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The Two-Pore-Domain K^+ Channels TREK-1 and TASK-3 Are Differentially Modulated by Copper and Zinc

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ABSTRACT

The "trace" elements copper and zinc are essential for life, and their role in the function of metalloproteins is well known. However, mounting evidence shows that these metals are also capable of modulating neuronal excitability under normal physiological conditions. They are present at high levels in the brain, are concentrated at nerve terminals, and are released at micromolar concentrations into the synaptic cleft after depolarization. They have been shown to affect the function of a number of different voltage- and ligand-gated ion channels, but their most important targets in the nervous system remain uncertain. In this study, we show that the two-pore-domain potassium channels TREK-1 and TASK-3 are potently modulated by both copper and zinc. Copper activates TREK-1 channels by 83 ± 11% with an EC_{50} of 3.0 ± 1.0 μM, whereas TASK-3 channels are potently inhibited, with an IC_{50} of 2.7 ± 0.4 μM. Zinc inhibits both channels but with very different affinities. The IC_{50} for inhibition of TREK-1 channels is 659 ± 94 μM whereas the IC_{50} for inhibition of TASK-3 is 12.7 ± 1.0 μM. Using site-directed mutagenesis, we show that Asp128 plays a critical role in the copper activation of TREK-1. These observations provide a novel explanation for how copper and zinc might affect neuronal excitability under both normal physiological conditions, as well as during diseases in which copper or zinc homeostasis has been disrupted.

The physiological importance of copper and zinc has been appreciated for centuries, with medicinal uses of copper dating back to ancient times. Both metals are found in the blood at a total concentration of about 15 μM, but they achieve much higher (up to 10-fold) concentrations in the brain (Kozma et al., 1981; Ono et al., 1997) and have been localized to several specific brain regions (Kozma et al., 1981; Sato et al., 1994; Ono et al., 1997). Copper and zinc are present in synaptic vesicles and, after depolarization, are released from nerve terminals (Assaf and Chung, 1984; Howell et al., 1984; Hartter and Barnea, 1988; Kardos et al., 1989) into the synaptic cleft, where they may achieve concentrations on the order of 100 μM or more. During the last few years, it has been shown that both metals can modulate (usually inhibit) several ligand-gated ion channels, including *N*-methyl-D-aspartate receptors (Vlachova et al., 1996; Paoletti et al., 1997), $GABA_A$ receptors (Sharonova et al., 1998; Hosie et al., 2003), glycine receptors (Bloomenthal et al., 1994; Wang et al., 2002), $5\text{-}HT_3$ receptors (Lovinger, 1991) and P2X receptors (Xiong et al., 1999), as well as affecting a variety of voltage-gated ion channels (Horning and Trombley, 2001).

In the course of experiments designed to study the modulation of two-pore-domain K^+ channels by general anesthetics (Gruss et al., 2004), we found that solutions delivered from glass syringe barrels with metal tips produced potent, but variable, degrees of inhibition. We subsequently identified that the problem was caused by micromolar concentrations of zinc being released from the metal tips (chromium-plated brass). During our investigation, we found that two-pore-domain K^+ channels were also sensitive to micromolar concentrations of copper, the other major component of brass.

Two-pore-domain K^+ channels play an important role in modulating neuronal excitability (Goldstein et al., 2001; Patel and Honoré, 2001) and are often described as "background" or "leak" channels. In humans, the superfamily has 15 known members, which have a complex and varied anatomical distribution and differ largely in the ways in which they can be modulated and regulated. They can be affected by internal and external pH, certain general anesthetics, membrane stretch, temperature, arachidonic acid, and membrane lipids, as well as by a variety of internal second messenger systems. There are reports that the TASK channels in this superfamily are inhibited by high concentrations of zinc (Leonoudakis et al., 1998; Buckler et al., 2000), but no work has been done on other members of the superfamily or, to our knowledge, with copper.

Herein, we show that TASK-3 (also known as KCNK9) and

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ABBREVIATIONS: TASK, acid-sensitive K^+ channel; TREK, weakly inwardly rectifying K^+ channel-related K^+ channel; HEK, human embryonic kidney.

TREK-1 (also known as KCNK2) channels are sensitive to both copper and zinc, although responses differ both qualitatively and quantitatively. TREK-1 channels are potently activated by copper, whereas TASK-3 channels are potently inhibited. In addition, both channels are inhibited by zinc, but TASK-3 channels are approximately 50 times more sensitive than TREK-1 channels. We show that the amino acids His98, which acts as a proton sensor (Rajan et al., 2000), and Glu70, which is required for inhibition by ruthenium red (Czirjak and Enyedi, 2003), are not involved in copper inhibition. In contrast, these two amino acids have previously been shown (Clarke et al., 2003) to be critical for the zinc inhibition of TASK-3. Using site-directed mutagenesis, we identified an amino acid (Asp128) that is critical for the copper-activation of TREK-1. We conclude that the differential modulation of two-pore-domain K^+ channels by copper and zinc may provide a novel explanation for some of the observed effects of these metal ions on neuronal excitability.

Materials and Methods

Tissue Culture. Modified HEK-293 cells (tsA 201) were maintained in 5% CO_2 and 95% air in a humidified incubator at 37°C in growth media [89% Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum, 1% penicillin (10,000 units/ml), and streptomycin (10 mg/ml)]. When the HEK-293 cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-D-lysine (1 mg/ml) to ensure good cell adhesion.

The HEK-293 cells were transiently transfected using the calcium phosphate method. One microgram of cDNA encoding a K^+ channel subunit was added to each 35-mm well, and 1 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing two-pore-domain K^+ channels. After a 24-h incubation period at 3% CO_2/97% air the cells were rinsed with saline, and fresh growth medium was added to the wells. The cells were incubated at 37°C with 5% CO_2/95% air for 12 to 72 h before electrophysiological measurements were made. A number of experiments were performed with mock-transfected cells; these were treated identically, except that the cDNA coding for the K^+ channel was replaced by water.

A cell-line of HEK-293 cells stably-transfected with human TREK-1 was maintained in 5% CO_2/95% air in a humidified incubator at 37°C in growth media [89.9% Dulbecco's modified Eagle's medium, 8.9% heat-inactivated fetal bovine serum, and 1.8% Geneticin (20 mg/ml)]. When the cells were 80% confluent, they were split and plated onto glass coverslips coated with poly-D-lysine (1 mg/ml) and stored in an incubator at 5% CO_2/95% air. The cells were used after 4 to 5 days for electrophysiological measurements.

HEK-293 cells stably expressing human TREK-1 two-pore-domain K^+ channels, and human TASK-3 (Chapman et al., 2000) and TREK-1 (Meadows et al., 2000) two-pore-domain K^+ channel clones in the pcDNA 3.1 vector were kindly provided by Dr. Helen Meadows at GlaxoSmithKline (Harlow, Essex, UK).

Mutations and Truncations. To generate mutations and truncations, point mutations were introduced into the K^+ channel clones using the QuikChange kit (Stratagene, Amsterdam, The Netherlands). A pair of short (33–45 bases) complementary oligonucleotide primers, incorporating the intended mutation, was synthesized (MWG-Biotech, Ebersberg, Germany). To aid identification of successful mutants, a diagnostic restriction site was included in the primer sequence. Mutant DNA constructs were sequenced (MWG-Biotech, Centre for Medical Education, University of Dundee, or Advanced Biomedical Services, Imperial College) to confirm the introduction of the correct mutated bases.

Solution Preparation. Stock solutions of $CuCl_2$ (100 μM) and $ZnCl_2$ (1 or 2.5 mM) were prepared in standard extracellular solution or high-K^+ solution for inside-out patch experiments and diluted to the test concentrations. Stock solutions of arachidonic acid (100 mM) in ethanol were diluted in external solution before the experiments. The stock solutions were kept under nitrogen, stored at −20°C and used within three days. For experiments with arachidonic acid, the control solution contained the same concentration of ethanol as the test solution. The halothane solution was prepared and used as described previously (Gruss et al., 2004).

Electrophysiology. The composition of the control extracellular solution was 140 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, titrated to pH 7.4 with NaOH; total Na^+ concentration was 145 mM. Glass microelectrodes were pulled from thick-walled borosilicate glass capillaries (GC150F-10; Harvard Apparatus, Edenbridge, Kent, UK) using a two-stage vertical puller (PP-830; Narishige, Tokyo, Japan). Fire-polished pipettes were back-filled with intracellular solution passed through a 0.2 μm filter (120 mM KCH_3SO_4, 4 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, 3 mM MgATP, and 0.3 mM Na_2GTP, titrated to pH 7.2 with KOH; total K^+ concentration was 155 mM. The pipette resistance ranged from 2 to 7 MΩ. Series resistance was compensated by 75 to 90%. Voltage-clamp recordings were made using outside-out patches, inside-out patches, or the whole-cell recording technique. Cells and patches were usually held at −80 mV, and 750-ms voltage-ramps from −120 to 0 mV or +40 mV were recorded every 10 s. For continuous recordings, cells or patches were clamped at a fixed potential (−50 mV, unless otherwise stated). Only whole-cell recordings with a positive holding current at −80 mV (indicating a negligible ohmic leak conductance) and an access resistance <10 MΩ were included in the analysis. Current-voltage curves from outside-out and inside-out patches were corrected for an ohmic leak using the equation $I_{corr} = I_{obs} - I_{rev}V_m/V_{rev}$ where I_{corr} is the corrected current, I_{obs} is the observed current at a membrane potential V_m and I_{rev} is the current measured at the K^+ reversal potential. For inside-out patches, the pipettes were filled with external solution, and the bath was perfused with a high potassium solution containing 150 mM KCl and 10 mM HEPES. The pH was adjusted to pH 7.2 by adding KOH giving a final potassium concentration of 153 mM. Inside-out patches were held at −80 mV and 1-s voltage-ramp from −120 to + 120 mV were recorded every 5 or 10 s. For analysis, the data were filtered at 1 kHz, three consecutive traces were averaged, and an ohmic leak was subtracted as described above. The slope conductance between −80 and +40 mV was measured, and the percentage change was calculated. In all cases, the percentage activation or inhibition was calculated relative to the average of controls taken just before and just after the test application.

Recordings were usually digitized at 10 kHz (2 kHz for continuous recordings) and the output of the patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA) was usually filtered at 5 kHz (corner frequency at −3 dB) using the four-pole Bessel filter of the patch-clamp amplifier. Continuous recordings were digitally filtered at 100 Hz (corner frequency at −3 dB) using an eight-pole Bessel filter (Axon Instruments), unless otherwise stated, before analysis using Clampfit software (Axon Instruments). All recordings were stored on a computer hard disk. All electrophysiological measurements were carried out at room temperature (21–23°C).

The concentration-response data for copper activating TREK-1 channels were fitted to a Hill equation of the form $I = [I_{max}\ C^{nH} \times 100]/[C^{nH} + (EC_{50})^{nH}]$, where I is the observed K^+ current, I_{max} is the maximum current, C is the concentration of metal ion, n_H is the Hill coefficient, and EC_{50} is the concentration for a half-maximal effect.

The concentration-response data for copper or zinc inhibiting the two-pore-domain channels were fitted to an inhibitory Hill equation of the form $I = [IC_{50}^{nH}]/[C^{nH} + (IC_{50})^{nH}]$, where I is the observed K^+ current, C is the concentration of metal ion, n_H is the Hill coefficient, and IC_{50} is the concentration for 50% inhibition.

Values throughout this paper are given as means ± SEMs. In

graphs in which error bars are not shown, they are smaller than the size of the symbols. Statistical significance was assessed using the Student's *t* test.

Results

Cells expressing human TREK-1 channels had resting potentials of around −85 mV and, under voltage clamp, passed large outward currents that were approximately 1000 times larger than those from mock-transfected HEK-293 cells, consistent with many previous descriptions of two-pore-domain K^+ currents (Goldstein et al., 2001; Patel and Honoré, 2001). These TREK-1 currents were markedly activated by micromolar concentrations of copper (see Fig. 1). The current-voltage curves (Fig. 1*A*) showed that the percentage activation was concentration dependent and showed a clear voltage dependence; the activation was significantly greater at more negative potentials (Fig. 1A, inset). The activation was rapid and completely reversible (Fig. 1B). The concentration-response curve for the activation is shown in Fig. 1C with the data fitted to a Hill equation (see *Materials and Methods*). The EC_{50} for the effect was 3.0 ± 1.0 μM, the V_{max} was 83 ± 11%, and the Hill coefficient was 1.7 ± 0.8. As shown by the error bars in Fig. 1C, the degree of activation was quite variable at concentrations of copper higher than 10 μM. Experiments with outside-out patches (Fig. 1D) showed a degree of activation that was comparable with that seen in whole-cell recordings, indicating that the intact cell is not required for the effect. Moreover, copper had no significant effect on inside-out patches (Fig. 1E), showing that copper must be acting on the external surface of the membrane.

In contrast to TREK-1 channels, TASK-3 channels were potently inhibited by copper ions (Fig. 2). The percentage inhibition was also reversible and concentration-dependent (Fig. 2, B and C), and there was a modest amount of voltage dependence (Fig. 2A, inset), with inhibition increasing slightly at more negative membrane potentials. The concentration dependence is shown in Fig. 2C with the data fitted to an inhibitory Hill equation (see *Materials and Methods*) with an IC_{50} of 2.7 ± 0.4 μM and a Hill coefficient of 1.8 ± 0.4. As with the activation of TREK-1 channels, the inhibition of TASK-3 channels by copper ions did not require the intact cell because channel currents in outside-out patches showed a sensitivity comparable with that seen with whole-cell recordings (Fig. 2D) but were unaffected by copper applied to the inside surface of the membrane (Fig. 2E).

The data in Fig. 3 show that TREK-1 and TASK-3 channels were both rapidly and reversibly inhibited by zinc ions but with very different sensitivities. TREK-1 channels (Fig. 3, A–C) were relatively insensitive to zinc, with an IC_{50} of 659 ± 94 μM and a Hill coefficient of 0.6 ± 0.1 (see Fig. 3C). TASK-3 channels were also inhibited by zinc (Fig. 3, D–F) but at much lower concentrations, with an IC_{50} of 12.7 ± 1.0 μM

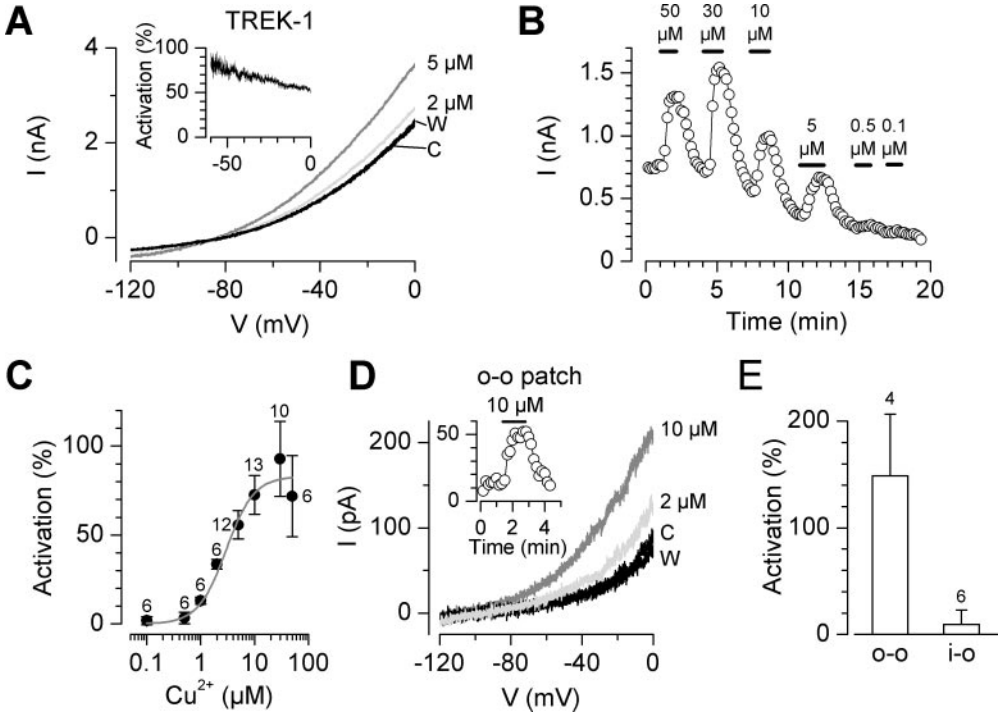

Fig. 1. Activation of human TREK-1 by Cu^{2+}. Cu^{2+} activates human TREK-1 at physiologically relevant concentrations. A, traces recorded from voltage-ramps (−120 to 0 mV, 750 ms) show concentration-dependent activation of TREK-1. The inset shows the activation (by 5 μM Cu^{2+}) is markedly voltage-dependent. "C" and "W" indicate control and washout traces, respectively. B, time course of activation of TREK-1 by different concentrations of Cu^{2+}. The activation is concentration-dependent, is maintained during the presence of Cu^{2+}, and is rapidly reversible. Values are measured at −50 mV from voltage-ramps as shown in A recorded every 10 s. C, concentration-response curve summarizing the activating effect of Cu^{2+} on TREK-1. Data represent means ± S.E.M. and the number of different cells for each concentration is shown next to each symbol. Data are fitted to the Hill equation (see *Materials and Methods*), the EC_{50} is 3.0 ± 1.0 μM, the V_{max} is 83 ± 11%, and the Hill coefficient is 1.7 ± 0.8. D, recordings from voltage-ramps (as in A) on TREK-1 in outside-out patches. As in whole-cell recording, Cu^{2+} activation of TREK-1 currents is concentration-dependent and rapidly reversibly. Traces were corrected for ohmic leak as described under *Materials and Methods*. The inset shows the time course of activation. E, summary showing the effects of 10 μM Cu^{2+} on TREK-1 outside-out and inside-out patches. In contrast to the substantial activation of TREK-1 channels in outside-out patches (149 ± 60%; $n = 4$), there is no significant effect on TREK-1 currents in inside-out patches (9 ± 14%; $n = 6$). Data were obtained and analyzed as described under *Materials and Methods*.

and a Hill coefficient of 1.7 ± 0.2 (see Fig. 3F). For both channels, the inhibition was essentially independent of voltage.

Because certain amino acids (Glu70 and His98) have been shown (Clarke et al., 2003) to be important in the zinc inhibition of TASK-3, we investigated their possible involvement in the copper inhibition of TASK-3. We found that mutating these amino acids (E70K and H98A, respectively) had no significant effect on the copper inhibition of TASK-3 (Fig. 4).

An amino acid in TREK-1, Glu306, that has been shown (Honoré et al., 2002) to be crucial in the modulation of the channel by arachidonic acid, membrane stretch, internal acidosis, and, most recently, gaseous anesthetics (Gruss et al., 2004) is also important in TREK-1 activation by copper. When this amino acid was mutated to an alanine, the copper (10 μM) activation was reduced by half (53.2 ± 6.3% activation, n = 9, for the wild-type compared with 22.5 ± 7.3%, activation, n = 5, for the E306A mutant).

Next, we investigated the possible location of copper binding sites on TREK-1 that might be responsible for the copper activation. We first mutated, in turn, each of the presumed extracellular histidine and cysteine residues (to alanine) because these amino acids are often involved in copper binding sites on proteins (Glusker, 1991). It is surprising that none of these mutations (H72A, H91A, C93A, H126A, and H232A) had any significant effect on copper activation (Fig. 5*A*), although the mean activations were all smaller than the control value, in no case did this reach statistical significance. We went on to mutate each of the presumed extracellular glutamic acid and aspartic acid residues, which would bear a negative charge at physiological pH. Only one of these mutations had a significant effect; the mutation of Asp128 to an alanine reduced the copper activation by about 60% (Fig. 5, B–D). Single mutations of four other residues (E69A, E234A, D240A, and D256A) and the double mutant (E69A/E73A) resulted in channel constructs that did not give measurable currents.

To provide confidence that the D128A mutation was disrupting copper binding per se, rather than perturbing the channel in a more general way, we determined the voltage-dependence, activation kinetics, arachidonic acid, and halothane activation and zinc sensitivity of the mutated channel and found them to be essentially indistinguishable from the wild-type channel (see Fig. 6).

Discussion

Zinc and copper are essential for human health, with a recommended daily intake of about 10 and 2 mg, respectively. The existence of highly selective zinc and copper transporters (Harris, 2002; Puig and Thiele, 2002) confirms the importance of zinc and copper homeostasis, and imbalances in the concentrations of these metals lead to serious pathological conditions, usually accompanied by disturbances of the nervous system. The physiological roles that these metals play in the nervous system, however, are poorly

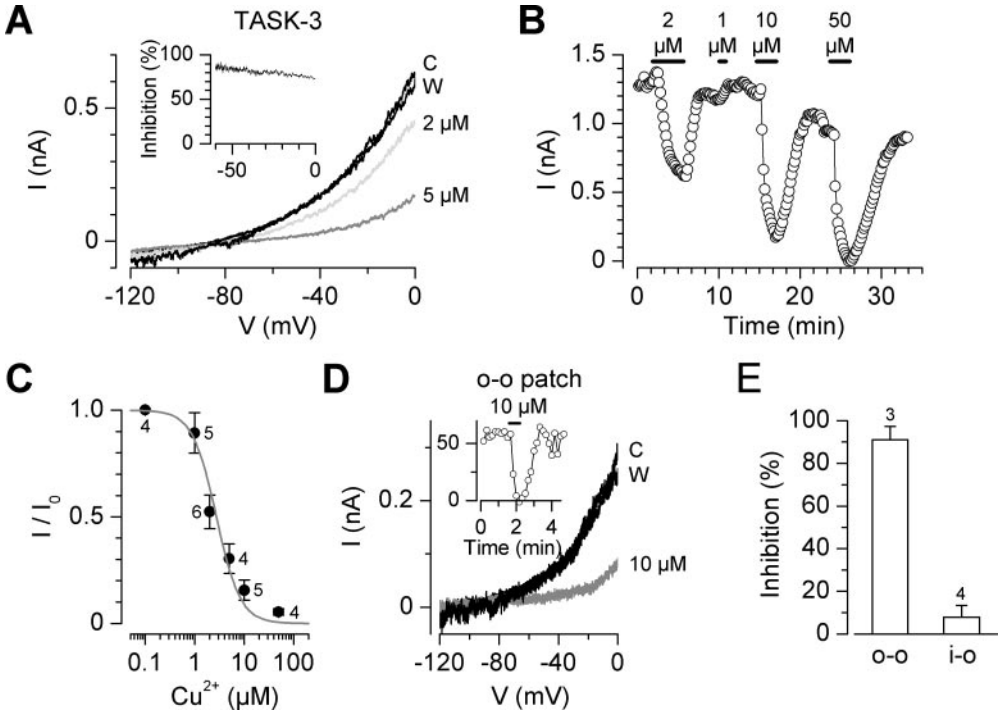

Fig. 2. Inhibition of human TASK-3 by Cu^{2+}. Cu^{2+} inhibits human TASK-3 at physiologically relevant concentrations. A, traces recorded from voltage-ramps (−120 to 0 mV; 750 ms) show concentration-dependent inhibition of TASK-3. The inset shows the inhibition (by 5 μM Cu^{2+}) is slightly voltage-dependent. B, time course of inhibition of TASK-3 by Cu^{2+}. The inhibition is maintained during the presence of Cu^{2+} and reversed upon washout. Values are measured at −50 mV from voltage-ramps as shown in A recorded every 10 s. C, concentration-response curve summarizing the blocking effect of Cu^{2+} on TASK-3. Data represent means ± S.E.M., and the number of different cells for each concentration is shown next to each symbol. Data are fitted to an inhibitory Hill equation (see *Materials and Methods*), the IC_{50} is 2.7 ± 0.4 μM and the Hill coefficient is 1.8 ± 0.4. D, recordings from voltage-ramps (as in A) on TASK-3 in outside-out patches. Copper inhibition of TASK-3 currents was concentration-dependent and rapidly reversible; the effect was significantly faster than in whole-cell recordings. Traces were corrected for ohmic leak as described under *Materials and Methods*. The inset shows the time course of activation. E, summary showing the effects of 10 μM Cu^{2+} on TASK-3 outside-out and inside-out patches. In contrast to the substantial inhibition of TASK-3 channels in outside-out patches (91 ± 6%; n = 3), there is no significant effect on TASK-3 currents in inside-out patches (8 ± 6%; n = 4). Data were obtained and analyzed as described under *Materials and Methods*.

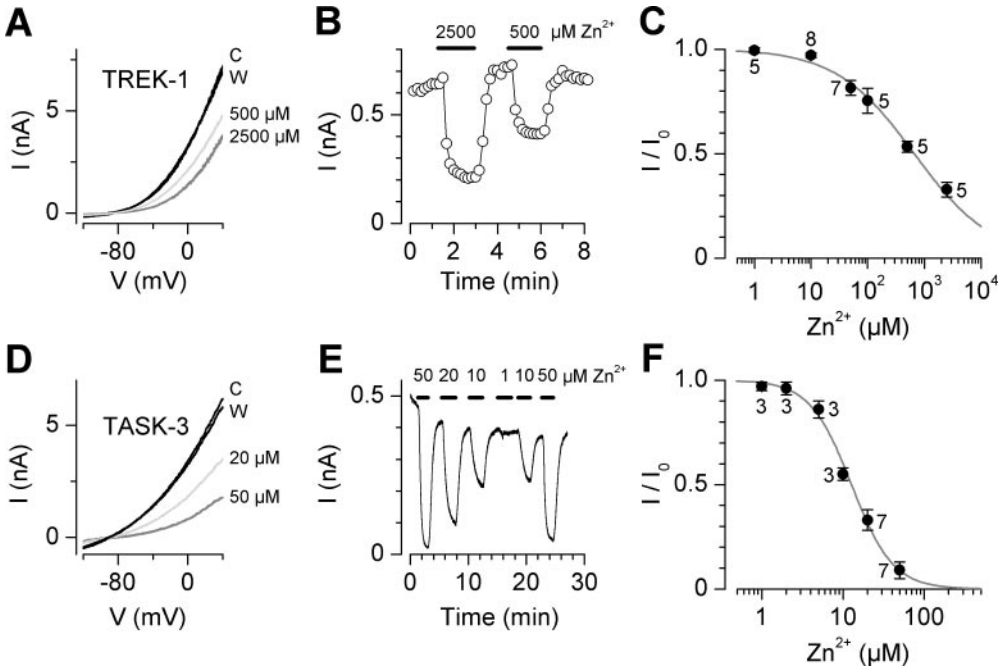

Fig. 3. Effects of Zn^{2+} on human TREK-1 and human TASK-3. A–C, Zn^{2+} inhibits human TREK-1 at millimolar concentrations. A, traces recorded from voltage-ramps (−120 to +40 mV; 750 ms) demonstrate concentration-dependent inhibition of TREK-1. B, time course of inhibition of TREK-1 by different concentrations of Zn^{2+}. The inhibition is dose-dependent, is maintained during presence of Zn^{2+}, and is reversible. Values are measured at −50 mV from voltage-ramps as shown in A recorded every 10 s. C, concentration-response curve summarizing the blocking effect of Zn^{2+} on TREK-1. Data represent means ± S.E.M., and the number of different cells for each concentration is shown next to each symbol. Data are fitted to an inhibitory Hill equation (see *Materials and Methods*), the IC_{50} is 659 ± 94 µM, and the Hill coefficient is 0.6 ± 0.1. D–F, Zn^{2+} inhibits human TASK-3 at micromolar concentrations. D, traces recorded from voltage-ramps (−120 to 0 mV; 750 ms) show concentration-dependent inhibition of TASK-3. E, time course of inhibition of TASK-3 by different concentrations of Zn^{2+}. The inhibition is concentration-dependent, is maintained during the presence of Zn^{2+}, and is rapidly reversible. The trace shows a recording from a cell continuously voltage-clamped at −50 mV. F, concentration-response curve summarizing the blocking effect of Zn^{2+} on TASK-3. Data represent means ± S.E.M., and the number of different cells for each concentration is shown next to each symbol. Data are fitted to an inhibitory Hill equation (see *Materials and Methods*), the IC_{50} is 12.7 ± 1.0 µM and the Hill coefficient is 1.7 ± 0.2.

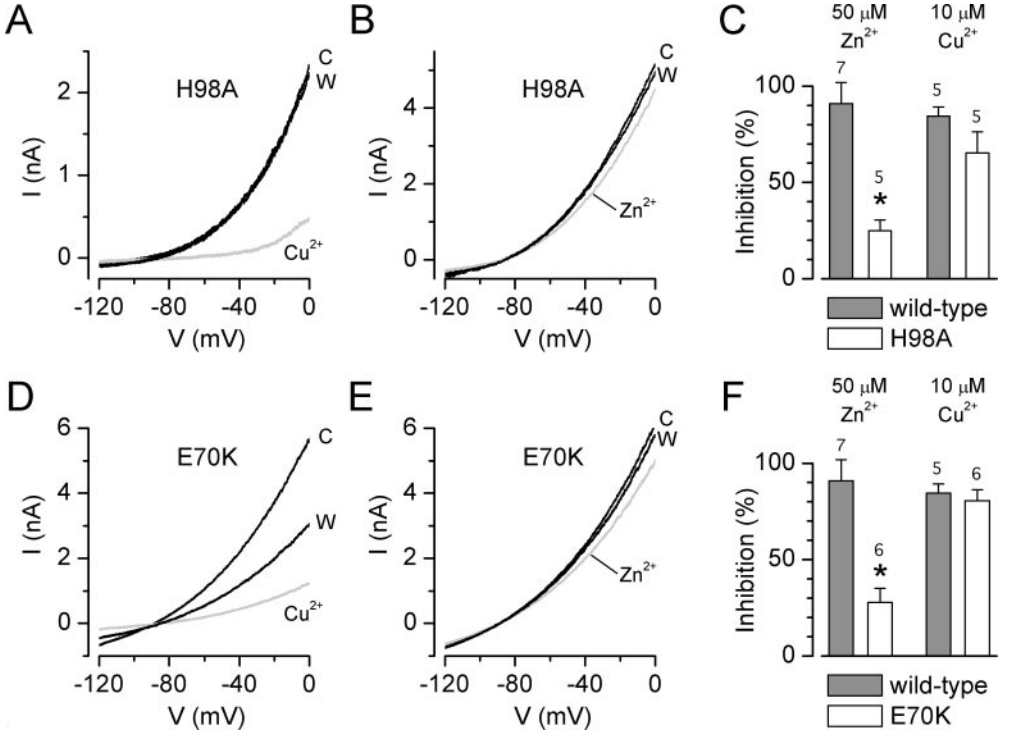

Fig. 4. TASK-3 H98A and TASK-3 E70K mutations prevent Zn^{2+} inhibition but do not affect Cu^{2+} inhibition. A and B, current-voltage curves showing the effects of 10 µM Cu^{2+} and 50 µM Zn^{2+} on a TASK-3 channel mutant where a histidine at position 98 is substituted by an alanine (H98A). C, summary showing that the inhibition of TASK-3 H98A (Clarke et al., 2003) by 50 µM Zn^{2+} is greatly reduced ($P < 0.001$), whereas its block by 10 µM Cu^{2+} is similar to inhibition of the TASK-3 wild-type channel ($P > 0.05$). D and E, current-voltage curves showing the effects of 10 µM Cu^{2+} and 50 µM Zn^{2+} on a TASK-3 channel mutant where a glutamate at position 70 is substituted by a lysine (E70K). F, summary showing that the inhibition of TASK-3 E70K by 50 µM Zn^{2+} is greatly reduced ($P < 0.001$), whereas its block by 10 µM Cu^{2+} is similar to the effect on the TASK-3 wild-type channel ($P > 0.05$).

understood. They are present in the blood at a total concentration of around 15 μM; almost all of this is complexed with proteins. The total concentration in the brain, however, is believed to be on the order of 100 μM, with both metals being found in many different brain regions (Kozma et al., 1981; Sato et al., 1994; Ono et al., 1997).

In the case of zinc, approximately 10% of brain Zn^{2+} is concentrated in the presynaptic terminals of certain gluta-

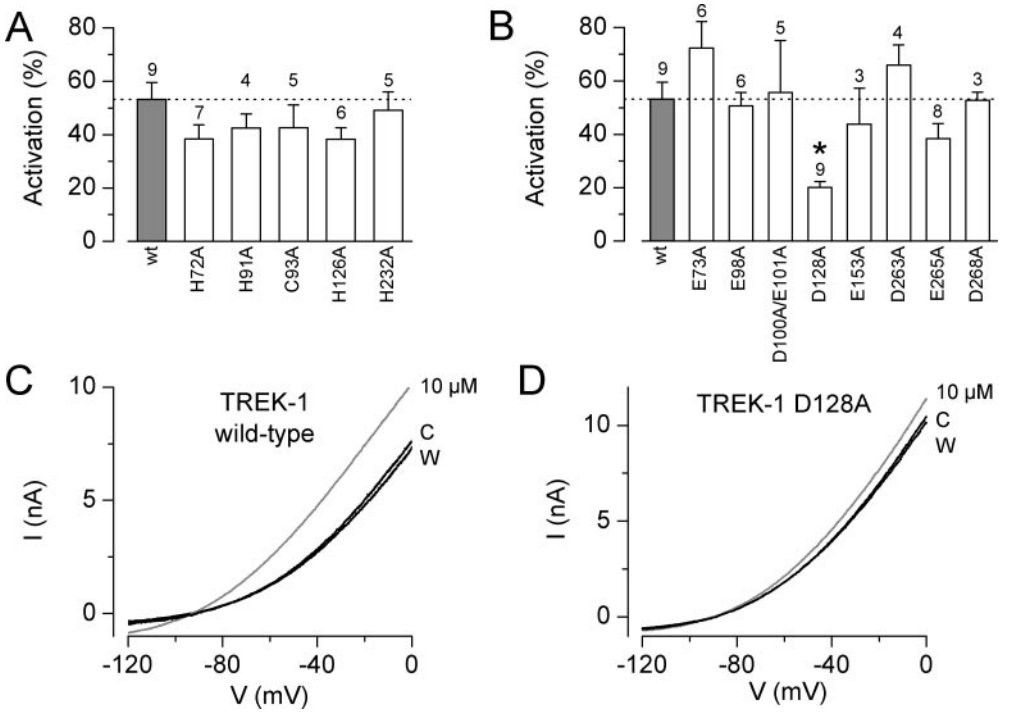

Fig. 5. Aspartate 128 is involved in Cu^{2+} induced activation of TREK-1. A, summary showing the lack of contribution of extracellular histidines (H) and cysteines (C) to the Cu^{2+}-induced activation of TREK-1. Copper (10 μM) activation of channels where single histidines or cysteines were mutated to an alanine is not significantly different from wild-type TREK-1. Data were measured at −50 mV from standard voltage-ramps. B, the effect of negatively charged extracellular amino acids [glutamic acids (E) and aspartic acids (D)] on the activation of TREK-1 by Cu^{2+}. Only D128A showed a significantly reduced activation by Cu^{2+} (20.1 ± 2.4% compared with 53.2 ± 6.3% for wild-type $P < 0.001$), whereas the other mutants were not significantly different from the wild-type. The dotted lines in A and B indicate the level of wild-type activation, data represent means ± S.E.M., and the number of different cells for each mutant is given above the columns. C and D, the activating effect of 10 μM Cu^{2+} is greatly reduced in a mutated channel in which an aspartic acid at position 128 is substituted with an alanine.

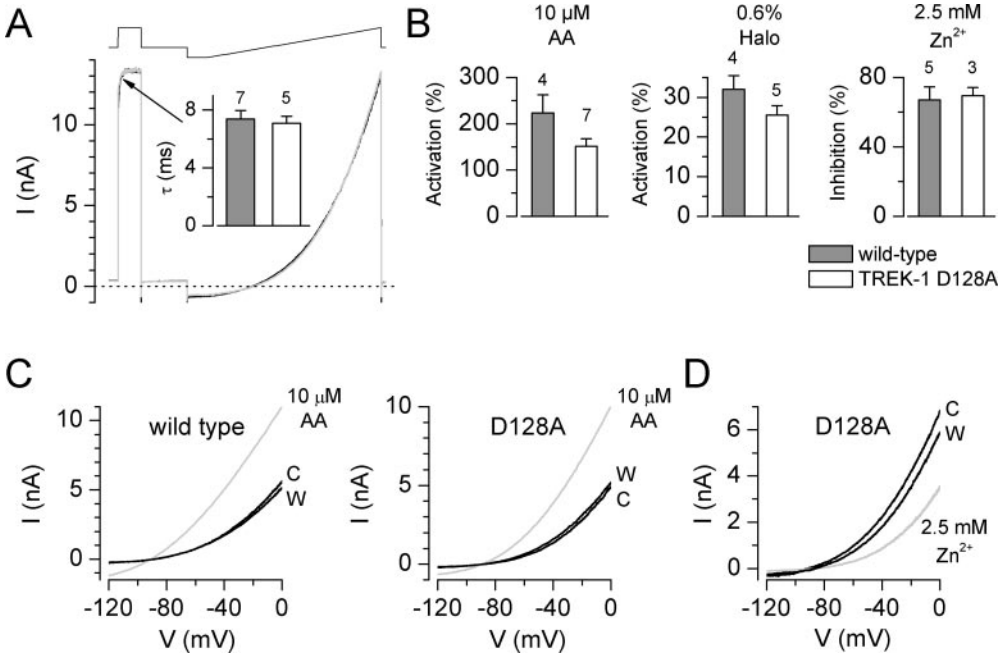

Fig. 6. The mutation D128A in TREK-1 does not affect channel gating or modulation by arachidonic acid, halothane, and Zn^{2+}. A, data showing that TREK-1 (black) and TREK-1 D128A (gray) currents have indistinguishable activation kinetics and voltage dependence. For comparison, the TREK-1 D128A recording was scaled to the same size as the wild-type current. The voltage protocol shows an initial depolarizing voltage step from −80 to 0 mV for 100 ms, followed by a short hyperpolarization to −120 mV and a 750-ms voltage-ramp from −120 to 0 mV. The inset shows the monoexponential time constants for the activation during the depolarizing voltage-step for the wild-type (τ = 7.4 ± 0.6; n = 7) and the D128A mutant (τ = 7.1 ± 0.5; n = 5). B, activation of TREK-1 D128A by 10 μM arachidonic acid (151 ± 17%; n = 7) and 0.6% halothane (26 ± 2%; n = 5) is not significantly different ($P > 0.05$) to activation of the wild-type (223 ± 40%; n = 4 for arachidonic acid, and 32 ± 3%; n = 4 for halothane) as is the degree of inhibition by 2.5 mM Zn^{2+} (70 ± 5%; n = 3 for TREK-1 D128A, and 67 ± 3; n = 5 for the wild-type). C and D, example recordings from standard voltage-ramps showing the reversible activation of wild-type TREK-1 and TREK-1 D128A by 10 μM arachidonic acid and the inhibition of TREK-1 D128A by 2.5 mM Zn^{2+}.

matergic synapses (Frederickson, 1989) and is released after depolarization (Assaf and Chung, 1984; Howell et al., 1984). This results in transient, local concentrations that are thought to be as high as 100 to 300 μM. The corelease of zinc with the neurotransmitter glutamate has been shown to modulate its actions on its postsynaptic receptors (Smart et al., 1994). However, because of the high concentrations of zinc released at glutamatergic synapses, it seems probable that other receptors and ion channels on neighboring cells would also be affected because of "spillover". Indeed, the effects of zinc (almost invariably an inhibition) have been reported for numerous ion channels and receptors; binding affinities vary over several orders of magnitude (Lovinger, 1991; Bloomenthal et al., 1994; Paoletti et al., 1997; Magistretti et al., 2003).

A similar picture is emerging with copper, although this has been much less intensively studied. As with zinc, copper is also released at synapses after depolarization, and concentrations as high as those reported for zinc have been estimated to occur in the synaptic cleft after neurotransmitter release (Hartter and Barnea, 1988; Kardos et al., 1989). Copper has also been shown to interact with a large number of molecular targets, again with widely varying affinities (Lovinger, 1991; Vlachova et al., 1996; Sharonova et al., 1998; Wang et al., 2002).

The data we present here on the effects of copper and zinc ions on two-pore-domain K^+ channels provide an additional explanation for some of the CNS effects of these metals. Two-pore-domain K^+ channels are found throughout the brain and have a complex anatomical distribution that is highly specific for each member of the superfamily (Talley et al., 2001), and it is clear that they play a central role in modulating neuronal excitability. Depending on the member of the superfamily, they can be modulated by internal and external pH, membrane stretch, temperature, phosphorylation, arachidonic acid, various complex lipids and certain general anesthetics (Goldstein et al., 2001; Patel and Honoré, 2001). There is very little information on how these channels respond to trace metals.

The data in Fig. 1 show that TREK-1 channels are activated by copper at micromolar concentrations. Almost all previous reports on the interactions of copper with ion channels or receptors show an inhibitory effect, with IC_{50} values generally on the order of tens of micromolar (for example, see Lovinger, 1991; Wang et al., 2002), although there are reports of binding sites with much higher affinities (Vlachova et al., 1996; Sharonova et al., 1998). The magnitude of the maximum activation of TREK-1 (Fig. 1) is modest (~80%) compared with the activation that is possible with agents such as arachidonic acid (Patel et al., 1998); nonetheless, this degree of channel activation will occur at concentrations that are likely to be encountered physiologically, and this would result in a very significant reduction in neuronal excitability in neurons expressing these channels. In qualitative contrast to this activation of TREK-1 channels, TASK-3 channels were equally potently inhibited by Cu^{2+} (Fig. 2), achieving an almost complete block at around 10 μM. The copper modulation of TREK-1 and TASK-3 is potent in the sense that it occurs in the micromolar range and is likely to have some physiological significance given the relatively high concentrations that are thought to be released at some synapses (Hartter and Barnea, 1988; Kardos et al., 1989). Thus, neuronal excitability could be markedly increased or reduced depending upon the type of two-pore-domain channel that was expressed as well as its cellular distribution.

Because the effects of Cu^{2+} on outside-out patches were so rapid, and because Cu^{2+} had no effect on inside-out patches, it is safe to assume binding sites on the external surface of the membrane. For both TREK-1 and TASK-3, the effects of Cu^{2+} were voltage-dependent, with this being particularly marked in the case of TREK-1. The activation of TREK-1 and the inhibition of TASK-3 both increased at more negative potentials, which is consistent with an external Cu^{2+} binding site that lies within the membrane field. However, the presence of other divalent cations, such as Mg^{2+}, have been shown to affect the current-voltage characteristics of TREK-1 (Maingret et al., 2002), so more work is required to understand the basis for this voltage-dependence.

The effects of zinc were qualitatively and quantitatively different. Zinc has been reported to inhibit TASK channels with an IC_{50} of around 200 μM (Leonoudakis et al., 1998; Buckler et al., 2000). We find (Fig. 3) that TASK-3 channels are more than an order of magnitude more sensitive [see also Clarke et al. (2003)]. Given that Zn^{2+} is thought to be released at synapses at concentrations considerably in excess of this IC_{50} value, it seems plausible that the Zn^{2+} sensitivity of TASK-3 channels is physiologically relevant. In contrast, TREK-1 channels were very much less sensitive, with an IC_{50} of 659 $\pm$ 94 μM. The high value of the IC_{50}, together with the low value of the Hill coefficient, is consistent with a number of very weak binding sites, and it seems unlikely that this effect is of physiological relevance.

We investigated certain mutations (Glu70 and His98) that have been shown to affect TASK-3 modulation by zinc (Clarke et al., 2003) to determine whether they also affected copper modulation. However, although we confirmed that mutating these amino acids almost completely eliminated the zinc inhibition of TASK-3, we found these mutations had no significant effect on the inhibitory effects of copper. Thus, whereas these amino acids clearly play a role in zinc modulation (Clarke et al., 2003), as well as in modulation by ruthenium red (Czirjak and Enyedi, 2003) and pH (Rajan et al., 2000), they are not involved in the inhibition of TASK-3 by copper.

We also investigated an amino acid (Glu306) in TREK-1 that has recently been shown (Honoré et al., 2002) to be critical in the mechanosensitivity of the channel and that we have found (Gruss et al., 2004) to be important for the activation of TREK-1 by anesthetic gases. We investigated here whether Glu306 was similarly important for the Cu^{2+} activation of TREK-1 by mutating this amino acid to an alanine. This mutation did indeed reduce the extent to which Cu^{2+} could activate the channel, but the loss of the glutamic acid did not have the dramatic effect that it has on the mechanosensitivity of the channel (Honoré et al., 2002) or its activation by anesthetic gases (Gruss et al., 2004).

There are many examples in which Cu^{2+} binding sites on proteins have been shown to involve interactions with histidine and/or cysteine groups (Glusker, 1991). For this reason, we investigated the roles of each of the histidine and cysteine groups that are presumed to lie on the extracellular surface of the membrane. There are five such amino acids, but the mutation of each, in turn, to an alanine had no significant effect on the extent to which Cu^{2+} was able to activate the

channel. We then investigated all of the presumed extracellular amino acids that would bear a negative charge at physiological pH and would thus be prime candidates to interact with Cu^{2+} ions. Although the roles of five amino acids could not be determined because their mutation to alanine resulted in nonfunctional (or nonexpressing) channels, we did find a single mutation D128A that caused a substantial reduction in activation by Cu^{2+}. This amino acid is near the first pore-forming loop (on the N-terminal side), and it seems probable that it is involved in Cu^{2+} binding; indeed, such a site might be expected to experience the membrane field and this would account for the clear voltage dependence of the activation. Moreover, the data in Fig. 6 show that the D128A mutation has no significant effect on the kinetics and voltage-dependence of the channel or on its sensitivity to arachidonic acid, halothane, or zinc. It therefore seems likely that Asp128 plays a direct role in coordinating copper in a binding site. A more complete analysis is required to identify other amino acids that are presumably necessary to co-ordinate the metal ion.

There are a number of well-known human disorders that are known to involve abnormal levels of Cu^{2+} in the brain, such as Wilson's disease and Menke's syndrome (Strausak et al., 2001), and there is increasing evidence (Horning et al., 2000; Weiss et al., 2000; Squitti et al., 2002) that the disruption of Zn^{2+} and Cu^{2+} homeostasis might be an important mediator of acute brain injury, as well as the neuronal degeneration that occurs during Alzheimer's disease. These disease states all involve complex and often severe neurological malfunction, and it is possible that the abnormal modulation of two-pore-domain potassium channels plays a direct role in their pathology.

In conclusion, our observation that Cu^{2+} and Zn^{2+} can potently modulate two-pore-domain potassium channels provides a novel explanation for how these ions might affect neuronal excitability under both normal physiological conditions as well as during diseases in which Cu^{2+} or Zn^{2+} homeostasis has been disrupted.

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