. for 3 or 4 experiments. *Significantly different from the value of the corresponding control, $P < 0.05$.

was similar to that reported in the ATP-desensitization experiment (Fig. 2).

Relationship between relaxations mediated by ATP and neurotensin in the proximal jejunum of Wistar rats

In our previous studies, neurotensin was reported to partially mediate NANC relaxation in the rat jejunum and ileum (23). We therefore examined the relationship between relaxations mediated by ATP and neurotensin. Relaxation at 10 Hz was inhibited by about 50% in the presence of SR48692, a neurotensin-receptor antagonist,

Fig. 4. Effects of A3P5PS on EFS-induced relaxations in various small intestinal regions. Relaxations were induced by EFS at various frequencies indicated in the absence (control) or presence of 100 μ M A3P5PS in segments of the duodenum, distal jejunum, and ileum. Data were expressed as a percentage of inhibition of the corresponding control. Values are the mean $\pm$ S.E.M. for 3 or 4 experiments. All values are significantly different from the value of the corresponding control, $P < 0.05$.

and the remaining relaxation was completely inhibited in the presence of A3P5PS (100 μ M) (Fig. 3). As noted above, the lower the frequency, the larger the participation of ATP was found. In contrast, the lower the frequency, the smaller the participation of neurotensin was found. A combination of both antagonists resulted in the complete inhibition of the relaxations induced at all frequencies, suggesting that ATP and neurotensin separately mediate the relaxation (Fig. 3B).

Effects of A3P5PS on NANC relaxation induced by EFS at different frequencies were also examined in the segments prepared from the duodenum, distal jejunum, and ileum of Wistar rats. In all the segments examined, A3P5PS completely inhibited the relaxation induced by EFS at 0.1 and 1 Hz and significantly inhibited the relaxation at 10 Hz, which were similar to the results reported for the proximal jejunal segments. However, the inhibition in relaxation at 10 Hz was smaller than that in the proximal jejunum, being about 40–50% inhibition (Fig. 4).

Examination of action mechanism of ATP in NANC relaxation of the Wistar rat proximal jejunum

Since NANC relaxation of the jejunal segments induced by EFS at 1.0 Hz for 10 s appeared to be solely mediated by ATP, the action mechanism of ATP in inducing NANC relaxation was studied under these conditions. It was previously shown that apamin significantly inhibited EFS (10 Hz)-induced relaxation of the jejunal segments by 83% (23). Apamin at 100 nM

Table 1. Effects of apamin and thapsigargin on EFS- and exogenous ATP-induced relaxation of the jejunal segments

	EFS-induced relaxation	% inhibition	
		ATP-induced relaxation	
		1 μ M ATP	10 μ M ATP
Apamin	100 (3)	77.2 $\pm$ 13.9 (5)*	72.2 $\pm$ 13.9 (3)*
Thapsigargin	81.3 $\pm$ 11.4 (5)*	67.0 $\pm$ 13.9 (5)*	100 (4)

Relaxations were induced by EFS at 1 Hz before (control) and after the treatment of the segments with apamin (100 nM) or thapsigargin (1 μ M). Relaxations were also induced by exogenously added ATP at 1 or 10 μ M before and after the treatments. Data are expressed as percentages of the corresponding control. Values are mean $\pm$ S.E.M. for the numbers of experiments noted in the parentheses. *Significantly different from the value of the corresponding control, $P < 0.05$.

completely inhibited the relaxation at 1.0 Hz (Table 1). Although exogenously added ATP at 100 μ M rapidly induced the desensitization, ATP at 1 or 10 μ M induced reproducible small or moderate relaxation, respectively. Therefore, effects of apamin on 1 and 10 μ M ATP-induced relaxation were also examined. The ATP-induced relaxations were also significantly inhibited in the presence of 100 nM apamin (Table 1). Thus, it seems that ATP mediates the NANC relaxation via activation of apamin-sensitive K^+ channels (SK channels) in proximal jejunal segments.

Since SK channels are activated by Ca^{2+} , we next examined the role of a store site of Ca^{2+} , the sarcoplasmic reticulum (SR), in NANC relaxation. The jejunal segments were treated with thapsigargin, an inhibitor of SR Ca^{2+} -ATPase, at a concentration of 1 μ M. EFS-induced NANC relaxation was gradually inhibited by this treatment, and a maximum inhibition of 81% was reached by 30–40 min after thapsigargin addition (Table 1). Exogenous ATP-induced relaxation was also inhibited by thapsigargin, although the magnitude of inhibition was less than that in EFS-induced relaxation (Table 1).

Effects of apamin and thapsigargin on changes in $[Ca^{2+}]_i$ induced by ATP

To examine the effect of ATP on $[Ca^{2+}]_i$, changes in muscle tension and $[Ca^{2+}]_i$ were measured simultaneously. Small strips of the jejunal longitudinal muscle that had been loaded with fura-2AM were pre-contracted with 1 μ M carbachol (CCh). These CCh-treated strips exhibited rapid contraction and subsequent spontaneous activity. The increase in $[Ca^{2+}]_i$ also showed a transient phasic component followed by a tonic sustained component (Fig. 5). When the tonic phases of contraction and the increase in $[Ca^{2+}]_i$ induced by CCh (1 μ M) reached

Fig. 5. Effects of apamin and thapsigargin on ATP-induced relaxation and decrease in $[Ca^{2+}]_i$ in the proximal jejunum. Muscle strips preloaded with fura-2AM were first contracted with 1 μ M carbachol (CCh). A: When the CCh-induced responses reached a constant level, the effects of 10 μ M ATP were examined in the absence (left side) or presence (right side) of 100 nM apamin. The second examination of an effect of ATP in the presence of apamin was carried out 10 min after the first examination. The response induced by 10 μ M ATP was reproducible under these conditions (data not shown). B: The effects of ATP were also examined before (left side) or after (right side) the treatment of the strips with 1 μ M thapsigargin for 1 h. Note that $[Ca^{2+}]_i$ gradually increased during 1 h thapsigargin-treatment, probably due to the capacitative calcium entry mechanism. The top panels in (A) and (B) show ATP-induced relaxation and the bottom panels show the change in $[Ca^{2+}]_i$ as indicated by the fluorescence ratio, F340/F380. Bars above and below the panels show the times of application of the indicated chemicals. *the artifact noise. Inset: high-speed recording of the change in $[Ca^{2+}]_i$ induced by ATP.

Table 2. Effects of apamin and thapsigargin on ATP-induced relaxation and decrease in $[Ca^{2+}]_i$ in the jejunal segments

	% inhibition	
	ATP-induced relaxation	ATP-induced decrease in $[Ca^{2+}]_i$
Apamin	100 (4)	89.6 $\pm$ 10.4 (4)*
Thapsigargin	96.7 $\pm$ 2.1 (6)*	90.8 $\pm$ 6.9 (6)*

Muscle strips preloaded with fura-2AM were first contracted with 1 μ M CCh. When the CCh-induced responses reached a constant level, the effects of 10 μ M ATP were examined in the absence or presence of apamin (100 nM) or thapsigargin (1 μ M). Data are expressed as percentages of the corresponding control. For further details, see the legend of Fig. 5. Values are mean $\pm$ S.E.M. for the numbers of experiments noted in the parentheses. *Significantly different from the value for the corresponding control, $P < 0.01$.

constant levels, ATP (10 μ M) added to the organ bath induced decreases in tension and $[Ca^{2+}]_i$ simultaneously. We next examined the effects of apamin (100 nM) and thapsigargin (1 μ M) on the changes induced by ATP. Pretreatment with apamin (100 nM) had no significant effect on these CCh-induced responses. However, the effects of ATP on $[Ca^{2+}]_i$ and tension were significantly inhibited by apamin-treatment (Fig. 5A, Table 2). Thapsigargin-treatment also resulted in almost complete inhibition of the ATP-induced changes in $[Ca^{2+}]_i$ and tension (Fig. 5B, Table 2).

Effects of A3P5PS on the EFS-induced i.j.ps and effects of apamin on ATP-induced hyperpolarization of longitudinal smooth muscle cells of the jejunum

The resting membrane potential of longitudinal muscle cells of the proximal jejunum was -52.9 ± 2.0 mV ($n = 23$). In the presence of atropine (1 μ M) and guanethidine (5 μ M), EFS with two pulses of 20-Hz frequency at 20 V induced mono-phasic rapid i.j.ps. The i.j.ps were completely inhibited by apamin as shown previously (23). SR48692 at 10 μ M also partially inhibited the i.j.ps and resulted in about 30% inhibition 20 min after treatment, i.j.ps in the absence or presence of SR48692 were 12.6 ± 1.6 mV ($n = 8$) or 8.8 ± 2.0 mV ($n = 8$, significantly different from the value in the absence, $P < 0.05$), respectively (Fig. 6). A3P5PS at 10 μ M completely inhibited the i.j.ps which persisted after SR48692-treatment ($n = 7$, Fig. 6).

Application of ATP at 10 μ M induced slow hyperpolarization of membrane potential of longitudinal muscle cells of the jejunum (2.9 ± 1.1 mV, $n = 7$). At 30 μ M, ATP induced greater hyperpolarization, 7.8 ± 1.3 mV ($n = 8$). Apamin (100 nM) completely inhibited the hyperpolarization (Fig. 7).

Fig. 6. Effects of SR48692 and A3P5PS on EFS-induced i.j.ps in the longitudinal muscle cell of the proximal jejunal segments. I.j.ps were induced by two pulses at 20 Hz in the absence or presence of SR48692. The segment was treated with 10 μ M SR48692 for 20 min. After the treatment, the segment was further treated with A3P5PS for 10 min. Note that SR48692 partially inhibited the i.j.ps and further addition of A3P5PS abolished the remained i.j.ps.

Fig. 7. Effects of ATP in the absence or presence of 100 nM apamin on membrane potential of longitudinal smooth muscle cell of the jejunum. Exogenously added ATP (30 μ M) induced slow hyperpolarization (upper trace). Inhibition of ATP-induced hyperpolarization by apamin (lower trace). Lines indicate the presence of drugs. Apamin was added 20 min before ATP-treatment. Records were from the same longitudinal muscle cell.

Discussion

In the present study, participation of ATP in NANC relaxation induced by EFS in the Wistar rat small intestine was observed. An essential role of ATP in the relaxation induced by EFS at lower frequencies was suggested. The magnitude of the effect of ATP in NANC relaxation was governed by the different frequencies of EFS studied. It has been reported that ATP is the most important transmitter at lower frequencies, but has a rather minor role at higher frequencies in longitudinal muscle-myenteric plexus preparations of the rat ileum (3). On the other hand, the participation of nitric oxide in the EFS-induced relaxation was shown to be of a similar magnitude in all relaxations induced at the various frequencies examined in the jejunum of 2- to 4-week-old

Wistar rats (24). Participation of VIP in the relaxation of the distal colon of 2- to 8-week-old Wistar rats was also demonstrated (28). Although participation of neurotensin in the relaxation induced at 1 Hz was shown to be of a similar magnitude to those at higher frequencies in the Wistar rat jejunum with the desensitization method (23), further detailed examination with a neurotensin receptor antagonist in the present study revealed little effect of neurotensin at 1 Hz, instead of a significant role at higher frequencies (Fig. 3A). These results suggest that the magnitude of participation of a specific NANC neuron in the EFS-induced relaxation sometimes depends on the frequency of EFS, but this is not a characteristic common to all NANC inhibitory neurons in the gastrointestinal tract. Thus, in the Wistar rat jejunum, it seems likely that ATP solely mediates NANC relaxation induced by EFS at lower frequencies, and that ATP and neurotensin separately mediate that induced at higher frequencies, since antagonists for purinergic and neurotensin receptors additively inhibited the relaxation (Fig. 3B).

Apamin completely inhibited EFS-induced NANC relaxation in the jejunal segments. Thapsigargin-treatment also resulted in significant inhibition of the relaxation. An important role of the thapsigargin-sensitive Ca^{2+} store site was also suggested in the nitric oxide donor-induced relaxation in the rat proximal colon (27). The Ca^{2+} increase might be localized just at the vicinity of the sites where SK channels are present since an increase in global Ca^{2+} to threshold levels induces contraction, not relaxation, of smooth muscle cells. In isolated myocytes of the murine colon, it was suggested that activation of P2Y receptors induces release of Ca^{2+} from the intracellular store, and the released Ca^{2+} ions activate SK channels, and then, hyperpolarization of the membrane potential is induced (29). A subsequent study further suggested that localized Ca^{2+} release mediates coupling between purinergic stimulation and SK channels and that localized Ca^{2+} release contributes to spontaneous transient outward currents (STOCs), consisting of charybdotoxin-sensitive and apamin-sensitive events, and also contributes to hyperpolarization induced by purinergic stimulation (30). Therefore, the present results suggest that activation of purinoceptors results in activation of SK channels via an increase in intracellular Ca^{2+} released from the thapsigargin-sensitive Ca^{2+} store site in the Wistar rat jejunum. Hyperpolarization of the membrane potential may result in a decrease in $[\text{Ca}^{2+}]_i$ and in turn, to a relaxation of smooth muscle cells. Indeed, ATP decreased $[\text{Ca}^{2+}]_i$, and induced relaxation. Both apamin and thapsigargin inhibited these ATP-induced responses (Fig. 6). It seems likely that ATP induces a decrease in $[\text{Ca}^{2+}]_i$ necessary

for the contractile machinery in smooth muscle cells to function.

The mediator of NANC relaxation was studied in the presence of atropine and guanethidine and the responses to EFS or exogenous ATP were recorded isotonicly. On the other hand, simultaneous changes in muscle tension with the $[Ca^{2+}]_i$ level were isometrically recorded after the muscle tone had been raised by CCh. However, exogenously added ATP induced relaxation in a concentration-dependent manner, similarly in both types of experiments with different recording methods.

ATP has been suggested to mediate apamin-sensitive fast i.j.ps in various intestinal regions of several animal species (9, 13, 14, 31). In the present study, apamin completely inhibited i.j.ps induced by two pulses of EFS at 20 Hz (this stimulation gave the most reproducible responses). Relaxation induced by EFS at low frequencies was also completely inhibited by apamin as described above. Although the effect of apamin on i.j.ps induced by more pulses was not examined for technical reasons, we have shown ATP-mediated apamin-sensitive relaxation concomitant with apamin-sensitive fast i.j.ps in the Wistar rat jejunum. We previously reported PACAP-mediated apamin-sensitive relaxation concomitant with apamin-sensitive fast i.j.ps in the Wistar-ST rat distal colon (32, 33) and neurotensin-mediated relaxation in the Wistar rat proximal jejunum (23). It was also suggested that an unknown peptide-like substance induced apamin-sensitive relaxation with apamin-sensitive fast i.j.ps in the Wistar-ST rat jejunum (34). However, in the Wistar-ST rat proximal colon (35) and rectum (36), apamin completely inhibited fast i.j.ps but did not affect relaxation induced by EFS. There is a clear dissociation in some tissues between the indication obtained from the electrophysiological study and that obtained by recording mechanical responses.

The most important question that remains unresolved is the localization of SK channels in jejunal tissue. Although coupling of activation of SK channels and relaxation of smooth muscle cells was suggested above, there is no direct evidence for the localization of SK channels in smooth muscle cells. We previously demonstrated the localization of SK3 channels in the interstitial Cajal cell-like cells, but not in smooth muscle cells in the rat intestine (37) and recently showed that these cells are fibroblast like cells neighboring and connecting with gap junctions to smooth muscle cells in the mouse small intestine (38). Therefore, when coupling of purinergic stimulation and SK channels to induce NANC relaxation is studied, the role of SK channels in cells other than smooth muscle cells is not excluded.

We reported nitric oxide-mediated relaxation con-

comitant with apamin-resistant slow i.j.ps in the Wistar rat ileum (23) and VIP-mediated relaxation in the Wistar-ST rat distal colon (25, 34). It is of interest that different mediators induce i.j.ps with different onset times. However, relaxations recorded in smooth muscle preparations including those in the present study can not be distinguished into plural components, that is, fast and slow relaxation. So, we would be remiss in not stating that there is a clear dissociation between time courses of mechanical responses and electrophysiological events.

Difference between the magnitude of the effects of thapsigargin on EFS- and exogenously added ATP-induced relaxations are reported in the present study. Namely, inhibition in exogenous ATP-induced relaxation after thapsigargin-treatment was only partial (about 45%), while that in EFS-induced relaxation was about 80% (Table 1). Some reports suggested that endogenous and exogenous purines activate different subtypes of purinoceptors (39, 40). It was also reported that the purinergic transmission is mainly mediated by P2Y1 receptors located on the smooth muscle cells and by P2X receptors located on both enteric neurons and on smooth muscle cells (41). Thus, the difference shown in the present study remains an interesting area to be clarified in future studies. In summary, ATP mediates NANC relaxation of longitudinal muscle of the jejunum of Wistar rats by activation of SK channels via release of Ca^{2+} from the thapsigargin-sensitive store site.

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