The novel structural class of mammalian channels with four transmembrane segments and two pore regions comprise background K⁺ channels (TWIK-1, TREK-1, TRAAK, TASK, and TASK-2) with unique physiological functions (1–6). Unlike its counterparts, TRAAK is only expressed in neuronal tissues, including brain, spinal cord, and retina (1). This report shows that TRAAK, which was known to be activated by arachidonic acid (3), is also opened by membrane stretch. Mechanical activation of TRAAK is induced by a convex curvature of the plasma membrane and can be mimicked by the amphipathic membrane crenator trinitrophenol. Cytoskeletal elements are negative tonic regulators of TRAAK. Membrane depolarization and membrane crenation synergize with stretch-induced channel opening. Finally, TRAAK is reversibly blocked by micromolar concentrations of gadolinium, a well known blocker of stretch-activated channels. Mechanical activation of TRAAK in the central nervous system may play an important role during growth cone motility and neurite elongation.

A key feature of all classes of K⁺ channel-pore-forming subunits is a conserved signature sequence constituting the pore segment (P) (7). Mammalian K⁺ channels can be divided into three major structural classes encoding channels with six transmembrane segments (TMS), 4TMS and 2TMS (7). The 6TMS class comprises voltage-gated as well as Ca²⁺-gated K⁺ channels. The inward rectifier IRK, the G protein-coupled GIRK and the ATP-gated K⁺ channels are members of the 6TMS structural class. The most recent structural class of 4TMS mammalian K⁺ channel subunits consists so far of five members (TWIK-1, TREK-1, TRAAK, TASK, and TASK-2) (1–4, 6). Besides the presence of 4TMS, the other major structural characteristic is the presence of two P regions as well as an extended M1P1 extracellular loop (8). Although these subunits display the same structural motif (4TMS/2P), they only share 25–40% sequence identity. These unusual K⁺ channel structures are associated with unique physiological properties. TWIK-1 is an ubiquitous subunit, which directs the expression of a time- and voltage-independent K⁺-selective weak inward rectifier in Xenopus oocytes (4, 8). TREK-1 is highly expressed in brain, heart, lung, kidney, and several other tissues and encodes a mammalian serotonin-sensitive-like K⁺ channel (3, 5). TASK and TASK-2 encode background K⁺ channels that are blocked by external acidification near the physiological pH (2, 6). TASK is highly expressed in heart and brain, while TASK-2 is mainly expressed in kidney (2, 6).

TRAAK is the only 4TMS subunit to be specifically expressed in the nervous system (1). TRAAK is opened by polyunsaturated fatty acids, including arachidonic acid (AA) (1). The present report demonstrates that TRAAK is also opened by membrane stretch and thus belongs to the class of mechano-gated ion channels (9–16).

MATERIALS AND METHODS

Cell Culture—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The cDNA for TRAAK was subcloned into the pIRES-Neo expression vector (CLONTECH), and the resulting construct was transfected in COS cells using the phosphate calcium method. After 48 h in normal medium, the transfected cells were dissociated and subcultured by 1:20 dilution in medium containing 500 µg/ml Geneticin (Life Technologies, Inc.). Antibiotic-resistant clones were selected at random after 2 weeks. A TRAAK-expressing clone was then identified by using an ⁸⁶Rb⁺ efflux assay as well as electrophysiology and expanded to maintain a stock culture.

Western Blots and ImmunocytoLOGY—Anti-TRAAK antibodies were raised against a glutathione S-transferase fusion protein containing the carboxyl-terminal 102 amino acids of TRAAK (residues Pro306 to Val407). The antibodies were purified by using a glutathione S-transferase fusion protein containing the same domain of TRAAK. The preparation of fusion proteins, rabbit immunization, and antibody purification were performed as described previously (8).

Proteins from COS cells and from synaptic plasma membranes of mouse spinal cord were prepared and analyzed in the presence of reducing agents (8). For immunocytochemistry, TRAAK immunodetection was performed as described previously (8), except that cells were permeabilized by adding 0.1% Triton X-100 in the blocking solution (phosphate-buffered saline supplemented with 2% BSA and 5% goat serum). Efflux of ⁸⁶Rb⁺—COS-7-TRAAK cells were plated at a density of 40,000 cells/well (Falcon, 24 wells) and used 2–3 days later. Cells were preloaded for 3 h with 0.5–1 µC/ml ⁸⁶Rb⁺ in 0.5 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 500 µg/ml Geneticin at 37 °C. Release experiments were then carried out in a standard (EXT) solution containing in (mM): 150 NaCl, 5 KCl, 1 CaCl₂, 10 Hepes, pH 7.4, with NaOH during consecutive intervals of 5 min at room temperature. For each point six independent experiments were performed at the same time. An hour washing with EXT solution was then performed before collecting 1-ml fractions. ⁸⁶Rb⁺ at 2–7 mCi/ml was from NEN Life Science Products. Fractional rates of release were calculated as ⁸⁶Rb⁺ released during each 5-min interval and expressed as the percentage of ⁸⁶Rb⁺ content in the cells at the beginning of the respective intervals. ⁸⁶Rb⁺ was counted on a Packard Tri-Carb with a Cerenkov program.

Patch Clamp Experimental Protocols, Recordings and Data Analysis—The electrophysiology procedure has been described elsewhere previously (5). For whole-cell experiments, bath solution (EXT) contained (in mM) 150 NaCl, 5 KCl, 3 MgCl₂, 1 CaCl₂, 10 Hepes, pH 7.4, with NaOH and pipette solution (INT) contained (in mM) 150 KCl, 3 MgCl₂, 5 EGTA, and 10 Hepes, pH 7.2, with KOH. For cell attached experiments, the EXT solution contained 150 KCl instead of 150 NaCl, and the pipette contained the EXT solution (150 mM NaCl). For inside-out experiments the pipette solution was EXT and the bath solution was

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‡ The abbreviations used are: P, pore segment; TMS, transmembrane segment(s); AA, arachidonic acid; BSA, bovine serum albumin; TNP, trinitrophenol, WT, wild type; Npo, number of channels × open channel probability.

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TRAAK Is a Mammalian Neuronal Mechano-gated K⁺ Channel*

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INT. Mechanical stimulation was applied through an open-loop pressure generating system and monitored at the level of the patch pipette throughout the experiment by a calibrated pressure sensor. This system provides a stable pressure pulse (5). Colchicine was dissolved daily in the saline solution at the concentration of 500 μM. Cytochalasin D was dissolved at the concentration of 1 mg/ml in Me2SO and kept at 20 °C. AA was dissolved in ethanol at the concentration of 100 mM, flushed with argon, and kept at 20 °C for a week. TNP was mixed with the saline solutions and pH adjusted. 4-Bromophenacyl bromide was dissolved daily at 100 mM in Me2SO and AACOCF3 at 100 mM in ethanol. All chemicals were obtained from Sigma.

RESULTS

TRAAK was transfected in COS cells, and a stable TRAAK cell line was characterized by immunoblot using affinity-purified antibodies directed against the carboxyl terminus of TRAAK (Fig. 1, A–C). A major band is detected with a relative molecular mass (Mr) of 54,000–64,000 and two minor bands of Mr 49,000 and 52,000 (Fig. 1A, lane 2). No signal is detected in nontransfected COS cells (Fig. 1A, lane 1). The difference between the observed Mr values and the calculated molecular mass of TRAAK (43,000 Da) is probably due to glycosylation. This hypothesis is supported by the presence of two consensus sites for N-linked glycosylation (residues 81 and 84) in a region of the channel that is expected to be extracellular (1). This probable glycosylation of TRAAK is also observed in the spinal cord (Fig. 1A, lane 3), a tissue that is known to express a high level of TRAAK transcript (1). Additional bands of Mr 49,000 and 52,000, which are detected in transfected COS cells, could correspond to incompletely glycosylated or partially degraded forms of the protein. The heterologous expression of TRAAK was confirmed by immunocytology (Fig. 1, B and C). A strong signal is observed at the plasma membrane of the cells expressing TRAAK (Fig. 1C), while it is absent in control WT COS cells (Fig. 1B).

The expression of exogenous K⁺ channel activity was initially detected in the TRAAK cell line using 86Rb⁺ efflux (Fig. 1, D–F). Control COS cells (WT) do not show any significant increase in 86Rb⁺ efflux in the presence of AA (Fig. 1D). However, cells expressing TRAAK display a reversible 3–4-fold increase in 86Rb⁺ efflux induced by AA (Fig. 1D and E). AA activation is reversed by the addition of micromolar concentrations of Gd³⁺ (IC₅₀: 5 ± 1 μM) (Fig. 1E).

The TRAAK cell line was then used for electrophysiological investigations. In the whole cell configuration, basal channel activity is low (TRAAK: 12.2 ± 1.3 pA/pF, n = 12; COS WT: 4.2 ± 0.5 pA/pF, n = 18; at 100 mV) (Fig. 2A). An outward current is slowly and reversibly induced at a holding potential of -100 mV (Fig. 2A). This current is blocked by Gd³⁺ (IC₅₀: 1.5 μM) (Fig. 2B). These results indicate that TRAAK is a mammalian neuronal mechano-gated K⁺ channel.
potential of 0 mV by AA superfusion (4.8 ± 0.4-fold, n = 12) (Fig. 2A, bottom inset). This current is absent in mock-transfected COS cells (n = 18) (5). The I-V curve of the current induced by AA displays a strong outward-going rectification (Fig. 2A). The reversal potential of the current activated by AA reverses at −83.7 ± 0.7 mV (n = 12), which is the predicted value for \( E_{K1} \). Opening of TRAAK is also reversibly induced by the amphipathic membrane crenator TNP (6.7 ± 1.1-fold, n = 6 with 400 μM TNP) (15, 17). The kinetics for current activation is faster in the presence of TNP compared with AA (Fig. 2A, insets). I-V curves in the presence of TNP and AA are similar. Finally, as observed with \(^{86}\)Rb\(^+\) efflux experiments, TRAAK activation by AA is also blocked by \( 10 \) μM Gd\(^{3+}\) (−64.2 ± 4.7%, n = 8 with 10 μM Gd\(^{3+}\)) (Fig. 2B).

In the cell-attached patch configuration, no channel activity is detected under resting conditions (NPo: 0.05 ± 0.03, n = 6). However, we observed that TRAAK opens when a negative pressure is applied to the patch pipette (Fig. 3A). No channel activity is observed in mock-transfected COS cells (5). In the experiment illustrated in Fig. 3, A and B, channel activity is absent at atmospheric pressure but three TRAAK 38-picosiemens channels readily and reversibly open during the application of a −66 mm Hg pressure. As reported previously for AA activation, TRAAK opening was characterized by flickering kinetics (1).

Both the number of active channels and the sensitivity to mechanical stretch are strongly enhanced after a treatment for 20 min with colchicine (500 μM) (Fig. 3, C–E). The threshold for mechanical activation is lowered from −70 to −20 mm Hg, and maximal channel activity is enhanced at all indicated pressures in the presence of colchicine (Fig. 3, C and D). Similarly, addition of cytochalasin D (5 μg/ml) enhances channel activity with, however, a weaker effect (Fig. 3E). Finally, excision of the patch in the inside-out configuration, which is known to disrupt cytoskeletal elements, produces an almost 10-fold increase in channel activation induced by a −70 mm Hg pressure (Fig. 3E). Moreover, the threshold for mechanical activation is also significantly lowered after excision of the patch (Fig. 4A). In the inside-out patch configuration, channel activity is basically absent at atmospheric pressure, and only negative, but not positive, pressure induces channel opening (Fig. 4B). The I-V curve performed with a voltage ramp protocol in physiological K\(^+\) conditions is outwardly rectifying and reverses at −80 mV, the predicted value for the equilibrium potential for K\(^+\) (Fig. 4B).

The absence of channel activation by positive pressure in the inside-out patch configuration suggests that channel opening is mediated by a specific membrane deformation. Indeed, in the outside-out patch configuration, TRAAK opening is only induced by positive pressure (Fig. 5A). The activation of TRAAK is purely pressure-dependent, and moreover, as observed in both the cell-attached and inside-out configurations, channel activity is absent at atmospheric pressure. Opening of TRAAK by positive pressure in outside-out patches is reversibly blocked by the addition of 30 μM Gd\(^{3+}\) (n = 3) (Fig. 5B). We then investigated the effect of voltage on TRAAK activation...
tion in the inside-out patch configuration. Fig. 6 shows that opening of TRAAK by membrane stretch is enhanced by membrane depolarization. The threshold for mechanical activation by negative pressure is lowered at depolarized voltage, and moreover maximal NPo is significantly enhanced (Fig. 6 C). The basal NPo measured at atmospheric pressure is also significantly increased at depolarized potential (Fig. 6, A–C). The steep modulation of TRAAK activation occurs at voltages between −25 and 50 mV.

We investigated the possible interaction between the membrane crenator TNP and membrane stretch. Fig. 7, A and B, show that in the inside-out configuration, internal application
of TNP has no effect at atmospheric pressure. However, TNP produces a significant reversible stimulation of the opening of TRAAK by negative pressure (Fig. 7, A and B). Interestingly, in the outside-out configuration, external TNP produces a significant activation of TRAAK at both atmospheric and positive pressures (Fig. 7, C and D).

Finally, to assay for the possible involvement of AA in the mechano-activation of TRAAK, we investigated the effect of 30 μM lipid-free BSA, which is known to bind fatty acids and remove them from membranes (18). Application of BSA on both sides of the membrane did not alter the activation of TRAAK by a −50 mm Hg pressure in the inside-out patch configuration (n = 6; data not shown). Moreover, the addition of the phospholipase A2 blockers 4-bromophenacyl bromide (30 μM) and arachidonyltrifluoromethyl ketone (AACOCF3) (30 μM) to the cytosolic face of inside-out patches for 5 min did not alter activation of TRAAK by a −50 mm Hg pressure (n = 5; data not shown).

DISCUSSION

TRAAK is a member of the novel K+ channel family with two P domains and four transmembrane segments (1). Despite a similar membrane topology, the 2P/4TMS channels display little sequence identity (about 25%). When compared with TWIK-1 and TREK-1, TRAAK has a shorter amino-terminal region but an extended C terminus (3, 4). TRAAK has a large extracellular loop between M1 and P1, with a cysteine residue analogous to the cysteine residue Cys69 involved in the disulfide-bridged homodimerization of TWIK-1 (8). TRAAK is only expressed in brain, retina, and spinal cord (1). The most intense levels of expression are present in the olfactory system, cerebral cortex, hippocampal formation, habenula, basal ganglia, and cerebellum. This laboratory demonstrated that TRAAK is opened by AA (1). The activation of TRAAK by AA is reversible, concentration-dependent, and direct (i.e., not via protein kinase C). Other polyunsaturated fatty acids also open TRAAK, while saturated fatty acids are without effect (1). TRAAK current is instantaneous and outwardly rectifying in a physiological K+ gradient. Finally, TRAAK is only inhibited by high concentrations of Ba2+ and is insensitive to the other classical K+ channel blockers, including tetraethylammonium, 4-aminopyridine, and Cs+ (1).

The anionic amphipath TNP has been shown previously to expand the exterior half of the lipid bilayer and thereby induce human erythrocytes to crenate (17). Moreover, TNP was shown to activate bacterial mechano-gated cationic channels, and this effect was attributed to membrane crenation (15). In the present study, we demonstrate that TNP is a strong activator of TRAAK. TRAAK opening by TNP is observed in the whole cell
as well as in the excised outside-out patch configurations, suggesting a rather direct mechanism (i.e. without second messenger). Interestingly, no significant effect of TNP (at the concentration used) is observed under resting conditions in the inside-out patch configuration. This observation suggests that TNP may act preferentially via the external side of the membrane. It has been hypothesized previously that because of the negative charges (mainly phosphatidylserine) of the inner leaflet, anionic amphipaths may preferentially insert in the external leaflet of the plasma membrane (15, 17). The differential insertion of these amphipaths in the external lipid monolayer induces a curvature of the membrane which generates transversal forces that may alter channel activity. This model implies that TRAAK may be sensitive to mechanical forces transmitted via the membrane.

In every configuration tested, basal channel activity measured at atmospheric pressure was basically absent. In the inside-out patch configuration, TRAAK is opened by negative pressure, while it is opened by positive pressure in the outside-out patch configuration. These results suggest that TNP may act preferentially via the external side of the membrane. It has been hypothesized previously that because of the negative charges (mainly phosphatidylserine) of the inner leaflet, anionic amphipaths may preferentially insert in the external leaflet of the plasma membrane (15, 17). The differential insertion of these amphipaths in the external lipid monolayer induces a curvature of the membrane which generates transversal forces that may alter channel activity. This model implies that TRAAK may be sensitive to mechanical forces transmitted via the membrane.

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Opening of TRAAK is clearly facilitated at depolarized potentials (between -25 and 50 mV). Depolarization lowers the threshold for mechanical activation and increases the maximal channel activity (NPo). The 2P/4TMS channels lack the positively charged S4 voltage sensor found in Shaker-type K⁺ channels (7). Thus the region responsible for the voltage-dependent modulation of TRAAK remains to be determined. An important physiological implication of these findings is that activation of TRAAK (induced mechanically or chemically) will be mostly efficient at positive potentials (i.e. during an action potential). The inside- and outside-out experiments indicate that TNP synergete with pressure activation. This result suggests that physiologically, mechanical activation of TRAAK may be most
effective when endogenous amphipathic crenators are produced.

In conclusion, this report demonstrates that the mammalian neuronal 2P/4TMS K\(^+\) channel TRAAK belongs to the mechano-gated ion channel family (9–16, 18, 20–23). Stretch-activated K\(^+\) channels have been described in several neuronal cell types, including snail and Aplysia neurons as well as in rat hippocampal neurons (20–24). The presence of stretch-activated K\(^+\) channels in the growth cones of Lymnaea stagnalis neurons suggested that these channels may be involved in the modulation of axonal pathfinding and guidance (22). Indeed, growth cone motility is a mechanical process where neurite membranes experience important tension changes. Activation of TRAAK during cell locomotion may participate via membrane hyperpolarization to the fine regulation of intracellular calcium, actin-myosin contractions and thus neurite elongation. Finally, the expression of TRAAK in dorsal root ganglia\(^2\) may suggest its possible role in more specialized sensory functions such as touch detection and/or pain sensation. We previously identified a charged region in the COOH terminus of TREK-1 (Arg\(^{297}\)-Glu\(^{306}\): RVISKTKKE), which is critically involved in channel activation by AA as well as mechanical stimulation. This region is conserved in TRAAK (Arg\(^{259}\)-Glu\(^{268}\): RAVSRTRAE) and may also fulfill an important function for TRAAK activation.

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