Molecular identification of a component of delayed rectifier current in gastrointestinal smooth muscles

FELICITAS SCHMALZ, JACQUELINE KINSELLA, SANG DON KOH, FIVOS VOGALIS, ANNE SCHNEIDER, ELAINE R. M. FLYNN, JAMES L. KENYON, AND BURTON HOROWITZ
Department of Physiology, School of Medicine, University of Nevada, Reno, Nevada 89557

K+ CHANNELS OF smooth muscle plasma membranes regulate contraction by setting the membrane potential, thus controlling the influx of Ca2+ through voltage-gated Ca2+ channels. Voltage-clamp studies of isolated myocytes have characterized K+ currents and identified two types of outwardly rectifying K+ channels: voltage-gated "delayed rectifier" channels that are insensitive to intracellular Ca2+, along with Ca2+- and voltage-activated channels (e.g., large-conductance Ca2+-activated K+ channels). Furthermore, the delayed rectifier current in canine colonic smooth muscle consists of three current components with characteristic voltage dependencies, kinetics, and pharmacology (4). In particular, I_{dK(n)} is a fast-activating current blocked by micromolar concentrations of 4-aminopyridine (4-AP), I_{dK(f)} is a slowly activating current blocked by tetraethylammonium (TEA), and I_{dK(t)} is a TEA-sensitive current that inactivates at negative potentials.

Although some of the functions of these channel types have been elucidated by pharmacological and physiological studies of intact smooth muscle cells (2, 10, 12, 34), much important information about their roles in the control of smooth muscle function remains to be revealed. This is due in part to difficulties interpreting data from preparations expressing several different kinds of K+ channels. The difficulty is compounded when the dissection is approached at the single-channel level (18). An alternate approach to these questions is the application of molecular biological techniques that identify genes encoding different K+ channels and that produce cells expressing only one kind of K+ channel for electrophysiological study. We (14, 22) have used this approach and probed mRNA isolated from gastrointestinal (GI) and vascular smooth muscles with degenerate oligonucleotide primers designed to hybridize to conserved sequences surrounding the pore region of members of the Kv1–4 family of K+ channels. This has led to the cloning and expression of two delayed rectifier K+ channels [Kv1.2 (14) and Kv1.5 (22)]. Heterotetramers of the proteins encoded by these genes probably underlie I_{dK(t)} in canine colonic smooth muscle (27).

In the present study, we describe the identification of transcripts encoding Kv2.2 channel in myocytes from several smooth muscles. This family, related to the shab channels in Drosophila, was first characterized in mammalian brain by Hwang et al. (16), who found that Kv2.1 and Kv2.2 transcripts were differentially localized in the central nervous system and that currents mediated by Kv2.2 activated relatively slowly and were inhibited by TEA. Slow activation is apparently a unique feature of Kv2 channels, as it has been observed in Kv2.1 (11) but not in other Kv families. In contrast, the sensitivity of the Kv2.1 channels to 4-AP varies among the members of the family: Frec et al. (11) found a high sensitivity, whereas Pak et al. (24) found that their channels were virtually insensitive to this agent. We describe the electrophysiological and pharmacological properties of Kv2.2 cloned from human and canine colonic smooth muscle. We also determine the transcriptional expression pattern of Kv2.2 and a β-subunit (β2) found to couple to Kv2.2 (9), in isolated myocytes of several GI, uterine, and vascular smooth muscles utilizing RT-PCR on mRNA prepared from isolated myocytes and Northern hybridization on mRNA prepared from tissue. We suggest that Kv2.2 may underlie a component of delayed rectifier current in many smooth muscles.

METHODS

Tissue dissection and mRNA preparation. Canine colonic circular and longitudinal muscles were dissected as previously described (3). Samples of human sigmoid colon were obtained from 23 volunteer patients during elective colon resections for nonobstructive neoplasms and provided to us by Byron McGregor (Department of Surgery, University of Nevada). Circular smooth muscle was prepared from human tissue as described previously (17). GI and other smooth muscles were isolated from freshly obtained tissues, conserved in liquid nitrogen, and transported to the laboratory. Circular and longitudinal muscles were dissected as previously described (3) and prepared for electrophysiological study. Cells were studied in an internal pipette solution (in mM): KCl, 120; NaCl, 10; MgCl2, 1; CaCl2, 1; HEPES, 5; and EGTA, 1; pH 7.4. Culture medium (in mM): KCl, 50; NaCl, 120; MgCl2, 2; CaCl2, 2; and HEPES, 20; pH 7.4. Internal pipette solution (in mM): KCl, 120; NaCl, 10; MgCl2, 1; CaCl2, 1; HEPES, 5; and EGTA, 1; pH 7.4. Culture medium (in mM): KCl, 50; NaCl, 120; MgCl2, 2; CaCl2, 2; and HEPES, 20; pH 7.4. Samples were placed on a vibrating platform in a recording chamber at 37°C under a microscope equipped with recording and digital video systems. Fluorescent FITC-conjugated dextran was used to identify cells expressing Kv2.2 channel and was excited by a mercury arc lamp (excitation, 450–490 nm; emission, 510 nm). Single cells were clamped at −80 mV, and currents were elicited by voltage steps for 1 sec to −60 mV (4-AP, 10 µM), −30 mV (bicuculline, 0.5 µM), or −10 mV (TEA, 5 mM).
muscle tissues were dissected free of fat and connective tissue under a dissecting microscope. Poly(A)^+ RNA was prepared from dissected tissue using the Fast Track kit (Invitrogen, San Diego, CA), according to the manufacturer’s instructions. Muscles from four to five animals (0.3–0.5 g) were pooled for each RNA preparation. Each sample of RNA was adjusted to the same concentration. Poly(A)^+ RNA was also isolated from canine brain using this method.

Total RNA preparation from isolated myocytes. Elongated smooth muscle cells were identified under magnification using the same criteria as that used for electrophysiological examination. These cells (50–60) were drawn up into a capillary pipette and expelled into a 0.5-ml tube. Only spindle-shaped cells (length 20–500 µm; diameter 5–60 µM) were selected. The cells were snap frozen in liquid nitrogen and stored at −70°C until RNA preparation. RNA was prepared from isolated cells using a modification of the guanidinium thiocyanate procedure (7). Polynucleotidic acid (20 µg) was added as a carrier (35). Myocytes from the circular and longitudinal muscle layers of tissues from several regions of the GI tract and other smooth muscles were prepared, as previously described (5).

cDNA isolation and nucleotide sequencing. Canine Kv2.2 (cKv2.2) and human Kv2.2 (hKv2.2) cDNAs were isolated with a modified RT-PCR method (30). First-strand cDNA was prepared from human and canine colonic circular smooth muscle-derived mRNA in a 60-µl reaction containing 100 ng of oligo(dT) primers or random oligonucleotides, 12 µl of 5× first-strand buffer (GIBCO-BRL, Gaithersburg, MD), 6 µl of 0.1 M dithiothreitol, 15 µl of 5 mM dNTPs (Invitrogen), 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL), 40 U of RNase inhibitor (Promega, Madison, WI), and 300 ng of poly(A)^+ RNA. The reaction was incubated at room temperature for 10 min and at 42°C for 50 min, then heated to 70°C for 15 min, and cooled on ice for 10 min. We added 20 U of RNase H (Promega), and the reaction was further incubated for 20 min.

PCR was performed utilizing several overlapping primer oligonucleotides. Initially, degenerate primers designed to amplify the conserved pore region for the mammalian Kv2 family were used to determine if any Kv2 homologue was expressed in this tissue. Primers corresponding to the cKv2.2 pore region was identified, oligo(dT) and specific primers designed to hybridize to sequence in the 3′ untranslated region were used as 3′ anchor-reverse primers in conjunction with several primers (in individual reactions) encoding regions of the rat Kv2.2 (rKv2.2) sequence (16) for the 5′ forward primer. Nucleotide positions (nt) are relative to the cKv2.2 coding sequence. The primers used were as follows: primer 1, (pore forward) 5′-CTCC-GAAITCGAATCGACAGT/GCTGCG-3′ (nt 847–871); primer 2, (pore reverse) 5′-TTTACAT/GAGAACAT/GATGATGCT/G, TGCC/GC-3′ (nt 1338–1358); primer 3, 5′-CCATGCGAGAAACGACCTCCTGCTG-3′ (nt –2–23); primer 4, 5′-GGGAACTCGCGAAGCGAAGTC/ATACGC-3′ (nt 184–208); primer 5, 5′-CCCAACTTGGTTGACAT/CCTGC-3′ (nt 985–1007); and primer 6, 5′-CCATGCGAGAAGCGCTTCCTGCTG-3′ (nt 2393–2417). In the 5′ region of rKv2.2 sequence, an anchor primer based on the sequence just upstream from the start codon was utilized (primer 3). This primer was used in conjunction with primers 2 and 5 (in individual reactions) encoding conserved regions of Kv2.2 structure for the 3′ reverse primer. In the 3′ region primer 5 was used as the anchor primer and paired with primers 1, 3, and 4.

PCR was performed in a reaction volume of 50 µl in buffer containing 2.3 mM MgCl_2, 0.1 mM of each dNTP, 0.2 µM of each primer, and 0.5 U of Taq polymerase (Promega). The reaction was performed with an initial denaturation of 5 min followed by 33 cycles (94°C for 30 s; 55°C for 30 s; 72°C for 20 s) and a final extension step of 10 min at 72°C in a thermal cycler (Coy). Reaction products (3 µl) were electrophoresed through a 1% agarose-40 mM Tris acetate, 2 mM EDTA gel and visualized with ethidium bromide.

Amplification products were subcloned into the PCR II vector (Invitrogen) using direct ligation in a 10-µl reaction containing 2 µl of the amplification products, 1 µl (10 ng) TA vector DNA, 1 µl 10× ligation buffer (Invitrogen), and 4 U of T4 ligase (Invitrogen). The ligation reaction was transformed into competent Escherichia coli and spread onto ampicillin plates. White colonies were picked (blue/white selection) and analyzed for amplification product inserts. Subclones were analyzed by nucleotide sequencing. Several were analyzed for each product subcloned, and overlapping regions were examined for sequencing accuracy. To determine transcriptional expression from isolated cells derived from GI, vascular, and uterine smooth muscles, a primer pair was designed from the deduced cKv2.2 nucleotide sequence. The region is specific for Kv2.2 and corresponds to nt 184–1007 (primers 4 and 5), generating an 823-nt amplification product. Expression of the Kv2.2 β-subunit (β2) (9) was determined by RT-PCR analysis using primers designed from the β2 sequence to be specific for this cDNA (forward, ATGCTCAAGGTATGGTCTGTAT, nt 1–24 of the coding sequence; reverse, CCAAGAGCACA-TATTTCACCCAC, nt 701–724 of the coding sequence). Expression of Kv8.1 was determined by RT-PCR analysis using primers designed from the Kv8.1 sequence to be specific for this cDNA (15) (forward, TCAACGTGGGGCGACGGCTCGTCTG, nt 290–317 of the coding sequence; reverse, CTTCTCTAAGTCCAGGCTTTCG, nt 646–670 of the coding sequence).

For expression of the complete Kv2.2 open reading frame (ORF), two clones that overlapped by 500 bp were joined by ligation at a common BamHI I site. This construct was subcloned into a Xenopus oocyte expression vector (pSP64T) (19) (kindly provided by D. Melton, Harvard University, Cambridge, MA) downstream from the SP6 promoter. The plasmid was linearized with KpnI, and capped transcripts were synthesized in vitro with SP6 RNA polymerase, as described previously (19). Transcripts were resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA at a final concentration of 1 µg/µl.

Northern analysis. RNA was size fractionated on 1.0% agarose-formaldehyde gels and transferred to Immobilon filters (33). Filters were baked and prehybridized in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 50 mM sodium phosphate, 5× Denhardt’s solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C overnight. Restriction endonuclease fragment (Bgl II) specific for Kv2.2 was prepared from the clone (nt 1–389), and the probe was labeled with ^32P by using a random-primer technique (8). Hybridization was performed under the same conditions overnight. The filters were washed at high stringency (3 times in 2× SSC at room temperature for 5 min each, then twice for 30 min each in 0.2× SSC, 0.1% SDS at 65°C) to ensure specificity of labeling. Specific for a Chinese hamster ovary (CHO) cell mRNA expressed at equivalent levels in all tissue examined (13), a cDNA of CHO-B was used as an internal standard to verify that equal amounts of poly(A)^+ RNA were applied. After hybridization, filters were exposed to intensifying screens and autoradiography was performed using a Bio-Rad PhosphorImager (Hercules, CA).

Oocyte injection and electrophysiological methods. Ovarian lobes were removed from anesthetized adult female Xenopus
laevis frogs (Xenopus 1, Ann Arbor, MI) under sterile conditions. The lobes were then mechanically opened, and the oocyte follicular layer was removed by incubation with collagenase (1 mg/ml) in Ca2+-free ND96 (see below) solution at room temperature for 2–3 h. The oocytes were then collected, rinsed, and stored in ND96 solution containing (in mM) 2.5 pyruvate, 96 NaCl, 1.5 CaCl2, 2 KCl, 1 MgCl2, and 5 HEPES plus antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 19°C for up to 24 h before injection. Only stage V and VI oocytes were selected for injection. mRNA (1 µg/µl) was injected in a total volume of 50 nl, and the oocytes were stored for 2–4 days until assay.

Whole cell K+ currents were recorded using the double-electrode voltage-clamp technique (GeneClamp 500, Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. Recordings were made at room temperature in a solution containing (in mM) 96 NaCl, 2 KCl, 2.8 MgCl2, and 5 HEPES, pH 7.4. Ca2+ was omitted from the solution to minimize the endogenous Ca2+-activated chloride currents. Single K+ channels were recorded in inside-out patches obtained from oocytes after mechanical removal of the vitelline membrane as described by Methfessel et al. (20). Currents were recorded by an Axopatch-1D amplifier (Axon Instruments) and filtered at 500 Hz before digitization. Patch pipettes were filled with (in mM) 140 KCl, 0.1 GdCl3, and 10 HEPES, pH 7.2. The bath solution contained (in mM) 140 KCl, 0.1 GdCl3, 10 HEPES, and 1 EGTA, pH 7.2. To establish the selectivity of the channels, 140 mM KCl was replaced with NaCl in some experiments. GdCl3 was included to inhibit stretch-activated channels. In all voltage-clamp experiments, data acquisition and analyses were done using PCCLAMP software (Axon Instruments). Data are expressed as means ± SE.

Native colonic myocytes were dispersed and patch-clamp recordings performed as previously described (4). Bath solution contained (in mM) 140 NaCl, 5 KCl, 2 MnCl2, 1.2 MgCl2, 10 dextrose, 10 HEPES, and 5 Tris, pH 7.4. Pipette solution contained (in mM) 20 KCl, 110 potassium gluconate, 5 MgCl2, 2.5 K2ATP, 0.1 Na2GTP, 2.5 disodium creatine phosphate, 5 HEPES, and 1 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (pH 7.2).

In some experiments, 4-AP (5 mM), TEA (10 mM), and quinine (1 µM) were added to the bath.

RESULTS

cDNA cloning and DNA sequence analysis. RT-PCR utilizing primers designed to generate several overlapping amplification products was used to construct the full ORF for the canine and human homologues of Kv2.2 cDNA. Several primer combinations resulted in amplification products overlapping in both the 5' and 3' orientations (see METHODS). Amplification products were separated on agarose gels and analyzed specifically for products of an unexpected size, which might indicate alternative splicing or homologous isoforms. Gels were also blotted and hybridized to previously isolated Kv2.2-specific amplification products as probes to detect low-level transcriptional expression of altered forms of Kv2.2. None were detected for these amplifications. At least five individual subclones were completely sequenced for each of the overlapping products to ensure the fidelity of the RT-PCR reaction. We constructed a full-length ORF using two amplification products overlapping by 823 nt (3' primers 5 and 6; 5' primers 3 and 4). The complete amino acid sequence of the cKv2.2 and hKv2.2 clones is shown in Fig. 1. The sequence is aligned with the translated rat brain cDNA sequence (16). Nucleotide sequence homology and amino acid sequence identity of cKv2.2 is 84.9% and 91.7% with hKv2.2 and 82.9% and 88.3% with rKv2.2. The majority of sequence divergence occurs in the carboxy-terminal domain.

Northern hybridization of cKv2.2 in visceral and vascular smooth muscles. Poly(A)- RNA was prepared from several dissected regions of the canine GI tract. Longitudinal and circular muscle layers were separated. Smooth muscle tissue from coronary artery (>300-µm-diameter vessels) was also analyzed to determine the distribution of this smooth muscle-derived K+ channel in a vascular smooth muscle. Canine brain tissue was also included to compare expression in the tissue source used for the initial cloning of Kv2.2 (16). A cDNA of CHO-B, specific for a CHO cell mRNA expressed at equivalent levels in all tissues examined (13), was used as an internal standard to verify that equal amounts of poly(A)- RNA were applied. A specific probe for Kv2.2 was used for the hybridization. Figure 2A displays the autoradiogram resulting from the hybridization. cKv2.2 hybridized to a 13- kb transcript in all canine tissues in which expression was detected. An additional transcript of ~6 kb hybridized in several tissues and was prominent in canine colon circular smooth muscle.

RT-PCR analysis of transcriptional expression of Kv2.2 in canine smooth muscle cells. To eliminate the possibility that detection of transcriptional expression of Kv2.2 was due to expression in a minor cell population in the smooth muscle (i.e., nerve, endothelial, interstitial cells), we prepared RNA from isolated smooth muscle cells. Myocytes were dispersed from several canine smooth muscles, including several regions of the GI tract, uterus, and vascular muscles. Fifty to sixty cells were identified based on criteria previously used for selection of cells for electrophysiological studies (4). Only spindle-shaped cells (length 20–500 µm; diameter 5–60 µm) were selected. Total RNA was prepared from the selected cells, and specific primers, designed to amplify an 823-nt product of cKv2.2, were used in the RT-PCR reaction. Although colonic longitudinal muscle displayed relatively little amplification product, product was detectable on longer photographic exposure. The Kv2.2 β3 auxiliary subunit was used to determine whether DNA contaminates the RNA prepared from the isolated cells. We did not perform the relative

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| cKv2.2  | 1 MAEACKPGLN RTKRSRTSLSL PEFPEVDRRS KTCSRVRKIN VGGLNHENVLW 50 |
| hKv2.2  | 1 MAEACKPGLN RTKRSRTSLSL PEFPEVDRRS KTCSRVRKIN VGGLNHENVLW 50 |
| rKv2.2  | 1 MAEACKPGLN RTKRSRTSLSL PEFPEVDRRS KTCSRVRKIN VGGLNHENVLW 50 |
| cKv2.2  | 51 RTDORLPRTR LGKLRDCTNH ESLLEVCDYY NDENEYFDF RHPGAFTSSIL 100 |
| hKv2.2  | 51 RTDORLPRTR LGKLRDCTNH ESLLEVCDYY NDENEYFDF RHPGAFTSSIL 100 |
| rKv2.2  | 51 RTDORLPRTR LGKLRDCTNH ESLLEVCDYY NDENEYFDF RHPGAFTSSIL 100 |
| cKv2.2  | 101 NFGYRTGKLMH MEECAEALGG YHELDYWGIDE Eylesccqar YHQQKEQMNE 150 |
| hKv2.2  | 101 NFGYRTGKLMH MEECAEALGG YHELDYWGIDE Eylesccqar YHQQKEQMNE 150 |
| rKv2.2  | 101 NFGYRTGKLMH MEECAEALGG YHELDYWGIDE Eylesccqar YHQQKEQMNE 150 |
| cKv2.2  | 151 ELRREAEMR DGEGEEDFNT CCEFLKRRKED DLLEKPNSSV AAATLAVSNS 200 |
| hKv2.2  | 151 ELRREAEMR DGEGEEDFNT CCEFLKRRKED DLLEKPNSSV AAATLAVSNS 200 |
| rKv2.2  | 151 ELRREAEMR DGEGEEDFNT CCEFLKRRKED DLLEKPNSSV AAATLAVSNS 200 |
| cKv2.2  | 201 LFIVLSTIAL SLNLPELQDE MDEFGQADDD FXLAHVEAVC HAWTTMYELL 250 |
| hKv2.2  | 201 LFIVLSTIAL SLNLPELQDE MDEFGQADDD FXLAHVEAVC HAWTTMYELL 250 |
| rKv2.2  | 201 LFIVLSTIAL SLNLPELQDE MDEFGQADDD FXLAHVEAVC HAWTTMYELL 250 |
| cKv2.2  | 251 RFLSSPNKWK FFKGPGLNVID LLARILYYVT PFLTESKSV LFQFONVRVV 300 |
| hKv2.2  | 251 RFLSSPNKWK FFKGPGLNVID LLARILYYVT PFLTESKSV LFQFONVRVV 300 |
| rKv2.2  | 251 RFLSSPNKWK FFKGPGLNVID LLARILYYVT PFLTESKSV LFQFONVRVV 300 |
| cKv2.2  | 301 KIFIRIMLRD ILCLLDNGRH LQSLGFTLRH SYNEGILGILFLPIGMIFS 350 |
| hKv2.2  | 301 KIFIRIMLRD ILCLLDNGRH LQSLGFTLRH SYNEGILGILFLPIGMIFS 350 |
| rKv2.2  | 301 KIFIRIMLRD ILCLLDNGRH LQSLGFTLRH SYNEGILGILFLPIGMIFS 350 |
| cKv2.2  | 351 SLVVFEEAEDK DATKFTISPA SFNWATITMT TVGDYDIYFK TLGCKTGGLG 400 |
| hKv2.2  | 351 SLVVFEEAEDK DATKFTISPA SFNWATITMT TVGDYDIYFK TLGCKTGGLG 400 |
| rKv2.2  | 351 SLVVFEEAEDK DATKFTISPA SFNWATITMT TVGDYDIYFK TLGCKTGGLG 400 |
| cKv2.2  | 401 CCIAGVURLIA LPIIFIIVVIP SEYKEQKRRV EKAIRKREAL EARKNSGIV 450 |
| hKv2.2  | 401 CCIAGVURLIA LPIIFIIVVIP SEYKEQKRRV EKAIRKREAL EARKNSGIV 450 |
| rKv2.2  | 401 CCIAGVURLIA LPIIFIIVVIP SEYKEQKRRV EKAIRKREAL EARKNSGIV 450 |
| cKv2.2  | 451 SMNLKDAFAR SMLIDIVAVE KAGEKBSK KSDKDNLSPS RKWSRANKLS 500 |
| hKv2.2  | 451 SMNLKDAFAR SMLIDIVAVE KAGEKBSK KSDKDNLSPS RKWSRANKLS 500 |
| rKv2.2  | 451 SMNLKDAFAR SMLIDIVAVE KAGEKBSK KSDKDNLSPS RKWSRANKLS 500 |
| cKv2.2  | 501 ETSSNKSDEN KYEQVSKGKD HEQLNNPTSS SPQHLSAQKLL EMLYNKTKT 550 |
| hKv2.2  | 501 ETSSNKSDEN KYEQVSKGKD HEQLNNPTSS SPQHLSAQKLL EMLYNKTKT 550 |
| rKv2.2  | 501 ETSSNKSDEN KYEQVSKGKD HEQLNNPTSS SPQHLSAQKLL EMLYNKTKT 550 |
| cKv2.2  | 551 QPESGNNDPC CEFRKPSAY EEEIEEMEEV CPOEQLAVAQ TCENVMDKST 600 |
| hKv2.2  | 551 QPESGNNDPC CEFRKPSAY EEEIEEMEEV CPOEQLAVAQ TCENVMDKST 600 |
| rKv2.2  | 551 QPESGNNDPC CEFRKPSAY EEEIEEMEEV CPOEQLAVAQ TCENVMDKST 600 |
| cKv2.2  | 601 SSIDSFTSCA TDFTETERSP LPPGASHLQ MARRDILDRCY DEHORUREFP 650 |
| hKv2.2  | 601 SSIDSFTSCA TDFTETERSP LPPGASHLQ MARRDILDRCY DEHORUREFP 650 |
| rKv2.2  | 601 SSIDSFTSCA TDFTETERSP LPPGASHLQ MARRDILDRCY DEHORUREFP 650 |
| cKv2.2  | 651 FIMLKPGP AADLELYA PDITVLDQ SGLRSEGKD LQKWSRANKLS 700 |
| hKv2.2  | 651 FIMLKPGP AADLELYA PDITVLDQ SGLRSEGKD LQKWSRANKLS 700 |
| rKv2.2  | 651 FIMLKPGP AADLELYA PDITVLDQ SGLRSEGKD LQKWSRANKLS 700 |
| cKv2.2  | 701 KSSLSGSPNL KSSLAVNFK ENRSGAFTPQ PSTARLPVP TADFLTPQ 750 |
| hKv2.2  | 701 KSSLSGSPNL KSSLAVNFK ENRSGAFTPQ PSTARLPVP TADFLTPQ 750 |
| rKv2.2  | 701 KSSLSGSPNL KSSLAVNFK ENRSGAFTPQ PSTARLPVP TADFLTPQ 750 |
| cKv2.2  | 751 LDSNSTILLEF FGCSRPGLP FRRKTVRNE FKGPCEFPP SNSLSDGDED 800 |
| hKv2.2  | 751 LDSNSTILLEF FGCSRPGLP FRRKTVRNE FKGPCEFPP SNSLSDGDED 800 |
| rKv2.2  | 751 LDSNSTILLEF FGCSRPGLP FRRKTVRNE FKGPCEFPP SNSLSDGDED 800 |
| cKv2.2  | 801 GGAFLK .................................................. 850 |
| hKv2.2  | 801 GGAFLK .................................................. 850 |
| rKv2.2  | 801 GGAFLK .................................................. 850 |
Kv2.2 expression in GI smooth muscle

Accordingly, we attribute them to the expression of injected Kv2.2 cRNA. Figure 3 summarizes the activation and steady-state inactivation properties of this current. Currents were elicited by 400-ms step depolarizations from −80 mV (Fig. 3A). A voltage-dependent conductance was activated at potentials positive to −20 mV. The midpoint of the conductance vs. voltage relationship was +5 ± 6 mV (n = 9), and the conductance increased e-fold per 12 ± 1.1 mV (n = 9). There was no inactivation apparent during 400-ms depolarizations, and the current-voltage relationship was constructed by averaging the currents from eight oocytes and plotting the current at 400 ms as a function of step potential (Fig. 3B).

The kinetics of activation of smooth muscle Kv2.2 channels resembled those of the rat brain Kv2.2 channel (16) in that there was a distinct delay before the conductance increased and the rate of activation was slow compared with Kv1 channels. We characterized the rate of activation by measuring the time to half maximum current (t½); t½ decreased as the test potential was made more positive over the range −10 to +50 mV. Furthermore, at a test potential of +10 mV, t½ from a holding potential of −80 mV was 97 ± 8.6 ms (n = 14).

Although there was no apparent inactivation over 400 ms, the Kv2.2 channels did inactivate during longer depolarizations. We measured the voltage-dependent inactivation of the channels using 20-s conditioning pulses to various potentials followed by 400-ms test steps to +20 mV (Fig. 3C, inset). Inspection of the currents during the conditioning steps revealed that a very slow inactivation process was still under way at the end of 20 s. Thus our results only approximate the true steady-state inactivation. We found that inactivation at the end of 20 s was well described by a Boltzmann function

\[
I = \frac{1 - C_{\min}}{1 + \exp\left(\frac{V - V_s}{h}\right)} + C_{\min}
\]

where \(C_{\min} = 0.42\) is the fraction of current that does not inactivate in 20 s, \(V_s = -16.3\) mV is the prepulse potential for half inactivation, and \(h = 4.8\) is the slope factor.

Pak et al. (24) found that the Kv2.1 channels encoded by Drosophila and mouse genes had different rates of recovery from inactivation so that Drosophila channels were nearly completely recovered after 1 s at −90 mV, whereas mouse channels took longer than 10 s to recover. We investigated this process in smooth muscle Kv2.2 channels using a similar protocol. In particular, oocytes were held at −60 mV and stepped for 10 s to +20 mV, for 1 s to −60 mV, and then to +20 mV for a 1-s

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**Fig. 1.** Amino acid sequence of canine and human homologues of Kv2.2 (cKv2.2 and hKv2.2, respectively). Sequences are compared with rat Kv2.2 (rKv2.2) (16), and divergent amino acids are highlighted. Putative transmembrane spanning segments are indicated by solid bars above the sequence with the H5-pore-lining region shown. ●, Serines within consensus protein kinase A phosphorylation sites. ●, Consensus tyrosine phosphorylation site. ●, Putative N-linked glycosylation site. Most species divergence occurs in the carboxy-terminal region of the protein.
Voltage-dependent activation and inactivation of Kv2.2 currents expressed in Xenopus oocytes. (A) A family of currents elicited by 400 ms depolarizations from a holding potential of −80 mV (inset). B: current–voltage relationship obtained by measuring the currents at the ends of the steps in A plotted as a function of step potential. C: the characterization of voltage-dependent inactivation in another oocyte. The test current normalized to the current elicited after the −80 mV conditioning step is plotted as a function of the conditioning potential. Inset, currents during 20 s conditioning pulses followed by test currents. V

Prepulse potential for half inactivation.

B

Fig. 4. Time course of recovery from inactivation. A: 5 superimposed traces during which the membrane potential was stepped from −80 to +20 mV for 10 s followed by recovery intervals of 2, 4, 6, 8, and 10 s before a 2 s test step to +20 mV. Runs were separated by 2 min rest periods to provide full recovery. B: the maximum current during the test step (I

Peak) divided by the peak current in the conditioning step (I

Peak) as a function of the recovery interval (○). The solid line has a time constant of 2 s and accounts for ∼60% of the recovery from inactivation.

Pharmacological characterization of Kv2.2 expressed in oocytes. In view of the differing pharmacological profiles of I

Kv1.5 and I

Kv1.1, it was of interest to characterize the response of the Kv2.2 currents to 4-AP and TEA. Figure 6, A and B, shows membrane currents elicited by steps from −60 to +50 mV in control and in the presence of 0.1 and 1 mM concentrations of 4-AP and TEA. Inhibition of Kv2.2 currents by 4-AP and TEA was dose dependent (Fig. 6, D and E). Furthermore, block by 4-AP was voltage dependent over the range +10 to +50 mV, being less effective at more positive potentials as has been demonstrated previously for Kv1.2 (14). At +10 mV, the IC

50 was 1.5 ± 0.1 mM (n = 10) for 4-AP and 2.6 ± 0.9 mM (n = 5) for TEA. In addition we tested the effect of quinine, a known blocker of delayed rectifier current. We previously found that quinine blocked Kv1.5 (IC

50 = 365 μM) (22). As shown in Fig. 6, C and F, quinine was a potent inhibitor of Kv2.2 with an IC

50 of 14 μM (n = 10). Block by quinine was not voltage dependent. Kv2.2 was not blocked by charybdotoxin (>300 nM) or iberiotoxin (>300 nM).
Delayed rectifier currents recorded from native myocytes. Because of the potent inhibition of Kv2.2 with quinine, we examined the sensitivity of the delayed rectifier current in native colonic myocytes to quinine in combination with 4-AP and TEA. 4-AP (5 mM) significantly increased the $t_{1/2}$ (23.5 ± 2.4 ms, n = 6) of the delayed rectifier current compared with control value (12.3 ± 1.6 ms) at +20 mV. However, TEA (10 mM) and quinine (1 µM) did not change $t_{1/2}$ [11.5 ± 1.2 vs. 12.3 ± 1.0 ms, TEA (n = 5) vs. quinine (n = 6), respectively] (Fig. 7, A, B, and C). 4-AP reduced the delayed rectifier $K^+$ current [35.1 ± 2.9%, n = 6, 413 ± 17 pA (control) vs. 286 ± 14 pA (4-AP)]. Application of quinine (10 µM) resulted in additional reduction of the current (56.5 ± 4.7%, n = 6, 179 ± 23 pA) in the presence of 4-AP (5 mM) (Fig. 7, D and F). However, application of quinine did not further reduce delayed rectifier current in the presence of TEA (10 mM) (57.1 ± 6.4% of inhibition, n = 7; 173 ± 28 pA) and did not show additional reduction before and after treatment of TEA (10 mM, 55.3 ± 5.1% of inhibition, n = 7; 171 ± 22 vs. 376 ± 41 pA of control) (Fig. 7, E and F). These data are consistent with quinine, at low concentration (10 µM), blocking the TEA-sensitive component of delayed rectifier current and blocking a slower activating current component.

DISCUSSION

Although the general importance of the plasmalemmal $K^+$ conductance to smooth muscle function is well accepted, the specific roles of individual $K^+$ channels are unclear and considerable effort is currently directed to the linking of $K^+$ channels to function in a variety of smooth muscles. One approach to this problem is based on the characterization of the components of $K^+$ current in smooth muscle myocytes and the molecular identification of the channels that mediate them. In the present study, we have identified a molecular component of the delayed rectifier current in GI smooth muscle cells, determined its transcriptional expression in several GI muscle types, and studied the properties of the channels expressed by Xenopus oocytes.

Because the goal of our study was the identification of $K^+$ channels in smooth muscle myocytes, it was important to consider that smooth muscle tissues contain several cell types that might contribute mRNA to the PCR reaction used to detect expression of the $K^+$ channel gene. Accordingly, we analyzed the distribution of Kv2.2 transcriptional expression by Northern analysis and collected and amplified mRNA from myocytes prepared using methods developed for electrophysiological studies (4, 21, 25, 34). Thus we know that the Kv2.2 expression described here is at levels sufficient to be detected by Northern hybridization and that Kv2.2 expression is localized to the smooth muscle myocytes. Interestingly, we found Kv2.2 is expressed in GI, vascular, and uterine smooth muscle myocytes, i.e., in all types examined. We conclude that, similar to Kv1.5 (22), Kv2.2 is ubiquitously expressed in smooth muscles. We note that we tested myocyte preparations for the expression of Kv2.1 using primers designed to distinguish between Kv2.1 and Kv2.2 but failed to find evidence for the expression of Kv2.1 mRNA (data not shown).
vascular smooth muscles. However, expression of Kv8.1, a new Kv channel with interesting regulatory properties toward the Kv2 family (15, 29), has not been detected in canine smooth muscles.

We find that Kv2.2 channels are activated at potentials positive to about $-20 \text{ mV}$ as are Kv2.2 channels from rat brain (16) and Kv2.1 channels (11, 31). This behavior is somewhat different from the $mShab$ (Kv2.1) channels that are activated at more negative potentials (23). For all of these channels, the rate of activation is slow compared with that of members of the Kv1 family. In particular, we reported that smooth muscle $cKv1.2$ and $cKv1.5$ channels have $t_{1/2}$ values of 7.6 and 5.5 ms, respectively (14, 22), similar to other Kv1 channels. In contrast, the smooth muscle Kv2.2 channel described here has a $t_{1/2}$ of 97 ms (at $-10 \text{ mV}$). This is similar to the behavior of Kv2.1 channels found by Frech et al. (11), faster than the channels described by Pak et al. (24), but slower than the activation of rat brain Kv2.2 (16).

We also studied the single channels encoded by the smooth muscle Kv2.2 and found that they were selective for $K^+$, were opened by depolarization, and that they had a single-channel conductance of 15.3 pS. This is similar to that reported by Fink et al. (9) for the Kv2.2 clone characterized by Hwang et al. (16) and similar to Kv1.2 and Kv1.5 smooth muscle $K^+$ channels, which have single-channel conductances of 14 and 9.8 pS, respectively (14, 22), making these channels underlying the delayed rectifier current difficult to dissect in native cells at the single-channel level.

The development of voltage-dependent inactivation of smooth muscle Kv2.2 qualitatively resembled that found for Kv2.1 channels (11, 31) in that a large fraction of the current is inactivated over many seconds. Interestingly, we found that the recovery from inactivation was very slow and followed a complex time course. That is, about one-half of the inactivation was reversed over 10 s, whereas full recovery of the current took longer than 2 min. In contrast, Pak et al. (24) found that recovery from inactivation by Drosophila Kv2.1 (fShab) channels was very fast (time constant of 0.4 s) compared with mouse channels (mShab, time constant of 4.2 s). For both channels, recovery was well described by a single exponential process. Thus the recovery from inactivation by smooth muscle Kv2.2 channels is uniquely complex and slow.

Although we have not characterized the recovery from inactivation in detail, it appears to have properties of cumulative inactivation described and modeled by Aldrich (1). Our results imply that although there is little inactivation during a single depolarization of 1- to 5-s duration [i.e., during a single slow wave, (32)], it is...
expected that inactivation will accumulate during rhythmic depolarizations separated by <10 s and that this inactivation will limit current through Kv2.2 channels in the steady state. An apparent continued development of inactivation during repolarization can be seen in the delayed rectifier currents of colonic smooth muscle myocytes [see Carl (4)].

Carl (4) used a pharmacological approach to identify the components of delayed rectifier K\textsuperscript{+} current in canine circular colonic smooth muscle and concluded that there are three components of this current: I\textsubscript{dK(f)}, I\textsubscript{dK(s)}, and I\textsubscript{dK(n)}. Carl (4) further reported that the current recorded in the presence of TEA [dominated by I\textsubscript{dK(f)}] activated relatively quickly, whereas the current recorded in the presence of 4-AP [dominated by I\textsubscript{dK(s)}] activated relatively slowly. The activation of smooth muscle Kv2.2 showed a sigmoidal time course as do other Kv1 and Kv2 currents. Although Carl (4) reported single exponential time constants to describe the activation of delayed rectifier currents in smooth muscle myocytes, those currents also showed a sigmoidal activation with \( t_{1/2} \) values at +10 mV of 20.6 ms in control [I\textsubscript{dK(f)} + I\textsubscript{dK(s)}], 30.3 ms in the presence of 4-AP [largely I\textsubscript{dK(s)}], and 13.9 ms in the presence of TEA [largely I\textsubscript{dK(s)}] (A. Carl, personal communication). Thus the rate of activation of I\textsubscript{dK(f)} is closer to that of the Kv1 channels, whereas the rate of activation of I\textsubscript{dK(s)} is closer to that of the Kv2 channels. Although there is an approximately threefold difference between \( t_{1/2} \) values of I\textsubscript{dK(s)} and Kv2.2, quantitative comparison of these parameters is difficult. In particular, the pharmacological dissection of the native currents is likely to be incomplete and some of the current recorded in the presence of 10 mM 4-AP may have been contributed by Kv1 channels. Such a contribution might add to the difference in \( t_{1/2} \) values seen here. Carl (4) found that the delayed rectifier current remaining in the presence of TEA [predominantly I\textsubscript{dK(s)}] was quite sensitive to 4-AP (IC\textsubscript{50} = 23 µM). Thus the sensitivity of I\textsubscript{dK(f)} to 4-AP resembles that of the Kv1 currents [IC\textsubscript{50} values < 211 µM, (14, 23)], and

<table>
<thead>
<tr>
<th>Channel</th>
<th>Conductance, pS</th>
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<tbody>
<tr>
<td>Kv1.2</td>
<td>14</td>
</tr>
<tr>
<td>Kv1.5</td>
<td>9.8</td>
</tr>
<tr>
<td>I\textsubscript{dK(f)}</td>
<td>20</td>
</tr>
<tr>
<td>I\textsubscript{dK(s)}</td>
<td>15.3</td>
</tr>
<tr>
<td>4-AP</td>
<td>14</td>
</tr>
<tr>
<td>Quinine</td>
<td>2.6 mM</td>
</tr>
<tr>
<td>TEA</td>
<td>2.2 mM</td>
</tr>
</tbody>
</table>

4-AP, 4-aminopyridine. TEA, tetraethylammonium. \( t_{1/2} \), Time to half maximum current. \( V_{th} \), the prepulse voltage at which half the current inactivates. \( V_s \), slope factor. I\textsubscript{dK(f)}, fast activating K\textsuperscript{+} current. I\textsubscript{dK(s)}, slow activating K\textsuperscript{+} current. ND, not determined.

Fig. 7. Delayed rectifier currents were obtained with averaged currents from 10 episodes with step depolarization from a holding potential of −80 mV to test potential of +20 mV. Interepisode interval was 115 s. A and B: effects of 4-AP (5 mM, n = 6), TEA (10 mM, n = 5), and quinine (10 µM, n = 6) in the same cell on the control delayed rectifier currents (n = 17). C: summary of time to half maximum current (\( t_{1/2} \)) of activation. 4-AP significantly increases \( t_{1/2} \) of activation. * \( P < 0.05 \). D: current was elicited by step depolarization of +20 mV from a holding potential of −80 mV in the presence of 4-AP and additional application of quinine. E: obtained from the same protocol and patch as in D in the presence of TEA and TEA + quinine. F: summary of % current inhibition. * \( P < 0.05 \).
this is one piece of evidence suggesting that \( \mathcal{I}_{K(I)} \) is mediated by a heterotetramer formed of Kv1.2 and Kv1.5 subunits (27). The low sensitivity of \( \mathcal{I}_{K(I)} \) to 4-AP is closer to the property of smooth muscle Kv2.2 (\( IC_{50} \) at +20 mV = 2 mM), suggesting that Kv2.2 may be a component of \( \mathcal{I}_{K(I)} \). The Kv2.2 channels from smooth muscle are very sensitive to quinine (\( IC_{50} = 14 \mu M \)) compared with 365 \( \mu M \) for Kv1.5 (22)). Therefore, we performed experiments on native colonic myocytes to examine if quinine at low concentrations would preferentially block \( \mathcal{I}_{K(I)} \). Figure 7, A–C, shows that in the presence of 5 mM 4-AP [a concentration that blocks all of \( \mathcal{I}_{K(I)} \), but only a portion of \( \mathcal{I}_{K(D)} \)], the overall delayed rectifier current is reduced and the \( \tau \) of the remaining current is significantly increased. Combining TEA (5 mM) or quinine (10 \( \mu M \)) agents that should only block \( \mathcal{I}_{K(D)} \) at these concentrations, with 4-AP further reduces the current and changes the \( \tau \) back to the original level in the absence of blockers.

Beech and Bolton (22) have reported delayed rectifier currents in single smooth muscle cells, and Nelson and colleagues (25, 26) have reported level combined with antisense knockout experiments in gastric smooth muscle cells from rabbit portal vein. J. Physiol. (Lond.) 418: 293–309, 1989.


