Functional evidence for purinergic inhibitory neuromuscular transmission in the mouse internal anal sphincter


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Functional evidence for purinergic inhibitory neuromuscular transmission in the mouse internal anal sphincter. Am J Physiol Gastrointest Liver Physiol 294: G1041–G1051, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00356.2007.—The neurotransmitter(s) underlying nitric oxide synthase (NOS)-independent neural inhibition in the internal anal sphincter (IAS) is still uncertain. The present study investigated the role of purinergic transmission. Contractile and electrical responses to electrical field stimulation of nerves (0.1–5 Hz for 10–60 s) were recorded in strips of mouse IAS. A single stimulus generated a 28-mV fast inhibitory junction potential (IJP) and relaxation. The NOS inhibitor Nω-nitro-l-arginine (l-NNA) reduced the fast IJP duration by 20%. Repetitive stimulation at 2.5–5 Hz caused a more sustained IJP and sustained relaxation. l-NNA reduced relaxation at 1 Hz and the sustained IJP at 2.5–5 Hz. All other experiments were carried out in the presence of NOS blockade. IJPs and relaxation were significantly reduced by the P2 receptor antagonists 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl(aza)-]1,3-benzenedisulfonic acid (PPADS) (100 μM), by desensitization of P2Y receptors with adenosine 5’-[β-thio]diphosphate (ADP-βS) (10 μM), and by the selective P2Y1 receptor blocker 2’-deoxy-N-methyl adenosine 3’,5’-diphosphate (MRS2179) (10 μM). Relaxation and IJPs were also significantly reduced by the K+ channel blocker apamin (1 μM). Removal of extracellular potassium (Ko) increased IJP amplitude to 205% of control, whereas return of Ko 30 min later hyperpolarized cells by 19 mV and reduced IJP amplitude to 50% of control. Exogenous ATP (3 mM) relaxed muscles in the presence of TTX (1 μM) and hyperpolarized cells by 15 mV. In conclusion, these data suggest that purinergic transmission significantly contributes to NOS-independent neural inhibition in the mouse IAS. P2Y1 receptors, as well as at least one other P2 receptor subtype, contribute to this pathway. Purinergic receptors activate apamin-sensitive K+ channels as well as other apamin-insensitive conductances leading to hyperpolarization and relaxation.

The internal anal sphincter (IAS) aids in maintaining fecal continence and permits evacuation of fecal contents during the rectoanal inhibitory reflex (RAIR). To fulfill these functions, the IAS usually exists in a state of contraction that can be relaxed by inhibitory nerves during the defecation reflex. In the present study we examined inhibitory motor innervation to the mouse IAS. The predominant neurotransmitters underlying inhibitory neural responses in the gastrointestinal tract include nitric oxide (NO), VIP, pituitary adenylate cyclase-activating polypeptide, and ATP (or a related compound). There is still controversy regarding the contribution of these neurotransmitters to neural inhibition in the IAS. Whereas some studies suggest that neural inhibition is almost exclusively due to NO in combination with VIP (40), earlier studies of the guinea pig (39), rat (14), and rabbit (32) IAS provided evidence that a purinergic neural pathway contributes as well.

Membrane hyperpolarization is an important mechanism contributing to inhibitory motor responses. Various enteric neurotransmitters hyperpolarize the postjunctional membrane to produce inhibitory junction potentials (IJPs). Membrane hyperpolarization in adjacent electrically coupled smooth muscle cells leads to the closing of voltage-dependent Ca2+ channels and contractile inhibition (43). To date, the electrical events underlying inhibitory transmission to the IAS have only been described in one species, i.e., the guinea pig (35, 39). Inhibitory nerves were reported to generate a fast IJP that could be reduced by purinergic antagonists and a slower IJP reduced by antagonists of the NO pathway. The authors concluded that both a purinergic and a nitrergic pathway contributed to neural inhibition in this species.

The mouse model is particularly useful for investigating neuromuscular transmission because of the availability of various transgenic knockout models. Previous contractile studies of the IAS in the neuronal NO synthase (nNOS) knockout mouse suggest that NO along with one or more other neurotransmitters participates in inhibitory motor innervation (13, 27, 41). One of these studies further suggests that the remaining NOS-independent transmitter is VIP (41). However, neither the electrical activity of the mouse IAS nor the electrical events accompanying inhibitory transmission have been described. Thus considerable uncertainty still exists with regard to NOS-independent neurotransmission in this model. In other regions of the mouse gastrointestinal (GI) tract there is strong evidence that a purinergic pathway contributes to inhibitory motor innervation [i.e., jejunum (15), colon (45, 47), stomach (36), and one study of multiple GI regions (21)]. Thus it is possible that a purinergic pathway contributes to neural inhibition in the mouse IAS as well.

The goal of the present study was twofold: 1) to characterize the basic electrical properties of the mouse IAS and the IJPs accompanying inhibitory neuromuscular transmission and 2) to examine the contribution of purinergic neurotransmission to the mouse IAS by testing various purinergic antagonists and agonists including PPADS, ADP-βS, MRS2179, and ATP, as well as the small conductance calcium-activated K+ (SK) channel blocker apamin. The role of K+ channels in generation of IJPs was also examined by manipulating extracellular K+ (Ko) concentration. Our results suggest that a purinergic neural pathway plays a significant role in both the electrical and functional properties of the mouse internal anal sphincter.
contractile events initiated by inhibitory motor neurons in this model.

MATERIALS AND METHODS

Tissue preparation. Animals used for these studies were maintained and the experiments performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Use and Care Committee at the University of Nevada approved all procedures used. Seventy-nine BALB/c mice (30–60 days old; Jackson Laboratory, Bar Harbor, MN) were euthanized with isoflurane (Baxter, Deerfield, IL) followed by cervical dislocation. The rectoanal region was removed and pinned in a dissection dish containing cold Krebs-Ringer bicarbonate (KRB) solution of the following composition (in mM): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 23.8 NaHCO₃, 1.2 KH₂PO₄, and 10.0 dextrose. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O₂-5% CO₂. All adhering skeletal muscle, glands, and mucosa were dissected away. The distal most extension of the IAS was identified, and a 2-mm-wide muscle strip including this edge was created.

Contractile measurements. Muscle strips were attached with suture to a stable mount and to a second wire connected to a Gould strain gauge. Muscles were immersed in 3-ml tissue baths containing oxygenated KRB solution maintained at 37°C. A basal tension of 0.3 g was applied. All experiments were performed in the presence of atropine (1 µM) and guanethidine (10 µM) to eliminate the possible contribution of cholinergic and sympathetic nerves. Electrical field stimulation (EFS) was applied by using a Grass S48 stimulator (0.1–5 Hz for 1 min, 15 V, 1-ms duration pulses). These stimulation parameters produced TTX-sensitive neural responses.

Intracellular measurements. Muscle strips were pinned submucosal side up to the base of an electrophysiological chamber. One end of the muscle remained free and was tied with suture to a hook connected to a Gould strain gauge. Muscle cells were impaled with glass microelectrodes filled with 3 M KCl with tip resistances ranging from 60 to 100 MΩ. Impalements were accepted on the basis of a rapid drop in potential upon entering the cell and return of potential to near zero upon removing the electrode from the cell. Membrane potential was measured with a high input impedance electrometer (WPI Duo 773), and outputs were displayed on an oscilloscope ( Nicolet 3091) and PC computer. Data was stored and analyzed by computer using the data acquisition program AcqKnowledge (Biopac systems, Goleta, CA). Nerves were stimulated (0.03–2.5 Hz; 70 V; 0.1 ms) via platinum electrodes placed on either side of the preparation. The stimulus parameters for these experiments differ from the isolated tissue bath experiments since the depth of fluid was less and the electrodes were closer. These stimulation parameters gave rise to TTX-sensitive neural responses. Potassium-free solution was created by replacing all KCl and KH₂PO₄ with NaCl and NaH₂PO₄, respectively. All experiments were carried out in the presence of atropine (1 µM) and guanethidine (10 µM).

Data analysis and statistics. The response to a single stimulus was determined as the area of contraction during the initial 1.5 s after the pulse (neural inhibition) and for 2.5 s during the subsequent rebound contraction. The overall response to nerve stimulation (1–5 Hz) was determined by measuring the area of contraction occurring during 1 min of nerve stimulation (g × s). The ATP-induced relaxation was determined as the contractile area for 15 s in the presence of ATP including the peak inhibitory response. All experimental contractile areas were normalized to control contractile areas during an equivalent amount of time. Poststimulus rebound at 1–5 Hz was determined as peak grams of contraction in the presence of blocker normalized to peak rebound contraction without blocker. The 75% single IJP duration was measured from 25% hyperpolarization to 75% repolarization. Significant differences between groups were determined using one-way ANOVA followed by a post hoc Dunn’s or Tukey’s test; n values indicate the number of animals. Means were considered significantly different at P < 0.05.

Drugs. TTX, atropine, N⁶-nitro-L-arginine (L-NNA), guanethidine, nifedipine, 4-[[(4-formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl]azo]-1,3-benzene disulfonic acid (PAPDS), apamin, adenosine 5'-[β-thio]diphosphate (ADP-BS), ATP, 8-sulfophenylthiophyline, and prostaglandin F2-α (PGF2-α) were purchased from Sigma-Aldrich (Saint Louis, MO). 2’-Deoxy-N⁶-methyl adenosine 3’,5’-diphosphate (MR52179) was purchased from Tocris Bioscience (Ellisville, MO).

RESULTS

Inhibitory motor innervation and the role of NO. The mouse IAS developed phasic contractile activity superimposed upon basal tone shortly after submersion in an isolated tissue bath as shown in Fig. 1A. Phasic contractions averaged 50.6 ± 3.2 counts per min (cpm) (n = 12) in frequency. Spontaneous activity persisted for the duration of the experiment (5–7 h) and could be blocked by either nifedipine (1 µM, Fig. 1B and Ref. 11) or sodium nitroprusside (10 µM, see Ref. 11).

To examine nerve-induced inhibition of contraction, nerves were stimulated with EFS (0.1–5 Hz, 1 min, 1-ms pulse duration, 15 V) in the presence of PGF2-α (1 µM). At 0.1 Hz, each stimulus elicited a transient relaxation followed by rebound contraction (Fig. 2A). The mean values for inhibition and rebound excitation are summarized in Fig. 2B. With increasing frequencies of stimulation, the time available for rebound contraction decreased, and, at 2.5 and 5 Hz, inhibition of contractile activity was sustained and rebound contraction was limited to the termination of EFS (Fig. 2A). Poststimulus rebound at 1–5 Hz is summarized in Fig. 2C. The area of contraction during 1 min of EFS was significantly reduced below control at frequencies of 1 Hz EFS and above (Fig. 2D). All responses to EFS were abolished by the neurotoxin TTX (1 µM, n = 3).

To evaluate the contribution of a nitricergic pathway to neural inhibition, we tested the NOS inhibitor L-NNA (100 µM). The transient relaxation produced with a single stimulus was not reduced by L-NNA (Fig. 2B). In contrast,

Fig. 1. Spontaneous activity in the mouse internal anal sphincter (IAS) is blocked by nifedipine. Sample traces showing spontaneous contractile activity in the mouse IAS. A: following initial submersion in an isolated tissue bath and application of 0.3 grams of tension (arrows labeled “St”), the mouse IAS developed both phasic and tonic contractile activity. Inset: the pattern of phasic contractions at a faster sweep speed (1 µM atropine and 10 µM guanethidine present throughout). B: both phasic and tonic contractions were abolished by addition of nifedipine (1 µM) to the bathing solution.
rebound contractions following 2.5 and 5 Hz EFS were significantly increased (Fig. 2C). L-NNA also significantly reduced the response to 1 Hz EFS but not 2.5 and 5 Hz (Fig. 2D). These data suggest that NO contributes to neural inhibition in the mouse IAS but that other neurotransmitters also play an important role.

Effect of P2 receptor blockade on nerve-evoked relaxation. To examine the role of P2 receptors in inhibitory transmission, we tested several different P2 receptor antagonists on contractions to EFS (0.1–5 Hz) in the presence of L-NNA (100 μM). The nonselective P2 receptor antagonist PPADS (100 μM, 30 min) significantly reduced responses to single stimuli (Fig. 3A), as well as both the EFS-induced reduction in contractile area (Fig. 3B) and rebound excitation (Fig. 3C) at 2.5 and 5 Hz. The role of P2Y receptors was further explored by desensitizing P2Y receptors with ADP-βS (10 μM). Initial exposure to ADP-βS caused a transient inhibition of contraction. Over the next 30 min in the continued presence of ADP-βS, spontaneous activity returned. The pattern of block with either ADP-βS (Fig. 3, D–F) or the selective P2Y1 receptor antagonist MRS2179 (Fig. 3, F–I) was very similar to that observed with PPADS although MRS2179 did not reduce neural responses to as great an extent as that observed with either PPADS or ADP-βS.

Effect of apamin on nerve-evoked relaxation. To examine the role of SK channels in neural inhibition we tested the SK channel blocker apamin. Surprisingly, apamin (1 μM) caused a marked increase in spontaneous contractile activity in muscle strips. This action of apamin occurred both in the absence (Fig. 4B) and presence of TTX (1 μM, n = 3, data not shown). Given the marked increase in contractile activity, it was not possible to reliably evaluate responses to single stimuli or poststimulus rebound. However, the area of contraction during EFS (1–5 Hz) was determined. Apamin converted the EFS-induced reduction in contractile area at 2.5 and 5 Hz to an increase in contractile area (Fig. 4B).

Nerve-induced hyperpolarization and the role of NO. Additional experiments were undertaken to examine the electrical activity in the mouse IAS and the IJPs associated with nerve stimulation. Membrane potential ($E_m$) was not constant, rather irregularly shaped membrane potential oscillations (MPOs) were observed with a frequency of ~45–60 cpm (Fig. 5A). Some MPOs gave rise to variable amplitude spikes. In some cases spikes were followed by a phasic contraction, whereas in other cases they were not (Fig. 5A). The average value for $E_m$ between MPOs was $-46.3 \pm 1.3$ mV ($n = 30$).

To characterize the electrical events underlying inhibitory motor responses, $E_m$ was measured and nerves were stimulated...
with EFS. Individual stimuli (0.1-ms-duration pulse, 70 V) gave rise to IJPs, which averaged 28.9 ± 0.7 mV in amplitude and a 75% duration of 771 ± 19 ms (n = 4). The IJP was followed by a burst of action potentials and rebound contractions (Fig. 5B). At 1 Hz, each IJP was followed by a single spike and a phasic contraction (Fig. 5C). Nifedipine abolished spikes and contraction but not IJPs (Fig. 5D). Resting $E_m$ “noise” persisted in the presence of nifedipine (Fig. 5D) as described for the mouse small intestine (31). At 2.5 Hz the IJP was more sustained (Fig. 6A), and contractile inhibition persisted for the duration of stimulation (Figs. 2A and 6A).

To investigate the role of NO in the electrical events accompanying nerve stimulation, l-NNA (100 μM) was again tested. NOS inhibition did not reduce the amplitude of the fast IJP (28.9 ± 0.7 mV control vs. 27.1 ± 3 mV with l-NNA, n = 4), but the duration of the IJP was significantly reduced from 771 ± 19 ms to 628 ± 22 ms (n = 4). This shortening of the IJP resulted in greater repolarization between stimuli at 2.5 Hz, but contractile inhibition was unchanged (Figs. 6A and 2D). The remaining $E_m$ measurements in this study were carried out in the presence of nifedipine (0.3–1 μM) to aid in maintaining impalements.

NOS inhibition did not significantly depolarize $E_m$ (Table 1), and its effects on IJPs in the presence of nifedipine were the same as those observed in the absence of nifedipine, i.e., the fast IJP amplitude was not reduced (28.1 ± 0.5 mV control vs. 26.4 ± 0.6 with l-NNA, Figs. 6B and 8A), whereas there was a significant reduction in IJP duration (713 ± 62 ms control vs. 525 ± 55 ms n = 4, Fig. 6B), resulting in greater repolarization between stimuli at 2.5 Hz (Fig. 6C). l-NNA also significantly reduced the amplitude of the sustained IJP at the end of 10-s EFS (Fig. 6C) from an average of 24 ± 2.2 mV to 17.8 ± 6.7 mV (Fig. 8B).

**Effect of P2 receptor blockade on nerve-evoked electrical events.** To explore the role of P2 receptors in inhibitory transmission, several different P2 receptor antagonists were tested on electrical responses to EFS. PPADS (100 μM) caused a small but significant depolarization of $E_m$ (3.6 mV, Table 1) and reduced the amplitude of the fast and the sustained IJP (Fig. 7, A and E and Fig. 8). In contrast, ADP-βS (10 μM) caused a 16.7-mV transient hyperpolarization to −62.5 ± 1.8 mV (n = 6, Table 1, Fig. 7B) followed by return of $E_m$ to the control level after ~8–10 min. ADP-βS greatly reduced both fast and sustained IJPs (Figs. 7F and 8). Finally we tested the P2Y1 blocker MRS2179. MRS2179 (10 μM) did not significantly affect resting $E_m$ (Table 1), but it reduced both the fast and sustained IJPs although the degree of inhibition was less...
Role of potassium channels in nerve-evoked IJPs. Additional experiments were undertaken to examine the role of small conductance SK channels in the generation of IJPs in the mouse IAS by testing the SK channel antagonist apamin (1 μM). Apamin (1 μM) caused a 3.6-mV depolarization (Table 1) and significantly reduced both fast and sustained IJPs (Fig. 7, D and H and Fig. 8). To further explore the role of K⁺ channels, we examined the effects of modifying Kᵋ by exchanging the superfusate with one containing no added K⁺. Thirty minutes later K⁺ was readded to the superfusate. IJPs were recorded throughout this time period at 0.05 Hz. Removal of K⁺ resulted in a rapid doubling of IJP amplitude (i.e., to 205 ± 25% of control) as well as a small transient hyperpolarization (Fig. 9, A and C). When K⁺ was returned, Eₘ hyperpolarized by 19 ± 4.5 mV and IJP amplitude was decreased to 50 ± 5.8% of control (n = 4, Fig. 9, B and C).

Effect of ATP on contractile and electrical activity. To determine whether exogenous ATP can mimic the effects of

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**Fig. 4.** Apamin enhances spontaneous contractions and blocks neural inhibitory responses. A: sample traces showing the contractile response to EFS in the absence (left) and presence (right) of apamin (1 μM atropine and 10 μM guanethidine present throughout). Apamin (1 μM) increased contractile activity to more than double that observed with PGF₂α alone. Later addition of PGF₂α in the continued presence of apamin further increased contractile activity. Under these conditions the response to 2.5 and 5 Hz EFS switched from inhibition to excitation. B: summary graph of the effects of apamin on the area of contraction during 1 min EFS (1–5 Hz, n = 10). The neurally evoked reduction in contractile area at 2.5 and 5 Hz was significantly reduced (*) by apamin. Shown are mean values ± SE.

**Fig. 5.** Spontaneous and nerve-evoked electrical activity in the mouse IAS. Shown are sample traces of membrane potential (Eₘ, upper traces) recorded simultaneously with contraction (lower traces). Atropine (1 μM) and guanethidine (10 μM) present throughout. A: Eₘ was not constant but rather irregular membrane potential oscillations (MPOs) occurred along with superimposed spikes of variable amplitude. Spontaneous spikes and phasic contractions were not entirely correlated in this recording. B: delivery of a single stimulus gave rise to an inhibitory junction potential (IJP) and contractile inhibition followed by rebound contraction and spiking activity. C: repetitive nerve stimulation at 1 Hz for 10 s. Note that each IJP was followed by a spike, which in turn was followed by a phasic contraction. D: nifedipine abolished spikes, MPOs, and contraction, but the IJP and a noisy electrical baseline remained.
endogenous transmitter, we tested the effects of 1 and 3 mM ATP on the contractile and electrical activity of the mouse IAS. ATP (1 and 3 mM) caused a 45.2 ± 12.5% (n = 6) and 86.3 ± 3.7% (n = 7) inhibition of contraction, respectively (Fig. 10A). This inhibition persisted in the presence of either TTX (1 μM, n = 3) or the adenosine receptor antagonist 8-sulfophenyltheophylline (100 μM, n = 3). ATP (1 mM) also hyperpolarized cells by 7.7 mV to −53.6 ± 0.7 mV, whereas ATP (3 mM) hyperpolarized cells by 15.4 mV to −61.7 ± 3.0 mV (n = 3, Fig. 10).

**DISCUSSION**

The IAS exists in a state of contracture that can be relaxed by activation of inhibitory motor nerves during defecation. Neural inhibition of the IAS is mediated by both NOS-dependent and NOS-independent pathways. The present study provides evidence that a purinergic pathway significantly contributes to NOS-independent inhibitory motor responses in the mouse IAS.

**Role of electromechanical coupling in inhibitory motor innervation.** The electrical properties of the mouse IAS have not previously been described. Our microelectrode measurements revealed that the IAS is relatively depolarized with an $E_m$ averaging −46 mV. Periodic action potentials (i.e., spikes) of variable amplitude occur as well as smaller $E_m$ fluctuations. Both spikes and contractile activity were abolished by the L-type Ca$^{2+}$ channel (Cav) blocker nifedipine, suggesting that this channel plays a fundamental role in the generation of spontaneous contractions. However, a one-to-one correlation between spikes and phasic contractions was not observed. This is likely due to the fact that $E_m$ was recorded from a single cell, whereas contraction was recorded from the full thickness of the muscle layer at one end of the IAS. Locally generated spikes do not propagate over long distances (44). In contrast, when the electrical activity of the tissue was synchronized by EFS (which stimulates all nerves simultaneously), spikes and phasic contractions were consistently coupled (Fig. 5).

Cav can give rise to a brief regenerative burst of Ca$^{2+}$ current (i.e., the spike) as well as smaller, more sustained “window current” when $E_m$ is within a range of voltages of −50−0 mV (19, 25, 33). Spiking activity can give rise to phasic contractions, whereas window current is associated with sustained tone in a variety of smooth muscles (10, 34, 49).

**Table 1. Effect of blockers on membrane potential**

<table>
<thead>
<tr>
<th>Blocker</th>
<th>Control $E_m$</th>
<th>$E_m$ With Blocker</th>
<th>Change in $E_m$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NNA 100 μM (5)</td>
<td>−46.4±0.7</td>
<td>−46.2±0.4</td>
<td>N/A</td>
<td>NS</td>
</tr>
<tr>
<td>*PPADS 100 μM (7)</td>
<td>−47.8±3.5</td>
<td>−44.2±3.4</td>
<td>+3.6</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>*ADP-βS 10 μM (6)</td>
<td>−45.8±1.2</td>
<td>−62.5±1.8</td>
<td>−16.7</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>*MRS2179 10 μM (5)</td>
<td>−46.2±0.6</td>
<td>−45.0±1.0</td>
<td>N/A</td>
<td>NS</td>
</tr>
<tr>
<td>*Avaspin 1 μM (10)</td>
<td>−45.5±2.3</td>
<td>−41.9±2.6</td>
<td>+3.6</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE. The n values are shown in parenthesis. *N*-nitro-L-arginine (L-NNA) present throughout. All experiments carried out in presence of atropine (1 μM), guanethidine (10 μM), and nifedipine (1 μM). $E_m$, membrane potential; NS, not significant; PPADS, 4-[[4-formyl-5-hydroxy-6-methyl-3-([phosphonooxy]methyl)-2-pyridinyl]azo]-1,3-benzene disulphonic acid; ADP-βS, adenosine 5'-[β-][thio]diphosphate; MRS2179, 2'-deoxy-N'-methyl adenosine 3',5'-diphosphate.
Thus the behavior of Cav channels is sufficient to account for both phasic and tonic contractile activity in the mouse IAS although the degree of contraction for a given level of Ca\textsuperscript{2+}/H\textsubscript{11001} entry is likely to be modulated by proteins such as Rho kinase (38). IJPs either transiently (i.e., the fast IJP) or persistently (i.e., the sustained IJP) hyperpolarized $E_m$ to levels at which most Cav channels are closed (i.e., to around $-60$ to $-75$ mV). Thus IJPs represent an important mechanism by which transmitter release is translated into contractile inhibition in the mouse IAS.

**Role of NO in inhibitory motor innervation.** There are conflicting reports regarding the role of NO as an inhibitory neurotransmitter in the mouse IAS. In the present study, blockade of NOS activity in vitro with L-NNA caused only a small decrease in neurally evoked relaxation in the adult BALB/c mouse IAS. This relative insensitivity to NOS blockade is similar to an in vitro study of wild-type littermates of W/W\textsuperscript{v} mice where nitro-L-arginine methyl ester (L-NAME) did not reduce IAS relaxation (2–8 Hz EFS)(13). In contrast, in the same study, L-NAME reduced IAS relaxation of wild-type nNOS$^{-/-}$ littermates by $50\%$ (13). An even greater decrease in IAS relaxation in wild-type nNOS$^{-/-}$ littermates ($70\%$) was reported by another group for L-NNA (41), whereas a third study of this mouse strain reported no significant effect of L-NNA on IAS relaxation at frequencies below 10 Hz (27). In vivo studies of nitrergic innervation are equally conflicting with one study reporting almost complete blockade of the RAIR at small distending pressures in nNOS$^{-/-}$ mice but only...
Hyperpolarization is seen along with a doubling of IJP amplitude.

Studies may be related to that the RAIR was absent (48). The differences between (13). In contrast, another study of this mouse model reported a 25% reduction in the reflex at larger distending pressures.

Fig. 9. The fast IJP amplitude is modified by changes in extracellular K concentration. A: sample trace showing the changes in IJP amplitude that occurred when K+ was omitted (0 K+) from the superfusate. A small transient hyperpolarization is seen along with a doubling of IJP amplitude. B: when K+ was returned to the bathing solution 30 min later, Edep hyperpolarized and IJP amplitude decreased to less than 50% of control. C: examples of single IJPs (corresponding to the numbered asterisks in A and B) displayed at faster sweep speed. Atropine (1 μM), guanethidine (10 μM), l-NNA (100 μM), and nifedipine (1 μM) present throughout.

A 25% reduction in the reflex at larger distending pressures (13). In contrast, another study of this mouse model reported that the RAIR was absent (48). The differences between studies may be related to 1) mouse strain differences, 2) age of animals studied, and 3) details of the conditions under which in vivo and in vitro measurements were made. Another important consideration is that 4) multiple inhibitory neurotransmitters and receptors appear to participate in responses in the mouse IAS; hence, different experimental conditions may favor one pathway over another. In spite of the discrepancies between studies, each suggests that under some conditions NO significantly contributes to neural inhibition in the mouse IAS.

A single stimulus gives rise to a large (i.e., 25–30 mV) fast (i.e., 75% duration of ~750 ms) IJP in the mouse IAS. Blockade of NOS changes neither the onset nor magnitude of the fast IJP, rather it causes a small (i.e., 20%) reduction in IJP duration. Thus NO only significantly contributes to the later phase of the IJP. Fast purinergic IJPs followed by slower nitrergic IJPs have been widely reported in a variety of GI muscles including the guinea pig IAS (39). The relatively minor role of NO as a participant in membrane hyperpolarization with a single stimulus in the mouse IAS is very similar to the mouse distal colon (46) but differs from the proximal colon where a large slow nitrergic IJP follows the initial fast IJP (46). Interestingly, the decline in slow nitrergic IJP amplitude from proximal to distal colon is associated with both a decrease in the density of NADPH diaphorase-positive neurons as well as an increase in the amplitude of the fast NOS-independent IJP (46). Thus there appears to be a shift in the relative contribution of NOS-dependent and -independent pathways from the proximal colon to the IAS in the mouse GI tract.

NOS blockade did not significantly reduce relaxations at 2.5 and 5 Hz EFS even though the sustained IJP amplitude at 10 s of EFS was significantly reduced (Fig. 8B). The inability of l-NNA to reduce neural inhibition was likely due to the fact that the remaining IJP (i.e., 18 mV after 10 s EFS) was still sufficient to keep Edep (~−65 mV) below the threshold level for activation of Cav channels. Thus the contribution of NO to neural inhibition of contraction may be underestimated due to the presence of a large sustained IJP in the mouse IAS. The amplitude of the NOS-independent sustained IJP decreased by only 35% after 10 s of EFS in mouse. This differs from the sustained IJP of human colon, which declines by ~75% after 10 s of EFS (28). The greater persistence of the IJP in mouse may be due to 1) purinergic receptors that desensitize less rapidly, 2) slower depletion of the Ca2+ stores needed for activation of Ca2+-dependent K+ channels, and/or 3) participation of other NOS- and purinergic-independent neural pathway(s).

Role of purinergic receptors in inhibitory motor innervation.

To evaluate purinergic motor innervation in the mouse IAS, we tested various blockers of this pathway. The nonselective P2 receptor antagonist PPADS, as well as desensitization of P2Y receptors with ADP-βS, significantly reduced contractile inhibition along with fast and sustained IJPs, suggesting that a purinergic pathway and, in particular, P2Y receptors play a prominent role in inhibitory transmission in the mouse IAS. Purinergic transmission, associated with fast IJPs and P2Y receptors, has also been described for other GI regions and species (17, 20, 21, 23, 39, 45, 51, 53, 55, 56). ADP-βS caused an initial transient hyperpolarization as well as contractile inhibition followed by repolarization and redevelopment of

Fig. 10. The putative neurotransmitter ATP causes relaxation and hyperpolarization in the mouse IAS. Shown are the contractile and electrical effects of 3 mM ATP. A: ATP blocked phasic and tonic contractile activity. After ATP removal, the bath solution was exchanged 2 additional times (w). B: sample trace of the effect of ATP on membrane potential. Downward deflections are fast IJP elicited at 0.05 Hz. A train of stimuli (2.5 Hz EFS, 10 s) was also applied once during the control period and once during superfusion with ATP. ATP caused a 21-mV hyperpolarization in this tissue and greatly reduced IJP amplitude. Both Edep and IJP amplitude were restored following washout of ATP.
contractile activity. These effects are commensurate with an initial stimulation of P2Y receptors followed by receptor desensitization, as suggested by others (20, 45, 53, 55). ADP-βS also inhibits fast IJP's in the mouse and human colon (20, 45, 55), mouse caecum (56), and human jejunum (53), suggesting similar mechanisms for generating fast IJP's in these muscles.

Fast and sustained IJP's as well as neural inhibition of contraction were all significantly reduced by the more selective P2Y1 receptor antagonist MRS2179 (7), providing evidence that P2Y1 receptors play an important role in purinergic transmission in the mouse IAS. This conclusion is in agreement with studies of several other GI regions and species (20, 37, 51). However, since ADP-βS caused a significantly greater reduction in the fast IJP than MRS2179 did, it suggests that other P2Y receptors may be involved in neural responses as well. This differs from the human and mouse colon and guinea pig small intestine where MRS2179 almost completely abolishes IJP's (20, 37, 51). Besides activation of P2Y receptors, it is possible that P2X receptors contribute to the generation of IJP's as proposed for the human jejunum (53). The P2X receptor agonist α,β methylene ATP causes relaxation in the presence of TTX in rat and guinea pig colon (50, 54) as well as in various mouse GI regions (22). We have observed a similar relaxation to α,β methylene ATP in the mouse IAS (K. Keef and R. Hamilton, unpublished observations). This relaxation has been suggested to be coupled to Ca²⁺ entry through P2X receptor cation channels leading to stimulation of SK channels and hence hyperpolarization (50). At present, eight P2Y receptor genes and seven P2X receptor genes have been described (3), and seven of these, i.e., P2Y1,2,4 and P2X1,2,3,5, have been identified in the mouse GI tract (9, 22, 42). Additional studies are required to determine which P2 receptor subtypes, besides P2Y1, participate in purinergic relaxation and hyperpolarization in the mouse IAS. Finally, there is evidence that other neurotransmitters such as VIP contribute to inhibitory neural responses in the IAS (40), including studies of the mouse IAS (41).

Exogenous ATP produced hyperpolarization as well as TTX-independent relaxation in the BALB/c mouse IAS, indicating that ATP can mimic the inhibitory effects of endogenous neurotransmitter. This observation is particularly relevant since previous studies of wild-type littermates of nNOS−/− mice (derived from C57BL/6 mice) have reported that ATP causes either no effect (13) or contraction (41). These differences may be due to differences in purinergic receptor populations and/or second messenger pathways between mouse strains. Indeed, although ATP caused relaxation in the BALB/c mouse IAS, the more selective P2Y2 and P2Y4 receptor agonist UTP causes contraction (K. Keef and R. Hamilton, unpublished observations). We have not pursued this issue further since bath application of drug does not faithfully mimic the neurally evoked response, i.e., neurotransmitter is released toward postjunctional receptors that may differ significantly from the receptor populations present on nonjunctional regions of the muscularis (for review see Ref. 52). Nonetheless, since both ATP and the P2Y receptor agonist ADP-βS caused inhibition of contraction and membrane hyperpolarization in the BALB/c mouse IAS, it lends support to the hypothesis that P2Y receptors are capable of producing inhibitory responses in this tissue.

At present the purine responsible for purinergic transmission is still controversial. Although ATP is a long standing candidate for transmission (6), recent studies of the mouse colon and ileum provide evidence that β-nicotinamide adenine dinucleotide (β-NAD) rather than ATP may be the transmitter (37). Since the aim of the present study was to determine whether purinergic transmission plays a role in the mouse IAS, we did not investigate this issue further but suggest that either ATP or a related purine such as β-NAD may be responsible for purinergic inhibitory motor responses.

Role of K⁺ channels in inhibitory motor innervation. The fast IJP in other GI muscles has been attributed to an increase in K⁺ channel conductance (e.g., Ref. 23). To investigate whether this is the case in the mouse IAS, we removed Kₑ. When K⁺ was removed from the bathing solution, IJP amplitude doubled. Removing Kₑ causes the K⁺ equilibrium potential (E钾) to become much more negative (e.g., E钾 = −182 mV for Kₑ = 0.1 mM) and greatly increases the driving force for K⁺ current. Thus the large increase in IJP amplitude is consistent with K⁺ channels underlying the IJP. When K⁺ was returned to the bathing medium, the IJP amplitude was greatly reduced. This effect is also consistent with K⁺ channels underlying the IJP since the driving force for K⁺ current is reduced by both the reduction in E钾 as well as the hyperpolarization that accompanies return of K⁺ (due to sodium pump stimulation, Ref. 8). In contrast, our results are not compatible with a decrease in Cl⁻ conductance underlying the IJP (12, 23) since extracellular Cl⁻ concentration remained unchanged throughout the experiment.

The fast IJP has specifically been attributed to the opening of small conductance SK channels (51, 55). The link between P2Y receptors and SK channel activation is suggested to involve release of Ca²⁺ from the sarcoplasmic reticulum (2, 4, 55). In the mouse IAS, we found that the SK channel blocker apamin (26) reduced IJP's by −50%, suggesting that these channels participate in generation of the fast IJP but that one or more other K⁺ channel contributes as well. Interestingly, although the effects of apamin on the IJP were very similar to those of MRS2179, apamin had a markedly greater effect on nerve-evoked contractile inhibition than MRS2179 did. This difference is likely related to the profound excitatory effect of apamin on spontaneous contractile activity (see Fig. 4) that occurred both in the presence and absence of TTX. This suggests that there is ongoing basal SK activity in the muscles that suppresses spontaneous contractions. Basal SK activity may be due in part to entry of Ca²⁺ through Cav. However, this pathway cannot account in total for basal SK channel activity since apamin still caused membrane depolarization in the presence of nifedipine (Table 1).

Mechanism of rebound excitation. The rebound contractions following single stimuli and trains of stimuli at 1, 2.5, and 5 Hz were also reduced by purinergic antagonists. This initially suggested the intriguing possibility that two opposing responses accompanied nerve stimulation, i.e., purinergic inhibition followed by purinergic excitation. However, the spikes and depolarization following the IJP were largely eliminated by nifedipine, particularly when E ['./hpjg.physiology.org/DownloadedFrom']
bound excitation in the mouse IAS is likely related to the spiking behavior of this muscle (e.g., Fig. 5). IJP s can further enhance spiking activity via “anode break excitation” (16). Rebound excitation may also be facilitated by other hyperpolarization-activated inward currents such as the voltage-dependent nonselective cation channel described for mouse colon (1). Interestingly, 1,NNNA reduced the rebound contraction following a single stimulus but increased rebound contraction following 1 min of stimulation at 2.5 or 5 Hz. The effect on single stimulus events may be related to the decrease in IJP duration caused by NOS blockade. This reduces the time available for activation of conductances participating in rebound excitation. In contrast, although NOS blockade causes some reduction in the sustained IJP amplitude, most hyperpolarization persists. The remaining hyperpolarization may still be sufficient to activate rebound conductances. The increase in rebound contraction under these conditions may occur because NOS blockade eliminates the NO normally present at the termination of repetitive stimulation, i.e., NO is no longer present to oppose rebound contraction.

In summary, this study has characterized the basic electrical and contractile activity of the mouse IAS and the junction potentials that underlie inhibitory motor innervation. Our results suggest that a purinergic pathway significantly contributes to inhibitory motor innervation in this animal model. This conclusion is in agreement with earlier microelectrode studies of the guinea pig IAS (35, 39) as well as previous contractile studies of the rabbit and rat IAS (14, 32). A purinergic inhibitory neural pathway has been described for the human colon (20, 28), and there is preliminary evidence that this pathway may contribute to neurally evoked relaxations in the human IAS as well (5). Purinergic transmission gives rise to membrane hyperpolarization that relaxes smooth muscle via the closing of voltage-dependent Ca2+ channels. Our results indicate that P2Y1 receptors and apamin-sensitive K+ channels contribute to this hyperpolarization; however, additional P2 receptors and K+ channels appear to participate as well.

REFERENCES


