Inhibition of neuronal K_V potassium currents by the antidepressant drug, fluoxetine

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1 The effect of the antidepressant drug, fluoxetine on neuronal delayed rectifier (K_V) potassium (K) currents was investigated using perforated-patch whole-cell electrophysiological recording methods.

2 Fluoxetine was an effective inhibitor of K_V currents in cerebellar granule neurons (CGNs) and also inhibited recombinant K_V1.1 channels expressed in Chinese hamster ovary (CHO) cells.

3 Fluoxetine had an IC_{50} of 11 μM in CGNs but was slightly less potent on K_V1.1 channels ($IC_{50} = 55$ μM). Interestingly, fluoxetine was a much more potent inhibitor of K_V1.1 expressed in mammalian cells than has been found previously for the same homomeric channel expressed in *Xenopus* oocytes.

4 At concentrations that produced around 50% block, the shape of the K_V currents in the presence of fluoxetine was simply scaled down when compared to control currents.

5 The effect of fluoxetine on K_V currents in CGNs was neither voltage-dependent nor dependent on the channels being in their open state. Both of these observations suggest that fluoxetine does not act as a simple open channel blocking agent.

6 It is concluded that block of K_V currents in mammalian neurons can occur at therapeutic levels of fluoxetine. This could lead to an increase in neuronal excitability and this effect may contribute to the therapeutic antidepressant action of fluoxetine.

Keywords: K_V; potassium current; fluoxetine; cerebellar granule neurons; K_V1.1

Abbreviations: CGN, cerebellar granule neurons; CHO, Chinese hamster ovary; FCS, foetal calf serum; K_V, delayed rectifier currents; K_A, A-type currents; MEM, minimum essential medium; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant

Introduction

Potassium (K) channels are among the most important signalling macromolecules in the nervous system. K channel activity determines the shape, frequency and duration of neuronal action potentials (Armstrong & Hille, 1998). In addition, K channels have a major role in the setting of the resting membrane potential of a neuron, which in turn may profoundly regulate neuronal excitability (Halliwell, 1990; Hille, 1992; Roeper & Pongs, 1996; Mathie *et al.*, 1998). A diverse number of K channels have been identified in different neurons by functional electrophysiological studies (Cull-Candy *et al.*, 1989; see also Rudy, 1988). These include the delayed rectifier currents (K_V), A-type currents (K_A), calcium-activated currents, inward rectifiers and M currents (Mathie *et al.*, 1998). Mutations of specific K channels in both animals and man have been connected to certain CNS disorders, such as epilepsy (see review by Sanguinetti & Spector, 1997).

There are a number of compounds which act to inhibit neuronal K channels, thereby altering the electrical properties of the neurons. Block of K channels has been proposed as a potential therapeutic mechanism in the treatment of certain clinical disorders such as myaesthenia gravis, multiple sclerosis, Huntington's chorea and Alzheimer's disease. However such an action is more often regarded as underlying some of the more serious side effects (such as convulsions and dysrythymias) of a number of compounds currently in therapeutic use (Mathie *et al.*, 1998). For example, previous

work from our laboratory has shown that a number of clinically used antidepressant drugs such as amitriptyline and imipramine, are potent inhibitors of certain K^+ currents in mammalian neurons, in particular the delayed-rectifier current, K_V (Wooltorton & Mathie, 1993; 1995).

In this study, the effects on K_V currents of a further clinically used antidepressant, fluoxetine, have been investigated. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), has been reported to have some blocking action on native K_V currents in cultured human lens epithelium (Rae *et al.*, 1995), on K_V currents in PC12 cells; which are often regarded as a good neuronal model cell line; (Hahn *et al.*, 1999), and on cloned K_V channels expressed in *Xenopus* oocytes (Tytgat *et al.*, 1997). In this study, we have examined the effects of fluoxetine on K_V currents found in native neurons in the mammalian central nervous system and compared the potency of these effects with that seen in a more artificial system. Firstly, we have studied its effects on K_V currents in rat cerebellar granule neurons (CGNs). These cells have been shown to have transient and sustained components to their whole-cell current which correspond to K_A and K_V conductances (e.g. Cull-Candy *et al.*, 1989; Jalonen *et al.*, 1990; Bardoni & Belluzzi, 1994; Watkins & Mathie, 1994). These can be studied in relative isolation with appropriate voltage protocols (see Methods and Watkins & Mathie, 1994). We have also examined the effects of fluoxetine in Chinese hamster ovary (CHO) cells permanently transfected with K_V1.1 channels. We have chosen this particular recombinant channel for two reasons. Firstly, CGNs are known to express high levels of the K_V channel subunit K_V1.1 (Veh *et al.*, 1995). Secondly, experiments using a behavioural model of anti-

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depressant action have suggested that inhibition of expression of K$_V$1.1 channels using antisense oligonucleotides has the same effect as treatment with antidepressant drugs such as amitriptyline and imipramine (Galeotti *et al.*, 1999).

Some of these results have been presented in abstract form (Yeung *et al.*, 1998).

Methods

Cell preparation

Cerebellar granule neurons (CGNs) CGNs were isolated and cultured from 6–9 day old rats, of either sex, as previously described (Watkins & Mathie, 1996). Briefly, animals were killed by decapitation, the cerebellum removed and dissociated with a series of enzymatic solutions (trypsin, trypsin inhibitor and DNAse) and then centrifuged. The cells were subsequently resuspended in MEM (minimum essential medium) containing 10% FCS (foetal calf serum), 2.5% chick embryo extract, 25 mM KCl, 39 mM glucose, 2 mM L-glutamine and 50 IU ml^{-1} penicillin/50 μg ml^{-1} streptomycin antibiotic solution. The resulting CGN cell suspension was plated onto poly-L-lysine coated coverslips (80 μl per coverslip) to give a density of 10^6 cells ml^{-1}. The coverslips were then incubated at 37°C in a humidified atmosphere of 95% O$_2$ and 5% CO$_2$. The cells were supplemented with 0.5 ml MEM and 10% FCS the next day, but were not used for at least 4 days post culture.

CHO cells expressing K$_V$1.1 CHO cells expressing the recombinant K$_V$1.1 channel, were kindly supplied by Dr David Owen (CeNeS Ltd.). Cells were grown in tissue culture in 25 ml flasks in a medium containing RPMI medium supplemented with 5% FCS, 4.1 mM L-glutamine, 50 mg ml^{-1} geneticin and 50 IU ml^{-1} penicillin/50 μg ml^{-1} streptomycin antibiotic solution. The flasks were maintained in an incubator at 37°C in a humidified atmosphere of 95% O$_2$ and 5% CO$_2$. The cells were diluted 1 in 10 every 3 days, plated onto poly-L-lysine coated coverslips and allowed to grow for at least 2 days prior to recording.

Solutions

The bathing (external) solution contained (mM): NaCl 120, KCl 2.5, MgCl$_2$ 2, CaCl$_2$ 0.5, glucose 5, HEPES 10, adjusted to pH 7.4 with NaOH. The pipette (internal) solution contained (mM): KCl 125, MgCl$_2$ 5, HEPES 5, BAPTA 0.1, adjusted to pH 7.4 with KOH. Both solutions were made up in distilled water.

Stock solutions of fluoxetine (HCl) were also made up in distilled water, and stored at −20°C until required. Perfusion solutions of fluoxetine were made up using the external solution, immediately before the experiment. Solutions were continuously perfused through the bath during recording at a rate of approximately 4–5 ml min^{-1}.

Reagents were purchased from Sigma Chemical Company and Life Technologies (Gibco).

Current recordings and analysis

Currents were recorded in the whole cell perforated patch-clamp configuration by use of an Axopatch-1D amplifier (Axon Instruments, Foster City) and amphotericin B (240 μg ml^{-1}) as the permeabilizing agent. All experiments were performed at room temperature. Patch pipettes were

made from borosilicate glass, which had resistances between 5 and 10 MΩ.

Voltage protocols The following two protocols were used to examine K$_V$ in isolation in CGNs and CHO cells expressing K$_V$1.1.

Protocol 1: Cells were held at −70 mV, prepulsed to −50 mV for 30 ms before being stepped to a depolarizing test potential of +10 mV for 150 ms once every 6 s to evoke the sustained K$_V$ current, before being stepped back to −50 mV for 30 ms and then back to the holding potential.

Protocol 2: Cells were held at −70 mV, prepulsed to −50 mV for 30 ms before being stepped to the test potentials of −40 to +40 mV in 10 mV increments. Each test potential was applied once every 6 s, before being stepped back to −50 mV for 30 ms then back to the holding potential.

For both protocols, the amplitude of K$_V$ current was measured as a mean over 15 ms, 134 ms following the step to +10 mV (except during experiments to generate current-voltage relationships when the step potential varied). For some cells, tail current relaxations following the step back to −50 mV were measured to give complete isolation of the K$_V$ current component. Linear components of leak current were calculated from the prepulse step from −70 to −50 mV and were subtracted off-line where appropriate.

Currents were low-pass filtered at 5 kHz and recorded and analysed using an IBM-compatible PC, pClamp (version 6.0.3) with a Digidata 1200 interface (Axon Instruments), Excel 97 (Microsoft) and Origin version 3.5 (Microcal). Results were expressed as means ± standard error of means (s.e.mean) and *n* as the number of cells. Statistical analyses were performed using Student's *t*-test. Results were considered significant at the $P < 0.05$ level.

Results

Inhibition of K$_V$ in CGNs by fluoxetine

The delayed rectifier, K$_V$, can be studied in relative isolation using a particular voltage protocol which is described in the Methods (protocol 1). The cells were held at a potential of −70 mV, prepulsed to −50 mV for 30 ms to obtain a measure of any background or leak currents (and during which K$_A$ channels are inactivated but K$_V$ channels remain closed), prior to stepping the current to a depolarizing potential of +10 mV for 150 ms to activate the K$_V$ current. The K$_V$ current was revealed by its distinctive profile, namely, a sustained current at a depolarized potential of +10 mV, which showed little or no inactivation over the duration of the step (see also Watkins & Mathie, 1994). This sustained component is blocked by tetraethylammonium ions and is sensitive to 4-aminopyridine (Watkins & Mathie, 1994).

K$_V$ was inhibited by fluoxetine, as illustrated in Figure 1. It can be seen that the inhibition of K$_V$ by fluoxetine was concentration-dependent. Figure 1A–C shows inhibition of K$_V$ by 3, 30 and 100 μM fluoxetine. As the concentration of fluoxetine added to the bath increased, the rate of inhibition increased. The time course of inhibition of K$_V$ in CGNs by 10 μM fluoxetine is illustrated in Figure 1D. With washout, there was some reversibility of the block at this concentration. The concentration-response relationship for block of the K$_V$ current by fluoxetine in CGNs is illustrated in Figure 1E. Concentrations of 3, 10, 30, 100 and 300 μM inhibited K$_V$ by 24±3% (mean±s.e.mean; *n*=6), 45±3% (*n*=6), 60±5% (*n*=7), 78±2% (*n*=5) and 83% (*n*=1) respectively. The fitted

curve gave an IC$_{50}$ for fluoxetine of 11 μM and a slope of 0.94. When the maximum response attainable was constrained to 83%, the fitted curve gave an IC$_{50}$ of 12.5 μM and a slope of 1.24. At concentrations producing close to 50% inhibition of the K$_V$ current, the current profile was not altered, the current was simply scaled down in the presence of the drug.

Inhibition of K$_V$ in CGNs is not voltage-dependent

Current-voltage relationships were obtained in CGNs using protocol 2 in the absence and presence of 10 μM fluoxetine (a concentration close to the IC$_{50}$ for fluoxetine in these cells) and are shown in Figure 2A. The control plot demonstrates a non-linear increase in current at voltage steps to potentials more depolarized than -20 mV. It appears that this voltage is the threshold for activation of K$_V$ channels in these cells (see also Watkins & Mathie, 1994). The percentage inhibition of K$_V$ by 10 μM fluoxetine was measured at test potentials between 0 and $+40$ mV. Mean data obtained from 12 cells showed no significant difference across the voltages (Figure 2B); for example, at a test potential of 0 mV, current was inhibited by 37 ± 6%, while at $+40$ mV, inhibition was 42 ± 4% ($n=12$). These results suggest that block of K$_V$ in CGNs by fluoxetine shows little voltage-dependence.

Inhibition of K$_V$ in CGNs does not require the channels to be open

For this set of experiments, a slightly modified version of protocol 1 was used to distinguish whether the block by fluoxetine depended on K$_V$ channels being in their open state. This was determined by ceasing the depolarizing pulses (and thus the opening of K$_V$ channels) during application of the drug. This is illustrated for a single experiment in Figure 3A,B.

The time course of the experiment is shown in Figure 3B. Depolarizing test pulses were stopped for 3 min and fluoxetine was simultaneously added to the bath. In control experiments (see Figure 1D), 3 min is sufficient to obtain maximal block by 10 μM fluoxetine. Immediately on resumption of pulses, block of K$_V$ was found to be fully established and was 51% in this cell (Figure 3B). In six cells, using an identical protocol, block of K$_V$ was 41 ± 5%. Thus block of K$_V$ in CGNs by fluoxetine occurs in the absence of depolarizing pulses, i.e. block occurs despite the channels being in the closed state.

Inhibition of K$_V$1.1 in CHO cells

The effects of fluoxetine on recombinant K$_V$1.1 channels were also investigated, as illustrated in Figures 4 and 5.
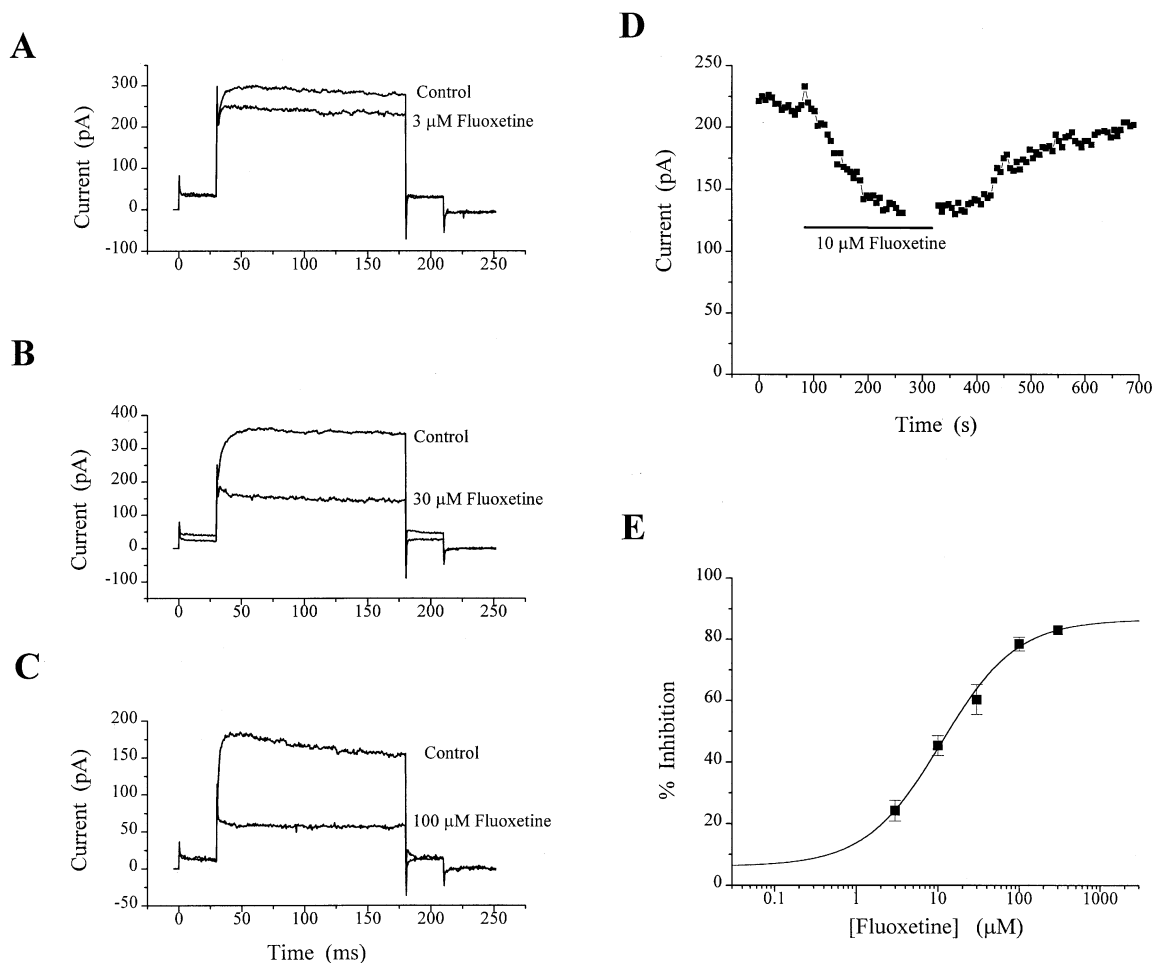
Figure 1 Concentration-dependent inhibition of K$_V$ currents in CGNs by fluoxetine. In the representative cells (A–C), 3, 30 and 100 μM fluoxetine produced 17, 59 and 63% inhibition respectively. Currents were activated by protocol 1 (see Methods). Each trace is the average current of four individual traces and all have been leak subtracted off-line. A time course plot in response to 10 μM fluoxetine is shown in (D). Each point is an average current taken over 15 ms, commencing 134 ms following the test step to $+10$ mV. The gap in the plot represents time during which an I-V relationship was obtained. The concentration-response curve for fluoxetine is shown in (E). Data points were fitted with a power function, giving a calculated IC$_{50}$ value of 11 μM, with a slope of 0.94 and a maximal inhibition of 87%.

A

B
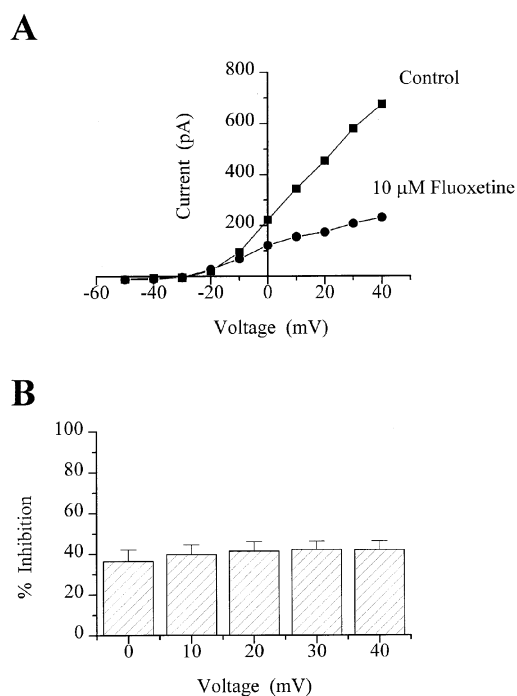
Figure 2 Inhibition of K_V in CGNs showed little or no voltage-dependence. Currents were activated by protocol 2. The current-voltage relationship (A) shows current amplitudes in the absence and presence of 10 μM fluoxetine in a single CGN. (B) shows the percentage inhibition of K_V by fluoxetine in 12 cells over the voltage range of 0 to +40 mV.

Recordings from CHO cells expressing $K_V1.1$ shows the classic delayed rectifier type profile (see also Robertson & Owen, 1993). Also illustrated is the time course of fluoxetine inhibition of $K_V1.1$ channels in these cells. It is clear that fluoxetine (10–300 μM) is not as potent at inhibiting recombinant $K_V1.1$ channels as it is on K_V in CGNs (Figure 4A–D; compare with Figure 1). Block of $K_V1.1$ with 30 μM was rapid, and was fully reversible (Figure 4C). However at the highest concentration used in CHO cells (300 μM) the inhibition was extremely rapid but irreversible over the time course of the recording (not shown).

A concentration-response curve for fluoxetine inhibition of $K_V1.1$ was obtained for concentrations of fluoxetine between 10 and 300 μM (Figure 4D). At concentrations of 10, 30, 50, 100 and 300 μM fluoxetine, inhibitions of the $K_V1.1$ current were calculated to be $7\pm1\%$ ($n=3$), $20\pm1\%$, ($n=3$), $48\pm6\%$ ($n=11$), $70\pm1\%$ ($n=3$) and $82\pm2\%$ ($n=3$) respectively. The inhibition of $K_V1.1$ at 10 and 30 μM was significantly less ($P<0.001$) than that seen for K_V in CGNs. The fitted data here gave an IC$_{50}$ of 55 μM (or 53 μM when the maximum response was constrained to 82%), which is significantly higher than that calculated for K_V in CGNs.

Application of 50 μM fluoxetine (as with the CGN experiments, a concentration close to the IC$_{50}$ for fluoxetine in these cells) produced an obvious decrease in current amplitude across a range of test voltages (Figure 5A–C) whether measuring absolute currents at the test potential or tail-current relaxations on stepping back to -50 mV (see Methods). The mean inhibition for three cells at test potentials ranging from -10 to $+40$ mV is shown in Figure 5D. Currents were inhibited by $46\pm9\%$ at -10 mV but $62\pm9\%$ at $+40$ mV.

A

B
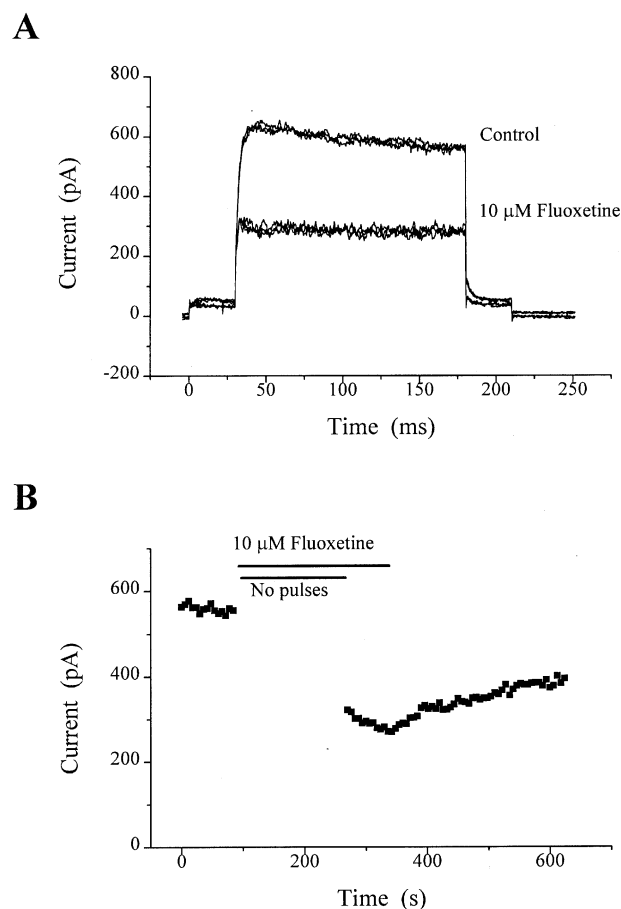
Figure 3 Inhibition of K_V in CGNs by 10 μM fluoxetine does not require the channels to be open. Currents were activated by adapting protocol 1 (see Results). Current recordings in (A) were taken under control and drug conditions. The latter represents maximal inhibition. Only three traces have been shown for clarity, which have not been leak subtracted. The time-course relationship (B) illustrates the protocol more clearly, and inhibition of K_V by fluoxetine during the 3 min period devoid of depolarizing pulses to +10 mV. This particular cell produced a 51% inhibition. Some recovery was possible upon wash-out.

Discussion

In this study, it has been shown that fluoxetine is an effective inhibitor of the K_V current in CGNs and that it also inhibited recombinant $K_V1.1$ channels expressed in CHO cells. The block of K_V currents in CGNs by fluoxetine is the first demonstration of the action of this compound on K_V currents in native mammalian neurons, however, the effects of fluoxetine have been investigated on K channels and other ion channels present in non-neuronal cells and in cell lines. It has been reported previously that fluoxetine blocks native K_V currents in human lens epithelium (Rae *et al.*, 1995) and K currents in both canine and human jejunal circular smooth muscle cells (Farrugia, 1996). Much less potently, fluoxetine (IC$_{50}$ of 600–700 μM) inhibits $K_V1.1$ channels transfected into *Xenopus* oocytes (Tytgat *et al.*, 1997). In addition fluoxetine inhibits K^+, Na^+ and Ca^{2+} currents in PC12 cells (Hahn *et al.*, 1999), while Pancrazio *et al.* (1998) reported that fluoxetine was as effective as tricyclic antidepressants (TCAs; e.g. amitriptyline) at inhibiting the Na^+ currents in bovine adrenal chromaffin cells. It has been shown by Lavoie *et al.* (1997) that fluoxetine is capable of inhibiting calcium uptake *via* calcium channels in rat hippocampal synaptosomes and more recently,
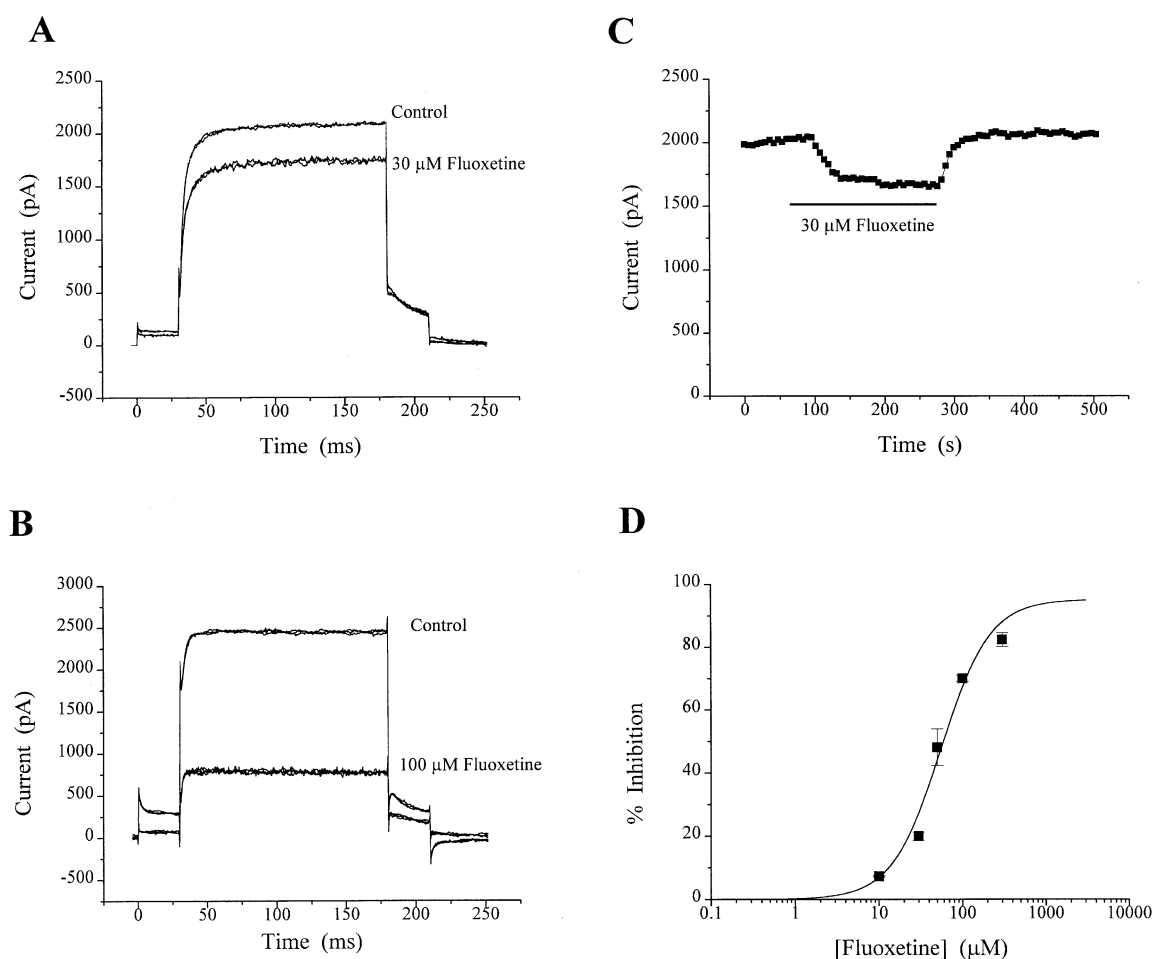
Figure 4 Inhibition of recombinant K$_V$1.1 in CHO cells by fluoxetine. Currents were activated by protocol 1. (A) shows recordings of control currents and the maximal inhibition by 30 μM fluoxetine. The corresponding time-course plot for this cell (C) shows rapid onset of action with full recovery of the block. Another current recording is shown in (B), but in response to 100 μM fluoxetine. (D) illustrates the concentration-response curve to fluoxetine. Each data point is the average inhibition of at least three cells. The IC$_{50}$ was calculated to be 55 μM, with a slope of 1.49 and a maximal inhibition of 95%.

it was reported by Maertens *et al.* (1999) that fluoxetine produces a reversible block of both the volume-sensitive chloride current and the Ca^{2+}-activated Cl$^-$ current in cultured bovine pulmonary artery endothelial cells. Thus fluoxetine has previously been shown to have blocking actions on a number of different ion channel types in non-neuronal cells and in cell lines.

A number of compounds, such as tetrapentylammonium ion and clofilium (French & Shoukimas, 1981; Reeve & Peers, 1992) block K$_V$ channels by binding to a site inside the pore of the channel and acting as open-channel blocking agents. For fluoxetine, however, the relatively consistent amount of block of K$_V$ currents in CGNs observed across the range of positive potentials tested suggests that it does not have a preference for open channels. In addition in the absence of depolarizing pulses, inhibition of the K$_V$ current by fluoxetine was still achieved. Again, this implies that fluoxetine can act to block channels whether they are open or closed.

Rae *et al.* (1995) suggested that the block of K$_V$ currents in human lens epithelium was due to an action of fluoxetine on segments of the channel located intracellularly, however, Maertens *et al.* (1999) altered the proportion of uncharged to charged fluoxetine by changing extracellular pH and found that it was the uncharged form of the molecule which mediates block of the voltage-regulated anion channel in endothelial cells and suggested that the most likely mechanism of action of

fluoxetine was that it had hydrophobic interactions with the channel within the membrane bilayer. The results in this study with fluoxetine are similar to those obtained in previous work on inhibition of neuronal K$_V$ currents by certain TCAs and related compounds (Wooltorton & Mathie, 1993; 1995). Wooltorton & Mathie (1995) demonstrated that amitriptyline and its permanently charged analogue, N-methylamitriptyline, when applied internally, had no effect on the K$_V$ current. Furthermore, Kuo (1998) showed that imipramine and a number of structurally related compounds inhibit the transient K$_A$ current of rat hippocampal neurons and hypothesized that there is a receptor for imipramine which consists of an aliphatic and aromatic site on the external side of the channel pore. By analogy with these experiments on TCAs, it seems reasonable to suggest that fluoxetine may act either by binding to an extracellular site on the channel or a hydrophobic site within the lipid bilayer. It seems less likely that fluoxetine acts intracellularly by gaining access to the channel region *via* the membrane. Finally, although unlikely, we cannot completely rule out the possibility that direct block of the K$_V$ channel by fluoxetine and related compounds may not be the only method of inhibition (see Farrugia, 1996). Intracellular biochemical processes such as tyrosine phosphorylation may also be affected which may, in turn, reduce the activity of K$_V$ channels (e.g. Lev *et al.*, 1995). However, in experiments using conventional whole-cell recording we found the same degree

A

B

C

D
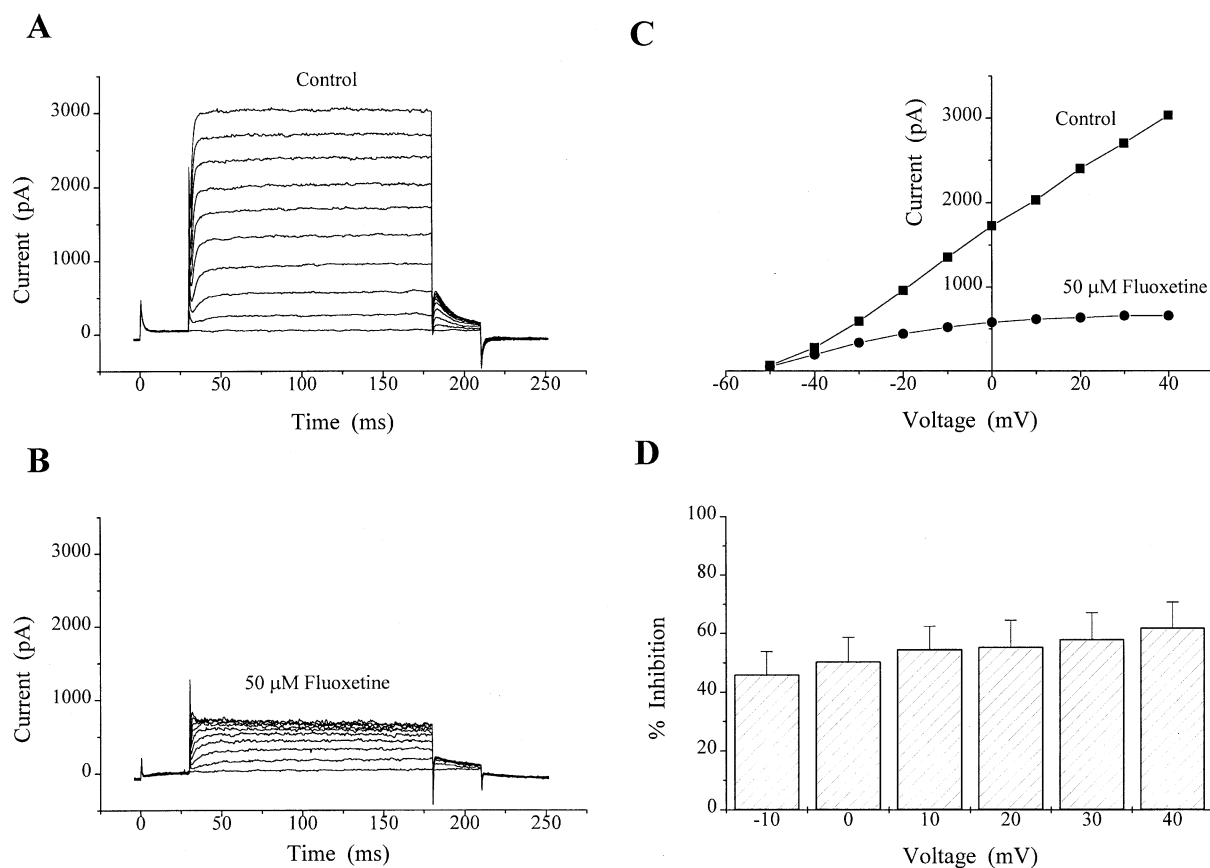
Figure 5 Fluoxetine inhibition of K$_V$1.1 at different voltages. Currents were evoked using protocol 2. (A and B) illustrate current recordings in the absence and presence of 50 μM fluoxetine in a single CHO cell. The corresponding current-voltage relationship is shown in (C). The degree of inhibition over the voltage range -10 to $+40$ mV for three cells is shown in (D).

of inhibition as that seen in perforated patch recordings which suggests that a diffusional biochemical messenger is unlikely to be involved.

The exact subunits constituting the K$_V$ channels in CGNs have not been identified, and the genes encoding many different K$_V$ channels have been found to be expressed to varying degrees throughout the developing rat brain (Kues & Wunder, 1992). However, since K$_V$1.1 was found to be particularly abundant in the granule layer of the cerebellum (Veh *et al.*, 1995), it was of some interest to compare the block by fluoxetine of native K$_V$ currents with that of recombinant K$_V$1.1 channels. When challenged with fluoxetine, block of K$_V$1.1 channels was observed but the drug was less potent than in CGNs (IC$_{50}$ = 55 μM compared to 11 μM). Variations between the sensitivity of recombinant K$_V$1.1 channels and K$_V$ channels in CGNs, may indicate that either a different subunit, or more likely, a more complex subunit composition exists in the native cells.

The effects of fluoxetine on K$_V$1.1 currents have previously been studied in *Xenopus* oocytes (Tytgat *et al.*, 1997). These authors showed that the inhibition of K$_V$1.1 by fluoxetine was much less potent (IC$_{50}$ between 600 and 700 μM at 0 mV) than observed in this study for K$_V$1.1 in a mammalian expression system. Moreover, while inhibition of K$_V$1.1 by fluoxetine was reversible in the mammalian expression system this was not the case in *Xenopus* oocytes. Thus there is a difference in potency of fluoxetine on K$_V$1.1 channels depending on the recombinant system used. It is possible that fluoxetine has easier access to the channel components in mammalian cells.

In man, following therapeutic doses of fluoxetine, the drug concentrations present in both plasma and brain have been measured. It was found that the latter contains approximately 20 times as much as in the blood (reaching levels ranging from 5 to 70 μM, see Karson *et al.*, 1993; Altamura *et al.*, 1994). This suggests that substantial block of K$_V$ currents in mammalian neurons can occur at therapeutic levels of fluoxetine and could lead to an increase in neuronal excitability. In clinical use, one noted side effect of fluoxetine in the CNS is that it can induce convulsions and the use of fluoxetine is cautioned in epilepsy and during concurrent electroconvulsive therapy. Furthermore, it is of some interest that Galeotti *et al.* (1999), using an established animal model to indicate antidepressant activity, have shown that inhibiting the expression of K$_V$1.1 channels through the use of antisense oligonucleotides has the same effect as treating animals with antidepressant drugs such as imipramine and amitriptyline. This suggests that block of K$_V$ channels such as K$_V$1.1 by antidepressant drugs, an effect which has been observed for all such drugs so far examined (see Mathie *et al.*, 1998), may be an important contributory factor to their antidepressant action.

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