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Inhibition of the human two-pore domain potassium channel, TREK-1, by fluoxetine and its metabolite norfluoxetine

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1 Block of the human two-pore domain potassium (2-PK) channel TREK-1 by fluoxetine (Prozac^R) and its active metabolite, norfluoxetine, was investigated using whole-cell patch-clamp recording of currents through recombinant channels in tsA 201 cells.

2 Fluoxetine produced a concentration-dependent inhibition of TREK-1 current that was reversible on wash. The IC_{50} for block was 19 μM. Block by fluoxetine was voltage-independent. Fluoxetine (100 μM) produced an 84% inhibition of TREK-1 currents, but only a 31% block of currents through a related 2-PK channel, TASK-3.

3 Norfluoxetine was a more potent inhibitor of TREK-1 currents with an IC_{50} of 9 μM. Block by norfluoxetine was also voltage-independent.

4 Truncation of the C-terminus of TREK-1 (Δ89) resulted in a loss of channel function, which could be restored by intracellular acidification or the mutation E306A. The mutation E306A alone increased basal TREK-1 current and resulted in a loss of the slow phase of TREK-1 activation.

5 Progressive deletion of the C-terminus of TREK-1 had no effect on the inhibition of the channel by fluoxetine. The E306A mutation, on the other hand, reduced the magnitude of fluoxetine inhibition, with 100 μM producing only a 40% inhibition.

6 It is concluded that fluoxetine and norfluoxetine are potent inhibitors of TREK-1. Block of TREK-1 by fluoxetine may have important consequences when the drug is used clinically in the treatment of depression.
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Abbreviations: 2-PK, two-pore domain potassium; PKA, protein kinase A; s.e.m., standard error of the mean; SPC, summary of product characteristics

Introduction

Background or 'leak' potassium (K) currents are present in almost all neurons and control the excitability and the resting membrane potential of these cells (Hille, 2001). It is becoming clear that the channel proteins underlying many of these leak K currents are members of the two-pore domain potassium (2-PK) channel family (Goldstein *et al*., 2001; O'Connell *et al*., 2002; Lesage 2003). Currently, there are known to be at least 16 mammalian channels in the 2-PK channel family. These can be divided, loosely, into six subfamilies on the basis of structural and functional similarities (Alexander *et al*., 2004). One of these subfamilies of 2-PK channels, the TREK subfamily, consists, at present, of three identified channel proteins, TREK-1, TREK-2 and TRAAK. In this study, we have considered the properties of one of these channels, TREK-1 (Fink *et al*., 1996), and its regulation by both the widely prescribed antidepressant agent, fluoxetine (Prozac^R), and its major active metabolite, norfluoxetine.

TREK-1 channels are expressed throughout the CNS (Fink *et al*., 1996; Hervieu *et al*., 2001; Talley *et al*., 2001; Gu *et al*., 2002) with a complex, species-dependent distribution (see Meadows *et al*., 2000), but with notable levels of expression in the hippocampus, cerebellum, cerebral cortex (Hervieu *et al*., 2001) and hypothalamus (Maingret *et al*., 2000a). TREK-1 channels are regulated by a wide range of physiological and pharmacological mediators. Physiologically, this regulatory activity will mean that the proportion of active TREK-1 channels is constantly varying and, as a consequence, the excitability of the neuron they are expressed in, will also vary. For example, TREK-1 channels are activated by membrane stretch (Patel *et al*., 1998), arachidonic acid (Patel *et al*., 1998), intracellular acidification (Maingret *et al*., 1999), heat (Maingret *et al*., 2000a), lysophospholipids (Maingret *et al*., 2000b), activation of cGMP-dependent protein kinase (Koh *et al*., 2003), copper ions (Gruss *et al*., 2004b) and a number of anaesthetic agents (Patel *et al*., 1999; Gruss *et al*., 2004a). The cytosolic C-terminal domain of the channel is critical for many of these activations (Patel *et al*., 1998; Maingret *et al*., 1999; 2000a; Gruss *et al*., 2004a). In addition, TREK-1 channels are inhibited by the antipsychotic agent, chlorpromazine (Patel *et al*., 1998), and by a number of G-protein-coupled neurotransmitter receptors (e.g. Chemin *et al*., 2003).

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A recent study using TREK-1 knockout mice (Heurteaux *et al*., 2004) has demonstrated the importance of TREK-1 channels for a number of primary CNS functions. TREK-1 channels have been shown to have an important role in neuroprotection against epilepsy and ischaemia. For example, the neuroprotective action of polyunsaturated fatty acids (Lauritzen *et al*., 2000; Blondeau *et al*., 2002) is lost in TREK-1 knockout animals (Heurteaux *et al*., 2004). Furthermore, these mice are resistant to anaesthesia by volatile anaesthetics. It follows that the use of any pharmacological agents that interfere with TREK-1 channel activity, such as we demonstrate here for fluoxetine, will have important consequences for CNS function.

Preliminary reports of some of these results have been published (Chumbley *et al*., 2003; Kennard *et al*., 2004).

Methods

Tissue culture

Modified HEK-293 cells (tsA 201) were maintained in 5% CO_2 in a humidified incubator at 37°C in growth media (89% Dulbecco's modified Eagle's medium; 10% heat-inactivated foetal bovine serum; 1% penicillin (10,000 U ml^{-1}) and streptomycin (10 mg ml^{-1}). When the tsA cells were 80% confluent, they were split and plated for transfection onto glass coverslips coated with poly-D-lysine (1 mg ml^{-1}) to ensure good cell adhesion. The tsA 201 cells were transiently transfected using the calcium phosphate method. cDNA (0.33 μg) encoding a TREK-1 or TASK-3 subunits was added to each 15 mm well, and 0.33 μg of a plasmid encoding the cDNA of green fluorescent protein was included to identify cells expressing two-pore domain channels. Following a 24 h incubation period at 3% CO_2, 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 for 12–72 h before electrophysiological measurements were made.

A cell line of HEK-293 cells stably transfected with human TREK-1 was maintained in 5% CO_2 in a humidified incubator at 37°C in growth media (89.3% Dulbecco's modified Eagle's medium; 8.9% heat-inactivated foetal bovine serum; 1.8% geneticin (20 mg ml^{-1})). When the cells were 80% confluent, they were split and plated onto glass coverslips coated with poly-D-lysine (1 mg ml^{-1}) and stored in an incubator at 5% CO_2. The cells were used after 1–2 days for electrophysiological measurements. HEK-293 cells stably expressing human TREK-1 two-pore channels and the human TREK-1 (Meadows *et al*., 2000) and TASK-3 (Meadows & Randall, 2001) two-pore channel clones in the pcDNA 3.1 vector were kindly provided by Dr Helen Meadows at GlaxoSmithKline, U.K.

Mutations and truncations

To generate mutations and truncations, point mutations were introduced into the TREK-1 channel clones using the Quikchange kit (Stratagene, Amsterdam, The Netherlands). A pair of short (25–35 bases) complementary oligonucleotide primers, incorporating the intended mutation, was synthesised (MWG-Biotech, Ebersberg, Germany). To aid identification of successful mutants, a diagnostic enzyme restriction site was included in the primer sequence. Mutant DNA constructs were sequenced (MWG-Biotech) to confirm the introduction of the correct mutated bases.

Electrophysiology

The composition of the control extracellular solution was (in mM) 145 NaCl, 2.5 KCl, 3 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, (titrated to pH 7.4 with NaOH). Glass microelectrodes were pulled from thick-walled borosilicate glass capillaries using a two-stage vertical puller (Narishige). Fire-polished pipettes were back-filled with 0.2-μm-filtered intracellular solution (composition in mM: 150 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES (titrated to pH 7.4 with KOH)). Voltage-clamp recordings were made using either outside-out patches or the whole-cell recording technique. Cells and patches were usually held at -80 mV, and, either 1 s voltage ramps from -120 to $+60$ mV or incrementing voltage steps from either -50 mV to $+40$ mV or -120 to $+120$ mV were recorded every 6 s. For the solutions above, the calculated potassium equilibrium potential was -103.4 mV. Therefore, only whole-cell recordings with positive holding current at -80 mV (thus where TREK-1 current dominates the resting conductance and there is a negligible leak conductance) were included in the analysis. Cells also had to have an access resistance <20 MΩ for inclusion.

Unlike whole-cell recording, outside-out patches from tsA 201 cells always contained a measurable ohmic leak conductance (see Gruss *et al*., 2004a). Current–voltage curves from outside-out patches were corrected for this 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 V_{rev}.

Recordings were usually digitised at 2 kHz and the output of the patch-clamp amplifier (Axopatch 1D; Axon Instruments, Union City, CA, U.S.A.) was usually filtered at 1 kHz using pClamp software. All recordings were stored on a computer hard disk. All electrophysiological measurements were carried out at room temperature (21–23°C).

Drugs were applied by bath perfusion with complete exchange of solution within 90 s. Control TREK-1 currents showed some run down with time during recordings, although this varied from cell to cell. We minimised the influence of this rundown on the measured effect of drugs, by calculating the percentage inhibition by fluoxetine or norfluoxetine relative to the average of controls taken just prior and just after the drug application. Values throughout this paper are given as mean ± standard error of the mean (s.e.m.). Statistical significance was assessed using the Student's *t*-test.

Results

Fluoxetine inhibition of hTREK-1 channel currents

Whole-cell currents through TREK-1 channels were recorded in tsA 201 cells transiently transfected with the 2-PK channel TREK-1. Ramp changes in membrane potential were applied from -120 to $+60$ mV. A typical control current obtained from such a ramp is shown in Figure 1a. The current is outwardly rectifying with a reversal potential at around

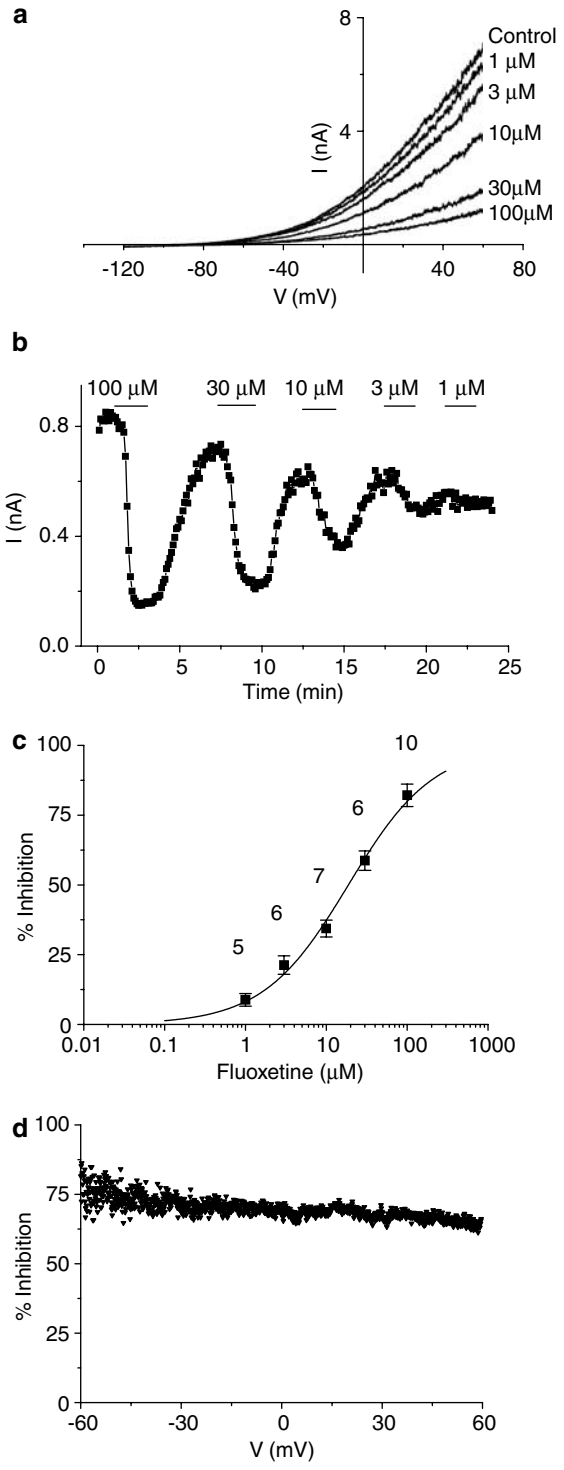

Figure 1 Fluoxetine inhibits TREK-1 channel currents. (a) TREK-1 currents evoked by ramp changes in voltage in control conditions and in the presence of increasing concentrations of fluoxetine. (b) Time-course plot of fluoxetine inhibition of TREK-1 current. Each point is a 5 ms average of the current at 0 mV taken from the ramp voltage protocol. (c) Concentration–response curve for fluoxetine inhibition of TREK-1 current. (d) Percentage inhibition of TREK-1 current by 30 μM fluoxetine is plotted as a function of voltage.

-100 mV, close to the calculated K equilibrium potential for the external and internal recording solutions used here (see Methods).

Bath application of fluoxetine (1–100 μM) produced a concentration-dependent inhibition of TREK-1 currents (Figure 1a and b), which was easily reversible on wash. The concentration–response curve (Figure 1c) gave an IC_{50} for inhibition of 19 ± 2 μM (mean $\pm$ s.e.m.) with a Hill coefficient of 0.9 ± 0.1 and a maximum inhibition, obtained from a free fit of the data, close to 100%. TREK-1 channels stably transfected into HEK-293 cells had very similar sensitivity to fluoxetine, with an IC_{50} of 14 ± 5 μM.

The voltage-dependence of block by fluoxetine was calculated by comparing a control ramp trace with one in the presence of 30 μM fluoxetine. The percentage inhibition at each voltage on the ramp protocol was calculated and plotted against the appropriate voltage (Figure 1d). The inhibition does not change significantly in the voltage range -60 to $+60$ mV, suggesting that block by fluoxetine is not voltage-dependent.

Although TREK-1 is a leak potassium conductance, it does show some voltage-dependence, the degree of which depends on the phosphorylation state of the channel (Bockenhauer *et al*., 2001). This is most clearly illustrated using step rather than ramp voltage protocols (Figure 2a), where a time-dependent component to the current is seen at test potentials of 0 mV and above. With step changes in voltage, the whole-cell TREK-1 current at $+60$ mV can be divided into an 'instantaneous' and a 'time-dependent' current (the former measured 0.5 ms after the step to $+60$ mV, while the total current was measured at the end of the test step). In such recordings, the instantaneous current at $+60$ mV was only 0.58 ± 0.08 ($n = 8$) of the total current. Fluoxetine (10 μM) was equally effective at inhibiting the instantaneous component of current ($47 \pm 8\%$, $n = 8$) and the total ($54 \pm 6\%$, $n = 8$) current (Figure 2a and b).

Fluoxetine inhibition of TASK-3 current

It is of interest that fluoxetine was much less effective at inhibiting a related human 2-PK channel from a different subfamily, TASK-3, compared with its action on TREK-1 channels. Fluoxetine (100 μM) produced a $31.4 \pm 5.5\%$ inhibition of these TASK-3 channels ($n = 6$; Figure 3). This was significantly smaller ($P < 0.01$) than block of TREK-1 channels, where 100 μM fluoxetine inhibited current by $84.1 \pm 3.3\%$ ($n = 10$). Thus, fluoxetine is more potent at blocking TREK-1 channels compared to TASK-3 channels.

Norfluoxetine inhibition of hTREK-1 channel currents

The effect of norfluoxetine (the active metabolite of fluoxetine) on TREK-1 channels was also investigated. Like fluoxetine, norfluoxetine produced a concentration-dependent block of the channel (Figure 4). The concentration response curve for norfluoxetine showed that the metabolite was even more potent than the parent fluoxetine molecule (Figure 4c) with an IC_{50} for inhibition of 9 ± 1 μM, a Hill coefficient of 1.3 ± 0.1 and, again, a maximum inhibition close to 100%. As for fluoxetine, block by norfluoxetine was voltage-independent (Figure 4d).

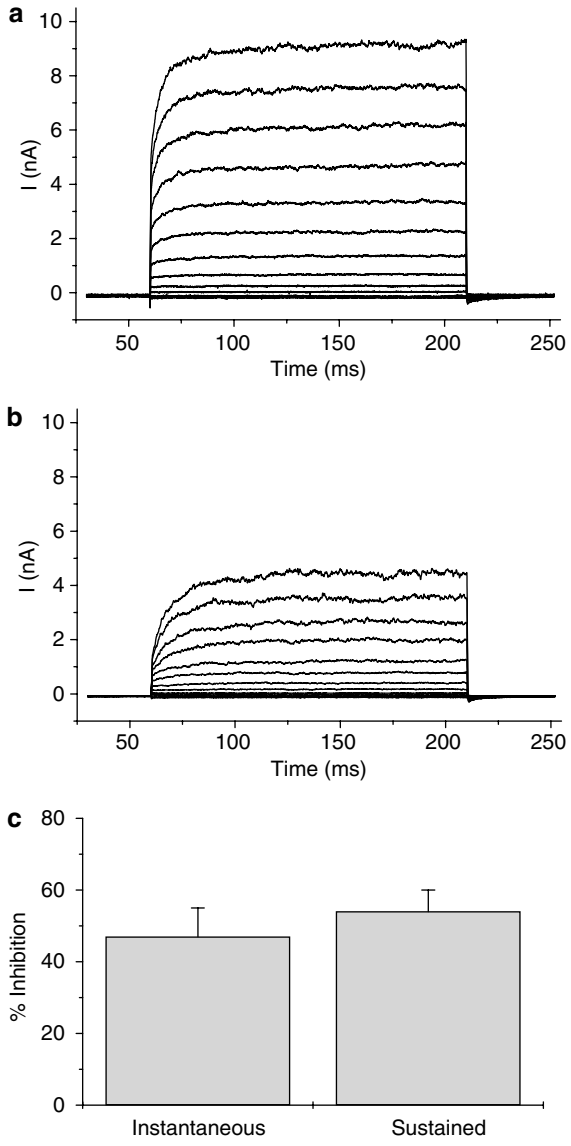

Figure 2 Fluoxetine is equally effective at blocking the instantaneous and sustained components of TREK-1 current. TREK-1 currents evoked by step changes in voltage from −120 to +120 mV in the absence (a) and presence (b) of 10 μM fluoxetine. (c) Percentage inhibition by 10 μM fluoxetine of the instantaneous and sustained components of TREK-1 current following depolarising voltage steps to +60 mV.

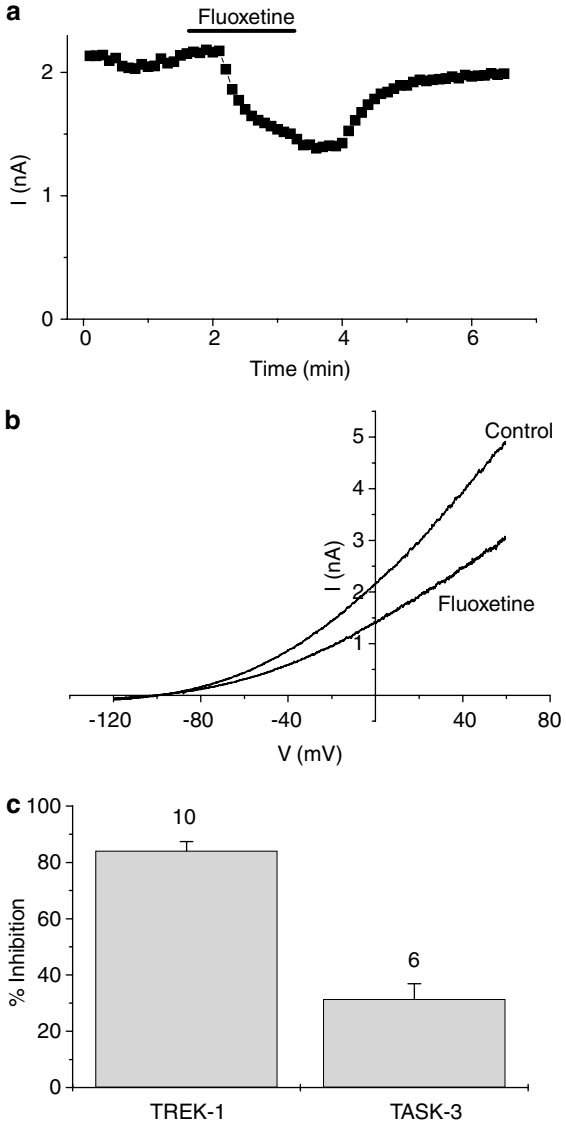

Figure 3 Fluoxetine is much less effective at inhibiting TASK-3 channel currents compared to TREK-1. (a) Time-course plot of fluoxetine inhibition of TASK-3 current. Each point is a 5 ms average of the current at 0 mV taken from the ramp voltage protocol. (b) TASK-3 currents evoked by ramp changes in voltage in control conditions and in the presence of 100 μM fluoxetine. (c) Comparison of the inhibition of TREK-1 and TASK-3 currents by 100 μM fluoxetine.

TREK-1 mutant channel properties

Many of the compounds that are known to interfere with TREK-1 channel activity (such as arachidonic acid, anaesthetic agents and phosphorylation by PKA) exert at least part of their action on the C-terminus of the channel (e.g. Patel *et al*., 1999). Similarly, at least part of the transduction of these effects relies on the amino acid E (glutamate) at position 306 (Honore *et al*., 2002). We have created a number of mutated TREK-1 channels and investigated whether these mutations have any effect on inhibition by fluoxetine. In doing so, we found that the mutations often affected the properties of the channels themselves.

In our hands, truncation of the last 89 amino acids (Δ89) of the C-terminus of TREK-1 resulted in a large reduction in channel function (Figure 5a–c). Conversely, making the mutation E306A resulted in a significant increase of the basal current measured at 0 mV from 98.2±12.3 pA/pF for wild-type channels to 189.7±39.7 pA/pF (Student's *t*-test, $P=0.03$) (see also Honore *et al*., 2002).

As well as increasing current density, the E306A mutation resulted in the loss of the slower phase of TREK-1 activation kinetics (Figure 5d and e). The instantaneous current measured at 0 mV was 0.86±0.02 of the total current for wild-type channels. This was significantly smaller ($P<0.01$) than that seen for E306A-mutated channels where the instantaneous current was 1.00±0.01 of the total current (Figure 5f).

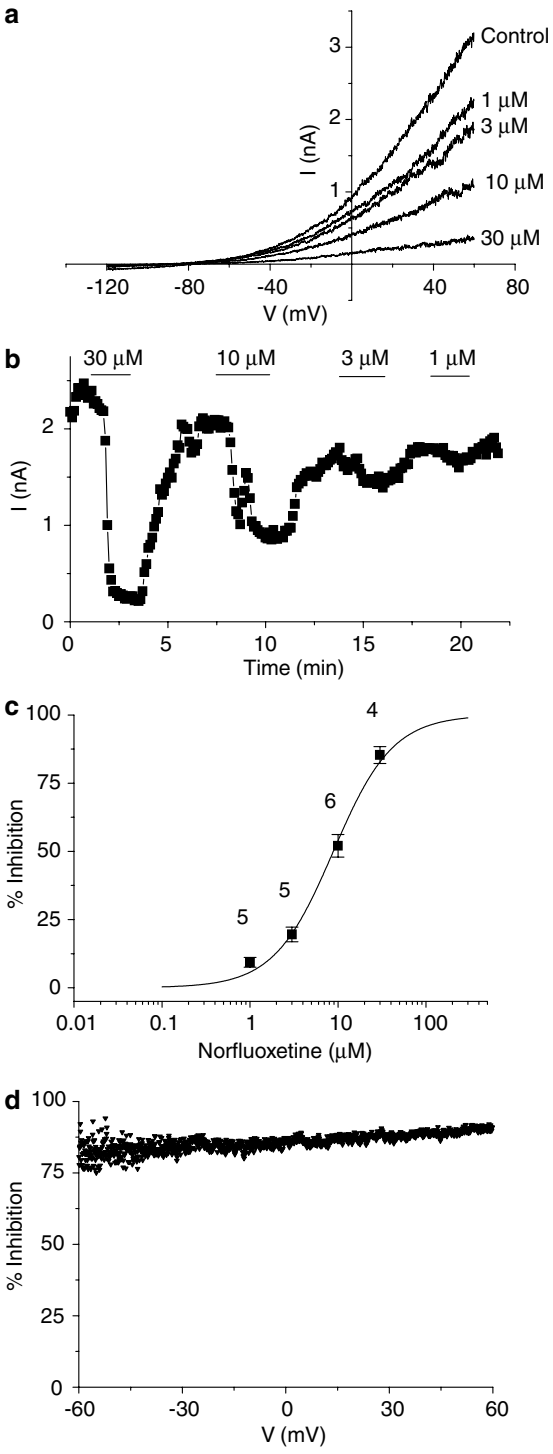

Figure 4 Norfluoxetine inhibits TREK-1 channel currents. (a) TREK-1 currents evoked by ramp changes in voltage in control conditions and in the presence of increasing concentrations of norfluoxetine. (b) Time-course plot of norfluoxetine inhibition of TREK-1 current. Each point is a 5 ms average of the current at 0 mV taken from the ramp voltage protocol. (c) Concentration–response curve for norfluoxetine inhibition of TREK-1 current. (d) Percentage inhibition of TREK-1 current by 30 μM norfluoxetine is plotted as a function of voltage.

The channel activity of Δ89 TREK-1 could be recovered in a number of ways. Acidification of the intracellular solution together with the use of outside-out macropatches (to ensure pH change close to the intracellular face of the channels) recovered channel activity (Figure 6b). Similarly, the channel activity of Δ89 could be recovered by introducing the E306A mutation to the truncated channel (see Figure 5c). Similar to the E306A mutation by itself, a significant ($P<0.01$) increase in the basal current to 197.7 ± 35.8 pA/pF was observed.

Fluoxetine inhibition of C-terminal-truncated TREK-1 channels

The C-terminus of TREK-1 is critical for the action of a number of compounds that regulate this channel (e.g. Patel *et al*., 1998; Gruss *et al*., 2004a). However, progressive deletion of the C-terminus did not affect fluoxetine inhibition of TREK-1. Unlike the longer truncation, Δ48 displays normal channel function. Fluoxetine (100 μM) produced a block of 94.9% (Figure 6a) comparable with that of wild-type TREK-1 channels.

After recovery of Δ89 TREK-1 channel function, using internal acidification to pH 5 in outside out patches, inhibition by fluoxetine was found to be 85.8 ± 2.8% (Figure 6b). This was not significantly different from wild type, which is inhibited by 84.1 ± 3.3%.

Fluoxetine inhibition of E306A TREK-1 channels

Mutation of amino acid E (glutamate) to A (alanine) has a profound effect on the function and regulation of TREK-1 channels (Honore *et al*., 2002). Similarly, we found that the magnitude of inhibition of TREK-1 by fluoxetine was significantly reduced by the E306A mutation with 100 μM fluoxetine producing a 39.5 ± 4.9 % inhibition ($P<0.01$) (Figure 6c and d).

Interestingly, the double mutant Δ89(E306A) also exhibited a significant ($P<0.01$) reduction in the inhibition by fluoxetine (100 μM) to 60.8 ± 4.7%. This suggests that the E306A mutation dominates the properties of the double mutant channels both in terms of current amplitude and degree of inhibition by fluoxetine.

Discussion

We have shown that the TREK-1 2-PK channel can be inhibited, potently, by fluoxetine, with an IC_{50} of 19 μM. The active metabolite of fluoxetine, norfluoxetine, was even more potent than the parent compound with an IC_{50} of 9 μM.

We have found that the TREK-1 channel is considerably more sensitive to block by fluoxetine than a related 2-PK channel from a different subfamily, TASK-3. It is interesting to note that a recent paper by Hajdu *et al*. (2003) found that fluoxetine also does not have a potent inhibitory action on another 2-PK channel, TASK-1. As we have found for TASK-3, 100 μM concentrations were needed to produce a significant inhibition of TASK-1. Thus, it appears that fluoxetine is a more potent inhibitor of TREK-1 channels, at least compared to members of the TASK subfamily of 2-PK channels.

Functional model of TREK-1 channel gating

TREK-1 channels exhibit two functional states. Firstly, they can behave as a leak conductance open across the

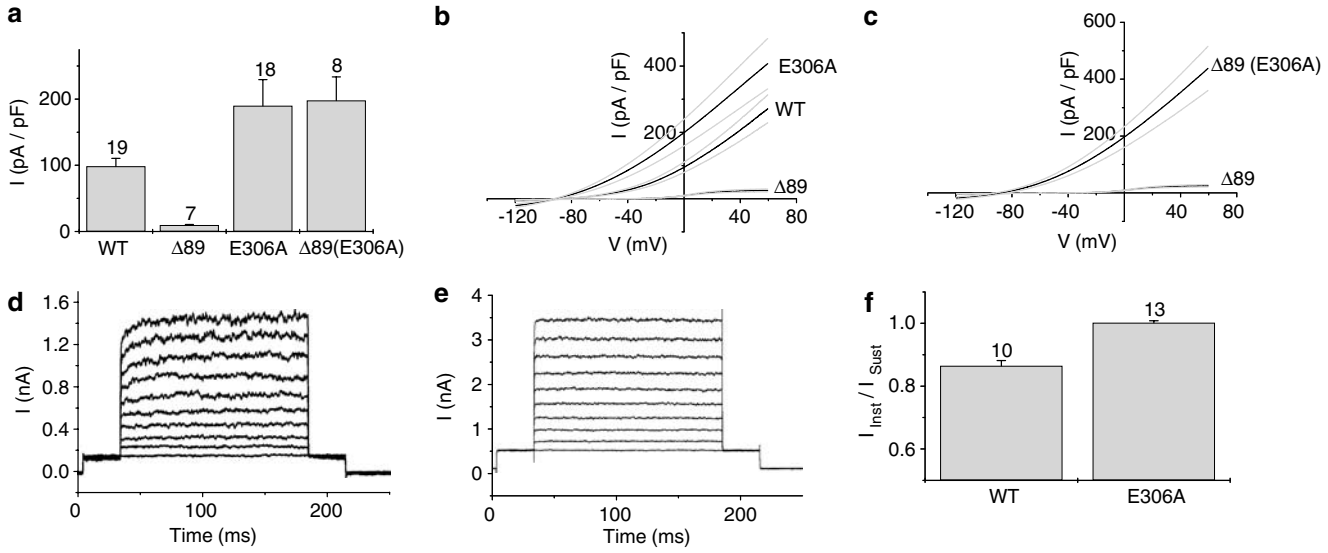

Figure 5 TREK-1 current density and channel kinetics are altered by channel mutations. (a) TREK-1 current density (pA/pF) at 0 mV for wild-type TREK-1 channels and mutated TREK-1 channels. (b and c) Average current density *versus* voltage plots for wild-type and mutated TREK-1 channels. Data are shown as mean ± s.e.m. (d, e) TREK-1 currents evoked by step changes in voltage from −50 to +40 mV for wild-type channels (d) and E306A TREK-1 channels (e). (f) Relative amplitude of the instantaneous component compared to the sustained component of current through wild-type TREK-1 channels or E306A-mutated channels following depolarising voltage steps to 0 mV.

physiological voltage range. In this state, the channels inhibit depolarisation and therefore decrease the excitability of the cell. Secondly, the channels can exist in a voltage-dependent state that preferentially passes an outward current at depolarised potentials. Thus, in this outward rectifier state, TREK-1 channels do not impede the depolarisation towards action potential firing threshold, but facilitate recovery and repetitive firing (Bockenhauer *et al*., 2001). The existence of these two states allows for a fine-tuning in the regulation of neuronal excitability. It has been shown that TREK-1 channels can be reversibly converted between their two phenotypes, *via* phosphorylation by protein kinase A (Bockenhauer *et al*., 2001). It is proposed that when the serine residue S333 in the C-terminal domain is phosphorylated, the channel becomes voltage-dependent. In normal, physiological conditions, the channel population exists as a mixture of phosphorylated and dephosphorylated channels and, following depolarising voltage-steps, two components to the activation of the current are observed – an instantaneous and a time-dependent component.

Another important residue close to the C-terminus of TREK-1 is E306, which has been proposed to act as an intracellular proton sensor. It has been shown that the mutation of the glutamate (E) to an alanine (A) at this position produces a channel that is a constitutively active leak conductance, mimicking the effects of intracellular acidosis (Honore *et al*., 2002, see also results). E306A can also rescue both lipid- and mechanosensitivity of a loss of function TREK-1 mutant (Honore *et al*., 2002).

Taking this information together, Honore *et al*. (2002) have proposed a kinetic model of TREK-1 channel gating containing two closed states (C_1 and C_2) and one open state (O) (see Figure 7). In the proposed model, the channel moves from C_1 to C_2 *via* internal acidosis or dephosphorylation of S333. Conversely, the channel can move from C_2 to C_1 by phosphorylation of S333. The instantaneous component of the whole-cell current following a voltage-step thus represents channels in C_2 moving to O, while the time-dependent component represents channels moving more slowly to O from C_1 (see also Honore *et al*., 2002). Since both the instantaneous and time-dependent components of the current were blocked equally well by fluoxetine, we propose that fluoxetine (and norfluoxetine) can bind to TREK-1 channels equally well, whether they are in C_1 or C_2 (Figure 7). Thus, we propose that fluoxetine acts as an allosteric blocker of TREK-1 channels binding to the channel in its closed states rather than acting as an open-channel blocking agent (see Hille, 2001 and below). A similar model has been proposed for a number of drugs that block potassium channels, such as ketoconazole block of $K_V1.5$ channels (Iftinca *et al*., 2001).

The mutation E306A is thought to mimic acidosis, and thus move the channel to C_2. Without the ability to return to C_1, the amount of time spent by the channel in O should be increased. In accordance with this, an increase in the basal current of E306A TREK-1 compared to the wild type was observed. This conversion of TREK-1 to a constitutively active channel demonstrates that the E306 residue is critical for gating of the channel. It was also observed that the activation kinetics of E306A TREK-1 were altered. Instead of the two components of activation seen in the wild type, only the instantaneous component was observed. Thus, the E306A mutation 'locks' the channel in the dephosphorylated, voltage-independent state (C_2). The inhibition of E306A by fluoxetine was shown to have the same time course and (lack of) voltage-dependence of inhibition as the wild type, but the magnitude of the inhibition was significantly reduced. In this mutation, fluoxetine can only bind to C_2, from which the channel has a higher probability of returning to O. A reduced inhibition of E306A by fluoxetine results from the higher probability of the channel escaping from C_2 (or drug bound C_2) to O.

The truncation of the C-terminus of TREK-1 (Δ89) resulted, in our hands, in a loss of function channel. We speculate that

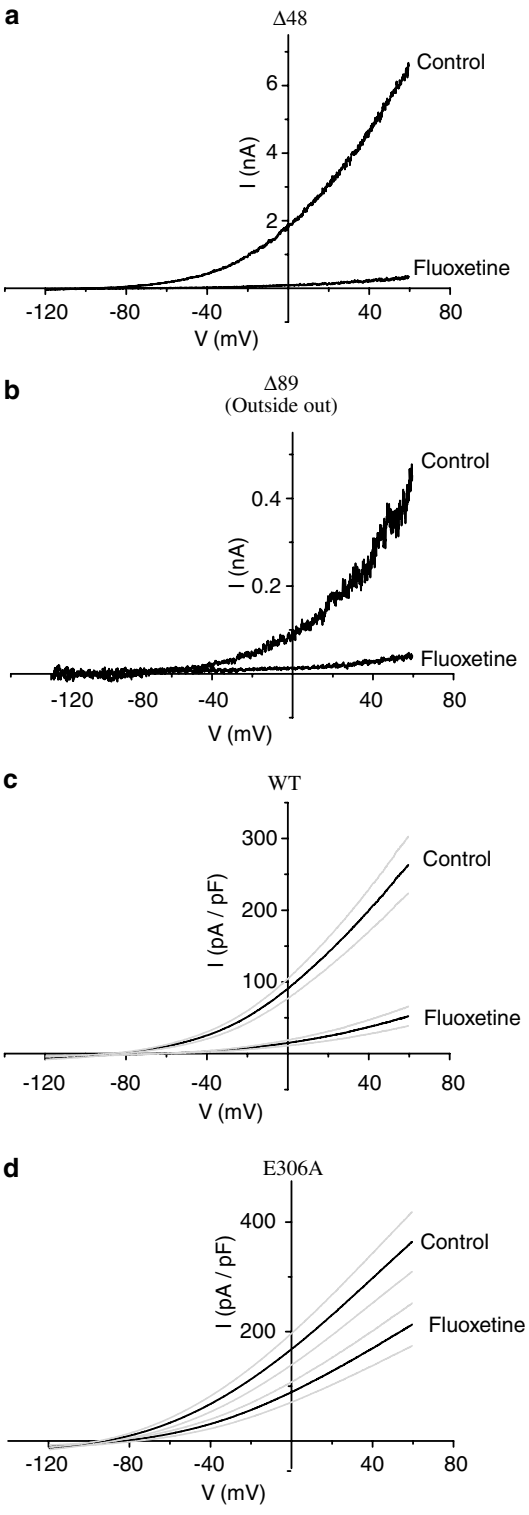

Figure 6 Fluoxetine inhibition of TREK-1 is unaffected by C-terminus truncations but reduced for E306A-mutated channels. (a) Whole-cell currents obtained by ramp changes in voltage for Δ48 TREK-1 channels in the presence and absence of fluoxetine (100 μM). (b) Current obtained in an outside-out patch recording for Δ89 TREK-1 channels in the presence and absence of fluoxetine (100 μM). The intracellular pH for this recording was set to 5.0. (c and d) Average current density *versus* voltage plots for wild-type (c) and E306A (d) TREK-1 channels in the presence and absence of fluoxetine (100 μM).

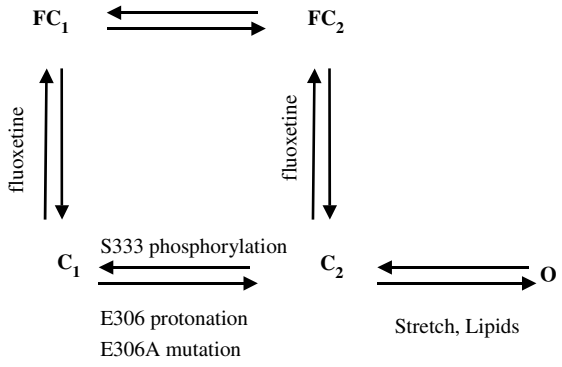

Figure 7 Model for TREK-1 gating (adapted from Honore *et al*., 2002).

the channel may be 'stuck' in C_1 and unable to reach O. Application of internal acidosis promotes the transition to C_2, and restores the equilibrium between the two closed states. Channel function and inhibition by fluoxetine are therefore restored as observed. It is also possible to recover the truncated channel function by introducing the E306A mutation. This is proposed to work in the same manner as internal acidosis. However, it is evident that the Δ89(E306A) mutant of TREK-1 is unable to restore wild-type behaviour precisely, as the inhibition by fluoxetine is lower than in the wild type.

Site of action of fluoxetine

There is mounting evidence that fluoxetine, like other antidepressant agents (see Mathie *et al*., 1998), can inhibit a variety of neuronal ion channels including voltage-gated Ca^{2+} channels (Deak *et al*., 2000), Na^{2+} channels (Pancrazio *et al*., 1998) and various K_V channels (Choi *et al*., 1999; 2001; Yeung *et al*., 1999). For all of these channels, an exact mechanism of block has yet to be determined, but there has been some evidence to suggest that in $K_V1.3$ channels, fluoxetine acts at an intracellular site to cause an open-channel block (Choi *et al*., 1999). In addition, it has been shown that norfluoxetine inhibits $K_V1.3$ channels with a higher potency than fluoxetine (Choi *et al*., 1999). A similar channel-blocking mechanism has been proposed for the inhibition of $K_V3.1$ by norfluoxetine (Choi *et al*., 2001) and, indeed, for the inhibition of HERG K channels by fluoxetine (Thomas *et al*., 2002). This is not, however, a universal mechanism. For example, Yeung *et al*. (1999) showed that block of native K_V channels in cerebellar granule cells by fluoxetine was neither voltage- nor use-dependent.

For TREK-1 channels, block by fluoxetine is not voltage-dependent. Furthermore, fluoxetine is less effective at blocking the E306A-mutated TREK-1 channels despite the channels having a higher open probability (see above and Honore *et al*., 2002). This suggests that the blocking site for fluoxetine in TREK-1 channels is unlikely to be in the channel pore itself and argues against an open-channel block mechanism for fluoxetine in this case.

The block of TREK-1 by fluoxetine also does not seem to involve the C-terminus of the channel. Truncation of the C-terminus by 48 or 89 amino acids, while interfering with channel function, did not appear to influence the degree of channel block by the drug. This can be contrasted with the effects of volatile anaesthetic agents such as chloroform and

halothane (Patel *et al*., 1999), and gaseous anaesthetic agents such as cyclopropane (Gruss *et al*., 2004a). Augmentation of TREK-1 currents by these agents was either completely abolished (Patel *et al*., 1999) or considerably reduced (Gruss *et al*., 2004a) by progressive deletion of the C-terminus of TREK-1.

While our data suggest that fluoxetine does not bind to either the pore region of TREK-1 channels or the C-terminus, it is not clear, at present, where fluoxetine does bind. Being a lipophilic compound, one intriguing possibility is that the mechanism of fluoxetine block may be analogous to that of antipsychotic agent chlorpromazine, which has been suggested to block TREK-1 channels by entering the lipid membrane and acting as a cationic amphipathic cup-former (Patel *et al*., 1998), thereby changing the curvature of the lipid bilayer. In this regard, it is of interest that both the high-affinity block of the voltage-dependent K channel (KvAP) and the block of a stretch-activated ion channel by two different toxins (VSTX1) and (GsMTx4) isolated from the venom of the tarantula *Grammostola spatulata* have been attributed to the ability of these toxins to partition into the lipid membrane (Lee & MacKinnon, 2004; Suchyna *et al*., 2004), a phenomenon termed 'mechanopharmacology' (Suchyna *et al*., 2004).

Possible clinical consequences of fluoxetine block of TREK-1 channels

The therapeutic plasma concentration of fluoxetine is estimated as between 0.15 and 1.5 μM (see Orsulak *et al*., 1988; Altamura *et al*., 1994). Under steady-state conditions, the plasma concentrations of norfluoxetine are higher than those of fluoxetine (Hiemke & Hartter, 2000) and, for both compounds, can reach much higher levels in the brain during chronic fluoxetine treatment (Karson *et al*., 1993). Fluoxetine is preferentially distributed from plasma into brain tissue because of its high lipophilicity (Karson *et al*., 1993). In addition, both compounds have long half-lives of elimination (4–6 days for fluoxetine and 4–16 days for norfluoxetine), so these compounds persist in the body for several weeks following discontinuation of treatment. Recent fluorine magnetic resonance spectroscopy measurements of fluoxetine have shown that the free concentration throughout the whole brain is around 5 μM for adults on an average dose of 24 mg per day (Strauss *et al*., 2002). It is worth noting that many patients take considerably higher doses than this; the recommended daily dose for major depressive episodes and obsessive compulsive disorder is 20–60 mg per day, while for bulimia nervosa it is 60 mg per day (www.emc.medicines.org.uk; summary of product characteristics (SPC) for Prozac^R). Thus, the steady-state concentrations of both fluoxetine and norfluoxetine in the brain are sufficient to produce significant block of TREK-1 channels.

Fluoxetine is thought to exert its antidepressant effects by selectively inhibiting the reuptake of 5-hydroxytryptamine (5-HT, serotonin) from the synaptic cleft (Wong *et al*., 1995). Fluoxetine inhibits 5-HT uptake in synaptosomal preparations of rat brain with a K_i of 8–20 nM, while norfluoxetine has a K_i of 17–45 nM (Wong *et al*., 1995). It is possible, however, that block of TREK-1 channels may contribute to the antidepressant action of this drug. Antidepressant drugs have been reported to decrease hypothalamic temperature and it has been suggested that this function, during sleep, may contribute to the antidepressant action of at least some of these agents (Duncan *et al*., 1995). Since TREK-1 is a heat-activated channel, which is highly expressed in the hypothalamus (Maingret *et al*., 2000a), it is possible that fluoxetine block of TREK-1 contributes to its antidepressant action. In addition, there are moderate levels of TREK-1 expression in the serotonergic, raphe nuclei, but little expression in the noradrenergic, locus ceruleus (Talley *et al*., 2001; Gu *et al*., 2002).

More likely, however, is that block of TREK-1 channels by fluoxetine and norfluoxetine may contribute to a number of the known side effects of fluoxetine treatment. Recent experiments on TREK-1 knockout mice have demonstrated the importance of these channels in neuroprotection against epilepsy and ischaemia, and in underlying at least part of the action of certain anaesthetic agents such as the volatile anaesthetics (Heurteaux *et al*., 2004). Since fluoxetine blocks TREK-1 channels, this may exacerbate any tendency to convulsions and may underlie the warning that fluoxetine should be 'introduced cautiously' in patients who have a history of seizures and avoided in patients with unstable seizure disorders/epilepsy (www.emc.medicines.org.uk; SPC for Prozac^R). This presents a particular management problem for clinicians, since epileptic patients often also suffer from depression (Salzberg & Vajda, 2001).

In conclusion, this study has shown that fluoxetine and norfluoxetine are inhibitors of the 2-PK channel TREK-1 and may block this channel in treated patients. Our results obtained from both wild-type and mutant TREK-1 channels are consistent with the functional model of TREK-1 gating proposed by Honore *et al*. (2002).

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