2-Aminoethoxydiphenyl borate activates the mechanically gated human KCNK channels KCNK 2 (TREK-1), KCNK 4 (TRAAK), and KCNK 10 (TREK-2)

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INTRODUCTION

KCNK channels (also known as K2P) for two-pore domain potassium channels) are potassium selective channels that tend to be constitutively open. Most of them behave as outward rectifiers under physiological K+ concentrations. Additionally, they also behave in an almost voltage-independent manner (Enyedi and Czirják, 2010). Because of these characteristics, they are considered the main channels responsible for the leak potassium current that helps maintain the resting membrane potential. This leak current exerts control over neuronal excitability by shaping the duration, frequency, and amplitude of action potentials (Goldstein et al., 2001). An increased K+ leak current stabilizes the cell at hyperpolarized voltages below the firing threshold, whereas leak suppression permits or facilitates depolarization. In this manner, KCNK channels play a role in such diverse processes as metabolic regulation, apoptosis, thermoperception, and chemoperception (Lotshaw, 2007). The mechanically gated TREK channels (for TWIK related K+ channels) family of KCNK channels is composed of three members: TREK-1 (KCNK2), TRAAK (KCNK4), and TREK-2 (KCNK10) with several functional splice variants reported for hTREK-1 and hTREK-2 (Gu et al., 2002; Xiao et al., 2006). Although members of the TREK subfamily differ slightly in their electrophysiological properties, they show similar characteristics, such as mechanos- and thermosensitivity. They also present overlapping pharmacological patterns, characterized by a mild sensitivity to standard potassium channel blockers such as quinidine and Ba2+ and sensitivity to volatile anesthetics like halothane (Lotshaw, 2007). TREK channels are widely expressed in both the peripheral and the central nervous system (CNS; Medhurst et al., 2001). Recent studies with TREK-1−/− mice have demonstrated that TREK channels are necessary for appropriate mechano- and thermoperception (Alloui et al., 2006; Noel et al., 2009).

2-Aminoethoxydiphenyl borate (2-APB) was originally described as a membrane-permeable modulator of Insc(1,4,5,8)-induced Ca2+ release (Maruyama et al., 1997). Although this function has been questioned, 2-APB is agreed to inhibit the store-operated current observed after depletion of ER Ca2+ stores (Dobrydneva and Blackmore, 2001); it also blocks certain transient receptor potential (TRP) channels belonging to the canonical (TRPC), and melastatin (TRPM) subfamilies (Hu et al., 2004) and certain voltage-gated potassium channels present in Limulus ventral photoreceptors (Wang et al., 2002). It is also a common activator for the TRPV1 (transient receptor potential vanilloid 1), TRPV2, and TRPV3 channels (Hu et al., 2004). Interestingly, the perception of noxious heat, in which TRPV1 and TRPV2 play a role, has been linked to the mechanosensory response of the afferent C-fibers (Koizumi et al., 2007). Additionally, 2-APB has been used to study native receptors in cell systems that endogenously express members of the TREK subfamily (e.g., rat dorsal root ganglia); our results thus warn against the use of 2-APB at high concentrations in these systems.

Keywords: 2-APB, KCNK channels, TREK, Xenopus oocyte, two-electrode voltage clamp
a key role, has been shown to be modulated or fine-tuned by KCNK2 and KCNK4 (Niel et al., 2009). Both the TREK and TRPV subfamilies have been shown to be coexpressed at the neuronal level in the rat trigeminal ganglia (Yamamoto et al., 2009). With this background in mind, we addressed the question of whether 2-APB has an effect on human KCNK channels of the TREK subfamily. We found that 2-APB activates all members of this subfamily in a dose-dependent manner and has the greatest effect on hKCNK10.

**EXPERIMENTAL PROCEDURES**

**MOLECULAR BIOLOGY**

hKCNK4 cloned into pBluescript SK−, hKCNK10 cloned into pCRA-TOP and hKCNK2 cloned into pDNA-dual were purchased from Imagenes (Berlin, Germany). hKCNK2 and hKCNK10 cDNA inserts were afterward subcloned into pSGEM for electrophysiological recordings in Xenopus oocytes. The plasmids were linearized with NheI for pBluescript SK− and PciI for pSGEM, providing linear cDNA templates for in vitro transcription. All three hKCNK inserts were subsequently subcloned into pCDNA3 for electrophysiological recordings in human embryonic kidney 293 (HEK293) cells.

hKCNK3 cloned into the expression vector pRES-CD8 was a gift from Dr. Fabrice Duprat (Institut National de la Santé et de la Recherche Médicale, Antipolis, France). hKCNK18 cloned into the plasmid pEKO was a gift from Professor Dr. P. Enyedi (Semmelweis University, Budapest, Hungary). hKCNK9 cloned into the expression vector pCRA-TOP was purchased from Imagenes (Berlin, Germany). The plasmids were linearized with the restriction enzymes BamHI, XhoI, and SphiI, respectively, and then used as templates for in vitro transcription.

Capped RNAs were synthesized in the presence of capping analog m7G(5′)ppp(5′)G using the AmpliCap-T7 Message Maker kit (Episcience, Madison, WI, USA). Complementary RNA (cRNA) was dissolved in nuclease-free water to give a final concentration of 500 ng/μL.

*Xenopus laevis OOCYTE PREPARATION*

Complementary RNAs were expressed in X. laevis oocytes essentially as described by Vogt-Külzer et al. (2007). Briefly, mature female X. laevis frogs were anesthetized with 0.15% MS-222, surgery was performed according to standard methods, and the extracted ovarian lobes were placed into Ca2+-free Barth’s solution containing collagenase type II (Worthington Biochemical Corporation) and incubated for 90 min on a shaker at 40 rpm (room temperature). After that time, healthy stage V and VI oocytes were selected for injection with cRNA coding for the proteins of interest, 30 nl of cRNA (100 ng/μL) for each channel was injected using a Microinjector Micro 4 TM (World Precision Instruments). After injection, the oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 2.5 mM sodium pyruvate, 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM, pH 7.2), and measurements were performed 24–72 h after injection.

The oocytes were placed in a 100 μL chamber perfused with frog Ringer’s solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES pH 7.2); this solution was also used to dissolve the applied chemicals. For recording, a Turbo Toc-03x (npi electronic GmbH) amplifier was used. Borosilicate glass capillaries were pulled with a Kopf vertical pipette puller. For the voltage clamp, the pulled glass capillaries were filled with 3 M KCl.

**TWO-ELECTRODE VOLTAGE CLAMP MEASUREMENTS**

A ramp series protocol was used to evaluate the activity of the channels. The voltage ramps consisted of a starting constant voltage of −100 mV for 300 ms, followed by a ramp to +50 mV in 700 ms, then a constant voltage at ±50 mV for 300 ms, and a final constant voltage at −40 mV for 300 ms. The time interval between the ramps was of 2 s. In order to evaluate the effect of a substance on the basal current, we took the average of the currents registered during the last 30 ms of the +50 mV constant from the three ramps that showed the maximal response to the applied substance and divided it by the average obtained from three ramps prior to the application.

The data were collected using the Cellworks Reader 3.7 software (npi instruments, Germany) and analyzed with Clampfit v10.2.0.14 (MDS Analytical Technologies). Curve fitting was performed using the Hill equation (Sigmaplot 8, Synstat software inc.). Data are expressed as means ±SEM (standard error of the mean). A paired t-test was used to evaluate the statistical significance of the results, and P < 0.05 was considered statistically significant and is marked with one star, P < 0.01 is marked with two stars, and P < 0.001 is marked with three stars.

**CELL CULTURE**

Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in a humidity-controlled incubator with 5% CO2.

**TRANSIENT EXPRESSION OF HUMAN KCNK2, KCNK4, AND KCNK10 IN HEK293 CELLS**

For transient expression of human KCNK2, KCNK4, and KCNK10 in HEK293 cells, we used the recombinant expression plasmid pCDNA3. HEK293 cells were transiently transfected (8 μg of hKCNK4 cDNA in pCDNA3 per dish) in 35-mm dishes (Falcon, BD Bioscience, Heidelberg, Germany) using the CaP precipitation method described previously by Dörner et al. (2007). These cells were cotransfected with pRES-EGFP (1 μg per dish), which served as a transfection marker. All recordings were performed 18–24 h after transfection.

**ELECTROPHYSIOLOGY IN HEK293 CELLS**

Recordings were performed using the whole-cell and inside-out modes of the patch-clamp technique. Cells were maintained in an extracellular recording solution containing (in mM) 140 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose, pH 7.4. Patch electrodes were pulled from borosilicate glass (1.2 mm OD × 1.17 mm ID; Harvard apparatus, Edenbridge, Kent, UK) and fire polished to 4–6 MQ tip resistance using a horizontal pipette puller (Zeitz Instruments, Munich, Germany) in order to obtain a patch of approximately 1–2 μm diameter.
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FIGURE 1

(A) Effect of 1 mM 2-APB on (from left to right) uninjected Xenopus oocytes and oocytes injected with cRNA coding for hKCNK2, hKCNK3, hKCNK4, hKCNK9, hKCNK10, and hKCNK18. For each group, the current registered at the final 50 ms of the +50 mV constant (see experimental procedures for a description of the ramp protocol used) was normalized to the current registered prior to the application of 2-APB (dotted line). All data are expressed as means ±SEM. Number of cells in each experiment is indicated above the bars.

(B) Absolute currents registered in uninjected Xenopus oocytes and oocytes injected with cRNA coding for hKCNK2, hKCNK4, and hKCNK10, before and after exposure to 1 mM 2-APB. (Continued)

Figure 1 | Continued

application of 1 mM 2-APB. (C) Representative I-V trace of a Xenopus oocyte expressing hKCNK10 before (black line) and after the application of 2-APB at 600 μM (green line), and 6 mM (blue line). (D) Ramp series measurement of a Xenopus oocyte expressing hKCNK10 that was repeatedly exposed to 1 mM 2-APB. The dotted green line represents zero current. (E) 1 mM 2-APB reversibly hyperpolarizes a Xenopus oocyte expressing hKCNK10. (F) Dose–response curve fitted to the 4-parameter Hill equation (n indicated above the points). (G) Effect of 2-APB at 100 μM (left) and 300 μM (right) on Xenopus oocytes injected with cRNA coding for hKCNK2, hKCNK4, and hKCNK10. Data were normalized to the current registered prior to the application of 2-APB (dotted line). All data are expressed as means ±SEM. Number of cells in each experiment is indicated above the bars. (H) Inside-out patch-clamp recordings of cell-excised patches from untransfected HEK293 cells (left) and HEK293 cells transfected with hKCNK10 (right) before and after exposure to 3 mM 2-APB.

For recordings, the pipette solution contained (in mM) 140 KCl, 1 MgCl2, and 10 HEPES, pH 7.4. All recordings were performed at room temperature with an EPC7 amplifier (List-Medical Electronic, Darmstadt, Germany). For whole-cell patch-clamp recordings, a step protocol consisting of a holding potential of −60 mV, followed by 20 mV steps of 400 ms duration from −110 mV to +50 mV was used. Because the current registered at the last two steps continued to increase and never presented a stable baseline, the last 30 ms of the +10 mV step were selected in order to build a dose–response curve. Cell-excised patches were evaluated in the inside-out configuration, at a holding potential of 0 mV. Data were acquired using Pulse software (HEKA, Lambrecht, Germany). All data are expressed as means ±SEM.

CHEMICALS

2-Aminoethoxydiphenyl borate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Munich, Germany). Stock solutions of 100 mM and 300 mM 2-APB diluted in DMSO were made and stored at −20°C; the latter was used for preparing solutions containing 3 and 10 mM 2-APB.

RESULTS

2-APB ACTIVATES ALL MEMBERS OF THE TREK SUBFAMILY

First, we studied the effect of 1 mM 2-APB on X. laevis oocytes injected with cRNAs coding for each member of the TREK subfamily (KCNK2, KCNK4, and KCNK10), two members of the TASK subfamily (KCNK3 and KCNK9), the only member of the TRESK subfamily (KCNK18) and a control group of non-injected oocytes (see Figure 1A). Oocytes injected with KCNK18 were not affected by 2-APB, whereas those injected with either member of the TASK subfamily were mildly inhibited by 2-APB. The currents presented by uninjected oocytes were negligible in comparison with the currents of oocytes injected with any of the aforementioned hKCNK channels; however, these currents were mildly but significantly inhibited by 1 mM 2-APB, reducing their basal activity to 70.3% (Figure 1B). 2-APB, however, increased the basal activity of hKCNK2, hKCNK4, and hKCNK10 by factors of 2.22 ± 0.2, 2.68 ± 0.2, and 4.35 ± 0.8, respectively.
2-APB ACTIVATES hKCNK10 CHANNELS IN A DOSE-DEPENDENT MANNER

We then proceeded to study in detail the effect of 2-APB on hKCNK10. hKCNK10, also known as TREK-2, is expressed throughout the human CNS. It is one of the major background K⁺ channels in dorsal root ganglion neurons (Kang and Kim, 2006), and it is also expressed in the kidneys, spleen, and small intestine (Medhurst et al., 2001). 2-APB produced a dose-dependent (Figure 1C) and reversible activation of hKCNK10 that was unaltered after repeated applications (Figure 1D). Consequently with this, when we recorded the membrane potential of oocytes expressing hKCNK10, we observed that 1 mM 2-APB produced a strong and quick hyperpolarizing effect that reversed almost completely after washout (Figure 1E). The dose–response curve gave an EC₅₀ of 1.22 ± 0.39 mM; the maximal effect (seen at around 3 mM) was a 14-fold increase in the basal current (Figure 1F). Lower concentrations, however, also led to significant changes in the basal activity of hKCNK10, as this was increased by factors of 1.33 ± 0.2 and 2.25 ± 0.4 by the application of 100 µM and 300 µM, respectively (Figure 1G). Because the highest concentration of 2-APB that was evaluated required a concentration of the vehicle compound DMSO higher than 1% (3.3% DMSO for 10 mM 2-APB), we evaluated the effect of this vehicle at its highest concentration used and compared this effect with both the basal current prior to application and basal current after the application of the same solution containing 2-APB (Figure 3B). We found no significant difference in the basal current prior and after the application of 3.3% DMSO for 10 s; this was true for both injected and uninjected oocytes (Figure 3A).

To determine whether this effect depended on the presence of any cytosolic factor, we proceeded to transfect HEK293 cells with hKCNK10 and measured cell-excised patches in the inside-out configuration, exposing them to 3 mM 2-APB (1% DMSO). Untransfected HEK293 showed no significant response to 3 mM 2-APB (Figure 1H, left). Transfected HEK293 cells showed open channel noise, as previously described (Bang et al., 2000), which was markedly increased after the application of 3 mM 2-APB (Figure 1H, right), demonstrating the direct nature of the interaction.

2-APB ACTIVATES hKCNK2 AND hKCNK4 CHANNELS IN A DOSE-DEPENDENT MANNER

We also investigated the effect of 2-APB on the other two members of the TREK subfamily. These are hKCNK2 (hTREK-1), which is the most thoroughly studied KCNK channel, and hKCNK4 (hTRAAK), which received its name because of its sensitivity to arachidonic acid, though it is also sensitive to other polyunsaturated fatty acids. Both channels are active at physiological body temperatures and have been shown to be necessary for appropriate thermoperception (Noël et al., 2009). Like KCNK10, they are expressed in both the central and peripheral human nervous system (Medhurst et al., 2001).

2-Aminoethoxydiphenyl borate induced dose-dependent and partially reversible activation of hKCNK2 and hKCNK4 in X. laevis oocytes expressing these channels (see Figure 2A). As for hKCNK10, 2-APB elicited more pronounced activation of these two channels at potentials above the Krev (Figure 2B). 2-APB had
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FIGURE 3 | (A) Comparison of the effects of applying 100 μL frog Ringer’s solution containing 3.3% DMSO vs. applying the same solution plus 10 mM 2-APB to uninjected oocytes (gray bars) or oocytes injected with cRNA coding for hKCNK2 (light green bars) and hKCNK10 (dark green bars) (n = 3–5 oocytes/group). The time of exposure was approximately 10 s. (B) Top: Representative voltage clamp recording from an oocyte expressing hKCNK2 exposed to vehicle solution (green rectangles) or the same solution plus 10 mM DMSO (blue rectangles). Bottom: Representative voltage clamp recording from an inside-out patch-clamp of a HEK293 cell expressing hKCNK10, before and after exposure to 1% DMSO (light green rectangle). (C) hKCNK4 currents recorded from a transfected HEK293 cell before (right) and after (left) the application of 300 μM 2-APB. Currents were elicited by voltage pulses from −110 to +50 mV in 20 mV steps, 400 ms in duration, from a holding potential of −60 mV.

an EC50 of 486 ± 135 μM for hKCNK2 (Figure 2C); however, an EC50 value for hKCNK4 could not be determined, although this channel was clearly activated in a dose-dependent manner by 2-APB in both X. laevis oocytes (Figure 2D) and transfected HEK293 cells (Figures 2E and 3C for original trace). Like hKCNK10, hKCNK4 also showed a marked increase in basal activity when treated with 2-APB in inside-out excised patches from transfected HEK293 cells, suggesting that this effect is independent of cytosolic factors (Figure 2F).

DISCUSSION

Members of the TREK subfamily of KCNK channels are widely expressed in the CNS (Medhurst et al., 2001), where they have been shown to play a key role in several processes ranging from...
long-term depression to neuroprotection (Heurteaux et al., 2006; Kim et al., 2011). This makes them valuable targets for pharmacological research, however, since their pharmacology, and common and stable activators are lacking. Here we have showed that the membrane-permeable substance 2-APB behaves as a highly effective activator for all the members of the mechanosensitive tandem-pore K+ channel family. J. Biol. Chem. 279, 17612–17619.

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