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Inhibition by fluoxetine of voltage-activated ion channels in rat PC12 cells

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Abstract

The effects of fluoxetine (Prozac) on voltage-activated K^+, Ca^{2+} and Na^+ channels were examined using the whole-cell configuration of the patch clamp technique in rat pheochromocytoma (PC12) cells. When applied to the external bath solution, fluoxetine (1, 10, 100 μM) decreased the peak amplitude of K^+ currents. The K^+ current inhibition by fluoxetine (10 μM) was voltage-independent and the fraction of current inhibition was 39.7–51.3% at all voltages tested (0 to +50 mV). Neither the activation and inactivation curves nor the reversal potential for K^+ currents was significantly changed by fluoxetine. The inhibition by fluoxetine of K^+ currents was use- and concentration-dependent with an IC_{50} of 16.0 μM. The inhibition was partially reversible upon washout of fluoxetine. The action of fluoxetine was independent of the protein kinases, because the protein kinase C or A inhibitors (H-7, staurosporine, Rp-cAMPS) did not prevent the inhibition by fluoxetine. Intracellular infusion with GDPβS or pretreatment with pertussis toxin did not block the inhibitory effects of fluoxetine. The inhibitory action of fluoxetine was not specific to K^+ currents because it also inhibited both Ca^{2+} (IC_{50} = 13.4 μM) and Na^+ (IC_{50} = 25.6 μM) currents in a concentration-dependent manner. Our data indicate that when applied to the external side of cells, fluoxetine inhibited voltage-activated K^+, Ca^{2+} and Na^+ currents in PC12 cells and its action on K^+ currents does not appear to be mediated through protein kinases or G proteins. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Fluoxetine; Ion channel, voltage activated; PC12 cell

1. Introduction

Fluoxetine (Prozac), the selective serotonin reuptake inhibitor has been shown to be effective in the treatment of depression (Stark et al., 1985; Wong et al., 1995). Besides its well-known inhibiting action on the neuronal serotonin reuptake (Fuller and Wong, 1990; Altamura et al., 1994), it has been demonstrated that fluoxetine exerted effects on a variety of ion channels. For instance, fluoxetine inhibited K^+ and Na^+ currents in lens and corneal epithelium, and the inhibitory effects of fluoxetine on ionic currents, however, were only observed in perforated-patch recordings but not in excised inside-out patch recordings (Rae et al., 1995). In canine jejunal smooth muscle cells, fluoxetine decreased the delayed rectifier K^+ current in amphotericin-perforated patch recordings and the effects of fluoxetine were blocked by PKC inhibitor, indicating that the actions of fluoxetine were dependent on PKC (Farrugia, 1996). Although these findings suggested that the effects of fluoxetine on the K^+ channels were mediated by a diffusible second messenger, data so far published were rather inconsistent and fluoxetine appears to inhibit ion channels in a complicated manner. More recent investigations have shown that fluoxetine directly blocked voltage-activated K^+ (Kv1.1) currents (Tytgat et al., 1997), nicotinic acetylcholine currents expressed in *Xenopus* oocytes (García-Colunga et al., 1997) and neuronal Na^+ channels in bovine adrenal chromaffin cells (Pancrazio et al., 1998). PC12 cells derived from rat adrenal medulla chromaffin cells represent a good neuronal model for electrophysiology and secretion. In addition, functional Ca^{2+} and Na^+ channels appear when the cells are differentiated with nerve growth factor (Garber et al., 1989). In this study, we investigated the effects of fluoxetine on voltage-activated K^+ channels and its cellular mechanisms underlying K^+ channels inhibition. It is also of interest to compare the

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relative effects of fluoxetine on other ionic currents, such as Ca^{2+} and Na^{+} channels in PC12 cells.

2. Materials and methods

PC12 cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated horse serum and 5% fetal bovine serum in a humidified 5% CO_2 incubator at 37°C. For recording voltage-activated Ca^{2+} and Na^{+} currents, cells were cultured in DMEM containing 2.5 S nerve growth factor (50 ng/ml, Sigma, MO, USA) for 5–7 days. Cells were plated on poly-L-lysine (Sigma) coated cover glasses (diameter: 12 mm, Fisher Scientific, Pittsburgh, PA, USA) in a petri dish for electrophysiology. Fluoxetine was obtained from Tocris Cookson (Bristol, UK), H-7, staurosporine from Sigma, GDPβS, pertussis toxin from Research Biochemicals International (Natick, MA, USA), and Rp-cAMPS from Biomol (Plymouth Meeting, PA, USA).

Voltage-clamp recordings were performed using the whole-cell configuration of the patch clamp technique at room temperature (Hamill et al., 1981). Patch pipettes (PG10165-4, WPI, Sarasota, FL, USA) had a resistance of 2–4 MΩ when filled with pipette solution. Whole-cell currents were amplified with an Axopatch 1D or 200B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 5 kHz and digitized at 10 kHz with a Digidata 1200A acquisition board (Axon Instruments). The data acquisition and analysis were performed with the pCLAMP 6.0 software (Axon Instruments). For K^{+} current: the external (bath) solution contained (in mM) NaCl 140, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1, HEPES 25, glucose 10 (pH 7.3 with NaOH), and the internal (pipette) solution contained (in mM) KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, EGTA 10, MgATP 2, (pH 7.3 with KOH). For Ca^{2+} current: the external, NaCl 120, CsCl 5, $BaCl_2$ 10, $MgCl_2$ 1, HEPES 10, tetrodotoxin 1 μM, glucose 10 (pH 7.3 with NaOH); internal, CsCl 120, $MgCl_2$ 1, HEPES 10, EGTA 10, MgATP 2, (pH 7.3 with CsOH). For Na^{+} current: the external, NaCl 120, CsCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1, $CoCl_2$ 1, HEPES 10, glucose 10 (pH 7.3 with NaOH); internal, CsCl 120, $MgCl_2$ 1, HEPES 10, EGTA 10, MgATP 2, (pH 7.3 with CsOH). During the recording, the cells were continuously perfused with test solution at a rate of 1 ml/min. Linear leak and capacitative currents were corrected on-line by using the P/4 subtraction method. Series resistance was approximately 5–10 MΩ, and series resistance was compensated if the current exceeded 1 nA. Concentration–response data were best fitted with the following logistic equation using Origin 5.0 software. $Y = 1/[1 + IC_{50}/F]^{n}$ where IC_{50} is the concentration of fluoxetine resulting in 50% inhibition, F is the fluoxetine concentration and n is the Hill coefficient. All the data were expressed as means ± S.E.M. Statistical significance was determined at the level of 0.05 using Student's t-test or one-way analysis of variance.

3. Results

Fig. 1A shows whole cell currents evoked by a series of depolarizing voltage steps ranging from −60 to +50 mV in 10 mV increments for 200 ms from a holding potential of −80 mV. K^{+} currents were activated rapidly and did not decay over the course of a 200 ms step. The peak outward currents were blocked by 5 mM tetraethylammonium to 22.0 ± 1.4% of the control values ($n = 10$) indicating a delayed rectifier K^{+} current, as previously reported for undifferentiated PC12 cells (Hoshi and Aldrich, 1988; Nakazawa et al., 1995). The current–voltage relationship of K^{+} currents in the presence of fluoxetine is shown in Fig. 1B. Fluoxetine (10 μM) decreased the peak amplitude at all the test voltages but the current–voltage relation did not appear to be affected (Fig. 1C). The degree of inhibition was relatively independent of voltage, for example 42.9 ± 3.4% at +10 mV and 47.4 ± 3.2% at +50 mV ($n = 6$). The voltage dependencies of the currents were described by a Boltzmann equation in the absence and presence of fluoxetine (Fig. 2). The activation curve gave $V_{50} = -0.3$ mV and $k = 15.5$ in control experiments and was not altered by fluoxetine ($V_{50} = 4.1$ mV, $k = 14.9$). Inactivation of the current was incomplete with 3-s long prepulses and reached a plateau level of about 65% ($n = 6$). The best fit yielded $V_{50} = -21.1$ mV and $k = 11.5$ in control experiment, and fluoxetine shifted the inactivation curve toward hyperpolarization ($V_{50} = -26.7$ mV, $k = 9.7$). This difference was not statistically significant. The reversal potential for K^{+} cur-

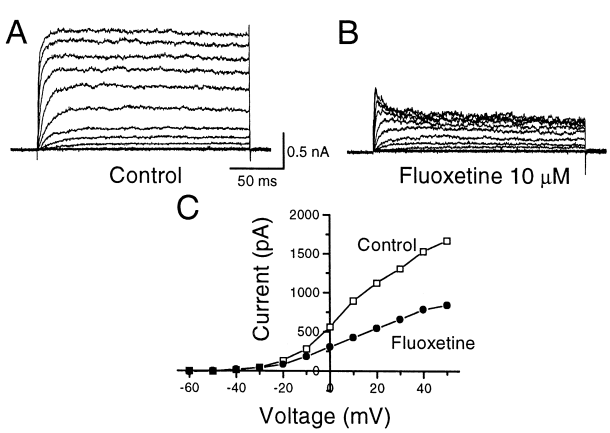

Fig. 1. The current–voltage relationship for K^{+} currents in control (A) and 10 μM fluoxetine (B). Whole-cell K^{+} currents were evoked by a series of depolarizing voltage steps ranging from −60 to +50 mV in 10 mV increments for 200 ms from a holding potential of −80 mV at 10 s intervals. (C) Effect of fluoxetine on the current–voltage relationship for peak K^{+} currents in the absence (□) and presence (●) of fluoxetine. Current amplitudes were plotted against test potentials.

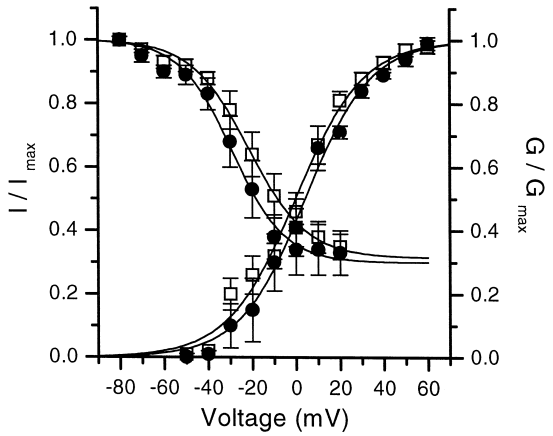

Fig. 2. Effects of fluoxetine on activation and inactivation of K^+ currents in the absence (□) or presence (●) of 10 μM fluoxetine. The curves are drawn with the Boltzmann equation. Conductance (G) was calculated from the peak current, assuming a reversal potential of −80 mV and normalized to its maximal value. I/I_{max} is the relative amplitude of current elicited by a test pulse to +40 mV, following a 3 s prepulse to the potential indicated by the ordinate. Peak amplitude of K^+ currents was normalized to maximal amplitude in each cell. Each point represents the mean ± S.E.M. of data from six cells.

rents was approximately −80 mV ($E_K = -83$ mV) and was not affected by fluoxetine (Fig. 3). We next examined whether fluoxetine displayed use-dependent inhibition. Use-dependence was assessed by applying a train of 20 depolarizing pulses of 100 ms from a holding potential of −80 to +40 mV at a rate of 1 or 2 Hz. Fig. 4 shows the effects of these pulse trains on the maximal peak currents in the absence and presence of fluoxetine (10 μM). Under control conditions, the maximal peak current decreased by 4.6 ± 1.2% at a rate of 1 Hz and by 10.6 ± 1.9% at a rate of 2 Hz after 20 depolarizing pulses. In the presence of fluoxetine, there was a significant use-dependent inhibition at both frequencies of stimulation that averaged 18.9 ± 3.8% and 30.9 ± 3.7% at 1 and 2 Hz, respectively.

Externally applied fluoxetine (1, 10, 100 μM) inhibited K^+ currents in a concentration-dependent manner (Fig. 5A). Fluoxetine reduced the peak current and accelerated

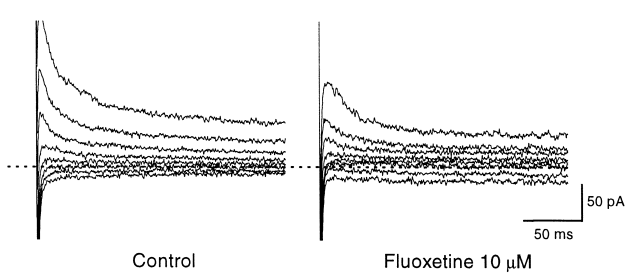

Fig. 3. The lack of effects of fluoxetine on reversal potentials for K^+ currents. Cells were depolarized to +40 mV for 80 ms from a holding potential of −80 mV and then repolarized to various voltages between −100 and −30 mV every 10 s. The dotted line represents the zero level of current. The obtained values for the reversal potentials were approximately −80 mV for K^+ currents in control and 10 μM fluoxetine ($n = 4$). Estimated reversal potential for K^+ was −83 mV.

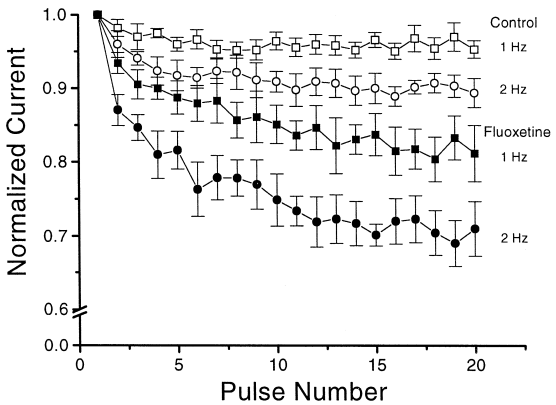

Fig. 4. Use-dependent inhibition of K^+ currents by fluoxetine. A 100-ms depolarizing pulse from −80 to +40 mV was applied at a rate of 1 or 2 Hz under control conditions and in the presence of 10 μM fluoxetine. Peak currents, normalized to the peak amplitude of the first pulse, are plotted against pulse number. Each point represents the mean ± S.E.M. ($n = 5$).

the rate of K^+ current decay at the most depolarized test pulse. Fluoxetine reduced the peak currents in the control by 10.5 ± 2.3, 41.6 ± 1.2 and 79.1 ± 1.8% at fluoxetine concentrations of 1, 10 and 100 μM, respectively ($n = 5$). The half inhibition (IC_{50}) of the currents was obtained with 16.0 μM fluoxetine (Fig. 5B). The time course of the fluoxetine inhibition was evaluated (Fig. 6). K^+ currents were elicited from a holding potential of −80 mV to a

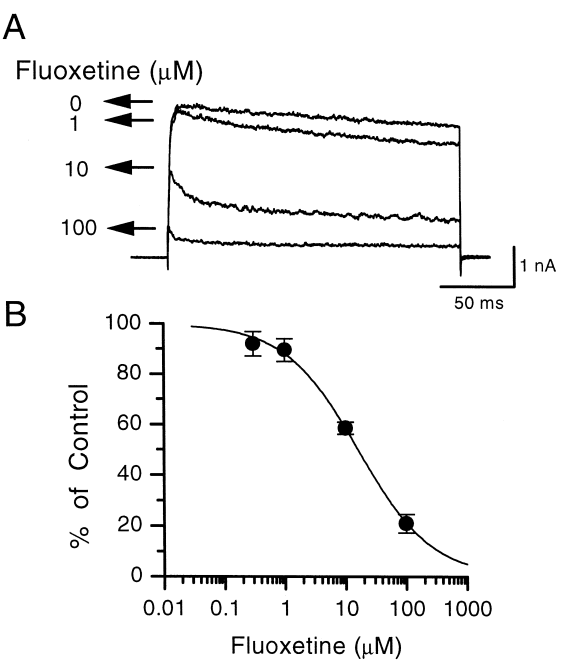

Fig. 5. Concentration-dependence of the effect of fluoxetine on K^+ currents. Whole-cell currents were elicited by 200 ms voltage step to +40 mV from a holding potential of −80 mV at 10 s intervals. The traces of the currents obtained in the absence and presence of 1, 10 and 100 μM of fluoxetine are shown (A). (B) Concentration–response curve. The data were fitted using the logistic equation given in the text, with an $IC_{50} = 16.0$ μM and a Hill coefficient = 0.7 ($n = 5$).

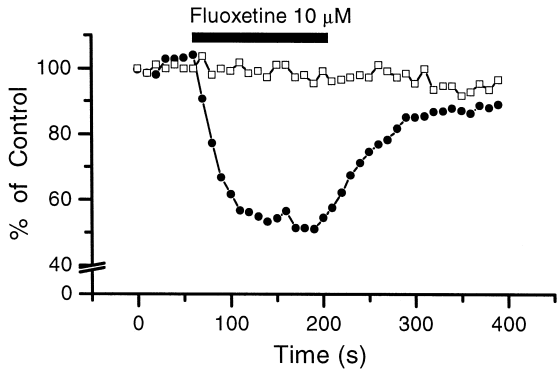

Fig. 6. Time course of fluoxetine inhibition in the absence (□) or presence (●) of 10 μM fluoxetine. Maximal inhibition occurred about 100 s after fluoxetine application began. A partial recovery from inhibition is observed after washout of the drug. Whole-cell currents were elicited by 200 ms voltage step to +40 mV from a holding potential of −80 mV at 10 s intervals. The peak amplitudes of the currents were plotted as a function of time. The bar indicates the time of application of fluoxetine.

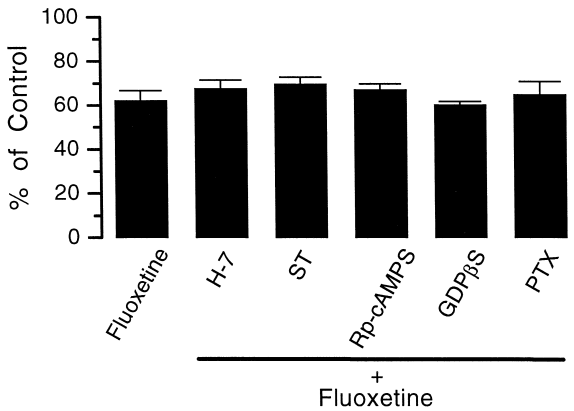

Fig. 7. Effects of various protein kinase inhibitors, GDPβS and pertussis toxin on the K^+ current inhibition induced by fluoxetine (10 μM). K^+ currents were evoked by a 200 ms depolarizing step to +40 mV from a holding potential of −80 mV. The amplitude of the peak K^+ current in the presence of fluoxetine was normalized to that just before the drug application. Cells were incubated with H-7 (10 μM for 30 min), staurosporine (ST, 1 μM for 30 min), pertussis toxin (PTX, 200 ng/ml for 24 h). Rp-cAMPS (1 mM) or GDPβS (1 mM) was included in intracellular pipette solution. The protein kinase inhibitors alone did not affect the amplitudes of K^+ currents during a 5-min recording period. Each column is the average of five cells.

command voltage of +40 mV every 10 s. When fluoxetine (10 μM) was applied to bath solution, K^+ currents were decreased and the inhibition was completed within 1 min. Upon washing fluoxetine, 88.5 ± 4.9% of the currents was recovered within 1–2 min in all cell tested ($n = 8$).

The effects of fluoxetine on delayed rectifier K^+ current in canine jejunum smooth muscle appeared to be mediated through inhibition of PKC (Farrugia, 1996). Hence, we examined whether the effects of fluoxetine were mediated by the protein kinases in conventional whole-cell and nystatin-perforated patch recordings. Pretreatment with H-7 (10 μM) or staurosporine (1 μM) did not prevent the inhibition of K^+ currents by fluoxetine (10 μM) in whole-cell recordings (Fig. 7). Similar results with H-7 (69.3 ± 4.8%) and staurosporine (64.9 ± 6.2%) were also obtained in perforated patch recordings ($n = 4$). Intracellular application of Rp-cAMPS (1 mM), a selective PKA inhibitor, also did not affect the inhibition of K^+ currents by fluoxetine. These results indicate that the inhibition of K^+ currents by fluoxetine is not mediated by either PKC or PKA. We also investigated whether the inhibitory effects of fluoxetine on K^+ currents were mediated via G proteins. Intracellular GDPβS (1 mM), the nonhydrolyzable GDP analog that inhibited G protein-mediated responses, failed to prevent the fluoxetine-induced inhibition of K^+ currents. The protein kinase inhibitors (H-7, staurosporine, Rp-cAMPS) or GDPβS alone did not show any effect on K^+ currents (data not shown). Similarly, the pretreatment of cells with pertussis toxin (200 ng/ml for 24 h) did not affect the inhibition of K^+ currents by fluoxetine. These results indicate that the possibility of G proteins involvement may be eliminated.

We also explored the effects of fluoxetine on Ca^{2+} and Na^+ currents in differentiated PC12 cells. Ba^{2+} ion was used as a charge carrier through voltage-activated Ca^{2+} channels. Cells were perfused sequentially with bath solution containing 10, 30, 100 μM fluoxetine. Like K^+ currents, the external application of different concentrations of fluoxetine led to a concentration-dependent inhibition of the peak amplitudes of Ba^{2+} (Fig. 8A) and Na^+ currents (Fig. 8B). Interestingly, fluoxetine (100 μM) completely abolished Ba^{2+} and Na^+ currents in contrast to incomplete inhibition of K^+ currents by 100 μM fluoxetine as illustrated in Fig. 5A. This finding may be related to the heterogeneity of different K^+ channel types in PC12 cells. Results of the cumulative concentration–response curves for fluoxetine are summarized in Fig. 8. Fig. 9

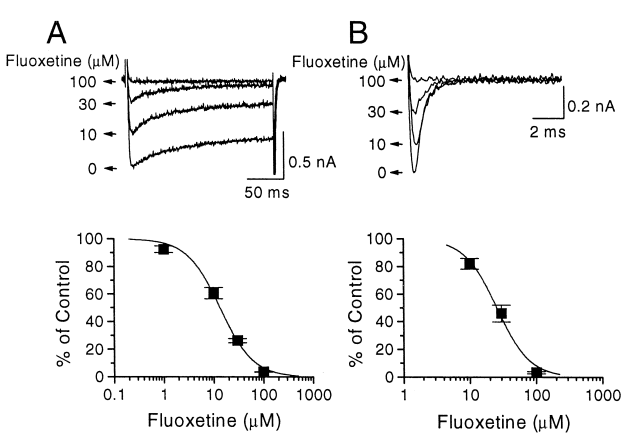

Fig. 8. Concentration-dependence of the effect of fluoxetine on Ba^{2+} (A) and Na^+ (B) currents. Whole-cell currents were elicited by 200 ms (Ba^{2+} current) and 10 ms (Na^+ current) voltage step to 0 mV from a holding potential of −80 mV at 10 s intervals. The traces of the currents obtained in the absence and presence of 10, 30 and 100 μM of fluoxetine are shown. The IC_{50} values for Ba^{2+} and Na^+ currents were 13.4 and 25.6 μM, and Hill coefficients were 1.3 and 1.8, respectively ($n = 5$).

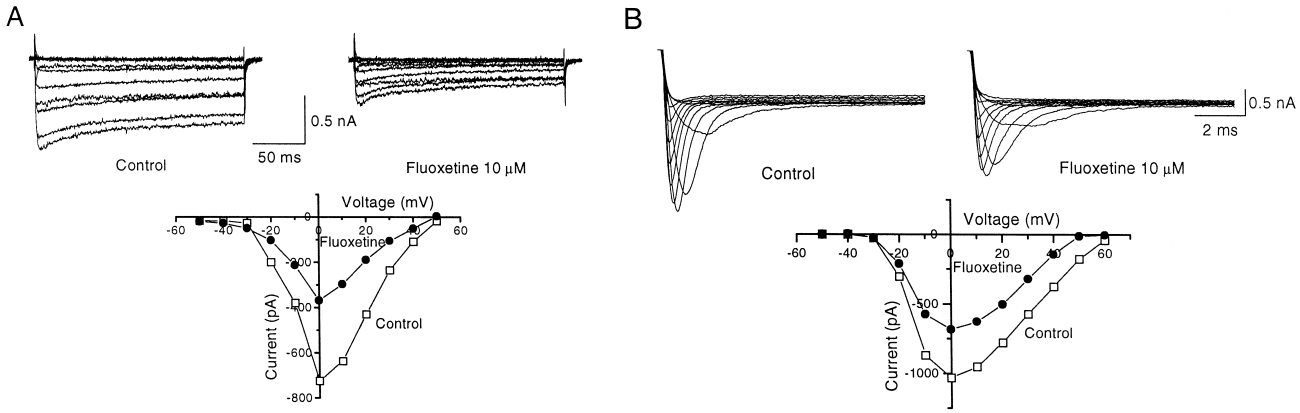

Fig. 9. Effect of fluoxetine on the peak current–voltage relationships of Ba^{2+} (A) and Na^{+} currents (B). Whole-cell currents were evoked by a series of depolarizing voltage steps ranging from −50 to +50 mV in 10 mV increments for 200 ms (Ba^{2+} current) and 10 ms (Na^{+} current) from a holding potential of −80 mV. Currents were measured before (□) and after (●) application of 10 μM of fluoxetine.

shows current–voltage relationship for Ba^{2+} and Na^{+} currents obtained with various depolarizing test potentials applied from a holding potentials of −80 mV. The current–voltage relationship exhibited a bell shaped curve with a peak at 0 mV in Ba^{2+} and Na^{+} currents. Fluoxetine (10 μM) inhibited both currents at all test potentials, and the current–voltage relationship was not affected by fluoxetine (10 μM) in any of the ionic currents examined as shown in Fig. 9.

4. Discussion

Our studies show that fluoxetine is a potent inhibitor of voltage-activated K^{+}, Ca^{2+} and Na^{+} channels in PC12 cells. The inhibition of K^{+} channels by fluoxetine was concentration-dependent but was not voltage-dependent. The inhibitory effects of fluoxetine on K^{+} currents were not abolished by inhibitors of protein kinases (H-7, staurosporine, Rp-cAMPS) or by GDPβS and pertussis toxin, indicating that protein kinases and G proteins were not involved in the inhibition of K^{+} currents by fluoxetine.

Previous study (Farrugia, 1996) showed that fluoxetine decreased the outwardly delayed rectifier K^{+} current in isolated canine jejunal smooth muscle cells in amphotericin-perforated patch recordings and the inhibition by fluoxetine of the K^{+} current was abolished by PKC inhibitor. In addition, the inhibitory effects of fluoxetine on K^{+} currents disappeared in conventional whole-cell recordings. These results suggested that the effects of fluoxetine on K^{+} currents were mediated via PKC inhibition. Modulation of ion channels through protein phosphorylation and G proteins has been well described for voltage-activated K^{+} channels (Reuter and Sigel, 1991; Levitan, 1994; Jonas and Kaczmarek, 1996). At present, it is not yet known if fluoxetine acts directly on the K^{+} channels in PC12 cells. Nevertheless, our results clearly show that this inhibition is not mediated via protein phosphorylation and/or G proteins and seems to be due to a direct effect of fluoxetine on the membrane or channels. This conclusion is supported by several observations. First, the inhibition of K^{+} currents by fluoxetine was not affected by protein kinase inhibitors (H-7, staurosporine, Rp-cAMPS), nor by intracellular dialysis associated with conventional whole-cell configuration. Moreover, the action of fluoxetine did not require ATP in the conventional whole-cell recordings because omission of ATP from the internal solution did not affect the action of fluoxetine (data not shown). Second, pretreatment with protein kinase inhibitors did not prevent the effect of fluoxetine on K^{+} currents in perforated patch recordings. Finally, internal application of GDPβS or exposure to pertussis toxin was without an effect. At present, we do not know the discrepancy between the results of Farrugia (1996) and the direct effect of fluoxetine on K^{+} channels in our study. The diversity of voltage-activated K^{+} channel types in a variety of cells may be an explanation for the different results.

Fluoxetine not only reduced the peak currents but also enhanced inactivation of currents associated with larger voltage steps. The accelerated inactivation may have resulted from several mechanisms, including the interactions with the open state of K^{+} channels or alteration of gating kinetics of K^{+} currents. Fluoxetine had no significant effect on the steady-state inactivation and activation properties of K^{+} currents. Furthermore, the inhibitory effects of fluoxetine were enhanced during repetitive depolarizations. These effects suggest that drug binding is tighter to the activated state of K^{+} currents. However, binding of fluoxetine to the activated closed state of the channel cannot be completely ruled out (Hoshi et al., 1994). More detailed experiments focusing on the inactivation kinetics are needed to determine the mechanisms of action.

In our experiment, fluoxetine inhibited K^{+} currents when applied from outside the cell. Since fluoxetine is lipid soluble and is able to penetrate cell membrane, it could act inside PC12 cells. It is difficult to know whether the inhibition of K^{+} currents by fluoxetine occurred from

the intracellular or the extracellular side of the membrane because of its highly lipid solubility. At this stage, the exact site of action of fluoxetine on K^+ channels is still unknown. Experiments including single-channel recordings will be required to obtain further insight into the mechanism of inhibition of K^+ currents by fluoxetine.

The action of fluoxetine on K^+ currents was not specific, and Ca^{2+} and Na^+ channels were also inhibited by fluoxetine at a concentration ranging from 1 to 100 μM. Judging from IC_{50} values (13–25 μM), three types of voltage-activated ion channels were equally sensitive to fluoxetine. Because the concentrations at which fluoxetine inhibited voltage-activated ion channels in our experiment were about 10–100 times higher than those of therapeutic plasma concentration (about 1 μM) (Altamura et al., 1994), it is unlikely that fluoxetine acts as an ion channel inhibitor within the range of therapeutic plasma concentration. However, concentrations of some drugs, especially lipid soluble antipsychotic drugs in the brain, can be 10–20 fold greater than those in the blood (Tsuneizumi et al., 1992; Karson et al., 1993). Since local concentration of fluoxetine may rise in the small extracellular space surrounding neurons, fluoxetine could act as an ion channel inhibitor in the brain. The overall effects of fluoxetine on neurons will be determined by the expression pattern of individual ion channels in different neurons, and this phenomenon may have pharmacological implications.

Acknowledgements

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