Potassium channels activated by membrane stretch may contribute to maintenance of relaxation of smooth muscle cells in visceral hollow organs. Previous work has identified K⁺ channels in murine colon that are activated by stretch and further regulated by NO-dependent mechanisms. We have screened murine gastrointestinal, vascular, bladder, and uterine smooth muscles for the expression of TREK and TRAAK mRNA. Although TREK-1 was expressed in many of these smooth muscles, TREK-2 was expressed only in murine antrum and pulmonary artery. TRAAK was not expressed in any smooth muscle cells tested. Whole cell currents from TREK-1 expressed in mammalian COS cells were activated by stretch, and single channel recordings showed that the stretch-dependent conductance was due to 90 pS channels. Sodium nitroprusside (10⁻⁶ or 10⁻⁵ M) and 8-Br-cGMP (10⁻⁴ or 10⁻³ M) increased TREK-1 currents in perforated whole cell and single channel recordings. Mutation of the PKG consensus sequence at serine 351 blocked the stimulatory effects of sodium nitroprusside and 8-Br-cGMP on open probability without affecting the inhibitory effects of 8-Br-cAMP. TREK-1 encodes a component of the stretch-activated K⁺ conductance in smooth muscles and may contribute to nitrergic inhibition of gastrointestinal muscles.

Many different K⁺ channels with diverse biophysical properties participate in the regulation of membrane potential in smooth muscles (for example see Ref. 1). Regulation of K⁺ conductances is also a major factor in the inhibitory control of smooth muscles that produces relaxation. K⁺ channels can be activated by inhibitory neurotransmitters such as nitric oxide (2, 3), ATP (4), endothelial factors (endothelial-derived relaxing factor and endothelial-derived hyperpolarizing factor) (5–7), and β-receptor agonists (8, 9). Finally, mechanical stimuli, such as cell stretch, can activate K⁺ channels (10–12) and may participate in the regulation of excitability in the bladder, uterus, and gastrointestinal tract in response to distension of the organ wall. Koh and Sanders (13) have recently identified stretch-dependent K⁺ (SDK) channels in murine colonic myocytes. The native SDK channels in murine colonic myocytes were inactive under atmospheric pressure and displayed a dramatic increase in open probability upon application of negative pressure to the patch pipette in the on-cell configuration. These channels were also activated in response to smooth muscle cell stretch, which was accomplished by attaching two patch pipettes to either end of the cell and elongating the cell.

Potassium channel proteins can be grouped according to transmembrane topology. Those with six transmembrane spans and a pore loop between segments S5 and S6 encode voltage-gated and calcium-sensitive K⁺ channels. Inwardly rectifying and ATP-sensitive K⁺ channel proteins have two transmembrane spanning segments and a pore loop between the two segments similar to that between S5 and S6 in the six-transmembrane span channels. Four individual subunits containing these topological structures assemble into a functional K⁺-selective ion channel. A recently identified family of K⁺-selective channels encodes two pore loops for each subunit, assembles into dimers, and has been referred to as two-pore domain (K₂P) potassium channels (see Ref. 14 for review). As more K₂P channels are identified at a molecular level, it is evident that the gene family encodes a highly diverse group of proteins that can be classified into four phylogenetic families based on homology. The gene names have adopted the prefix KCNK, whereas the original channel names describe aspects of their regulation or electrophysiological properties. The THIK family members (KCNK12 and KCNK13) have the common property of being activated by arachidonic acid and inhibited by the volatile anesthetic halothane (15). The TASK family members (KCNK3 and KCNK9) are acid-sensitive and display currents consistent with background conductances (16). TWIK channels (KCNK1, KCNK5, KCNK6, and KCNK7) are weakly inwardly rectifying (16, 17), and TREK (KCNK2 and KCNK10) or TRAAK (KCNK4) channels are mechanosensitive (18, 19).

Molecular identification of ion channels important to smooth muscle function allows analysis of their biophysical, pharmacological, and regulatory properties in heterologous systems (20). In the present study, we have identified the molecular entity responsible for a component of stretch-activated K⁺ channels in gastrointestinal smooth muscle cells. These channels appear to be responsible for one of the nitric oxide-sensitive conductances in gastrointestinal muscles (13) and possibly vascular smooth muscles.

EXPERIMENTAL PROCEDURES

Tissue Dissections and Enzymatic Isolation of Smooth Muscle Cells—BALB/c mice were sacrificed by cervical dislocation, and incisions were made along the abdomen. Segments of gastrointestinal tissue were isolated, and cells were collected as described previously (21) and detailed below. Strips of muscle were removed and placed into Krebs solution containing 120.35 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM
MgCl₂, 15.5 mM NaHCO₃, 1.2 mM Na₂HPO₄, and 11.5 mM glucose. Segments of tissue were pinned in a dissecting dish with the mucosa facing upward. The mucosa and submucosa were removed by sharp dissection. Small portions of the circular smooth muscle tissue were placed into a Ca²⁺-free Hanks’ solution containing 125 mM NaCl, 5.96 mM KC1, 15.5 mM NaHCO₃, 0.44 mM KH₂PO₄, 2.9 mM sucrose, and 11 mM HEPES. Strips of tissue were incubated in a Ca²⁺-free Hanks’ solution containing 230 units of collagenase (Worthington Biochemical Co.), 2 mg of fatty acid-free bovine serum albumin (Sigma), 2 mg of trypsin inhibitor (Sigma), and 0.11 mg of ATP (Sigma). Incubation in this enzyme was carried out at 37 °C for 8–12 h. The tissues were washed with Ca²⁺-free Hanks’ solution, and gentle trituration resulted in the isolation of individual myocytes. The cells were transferred to the stage of a phase contrast microscope and allowed to adhere to the glass coverslip bottom for 5 min. Smooth muscle cells were distinguished by their characteristic morphology. Single cells were collected through applied suction by aspiration them into a wide bore patch-clamp pipette (borosilicate glass; Sutter Instruments, CA). Approximately 60 smooth muscle cells were collected, flash-frozen in liquid nitrogen, and stored at −80 °C until use.

**Total RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**—Total RNA was prepared from tissue and isolated smooth muscle cells using SNAP Total RNA isolation kit (Invitrogen, San Diego, CA) as per the manufacturer’s instructions including the use of polyethylene glycol (20 μg) as an RNA carrier. First strand cDNA was prepared from the RNA preparations using the Superscript II Reverse Transcriptase kit (Life Technologies, Inc.), and 500 μg/μl of oligo(dT) primers were used to reverse transcribe the RNA sample. The cDNA reverse transcription product was amplified with channel-specific primers by PCR. The amplification profile for these primer pairs were: 95 °C for 10 min to activate the amplification polymerase (PerkinElmer Biosystems), Foster City, CA), 95 °C for 15 s, and 60 °C for 1 min, each of 40 cycles. The amplified products (5 μl) were separated by electrophoresis on a 4% agarose, 1× Tris, acetic acid, EDTA gel, and the DNA bands were visualized by ethidium bromide staining. RT control on each RNA sample used a DNA reaction as template for which the reverse transcriptase was not added, controlling for genomic DNA contamination in the source RNA. These negative controls were subjected to a second round of amplification to assure specificity of the reactions and the quality of the reagents.

**Primer Design**—The following PCR primers were used (the GenBank™ accession number is given in parentheses for the reference nucleotide sequence used): TREK-1 (KCNK2) (accession number U73488), nucleotides 506–527 and 803–822, amplicon = 316 base pairs; TREK-2 (KCNK10) (accession number NM_021161), nucleotides 1652–1675 and 1806–1826, amplicon = 174 base pairs; TRAAK (KCNK4) (accession number NM_016611), nucleotides 618–640 and 697–718, amplicon = 101 base pairs; and β-actin (V01217), nucleotides 2384–2402 and 3071–3091, amplicon = 488 base pairs. Full-length TREK-1 was amplified using primers designed to hybridize within the 5′- and 3′-untranslated sequences (nucleotides 463–485 and 1786–1761).

**Quantitative RT-PCR**—Real time quantitative PCR was performed using Syber Green chemistry on an ABI 5700 sequence detector (PerkinElmer Biosystems). Regression analysis of the mean values of eight multiplex RT-PCRs for the log₁₀ diluted cDNA was used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated yielding transcriptional quantitation of gene products relative to the endogenous standards (β-actin and glyceraldehyde-3-phosphate dehydrogenase). The reproducibility of the assay was tested by analysis of variance comparing repeat runs of samples, and the mean values generated at individual time points were compared by Student’s t test.

**TREK-1 Expression in Xenopus Oocytes**—Whole cell potassium currents from oocytes injected with in vivo transcribed cDNA were recorded using the two-microelectrode voltage-clamp technique as described previously (22). Briefly, microelectrodes were filled with 3 M KCl (resistances between 1 and 3 MΩ), and oocytes were superfused with a solution containing 96 mM NaCl, 2 mM KCl, 2.8 mM MgCl₂, and 5 mM HEPES, pH 7.4. Linear leak and capacitance currents were removed from the recorded currents by applying five hyperpolarizations of one fifth of the test amplitude, summing these, and subtracting the result to the current elicited by the test pulse (i.e. −P₀ protocol). Solution of tetraethylammonium chloride (1 mM; Sigma), 4-amino-pyridine (0.1 μM; Sigma) were prepared in distilled water. Immediately prior to use, solution was diluted to the desired concentration in the superfusate. Each experiment was performed at room temperature (24–28 °C) on oocytes collected from one more frog.

**Heterologous Expression of TREK-1 in COS Cells**—COS cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in modified RPMI medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum (Summit Biotechnology, Fort Collins, CO) and 1% glutamine (Life Technologies, Inc.) in a humidified 5% CO₂ incubator at 37 °C. The cells were subcultured twice a week by treatment with trypsin-EDTA (Life Sciences, Inc.). The TREK-1 DNA was transfected into COS cells by electroporation. After harvesting COS cells by trypsin-EDTA, the cells were washed twice with phosphate-buffered solution and resuspended in ice-cold phosphate-buffered solution at a density of 5 × 10⁶ cells/ml in the cuvette for electroporation (Electroporator II, Invitrogen, CA). A green fluorescent protein reporter plasmid and pcDNA3.1 containing TREK-1 were transfected at a 10:1 ratio of cDNAs. COS cells expressing TREK-1 were subcultured on glass coverslips for electrophysiological recordings. Current recordings were performed 1–3 days after the electroporation procedure.

**Voltage-clamp Experiments in COS Cells**—We performed the patch-clamp technique to measure membrane currents in whole cell and single channel configurations. The patch pipettes were made from borosilicate glass capillaries pulled with micropipette puller (P-80/PC, Sutter, CA) and heat polished with a microforge (MF-83, Narishige, Japan). The pipette resistances were 1–3 MΩ for whole cell recordings and 5–8 MΩ for single channel recordings. The averaged cell capacitance was 12 ± 5 picofarad.

Currents were amplified with a List EPC-7 amplifier and/or Axopatch-1A amplifier and digitized with a 12-bit analog to digital converter (Model TL-1, DME interface, Axon Instrument). The data were stored on videotape or directly digitized on-line using pClamp software (version 5.5.1 or 6.03 Axon instrument). The data were sampled at 2 kHz for whole cell and 1–5 kHz for single channel recordings with low pass filtered at 0.2–1 KHs using an eight-pole Bessel filter. The data were analyzed using pClamp (version 6.2, Axon Instrument) and Origin software (MicroCal Software) to obtain amplitude histogram and channel activity (Npₑₛ), where N is the number of channels in the patch, and
**P** is the probability of channel being open. **NP** was determined from 1 min of channel recording.

**Application of Negative Pressure and Mechanical Stretch**—Application of negative pressure to on-cell patches is thought to pull the plasma membrane into the patch pipette, single channel recordings from stretched membrane were recorded. Membrane stretch was elicited by applying suction (negative pressure) to the back end of the patch pipette. The amount of negative pressure was calibrated with a pressure transducer. The negative pressure and volume relation was linear.

**Solutions**—For whole cell recordings COS cells were bathed in a solution 5 mM KCl, 135 mM NaCl, 2 mM CaCl2, 10 mM glucose, 1.2 mM MgCl2 and 10 mM HEPES, adjusted to pH 7.4 with Tris. CaCl2 was replaced with MnCl2 for some experiments. The pipettes solution was 130 mM KCl, 5 mM MgCl2, 2.7 mM ATP, 0.1 mM Na2GTP, 2.5 mM creatine phosphate disodium salt, 0.1 mM EGTA and 5 mM HEPES, set to pH 7.2 with Tris. For the perforated whole cell patch-clamp experiments, the composition of the pipette solution was 140 mM KCl, 0.5 mM EGTA, and 5 mM HEPES, adjusted pH 7.2 with Tris. Amphotericin B (90 mg/ml) was dissolved with Me2SO, sonicated, and diluted in the pipette solution to give a final concentration of 270 μg/ml. The external solution for these experiments was the same as for the dialyzed whole cell patch experiments. For single channel recordings in cell-attached or excised patches, the bath solution was 140 mM KCl, 1 mM EGTA, 0.61 mM CaCl2, and 10 mM HEPES adjusted to pH 7.4 with Tris. The pipette solution for asymmetrical K+ gradients was 5 mM KCl and 135 mM NaCl, and for symmetrical K+ experiments it was 140 mM KCl including 200 nM charybdotoxin to inhibit large conductance Ca2+-activated K+ channels. The bath solution was 140 mM K+. Sodium nitroprusside, 8-Br-cGMP, and 8-Br-cAMP were added to the bath solution in some experiments. We also tested the effect of the catalytic subunit of PKG and PKA on TREK-1 from inside-out patches.

**Construction of S351A Mutation in TREK-1**—The mutant S351A of mouse TREK-1 was created by PCR-based site-directed mutagenesis using an ExSite mutagenesis kit (Stratagene, CA) as described in the protocol with a minor modification. Linearized TREK-1 plasmid was modified and amplified simultaneously by PCR. Two primer pairs used for PCR are a forward primer (5'-AGCTCGCCGACAGCTGCGGG-3') containing the S351A mutation spanning nucleotides 1529–1550 of mouse TREK-1 and a reverse primer (5'-TCGCCCTACGGATGTTG-GACGG-3'), which was 5'-phosphorylated, complementary to nucleotides 1507–1528. PCR was performed in a 25-μl PCR mixture containing 2.5 μl of 10× mutagenesis buffer, 1 mM dNTP mix, 2 μg of TREK-1

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**FIG. 2.** Functional properties of mTREK-1 expressed in Xenopus oocytes. Representative current responses evoked from a holding potential of −80 mV with 400-ms voltage pulses ranging from −150 to +50 mV in 20-mV increments, in A mTREK-1 injected oocytes were recorded in 5 and 98 mM potassium bathing solutions. mTREK-1 current-voltage (I-V) relations obtained from the protocol described in A were recorded in 5 mM potassium (open circles) and 98 mM potassium (closed circles). B-E show pharmacological properties of mTREK-1 expressed in Xenopus oocytes. The left and middle panels show representative current responses evoked from a holding potential of −90 mV with 400-ms voltage pulses ranging from −150 to +50 mV in 20-mV increments, recorded in 5 mM potassium before and after application of various compounds. The right panels summarize mTREK-1 current-voltage relations before (open circles) and after (closed circles) drug application.
DNA in pcDNA3.1 expression vector as a template, 15 pmol of each primer, and 1 μl of ExSite DNA polymerase blend. The cycling parameters were as follows: 1 cycle of 4 min at 94 °C, 2 min at 50 °C, and 4 min at 72 °C followed by 8 cycles of 1 min at 94 °C, 2 min at 56 °C, and 2 min at 72 °C with a final cycle of 5 min at 72 °C. Following completion of the PCR, 10 units of DpnI and 1.25 units of Pfu DNA polymerase were added directly to the reaction. The reaction mixture was mixed and incubated at 72 °C for 30 min. The DpnI, Pfu DNA polymerase-treated plasmids were transformed into Escherichia coli XL1-Blue competent cells (Stratagene, CA), and the cells were grown on a LB plate containing 60 μg/ml ampicillin overnight. Each colony grown on the plate was used for a colony-directed PCR (23). The entire mutant TREK-1 gene (S351A) in the isolated plasmid was sequenced. This plasmid was used for heterologous expression in COS cells.

RESULTS

Expression of Stretch-activated K_{AP} Channels in Smooth Muscles—We recently reported the expression of 90 pS, K'-selective, stretch-activated channels in murine and canine colonic myocytes (13). These observations led us to examine the expression of stretch-activated K_{AP} channels in smooth muscles. The stretch-activated K_{AP} channels belong to the TRENK family (see Ref. 15 for an up-to-date dendrogram) and include TREK-1, TREK-2, and TRAAK (KCNK2, KCNK4, and KCNK10, respectively). Primers were designed to specifically amplify these cDNAs (see “Experimental Procedures”). RT-PCR was performed on isolated smooth muscle cells derived from several regions of the murine gastrointestinal tract, as well as vascular vessels, urinary bladder, and uterine muscles. The latter two tissues were included because of their roles in hollow organs that function as expandable reservoirs. The expression pattern for the three stretch-activated K_{AP} channels in smooth muscles is shown in Fig. 1A. TREK-1 was expressed in all the smooth muscles tested except bladder and uterus. TREK-2 was only expressed in antrum and pulmonary artery. TRAAK could not be detected in any of the smooth muscles tested. The expression level of TREK-1 expression relative to β-actin in smooth muscles is shown in Fig. 1B. There was little difference in expression levels in smooth muscles and approximately a 20-fold difference in expression between smooth muscle and brain tissue. The results are expressed as the means ± S.E. TREK-1 expression relative to β-actin (arbitrary units) was 0.564 ± 0.051 for murine brain, 0.023 ± 0.0054 for murine colon, 0.040 ± 0.0025 for murine jejunum, 0.013 ± 0.0011 for murine fundus, 0.048 ± 0.0062 for murine antrum, 0.062 ± 0.0041 for murine portal vein, and 0.034 ± 0.0059 for murine pulmonary artery (n = 3 for all of these data).

Cloning and Expression of TREK-1 cDNA Cloned from Murine Colonic Smooth Muscle—Full-length cDNA was amplified from mouse colonic smooth muscle RNA using primers directed to the 5'- and 3'-untranslated sequences. Several independent clones were sequenced and found to be identical to the previously cloned TREK-1 from murine brain (19). TREK-1 was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad CA) and either in vitro transcribed and injected into Xenopus oocytes or transiently transfected into COS cells.

Expression of TREK-1 in Oocytes and COS Cells—To compare the properties of mTREK-1 cDNA cloned from murine smooth muscle to previous reports as well as the native SDK currents from smooth muscles, mTREK-1 was expressed in oocytes and COS cells. Voltage-clamp recordings from oocytes expressing TREK-1 revealed a rapidly activating and noninactivating current (Fig. 2A). Similar currents were not detected in control-injected oocytes. Typically, resting membrane potential in cells expressing TREK-1 was hyperpolarized by 30.2 mV (i.e. –63.9 ± 1.48 mV (n = 10)) as compared with water injected oocytes (–33.7 ± 1.48 mV (n = 9), p < 0.01), as expected if the background potassium conductance was increased. The ion selectivity of the current was examined by replacing external sodium with potassium such that extracellular potassium concentration ([K']_o) was increased from 5 to 98 mM. Increasing
The effects of K+ channel blockers on mTREK-1 currents. We examined the conductance and the effects of SNP and 8-Br-cGMP on mTREK-1 using on cell patches. In symmetrical K+ gradients (5 mM/140 mM), the amplitude of unitary current was 2.1 ± 0.4 pA at 0 mV under asymmetrical K+ conditions (5 mM K+ in the patch solution (n = 4)).

In the present study we tested the effects of sodium nitroprusside (SNP) and 8-Br-cGMP on TREK-1 expressed in COS cells. SNP (10^-6 M) did not significantly increase outward current in dialyzed cells under whole cell recording conditions (e.g. +50 mV the average current was 1.06 ± 0.29 nA in control recordings and 1.10 ± 0.40 nA after addition of SNP (n = 4)). Application of 8-Br-cGMP similarly had no significant effect on mTREK-1 current in dialyzed cells (data not shown). However, when the perforated patch technique was used, a significant increase in outward currents in cells expressing mTREK-1 was observed in response to SNP (Fig. 4). The mean peak currents at +50 mV was 1.28 ± 0.42 nA (n = 4) under control conditions and increased to 2.31 ± 0.39 nA in the presence of SNP (10^-6 M; p < 0.05). Application of 8-Br-cGMP (1 mM) also significantly increased outward currents (n = 4, p < 0.05 by analysis of variance).

Effects of SNP and cGMP on Single Channel Recordings of TREK-1—We examined the conductance and the effects of SNP or 8-Br-cGMP on mTREK-1 using on cell patches. In symmetrical K+ gradients (140 mM K+ in the patch solution), the conductance of mTREK-1 channels was 95 ± 2 pS (n = 4). The amplitude of unitary current was 2.1 ± 0.4 pA at 0 mV under asymmetrical K+ conditions (5 mM K+ in the patch solution (n = 4)).

We tested the responsiveness of mTREK-1 channels to negative pressure applied to the patches (Fig. 5A). To avoid contamination from native nonselective cation current, the cells were held at 0 mV during these experiments. Application of negative pressure (~20 or ~40 cm H2O) caused a significant increase in the open probability of the 53 pS mTREK-1 channels at 0 mV in asymmetrical K+ gradient (5 mM/140 mM) (i.e. NPo increased from 0.08 ± 0.04 to 1.35 ± 0.20 (n = 4); p < 0.01 in response to ~40 mm Hg). After releasing the negative pres-
sure (i.e. restoring atmospheric pressure) to the pipette, open probability returned to the control level (Fig. 5A).

We also tested the effects of SNP and 8-Br-cGMP on the open probability of mTREK-1 channels. In on-cell patches, the open probability of TREK-1 channels was 0.13 ± 0.12 (n = 3) at 0 mV in asymmetrical K+ gradient. Application of SNP (10^{-6} M) induced an increase of NP_o (0.89 ± 0.10; Fig. 5B). The application of 8-Br-cGMP (10^{-4} M) resulted in increased openings of mTREK-1 channel.

**Fig. 5. Activation of mTREK-1 channel by negative pressure, SNP, and 8-Br-cGMP in single channel recordings.** To remove contaminating currents, the cells were held at 0 mV at asymmetrical K+ (5/140 mM). A shows application of negative pressure (~40 cm H2O) to patch pipettes increased channel activity in cell attached patches. B is from the same cell; application of SNP (10^{-6} M) resulted in openings of mTREK-1 channel. C shows application of negative pressure (~20 cm H2O) to patch pipettes increased channel activity in cell attached patches. D is from the same cell; application of 8-Br-cGMP (10^{-4} M) resulted in increased openings of mTREK-1 channel.

**Effects of cGMP and cAMP on TREK-1 S351A—Activation of TREK-1 channels by cGMP has not previously been reported; however, others have found that cAMP-dependent mechanisms suppress TREK-1 currents (24, 25). Therefore we tested 8-Br-cAMP effects on mTREK-1. From an average control current of 0.52 nA at 30 mV, addition of 8-Br-cAMP decreased the currents initially (e.g. to 0.31 nA after 5 min). With sustained exposure to cAMP, we observed a substantial increase in the current (i.e. to 2.32 nA at +30 mV, 15 min after application).

**Fig. 6. Effects of 8-Br-cAMP on mTREK-1 currents.** Membrane potential was stepped from −80 to +70 mV in 10-mV increments for 400 ms. A shows representative currents of TREK-1 transfected COS-cells. B shows that 5 min after application of 8-Br-cAMP (10^{-3} M) decreased outward current. C shows that 15 min after application of 8-Br-cAMP (10^{-3} M) increased outward current. D is the washout of 8-Br-cAMP that restored currents to control levels. E shows that changes in peak outward currents as a function of time caused by 8-Br-cAMP. The cells were held at −80 mV and stepped to +30 mV every 1 min. F shows the current-voltage (I-V) relationship before (○) and after 5 min (●) and 15 min (□) 8-Br-cAMP application from four cells.
The currents returned to control level after washout of cAMP. Representative traces are shown in Fig. 6 (A–D). Time courses of the cAMP effects are shown in Fig. 6E, and averaged current-voltage curves for these experiments are shown in Fig. 6F (n/H11005 4).

The amino acid sequence of TREK-1 has two consensus sequences for PKG phosphorylation, and these could also serve as PKA phosphorylation sites. Therefore, we created a point mutation in mTREK-1 in which Ala replaced Ser-351 (S351A). The amino acid sequence containing the two consensus sequences are shown in Fig. 7A. Cells transfected with mTREK-1 (S351A) did not respond to 8-Br-cGMP (1 mM; Fig. 7, B–F). Application of 8-Br-cAMP to cells with mTREK-1 (S351A) caused a significant and sustained suppression of current (Fig. 8). For example, the mean peak current at +50 mV in these cells was 2.30 ± 0.58 nA under control condition (n = 3), and this decreased to 0.80 ± 0.22 nA after application of 8-Br-cAMP (Fig. 8, A and B). The time course and mean current-voltage curve for this effect is shown in Fig. 8 (C and D). Thus the typical biphasic (time-dependent) response of mTREK-1 channels to the cAMP analog was changed to a monotonic decrease in current in cells with the S351A mutant.

These effects of 8-Br-cGMP and 8-Br-cAMP were also apparent at the single channel level. In cells with TREK-1 (S351A), we confirmed the expression of TREK-1-like currents by the application of negative pressure (−40 cm H2O) to the pipettes. This increased NP0 from 0.08 ± 0.02 to 1.15 ± 0.21 (p < 0.05 similar to the effect of negative pressure on patches in cells with wild type TREK-1). After restoration of atmospheric pressure and control level NP0, 8-Br-cGMP (10−4 and 10−3 M) had no effect on NP0 in cells transfected with TREK-1 (S351A) (Fig. 9B). Another group of cells was exposed to sustained negative pressure before exposure to 8-Br-cAMP. The increase in NP0 of mTREK-1 (S351A) caused by negative pressure was inhibited from 1.04 ± 0.21 to 0.21 ± 0.11 by 8-Br-cAMP (n = 3, Fig. 9C).

DISCUSSION

Information describing K2P channels has been emerging as the biophysical and regulatory properties of these K+ channels are elucidated. TREK-1 has been described as a mechano-gated S-like channel in sensory neurons important to presynaptic facilitation and behavioral sensitization (24). Functional roles for K2P channels have not been determined for smooth muscles, and little detailed information concerning smooth muscle specific expression of this class of K+ channels has been reported.

In the present study we determined that TREK-1 is the predominant stretch-activated TREK family member expressed in smooth muscle cells, although TREK-2 was expressed in cells of the murine antrum and pulmonary artery. Expression of colonic mTREK-1 in oocytes or COS cells resulted in a conductance with similar electrophysiological properties as the brain form analyzed in previously studies (19). TREK-1 was activated by cell or membrane stretch maneuvers, and it was K+-selective and nonrectifying under symmetrical K+ conditions with a slope conductance of −90 pS. These properties are
FIG. 8. Effects of 8-Br-cAMP on mTREK-1 (S351A). Membrane potential was stepped from −80 to +70 mV in 10-mV increments for 400 ms. A shows representative currents mTREK-1 (S351A) transfected COS-cells. B shows that application of 8-Br-cAMP (10⁻³ M) decreased outward current. C shows changes in peak outward currents as a function of time caused by 8-Br-cAMP. The cells were held at −80 mV and stepped to +30 mV every 1 min. D shows current-voltage relationship before (●) and after (○) 8-Br-cAMP from three cells.

FIG. 9. Effects of 8-Br-cGMP and 8-Br-cAMP on mTREK-1 (S351A) channel in single channel recordings. To remove contaminating currents, the cells were held at 0 mV at asymmetrical K⁺ (5/140 mM). A shows that application of negative pressure (−40 cm H₂O) to patch pipettes increased channel activity in cell-attached patches. B shows from the same cell; application of 8-Br-cGMP (10⁻³ M) did not increase the activity of mTREK-1 mutant channel. C shows that after application of negative pressure (−40 cm H₂O) to patch pipettes, 8-Br-cAMP (10⁻³ M) decreased channel activity in cell attached patches.

identical to the characteristics of the SDK channels identified in murine and canine colonic myocytes (13), suggesting that TREK-1 may encode SDK in these muscles.

NO stimulates the activity of guanylyl cyclase, increasing cGMP concentrations and the activity of PKG (27). NO relaxes smooth muscle cells in vascular (28) and visceral (29) tissues, and much of the relaxation response is mediated via cGMP-dependent mechanisms (30, 31). A portion of NO-dependent relaxation is due to hyperpolarization and/or reduced excitability that is due to activation of K⁺ channels. NO activates several classes of K⁺ channels. For example, NO stimulation can target phosphorylation of large conductance Ca²⁺-activated K⁺ channels (BK channels), resulting in increased channel open probability (32, 33). However, increased BK channel opening alone cannot explain NO-mediated hyperpolarization because BK channel blockers such as iberiotoxin do not eliminate the inhibitory response to NO (30). In isolated murine colonic smooth muscle cells three distinct K⁺ channels are activated by NO-dependent mechanisms (34). BK underlies the large conductance channel (−220 pS), and there are also 90 and 2–4 pS channels activated by NO (34). The molecular identity of the latter two conductances has not been identified. We have previously shown that the 90 pS channels activated by NO- and cGMP-dependent mechanisms in colonic myocytes are the stretch-dependent K⁺ channels (13). The data in the present study show that mTREK-1 channels are also activated by NO and cGMP-dependent mechanisms. These data further suggest that mTREK-1 encodes SDK channels of colonic myocytes.

We investigated the mechanisms for cyclic nucleotide-dependent regulation of TREK-1. TREK-1 has two potential consensus sites for PKA/PKG phosphorylation. These sites reside in the carboxyl terminus at amino acids 333 and 351. In a previous study, mutation of the serine 333 eliminated channel cAMP-mediated inhibition and inhibition by serotonin in 5HT-receptor/TREK-1 cotransfected cells (24). However, changing the serine 351 to alanine did not eliminate PKA-mediated regulation. We found that the response of wild type mTREK-1 was biphasic. Stimulation with 8-Br-cAMP caused transient depression in the mTREK-1 current followed by significant stimulation within minutes. The latter response was completely blocked by the serine to alanine mutation at 351. These data demonstrate that cAMP-dependent regulation at serine 333 is likely to be inhibitory, but subsequent phosphorylation at serine 351 may counteract the inhibitory effects of phosphorylation of serine 333.

We also identified the molecular site of regulation of mTREK-1 by a cGMP-dependent mechanism. Exposure of cells with wild type channels to either the NO donor or 8-Br-cAMP resulted in a sustained increase in open probability of mTREK-1 channels. Mutation of the PKG consensus site (S351A) in mTREK-1 eliminated the increase in open probability caused by SNP and 8-Br-cGMP. Serine 351 might provide a site for phosphorylation by both PKG and PKA. Taken together, our data suggest that the initial decrease in channel activity after exposure to 8-Br-cAMP is due to PKA phosphorylation at Ser-333, and channel activation is due to PKG or PKA phosphorylation at Ser-351. The initial decrease in channel activity after 8-Br-cAMP, presumably because of phospho-
In conclusion, this study suggests that TREK-1 encodes the 90 pS K⁺ channel in smooth muscles. These are important channels in gastrointestinal muscles that appear to have multiple regulatory pathways in control of their open probability. At a minimum, we have observed that TREK-1 channels are regulated by mechanical stimulation and by NO and cGMP-dependent mechanisms. Our data are consistent with the following scheme: NO activation of TREK-1 is mediated via PKG phosphorylation at Ser-351. Inhibition of TREK-1 appears to be regulated by mechanical stimulation and by NO and cGMP-dependent mechanisms. Our data are consistent with the following scheme: NO activation of TREK-1 is mediated via PKG phosphorylation at Ser-351. Inhibition of TREK-1 appears to be mediated via phosphorylation at Ser-333, but this inhibitory effect can be overridden by subsequent phosphorylation at Ser-333. Either PKG or PKA may be able to provide activating phosphorylation at Ser-351, but the site at Ser-333 is restricted to regulation by PKA. Thus, phosphorylation-dependent regulation at the carboxyl terminus of TREK-1 appears to finely tune channel open probability.

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TREK-1 Regulation by Nitric Oxide and cGMP-dependent Protein Kinase: AN ESSENTIAL ROLE IN SMOOTH MUSCLE INHIBITORY NEUROTRANSMISSION
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