Xue, L., G. Farrugia, and J. H. Szurszewski. Effect of exogenous ATP on canine jejunal smooth muscle. Am J Physiol Gastrointest Liver Physiol 278: G725–G733, 2000.—Intracellular recordings were made from the circular smooth muscle cells of the canine jejunum to study the effect of exogenous ATP and to compare the ATP response to the nonadrenergic, noncholinergic (NANC) inhibitory junction potential (IJP) evoked by electrical field stimulation (EFS). Under NANC conditions, exogenous ATP evoked a transient hyperpolarization (6.5 ± 0.6 mV) and EFS evoked a NANC IJP (17 ± 0.4 mV). Conotoxin GVIA (100 nM) and a low-Ca²⁺, high-Mg²⁺ solution abolished the NANC IJP but had no effect on the ATP-evoked hyperpolarization. The ATP-evoked hyperpolarization and the NANC IJP were abolished by apamin (1 µM) and N⁶-nitro-L-arginine (100 µM). Oxyhemoglobin (5 µM) partially (38.8 ± 5.5%) reduced the amplitude of the NANC IJP but had no effect on the ATP-evoked hyperpolarization. Neither the NANC IJP nor the ATP-evoked hyperpolarization was affected by P2 receptor antagonists or agonists, including suramin, reactive blue 2, 1-(N,O-bis-[5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid, α,β-methylene ATP, 2-methylthioadenosine 5'-triphosphate tetrasodium salt, and adenosine 5'-O-2-thiodiphosphate. The data suggest that ATP evoked an apamin-sensitive hyperpolarization in circular smooth muscle cells of the canine jejunum via local production of NO in a postsynaptic target cell.

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MATERIALS AND METHODS

Adult mongrel dogs of either sex were anesthetized with thiopental sodium, using a protocol approved by the Institutional Animal Care and Use Committee. After the abdomen was opened, a segment of jejunum ~20 cm distal to the ligament of Treitz was removed and placed in oxygenated Krebs solution at room temperature. Other organs were removed by other investigators for other studies. The jejunal segments were opened along their antimesenteric border and transferred to fresh oxygenated Krebs solution at room temperature. The mucosa was removed under a binocular microscope, and full-thickness muscle strips (1 mm x 10 mm) were prepared with the long axis cut parallel to the circular muscle layer. Muscle strips were then placed in a recording chamber and allowed to stabilize for at least 2 h before experiments were performed.
chamber with the circular muscle facing up and pinned to a Sylgard (Dow Corning, Midland, MI)-coated floor of the chamber to record intracellular electrical activity. The chamber (3-ml volume) was perfused with prewarmed (37°C) and preoxygenated Krebs solution at a constant flow rate of 3 ml/min. After an equilibration period of at least 2 h, the muscle strips were stretched to an initial tension of 250 mg from baseline tension. Recordings of intracellular electrical activity from smooth muscle cells were obtained using glass capillary microelectrodes that were filled with 3 M KCl and had resistances ranging from 30 to 80 MΩ. Intracellularly recorded potentials were amplified using a WPI M-707 amplifier (WPI, New Haven, CT), displayed on an oscilloscope (Tektronix 5113; Tektronix, Beaverton, OR), and recorded on chart paper (Gould 220; Gould, Cleveland, OH) and also with an FM tape recorder (Hewlett Packard 3964A; Hewlett Packard, San Diego, CA). Two platinum wires placed parallel to the long axis of the preparation and connected to a square wave stimulator (Grass SIU 5A) were used to apply EFS. Individual electrical pulses were of 0.35-ms duration and 100- to 150-V intensity. The range of frequencies used was 1–30 Hz. ATP was administered by a pressure application device (Picospritzer; General Valve, East Hanover, NJ). A micropipette (10-µm diameter) filled with 0.1 M ATP was placed as close as possible to the recording microelectrode site. Pressure pulses at 12 psi and 60-ms duration were used to deliver ATP. NO solutions were prepared and applied using methods similar to those reported previously (31).

RESULTS

General observations. Spontaneously occurring electrical slow waves were recorded with a mean frequency of 8.9 ± 0.2 min (n = 10 cells from 10 preparations) and a mean amplitude of 10.5 ± 1.7 mV (n = 10 cells from 10 preparations). The mean maximum membrane potential between slow waves was −59.4 ± 0.3 mV (n = 327 cells from 108 preparations). Application of EFS (0.35-ms pulse width; 100 V) with frequencies ranging from 1 to 30 Hz elicited a frequency-dependent, fast monophasic NANC IJP, as previously described (32). The mean peak amplitude of the NANC IJP at 30 Hz was 17 ± 0.4 mV (n = 120 cells from 90 preparations); the mean duration measured from onset of EFS to the end of the IJP was 2,560 ± 66 ms (n = 120 cells from 90 preparations). TTX (1 µM) abolished NANC IJPs (17 ± 1.2 mV in control vs. 0 ± 0 mV in the presence of TTX; P < 0.05; n = 3). NANC IJPs were usually, but not invariably, followed by a rebound depolarization (Fig. 1), a common feature of NANC nerve-evoked IJPs in gastrointestinal smooth muscle (2). We have no explanation for why some preparations generated this rebound depolarization and others did not.

L-NNA (100 µM) completely inhibited NANC IJPs after the tissue was incubated with this drug for 20 min (Fig. 1A; cf. Fig. 6). However, L-NNA had no effect on the rebound depolarization when one occurred (Fig. 1A). In normal Krebs solution, the mean amplitude of the rebound depolarization was 6.8 ± 0.9 mV (n = 7 cells from 7 preparations). In the presence of L-NNA (100 µM), it was 5.8 ± 1.1 mV (P > 0.05; n = 7 cells from 7 preparations). OxyHb (5 µM) significantly reduced the amplitude of NANC IJPs (19.6 ± 3.4 mV in control vs. 11.7 ± 2.3 mV in the presence of OxyHb; P < 0.001; n = 6 cells from 6 preparations; Fig. 1B; cf. Fig. 6) but had no significant effect on the amplitude of the rebound depolarization (6.2 ± 0.4 mV in control vs. 7.1 ± 1.1 mV in the presence of OxyHb; P > 0.05; n = 5 cells from 5 preparations). Increasing the concentration of OxyHb to 10-fold (50 µM) failed to further reduce the amplitude of the NANC IJP. Neither L-NNA nor OxyHb had any effect on membrane potential or electrical slow wave frequency and amplitude.

NANC IJPs were completely inhibited by apamin (1 µM; Fig. 1C; cf. Fig. 6), a blocker of a class of small- and intermediate-conductance Ca2+-activated K+ channels. Apamin slightly depolarized the membrane potential (range = 1–4 mV). In some preparations, an increase in the amplitude of the electrical slow wave was observed (Fig. 1C).

Response to exogenous ATP. ATP (0.1 M) applied between electrical slow waves by a Picospritzer evoked an immediate and transient (4–10 s) membrane hyperpolarization. The mean amplitude of the hyperpolarizing response was 6.5 ± 0.6 mV (n = 30 cells from 10 preparations). The amplitude of the ATP-evoked response was significantly less compared with the NANC IJP amplitude recorded from the same cells (6.5 ± 0.6 mV vs. 16 ± 0.6 mV; P < 0.05). The times to 50% maximum amplitude and to peak amplitude of the ATP-evoked hyperpolarizing response were 674 ± 45 ms and 945 ± 26 ms, respectively (n = 10 cells from 10 preparations). For the NANC IJP (30 Hz), these values
were 556 ± 34 ms and 781 ± 52 ms, respectively (n = 10 cells from 10 preparations). A typical hyperpolarizing response to exogenous ATP is shown in Fig. 2B. Note that a rebound depolarization followed the ATP-evoked hyperpolarization. In 19 cells from 10 preparations, the mean amplitude of the rebound depolarization was 4.4 ± 0.6 mV. The hyperpolarizing response evoked by ATP (0.1 M) and the NANC IJP recorded in the same cell were compared by superimposition (Fig. 2C) and by measuring the rate of membrane hyperpolarization between 25% and 75% of the maximum amplitude. The rate of hyperpolarization evoked by ATP was 13.7 ± 2.5 mV/s, whereas the rate of hyperpolarization of the NANC IJP was 36.5 ± 3.9 mV/s (P < 0.05). The significantly slower rate of hyperpolarization of the ATP response compared with the NANC IJP most likely was due to diffusional delay of the exogenously applied ATP to its receptor site. ATP-evoked responses
were unaffected by TTX (1 µM; 5.1 ± 1.2 mV in control vs. 5.5 ± 1.1 mV in the presence of TTX; P > 0.05; n = 2 cells from 2 preparations). Application by a Picospritzer of the vehicle used to dissolve ATP had no effect on membrane potential and no effect on electrical slow waves (n = 3).

To further clarify the role of ATP in NANC neurotransmission and the possible interaction between ATP and NO generation, the response to ATP was tested in the presence of apamin, L-NNA, and OxyHb. The effect of these drugs on the NANC IJP recorded from the same cells also was tested. In five of six preparations, apamin (2 µM) abolished the response to ATP. In the sixth preparation, the recording microelectrode was dislodged before the response to ATP was completely blocked. The effect of apamin is shown in Fig. 3A, and the results from a series of experiments are summarized in Fig. 6A. Similarly, L-NNA (100 µM) abolished the response to ATP (Figs. 3B and 6A). Apamin (1 µM) and L-NNA (100 µM) also abolished the NANC IJP (Fig. 6A). These data suggested that the apamin-sensitive ATP-evoked hyperpolarization was via a pathway that involved the generation of NO. When the response to ATP was tested in the presence of OxyHb (5 µM), the response to ATP was unaffected (Figs. 3C and 6A), suggesting that NO generated by ATP was not released into the extracellular compartment.

To clarify the site of action of exogenously applied ATP, the effect of ω-conotoxin GVIA (an N-type Ca²⁺ channel blocker) and the effect of a low-Ca²⁺, high-Mg²⁺ solution were studied. Although ω-conotoxin GVIA (500 nM) significantly inhibited the NANC IJP (from 18 ± 2.1 mV to 2.3 ± 0.3 mV; P < 0.05; n = 3 cells from 3 preparations), it had no effect on the ATP-evoked hyperpolarization (5 ± 0.5 mV vs. 4.6 ± 0.13 mV; n = 2 cells from 2 preparations; P > 0.05; Fig. 4A). Similarly, although the low-Ca²⁺, high-Mg²⁺ solution significantly inhibited the NANC IJP (15 ± 1.5 mV in normal Krebs solution vs. 0 ± 0 mV in the low-Ca²⁺, high-Mg²⁺ solution; P < 0.05; n = 3 cells from 3 preparations), it had no effect on the ATP-evoked hyperpolarizing response (3.6 ± 0.4 mV in normal Krebs solution vs. 3.7 ± 0.2 mV in the low-Ca²⁺, high-Mg²⁺ solution; P > 0.05; n = 3 cells from 3 preparations). In the low-Ca²⁺, high-Mg²⁺ solution, the membrane potential depolarized by 2–5 mV in the different preparations and the electrical slow wave frequency and amplitude were slower and smaller, respectively (Fig. 4B).

Response to exogenous NO. NO (1% vol/vol) applied between slow waves by a Picospritzer evoked a membrane hyperpolarization similar to that previously described (31, 32). The amplitude of the hyperpolarizing response was 9.8 ± 0.9 mV (n = 3). In the presence of apamin (1 µm), the NO-evoked hyperpolarization was significantly reduced to 1.3 ± 0.3 mV (P < 0.05; n = 3). Effect of purinergic receptor agonists and antagonists on the response to EFS and exogenous ATP. In visceral tissues, the apamin-sensitive component of the NANC IJP is thought to be mediated by ATP via a P2 receptor (40). We tested for the possibility that the P2 receptor mediated the apamin-sensitive ATP-evoked hyperpolarizing response and the apamin-sensitive NANC IJP.
Preparations were superfused with suramin or reactive blue 2, two putative nonselective P2 receptor antagonists (13). Suramin up to 100 µM had no effect on the NANC IJP (Figs. 5A and 6B) and also had no effect on the ATP-evoked hyperpolarization (Figs. 5A and 6A). Reactive blue 2 up to 300 µM also failed to inhibit the NANC IJP and the hyperpolarizing response to exogenous ATP (Figs. 5B and 6). Preincubation with α,β-MeATP, a P2X receptor agonist (13), for 30 min to desensitize the receptor had no effect on the NANC IJP and no effect on the ATP-evoked hyperpolarization (Figs. 5C and 6). Other agonists and antagonists of the P2 receptor subtype, including the P2X antagonist PPAD (50 µM), the putative P2Y agonists 2-MeSATP (50 µM) and ADPβS (100 µM), and the P2Z/P2X antagonist KN-62 (10 µM), were tested in 28 preparations. None of these agonists nor this antagonist had an effect on either the NANC IJP (Fig. 6B) or the ATP-evoked hyperpolarization (Fig. 6A).

The peptides VIP (18) and PACAP (28) are reported as candidate NANC neurotransmitters in the guinea pig gut and opossum internal anal sphincter. The existence of colocalization of NOS and VIP or PACAP in myenteric ganglion neurons has also been reported in some species (16, 28). To check the possibility that either of these two peptides might mediate NANC neurotransmission in canine jejunal circular smooth muscle, the effects of the agonists and antagonists of VIP and PACAP on the resting membrane potential and NANC IJP were investigated. Neither VIP (100 µM) nor PACAP-(1–27) (100 µM) had any effect on the resting membrane potential of smooth muscle cells when exogenously applied by a Picospritzer. VIP-(6–17), an antagonist of VIP receptors, had no effect on the shape and size of the NANC IJP (15 ± 0.5 mV vs. 15.5 ± 0.2 mV in control; P > 0.05; n = 3). PACAP-(6–38), a PACAP receptor antagonist, increased the NANC IJP amplitude (12.4 ± 0.19 mV vs. 10.5 ± 0.13 mV in control; P < 0.05; n = 3) and partially inhibited the rebound responses that followed NANC IJPs (6.1 ± 0.31 mV vs. 7.9 ± 0.04 mV in control; P < 0.05; n = 3). Because the local application of PACAP did not change the resting membrane potential, the increased amplitude of IJP caused by PACAP-(6–36) was most likely the result of the inhibition of the rebound response by the PACAP antagonist.

Effect of SP. To determine the basis of the rebound depolarization that followed the NANC IJP, we tested the effect of desensitization of the tissue to exogenously applied SP. In four experiments, superfusion with SP (1 µM) depolarized the membrane by 13.2 ± 2.5 mV. After 30 min of pretreatment with SP, the membrane potential returned to the control level, and in the continuous presence of SP the amplitude of the rebound depolarization was 3.3 ± 0.3 mV, a value not significantly different from control (3.8 ± 0.5 mV; P > 0.05).
DISCUSSION

The two key observations made in the present study were 1) L-NNA, a specific inhibitor of NOS, completely inhibited both the NANC IJP and the hyperpolarizing response to exogenously applied ATP, and 2) OxyHb reduced but did not completely inhibit the NANC IJP and had no effect on the ATP-evoked hyperpolarization. The effect of L-NNA implies a role for NO in both the NANC IJP and the ATP-evoked hyperpolarization, whereas the effect of OxyHb implies at least two different sites where NO was generated. The OxyHb-sensitive component most likely was due to NO released from intrinsic nerve fibers since there is ample evidence for the existence of nitrergic nerves in the circular muscle layer of canine (36), human (34), and guinea pig (15) intestines. Previous studies of mechanical and intracellular electrical activities have shown that the NANC inhibitory innervation is in part mediated by NO released from intrinsic nerves (5, 6, 31, 32). The present work confirms these observations.

The OxyHb-insensitive (but L-NNA-sensitive) component suggests that NO was formed, released, and physiologically active in target cells postsynaptic to the innervating NANC inhibitory nerves. The mediator of the OxyHb-insensitive (but L-NNA-sensitive) component of the NANC IJP became the focus of this study. Because ATP-containing nerves are present in the small intestine (1), and because there is ample evidence that ATP also functions as a mediator of the NANC IJP (10, 40), we investigated whether exogenously applied ATP could evoke a hyperpolarization in canine jejunal circular smooth muscle cells and whether the ATP response was also OxyHb insensitive and L-NNA sensitive. The ATP hyperpolarizing response was completely inhibited by L-NNA and was OxyHb insensitive. These data suggest that ATP may function as a neurotransmitter mediating a part of the NANC IJP and that when ATP is released from intrinsic nerves, it causes the production of NO in the target cell. In a previous study on the canine ileum, Boeckstaens et al. (5) found that exogenous ATP evoked an L-NNA-sensitive relaxation that was blocked by TTX and only partially inhibited by OxyHb. Data similar to that obtained in the present study, Boeckstaens et al. (5) suggested that the portion of the ATP-evoked inhibitory response that was OxyHb insensitive was due to the inability of OxyHb to penetrate all actively transmitting sites at which NO was released. On the basis of the data obtained in the present study, we suggest that in the canine jejunum NO was inaccessible to OxyHb because it was generated in the target cell. In previous studies in the guinea pig gut, the NO-evoked component of the NANC IJP...
Since ATP causes formation of NO, the effect of ATP would also be blocked by apamin, as observed. The target cell for ATP was not intrinsic nerve fibers because TTX, α-conotoxin GVIA, and a low-Ca\(^{2+}\)/high-Mg\(^{2+}\) solution had no effect on the ATP-hyperpolarizing response. These data also indicate that the NO formed by the action of ATP did not diffuse back to act presynaptically to enhance further release of neurotransmitter(s). The target cell most likely was either the smooth muscle cell or the network of the interstitial cells of Cajal (ICC). Our results do not provide definitive evidence either way. Although there is no information regarding the ability of ATP to generate NO in small intestinal smooth muscle cells, it is known that other inhibitory neurotransmitters such as VIP stimulate NO production in smooth muscle cells (19), that endothelial NOS is present in human and rabbit gastrointestinal smooth muscle (33), and that inducible macrophage NOS is present in vascular (17) and uterine myometrial smooth muscle cells (21). The possibility that the target cells were the ICC also has merit because ICC in the canine large intestine and rat small intestine may contain the constitutive form of NOS (27, 37). The OxyHb-insensitive component of the NANC IJP and the OxyHb insensitivity of the ATP-hyperpolarizing response would indicate that if NO was produced by ATP in ICC, then the diffusional path of NO would have had to have been across an OxyHb-inaccessible pathway.

The nature of the receptor mediating the response to exogenously added ATP is uncertain. The failure of the P2 receptor agonists and antagonists tested, including α,β-MeATP, 2-MeSATP, ADPβS, suramin, reactive blue 2, PPAD, and KN-62, to inhibit the NANC IJP and the hyperpolarizing response to exogenous ATP was a surprise. One explanation for the failure of these agents to alter the NANC IJP and the response to exogenous ATP is that none is completely selective for the P2 receptor. An alternative explanation is that the action of ATP was not mediated by a “classical” purinergic receptor. Although we have no further evidence on this point, it is interesting to note that in bovine brain arteries, activation of a K\(^+\) channel and enhancement of intracellular Ca\(^{2+}\) concentration induced by ATP appear to be caused by a direct action of ATP on the G protein β-subunits (24).

In many but not all myenteric neurons, NO is colocalized with VIP and/or PACAP, raising the possibility that these peptides, along with ATP, might also be released during EFS. The failure of either VIP or PACAP to evoke hyperpolarization as well as the failure of VIP and PACAP receptor antagonists to alter the NANC IJP rule out the possibility that either of these peptides was involved in inhibitory transmission. There remains to be discussed the rebound depolarization seen following the NANC IJP and following the hyperpolarizing response to exogenous ATP. Similar effects of local application of ATP have been reported in the chicken rectum and guinea pig urinary bladder, suggesting that ATP may not only function as an inhibitory neurotransmitter but may also have a role as
an excitatory neurotransmitter (26). Another possible explanation for the rebound depolarization may be related to the phenomenon known as purine-related rebound excitation (22). The messengers involved in this type of rebound excitation are unclear, but in the guinea pig taenia coli, mouse colon, and rat duodenum, it appears to involve prostaglandin synthesis since the cyclooxygenase inhibitor indomethacin attenuates the rebound effect (3). NO is also considered to be a possible candidate mediator for the rebound excitation because inhibitors of NOS greatly reduce the rebound excitatory response in canine colon (35). However, in the present study, the failure of L-NNA and OxyHb to block the rebound depolarization following the NANC IJP excludes the possibility of NO mediating this electrical phenomenon. Another possibility is that the rebound depolarization was mediated by the release of SP during EFS. However, this does not appear to be the case, since the rebound response was unaffected when the muscle was desensitized by prolonged exposure to SP.

In summary, exogenous application of ATP evoked an apamin-sensitive membrane hyperpolarization in canine jejunal circular smooth muscle cells. ATP appears to be involved in generation of the NANC IJP via production of NO in a postsynaptic target cell. The receptor mediating this action of ATP is unknown.

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