Kir3.1/3.2 encodes an I\textsubscript{KACH}-like current in gastrointestinal myocytes

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Kir3 family members encode G protein-gated inwardly rectifying K\textsuperscript{+} channels (I\textsubscript{KACH}) in other tissues, including the heart and brain. In the GI tract, I\textsubscript{KACH} could act as a negative feedback mechanism to temper the muscarinic response mediated primarily through activation of nonselective cation currents and inhibition of delayed-rectifier conductance. Kir3 channel subfamily isoforms expressed in GI myocytes were determined by performing RT-PCR on RNA isolated from canine colon, ileum, duodenum, and jejunum circular myocytes. Qualitative PCR demonstrated the presence of Kir3.1 and Kir3.2 transcripts in all smooth muscle cell preparations examined. Transcripts for Kir3.3 and Kir3.4 were not detected in the same preparations. Semi-quantitative PCR showed similar transcriptional levels of Kir3.1 and Kir3.2 relative to β-actin expression in the various GI preparations. Full-length cDNAs for Kir3.1 and Kir3.2 were cloned from murine colonic smooth muscle RNA and coexpressed in Xenopus oocytes with human muscarinic type 2 receptor. Superfusion of oocytes with ACh (10 µM) reversibly activated a Ba\textsuperscript{2+}-sensitive and inwardly rectifying K\textsuperscript{+} current. Immunohistochemistry using Kir3.1- and Kir3.2-specific antibodies demonstrated expression in circular and longitudinal smooth muscle cells. We conclude that an I\textsubscript{KACH} current is expressed in GI myocytes encoded by Kir3.1/3.2 heterotetramers.

Electrical rhythmicity of gastrointestinal (GI) smooth muscles modulates contractile activity of the muscle, leading to GI motility. The slow wave cycle ensures a period of relaxation between contractions to allow mixing and movement of luminal contents. Ionic conductances expressed in interstitial cells of Cajal and smooth muscle cells determine, to a large degree, the properties of the electrical slow wave (6). Resting membrane potential in canine colonic smooth muscle at the submucosal border is approximately −80 mV, close to the K\textsuperscript{+} equilibrium potential (27). The Na\textsuperscript{+} pump has been suggested to participate in the generation of the very hyperpolarized resting potential in this tissue (2) but may not play a significant role in the generation of pacemaker activity (1). However, another factor that may influence resting potential in these cells is K\textsuperscript{+} conductance in the form of strongly inwardly rectifying K\textsuperscript{+} channels (Kir2.1) (5). This study found that micromolar concentrations of Ba\textsuperscript{2+} inhibited slow wave activity in the canine colon and depolarized resting membrane potential. The identification of inwardly rectifying K\textsuperscript{+} conductance in colonic smooth muscle led us to question whether inwardly rectifying K\textsuperscript{+} channels might participate in the response of the tissue to neurotransmitters.

ACh is an excitatory neurotransmitter in the gut (8). Application of 1 µM ACh to strips of colonic smooth muscle increases slow wave duration and contractile activity (9). On the other hand, even under this excitatory influence rhythmicity is not abolished and tonic contraction does not occur. The primary targets of muscarinic stimulation are nonselective cation channels (14). These are probably activated through G protein α-subunits and pass inward current carried by Na\textsuperscript{+} and Ca\textsuperscript{2+}, depolarizing membrane potential and activating L-type Ca\textsuperscript{2+} channels. In addition to this depolarizing current, muscarinic activation leads to the inhibition of delayed-rectifier K\textsuperscript{+} channels in GI smooth muscles (28), potentiating the excitatory response through a prolongation of slow wave duration. Our hypothesis is that a hyperpolarizing current may also be activated during muscarinic stimulation to provide repolarization during excitatory stimulus, maintaining rhythmicity and preventing a tonic contraction. Maintenance of rhythmic contractile activity allows for increased motility under excitatory conditions.

Members of the Kir3 family encode G protein-gated inwardly rectifying K\textsuperscript{+} channels (GIRKs) (4, 12). In cardiac myocytes, I\textsubscript{KACH} has been identified and characterized (3, 19, 23). Activation of this current slows heart rate and acts to hyperpolarize membrane potential. Krapivinsky et al. (11) identified the molecular components underlying this current to be a combination of Kir3.1 and Kir3.4. However, other combinations of Kir3 family members can encode a similar current (7, 18). I\textsubscript{KACH} could provide the hyperpolarizing current hypothesized to maintain rhythmic GI electrical and contractile activity during excitatory stimulus. We examined GI smooth muscles for the expression of Kir3 family members and demonstrated the presence of Kir3.1 and Kir3.2 but did not detect Kir3.3 or Kir3.4. We have cloned the GI smooth muscle forms of Kir3.1 and Kir3.2...
and determined the properties of these channels expressed in oocytes. We conclude that these GIRKs encode an I\textsubscript{K,ACH} like conductance in GI myocytes.

**MATERIALS AND METHODS**

Isolation and collection of GI tissues and cells. Mongrel dogs were overdosed with pentobarbital sodium (100 mg/kg), and a midline incision was made along the abdomen. The stomach, small bowel, and proximal colon were removed and immediately placed in Krebs solution containing (in mM) 120.35 NaCl, 5.9 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 15.5 NaHCO\textsubscript{3}, 1.2 Na\textsubscript{2}HPO\textsubscript{4}, and 11.5 glucose (pH 7.4 after equilibration with 95% O\textsubscript{2}-5% CO\textsubscript{2} at 37°C). Segments of stomach, small bowel, and proximal colon were pinned in a dissecting dish with the mucosa facing upwards, and the overlying mucosa and submucosa were removed by sharp dissection. Strips of circular smooth muscle (1 mm × 10 mm) were cut parallel to the circular muscle axis (26). Smooth muscle cells from the circular muscle layer of the proximal colon, duodenum, ileum, and jejunum were enzymatically dispersed as previously described by Langton et al. (13). Smooth muscle cells were transferred to the stage of a phase contrast microscope and allowed to adhere to the glass coverslip bottom of the chamber for 5 min. Smooth muscle cells were differentiated from other cell types by their characteristic morphology: spindle-shaped cells with a length of 50–100 µm and a width of 5–10 µm. Through applied suction, single smooth muscle cells were collected by aspirating them into a wide-bore patch-clamp pipette (borosilicate glass; Sutter Instruments). Approximately 60 smooth muscle cells were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use.

RNA isolation and RT-PCR. Total RNA was prepared from various tissues and smooth muscle cells by use of the SNAP Total RNA Isolation kit (Invitrogen) per the manufacturer’s instructions. Polynucleosinic acid (a carrier of RNA, 20 µg) was added to lysates because RNA was isolated from small amounts of tissue (5–20 mg) or isolated smooth muscle cells (~60 cells). First-strand cDNA was synthesized from RNA preparations using SuperScript II RNase H Reverse Transcriptase ( Gibco BRL). RNA (1 µg for either quantitative or qualitative PCR studies performed on tissue; 1 µg for qualitative PCR on 60 single smooth muscle cells) was reversely transcribed by use of an oligo(dT)\textsubscript{20} primer (500 µg/µl). To perform PCR, the following sets of primers were used: Kir3.1 sense nt 430–450 and antisense nt 751–770 (GenBank accession no. U39196); Kir3.2 sense nt 853–872 and antisense nt 1161–1180 (L78480); Kir3.3 sense nt 960–980 and antisense nt 1382–1402 (L77929); Kir3.4 sense nt 920–940 and antisense nt 1193–1213 (L47208); and β-actin sense nt 2383–2402 and antisense nt 3071–3091 (V01217). PCR primers for β-actin were used to assess the viability of RNA samples as well as to detect genomic DNA contamination, whereby the primers were designed to span an intron in addition to two exons. In addition, c-Kit primers (X016128, sense nt 2259–2283 and antisense nt 2873–2897) were used to detect interstitial cell contamination and PGP9.5 primers (PGP9.5; D10699, sense nt 34–53 and antisense nt 344–363) were used to detect neuronal contamination. Complimentary DNA (20% of the first-strand reaction) was combined with sense and antisense primers (20 µM), 1 mM dNTPs, 40 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 units Taq (Promega, Madison, WI), 1 AmpliWax Gen 100 (Perkin Elmer, Foster City, CA), and RNase-free water to a final volume of 50 µl. PCR was performed in a Perkin Elmer 2400 Thermal Cycler under the following conditions: 32 cycles at 94°C for 15 s, 57°C for 20 s, 72°C for 1 min, and then incubation at 72°C for 10 min. For single-cell PCR, if no amplification product was detected in the first round of amplification 10% of the first-round PCR products were added to a new reaction mixture containing all of the components listed above and 32 additional cycles of PCR were then performed. All PCR products were separated by 2% agarose gel electrophoresis and sequenced by use of an automated nucleotide sequencer (Applied Biosystems, model 310). In every case throughout the study, amplification products of the predicted size for the primer pairs were gel extracted and sequenced to confirm their identity.

Quantitative PCR. Quantitative PCR was performed by use of the PCR MIMIC construction kit (Clontech), which is based on a competitive PCR approach; nonhomologous engineered DNA standards (referred to as PCR MIMICs) compete with target DNA for the same gene-specific primers. PCR MIMICs were constructed for Kir3.1, Kir3.2, and β-actin. Competitive PCR was carried out by titration of sample cDNA with known amounts of the desired nonhomologous PCR MIMIC constructs; 10-fold serial dilutions of these constructs were then added to PCR amplification reactions. After PCR, products were separated by 2% agarose gel electrophoresis and quantified by use of Molecular Analyst.

Cloning and in vitro transcription of Kir3.1 and Kir3.2. Kir3.1 and Kir3.2 were cloned from colonic smooth muscle in the presence of gene-specific primers for Kir3.1 (sense nt 44–63 and antisense nt 1534–1553; D45022) and Kir3.2 (sense nt 474–498 and antisense nt 1936–1959; U11859), respectively. Full-length cDNA fragments were ligated into pCR2.1 vector constructs (Invitrogen) and transformed by use of the TA cloning kit (Invitrogen). Clones were then sequenced by use of an automated nucleotide sequencer (Applied Biosystems, Model 310). Kir3.1 and Kir3.2 capped RNA (cRNA) were transcribed in vitro from pCR2.1 plasmids containing full-length Kir3.1 or Kir3.2 cDNA by use of the Ambion mMessage mMachine transcription kit. Briefly, reactions contained cRNA (5–25 µg of linearized template DNA, 500 µM ribonucleotides, 1x transcription buffer, and 40 units T7 RNA polymerase; the final concentration of cRNA was adjusted to 1 ng/µl).

Oocyte isolation and injection. Adult female Xenopus laevis from Xenopus Express, Homosassa, FL and Xenopus 1, Dexter, MI) were anesthetized in chilled 0.17% 3-aminobenzoic acid ethyl ester solution (Sigma). The ovarian lobes were removed and placed in ND96 solution plus 100 µg/ml gentamicin (Sigma). ND96 solution contained (in mM) 2.4 sodium pyruvate, 96 NaCl, 2 KCl, 1 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, and 5 HEPES, pH 7.4. The lobes were mechanically opened and incubated in collagenase (type IA, 1.2 mg/ml; Sigma) in ND96 solution at room temperature for 2–3 h to remove the follicular layer. The oocytes were collected, rinsed, and stored in ND96 solution plus gentamicin (100 µg/ml) at 19°C for up to 24 h before injection. Stage V and VI oocytes were injected with 50 nl of mRNA encoding Kir3.1 and/or Kir3.2 plus the human M2 muscarinic receptor (hM\textsubscript{2}) to a total volume of 50 nl using a Drummond Nanoject microinjector (Drummond Scientific, Broomall, PA). The ratio of cRNA was adjusted so that the concentration of each cRNA was equal and a total volume of 50 nl was injected. The oocytes were then stored at 19°C for 2–5 days until electrophysiological assay.

Electrophysiological methods. Whole cell K\textsuperscript+ currents were recorded using the two-microelectrode voltage-clamp technique (GeneClamp 500, Axon Instruments, Foster City, CA). Microelectrodes were pulled from glass capillaries (Kimas-51, Kimble Products) with resistances of 1–3 MΩ when filled with 3 M potassium aspartate. Oocytes were superfused with a low-C\textsubscript{1} solution designed to minimize the endog-
enous Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current in oocytes. This solution contained (in mM) 90 potassium methanesulfonate, 2 MgCl\textsubscript{2}, 5 HEPES, and 0.05 niflumic acid, pH 7.4. In some cases, K\textsuperscript{+} was replaced by equimolar Na\textsuperscript{+}. Reagents were applied to the bath (volume 0.5 ml) via a gravity-fed perfusion system. The dead time to exchange solutions was ~30 s. Each experiment was performed at room temperature (24–28°C) on oocytes collected from more than one frog. In some experiments, potassium methanesulfonate was replaced with equimolar sodium methanesulfonate to examine the K\textsuperscript{+} selectivity of the channel. Data were collected using a GeneClamp 500 amplifier connected to a DigiData 1200 A/D converter (Axon Instruments) interfaced to a PC clone microcomputer. Voltage protocols were applied using pCLAMP 6.0 software (Axon Instruments). In short, 400-ms voltage steps were applied from a holding potential of ~10 mV to test potentials ranging from −100 mV to +50 mV in 10-mV increments. No correction for leak subtraction was applied. Data were analyzed with Microcal Origin software and expressed as means ± SE, with n representing the number of oocytes. Statistical analysis was performed using the Student’s paired or unpaired t-test, and P values of <0.05 were regarded as significant.

Solutions and drugs. Stock solutions of ACh, atropine (1 mM; Sigma), and Ba\textsuperscript{2+} (1 M; Sigma) were prepared in distilled water. Immediately before use, stock solutions were diluted to the final desired concentration in low-CI\textsuperscript{-}, Ca\textsuperscript{2+}-free solution.

Data analysis and statistical treatments Qualitative PCR was performed on smooth muscle cells isolated from the circular layer of the canine colon, duodenum, ileum, and jejunum from at least three different dogs. For quantitative PCR studies, tissue samples from two different animals contributed to each RNA preparation and at least three different RNA isolations were performed. The concentration of the target DNA as well as β-actin was determined on each sample; target DNA concentration was then normalized to β-actin expression. All results are expressed as means ± SE, and n = number of experiments. Data for quantitative PCR were analyzed by one-way ANOVA, and differences between tissues were illustrated by Newman–Keuls multiple comparison tests. P values <0.05 were considered significant.

Immunohistochemistry. Tissues from the proximal colon and jejunum were opened, and luminal contents were washed with Krebs-Ringer-bicarbonate solution. Tissues were pinned to the base of a Sylgard dish mucosal side up and fixed in 4% paraformaldehyde (wt/vol) made up in 0.01% PBS (0.1 M, pH 7.4) for 30 min at 4°C. After fixation, tissues were cut into slices longitudinally along the lumen and transversely across the lumen of the intestine using a scalpel. Tissues were then washed for 3 × 30 min in PBS. Tissues were cut into small muscle strips (2 × 10 mm) and cryoprotected in a graded series of sucrose solutions (5, 10, 15, and 20% wt/vol made up in PBS, 1 h each). Tissues were subsequently embedded overnight in a solution containing Tissue Tek (Miles) and 20% sucrose in PBS (1 part/2 parts vol/vol), and the following day they were rapidly frozen in isopentane precooled in liquid nitrogen. Cryosections were cut on a cryostat (Leica CM3050) at a thickness of 8 µm and collected on Vectabond-treated slides (Vector Laboratories, Burlingame, CA). Nonspecific antibody binding was reduced by incubation in 10% goat serum for 1 h at room temperature. Tissues were incubated overnight with either polyclonal anti-Kir3.1 (Alomone Labs) or polyclonal anti-Kir3.2 (Alomone Labs), both raised in rabbit at manufacturer’s recommended dilutions. For negative control, primary antibody was omitted and PBS was added in its place. Immunoreactivity was detected using fluorescein FITC-conjugated secondary antibody (FITC anti-rabbit) at a dilution of 1:200 in PBS for 1 h at room temperature. Sections were then washed 3 × 15 min in PBS and mounted with an aqueous mounting medium (Aquamount, Pittsburgh, PA). Mounted slides were viewed, and fluorescence photomicrographs were taken using a Nikon eclipse E600 fluorescence microscope with appropriate excitation and emission wavelengths for fluorescein FITC.

RESULTS

Expression of Kir3 family members in GI smooth muscles. The presence of the Kir3 channel subfamily in canine GI smooth muscle cells was determined by performing RT-PCR on total RNA isolated from proximal colon, duodenum, ileum, and jejunum circular smooth muscle cell preparations. Qualitative RT-PCR was performed on myocytes that were individually selected in an attempt to eliminate other contaminating cell types from the analysis. Transcripts for Kir3.1 and Kir3.2 (Fig. 1, A and B, respectively) were observed in proximal colon, duodenum, ileum, and jejunum smooth muscle cell preparations.

To test for genomic DNA contamination in the RNA preparations, RT-PCR was performed in the presence of β-actin primers spanning an exon. Transcript amplification should generate only a 498-bp band; the presence of genomic DNA would have yielded a 708-bp band (Fig. 1C). Any RNA preparations resulting in amplification of a 708-bp product were discarded. In addition, PCR reactions were performed on aliquots of the RNA prepared from cells in which RT was not added during the cDNA synthesis step (RT−). Any preparations that demonstrated amplification products from the RT− reactions were discarded. Although qualitative PCR demonstrated the isoforms of Kir3 that were present in

![Fig. 1. RT-PCR detection of Kir3 channel subfamily members in canine gastrointestinal (GI) circular smooth muscle cell preparations. Amplified PCR products generated by use of gene-specific primers for Kir3.1 (A), Kir3.2 (B), and β-actin (C) were fractionated on 2% agarose gels; size markers were used to indicate size of experimental fragments. RT-PCR yielded visible amplified products of Kir3.1 and Kir3.2 in mRNA isolated from proximal colon, duodenum, ileum, and jejunum circular myocytes. RT-PCR performed in the presence of β-actin gene-specific primers demonstrated that amplification products were representative of RNA (denoted by 498-bp band; C) and not genomic DNA (absence of 708-bp band; C).](image-url)
canine GI myocytes, differences in expression levels of the detected transcripts in the various GI regions were determined by semiquantitative RT-PCR. A representative competitive PCR gel is shown in Fig. 2A, and the actual concentrations of the Kir3.1 and Kir3.2 cDNA concentration in the various preparations relative to β-actin cDNA concentration are presented in Fig. 2, B and C, respectively (arbitrary units). The transcriptional levels of Kir3.1 and Kir3.2 varied significantly among the various GI preparations (P < 0.05). Kir3.1 mRNA concentration was statistically greater in canine jejunum isolated circular smooth muscle preparations (1.43 × 10⁻³ ± 8.09 × 10⁻⁵; n = 4) compared with transcriptional levels measured in proximal colon (1.28 × 10⁻⁴ ± 4.67 × 10⁻⁷; n = 3, P < 0.001), duodenum (1.08 × 10⁻³ ± 6.47 × 10⁻⁵; n = 3, P < 0.01), or ileum (1.18 × 10⁻³ ± 6.70 × 10⁻⁵; n = 3, P < 0.05) isolated circular preparations. Statistically significant differences were also detected between the level of Kir3.1 transcripts in proximal colon compared with either duodenum or ileum smooth muscle preparations (n = 3, P < 0.001). In contrast, no significant differences were detected between the amount of Kir3.1 mRNA in duodenum and ileum circular smooth muscle preparations (n = 3, P > 0.05). The level of Kir3.2 expressed in the circular smooth muscle layer of the canine jejunum (1.42 × 10⁻² ± 1.23 × 10⁻⁴; n = 3) was significantly greater than those detected in the canine proximal colon (1.17 × 10⁻⁴ ± 1.04 × 10⁻⁵; n = 3, P < 0.001), duodenum (1.00 × 10⁻³ ± 4.12 × 10⁻⁵; n = 3, P < 0.001), or ileum (1.14 × 10⁻⁴ ± 3.03 × 10⁻⁶; n = 3, P < 0.001). Furthermore, a statistically significant difference was also observed between the amount of Kir3.2 transcript in canine duodenum compared with that measured in either canine ileum (n = 3, P < 0.001) or proximal colon (n = 3, P < 0.001) circular smooth muscle preparations. The concentration of β-actin was ~1 amol/µl in all canine mRNA preparations tested.

Immunohistochemical localization of Kir3.1 and Kir3.2 in GI smooth muscles. Antibodies raised against Kir3.1 and Kir3.2 both exhibited positive immunoreactivity within myocytes of both the jejunum of the small intestine and the proximal colon of the large intestine. Positive immunoreactivity was exhibited throughout the circular and longitudinal muscle layers of the external muscularis (Fig. 3, A–D), within the muscularis mucosa, and in a range of vasculature including both arteries and veins (Fig. 3, A and D).

Positive immunoreactivity was also observed in non-muscle cells such as nerve trunks, in the enteric ganglia of Meissner's and Schabadasch's plexus within the submucosa (not shown), and in Auerbach's (myenteric) plexus between the longitudinal muscularis and circular muscularis (Fig. 3, A–D). Interestingly, a cell type at the submucosal and circular muscle border exhibited positive staining; the position and morphology of this cell type was indicative of interstitial cells of Cajal (not shown). However, double labeling with c-Kit specific antibody was not performed in this study. At higher magnification, it was evident that both Kir3.1 and Kir3.2 antibodies labeled smooth muscle cells throughout the cell, with more intense labeling at the plasma membrane (Fig. 3, E and F).

Heterologous expression of Kir3.1 and Kir3.2 cloned from GI smooth muscle. Both Kir3.1 and Kir3.2 full coding sequences were cloned using RT-PCR from RNA isolated from murine proximal colon smooth muscle (see MATERIALS AND METHODS for details). The DNA sequences were identical to those previously cloned from murine brain RNA. Xenopus oocytes were injected with cRNA encoding hM2 alone, hM2 + Kir3.1, hM2 + Kir3.2, or hM2 + Kir3.1 and Kir3.2. Figure 4 shows representative currents elicited in oocytes injected with cRNA encoding these Kir channels in control and during exposure to 10 µM ACh, as well as the ACh-induced difference current (i.e., the difference between

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**Fig. 2.** Differential transcriptional expression of Kir3.1 and Kir3.2 in GI circular smooth muscle. Representative gel of semiquantitative RT-PCR is shown (A); 10-fold serial dilutions of mimic DNA were included in PCR reactions, whereas target cDNA (in this case, Kir3.1) concentration remained constant. Actual concentrations of Kir3.1 and Kir3.2 cDNA were calculated and expressed relative to β-actin cDNA concentration (B and C, respectively). Results are expressed as means ± SE. Transcriptional levels of Kir3.1 and Kir3.2 varied significantly among the various GI preparations (P < 0.05).
control currents and currents in the presence of ACh). Membrane currents in oocytes injected with hM2 + Kir3.1 or hM2 + Kir3.2 were not significantly different from those observed in oocytes injected with hM2 alone. In contrast, injection with cRNA encoding hM2 + Kir3.1 and Kir3.2 resulted in the heterologous expression of inwardly rectifying currents (Fig. 4 and Fig. 5C). These data suggest that Kir3.1 and Kir3.2 have, at best, limited ability to form functional channels on their own and that coexpression results in the expression of significantly larger current (17). We also observed that inward currents in the hM2 + Kir3.1 and Kir3.2 oocytes peaked later than the inward currents in the other oocytes, suggesting that the kinetics of the endogenous currents differ from those of the heterologously expressed inwardly rectifying currents.

We investigated the effect of changing extracellular K+ on the current-voltage relationship of the currents measured at the end of 300-ms test steps in oocytes injected with cRNA encoding Kir3.1 and Kir3.2. Results...
from one oocyte are shown in Fig. 5A. In this cell, membrane current reversed at −5 mV in 90 mM extracellular K⁺ and at −20 mV in 40 mM extracellular K⁺. In four oocytes, the reversal potentials were −5.5 ± 0.6 mV in 90 mM K⁺ and −22 ± 2.5 mV in 40 mM K⁺. These values are close to the equilibrium potentials for K⁺ calculated assuming an intracellular K⁺ concentration of 90 mM (0 and −21 mV) and indicate that the expressed channels are highly selective for K⁺.

Exposure to ACh increased the initial currents elicited by hyperpolarizations in oocytes injected with cRNA encoding hM2 alone, hM2 + Kir3.1, or hM2 + Kir3.2 (Fig. 4). ACh had no effect on the amplitude of the currents at the end of the 300-ms test pulses to −100 mV in oocytes injected with cRNA encoding hM2 alone or hM2 + Kir3.1 but caused a small increase in the current in oocytes injected with cRNA encoding hM2 + Kir3.2 (Fig. 5C). In contrast, ACh greatly increased the late current at −100 mV in oocytes injected with cRNA encoding hM2 + Kir3.1 and Kir3.2 (Fig. 5C). This effect developed over several minutes and was slowly reversed during washout of ACh (Fig. 5B). Furthermore, the magnitude of the increase in oocytes expressing Kir3.1 and Kir3.2 was significantly greater than the increase in oocytes injected with cRNA encoding Kir3.2 (Fig. 5C). ACh had no effect on the currents in oocytes injected with cRNA encoding Kir3.1 and Kir3.2 without cRNA encoding the hM2 receptor (data not shown).

Inwardly rectifying K⁺ currents are characterized by the sensitivity to blockage by external Ba²⁺ ions. We found that low concentrations of Ba²⁺ blocked the currents expressed by oocytes injected with cRNA encoding Kir3.1 and Kir3.2 as shown in Fig. 6. Figure 6A shows currents from a typical oocyte elicited by test steps to −100 mV in control, 50 µM Ba²⁺, and 500 µM Ba²⁺. The currents blocked by 500 µM Ba²⁺ are shown in Fig. 6B. Ba²⁺ strongly inhibited inward currents but had little or no effect on outward currents, consistent with the characteristic voltage dependence of the blockage of inward rectifiers by Ba²⁺ (20). Interestingly, the dose dependence of the blockage of currents at −100 mV found an IC₅₀ of 44 ± 4.9 µM (n = 4), lower than the IC₅₀ of 106 µM reported by Lesage et al. (17) for Kir3.1/3.2 donor from mouse brain.

**DISCUSSION**

We have shown that the IₖACh channels Kir3.1 and Kir3.2 are expressed in GI smooth muscles as well as myocytes using a combination of RT-PCR on RNA from isolated cells, quantitative RT-PCR on RNA from bulk tissue, and immunohistochemistry using commercially available specific antibodies. Other known members of the Kir3 family (e.g., Kir3.3 and Kir3.4) have not been detected in these cells. This is the first report of Kir3 expression in smooth muscles. When expressed heterologously, the smooth muscle forms of Kir3.1 and Kir3.2 are inwardly rectifying K⁺ channels that are activated through muscarinic receptors. When they are expressed individually, very little current is displayed. Only when they are expressed together, presumably as a heterotetramer (11), are large inward rectifying currents produced. In addition, currents are observed when Kir3.1 and Kir3.2 are expressed without coexpression of the muscarinic receptor hM2 but are unaffected by ACh application. When coexpressing hM2 with Kir3.1 and Kir3.2, a large basal current is observed that can be potentiated by application of ACh. The properties of this heterotetrameric channel differed only slightly from a previous report (17). The Ba²⁺ sensitivity of our clones is higher (44 µM vs. 105 µM).

Muscarinic stimulation of GI muscles leads to an increased force of contraction and an increased rate of GI motility (24, 25). The mechanism proposed for mediating the increased excitability involves neurotransmitter binding to M₂ and M₃ receptors on the smooth muscle cell surface (30, 31). M₂ receptors are coupled to the Gq/11 family of G proteins, whereas M₃ receptors are coupled to pertussis toxin-insensitive Gq/11 that activates membrane-bound phospholipase C-β (PLC-β). M₂ receptor stimulation leads to the inhibition of adenyl cyclase and protein kinase A (PKA), which would have an excitatory effect due to the stimulatory influence of PKA on several K⁺ channels in...
GI muscles (10). M₃ receptor stimulation coupling through PLC-β and G_q would have at least two targets that both eventually lead to the activation of L-type Ca²⁺ channels and release of Ca²⁺ from internal stores. Nonselective cation currents are activated by excitatory neurotransmitters, such as ACh, that result in depolarization and activation of L-type Ca²⁺ channels (14, 16). In addition, PLC-β stimulation leads to protein kinase C activation that inhibits delayed-rectifier currents in GI smooth muscles, also contributing an excitatory influence (28). The concomitant activation of I_KACh (Kir3.1/3.2) in these muscles would temper the excitatory response and prevent the tonic contraction that would inhibit motility. Unlike tonic smooth muscles, phasic muscles require rhythmic contractions to maintain functional activity. With the inhibition of delayed-rectifier K⁺ channels as a result of muscarinic activation, I_KACh may provide the hyperpolarization necessary for rhythmic oscillations in membrane potential. Activation of I_KACh is proposed to be through direct G protein βγ-stimulation (29) that in GI muscles could result from stimulation of M₂ or M₃.

We observed basal activity of Kir3.1/3.2 when expressed with the M₂ muscarinic receptor. These results are consistent with those of other investigators (12, 29). The cardiac muscarinic K⁺ channel is not active in the...
absence of either receptor stimulation or direct G protein βγ-subunit application (19). Neuronal G protein-gated inwardly rectifying K⁺ channels are also inactive under basal conditions and can be isolated from the more Ba²⁺-sensitive and strongly rectifying channels of the Kir2 family (21, 22). In GI smooth muscle cells, the effects of muscarinic stimulation on K⁺ currents at very hyperpolarized potentials have yet to be examined. In addition, the possibility of a basally active I_KACh-like conductance contributing to resting membrane potential along with Kir2.1 currents (5) is difficult to assess because of the lack of appropriate pharmacological tools. However, the molecular identification of the components for an I_KACh-like current suggests a role for this conductance during excitatory neurotransmission. The results from this molecular study should stimulate interest in I_KACh as a component of the muscarinic response in GI smooth muscles and lead to investigations of the regulation of this conductance in native myocytes.

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