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TREK-2, a New Member of the Mechanosensitive Tandem-pore K^+ Channel Family*

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Recently, several mammalian K^+ channel subunits (TWIK, TREK-1, TRAAK, and TASK) possessing four transmembrane segments and two pore-forming domains have been identified. We report the cloning of a new member of this tandem-pore K^+ channel from a rat cerebellum cDNA library. It is a 538-amino acid protein and shares 65% amino acid sequence identity with TREK-1. Therefore, the new clone was named TREK-2. Unlike TREK-1, whose mRNA has been reported to be expressed in many different tissues, TREK-2 mRNA is expressed mainly in the cerebellum, spleen, and testis as judged by reverse transcriptase-polymerase chain reaction and Northern blot analysis. Expression of TREK-2 in COS-7 cells induced a time-independent and non-inactivating K^+-selective current. TREK-2 was partially blocked (36%) by 2 mM Ba^{2+}. In symmetrical 150 mM KCl, the single-channel conductances were 110 picosiemens at −40 mV and 68 picosiemens at +40 mV, and the mean open time was 0.9 ms at −40 mV. TREK-2 was activated by membrane stretch or acidic pH. At −40 mm Hg pressure, channel activity increased 10-fold above the basal level. TREK-2 was also activated by arachidonic acid and other naturally occurring unsaturated free fatty acids. These results show that TREK-2 is a new member of the tandem-pore K^+ channel family and belongs to the class of mechanosensitive and fatty acid-stimulated K^+ channels.

Molecular cloning has identified a large number of K^+ channel subunits consisting of two, four, six, and eight transmembrane segments (1–3). Despite the structural diversity, all K^+ channels share a conserved pore-forming domain that is essential for providing K^+ selectivity (4, 5). The most recently identified members of the K^+ channel family are those that possess two pore (2P)[1]-forming domains and four transmembrane (4TM) segments (3, 6–8). The genes for the 2P/4TM K^+ channels were first identified in the nematode *Caenorhabditis elegans* (9) and subsequently in other organisms. Numerous members of the 2P/4TM K^+ channel family (KCNK*x*) have now been identified in mammalian tissues and one member from *Drosophila* (ORK1) (6). Some of the cloned 2P/4TM K^+ channel subunits form functional K^+ channels when expressed either in *Xenopus* oocytes or in mammalian cell lines and exhibit properties of a background K^+ current (7, 10–16). Those that do not express in the plasma membrane may be targeted to intracellular organelles or need a partner for functional expression (17, 18).

In the 2P/4TM K^+ channel family, two members have been named TREK-1 (TWIK-related K^+ channel) and TRAAK (TWIK-related arachidonic acid-stimulated K^+ channel). TREK-1 and TRAAK are activated by either membrane stretch or free fatty acids (19, 20). Thus, they may represent the native mechanosensitive and fatty acid-sensitive K^+ channels that were identified earlier in various cell types (21–24). In cardiac and neuronal cells, arachidonic acid has been shown to cause opening of three types of K^+ channels (K_{FA} channels) whose current-voltage relationships are outwardly rectifying, inwardly rectifying, or linear (24). Based on the reported electrophysiological and pharmacological characteristics (10, 12), TREK-1 is most likely to encode the K^+ channel that has an outwardly rectifying current-voltage relationship.

In this study, we report the cloning of a new member (TREK-2)[2] of the 2P/4TM K^+ channel family whose single-channel current-voltage relationship shows inward rectification. TREK-2 shares 65 and 45% amino acid identities with TREK-1 and TRAAK, respectively, but <30% with other 2P/4TM K^+ channels. Our results show that TREK-2 is mainly expressed in the cerebellum. When expressed in COS-7 cells, TREK-2 exhibits an instantaneous and non-inactivating K^+-selective current with high sensitivity to mechanical stretch and free fatty acids and shows biophysical properties nearly identical to those of the inwardly rectifying K^+ channel previously described in the rat brain (24).

MATERIALS AND METHODS

Library Screening—Total RNA from rat cerebellum was reversed-transcribed using oligo(dT) to generate first strand cDNA (Superscript pre-amplification system, Life Technologies, Inc.). A DNA fragment of 421 bp was obtained by RT-PCR using TRAAK-specific primers (5′-CAATAGCAGCAACCACTC-3′ and 5′-GTACATAATCGCCAAAGC-3′). The DNA fragment was labeled with ^{32}P and used to screen the rat cerebellum cDNA library (λZAP II, Stratagene, La Jolla, CA). Hybridization was carried out in 20% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 0.2 mg/ml salmon sperm DNA at 42 °C for 15 h. Filters were washed twice with the solution containing 2× SSC and 0.1% SDS for 15 min at room temperature. Of 1×10^6 phages screened, 12 positive clones were obtained from the cerebellum cDNA library. DNA inserts were excised from the λZAP II vector into pBluescript SK^-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF196965.

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[1] The abbreviations used are: 2P, two pore; 4TM, four transmembrane; bp, base pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase(s); GFP, green fluorescent protein; AA, arachidonic acid.

[2] TREK-2 has been assigned the gene name *KCNK10* (approved by the Human Genome Organization).

and analyzed by restriction analysis. Four DNA inserts were sequenced using the dideoxynucleotide chain termination method. One clone (1.45 kb) contained a partial sequence of a new 4TM K^+ channel as judged by the presence of two pore-forming domains. The same cDNA library was screened again with a 921-bp DNA fragment obtained by cutting with *Eco*RI. Two positive clones included the entire coding region of a new 4TM K^+ channel (pBS-TREK-2).

Northern Blot Analysis and RT-PCR—Rat multiple-tissue Northern blots were purchased from CLONTECH (Palo Alto, CA) and OriGene Technologies, Inc. (Rockville, MD). The membranes were prehybridized for 30 min at 65 °C and hybridized for 3 h at 65 °C in ExpressHyb solution (CLONTECH) with a ^{32}P-labeled 597-bp DNA fragment following the manufacturer's protocol. This DNA fragment is part of the C terminus of TREK-2 and was obtained by PCR using pBS-TREK-2 as template and TREK-2-specific primers (5′-GGCTAATGTCA CTGCT-GAGTTCC-3′ and 5′-AAGCCACACTTTAGTCCAGCT CC-3′). The membranes were rinsed with solution containing 2× SSC and 0.05% SDS for 40 min at room temperature. A second washing was performed in solution containing 0.1× SSC and 0.1% SDS for ~50 min at 50 °C. The membranes were exposed to an x-ray film and developed 24–36 h later. The membranes were probed again with ^{32}P-labeled β-actin DNA.

For RT-PCR, total RNAs were extracted from 14 rat tissues (RNA STAT-60, TEL-TEST Inc.), and their integrity was checked by gel electrophoresis. Total RNAs were reverse-transcribed using an oligo(dT) primer using the Superscript pre-amplification system. The first strand cDNA was used as template for PCR amplification using rat TREK-2 primers that yield the 597-bp TREK-2 fragment (see above). As a control, glyceraldehyde-3-phosphate dehydrogenase was amplified using specific primers (CLONTECH). PCR amplification was performed as follows: initial denaturation at 94 °C for 3 min; 30 cycles at 94 °C for 45 s, 55 °C for 1 min (60 °C in the case of glyceraldehyde-3-phosphate dehydrogenase), and 72 °C for 2 min; and a final extension step at 72 °C for 8 min.

Transfection of TREK-2 into COS-7 Cells—For transfection into COS-7 cells, the coding region of rat TREK-2 was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) by ligating into the *Eco*RV-*Xho*I site after cutting pBS-TREK-2 with *Sma*I and *Xho*I. Cells were seeded at a density of 2×10^5 cells/35-mm dish 24 h prior to transfection. pcDNA3.1/TREK-2 and pcDNA3.1/GFP were cotransfected into COS-7 cells with LipoTaxi reagent (Life Technologies, Inc.). Green fluorescence from cells expressing GFP was detected using a Nikon microscope equipped with a mercury lamp light source. Cells were used 1–3 days after transfection.

Electrophysiological Studies—Electrophysiological recording was performed in the whole-cell, cell-attached patch, inside-out patch, and outside-out patch configurations using a patch clamp amplifier (Axopatch 200, Axon Instruments, Inc., Foster City, CA). In experiments using excised patches, pipettes and bath solutions contained 150 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.3). In whole-cell recordings, the bath solution contained 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.3). All recordings were performed at room temperature (22–24 °C). Currents were digitized with a digital data recorder (VR10, Instrutech, Great Neck, NY) and stored on videotape. The recorded signal was filtered at 5 kHz using an 8-pole Bessel filter (−3 dB; Frequency Devices, Haverhill, MA) and transferred to a computer (Dell) using the Digidata 1200 interface (Axon Instruments, Inc.) at a sampling rate of 20 kHz. Whole-cell and single-channel currents were analyzed with the pClamp program (Version 7). Data were analyzed to obtain a duration histogram, an amplitude histogram, and relative channel activity (relative NP_o). The filter dead time was ~100 μs (0.3/cutoff frequency). Therefore, events shorter than ~50 μs will be missed in our analysis. When studying the effect of membrane tension and free fatty acids using patches that contain many channel openings (more than five channel levels), currents were integrated over time to determine the relative channel activity. N is the number of channels in the patch, and P_o is the probability of a channel being open. NP_o was determined from ~1 min of current recording. Current tracings shown in the figures were filtered at 1 kHz. Data are represented as mean ± S.D. Student's t test was used to test for significance at the level of 0.05. All free fatty acids in liquid form were first dissolved in chloroform and kept in a −80 °C freezer. The solvent (chloroform) was evaporated, and free fatty acids were dissolved by sonicating for 10 min (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) in bath recording solution at a desired concentration. Free fatty acids in powder form were dissolved in ethanol. The final ethanol or Me_2SO concentration in the perfusion solution used was <0.1% and had no effect on the TREK-2 channel activity. All free fatty acids were purchased from Sigma. Negative pressure was applied using a plastic syringe via tubing connected to the pipette holder and monitored using a mercury manometer attached in parallel to the tubing. A steady negative pressure could be obtained using this system. H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) was purchased from Calbiochem. All other chemicals and drugs were from Sigma.

RESULTS

Cloning of TREK-2—A 421-bp DNA fragment from rat TRAAK was used to screen a rat cerebellum cDNA library under mild stringency conditions. Two positive clones containing a coding region of a new 2P/4TM K^+ channel subunit was identified. The DNA sequence revealed an open reading frame of 1617 bases that encodes a 538-amino acid polypeptide with a calculated molecular mass of 60 kDa (Fig. 1*A*). A stop codon (TAA) was present in the 5′-region upstream of the first methionine, indicating that the DNA contains the complete coding sequence for a protein. Hydrophobicity analysis (25) of the amino acid sequence showed that the new clone belongs to the K^+ channel family with two pore-forming domains and four transmembrane segments (Fig. 1*B*). We placed the N terminus in the intracellular side, similar to those of other tandem-pore K^+ channels. Thus, the putative K^+ channel subunit has a short N terminus, an extended extracellular loop between M1 and P1, and a long C terminus, structural features typical of nearly all 4TM K^+ channels (Fig. 1*C*). Two *N*-glycosylation sites (Asn^{144} and Asn^{147}) are present in the extracellular loop between M1 and P1. The amino acid sequence of the new clone shows potential phosphorylation sites for protein kinases A and C in the C terminus (Ser^{359} and Thr^{475}) as indicated.

Searching the GenBank™ Data Bank using the BLAST sequence alignment program (26) identified a human homologue of rat TREK-2 in the genomic sequences of chromosome 14 (accession numbers AL133279 and AL122021) with 93% amino acid identity. The human TREK-2 DNA was distributed in eight different locations with seven intervening sequences, as illustrated in the gene map (Fig. 2*C*). In the dbest data base, we found one expressed sequence tag sequence (accession number AI073392) of which 147 bp was similar to the M2 region of TREK-2 (851–991 bp of the open reading frame). The search also revealed that the DNA sequence of the new clone is similar to those of TREK-1 and TRAAK, which are 2P/4TM K^+ channel subunits (10, 12). Alignment of the DNA sequence of the new clone with those of TREK-1 and TRAAK shows high homology within the transmembrane and pore-forming domains (Fig. 2*A*). The new clone shares 65 and 45% amino acid sequence identities with TREK-1 and TRAAK, respectively. Therefore, the new clone is a third member of the TREK/TRAAK family of 4TM K^+ channels. The dendrogram of all tandem-pore K^+ channels identified to date in the mammalian system is shown in Fig. 2*B*. Rat TASK-3 (KCNK9) was just recently identified in our laboratory (GenBank™ accession number AF192366). The percentage indicates amino acid identity between two subunits. Thus, closely related subunit groups exist within the 2P/4TM K^+ channel family such as TWIK, TASK, TREK, and KCNK6/7 groups. Homology between different groups of subunits is very low (~20%).

Tissue Distribution of TREK-2 mRNA—The expression of TREK-2 mRNA in adult rat tissues was examined by Northern blot analysis and RT-PCR. TREK-2-specific primers were used to generate a 597-bp DNA fragment in the C terminus of TREK-2 and used as probe. The first blot shows that of the eight tissues, the brain shows a distinct band with an estimated size of 7.5 kb. Interestingly, a diffuse band located between 3 and 5 kb was present in the pancreas. To further localize the expression of TREK-2 within the brain, a blot in which total RNAs from six different regions of rat brain were separated was used. A major RNA transcript with an estimated size of ~7.5 kb was detected in the cerebellum. RT-PCR was

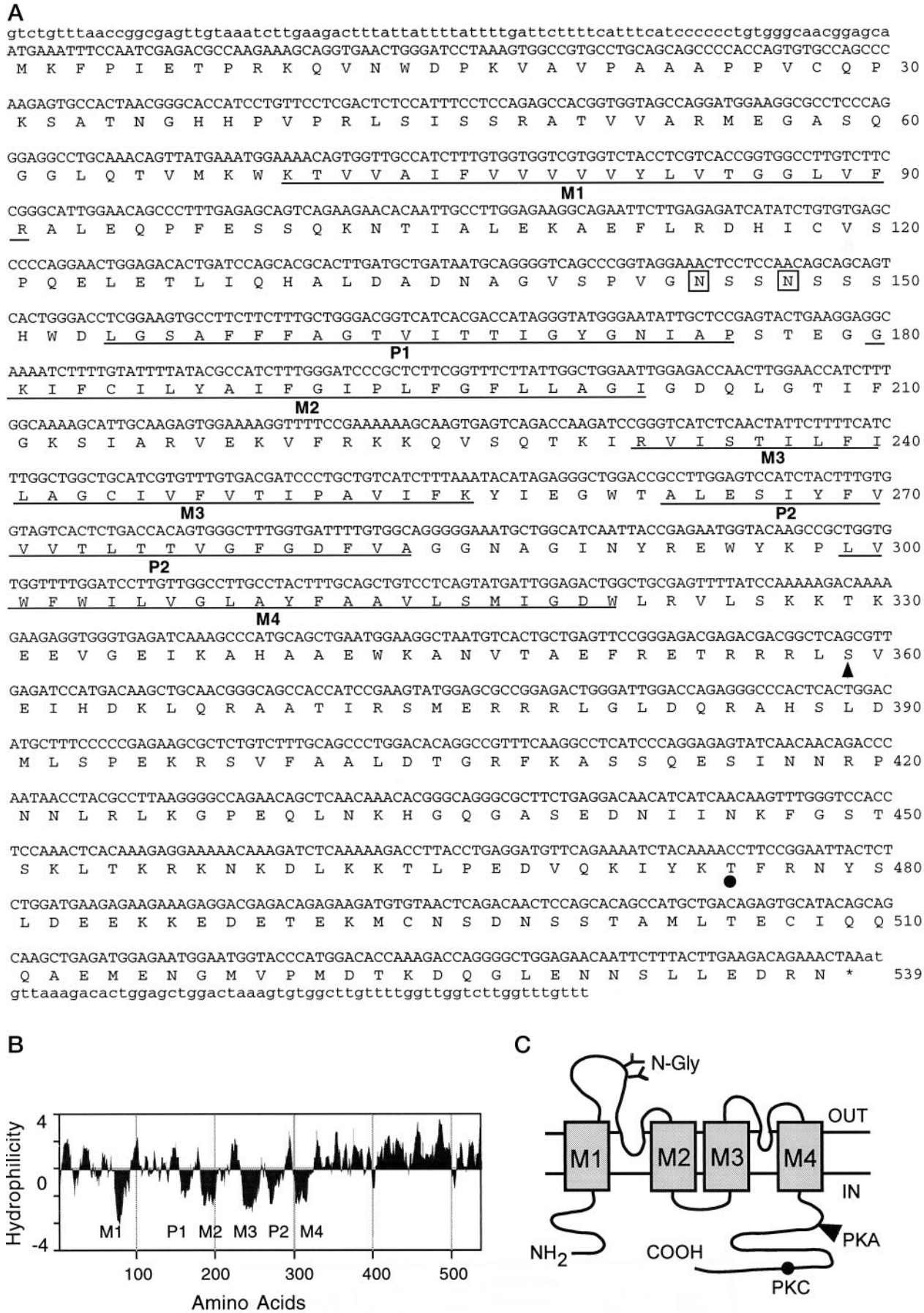

FIG. 1. **TREK-2 sequences and predicted membrane topology.** *A*, nucleotide and amino acid sequences of rat TREK-2. Four transmembrane segments and two pore-forming domains are *underlined*. Consensus sites for *N*-glycosylation (*boxed*) and phosphorylation by protein kinase

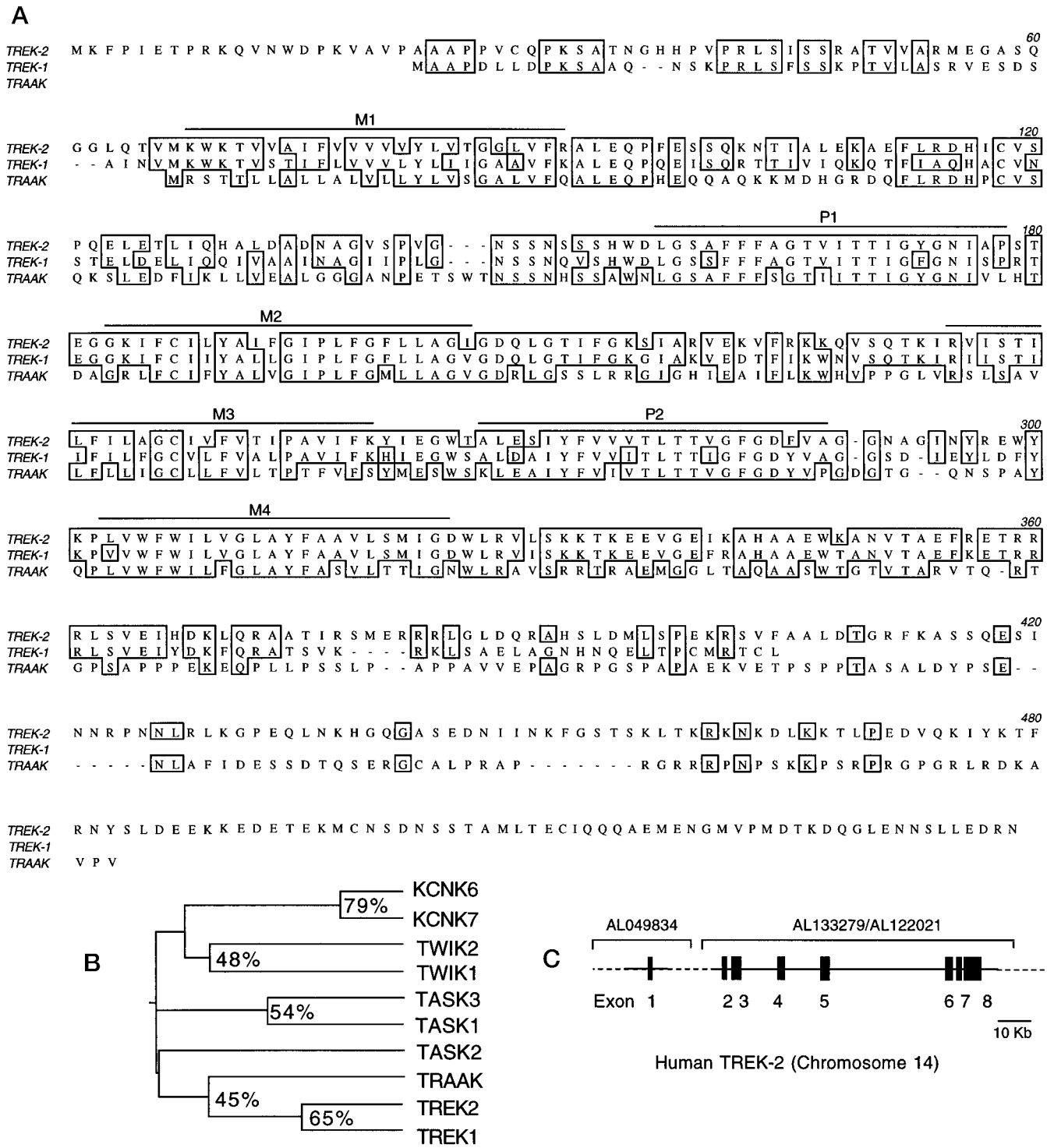

FIG. 2. **Alignment of amino acid sequences of TREK-2, TREK-1, and TRAAK.** *A*, three sequences are aligned, and identical amino acids are *boxed*. *Dashes* indicate gaps in alignment. Four transmembrane segments and two pore-forming regions are indicated by *solid lines* above the amino acids. *B*, a proposed phylogenetic tree of mammalian 4TM K^+ channels is shown. The percent values indicate amino acid identity between two subunits. The accession number for TREK-2 is AF196965. *C*, shown is a gene map of human TREK-2 based on the three human chromosome 14 sequences (accession numbers AL133279, AL122021, and AL049834). The first 51 bases of TREK-2 are located in the AL049834 sequence. The map shows seven exons within the span of ~90 kb of DNA. The sizes of exons from left to right are 51, 57, 258, 122, 156, 188, 148, and 623 bp.

carried out to further determine the expression of TREK-2 mRNA in various tissues (Fig. 3*B*). The expected 597-bp PCR products of TREK-2, confirmed by sequencing, were detected in three tissues. The strongest signal was present in the cerebellum, confirming the results of the Northern blot analysis. Spleen and testis showed relatively weak signals. Control re-

A (▲) and protein kinase C (●) are shown. These sites were identified using the MacVector program. *B*, hydropathy plot of TREK-2 amino acid sequence analyzed using the Kyte-Doolittle algorithm (25) shows four potential transmembrane segments and two potential pore-forming regions. *C*, deduced topology of TREK-2. *N-Gly*, *N*-glycosylation; *PKA* and *PKC*, protein kinase A and C, respectively.

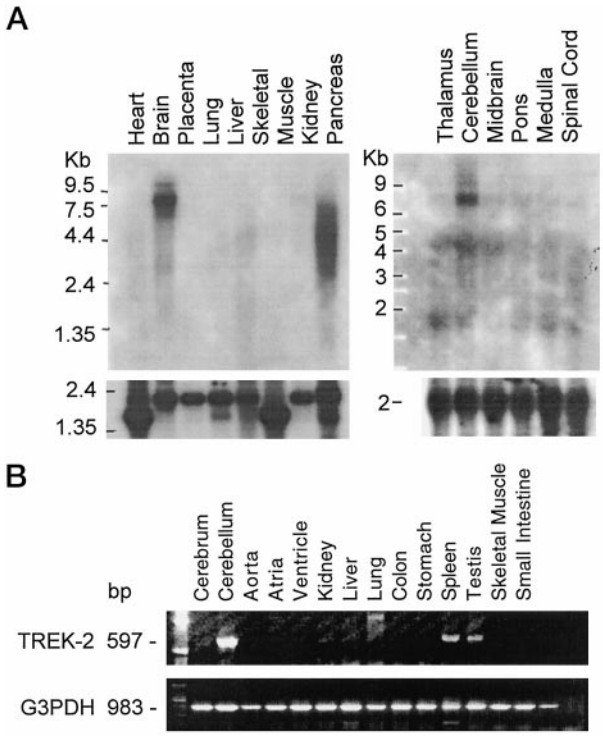

FIG. 3. **Tissue distribution of TREK-2 in the rat.** *A*, Northern blot analysis was performed using a TREK-2-specific probe and β-actin (control). The first blot (CLONTECH) after 24 h of exposure shows a major band at ~7.5 kb expressed in the brain. The second blot (OriGene) after 36 h of exposure shows that, of the six regions of the brain, the cerebellum expresses TREK-2 mRNA. Longer exposure (5 days) did not show any other distinct bands. *B*, reverse transcriptase-PCR analysis of TASK-3 in rat tissues is shown. Total RNA from each tissue was used to prepare first strand cDNAs. TREK-2-specific primers were used to generate the expected PCR product of 597 bp. The amplified PCR products were subcloned into the pCR2.1 vector (Invitrogen) and sequenced on one strand for confirmation. Two controls (*upper panel*, *last two lanes*) included one with no DNA and one with no enzyme. The quality of cDNA was checked using glyceraldehyde-3-phosphate dehydrogenase (*G3DPH*)-specific primers. Two controls included the template DNA for glyceraldehyde-3-phosphate dehydrogenase and one that has no DNA (*lower panel*, *last two lanes*).

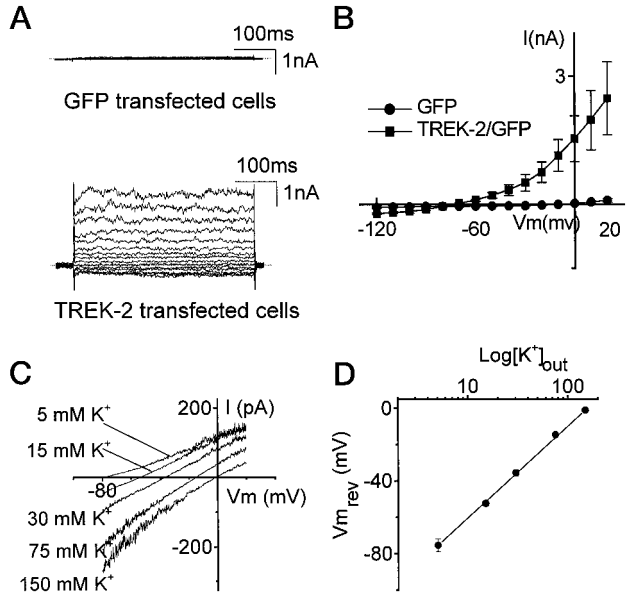

FIG. 4. **Expression of TREK-2 in COS-7 cells.** *A*, whole-cell currents were recorded from a cell transfected with GFP only or with TREK-2/GFP. Pipette and bath solutions contained 150 and 5 mM KCl, respectively. The membrane potential was held at −80 mV and stepped to various potentials ranging from −120 to +20 mV. The *dotted line* indicates the zero current level. *B*, current-voltage relationships are shown as indicated (mean ± S.D. of three cells). *C*, outside-out patches were formed, and $[K^+]$ in the bath solution was changed as indicated. Ramp pulses (−80 to +20 mV) were applied at each $[K^+]$. Typical tracings from one experiment are shown. *D*, reversal potentials from three patches were determined and are plotted as a function of $[K^+]$. Experimental values were fitted by linear regression (slope, 53 mV/decade).

actions performed in the absence of reverse transcriptase in four tissue samples (cerebellum, spleen, testis, and kidney) did not yield any visible bands under identical conditions.

Basic Electrophysiological and Pharmacological Properties—To determine whether TREK-2 forms a functional ion channel, the coding region of TREK-2 was subcloned into a mammalian expression vector (pcDNA3.1) and transiently transfected along with DNA that encodes GFP into COS-7 cell. Fig. 4*A* shows whole-cell currents recorded in nontransfected and transfected COS-7 cells in bath solution containing 5 mM K^+ and in pipette solution containing 150 mM K^+. Cell membrane potential was held at −80 mV and stepped to various potentials for 430-ms duration. Cells transfected with GFP alone showed small currents of <200 pA (95 ± 40 pA at +20 mV, $n = 5$). Cells that were transfected with TREK-2/GFP showed instantaneous and non-inactivating currents in the nA range. The averaged current at +20 mV in cells transfected with TREK-2 was 2.5 ± 0.9 nA ($n = 4$). Thus, TREK-2 forms a functional ion channel in the plasma membrane of COS-7 cells. Current-voltage relationships obtained from cells transfected with GFP alone and GFP/TREK-2 are shown in Fig. 4*B*. In nontransfected cells, the reversal potential was found to be close to zero (−9 ± 7 mV, $n = 4$), indicating that only small background leak current was present. The reversal potential shifted to −74 ± 4 mV ($n = 3$) after transfection with TREK-2, as expected if functional K^+ channels were expressed.

Ion selectivity of TREK-2 was studied using large outside-out patches containing many channels by changing the concentration of K^+ in the bath solution from 5 to 150 mM while maintaining the pipette $[K^+]$ constant at 150 mM. A voltage ramp from −80 to +20 mV was applied to the patch, and currents from three trials were averaged for each concentration of K^+. The reversal potential shifted to the right as $[K^+]$ in the bath solution was elevated, as expected of an ion channel that is permeable to K^+, but not to Cl^- (Fig. 4*C*). A plot of the reversal potential as a function of $[K^+]_{out}$ showed that the slope was 53 ± 2 mV ($n = 4$) per 10-fold change in $[K^+]_{out}$, close to the calculated Nernst value of 59 mV at 22 °C (Fig. 4*D*). In patches obtained from cells transfected with GFP alone, a basal current level of <5 pA was present at +20 mV. These results show that TREK-2 is a relatively K^+-selective ion channel, similar to other two-pore K^+ channels.

In cell-attached and inside-out patches, channel openings with marked open channel noise were present in cells transfected with TREK-2/GFP, but not with GFP alone. Channel openings at different membrane potentials from an inside-out patch are shown in Fig. 5*A* when both pipette and bath solutions contained 150 mM KCl. An open-time histogram was determined from patches showing only one level of opening at −40 mV such as that shown in Fig. 5*A* (Fig. 5*B*). The mean open time of TREK-2 determined from five patches was 0.9 ± 0.1 ms. Mean open time at positive potentials could not be determined due to clustered multiple openings. Amplitude histograms obtained from channel openings at +40 and −40 mV are shown in Fig. 5*C*. Despite the open channel noise, amplitude levels could be visually identified, as indicated by *dotted lines*. Single-channel current-voltage relationship determined using the mean amplitude values at different voltages shows that TREK-2 is an inwardly rectifying K^+ channel in symmet-

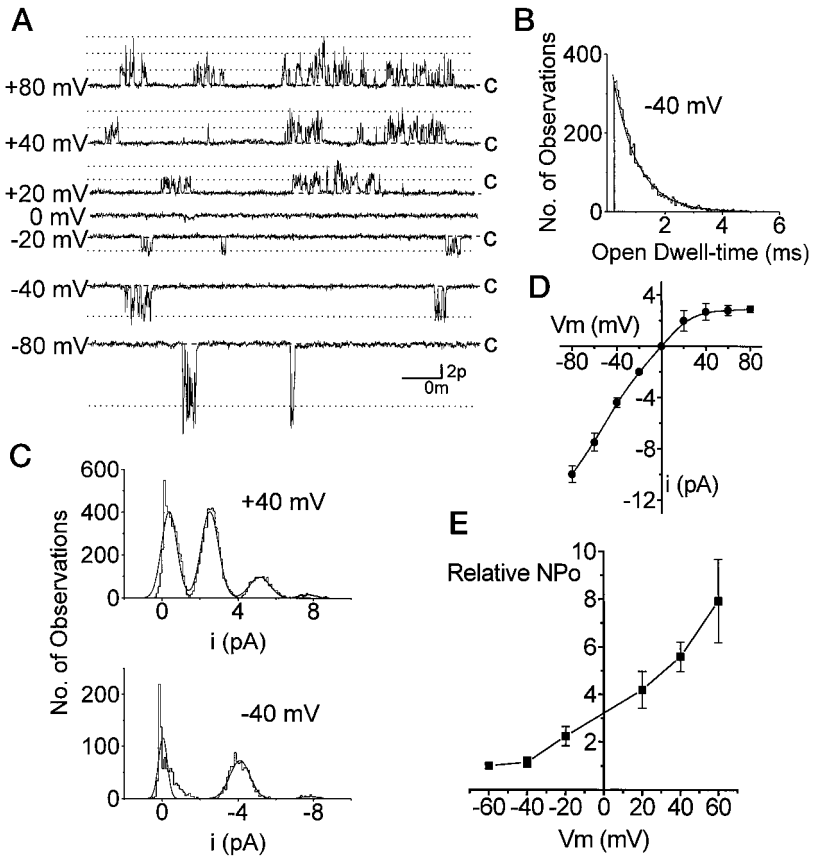

FIG. 5. **Single-channel properties of TREK-2.** *A*, TREK-2 channels in an inside-out patch was recorded at various membrane potentials as shown. *c* indicates the closed state. The *dotted lines* indicate the open state and were drawn by eye. *B*, a typical open-time histogram determined from openings at −40 mV is shown. *C*, amplitude histograms at two membrane potentials (−40 and +40 mV) are shown. *D*, current amplitudes from the first open level were determined to obtain the current-voltage relationship (mean ± S.D. of three values). *E*, relative channel activity at different membrane potentials is shown. In this experiment, the channel activity at −60 mV was 0.10 ± 0.02 (n = 5) and was taken as 1.0 for determining relative NP_o at other membrane potentials.

rical 150 mM K^+. The single-channel conductances were 68 ± 16 picosiemens at +40 mV and 110 ± 9 picosiemens at −40 mV (n = 3) (Fig. 5*D*). TREK-2 channel activity was always more active at depolarized than at hyperpolarized potentials. Therefore, channel activities were determined at various potentials from five patches and are plotted in Fig. 5*E*. The data clearly show that the open probability increases as the cell membrane depolarizes. This explains the linear or slightly outwardly rectifying whole-cell current-voltage relationships of TREK-2 currents in COS-7 cells (Fig. 4), despite the inward rectification of single-channel currents.

The effects of various pharmacological agents and pH were examined on TREK-2 current using large outside-out patches from COS-7 cells in symmetrical 150 mM KCl. TREK-2 was insensitive to low concentrations of Ba^{2+} (100–500 μM) and blocked only at high concentrations. Ba^{2+} at 2 mM applied extracellularly blocked the inward TREK-2 currents by 36 ± 5% (−40 mV, n = 3). TREK-2 was insensitive to 1 mM tetraethylammonium, 100 μM quinidine, 1 mM lidocaine, 100 μM bupivacaine, and 100 μM gadolinium when applied extracellularly (<5% change; −80 and +80 mV, n = 4 each). We examined the effect of changes in intracellular pH on TREK-2 using inside-out patches. At −40 mV, changes in pH_i from 7.3 to 6.8 and 7.8 resulted in 4.0 ± 1.4- and 0.4 ± 0.2-fold changes from basal channel activity observed at pH 7.3 (basal NP_o = 0.17 ± 0.15, n = 8), respectively. Thus, TREK-2 is stimulated markedly by acidic pH and inhibited mildly by alkaline pH.

TREK-2 possesses potential phosphorylation sites for both protein kinases A and C. Therefore, the effects of activators of protein kinases A and C were tested on TREK-2 in bath solution containing 5 mM K^+ and 1 mM Ca^{2+} using cell-attached patch configuration. Membrane potential was held at −60 mV, and changes in channel activity was determined. Extracellular application for 5 min of phorbol 12-myristate 13-acetate (100 nM), an activator of protein kinase C, failed to significantly alter TREK-2 current in three cells. Application of 8-(4-chlorophenylthio)-cAMP (200 μM), a permeant derivative of cAMP, to increase intracellular cAMP levels resulted in a 66 ± 13% (n = 4) decrease in channel activity at the end of a 3-min period. This inhibition by 8-(4-chlorophenylthio)-cAMP was reduced by 58 ± 12% (n = 3) by pretreatment with H-7 (25 μM), a protein kinase A inhibitor. Application of 1-methyl-3-isobutylxanthine (200 μM), which should also increase cAMP concentration in the cell and activate protein kinase A, resulted in 85 ± 5% (n = 3) inhibition of channel activity after 3 min of perfusion. Thus, phosphorylation by protein kinase A caused a significant reduction of TREK-2 current, whereas phosphorylation by protein kinase C had no effect.

Mechanosensitivity of TREK-2—Whether TREK-2 is sensitive to membrane stretch was assessed by applying negative pressure to the patch membrane. In cell-attached patches, a basal level of channel activity was usually present under normal atmospheric pressure. Applying negative pressure (−40 mm Hg) to the patch membrane increased channel activity in every cell that contained TREK-2. In 11 out of 12 patches obtained from cells transfected with GFP only, application of negative pressure even up to 80 mm Hg failed to activate channels. In one patch, a small conductance channel (15 picosiemens) was activated by negative pressure. In inside-out patches from cells transfected with TREK-2, the basal activity at atmospheric pressure was also observed, but tended to be higher than that in the cell-attached patch in most patches. Using inside-out patches with relatively low basal activity, we tested the effect of negative pressure on TREK-2. At a holding potential of −40 mV, application of negative pressure (0 to −80 mm Hg) produced a rapid increase in channel activity in every patch studied (Fig. 6*A*). Return of pressure to the atmospheric level (0 mm Hg) resulted in a quick return of TREK-2 to basal activity. No desensitization of channel activity was observed when the negative pressure (−40 mm Hg) was held constant for

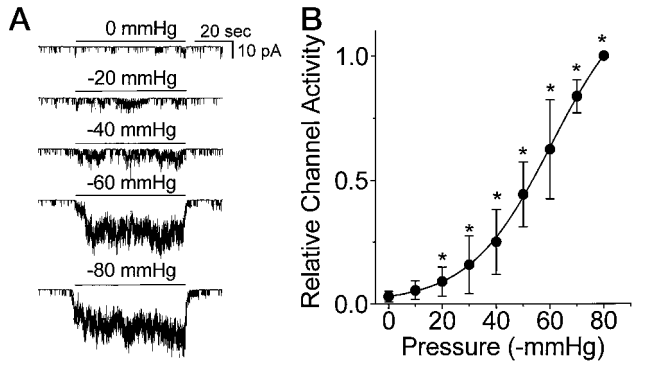

FIG. 6. **Mechanosensitivity of TREK-2.** *A*, inside-out patches were formed, and then negative pressure (0 to −80 mm Hg) was applied to the patch membrane. Patches that showed low basal channel activity at atmospheric pressure were used. Activation by pressure was reversible in every patch. The holding potential was −40 mV. *B*, channel activity at −80 mm Hg was taken as 1.0, and relative channel activities at various pressures were then plotted as a function of pressure. Each point is the mean ± S.D. of three determinations. *Asterisks* indicate a significant difference from the value at atmospheric pressure (0 mm Hg).

>3 min. Relative channel activity plotted as a function of applied pressure is shown in Fig. 6*B*. We were unable to determine channel activity at pressures less than −80 mm Hg, as patches became leaky and eventually broke. Therefore, the pressure at which half-maximal activation occurs could not be determined. These results show that TREK-2, like TREK-1 and TRAAK, is a mechanosensitive K^+ channel.

Activation of TREK-2 by Free Fatty Acids—Whether TREK-2 is sensitive to free fatty acids was tested using inside-out and outside-out patches. As shown in Fig. 7*A*, arachidonic acid (AA) increased channel activity in a concentration-dependent manner when applied to the cytoplasmic side of inside-out patches. Onset of activation was generally rapid, and steady-state activation was observed within 30 s after application. High concentrations of AA (30–50 μM) did not further increase channel activity above that produced by 20 μM and generally caused patches to become unstable and leaky. In Fig. 7*B*, relative channel activities were plotted as a function of [AA]. With the reasonable assumption that 20 μM AA produces maximal activation of TREK-2, averaged data from eight patches were fitted to a Hill equation of the following form: $y = 1/(1 + (K_{1/2}/[AA])^n)$, where $K_{1/2}$ is the apparent concentration of AA that produces half-maximal activation and n is the Hill coefficient (apparent $K_{1/2}$ = 7.3 μM, Hill coefficient = 2.2). We tested whether other free fatty acids are also able to activate TREK-2. When applied to the cytoplasmic side of inside-out patches, docosahexaenoic acid, eicosapentaenoic acid, linolenic acid, linoleic acid, and oleic acid all strongly and reversibly stimulated TREK-2 current at 20 μM (Fig. 7*C*). Elaidic acid, the *trans*-isomer of *cis*-oleic acid, failed to activate TREK-2, indicating that structural specificity exists for TREK-2 activation. Saturated free fatty acids such as stearic acid and palmitic acid did not increase TREK-2 channel activity even up to 100 μM. We also tested whether free fatty acids could activate TREK-2 from the extracellular side of the membrane. In outside-out patches, extracellular application of linoleic and oleic acids caused 9 ± 3- and 6 ± 2-fold increases above the basal level, respectively (n = 3 each). Elaidic acid, stearic acid, and palmitic acid failed to significantly increase TREK-2 activity from the extracellular side of the membrane (<2% change from three patches each). Examples of activation by AA, linoleic acid, and oleic acid in inside-out and outside-out patches are shown in Fig. 7*D*. These results show that TREK-2 is activated by long-chain unsaturated free fatty acids.

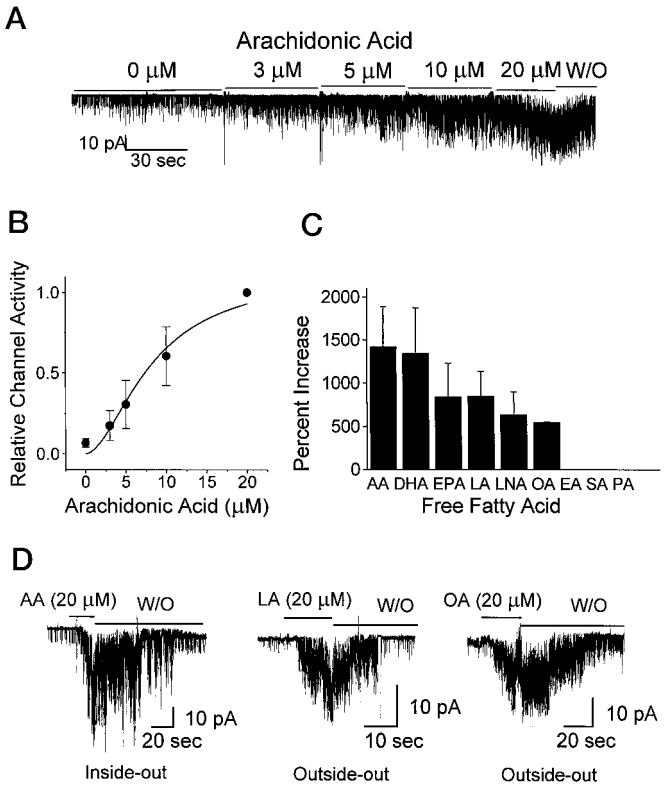

FIG. 7. **Activation of TREK-2 by free fatty acids in COS-7 cells.** *A*, in an inside-out patch, AA was applied to the cytoplasmic side of the membrane. The concentration of AA was increased in steps from 0 to 20 μM. *B*, relative channel activity is plotted as a function of [AA]. Channel activity determined at 20 μM AA was taken as 1.0. Each point is the mean ± S.D. of eight values. The points were fitted to the Hill equation to obtain the apparent $K_{1/2}$ (7.3 μM) and Hill coefficient (2.2). *C*, shown is the percent increase in TREK-2 activity by different free fatty acids applied at 20 μM (n = 3 each). AA, docosahexaenoic acid (*DHA*), eicosapentaenoic acid (*EPA*), linoleic acid (*LA*), linolenic acid (*LNA*), oleic acid (*OA*), elaidic acid (*EA*), stearic acid (*SA*), and palmitic acid (*PA*) were used. *D*, examples of reversible activation by AA, linoleic acid, and oleic acid in inside-out and outside-out patches are shown as indicated. *W/O*, washout.

DISCUSSION

In this study, we report the cloning and expression of TREK-2, a new member of the 2P/4TM K^+ channel family. TREK-2 forms a functional K^+ channel when expressed in COS-7 cells and is activated by membrane stretch and unsaturated free fatty acids, a hallmark of the TREK/TRAAK group of the 2P/4TM K^+ channel subfamily (12, 19, 20). In the rat, the distribution of TREK-2 mRNA was mainly in the cerebellum, suggesting that TREK-2 may have a unique role in this part of the brain.

Native K_{FA} Channels and TREK/TRAAK—We have previously identified and characterized a family of K^+ channels in cardiac and neuronal cells and have referred to them as K_{AA} and K_{FA} channels, as they were activated by arachidonic acid and other free fatty acids (23, 24, 27). It was also found that membrane stretch can cause activation of these K^+ channels (24, 28). K^+ channels with similar properties were also identified in smooth muscle cells and in a colonic secretory cell line (21, 29). The gating kinetics of the K_{FA} channels are unique in that they show extremely high open channel noise even in the absence of any channel blocker or divalent cations. Furthermore, none of the organic K^+ channel blockers tested (1 mM) caused a significant inhibition of K^+ channel activity. Therefore, the K_{FA} channels were thought to belong to a new family of K^+ channels with distinct function and structure. Recent cloning and expression studies show that the TREK/TRAAK

members of the 2P/4TM K^+ channel family are likely to encode the K_{FA} channels. TREK-1 and TRAAK have been shown to be activated by membrane stretch and free fatty acids and have biophysical properties similar to those of native K_{FA} channels. Thus, both K_{FA} channels and TREK/TRAAK show marked open channel noise, opening in bursts, lack of block by organic K^+ channel blockers, and large single-channel conductance (100–130 picosiemens at negative potentials).

We have previously identified in isolated neurons from rat brain three different types of K_{FA} channels by applying either suction or free fatty acids (24). The current-voltage relationships of the three K_{FA} channels were outwardly rectifying, inwardly rectifying, and linear, allowing unambiguous identification of each type of K_{FA} channel. TREK-1 shows an outwardly rectifying single-channel current-voltage relationship in high [K^+] solution, with conductances similar to those of the native K_{FA} channel (10, 30). Single channels of TREK-2, on the other hand, shows clear inwardly rectifying behavior in high [K^+] solution, with conductances similar to those of another native K_{FA} channel described earlier (24). Therefore, TREK-1 and TREK-2 probably represent two of the three K_{FA} channels that we have identified in the neurons. At present, a native K_{FA} channel whose single-channel kinetic properties are similar to those of TRAAK has not yet been described.

Activation by Pressure and Free Fatty Acids—When TREK-2 is expressed in mammalian cells such as COS-7 cells, a basal level of channel activity is normally present. Similarly, TREK-1 exhibits some basal current when expressed in COS cells (10, 19). The basal current that is present at resting membrane potential is expected to drive the membrane potential toward the K^+ equilibrium potential. TREK/TRAAK current is activated instantaneously by a voltage step, does not inactivate with time, and shows no voltage threshold for activation. These properties of TREK/TRAAK suggest that they would serve as background currents that help to set the resting membrane potential. In addition, they might serve as sensors for changes in osmotic pressure and free fatty acids and be involved in mechano- and metabolism-electrical coupling, respectively. Mechanosensitive ion channels have been identified in various mammalian cell types, but how membrane tension opens and modulates ion channels is not known (31–35).

In summary, we have cloned TREK-2, an inwardly rectifying K^+ channel that belongs to the 2P/4TM K^+ channel family. The biophysical properties of TREK-2, together with its sensitivity to membrane stretch and free fatty acids, indicate that the *TREK-2* gene probably encodes an inwardly rectifying K_{FA} channel that we have identified earlier in neurons. The physiological significance of K_{FA}/TREK/TRAAK channels is not evident at present, although they are expected to be involved in the regulation of resting membrane potential, if they are active at rest *in vivo*. One could speculate that activation of these channels in specific tissues might help to protect the cell against damage produced by hypoxia or ischemia, which causes a rise in intracellular free fatty acids and cell swelling and intracellular acidosis (36–39). Further studies are clearly needed to address this important question.

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TREK-2, a New Member of the Mechanosensitive Tandem-pore K^+ Channel Family

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