TWIK-2, a New Weak Inward Rectifying Member of the Tandem Pore Domain Potassium Channel Family

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Potassium channels are found in all mammalian cell types, and they perform many distinct functions in both excitable and non-excitatory cells. These functions are suberved by several different families of potassium channels distinguishable by primary sequence features as well as by physiological characteristics. Of these families, the tandem pore domain potassium channels are a new and distinct class, primarily distinguished by the presence of two pore-forming domains within a single polypeptide chain. We have cloned a new member of this family, TWIK-2, from a human brain cDNA library. Primary sequence analysis of TWIK-2 shows that it is most closely related to TWIK-1, especially in the pore-forming domains. Northern blot analysis reveals the expression of TWIK-2 in all human tissues assayed except skeletal muscle. Human TWIK-2 expressed heterologously in Xenopus oocytes is a non-inactivating weak inward rectifier with channel properties similar to TWIK-1. Pharmacologically, TWIK-2 channels are distinct from TWIK-1 channels in their response to quinidine, quinine, and barium. TWIK-2 is inhibited by intracellular, but not extracellular, acidification. This new clone reveals the existence of a subfamily in the tandem pore domain potassium channel family with weak inward rectification properties.

Potassium (K⁺) channels are the most diverse class of ion channels discovered. In humans over 50 distinct channels have been identified in both excitatory and non-excitatory cell types. These channels are involved in the control of a variety of cellular functions, including neuronal firing, neurotransmitter and hormone secretion, and cellular proliferation. All of these channels contain a pore-forming (P) domain with a sequence motif common to all K⁺ channels, the tripeptide sequence GYG/FG, found in this domain. The residues immediately adjacent to either side of this motif are also well conserved. It is believed that four of these P domains contribute to the formation of a functional K⁺-conductive pore (1–3).

Analysis of the K⁺ channels in the Caenorhabditis elegans genome suggests eight distinct families of K⁺ channels (4). These channels contain 2, 4, or 6 transmembrane domains and 1 or 2 P domains, with both termini oriented toward the cytoplasm. The presence of other features in the primary sequence differentiates the families into distinctive functional subtypes. For example, among the six transmembrane domain channels, members of the K₁ channel family have a charged S4 transmembrane domain characteristic of voltage-gated channels. K⁺ channels with only two transmembrane domains surrounding a single P domain are all inward rectifiers (Kᵢ). Mammalian homologues of each of these classes have been identified.

The most recently discovered class of potassium channels is the tandem pore domain potassium (K₁) channel family. Kᵢ channels have four putative transmembrane domains and two P domains. The P domains are separated by the second and third transmembrane domains. Although all these Kᵢ channels have a conserved core region between M1 and M4, the amino- and carboxyl-terminal domains are quite diverse. Kᵢ channels represent the most abundant class of K⁺ channels in C. elegans, with at least 39 distinct members (5). Four mammalian Kᵢ channels have been cloned to date. All the mammalian channels in this family exhibit nearly instantaneous, non-inactivating K⁺ currents. The first mammalian Kᵢ channel discovered was the weak inward rectifier TWIK-1 (6). Although TWIK-1 is sensitive to some classical K⁺ channel blockers (e.g. Ba²⁺), it is relatively insensitive to others (e.g. tetraethylammonium and 4-aminopyridine). TWIK-1 activity is indirectly inhibited by acidification and stimulated by the phorbol ester PMA. The second pore domain, P2, has an atypical sequence, GLG, often seen in the P2 domains of Kᵢ channels in C. elegans. Northern blot analysis shows that TWIK-1 mRNA is widely expressed.

We report here the discovery of a new Kᵢ channel cloned from human brain. We have named this channel TWIK-2 on the basis of three criteria. First, TWIK-2 shares significant sequence homology to TWIK-1, especially in the P domains. Second, the tissue distribution of TWIK-2 is similar to that of TWIK-1. Finally, the TWIK-2 channel is a weak inward rectifier whose electrophysiologic properties are similar to those of TWIK-1.

EXPERIMENTAL PROCEDURES

Molecular Cloning of TWIK-2—The basic local alignment search tool (7) was used to identify human expressed sequence tag entries similar to the mammalian Kᵢ channels TWIK-1, TREK-1, and TASK. The translation product of one of the returned ESTs, AA604914, is most closely related to human TWIK-1 (E = 3 × 10⁻⁵) and human TASK (E = 2 × 10⁻⁵) by BLAST-X analysis. No overlapping EST clones were found by BLAST-N analysis.

The sequence reported for AA604914 was used to isolate cDNA clones...
using a modified solution capture method (8). Briefly, three oligonucleotides were designed, with two unmodified oligonucleotides flanking a third, biotinylated 30-mer oligonucleotide (Keystone, Camarillo, CA). These oligonucleotides were incubated with a NaOH-denatured human brain SuperScript cDNA library (Life Technologies, Inc.). The solution was neutralized and the hybridization proceeded overnight at 40°C. The annealed plasmid-biotinylated oligonucleotide complexes were isolated with streptavidin-coated magnetic beads (Dynal, Lake Success, NY). The plasmids were eluted from the beads and were used to transform bacteria. The resultant bacterial colonies were transferred onto Hybond-N filters (Amersham Pharmacia Biotech) and probed with a radiolabeled oligonucleotide derived from AA604914. After extensive washing in 2× SSPE, 0.5% SDS, the filters were exposed to BioMax film (Amersham Pharmacia Biotech). Individual bacterial clones were picked from the original dish, and the plasmid was recovered from overnight mini-cultures. Bidirectional sequencing demonstrated an ORF that contained the nucleotide sequence found in EST AA604914. Sequence analysis and alignments were performed with the LaserGene suite (DNASTar, Madison, WI), National Center for Biotechnology Information resources2 and the Prosite internet resource.3

The TWIK-2 cDNA was subcloned into a Xenopus oocyte expression plasmid, pOIX, a generous gift from Dr. Tim Jegla (Stanford University). This vector contains the 5'- and 3'-untranslated regions of the Xenopus β-globin gene flanking the insert (9).

Northern Blot Analysis—A 510-base pair fragment was amplified from the TWIK-2 sequence by the polymerase chain reaction and was used to probe a human multiple tissue Northern blot (CLONTECH, Palo Alto, CA). The cDNA fragment was labeled with a[α-32P]dCTP (Amersham Pharmacia Biotech) with the Rediprime labeling kit (Amersham Pharmacia Biotech) and hybridized to the Northern blot membrane with the high efficiency hybridization system (Molecular Research Center, Cincinnati, OH) under conditions specified by the manufacturer. The washed blot was exposed to film (Amersham Pharmacia Biotech).

Transcript Preparation and Oocyte Electrophysiology—Transcripts were synthesized from linearized cDNA templates with T3 RNA polymerase (Promega). The washed blot was exposed to film (Amersham Pharmacia Biotech). Individual bacterial clones were sequenced using a modified solution capture method (8). Briefly, three oligonucleotides were designed, with two unmodified oligonucleotides flanking a 30-mer oligonucleotide (Keystone, Camarillo, CA). These oligonucleotides were incubated with a NaOH-denatured human brain cDNA library (Life Technologies, Inc.). The solution was neutralized and the hybridization proceeded overnight at 40°C. The annealed plasmid-biotinylated oligonucleotide complexes were isolated with streptavidin-coated magnetic beads (Dynal, Lake Success, NY). The plasmids were eluted from the beads and were used to transform bacteria. The resultant bacterial colonies were transferred onto Hybond-N filters (Amersham Pharmacia Biotech) and probed with a radiolabeled oligonucleotide derived from AA604914. After extensive washing in 2× SSPE, 0.5% SDS, the filters were exposed to BioMax film (Amersham Pharmacia Biotech). Individual bacterial clones were picked from the original dish, and the plasmid was recovered from overnight mini-cultures. Bidirectional sequencing demonstrated an ORF that contained the nucleotide sequence found in EST AA604914. Sequence analysis and alignments were performed with the LaserGene suite (DNASTar, Madison, WI), National Center for Biotechnology Information resources2 and the Prosite internet resource.3

This vector contains the 5'- and 3'-untranslated regions of the Xenopus β-globin gene flanking the insert (9).

Two distinct P and transmembrane domains, coupled with the lack of an amino-terminal signal sequence and a large carboxyl-terminal domain. The TWIK-2 channel (Figs. 1 and 2; underlined of EST, AA604914, is most similar to TWIK-1. In TWIK-1, a specific structural features most similar to TWIK-1. In TWIK-1, a specific structural feature is a transmembrane topology identical to that of other K+ channels. Of the known mammalian K+ channels, TWIK-2 has structural features most similar to TWIK-1. In TWIK-1, a specific cysteine residue found in the M1-P1 loop (Cys-69) is implicated in the formation of homodimers by extracellular disulfide bond formation (11). A similarly placed cysteine is found in TWIK-2 domains are located between pairs of transmembrane domains identified by hydropathy analysis (Kyte/Doolittle). This core region is preceded by a 3-amino acid amino-terminal domain and followed by a 57-amino acid carboxyl-terminal domain. The arrangement of P and transmembrane domains, coupled with the lack of an amino-terminal signal sequence and a large carboxyl-terminal domain between transmembrane domain M1 and the first P domain, suggests a transmembrane topology identical to that of other K+ channels.
TWIK-2 Potassium Channel

**A**

|      |       |       |
|------|-------|-------|
| TWIK-1 | TWIK-2 | TREK-1 |
| TASK  | TRAAK |       |

**B**

|      |       |       |
|------|-------|-------|
| TWIK-1 | TWIK-2 | TREK-1 |
| TASK  | TRAAK |       |

**FIG. 3.** K_+ channel pore domain alignments. P regions from the indicated K_+ channels were aligned as in Fig. 2. A, P1 regions. B, P2 regions. Highlighted residues indicate identity with the TWIK-1 sequence.

**FIG. 4.** Northern blot analysis. Human multiple tissue Northern blots were probed with radiolabeled cDNA derived from TWIK-2 as described under “Experimental Procedures.” PBL, peripheral blood leukocytes; S INTESTINE, small intestine; Sk MUSCLE, skeletal muscle. Markers represent migration of RNA standards of the indicated sizes (kb).

**FIG. 2.** Amino acid sequence of TWIK-2. The P domains (underlined), potential glycosylation sites (*), and potential phosphorylation sites (boxed) described in the text are indicated. The putative transmembrane domains are highlighted.

(Cys-53). TWIK-2 has two N-glycosylation consensus sequences (Asn-79 and Asn-85; Fig. 2), whereas TWIK-1 has a single glycosylation site in the M1-P1 loop. Two potential phosphorylation sites have been identified in TWIK-2 (Fig. 2). Like TWIK-1, TWIK-2 has a single casein kinase II recognition sequence (Ser-304) but lacks a Ca^{2+}-calmodulin kinase phosphorylation site. TWIK-2 has a potential PKC phosphorylation site (Ser-158) that is located in the putative M2-M3 cytoplasmic loop. In TWIK-1 this PKC site is also found in the M2-M3 loop at residue Thr-161. We have named the new gene TWIK-2 because of its greatest overall sequence similarity with TWIK-1 at residue Thr-161. We have named the new gene TWIK-2 because of its greatest overall sequence similarity with TWIK-1 (53.8 versus 24–33%). In addition, 19 consecutive amino acids in its P2 domain are identical with amino acids within the P2 domain in TWIK-1. The P1 and P2 domains of TWIK-2 are more closely related to their counterparts in TWIK-1 (72 and 88% amino acid identity, respectively) than any other K_+ channel (Fig. 3, A and B).

Expression Pattern of TWIK-2—To determine the expression pattern of TWIK-2 in human tissues, a human multiple tissue Northern blot was hybridized with a cDNA probe derived from the TWIK-2 nucleotide sequence. A 2.6-kb species, similar in size to the full-length TWIK-2 cDNA described above, was present in a variety of tissues, primarily in placenta, heart, colon, and spleen (Fig. 4). Lower levels of TWIK-2 mRNA could be detected in peripheral blood leukocytes, lung, liver, kidney, and thymus, with the lowest detectable levels found in brain. Two additional species, 6.8 and 1.35 kb, were also detected. The relative expression levels for these two species in these tissues closely paralleled the level of the 2.6-kb species. Significant TWIK-2 expression was also detected in pancreas (data not shown). TWIK-2 could not be detected in skeletal muscle by Northern blot analysis. Although the hierarchy of expression levels in these tissues is different for TWIK-1 and TWIK-2, TWIK-2 is expressed in the tissues in which TWIK-1 expression has been detected (6).

Electrophysiology—Electrophysiologic properties of heterologously expressed TWIK-2 channels were measured in *Xenopus* oocytes. TWIK-2 currents activated instantaneously, did not inactivate, and deactivated within the resolution of two-electrode voltage clamp (Fig. 5, A and B). Weak inward rectification of TWIK-2 currents was observed (Fig. 5C). However, unlike both human and mouse TWIK-1 (6, 12), TWIK-2 currents did not saturate at depolarized potentials in either FR (Fig. 5, A and C) or ND96 (data not shown). The slope of the plot of reversal potential versus K_+ concentration was 53 ± 3 mV per 10-fold change in K_+ concentration (Fig. 5C).

Pharmacology—Pharmacologic modulators of other cloned tandem pore domain potassium channels were examined, in addition to non-selective potassium channel inhibitors. To test the sensitivity of TWIK-2 currents to changes in extracellular pH, oocytes expressing TWIK-2 were incubated in FR whose pH was titrated with either HCl or NaOH. TWIK-2 currents were not sensitive to extracellular pH over the range of pH 5.6 to 8.4 (n – 4–6 oocytes at each extracellular pH, data not shown). However, pharmacologic treatments (1 mM 2,4-dinitrophenol or 100% carbon dioxide) known to lower intracellular pH (13) reduced TWIK-2 currents substantially (Fig. 6A). TWIK-2 currents were not inhibited by quinidine (100 μM, n = 5) or quinine (100 μM, n = 4, data not shown). TWIK-1 currents are inhibited with these treatments (6). TWIK-2 currents were also relatively insensitive to barium (Fig. 6B). Zinc (100 μM) slightly inhibited TWIK-2 currents (∼10 ± 1%, n = 3, p = 0.005 by paired t test). Currents observed in TWIK-2-
injected oocytes were slightly larger after treatment with the PKC activator PMA (50–100 nM; 24 ± 3%; n = 5, p = 0.001 by paired t test). Pharmacologic treatments known to activate protein kinase A (forskolin, 10 μM with 3-isobutyl-1-methyl-xanthine, 1 mM, n = 4; or 8-bromo-cyclic AMP, 300 μM, n = 4) had no effect on TWIK-2 currents (data not shown).

Unlike TREK-1 currents, TWIK-2 currents were not inhibited by N-methyl D-glucamine substitution for sodium in frog Ringer’s solution (n = 5, data not shown). The following pharmacologic treatments had no or only minimal effect on TWIK-2 currents (data not shown): 4-aminopyridine (1 mM, n = 3), cesium chloride (1 mM, n = 3), ethanol (17 mM, n = 3), n-octanol

FIG. 5. Biophysical properties of TWIK-2 currents in Xenopus oocytes studied with two-electrode voltage clamp. Whole cell currents of TWIK-2 cRNA-injected oocytes perfused with either frog Ringer’s solution (FR, A) or high potassium frog Ringer’s solution (115 mM K⁺; HK, B). Voltage pulses (1-s duration) are from −120 to +40 mV from a holding potential of −80 mV (inset). Data also are shown for control water-injected oocytes. C, shows average current-voltage curves of TWIK-2-injected oocytes (n = 6) at different levels of extracellular potassium and a plot of the reversal potentials obtained at these varying extracellular potassium concentrations. Error bars represent S.E.
(1 mm, n = 3), arachidonic acid (10 μM, n = 6), histamine (100 μM, n = 2), isoflurane (500 μM to 1 mm, n = 6), bupivacaine (10 μM, n = 3), tetraethylammonium (10 mm, n = 6), acetylcholine (1 mm, n = 6), secretin (1 mm, n = 4), apamin (1.0 μM, n = 4), glibenclamide (30 μM, n = 5), magnesium (5 mm, n = 5), and gadolinium (100 μM, n = 3). Changes in perfusate osmolality (200–400 mmol/kg) from addition of sucrose had minimal effect (<10%, n = 3) on TWIK-2 currents.

**DISCUSSION**

We describe the cloning and functional expression of a new member of the mammalian K_\text{ir} family of channels, TWIK-2. This new channel is the smallest of the K_\text{ir} channels yet cloned but possesses structural features that define this family of potassium channels, i.e. two P domains, each bounded by a pair of transmembrane domains. For each of the P domains, TWIK-2 is most closely related to TWIK-1. The P2 domain in TWIK-2 is strikingly homologous to the P2 domain in TWIK-1 (Fig. 3B). Only TWIK-1 and TWIK-2 have a GLG sequence motif in the second P domain; all of the other cloned mammalian K_\text{ir} channels have a GFG sequence in this position.

Besides the high primary sequence similarity of TWIK-2 to TWIK-1, we also found physiologic and pharmacologic similarities between the two channels. Both channels show weak inward rectification and have similar current-voltage relationships. Like TWIK-1 channels, TWIK-2 channels are inhibited by intracellular acidity. The only K_\text{ir} channels inhibited by intracellular acidity are TWIK-1 (6) and TOK1 (14), an outwardly rectifying K_\text{ir} channel from *Saccharomyces cerevisiae* (14, 15). In contrast to TOK1 and TWIK-1, TWIK-2 is not sensitive to barium. Although our data suggest a role for modulation of TWIK-2 by PKC, the effect we observed in oocytes was small. Treatment of oocytes expressing TWIK-1 with the phorbol ester PMA has been shown to activate TWIK-1 channel activity, presumably by activating PKC (6). The single PKC site in TWIK-1 is located within the M2-M3 loop, but mutation of this site (Thr-161) to an alanine residue did not alter the PMA sensitivity of TWIK-1. The PKC site in TWIK-2 is in the same loop. Therefore, like TWIK-1, the sensitivity of TWIK-2 to PMA may reflect an indirect effect of PKC on the channel.

TWIK-2 is distinct from the other members of the K_\text{ir} family. The second cloned mammalian K_\text{ir} channel, TREK-1, is an outward rectifier K_\text{ir} channel (16). Expressed in many brain regions and the lung, as well as in kidney, heart, and skeletal muscle, this channel, unlike TWIK-2, is insensitive to acidification. TASK, the first mammalian K_\text{ir} channel found to satisfy all the characteristics of a background channel, is a voltage-insensitive open rectifier whose activity is inhibited by extracellular acidification (10, 17). Most recently, a fourth mammalian K_\text{ir} channel, TRAAK, was reported (18). This open rectifier also has characteristics of a background K^+ conductance and is expressed only in the central nervous system, including the retina and spinal cord.

Like TWIK-1, TWIK-2 has a widespread distribution. Of the tissues examined, TWIK-2 expression was absent from skeletal muscle only. TWIK-2 is abundantly expressed in pancreatic tissue, and heterologous TWIK-2 currents resemble physiologic base-line potassium channels that maintain the resting membrane potential of acinar cells (19–21). Like the physiologic base-line potassium channels expressed in pancreatic acinar cells, TWIK-2 is inhibited by intracellular acidity but is not sensitive to a number of potassium channel inhibitors, including tetraethylammonium, Ba^{2+}, and 4-aminopyridine (20). In addition, barium-insensitive potassium currents have been described in the kidney (22–24). Weak inward rectifiers have also been reported in hepatocytes (25) as well as in eosinophils (26).

TWIK-2 is a weak inward rectifier whose zero current potential follows the reversal potential for K^+. For some potassium channels, the primary sequence provides clues to potential mechanisms of inward rectification. Intracellular polyamines and magnesium control the degree of inward rectification of K_\text{ir}.
channels by interacting with acidic residues within the second transmembrane domain (27, 28). Although the degree of inward rectification of TWIK-1 is known to depend on intracellular magnesium levels, in general the mechanisms of rectification within the tandem pore domain family remain unknown.

One area of future study is the formation of K+ channel dimers. TWIK-1 forms homodimers via a disulfide bridge between subunits involving identical extracellular cysteine residues (Cys-69) found in the M1-P1 linker region (11). TWIK-2 has a similarly placed cysteine residue, Cys-53. A TWIK-2 mutant in which Cys-53 has been mutated into an alanine residue did not show channel activity after heterologous expression in oocytes (data not shown). TWIK-2 may therefore form functional homodimeric channels via a disulfide bridge. Northern blot analysis of TWIK-2 expression demonstrates significant overlap with the pattern of TWIK-1 expression. It is therefore possible that TWIK-1/TWIK-2 heterodimers may form in those tissues, suggesting a mechanism for further functional diversity within the tandem pore domain K+ channel family.

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