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Effect of fluoxetine on a neuronal, voltage-dependent potassium channel (Kv1.1)

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1 Fluoxetine (Prozac) is widely used as an antidepressant drug and is assumed to be a selective 5-hydroxytryptamine (5-HT) reuptake inhibitor (SSRI). Claims that its beneficial psychotropic effects extend beyond those in treatment of depression have drawn clinical and popular attention to this compound, raising the question of whether there is anything exceptional about the supposed selective actions.

2 We have used the voltage clamp technique to study the effect of fluoxetine on a neuronal, voltage-dependent potassium (K^+) channel (RCK1; Kv1.1), expressed in *Xenopus laevis* oocytes. This channel subunit is abundantly expressed in the central nervous system and K^+ channels containing this subunit are involved in the repolarization process of many types of neurones.

3 Blockade of the K^+ currents by fluoxetine was found to be use- and dose-dependent. Wash-out of this compound could not be achieved. Fluoxetine did not affect the ion selectivity of this K^+ channel, as the reversal potential was unaltered.

4 Slowing of both activation and deactivation kinetics of the channel by fluoxetine was observed, including tail current crossover upon repolarization.

5 Hodgkin-Huxley type of models and more generalized Markov chain models were used to fit the kinetics of the data. Based upon a Markov kinetic scheme, our data can be interpreted to mean that blockade of fluoxetine consists of two components: a voltage-independent occurring in the last closed, but available state of the channel, and a voltage-dependent occurring in the open state.

6 This study describes the first biophysical working model for the mechanism of action of fluoxetine on a neuronal, voltage-dependent K^+ channel, RCK1. Although this channel is not very potently blocked by fluoxetine when expressed in oocytes, this study may help us to understand some of the clinical symptoms seen with elevated serum concentrations of this SSRI.

Keywords: Fluoxetine; potassium channels; oocytes; voltage clamp

Introduction

Fluoxetine, HCl (Prozac, Flu) a substituted propylamine prescribed for the treatment of depression, is assumed to derive its antidepressant activity through its ability to block the reuptake of 5-hydroxytryptamine (5-HT), with minimal effect on other neurotransmitter reuptake systems (Richelson & Pfenning, 1984; Wong *et al*., 1995). The potency of this blockade has been used to designate Flu, along with its congeners sertraline, paroxetine and fluvoxamine, as a selective 5-HT reuptake inhibitor (SSRI) (Asberg *et al*., 1986). During the last couple of years, Flu has acquired public awareness that is not evident with other SSRIs. Claims that its beneficial psychotropic effects extend beyond those in treatment of depression, like obsessive compulsive disorders (OCDs) and bulimia nervosa (Wong *et al*., 1995), have drawn further clinical and popular attention to this compound. This raises the question of whether there really is anything exceptional about the supposed selective actions of Flu on the 5-HT-mediated system. In particular, several other possible effects of Flu ($\geqslant 5\ \mu$M) have been suggested, like inhibition of [^{3}H]-5-HT release from rat spinal cord and cortical synaptosomes via Ca^{2+} channel inhibition (Stauderman *et al*., 1992), and partial inhibition of α_1-adrenoceptors, muscarinic, dopamine D_2 and histamine H_1 receptors (Wong *et al*., 1983; Richelson & Nelson, 1984; Cusack *et al*., 1994). Furthermore, it was found that Flu ($10-100\ \mu$M) blocks a potassium-dependent component of Cl^--originated short-circuit current in the frog corneal epithelium (Zamudio & Candia, 1993), and that the compound also inhibits several types of voltage-dependent K^+ and Na^+ channels in rabbit corneal epithelium (Rae *et al*., 1995).

Although higher doses of Flu may be needed to observe the different types of effects, in contrast to the dose needed for a SSRI effect, it is clear that the drug can have numerous and complicated mechanisms of action with different types of receptors and ion channels. Here we used the voltage clamp technique to study the effect of Flu on a neuronal, voltage-dependent K^+ channel, RCK1(Kv1.1), belonging to the *Shaker* subfamily, expressed in oocytes of *Xenopus laevis*. Since this channel subunit is abundantly expressed in the central nervous system and primarily involved in the repolarization of many types of neurones (Wang *et al*., 1994; Veh *et al*., 1995), the rationale was that if Flu inhibited this channel, the spontaneous repolarization of pre- and/or postsynaptic neurones under physiological conditions would be impaired. This could lead to an increased excitatory response (e.p.s.p.), possibly underlying the symptoms of the antidepressive potential of Flu (Wong *et al*., 1995) and also of the 5-HT syndrome (Sternbach, 1991; Sporer, 1995). In addition, among the myriad studies published so far on the (mostly clinical) effects of Prozac, not a single one deals with the biophysical effects of the compound at the level of ion channels and biomembranes. Therefore, this study also determined, for the first time, the biophysical characterization of Flu.

Methods

The cDNA encoding the neuronal RCK1 K^+ channel (Baumann *et al*., 1988) was subcloned in a 3 kb high-expression vector, called pGEM-HE, based on pGEM-3Z (Promega, U.S.A.) containing 5′ and 3′ non-translated sequences of a *Xenopus* β-globin gene flanking the channel cDNA (Liman *et al*., 1992). For *in vitro* transcription, the plasmid was first lin-

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earized with *Pst*l (New England Biolabs, U.S.A.) 3′ to the 3′ non-translated β-globin sequence and then transcribed with the Riboprobe Gemini System (Promega, U.S.A.) by use of T7 RNA polymerase and a cap analogue diguanosine triphosphate (Boehringer, Germany). Stage V-VI *Xenopus laevis* oocytes were isolated by partial ovariectomy under anaesthesia (tricaine, 1 g l^{-1}). Anaesthetized animals were kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg ml^{-1} collagenase (Boehringer, Germany) in zero calcium ND-96 solution. The ND-96 solution contained (in mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5; pH 7.5, supplemented with 1 ml of 50 mg ml^{-1} gentamycin sulphate (for incubation only). Between 1 h and 24 h after defolliculation, oocytes were injected with 50 nl of 1–100 ng μl^{-1} mRNA. The oocytes were then incubated in ND-96 solutions at 18°C for 1–5 days. Whole-cell currents from oocytes were recorded by use of the two-microelectrode voltage clamp technique (GeneClamp 500, Axon Instruments, U.S.A.). Resistances of voltage and current electrodes were kept as low as possible (voltage electrode: 0.1–0.5 MΩ, current electrode: 0.1 MΩ) and were filled with 3 M KCl. Current records were sampled at 200 or 400 μs intervals and filtered at 2 or 1 kHz, respectively, by means of a four-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled, as measured near the outside surface of the oocyte by means of a two-electrode virtual-ground circuit (also called 'bath clamp'). Voltage records were carefully monitored on an oscilloscope (Hameg, Germany) to ensure fast and proper clamping, and only adequately clamped oocytes were qualified for storage and off-line analysis on an IBM-compatible PC. Linear components of capacity and leak currents were subtracted by use of a P/4 protocol (Bezanilla & Armstrong, 1977). In non-injected or H_2O-injected oocytes ($n=20$), endogenous currents observed in the tested voltage range amounted only to $\leqslant 1\%$ of the amplitude of RCK1 currents. All experiments were performed at room temperature (19–23°C). Flu ((±)-N-methyl-3-phenyl-[(α,α,α-trifluoro-p-tolyl)-oxy]-propylamine hydrochloride) was obtained as a white, crystalline solid (kindly provided by Lilly Co., U.S.A.) and as commercially available Prozac capsules (14 × 20 mg, Lilly Co., U.S.A.). The compound was always applied extracellularly. Starting either from the pure product or from Prozac, the same results were obtained and therefore pooled for statistical analysis.

Results

Oocytes injected with mRNA coding for RCK1 were clamped at a holding potential (V_{hold}) of −90 mV and 100 ms depolarizing steps from test potentials (V_{test}) −50 to 10 mV were applied every 3 s, in the absence and presence of 50 μM Flu after 10 and 20 min equilibration (Figure 1a). A slowly developing, time-dependent reduction of the K^+ current was observed in the presence of Flu. This reduction in current was absent in control oocytes, i.e. in the absence of Flu, as measured for up to 30 min ($n>10$). The inhibition of K^+ current was prominent at V_{test}-values positive to −30 mV (Figure 1b), and steady-state block could not be achieved within the time frame of the experiment.

To address the phenomenon of possible use-dependent block of fluoxetine, wash-in of 500 μM of the drug was performed by means of two different protocols, i.e. wash-in during a period of time with repetitive depolarizations vs during a period of time devoid of any depolarization (Figure 2). In contrast to the slowly developing, time-dependent block illustrated in Figure 1, a more pronounced and much faster developing block was seen when fluoxetine was administered during a train of repetitive depolarizations from V_{hold} −90 mV to V_{test} 0 mV applied at 5 kHz. Block amounted to 40.6±5.2% ($n=6$) (a and c). A much smaller block was obtained when fluoxetine was adminstered during 1 min when the oocyte was clamped continuously at V_{hold} −90 mV (8.6±1.5%, $n=4$, b). Successively a similar repetitive train of depolarizations was applied in the presence of fluoxetine which resulted in a more pronounced and faster developing block of the RCK1 channel, similar to the protocol in (a). These results are indicative of use-dependent block. Interestingly, the development of use-dependent block coincided with tail current crossover, as measured at a tail potential (V_{tail}) of −50 mV (c), an effect which will be detailed later.

To investigate whether the inhibition was caused by a positive shift of the steady-state activation curve in the presence of Flu, the oocytes were clamped at V_{hold} −90 mV, every 3 s to V_{test} −50 to 10 mV, and to V_{tail} −50 mV (Figure 3). Plotting the peak tail current amplitudes from (a) as a function of V_{test}

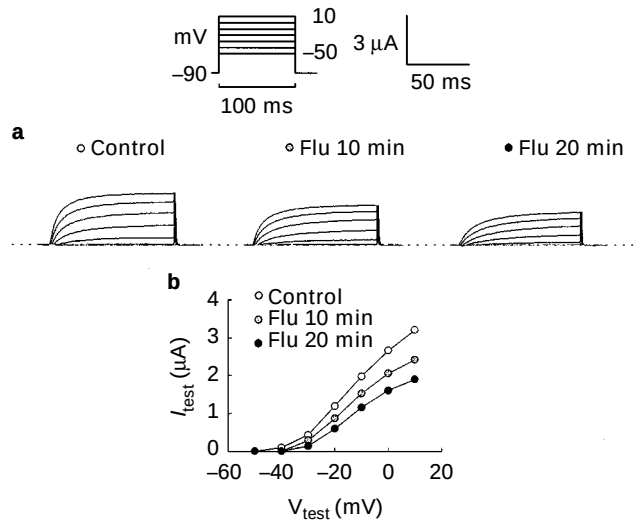

Figure 1 Effect of fluoxetine (Flu) on RCK1, expressed in *Xenopus laevis* oocytes. (a) The oocyte was clamped at V_{hold} −90 mV and 100 ms depolarizing steps from V_{test} −50 to 10 mV were applied every 3 s (see boxed inset), in the absence (control) and presence of 50 μM Flu after 10 and 20 min equilibration. (b) The inhibition of the K^+ current induced by Flu was prominent at V_{test} -values positive to −30 mV.

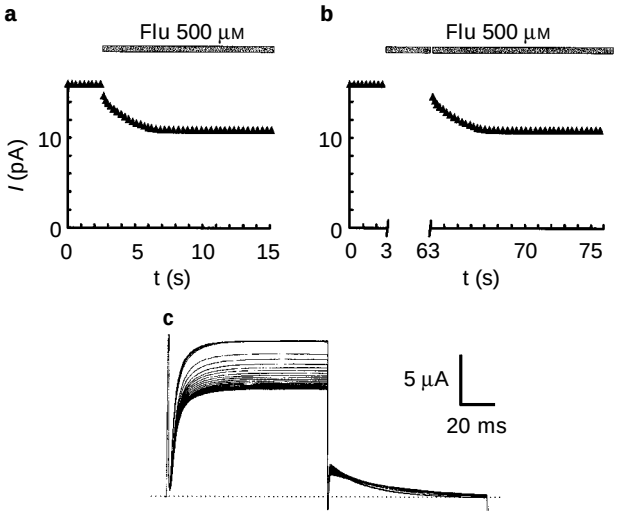

Figure 2 Use-dependence of fluoxetine (Flu). Flu 500 μM was washed-in during application of 50 depolarizations from V_{hold} −90 mV to V_{test} 0 mV at 5 kHz (a) or during a 1 min period of time devoid of any depolarization at V_{hold} −90 mV (b), followed by the same train of repetitive depolarizations as in (a). (c) Representative current traces obtained from the protocol in (a).

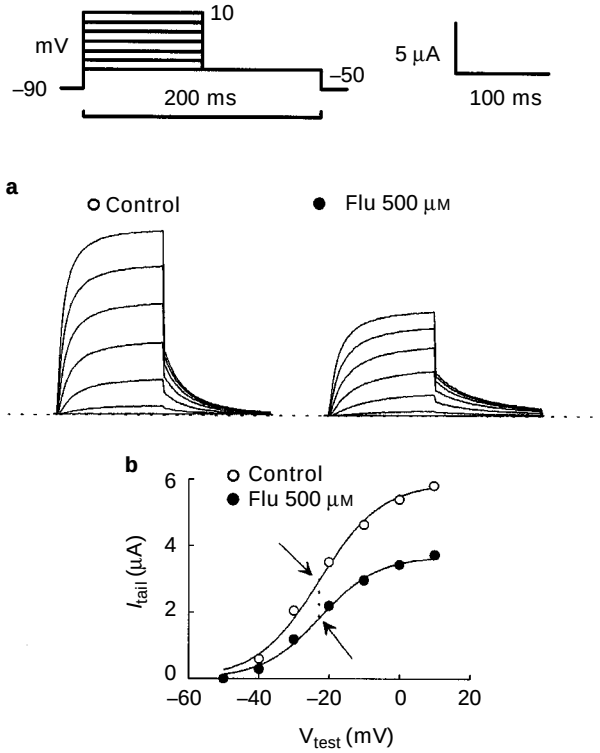

Figure 3 Effect of fluoxetine (Flu) on the steady-state activation curve of RCK1. (a) The oocyte was clamped at V_{hold} −90 mV, to V_{test} −50 to 10 mV, and then to V_{tail} −50 mV. This protocol was applied both in the absence and presence of 500 μM Flu after 5 min equilibration. (b) Steady-state activation curves in control and in the presence of Flu obtained after fitting with a Boltzmann function (equation (1)). The $V_{1/2}$-value was not shifted by Flu as illustrated by the arrows.

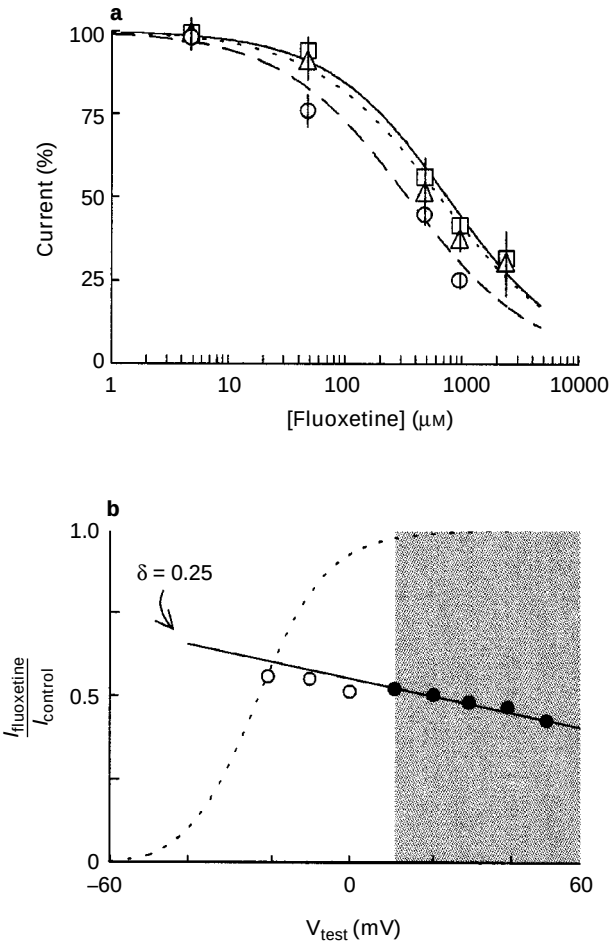

Figure 4 Dose-response relationship and voltage-dependent block of fluoxetine (Flu). (a) Concentration- and voltage-dependence of Flu-induced block of RCK1. Reduction in current (relative to control) at the end of depolarizing steps from V_{hold} −90 mV to V_{test} −20, 0 and 20 mV was used as index for block. Data are mean and vertical lines show s.e.mean (n varies between 3–14 for individual concentrations tested). The fits of the experimental data were obtained by means of equation (2). At V_{test} -values of −20, 0, and 20 mV, K_D-values were 748, 640 and 342 μM, respectively, with a Hill coefficient of 0.8. (b) Relative current expressed as $I_{Flu}/I_{control}$. The dashed line represents the steady-state activation curve, as obtained from the date in Figure 3b. Block was shallower in the voltage range of activation of the channel (open circles), than in the voltage range positive to 10 mV (solid circles, grey shaded area). The voltage-dependence, representing the effect of the membrane electrical field on the interaction between Flu and RCK1, is represented by the straight line and was fitted with equation (3) yielding $\delta = 0.25$.

resulted in steady-state activation curves which were fitted with a Boltzmann function given by the equation:

$$I = \frac{I_{max}}{1 + \exp(-(V_{test} - V_{1/2})/s)} \quad (1)$$

where $V_{1/2}$ is the midpoint of activation and s the slope. In the control ($n = 5$), $V_{1/2}$ was -20.0 ± 2.6 mV and s was $8.5 \pm$ mV. In the presence of Flu ($n = 5$), $V_{1/2}$ was -18.8 ± 3.3 mV and s was 8.1 ± 0.4 mV. These results demonstrate that Flu does not significantly affect the steady-state activation curve of RCK1.

The potency of Flu-induced inhibition of RCK1 K^+ currents was investigated by studying 5 different doses of Flu in the range of 5–2000 μM (Figure 4a). The dose-response curve summarizes the remaining current (in %) as measured at V_{test} −20, 0 and 20 mV in the presence of the different concentrations of Flu after 3–5 min equilibration. The most potent inhibition was observed with the most depolarized V_{test}-values, but it was not significantly different for some of the tested concentrations (e.g. 50 μM, V_{test} −20 vs 0 mV). The apparent dissociation constant, K_D, and Hill coefficient, n_H, were obtained from fitting of the remaining current, Y, at various drug concentrations [C]:

$$Y = 100 - \frac{100}{1 + (K_D/[C])^{n_H}} \quad (2)$$

At V_{test}-values of −20, 0 and 20 mV, K_D-values were 748, 640, and 342 μM, respectively, with a Hill coefficient of 0.8. To quantify the voltage-dependence of RCK1 block, the relative current $I_{Flu}/I_{control}$ was plotted as function of membrane potential (Figure 6b). It was found that block increased with stronger depolarizations, and also that voltage-dependence of block in the voltage range corresponding to channel opening (dashed line) was shallower than in the voltage range where activation had reached saturation and all channels were open (shaded area). We then determined the voltage-dependence of block observed in the range of V_{test}-values between 10 and 60 mV by a Boltzmann relationship based on the Woodhull model:

$$f = \frac{[C]}{[C] + K_{D'} \times \exp(-\delta zFV/RT)} \quad (3)$$

where f = 1 - ($I_{Flu}/I_{control}$), z, F, R, and T have their usual meaning, δ represents the fractional electrical distance, i.e. the fraction of the transmembrane electrical field sensed by a single charge (z = 1) at the receptor site (Woodhull, 1973), and $K_{D'}$ represents the apparent dissociation constant at the reference

potential (0 mV). The solid, straight line in Figure 4b represents the fit of this Boltzmann equation to the data points positive to 0 mV (solid circles). This latter restriction was required to quantify the voltage-dependence independent from the voltage-dependence of channel opening. Using this analysis, we obtained a value of 0.25 for the fractional electrical distance (δ).

The effect of Flu on the kinetics of activation and deactivation of RCK1 channels was studied by clamping the oocytes from V_{hold} −90 mV to V_{test} −50 to 10 mV for 100 ms, and clamping back the cells to V_{tail} −50 mV. This protocol enabled us to study both the voltage-dependent time course of activation and deactivation, in the absence and presence of 500 μM Flu after 5 min equilibration. Figure 5a and b illustrates the currents in control and in the presence of Flu, respectively. Next we superimposed the traces in the absence and presence of the drug obtained at V_{test} 10 mV (Figure 5c). It is evident that in the presence of Flu, the outward K^+ current evoked during V_{test} was partly inhibited. However, more interestingly, was the tail current crossover upon deactivation of the channels (arrow), indicating that in the presence of Flu, the channels deactivate more slowly (see further and Figure 7). In Figure 5d, the same traces as in (c) are superimposed, but now scaled to obtain the same current amplitudes at the end of V_{test}. This procedure enabled us to show that the activation kinetics were also slowed down as compared to control conditions (arrow). To quantify this shift in activation kinetics, the current trace obtained at V_{test} 10 mV in the presence of Flu was compared with several traces obtained in control conditions: one obtained at V_{test} 0 mV (e), −10 mV (f), and −20 mV (g). The activation kinetics of the control trace at V_{test} −10 mV matched those of the trace at V_{test} 10 mV in the presence of Flu. This indicates that 20 mV must be added to the depolarization to make activation in the presence of Flu as fast as in control conditions. The same 20 mV shift was also observed when other current traces were compared between control and Flu conditions, respectively, for instance V_{test} −30 and −10 in control and in the presence of Flu (Figure 5h).

Interestingly, the 2 measurable parameters of gating we have studied thusfar, i.e. fraction open channels/membrane voltage (or activation curve) and activation kinetics (or activation rate constants) were not shifted equally: the first parameter was not affected by Flu, whereas the latter was shifted by 20 mV in the depolarizing direction. This discrepancy is explained in the description of the model shown in Figure 8.

It is known that the voltage-dependent K^+ currents develop with a delay and show a sigmoidal onset, especially prominent at moderate depolarizations. Figure 6a illustrates this phenomenon in control conditions for V_{test} −30, −10, and 10 mV. Two types of models were used to fit the time courses of activation: a Hodgkin-Huxley type model (Hodgkin & Huxley, 1952) with power functions of the form:

$$I_t = A \times (1 - \exp(-(t - d)/\ ))^{\hat{}n} + c \qquad (4)$$

with I_t the macroscopic and time-dependent current, A the current predicted at steady-state, d the delay, τ the time constant, n the power, and c a constant; alternatively, time courses of activation were also fitted with a more generalized Markov chain model (Colquhoun & Hawkes, 1995) by use of exponential functions of the form:

$$I_t = \Sigma A_n \times (\exp(-(t - d)/\ _n)) + c \qquad (5)$$

where A_n is the amplitude of the n^{th} component represented by time constant τ_n. In the Hodgkin-Huxley type model, a single exponential function up to the fourth power ($n = 4$) failed to

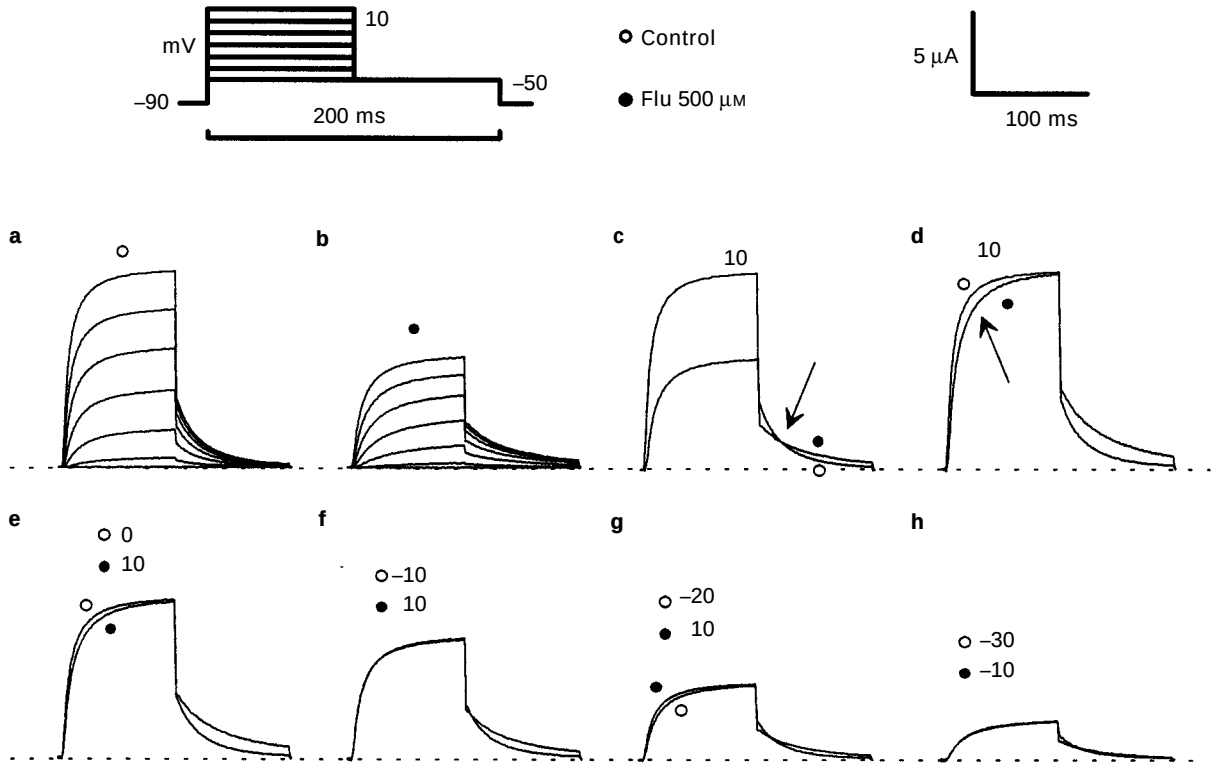

Figure 5 Kinetics of RCK1 and the effect of fluoxetine (Flu). The oocyte was clamped at V_{hold} −90 mV, to V_{test} −50 to 10 mV, and then to V_{tail} −50 mV. This protocol was applied both in the absence (a) and presence of 500 μM Flu after 5 min equilibration (b). (c) Superposition of the traces at V_{test} 10 mV from (a) and (b). The slower decaying tail current in the presence of Flu with a concomitant crossover can clearly be seen (arrow). (d) Superposition and scaling of the traces at V_{test} 10 mV from (a) and (b). The kinetics of activation were slowed in the presence of Flu (arrow). Superposition and scaling of the trace at V_{test} 10 mV in the presence of Flu with the ones in control at V_{test} 0 mV (e), −10 mV (f), and −20 mV (g). (h) Superposition, scaling and comparison of the control trace at V_{test} −30 mV revealed the same activation kinetics as the trace in the presence of Flu at V_{test} −10 mV. A 20 mV difference is thus present at all V_{test}-values when the kinetics of activation were compared in the absence and presence of Flu.

describe adequately the initial delay and the continued activation at the end of a 100 ms pulse for V_{test}-values in the range of -30 to 10 mV (dashed lines, Figure 6a). In a Markov chain model, mono- and bi-exponential functions also could not account for the initial delay in the current (data not shown). However, a tri-exponential function described the activation kinetics, both in control and in the presence of 500 μM Flu, significantly better for all V_{test}-values (Figure 6b). In (c), means of the 3 time constants for activation, τ_1, τ_2 and τ_3, in control and in the presence of Flu are illustrated as a function of V_{test} ($n=5$). It can be seen that the 3 time constants become smaller with stronger depolarizations, for instance the channels activated significantly faster at V_{test} 10 mV than at -30 mV. Flu slowed down the kinetics at all potentials, but only significantly for the smallest time constant, τ_1. Relative areas of each of the three time constants did not change proportionally after Flu application (ratios A_1/A_2, A_1/A_3 and A_2/A_3 were not significantly different in control and in the presence of Flu).

The effect of Flu on the tail current deactivation was studied by clamping the oocytes first from V_{hold} -90 mV to V_{test} 10 mV in order to activate fully the K^+ channels. Then the oocytes were repolarized to V_{tail}-values ranging from -50 to -120 mV (Figure 7). The kinetics of the tail currents could adequately be described as a mono-exponential decay, by use of equation (5) with $n=1$, both in control and in the presence of 500 μM Flu (dashed lines superimposed on data traces, Figure 7a). The time course of the tail currents was slowed in the presence of Flu, relative to control. A summary of the time constants obtained at V_{tail} -120 to -50 mV is shown in Figure 7c ($n=6$). The process of deactivation was much faster at more negative tail potentials, for instance the time constant in control ($n=6$) decreased from 22.24 ± 1.47 ms to 2.0 ± 0.6 ms for V_{tail}-values of -50 and -120 mV, respectively. At all tested potentials, Flu increased the time constant of deactivation. Additionally, Flu reduced the peak tail current amplitude recorded on return to the different V_{tail}-values, re-

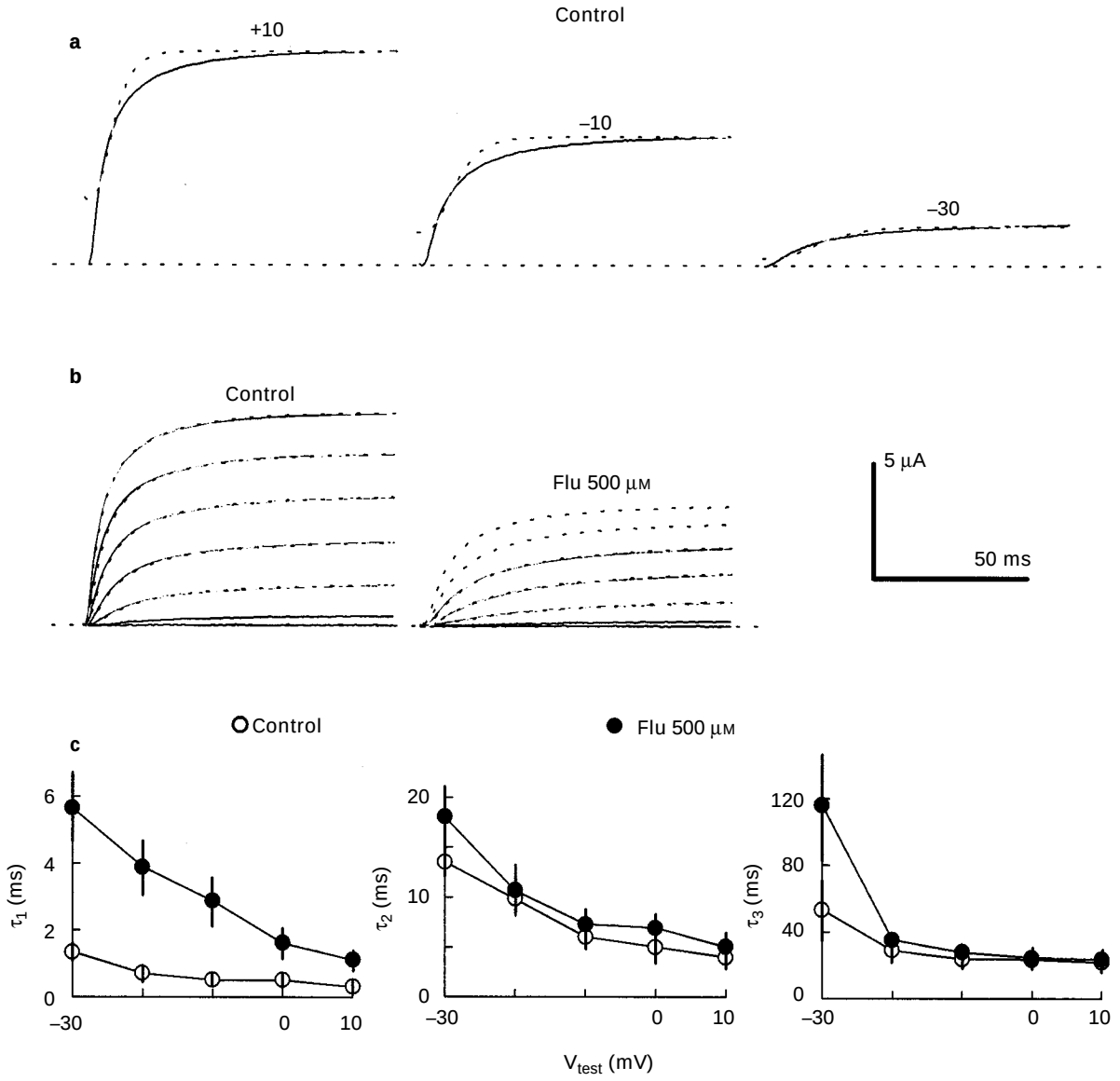

Figure 6 Effect of fluoxetine (Flu) on the activation kinetics of RCK1. (a) Failure of Hodgkin-Huxley type of model with $n=4^{th}$ power to fit the activation kinetics in control for V_{test}-values of 10, -10 and -30 mV (dashed lines superimposed on data traces, V_{hold} -90 mV), by use of equation (4). For this experiment, values for V_{test} 10 mV for τ, A and c were 4.35 ms, 13.3 μA and 5.22 μA, respectively; for V_{test} -10 mV, 5.97 ms, 8.57 μA and 2.49 μA, respectively; for V_{test} -30 mV, 9.67 ms, 2.94 μA and 0.51 μA, respectively; d was 1.2 ms in all cases. Since these fits were not successful in control conditions, no attempts were made to use equation (4) in the presence of Flu. (b) Successful fits (dashed line) in control and in the presence of 500 μM Flu (5 min presence), V_{hold} -90 mV and V_{test} -50 to 10 mV, obtained with a Markov chain type of model using equation (5) with 3 time constants ($\tau_1-\tau_3$). (c) The 3 time constants are summarized as a function of V_{test}. Data are mean with vertical lines showing s.e.mean ($n=5$); d was 1.2 ms in all cases; the 30 different values for A in control and in the presence of Flu are not shown.

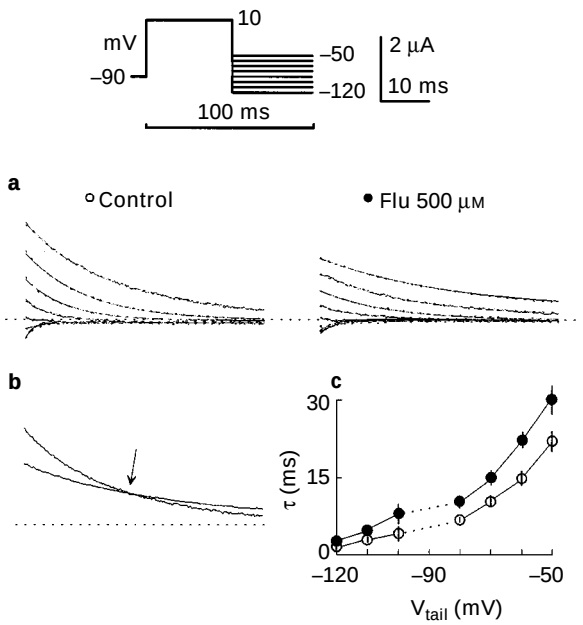

Figure 7 Effects of fluoxetine (Flu) on the deactivation kinetics of RCK1. (a) Tail currents obtained at V_{tail}-values -50 to -120 mV, after clamping from V_{hold} -90 mV to V_{test} 10 mV to activate fully the channels, both in control and in the presence of 500 μM Flu (5 min presence). A single, mono-exponential process adequately described the deactivation kinetics for all test V_{tail}-values. The fitted lines obtained by use of equation (5) with n = 1 are superimposed on the data traces. (b) Tail current crossover phenomemon (arrow) seen at V_{tail} -50 mV in the presence of Flu as compared to control conditions. (c) Plot of the time constants of deactivation as a function of V_{tail}. Data are mean with vertical lines showing s.e.mean ($n = 6$). No τ value could be calculated at V_{tail} -90 mV, because of the small current amplitude evoked at -90 mV which is close to equilibrium potential for K^+ions.

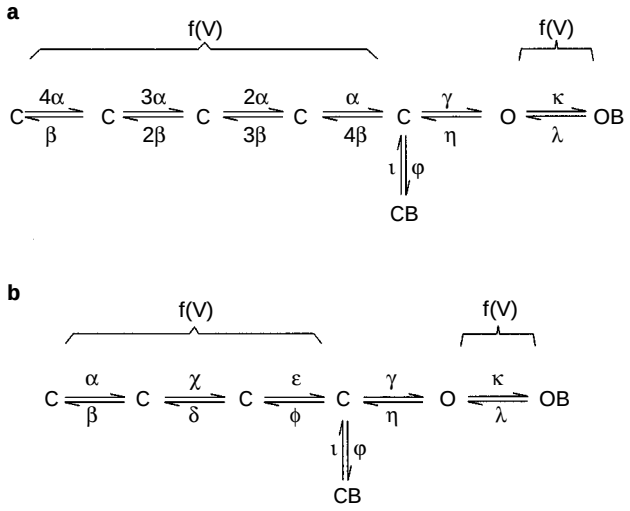

Figure 8 Mathematical models of interaction of fluoxetine (Flu) with RCK1 channels. (a) Hodgkin-Huxley type model. Voltage-dependent channel gating is governed by voltage-dependent forward and backward rate constants, α and β, respectively. Each of these 4 transitions represents a conformational change in the K^+ channel protein, associated with charge movement. The final opening rate, γ, and the initial closing rate, η, govern the actual opening and closing of the channel (C$\rightleftharpoons$O). These transitions are voltage-independent and are very fast as compared to α and β. In the presence of Flu (=B), the channel can be blocked in the last closed, but available state (C$\rightarrow$CB), and also when it is in the open state (O$\rightarrow$OB). Inactivated states are not included. As mentioned in the text and illustrated in Figure 6a, this type of model could not be used adequately. (b) Markov type model. Voltage-dependent channel gating is governed by 6 different, voltage-dependent forward and backward rate constants, α to ϕ. The final opening rate, γ, and the initial closing rate, η, govern the actual opening and closing of the channel (C$\rightleftharpoons$O). These transitions are voltage-independent and are fast as compared to α, β, γ, δ, ε and ϕ. In the presence of Flu (=B), the channel can be blocked in the last closed, but available state (C$\rightarrow$CB). This transition, determined by the large rate constant, φ, is assumed to be very fast and therefore produces scaling down of the current amplitudes. The dissocation rate constant, ι, is very small. The classical open channel block (O$\rightarrow$OB) is voltage-dependent. Both forward and backward transitions, governed by κ and λ, are fast, produce use-dependence and slowing of tail current deactivation upon repolarization and crossover in the presence of Flu (see Figure 7b).

sulting in a crossover phenomenon (arrow, Figure 7b). The currents reversed around V_{tail} -93 mV, indicating that they were carried by K^+ ions and correspond to RCK1 under the ionic conditions used. Furthermore, it was found that Flu did not alter the ion selectivity of the channel, as the reversal potential was not changed in the presence of the drug: -92.8 ± 1.3 mV in control ($n = 5$) and -93.8 ± 1.7 mV in the presence of Flu ($n = 5$).

Discussion

The experimental results obtained in this study were interpreted in a simplified kinetic state diagram (Figure 8). The delayed, sigmoidal activation seen for RCK1 channels with depolarizations requires multiple, non-conducting states before the open state. Most transitions, if not all, between these states are governed by voltage-dependent rate constants (Koren *et al*., 1990). Based on the four-subunit composition of this channel (MacKinnon, 1991; Liman *et al*., 1992), we first fitted the activation kinetics by use of a Hodgkin-Huxley formalism with n = 4 (equation (4)). As can be concluded from Figure 6a, these fits were not satisfactory. Hence, a Hodgkin-Huxley-type kinetic state diagram with voltage-dependent forward and backward rate constants, α and β, respectively, multiplied with factors 1 to 4 representing the number of subunits which have to undergo the voltage-dependent transitions, could not be used (Figure 8a). In contrast, superior fits of the data were obtained with a tri-exponential function (Figure 6b; equation (5)), as in the case of the I_{sK} protein (Hice *et al*., 1994). The 3 independent time constants represent a Markov chain model with at least 3 closed states, 1 open, and no inactivated states (Figure 8b). The presence of $\geqslant 3$ closed states does not contradict the proposed four fold subunit composition of Kv channels, since our macroscopic results cannot discriminate reliably between the exact number of activation subunits.

In order to use the most simple model compatible with our data, we therefore have included 4 closed states, of which the 3 first states represent the channel in the resting conformation, and the fourth closed state the channel in a closed, but available conformation. Between all these closed states, voltage-dependent rate constants (α to ϕ) govern the equilibrium. Transitions between the last closed, available state and the open state are governed by voltage-independent rate constants, γ and η. Inactivated states are not included, because at the whole-cell level no significant inactivation was apparent during test pulses of 100–200 ms.

It is unlikely that the voltage-dependence of the block observed at membrane potentials positive to 10 mV was due to channel gating or use-dependent block, because RCK1 activation had reached saturation over this voltage range with time constants of less than 20 ms and voltage-dependence was studied with depolarizing test pulses at low frequencies (0.33 or 0.2 Hz), during which no use dependence was observed. Voltage-dependence of block could result from the influence of the transmembrane electrical field on the interaction between ca-

tionic Flu and the channel receptor, since Flu is predominantly charged at pH 7.5 ($pK_a = 9.5$).

When voltage-dependent channel activation structurally alters the receptor such that the receptor becomes available for high affinity binding of a ligand in the voltage range of channel activation, the voltage-dependence of ligand binding as well as of channel activation coincides and thus superimposes. As a consequence, the steepness of the voltage-dependence of open channel block will be highest at V_{test}-values close to $V_{1/2}$ of the activation curve and lowest at V_{test} values where all channels are activated. This phenomenon has clearly been observed for stereoselective block of hKv1.5 by bupivacaine enantiomers (Valenzuela *et al*., 1995), but is absent in our study on Flu (Figure 4b). One explanation could be that binding of Flu on RCK1 is entirely voltage-independent. However, this seems difficult to reconcile with the voltage-dependence seen in the voltage range positive to 10 mV, i.e. where the channel is fully open, consistent with an equivalent electrical distance δ of 0.25. Alternatively, our data support a model wherein the absence or presence of voltage-dependence of the binding of Flu is a function of the state of the channel. In other words, if block by Flu occurs in the available, closed state (C→CB) and the apparent blocking rate exceeds that of channel opening (C→O), which is likely the case in our experiments with a high concentration (500 μM) of Flu, then the current recorded in the presence of the drug would be expected to be scaled down without any effect on the steady-state activation curve ($V_{1/2}$ unchanged, see Figure 3b). This component of block is thus characterized by (1) high association rate constant, (2) very small dissociation rate constant, (3) high affinity, and (4) voltage-independence. The irreversible mechanism of action of Flu, i.e. the fact that wash-out could never be achieved, also supports the very small dissociation rate constant, ι, in our model. The other component of block is located in the transition O→OB and is characterized by (1) high association rate constant, (2) high dissociation rate constant, (3) lower affinity than C→CB, and (4) voltage-dependence (with fractional electrical distance, $\delta = 0.25$). This type of block is called open channel block and is known to influence the time course of the deactivating currents upon repolarization leading to crossover (Figure 7b) (Follmer *et al*., 1992; Snyders *et al*., 1992; Carmeliet, 1993; Choi *et al*., 1993). The use-dependence illustrated in Figure 2 also supports this phenomenon: the more block built-up during consecutive depolarizations, the more crossover is pronounced (Figure 2c). Furthermore, crossover, together with slowing of the activation kinetics, rules out any effect on voltage-dependent gating caused by changes in surface charge on the cell membrane. In such a case, slowing of the activation kinetics should be accompanied by increased kinetics of deactivation, as is seen in the presence of typical surface charge screening ions like external Ca^{2+} (Frankenhaeuser & Hodgkin, 1957) and La^{3+} (Sanguinetti & Jurkiewicz, 1990; Tytgat & Daenens, 1997). The absence of a shift of the steady-state activation curve of RCK1 by Flu, as seen in Figure 3b, is another argument against surface charge changes mediating the effects on channel gating. Currently, our study does not comprise mathematical simulations to test whether the proposed model (Figure 8b) is valid or not. The main reason is that our macroscopic results cannot discriminate reliably between the exact number of activation subunits (i.e. closed states). From the many alternative models which can be proposed, we have given here only the most simple Markov type of model which, in our opinion, represents a plausible working scheme.

The Flu concentrations that we used in our experiments are 5 to as much as 2000 fold higher than the anticipated plasma concentrations in patients being treated for depression with these compounds (Preskorn *et al*., 1991). Therefore, we would not expect to see in man the kind of effects described here unless toxic concentrations are being used. However, it should be stressed that for drugs with an intracellular site of action, K_D-values obtained from oocyte experiments can be 100 fold greater than those obtained from cultured, mammalian cell lines (e.g. quinidine block of Kv1.5 and Kv1.4; Deal *et al*., 1996). It appears that the variation is due to the charged hydrophobic yolk in the oocyte which probably complicates drug equilibration. If this is true for Flu, then it would be interesting to determine the K_D in a mammalian cell line (e.g. mouse fibroblasts) and to correlate this value with plasma concentrations in man. Although use of the oocyte expression system implies a drawback for quantitative studies of drugs like Flu, it has to be stressed that Flu affects RCK1 channels at concentrations that are easily as low as those affecting several other type of receptors, e.g. α_1-adrenoceptor, muscarinic, dopamine D_2 and histamine H_1 receptors.

In conclusion, the biophysical analysis of the effects induced by fluoxetine on a neuronal, voltage-dependent RCK1 (Kv1.1) channel expressed in oocytes has revealed that the drug shows a time- and dose-dependent block, with clear changes on both the activation and deactivation kinetics of the channel, but without changing the ion selectivity of the K^+ channel. Based upon a Markov kinetic scheme, our data can be interpreted to mean that blockade consists of two components.

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References

ASBERG, M., ERIKSSON, B., MARTENSSON, B., TRASKMAN-BENDZ, L. & WAGNER, J. (1986). Therapeutic effects of serotonin uptake inhibitors in depression. *J. Clin. Psychiatry*, **47,** 23–35.

BAUMANN, A., GRUPE, A., ACKERMANN, A. & PONGS, O. (1988). Structure of the voltage-dependent potassium channel is highly conserved from Drosophila to vertebrate central nervous system. *EMBO J*., **7,** 2457–2463.

BEZANILLA, F. & ARMSTRONG, C.M. (1977). Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol*., **70,** 549–566.

CARMELIET, E. (1993). Use-dependent block and use-dependent unblock of the delayed rectifier K^+ current by almokalant in rabbit ventricular myocytes. *Circ. Res*., **73,** 857–868.

CHOI, K., MOSSMAN, C., AUBE, J. & YELLEN, G. (1993). The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron*, **10,** 533–541.

COLQUHOUN, D. & HAWKES, A.G. (1995). The principles of the stochastic interpretation of ion-channel mechanisms. In *Single-Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 397–482. London: Plenum Press.

CUSACK, B., NELSON, A. & RICHELSON, E. (1994). Binding of antidepressants to human brain receptors: focus on newer generation compounds. *Psychopharmacology*, **114,** 559–565.

DEAL, K.K., ENGLAND, S.K. & TAMKUN, M.M. (1996). Molecular physiology of cardiac potassium channels. *Physiol. Rev*., **76,** 49–67.

FOLLMER, C., CULLINAN, C. & COLATSKY, T. (1992). Differential block of cardiac delayed rectifier current by class Ic antiarrhythmic drugs: evidence for open channel block and unblock. *Cardiovasc. Res*., **26,** 1121–1130.

FRANKENHAEUSER, B. & HODGKIN, A.L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol*., **137,** 218–244.

HICE, R.E., FOLANDER, K., SALATA, J.J., SMITH, J.S., SANGUINETTI, M.C. & SWANSON, R. (1994). Species variants of the I_{sK} protein: differences in kinetics, voltage dependence, and La^{3+} block of the currents expressed in Xenopus oocytes. *Pflügers Arch*., **426,** 139–145.

HODGKIN, A.L. & HUXLEY, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.*, **117,** 500 – 544.

KOREN, G., LIMAN, E.R., LOGOTHETIS, D.E., NADAL-GINARD, B. & HESS, P. (1990). Gating mechanisms of a cloned potassium channel expressed in frog oocytes and mammalian cells. *Neuron*, **4,** 39 – 51.

LIMAN, E.R., TYTGAT, J. & HESS, P. (1992). Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron*, **9,** 861 – 871.

MACKINNON, R. (1991). Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature*, **350,** 232 – 235.

PRESKORN, S.H., SILKEY, B., BEBER, J. & DOREY, C. (1991). Antidepressant response and plasma concentrations of fluoxetine. *Ann. Clin. Psych.*, **3,** 147 – 151.

RAE, J.L., RICH, A., ZAMUDIO, A.C. & CANDIA, O.A. (1995). Effect of Prozac on whole cell ionic currents in lens and corneal epithelia. *Am. J. Physiol.*, **269,** C250 – C256.

RICHELSON, E. & NELSON, A. (1984). Antagonism by antidepressants of neurotransmitter receptors of normal human brain in vitro. *J. Pharmacol. Exp. Ther.*, **230,** 94 – 102.

RICHELSON, E. & PFENNING, M. (1984). Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: most antidepressants selectively block norepinephrine uptake. *Eur. J. Pharmacol.*, **104,** 277 – 286.

SANGUINETTI, M.C. & JURKIEWICZ, N.K. (1990). Lanthanum blocks a specific component of I_K and screens membrane surface in cardiac cells. *Am. J. Physiol.*, **259,** H1881 – H1889.

SNYDERS, D.J., KNOTH, K., ROBERDS, S. & TAMKUN, M. (1992). Time-, state- and voltage-dependent block by quinidine of a cloned human cardiac channel. *Mol. Pharmacol.*, **41,** 332 – 339.

SPORER, K.A. (1995). The serotonin syndrome. Implicated drugs, pathophysiology and management. *Drug Safety*, **13,** 94 – 104.

STAUDERMAN, K.A., GANDHI, V.C. & JONES, D.C. (1992). Fluoxetine-induced inhibition of synaptosomal [^{3}H]5-HT release: possible Ca^{2+}-channel inhibition. *Life Sci.*, **50,** 2125 – 2138.

STERNBACH, H. (1991). The serotonin syndrome. *Am. J. Psychiatry*, **148,** 705 – 713.

TYTGAT, J. & DAENENS, P. (1997). Effect of lanthanum on voltage-dependent gating of a cloned mammalian neuronal potassium channel. *Brain Res.*, **749,** 232 – 237.

VALENZUELA, C., DELPON, E., TAMKUN, M.M., TAMARGO, J. & SNYDERS, D.J. (1995). Stereoselective block of a human cardiac potassium channel (Kv1.5) by bupivacaine enantiomers. *Biophys. J.*, **69,** 418 – 427.

VEH, R.W., LICHTINGHAGEN, R., SEWING, S., WUNDER, F., GRUMBACH, I.M. & PONGS, O. (1995). Immunohistochemical localization of five members of the Kv1 channel subunits: contrasting subcellular locations and neuron-specific co-localizations in rat brain. *Eur. J. Neurosci.*, **7,** 2189 – 2205.

WANG, H., KUNDEL, D.D., SCHWARTZKROIN, P.A. & TEMPEL, B.L. (1994). Localization of Kv1.1 and Kv1.2, two K channel proteins, to synaptic terminals, somata, and dendrites in the mouse brain. *J. Neurosci.*, **14,** 4588 – 4599.

WONG, D.T., BYMASTER, F.P. & ENGLEMAN, E.A. (1995). Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci.*, **57,** 411 – 441.

WONG, D.T., BYMASTER, F.P., REID, L.R. & THRELKELD, P.G. (1983). Fluoxetine and two other serotonin uptake inhibitors without affinity for neuronal receptors. *Biochem. Pharmacol.*, **32,** 1287 – 1293.

WOODHULL, A. (1973). Ionic blockade of sodium channels in nerve. *J. Gen. Physiol.*, **61,** 687 – 708.

ZAMUDIO, A.C. & CANDIA, O.A. (1993). Antidepressants and an H1 blocker reduce CI transport in frog corneal epithelium by decreasing K permeability. *Invest. Ophthalmol. Visual. Sci.*, **34,** 1400.

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