Effects of Compounds That Influence $I_K$ (KCNN4) Channels on Afterhyperpolarizing Potentials, and Determination of $I_K$ Channel Sequence, in Guinea Pig Enteric Neurons

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INTRODUCTION

Contractile activity of the mammalian intestine is controlled through the enteric nervous system. Within this system, the intrinsic primary afferent neurons (IPANs) play a critical role in determining the excitability and patterns of reflex activity (Thomas and Bornstein 2003). Drugs that target IPANs are therefore of interest as therapeutic agents (Clerc et al. 2002). IPANs respond to changes in luminal chemistry, mucosal deformation and tension of the gut wall (Bertrand et al. 1997; Kirchgeissner et al. 1992; Kunze et al. 1998), and they connect with interneurons and motor neurons that complete the reflex circuits for motility, secretion, and local blood flow control (Furness 2006). In the guinea pig small intestine, the IPANs have AH electrophysiological characteristics and Dogiel type II morphology (Furness et al. 2004; Gershon 2005). A defining feature of AH/Dogiel type II neurons is the prolonged hyperpolarization of the late afterhyperpolarizing potential (AHP) that follows action potentials (Hirst et al. 1974). The late AHP inhibits further action potential firing of AH neurons for a period of several seconds after an action potential or a group of action potentials is elicited (North 1982). This period of quiescence, combined with excitation through synaptic connections that join the AH neurons into networks, has the potential to synchronize AH neuron firing. In the CNS, synchronized firing is believed to be important in determining that assemblages of neurons provide appropriate outputs (Singer 1999; Somogyi and Klausberger 2005). It is thus feasible that the channels of the late AHP could be targeted to change intestinal function (Clerc et al. 2002; Thomas and Bornstein 2003).

Studies of the biophysical properties of the late AHP current and of the underlying channels in guinea pig and mouse enteric neurons have suggested that the channels of the late AHP are intermediate-conductance potassium ($I_K$) channels (Mao et al. 2006; Vogalis et al. 2002a). Furthermore, enteric neurons in the rat, mouse and human show immunoreactivity for $I_K$ channels, and the late AHP in mouse enteric neurons is blocked by the $I_K$ channel blocker, clotrimazole (Arnold et al. 2003; Furness et al. 2003; Mao et al. 2006; Neylon et al. 2004b). However, a recent study has shown that a blocker of $I_K$ channels, TRAM39, does not affect the AHP in guinea pig enteric neurons (Davies et al. 2006). These authors suggest that $I_K$ channels in enteric neurons of guinea pigs may have different properties to $I_K$ channels elsewhere. Because of the potential importance of this observation, we have investigated the sensitivity of the AHP to the more potent $I_K$ channel antagonist, TRAM34, to another antagonist, clotrimazole, and to the $I_K$ channel activator, DC-EBIO. We have also extracted RNA from guinea pig enteric ganglia to test the presence of $I_K$ channel transcripts, and we have determined the sequence of the guinea pig $I_K$ channel.
METHODS

Electrophysiology

Guinea pigs of either sex (180–300 g) were stunned by a blow to the head and killed by severing their carotid arteries and spinal cord. All procedures conformed to National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee. Segments of ileum were placed in oxygenated (95% O2-5% CO2) physiological saline of the following composition (in mM): 118 NaCl, 4.8 KCl, 25 NaHCO3, 1.0 NaH2PO4, 1.2 MgSO4, 11.1 glucose, and 2.5 CaCl2. The physiological saline contained nicardipine (3 μM) and scopolamine (1 μM) to eliminate muscle movements (both compounds from Sigma-Aldrich, Sydney, Australia). The segments were cut along the line of mesenteric attachment and pinned flat under moderate tension with the mucosa uppermost. The mucosa, submucosa and circular muscle were removed to expose the myenteric plexus attached to the longitudinal muscle layer. The preparation of longitudinal muscle plus myenteric plexus was transferred to a warmed recording chamber mounted on an inverted microscope. The recording chamber was perfused with the physiological saline at 4 ml/min and was maintained at 33–34°C. TRAM34 [1-{[2-chlorophenyl]diphenyl]imethyl]-1H-pyrazole; synthesized in house, according to the method of Wulf et al. (2000), clotrimazole (Sigma) and DC-EBIO (5,6-dichloro-1-ethyl-2-benzimidazolone; Tocris Bioscience, http://www.tocris.com) were applied into the bath perfusate. A 10 mM stock solution of TRAM34 was prepared by dissolving the salt in pure dimethyl sulfoxide (DMSO) contained in acid-washed glassware. Complete solubilization was achieved by agitation using a vortex mixer for 15 min at room temperature. The stock was stored at −20°C and was renewed every 2 wk. Working dilution solutions for experiments were made up from the stock on the day of experiment. Dissolution of stock solution was by mixing for 30 min in physiological saline that had been gassed with 95% O2-5% CO2. TRAM34 needed to be added slowly into the working solution and to be mixed gently. The solution must be gassed for ≥30 min for TRAM34 to dissolve completely. If there was insufficient mixing, TRAM34 precipitated on glass or plastic surfaces, making the true concentration much lower than that calculated. If glassware and plastic tubes were not cleaned, there was a tendency to precipitation and adherence of TRAM-34 to container walls. Stock solutions of clotrimazole and DC-EBIO were made up at 10 mM in DMSO and stored at −20°C for ≤6 mo. Stock solutions were diluted in physiological saline. The final concentration of DMSO in the working solutions was 1:1000 to 1:100,000. These concentrations of DMSO had no effect on electrophysiological properties of enteric neurons.

Glass microelectrodes used for recording were filled with 1.0 M KCl and had resistances in the range of 50–90 MΩ (voltage-clamp experiments) and 100–120 MΩ (current-clamp experiments). Signals were recorded under single-electrode voltage and current-clamp conditions, using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) or an NPI SEC05LX amplifier (NPI Electronic GmbH, Tamm, Germany). Recordings were digitized at 10 or 20 kHz and stored using PC-based data acquisition software (Clampex 8.1, Axon Instruments). All electrophysiological data were analyzed on Igor Pro v4.0 (Wavemetrics) and Clampfit v8.1 (Axon Instruments). ANOVA, or paired t-test, with a significance level of P < 0.05, were used to compare data sets.

Isolation of ganglia

Segments of ileum were kept in physiological saline on ice for 30 min. The solution contained 3 μM nicardipine and 1 μM scopolamine (Sigma-Aldrich) to inhibit muscle contraction. Segments (2 cm long) were opened along the mesenteric border and pinned in a elastomer-based dish. All glassware and dissecting instruments were sterilized with absolute alcohol and rinsed with distilled water before the dissection. Tips of forceps were soaked in alcohol and rinsed with sterile cold physiological saline before dissecting each ganglion. The preparations were continuously superfused (10 ml/min) with cold physiological saline during the dissection. The mucosa, submucosa, and circular smooth muscle were carefully dissected away to expose the myenteric plexus. The ganglia were dissected manually from the tissue using fine forceps under an inverted microscope. Only healthy ganglia in the center of the tissue, without muscle overlying them, were selected and the time for dissecting each ganglion was kept at <30 s. Twenty to 30 ganglia were collected in cold extraction solution (RNAlater, Ambion, Austin, TX) from each sample and mRNA was extracted immediately after the dissection.

RNA extraction and sequencing

Guinea pigs were taken as described in the preceding text, and segments of ileum and bladder were placed in phosphate-buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing 1 μM nicardipine to prevent muscle contraction during dissection. Tissues were pinned out in silicone elastomer (Sylgard) dishes on ice to enable dissection into layers to yield either ileum external muscle plus myenteric plexus (EM/MP) or bladder urothelium. Samples were placed in RNAlater and stored at −20°C prior to use. Total RNA was later extracted using the RNaseasy Mini Kit (Qiagen, Melbourne, Australia).

RNA concentration was determined spectrophotometrically, and ~15 ng to 1 μg was then reverse-transcribed using Superscript III reverse transcriptase (Invitrogen, Melbourne, Australia) and oligo(dT) primers in a 20 μl reaction volume. Reactions for each RNA sample were also run in the absence of Superscript III to probe for contaminating genomic DNA.

The resultant genomic DNA free cDNA samples were then amplified by PCR using Platinum Taq DNA polymerase (Invitrogen) and primer pairs for IK channels, 14F&19R, IK 18F&38R, IK 18F&28R (Table 1). Primers were based on alignments of rat, mouse, and human nucleotide sequences (Accession Nos.: human, AF022150; mouse, NM008433; rat, NM023021) because the guinea pig sequence was unknown. Primers against the muscle specific gene, smoothelin, were used to confirm that the microdissected ganglia preparations were free of muscle. Smoothelin primers 7Fand8R (Table 1) were based on regions conserved between all smoothelin splice variants in human and mouse (mouse, NM013870; human, NM132470; human 2, NM132469; human 3, NM006932).

Touchdown PCR was performed using annealing temperatures ranging from either 74–57 or 70–50°C. PCR products were resolved by electrophoresis on 1.7% agarose gels. Sequencing was carried out using ABI PRISM Big Dye Terminator V3.1 (Applied Biosystems, Melbourne, Australia). Gel separation and sequencing were conducted by the Australian Genome Research Facility, Melbourne, Australia.

TABLE 1. Primers used to probe for I K channels and smoothelin (SMTH)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Base Positions</th>
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<tbody>
<tr>
<td>IK-014F</td>
<td>GCACGAGAAGCTGGCTAG</td>
<td>−37 to −20</td>
</tr>
<tr>
<td>IK-20F</td>
<td>GCCTCAACCAAATCCCTTTC</td>
<td>552 to 571</td>
</tr>
<tr>
<td>IK-019R</td>
<td>GCATTCTGAGAATGTCGAC</td>
<td>1105 to 1123</td>
</tr>
<tr>
<td>IK-018F</td>
<td>CAACAGGCGGAGAAACACG</td>
<td>873 to 892</td>
</tr>
<tr>
<td>IK-028R</td>
<td>TGGCCTCTGCTGTTGGTTCTG</td>
<td>1654 to 1675</td>
</tr>
<tr>
<td>IK-038R</td>
<td>GGCTCCTGGACCTGAGGTTG</td>
<td>+91 to +109</td>
</tr>
<tr>
<td>SMTH-007F</td>
<td>CACTCAATAAGTGCCAC</td>
<td>2238 to 2254</td>
</tr>
<tr>
<td>SMTH-008R</td>
<td>CAGTGTGGAGGAGAATTC</td>
<td>2684 to 2701</td>
</tr>
</tbody>
</table>

Primers used are based on human, mouse, and rat sequences (see METHODS). Positions given are relative to the human gene sequence. −, before the coding region; +, after the coding region. All primer pairs that were used were intron-spanning.
RESULTS

Effects of \( I_K \) channel active drugs on the late AHP

AH neurons in the myenteric plexus of the small intestine were identified by the presence of a late slow AHP that followed the action potential and a hump on the repolarizing phase of the action potential (Figs. 1 and 2).

It was necessary to use the protocols given in METHODS to dissolve the \( I_K \) channel blocker TRAM34 and to maintain it in solution. This included using acid-washed glassware, making stock solution in DMSO and thorough mixing. TRAM34 was very slow to dissolve directly in aqueous solutions.

TRAM34 (0.1 \( \mu \)M in the bath perfusate) substantially reduced the size of the AHP that followed a single action potential in AH neurons (Fig. 1). Partial block was observed after 10-min exposure and maximum block was achieved by 20 min. All measurements of changes caused by TRAM34 were made after 20 min of exposure to the compound. The integrated potential change (area under the curve, AUC) of the AHP following a single action potential was reduced from 101 ± 17 to 37 ± 20 (SE) mV.s \((P < 0.01, n = 8)\). At a concentration of 1 \( \mu \)M, there was further reduction to 13 ± 6 mV.s \((P < 0.01, n = 9)\). The peak amplitude of the AHP was also reduced: to 56% of control with TRAM34 at 0.1 \( \mu \)M and to 38% with 1 \( \mu \)M TRAM34 (Fig. 1). TRAM34 did not modify the action potential height or shape or the brief AHP (early AHP) that follows it (Fig. 1). TRAM34 (0.1 \( \mu \)M) caused a small depolarization of 6.1 ± 1.3 mV \((n = 8; \text{Fig. } 1E)\) and increased the input resistance from 166 ± 14 to 232 ± 32 M\( \Omega \) (Fig. 1F). In the presence of 1 \( \mu \)M TRAM34, the depolarization was 10.9 ± 2.2 mV \((n = 9)\) and the input resistance increased to 246 ± 17 MΩ. These differences from control are significant \((P < 0.01, n = 8 \text{ for } \text{TRAM34, } 0.1 \mu \text{M, and } n = 9 \text{ for } \text{TRAM34, } 1 \mu \text{M})\).

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**FIG. 1.** Effects of TRAM34 on electrophysiological properties of AH/Dogiel type II neurons in the guinea pig ileum. A: single action potential, prior to TRAM34, elicited a late afterhyperpolarization (AHP). The AHP was blocked by 1 \( \mu \)M TRAM34 (bottom). B and C: TRAM34 did not change the shape of the action potential, in particular, it did not alter the hump on the declining phase of the action potential (arrow), which is shown by the inflection in the first derivative of the trace (right, arrow). Top: control; bottom: TRAM34. D–G: changes in electrophysiological parameters caused by TRAM34 (0.1 and 1 \( \mu \)M). Data shown as means ± SE, control \((n = 12 \text{ neurons})\), and during exposure to 0.1 \( \mu \)M \((n = 8 \text{ neurons})\) and 1 \( \mu \)M \((n = 9 \text{ neurons})\). Five of the neurons were exposed to both concentrations. TRAM34 reduced the AHP amplitude \((D)\) and the integrated AHP (area under the curve, E). TRAM34 reduced membrane polarization, 1 \( \mu \)M causing a depolarization of ~10 mV \((F)\). TRAM34 increased input resistance by ~50% \((F)\). All changes were statistically significant in comparison with control \((P < 0.02)\).
The excitability of neurons was increased after TRAM34, which is a predictable consequence of the block of the AHP current and the increase in input resistance. This was demonstrated by increased numbers of action potentials evoked by intracellular depolarizing current injection.

Clotrimazole (10 μM) caused similar changes to TRAM34, with maximum effect being observed after 20-min exposure (Fig. 2). The integrated AHP potential change (AUC) following a single action potential was reduced from 80 ± 14 to 19 ± 5 mV·s (P < 0.01, n = 13). Clotrimazole depolarized the neurons by 9.5 ± 2.5 mV (n = 10) and increased the input resistance from 234 ± 40 to 364 ± 42 MΩ (n = 10). Clotrimazole did not significantly alter the amplitude of the action potential or change the hump on its descending phase. However, it slightly slowed the maximum rate of rise of the action potential (Fig. 2), from 272 ± 16 to 223 ± 24 V/s (P < 0.05, n = 11). The decline from peak, which represents the Ca²⁺ hump, was not changed. The slope of the decline was −29 ± 3 V/s before and −26 ± 4 V/s after 20-min exposure to clotrimazole (10 μM). Like TRAM34, clotrimazole increased neuronal excitability.

DC-EBIO (100 nM), which increases the opening probability of I_K channels in the presence of Ca²⁺, enhanced AHP amplitude and caused an increase in the underlying current (Fig. 3). DC-EBIO did not change the action potential shape or amplitude (Fig. 3, A and B, insets). In many but not all neurons, the AHP repolarized to resting membrane potential more quickly in the presence of DC-EBIO. This change in the rate of AHP repolarization could be a consequence of a greater involvement of the hyperpolarization-activated I_h current in the return of the membrane potential to its resting value. The I_h current is prominent in 80% of AH neurons (Rugiero et al. 2002). To avoid activation of I_h, the AHP current (I_AHP) was recorded in voltage clamp mode (Fig. 3, C and D). A group of three step depolarizations to −50 mV (5 ms, 20 ms apart) were passed through the recording electrode each 20 s to cause an inward Ca²⁺ movement to elicit the I_AHP. In the presence of DC-EBIO (100 nM), the resultant I_AHP increased in amplitude over a period of 30 min (Fig. 3), from 65 ± 7 to 95 ± 13 pA (significantly different, P < 0.02, n = 6). When DC-EBIO was applied for longer than 60 min, the amplitude and AUC of the I_AHP gradually declined. A more rapid decline in response was observed at a higher concentration of DC-EBIO (1 μM). Thus the effect of DC-EBIO follows a bell-shaped curve, as previously reported (Koegel et al. 2003). DC-EBIO (100
nM) caused a hyperpolarization of 3.1 ± 1.2 mV (n = 6) and decreased the input resistance by 47 ± 12 MΩ (n = 6).

Identification and sequence determination of I\(_K\) channel gene transcripts

RT-PCR products, from preparations of isolated myenteric ganglia, myenteric ganglia/external muscle and from bladder urothelium, in each case from at least three different guinea pigs, showed expression of I\(_K\) channel mRNA that resolved on agarose gels at predicted sizes (Fig. 4). RT-PCR products for the smooth muscle protein, smoothelin, were absent from extracts of isolated ganglia although they were found in the extracts from myenteric ganglia/external muscle (Fig. 4). Moreover, contamination by smooth muscle should not be an issue, as I\(_K\) channels do not occur in mature smooth muscle cells in the gastrointestinal tract or elsewhere, although they are expressed by the proliferative, but not the mature contractile smooth muscle phenotype of the vascular wall (Chen et al. 2004; Köhler et al. 2003; Neylon et al. 1999; Thompson-Vest et al. 2006). Overlapping primer pairs, 14F&d19R: 1,160 bp; 20F&19R: 572 bp; 18F&38R: 521 bp (Table 1), encompassing the entire coding region, were used to amplify and sequence guinea pig I\(_K\) from preparations of ileum EM/MP and from urothelium. The number of animals was at least four for each product with sequence obtained from both bladder and ileum for each product. Sequences were obtained in both the forward and reverse directions with the total number of clean sequences at least five for each product. Sequences were combined to generate a consensus sequence and the amino acid sequence was then deduced (Fig. 5).

The potassium selectivity GYG motif, the N-glycosylation site, the clotrimazole/TRAM34 binding site and the charybdotoxin binding site were all conserved. The sequences shown to be necessary for correct channel localization are also conserved (K198, also leucine zipper in the C terminal tail and L18, L19 and L25 in the N terminal). The predicted phosphorylation sites were conserved except that the PKA site at S332 (rat, 334 in human) is not conserved, being proline in guinea pig. Previous experiments with rat I\(_K\) indicate this site to be involved in the regulation of the channel (see DISCUSSION).

**Discussion**

The present work shows that TRAM34 (0.1–1 µM) and clotrimazole (10 µM), both antagonists of I\(_K\) channels (Wulff et al. 2001), inhibit the late AHP in AH neurons of the guinea pig small intestine and that DC-EBIO (100 nM), an I\(_K\) channel opener (Singh et al. 2001), enhances the AHP current. We sequenced the channel transcript and showed that the binding sites for clotrimazole and TRAM34 were both present in the guinea pig channel. These sites are more accessible from the

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**Fig. 3.** Effects of DC-EBIO on the AHP in AH/Dogiel type II neurons of the guinea pig ileum. Records from an AH/Dogiel type II neuron are shown before (A) and in the presence (B) of DC-EBIO (100 nM). Before DC-EBIO was applied, the AHP amplitude was ~10 mV on average, and after 20-min exposure to the compound, it was ~15 mV and was greatly prolonged. DC-EBIO did not change the properties of the action potential; in particular, it did not change the Ca\(^{2+}\) hump on its falling phase (action potentials shown at greater sweep speed in the insets). C and D: effect of DC-EBIO (100 nM) on the late AHP current, recorded by single-electrode voltage clamp. E: average increase in peak AHP current caused by 100 nM DC-EBIO (mean ± SE, n = 6 neurons). F: increase in the integrated current (area under the curve) caused by DC-EBIO. Changes in the AHP were statistically significant (P < 0.02).
cytoplasmic face of the channel (Dunn 1998; Wulff et al. 2001). Thus when applied to intact cells, these compounds need to penetrate the cell membrane to produce channel block. In transfected cells, TRAM34 reduces $I_K$ currents to 40% at a concentration of 1 μM and to ~15% at 5 μM (Wulff et al. 2001). We have found that a concentration of 0.1 μM significantly reduced the AHP (to ~40%) after a single action potential and that 1 μM reduced the integrated potential change to ~15%. We attribute the greater potency that we have observed to a more effective solubilization of the compound. TRAM34 has a 200 – 1,500 selectivity for $I_K$ channels over other $K^+$ channels (Wulff et al. 2000), which is consistent with our observation that TRAM34 (0.1 and 1 μM) had no effect on the repolarization of the action potential or the early AHP. The repolarization is due to activation of $K_{Ca}$ delayed rectifier channels and also BK channels; block of the delayed rectifier channels with TEA substantially prolongs the action potential (Hirst et al. 1985; Rugiero et al. 2002). Likewise, the BK channel inhibitor, charybdotoxin, prolongs the action potential (Vogalis et al. 2002b). An A-like current that contributes to the early AHP and is blocked by 4-AP is observed in these neurons (Galligan et al. 1989). TRAM34 neither prolonged the action potential nor reduced the early AHP, confirming its selectivity for the late AHP channels. Our data are also consistent with recordings of channel openings in guinea pig AH neurons. After an action potential there is enhanced activity of intermediate conductance $Ca^{2+}$-activated $K^+$ channels the opening of which is inhibited by clotrimazole (Vogalis et al. 2002a,c). $I_K$ channels also open after action potentials in AH neurons in the mouse (Mao et al. 2006). In confirmation of the single-channel data, we found that clotrimazole (10 μM) substantially reduced the AHP. We also found that the $I_K$ channel opening compound, DC-EBIO (Singh et al. 2001) caused a large increase in AHP current amplitude without affecting the action potential. Thus our pharmacological data support earlier investigation of single-channel activity to conclude that the current of the AHP in the guinea pig small intestine is caused by the opening of $I_K$ channels.

Other data also point to the channels of the late AHP being $I_K$ channels. The AHP current in myenteric neurons in the guinea pig is well established to be a $Ca^{2+}$-activated $K^+$ current (Hirst et al. 1985; Morita et al. 1982; North 1973). There are three families of $Ca^{2+}$-activated $K^+$ channels, BK (large conductance), $I_K$ (intermediate conductance), and SK (small conductance) channels. BK channels are highly voltage sensitive and include those BK channels that occur in enteric AH neurons (Kunze et al. 2000; Vogalis et al. 2002a), whereas the late AHP is almost voltage insensitive, its amplitude being related to the potassium gradient across the membrane by the Goldman-Hodgkin-Katz equation (Hirst et al. 1985; Morita et al. 1982; Rugiero et al. 2002). The intermediate conductance channels of the late AHP are also voltage insensitive and have conductances of ~60 pS, and the ensemble averaged time course of their opening after an action potential matches the AHP (Mao et al. 2006; Vogalis et al. 2002a). SK channels have conductances of 4–14 pS (Vergara et al. 1998). In contrast to $I_K$ channels, SK channels are insensitive to clotrimazole and TRAM34 (Wulff et al. 2000). In addition, SK channels are generally sensitive to apamin, which does not affect the AHP (Tack and Wood 1992; Vogalis et al. 2002a). In summary, the voltage insensitivity of the late AHP current and of underlying channel opening, unitary conductance amplitudes of underlying channels, $Ca^{2+}$ dependence, selective block by clotrimazole and TRAM34 and enhancement by DC-EBIO, all indi-
Our data, and the other published data that are reviewed in the preceding text, are in apparent contradiction with the observation made by Davies et al. (2006), who found that a different I\(\text{K}\) channel blocker, TRAM39 (1 \(\mu\)M) did not affect the AHP. TRAM39 has about a third the potency of TRAM34, is poorly soluble, and has the additional disadvantage that it causes significant inhibition of cytochrome P450 (Wulff et al. 2000). These authors found that TRAM39 inhibited the AHP in sympathetic ganglia and suggested that the form of the channel may be different in enteric neurons. This was also suggested by Vogalis et al. (2003), who in particular pointed to the differences in sensitivity to kinases of the I\(\text{K}\)-like channels of enteric neurons and the I\(\text{K}\) channels in epithelial cells. The present results indicate that the I\(\text{K}\) channel sequence in enteric neurons and epithelial cells is the same, and that the guinea pig sequence has only minor differences from the rat, mouse, and human I\(\text{K}\) channel sequence. Nevertheless, it is possible that the pharmacological properties may be different because of the molecular environment of the I\(\text{K}\) channel in enteric neurons.

TRAM34 depolarized the AH neurons and increased input resistance. This implies that IK channels are active at rest and that the AHP current contributes \(-9 \text{ mV}\) to the resting membrane potential. These data are consistent with earlier data in which depolarization was observed when Ca\(^{2+}\) was removed from the solution (North and Tokimasa 1987) or the current was blocked with charybdotoxin (Kunze et al. 1994). Neurons were hyperpolarized by DC-EBIO, as would be expected, as clearly the AHP current is not maximally activated at rest. The degree of hyperpolarization is probably not a true reflection of the degree of activation of the channels, as it was almost certainly curtailed by the concomitant activation of I\(\text{h}\) in response to hyperpolarization (Galligan et al. 1990).

We identified IK mRNA in extracts of myenteric ganglia from guinea pig small intestine, which is consistent with our pharmacological analysis of the AHP and its underlying current. We also used the urothelium, which is rich in I\(\text{K}\) channel expression (Thompson-Vest et al. 2006), as a source of guinea pig I\(\text{K}\) channel mRNA. Determination of the full amino acid sequence of the guinea pig I\(\text{K}\) channel revealed high sequence homology with the channel from other mammals. This sequence is entered into GenBank: Accession No. DQ911473. The sequence contains the binding sites for clotrimazole/TRAM34, Thr\(^{250}\) and Val\(^{275}\), that are absent from SK channels (Neylon et al. 1999; Wulff et al. 2001). The presence of these sites is consistent with our pharmacological observations. It surprised us that the serine at position 332 (rat, this is 334 in human), which has been proposed to be the major phosphorylatable site that is targeted by PKA to reduce channel opening (Neylon et al. 2004a), is substituted by proline in the guinea pig channel and arginine in dog (Fig. 5). Single-channel and whole cell current recording from guinea pig myenteric neurons indicate that the I\(\text{K}\) channel is closed by activation of PKA or by direct exposure to catalytic subunits of PKA (Vogalis et al. 2003). Thus there may be another mechanism of PKA-mediated inhibition of the channel in the guinea pig. This possibility remains to be investigated.

**Conclusion**

This study demonstrates that the pharmacological profile of the late AHP current is consistent with previous electrophysiological analysis that indicates that this current is carried by I\(\text{K}\).
intermediate conductance, $K_{Ca}$ channels. It also shows that the guinea pig $I_K$ channel is similar in sequence to that of other mammals. It contains the binding sites for $I_K$ channel inhibitors, but it lacks what may be a critical regulatory site. The sensitivity of the AHP to the $I_K$ channel blockers and openers suggests that such compounds may be used to modulate patterns of activity of the gastrointestinal tract.

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GRANTS

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