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Fluoxetine Inhibits K^+ Transport Pathways (K^+ Efflux, Na^+-K^+-$2Cl^-$ Cotransport, and Na^+ Pump) Underlying Volume Regulation in Corneal Endothelial Cells

E. Hara[1], P.S. Reinach[1,2,3], Q. Wen[1], P. Iserovich[1], J. Fischbarg[1,2]

[1]Departments of Ophthalmology and [2]Physiology and Cellular Biophysics, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA

[3]Department of Biological Sciences, SUNY, College of Optometry, New York, NY 10010, USA

Abstract. We have studied regulatory volume responses of cultured bovine corneal endothelial cells (CBCEC) using light scattering. We assessed the contributions of fluoxetine (Prozac) and bumetanide-sensitive membrane ion transport pathways to such responses by determining K^+ efflux and influx. Cells swollen by a 20% hypoosmotic solution underwent a regulatory volume decrease (RVD) response, which after 6 min restored relative cell volume by 98%. Fluoxetine inhibited RVD recovery; 20 μM by 26%, and 50 μM totally. Fluoxetine had a triphasic effect on K^+ efflux; from 20 to 100 μM it inhibited efflux 2-fold, whereas at higher concentrations the efflux first increased to 1.5-fold above the control value, and then decreased again. Cells shrunk by a 20% hyperosmotic solution underwent a regulatory volume increase (RVI) which also after 6 min restored the cell volume by 99%. Fluoxetine inhibited RVI; 20 μM by 25%, and 50 μM completely. Bumetanide (1 μM) inhibited RVI by 43%. In a Cl^--free medium, fluoxetine (50–500 μM) progressively inhibited bumetanide-insensitive K^+ influx. The inhibitions of RVI and K^+ influx induced by fluoxetine 20 to 50 μM were similar to those induced by 1 μM bumetanide and by Cl^--free medium. A computer simulation suggests that fluoxetine can interact with the selectivity filter of K^+ channels. The data suggest that CBCEC can mediate RVD and RVI in part through increases in K^+ efflux and Na-K-2Cl cotransport (NKCC) activity. Interestingly, the data also suggest that fluoxetine at 20 to 50 μM inhibits NKCC, and at 100–1000 μM inhibits the Na^+ pump. One possible explanation for these findings is that fluoxetine could interact with K^+-selective sites in K^+ channels, the NKC cotransporter and the Na^+ pump.

Correspondence to: J. Fischbarg

Key words: Na^+-K^+-$2Cl^-$ cotransporter — Na^+/K^+ ATPase — Prozac (fluoxetine) — Regulatory volume decrease (RVD) — Regulatory volume increase (RVI) — Corneal endothelium

Introduction

The cornea is composed of the epithelium, the underlying stroma, and the endothelium. The stromal ground substance can imbibe fluid, which would result in corneal swelling and loss of transparency. However, metabolically dependent mechanisms in the corneal limiting layers transport fluid out of the stroma and maintain normal vision. Fluid transport across the endothelium is secondary to ionic transport across the layer; such transport depends on endothelial Na^+:K^+ATPase activity (Trenberth & Mishima, 1968) and includes a bicarbonate secretory mechanism (Hodson & Miller, 1976).

It has been hypothesized that fluid transport across epithelial layers may result from cyclic regulatory volume responses (Fischbarg, 1997). During a cycle, polarized epithelial cells would first gain fluid across one side during RVI, and would then expel it across the opposite side during RVD. It is currently difficult to assess the validity of this hypothesis for the corneal endothelium. Volume regulation and its underlying ionic transport mechanisms in this layer have been identified but not yet extensively described (Fischbarg et al., 1993; Srinivas & Bonanno, 1997).

In many other systems, membrane ion transport mechanisms underlying RVD include an increase in the loss of K^+ and/or Cl^- which can occur through parallel K^+ and anion channels or via KCl symport activity (Hallows & Knauf, 1994). Likewise, mechanisms underlying RVI include the bumetanide-sensitive NKC cotrans-

porter which is dependent on the Na^+ gradient maintained by the Na^+:K^+ ATPase (Hallows & Knauf, 1994). We report here that CBCEC do undergo classical RVD and RVI responses to aniso-osmotic challenges; RVD is associated with K^+ efflux (a variety of K^+ channels are present in corneal endothelium (Rae & Watsky, 1996)) whereas RVI is linked to the NKCC activity also known to exist in CBCEC (Diecke et al., 1998). Since fluoxetine has been reported to inhibit Ca^{2+} channels (Stauderman, Gandhi, & Jones, 1992), K^+ and Na^+ conductances in cornea and lens epithelium (Rae et al., 1995), and the Kv11.1 K^+ channel in neurons (Tytgat, Maertens, & Daenens, 1997), we used it as a probe to characterize K^+ channel activity during RVD. We confirmed that fluoxetine can block K^+ efflux, and found that this inhibition is associated with suppression of the endothelial RVD response. We also describe that higher fluoxetine concentrations result in paradoxical increases in K^+ efflux without compromising cell viability. Lastly, we report for the first time that fluoxetine can also inhibit the NKC cotransporter and the Na^+ pump. Based on our computer simulations employing the recently solved structure of the Kcs A K^+ channel (Doyle et al., 1998), one possible mode of action is that fluoxetine could bind to the K^+ recognition sites known for K^+ channels and postulated for the NKC cotransporter and the Na^+ pump.

Materials and Methods

Culture of Bovine Corneal Endothelial Cells

Bovine eyes were obtained from a local slaughterhouse and rapidly transported to the laboratory on ice. They were washed with Dulbecco's Phosphate-Buffered Saline (GIBCO, Grand Island, NY). The corneas were excised at the limbus under sterile conditions, and were placed into a matching hemispherical depression. The corneal endothelial layers were covered with 0.25% trypsin-EDTA (GIBCO) for 8 min, after which the endothelial surface was gently scraped away with a rounded glass spatula. The detached cells were aspirated and added to Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; GIBCO), 2 ng/ml bFGF (SIGMA, St. Louis, MO) and antibiotics (Penicillin, 100 U/ml, + streptomycin, 2.5 μg/ml, GIBCO) in 25 ml tissue culture flasks (Falcon, Lincoln Park, NJ). The cells from 3 corneas were pooled into one flask and were cultured at 37°C in a CO_2 incubator. The medium was changed twice a week. First passages of CBCEC were used for the light scattering measurements and second or third passages were used for measuring $^{86}Rb^+$ efflux and influx.

The composition of NaCl Ringer's solution was (mmol/l): 122.8 NaCl, 26.2 $NaHCO_3$, 4.7 KCl, 1.0 NaH_2PO_4, 0.39 $MgSO_4$, 1.8 $CaCl_2$, 5.6 glucose, 5.3 HEPES Na. The composition of the Cl^--free Ringer's solution was (mmol/l): 142 Na-gluconate, 2.5 K_2S0_4, 2 $MgSO_4.7H_20$, 5 glucose, 5.4 $CaSO_4.2H_2O$, 5.3 NaHEPES (Sigma, St. Louis, MO). In both cases, the pH was 7.4 (tonicity: 300 mOsm). Osmolarity was varied by changing the NaCl (or Na gluconate) concentration. Supplementation of these Ringer's solutions with three active substances described in a prior (De Smet, Simaels, & van Driessche, 1995) report (5 μg/l insulin, 5 μg/l transferrin, 5 ng/l sodium selenite) was found to be essential for CBCEC to consistently undergo RVD. Ouabain, bumetanide (both from Sigma, St. Louis, MO) and fluoxetine hydrochloride (Ely Lilly, Indianapolis, IN) were dissolved directly in the experimental solutions.

Cell viability

This parameter was evaluated based on measurements of trypan blue dye exclusion and protein release into the bathing solution.

Cell Volume Measurement by Light Scattering

Relative cell volume as a function of bathing solution osmolality was evaluated by light scattering as described (Fischbarg et al., 1993). Cells were detached from flasks with 0.05% trypsin and then subcultured on rectangular glass coverslips in DMEM including 10% FBS, bFGF and antibiotics. They were used after the cells had reached about 90% confluence (1 day later). The coverslips were affixed to a plastic holder which was inserted into a round glass vial, and light scattering intensity was subsequently monitored. Cells were preequilibrated in iso-osmotic solution at 37°C until a stable baseline was obtained (30–45 min). Subsequently, the solution in the vial was exchanged with either a 20% hypo-osmotic or 20% hyperosmotic medium to obtain control regulatory volume responses. Typically, after volume stabilization, the aniso-osmotic solution was replaced with iso-osmotic solution; the same coverslip could be exposed to several such volume regulatory cycles during an experiment.

To quantify relative cell volume changes, we followed Iserovich's procedure (Wu et al., 1997; Iserovich et al., 1998). The experimental data $V(t)$ arising from the transient changes in the intensity of light scattering cannot be normalized directly because the volume transient results from at least two simultaneous processes, one osmometric, one regulatory. To deconvolve them, the first step was to fit the experimental data $V(t)$ to the function:

$$V(t) = V_0 + A\left[1 - e^{\frac{-t}{\tau_{osm}}}\right] - B\left[1 - e^{\frac{-t}{\tau_{vr}}}\right] \quad (1)$$

We found it expedient to use a fitting procedure (to an exponential associate function) in the Origin™ program (Microcal Software, Northampton, MA). The parameters were: V_0, volume at zero time; A, amplitude of the osmotic response (swelling or shrinking); B, amplitude of regulated volume response; τ_{osm}, characteristic time of the osmotic response; τ_{vr}, characteristic time of RVD or RVI responses. $V(t)$, V_0, A and B were in arbitrary units. This procedure already allows one to compute the degree of volume regulatory recovery (ρ) as simply a ratio:

$$\rho = \frac{B}{A} \quad (2)$$

For comparisons, it is useful to be able to plot all transients on the same scale (as done in Fig. 1). To achieve this, we normalized the data by scaling each set of them to the ideal osmometric component. The observed amplitude of the osmometric component in arbitrary units was A in each case, while for an ideal osmometer, the expected relative volume change ΔV_r is:

$$\Delta V_r = \frac{V_f - V_0}{V_0} = \frac{C_0 - C_f}{C_f} \quad (3)$$

Therefore, the normalized volume data $V_n(t)$ were calculated as:

$$V_n(t) = 1 + (V(t) - V_0)\frac{\Delta V_r}{A} \quad (4)$$

Data are reported as means ± SEM. Analyses were performed using (one-way) repeated ANOVA; $P < 0.05$ was considered significant.

K^+ Efflux During Regulatory Volume Decrease

K^+ efflux was determined using $^{86}Rb^+$ (New England Nuclear, Boston, MA) as a congener for K^+. CBCEC were cultured in four-well plates (Nunc, Naperville, IL). Cells were preloaded in iso-osmotic solution containing 3 μCi $^{86}Rb^+$ per well at 37°C for 3 hr. Three minutes prior to the beginning of an efflux experiment, cells were preincubated in iso-osmotic solution containing 1 mM ouabain. Cells were then washed three times with 1 ml iso-osmotic solution, incubated in 0.5 ml of a 20% hypo-osmotic solution containing 1 mM ouabain and 20 to 1,000 μM fluoxetine. For up to one hour, every 6 min the solution in each well was collected and replaced with fresh solution. At the end, residual $^{86}Rb^+$ radioactivity retained by the cells was extracted with 5% TCA and determined by scintillation counting. The residue after extraction was dissolved in 5% SDS and 0.5 N NaOH to determine the protein contents with a modified form of the Lowry (Lowry et al., 1951) assay.

K^+ Influx During Regulatory Volume Increase

Fluoxetine-inhibitable K^+ influx was assessed by comparing the extent of inhibition due to fluoxetine (5 μM–1 mM) to that measured in the presence and absence of bumetanide (25 nM–1 μM) and ouabain (1 mM). This comparison was done because more than 90% of the K^+ influx is accounted for by the sum of NKCC and Na^+ pump activity. Experiments were done with CBCEC grown in four-well plates at 37°C. Before an experiment, the cells were preincubated in iso-osmotic medium (either NaCl or Cl^--free medium) for 90 min to achieve equilibration with their new environment. Solution osmolality was then increased by 20% and the cells were incubated for another 10 min with 1 μCi ^{86}Rb. To terminate an experiment, cells were washed 3 times with 1 ml of ice-cold iso-osmotic 0.1 M $MgCl_2$ solution. Cellular $^{86}Rb^+$ was then extracted and counted, and protein contents were measured as above.

Results

Regulatory Volume Responses to Hypo-osmotic and Hyperosmotic Challenges

Figure 1 shows the regulatory volume responses that occur during exposure to 20% hypo-osmotic (top curves) and 20% hyperosmotic solutions (bottom curves). The smooth curves are fitted to exponential buildup functions representing osmometric swelling (or shrinkage) and a regulatory volume response. Preequilibration was done for 20 min in isosmotic solution. Figure 1 shows that subsequent substitution of either 20% hypo-osmotic or hyperosmotic solutions caused initially a rapid relative cell volume increase (decrease) and the onset of RVD (RVI). Under control conditions, the extent of RVD was (Fig. 2) 98 ± 3% ($n = 10$), and that of RVI (Fig. 2) 101 ± 2% ($n = 14$).

For our fitting procedure, as stated in Materials and Methods, we normalize the volume displacement to an ideal osmometric shift devoid of any superimposed regulatory volume components. Hence, based on osmometry, 20% hypo-osmotic and hyperosmotic challenges would have resulted in relative cell volumes of 1.25 and 0.83, respectively. As Fig. 1 shows, when RVD and RVI were completely inhibited (both by 50 μM fluoxetine, *see* below), the maximal volume displacements were largest. This suggests that regulatory volume responses are activated as soon as the cells are challenged, and hence during the initial 10–20 sec, RVD and RVI begin while the osmometric response is still ongoing (Fig. 1). Consistent with this, in the control RVD and RVI curves, the peak cell volumes after aniso-osmotic challenge were 1.21 and 0.84 (as compared to the osmometric maximal volumes of 1.25 and 0.83 above). Interestingly, the peak cell volume was affected more in RVD than in RVI. There was also a disparity in the time course of control RVD and RVI responses; with reference to Eq. 1, the characteristic volume regulatory times (τ_{vr}) for the fitted responses were $\tau_{RVD} = 2.44 \pm 0.01$ min ($n = 10$), and $\tau_{RVI} = 2.65 \pm 0.01$ min ($n = 14$).

Inhibition of Volume Regulatory Responses by Fluoxetine and Bumetanide

In general, cells responded with remarkable reproducibility to successive anisosmotic challenges. In a typical control experiment, under sustained challenge, cell volume returned to baseline (Fig. 1). The bathing solution was then made isosmotic, which resulted in post-regulatory complementary volume regulatory changes (*not shown*). The cells were then left to recover for ~30 min in isosmotic solution, after which a new anisosmotic challenge could be imposed. It was commonplace to elicit three successful cycles of regulation and recovery in given preparations (meaning a set of plated cells). Making use of that, inhibitory agents were tested in given experiments only after a proper control response had been priorly obtained. The protocol involved preincubating the cells with the inhibitor for 15 min before anisosmotic challenge; the anisosmotic solutions also contained the inhibitors. Both 20 and 50 μM fluoxetine inhibited RVD (Figs. 1, 2). With 20 μM fluoxetine, the extent of RVD recovery decreased to $\rho_{RVD} = 74 \pm 3\%$ ($n = 4$, $p < 0.05$), and with 50 μM fluoxetine, the RVD response was abolished ($\rho_{RVD} = -7 \pm 21\%$, $n = 4$, $p < 0.05$). Inhibitory effects on RVI (Figs. 1, 2) were seen with 20 and 50 μM fluoxetine, and with 1 μM bumetanide. With 20 μM fluoxetine, the extent of RVI recovery decreased (Fig. 2) to $\rho_{RVI} = 75 \pm 8\%$ ($n = 7$, $p < 0.05$). With 50 μM fluoxetine, the recovery was es-

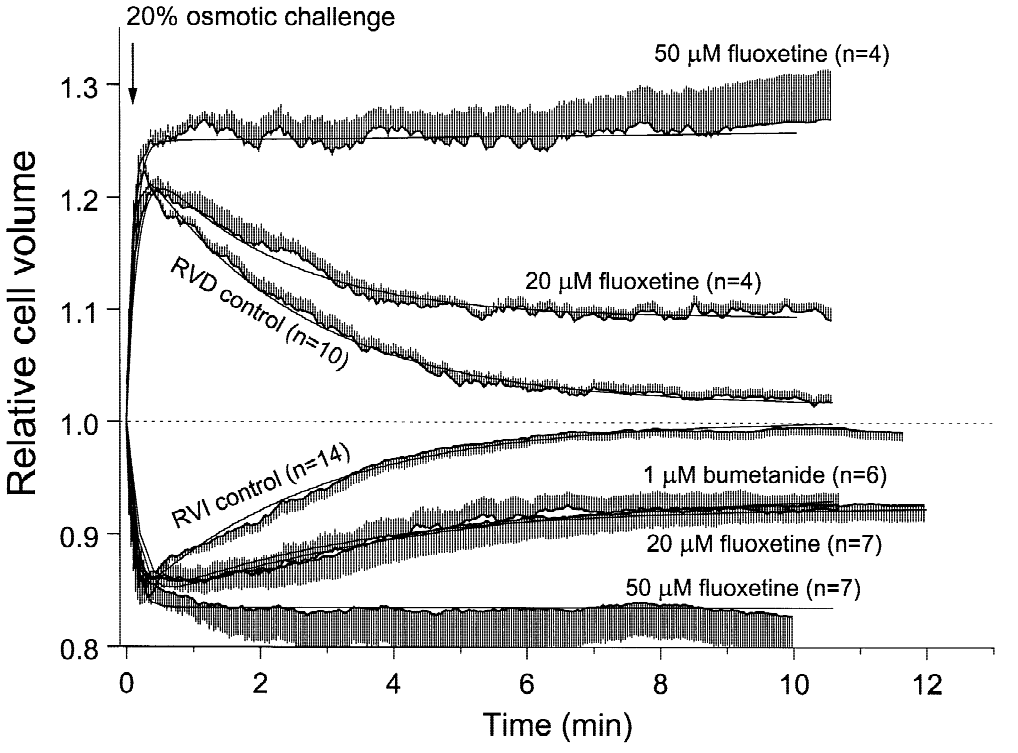

Fig. 1. Regulatory volume responses of CBCEC after a 20% anisosmotic challenge. Data were determined by light scattering. Each curve represents an average of the number of experiments (*n*) shown in each case. Vertical lines represent deviations (SEM). Smooth curves were fitted as described in Materials and Methods (Eqs. 1–4). *Positive deflections:* regulatory volume decrease (RVD) following a 20% hypo-osmotic (240 mOsm) challenge. The RVD responses include those under control conditions as well as those showing the effects of 20 and 50 μM fluoxetine. At 20 μM fluoxetine, RVD was partially inhibited whereas 50 μM fluoxetine completely suppressed this response. *Negative deflections:* regulatory volume increase (RVI) following a 20% hyperosmotic (360 mOsm) challenge. Along with the control response, inhibitory effects are shown for 20 and 50 μM fluoxetine as well as 1 μM bumetanide.

sentially eliminated ($\rho_{RVI} = -10 \pm 12\%$, $n = 7$, $p < 0.05$). One μM bumetanide decreased the extent of RVI to $\rho_{RVI} = 57 \pm 19\%$ ($n = 6$, $p < 0.05$; Fig. 2). This particular concentration of bumetanide was chosen because the extent of inhibition of K^+ influx was unchanged from that observed at higher bumetanide concentrations (*see* below).

Figure 2 also shows that the time constants for cell swelling (τ_{osm}) and volume recovery (τ_{vr}) went largely unaffected by fluoxetine. However, bumetanide lengthened both time constants. The bumetanide effect on τ_{vr} is consistent with the inhibition of NKCC cotransport reported below. The bumetanide affect on τ_{osm} may indicate a partial inhibitory effect of this compound on water channels such as the AQP1 known to be present in corneal endothelial cells (Nielsen et al., 1993; Li et al., 1999). This unexpected finding will require further corroboration.

K^+ Efflux: Triphasic Effects of Fluoxetine

The complex effects of fluoxetine from 20 to 1,000 μM on K^+ efflux from swollen cells (*see* Materials and Methods) are shown in Fig. 3. The main graph shows the time course of cumulative K^+ loss, while the inset depicts the initial K^+ efflux *vs.* [fluoxetine]. Increasing [fluoxetine] from 20 to 100 μM inhibited K^+ efflux by more than 2-fold. However, further increases in [fluoxetine] resulted in a rebound; K^+ efflux increased and even surpassed control levels, peaking at ~185% of control at [fluoxetine] = 500 μM. Larger [fluoxetine] once more inhibited K^+ efflux (Fig. 3, inset); at 1,000 μM fluoxetine, the efflux decreased back to the same value observed at 250 μM. The possibility that the increased efflux at fluoxetine concentrations larger than 100 μM was due to a toxic effect was evaluated by determining Trypan dye exclusion and protein release into the medium after 1 hr of exposure at all fluoxetine concentrations tested between 100 and 1,000 μM. In all cases, cell viability was unaffected (*data not shown*).

K^+ Influx. (A) Inhibition of the Na^+-K^+-$2Cl^-$ Cotransporter by Bumetanide

The data for this series were collected from some 100 wells; as detailed in Materials and Methods, K^+ influx

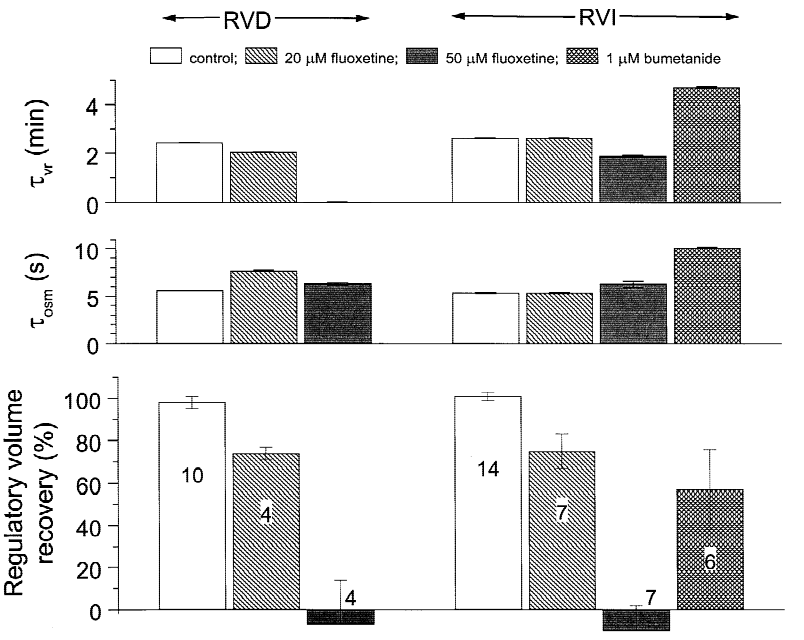

Fig. 2. Effects of fluoxetine and bumetanide on RVD and RVI recovery, and on the time constants for osmotic volume change and for recovery. Deviations for the extent of volume recovery correspond to the whole range of values in each experimental series. Deviations for the time constants are those for the fits shown in Fig. 1.

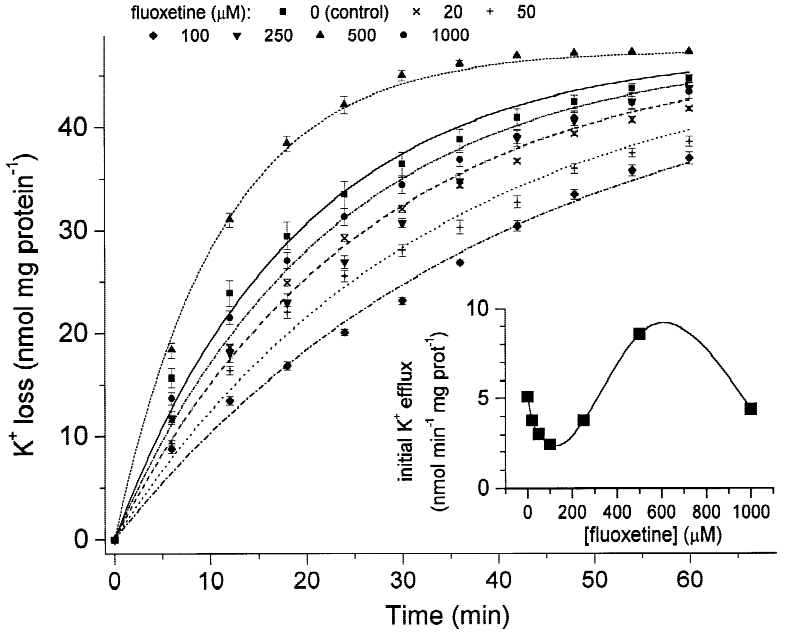

Fig. 3. Complex concentration-dependent effects of fluoxetine on K^+ loss. For each fluoxetine concentration, an exponential buildup function was fit to the data using Origin™. The initial K^+ efflux depicted in the insert is the derivative at zero time computed for each fitted curve. The results represent the average ± SEM of four wells for each time point at each concentration.

measurements were done in shrunken cells. Figure 4*A* shows results from a representative batch depicting the effects of bumetanide on K^+ influx. In Cl^--containing medium, bumetanide (0.2 μM) decreased K^+ influx by 26% and this decline remained essentially constant at bumetanide concentrations up to 50 μM. As shown previously (Diecke et al., 1998), under these conditions the inhibition of K^+ influx resulting from exposure to low bumetanide concentrations is indicative of complete inhibition of NKCC activity in these cells. In keeping with this, the bumetanide-induced decline in K^+ influx in NaCl medium was essentially the same as the one that resulted from Cl^- removal from the bathing solution (Fig. 4*A*). In addition, consistent with what has also been previously reported (Diecke et al., 1998), when the Na^+ pump is completely inhibited in the presence of 1 mM ouabain (Fig. 4*A*, bottom curve), bumetanide concentrations of 1 μM or less are sufficient to inhibit the residual K^+ influx, pointing to selective inhibition of the NKCC activity (IC_{50} = 46 ± 7 nM, n = 4).

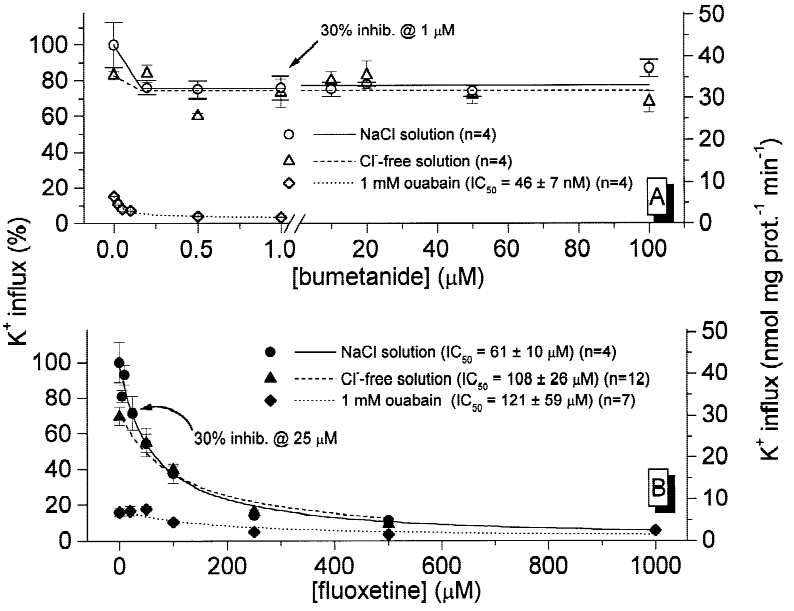

Fig. 4. Concentration-dependent effects of bumetanide (top) and fluoxetine (bottom) on K^+ influx. In each case, influx was measured under the three conditions shown in the figure. *N* represents the number of wells utilized for every concentration.

K^+ Influx. (B) Inhibition of the Na^+-K^+-$2Cl^-$ Cotransporter and the Na^+ Pump by Fluoxetine

Having found that 50 μM fluoxetine completely inhibited RVI (Fig. 1), we evaluated the individual declines in NKCC activity and Na^+ pumping that may account for the effect of fluoxetine on this response. For this, we determined the dependence of K^+ influx on [fluoxetine] in (*a*) Cl^--containing medium; (*b*) Cl^--free medium; and (*c*) in the presence of 1 mM ouabain. As can be seen in Fig. 4*B*, in the Cl^- containing medium, fluoxetine (5 to 1,000 μM) inhibited K^+ influx in a dose-dependent fashion (IC_{50} = 121 ± 59 μM, n = 4, ● symbols). We then tested whether fluoxetine could be inhibiting NKCC by performing influx measurements in cell layers which had been preincubated for 10 min in 1 mM ouabain (Fig. 4*B*, ♦ symbols). Such preincubation reduced influx by 85%, which is consistent with inhibition of the Na^+ pump and a subsequent indirect effect on NKCC via quick partial dissipation of the Na^+ gradient (Kuang, Cragoe, & Fischbarg, 1993). Fluoxetine concentrations above 25 μM further inhibited the K^+ influx. This effect is attributable to inhibition of the small residual activity of the NKC cotransporter remaining after ouabain inhibition (Fig. 4*B*, ♦ symbols).

Figure 5 depicts the results of other experimental series in which the individual and combined effects of bumetanide, fluoxetine and ouabain were determined on K^+ influx in the presence and absence of Cl^-. It is evident that, in Cl^--free medium, 500 μM fluoxetine decreased K^+ influx to the same extent as 1 mM ouabain. Furthermore, 500 μM fluoxetine plus either 1 μM bumetanide or 1 mM ouabain did not decrease K^+ influx any more than 500 μM fluoxetine alone.

Simulation of Fluoxetine Interaction with a K^+ Channel

Given its inhibitory effect on K^+ efflux, we postulated that fluoxetine might interact with K^+ channels. The three-dimensional structure of the Kcs A K^+ channel has been recently described to a 3.2 Å resolution (Doyle et al., 1998), so we explored whether fluoxetine could conceivably interact with the vestibular or pore regions of this channel structure. We worked in the Hyperchem 5.1 environment for Windows (Professional version; Hypercube, Gainesville, FL), running in a 330 Mhz Pentium machine. We displayed the K^+ channel, and removed the outermost of the three K^+ ions found in it (Doyle et al., 1998). We placed fluoxetine in line with the pore axis and some 5 Å away from the exofacial vestibule, and then ran a molecular mechanics simulation *in vacuo* with the MM+ force field (Hypercube's extension of the MM2 field developed by Allinger and colleagues (Burkert & Allinger, 1982)). To save time, the energy minimization involved fluoxetine and only the Kcs A residues forming part of the exofacial surface of the tetramer and the pore wall; the rest of the protein was immobilized. As the simulation converged to an energy minimum, fluoxetine moved and lodged itself with its amino end terminal inside the pore and its aromatic rings seemingly interacting with the pore vestibule (Fig. 6), a position in which the pore is blocked. Similar results were obtained with both the ternary (neutral) and the quaternary (cationic) forms of fluoxetine. Once the simulation had converged, we moved fluoxetine some 30 Å away from the pore entrance; the energy difference in favor of binding was −29.8 Kcal/mol. Using a similar procedure, we verified that the K^+ channel blocker tet-

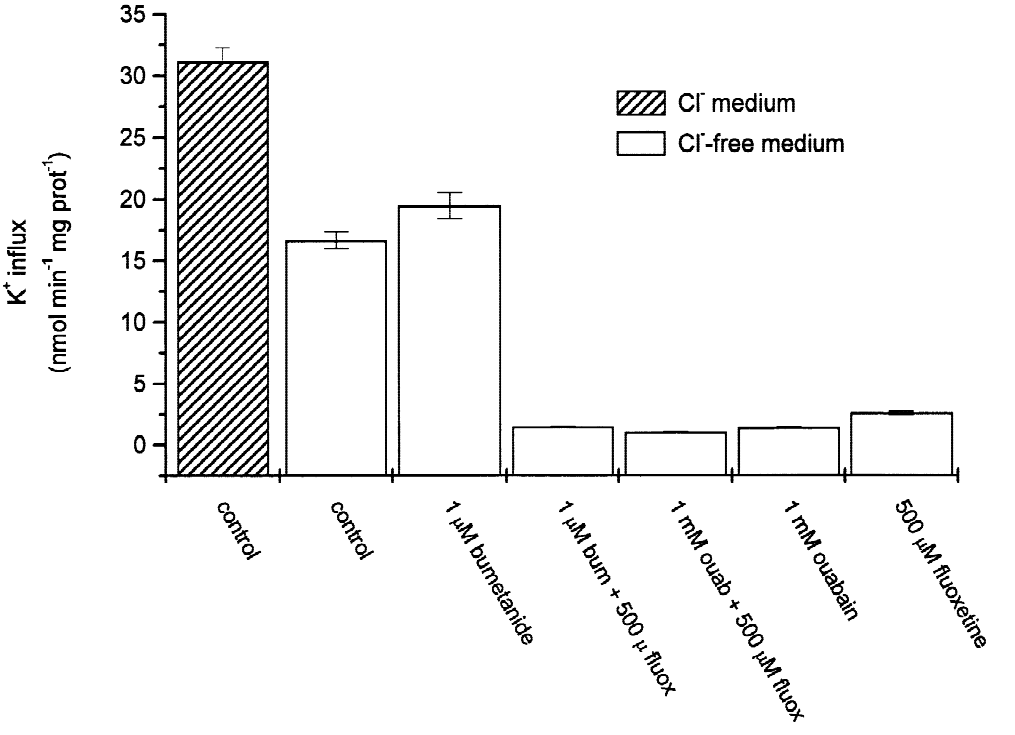

Fig. 5. Fluoxetine inhibition of the Na^+ pump. Four wells per column; data representative of some 100 wells.

raethyl-ammonium (TEA) also lodged itself at the channel outer entrance. Moving the TEA molecule 30 Å away from the channel entrance, the energy difference favoring binding was −11.2 Kcal/mol.

For comparison, using K_I values of 100 μM for fluoxetine (Fig. 3, inset) and 2.8 mM for TEA (Gomez-Hernandez et al., 1997) and assuming that the binding energies of these inhibitors (ΔF_b) are related to their K_I by: $\Delta F_b = RT\ ln\ (K_I/1\ mol\ L^{-1})$, we compute binding energies of −5.7 and −3.6 Kcal/mol, respectively. Given that these numbers refer to standard conditions rather than to a simulation in vacuum, the qualitative agreement appears satisfactory, and is consistent with both compounds blocking K^+ channels by interacting with the mouth of the selectivity filter, as proposed in Fig. 6.

Discussion

Ionic Transport Mechanisms Underlying Regulatory Volume Responses

This study documents the characteristics of the time course and identifies some ionic transport mechanisms underlying regulatory volume responses by CBCEC. Exponential time constants (τ) have been reported for RVD processes for ciliary epithelium (Yantorno et al., 1989; De Smet, Li, & van Driessche, 1998); we report here time constants for both RVD and RVI (Figs. 1 and 2). We find that RVD is some 8% than RVI (in minutes: $\tau_{RVD} = 2.44 \pm 0.01$; $\tau_{RVI} = 2.65 \pm 0.01$). Since in these cells RVD appears to be mediated by channels (Figs. 1 and 3) and RVI by transporters (Figs. 1 and 4), that $\tau_{RVD} < \tau_{RVI}$ is consistent with a faster rate of salt efflux during RVD than salt influx during RVI. However, whether such faster rate could be due to the sole involvement of channels requires further studies since, for instance, a KCl cotransporter has not been ruled out for these cells. It may be also noted that upon hypo-osmotic challenge, the percent cell volume change (25%) was somewhat larger than that upon hyperosmotic challenge (17%). Whether that asymmetry could account for differing regulatory volume rates is unknown at this time.

RVD

As in other tissues (rabbit corneal epithelium (Rae et al., 1995) and smooth muscle cells (Farrugia, 1996) K^+), CBCEC have K^+ efflux pathways which are fluoxetine-sensitive (Figs. 1 and 2). As to its specificity, as detailed below, fluoxetine reportedly inhibits several transport systems plus kinase activities and oxidative metabolism. However, its effects on K^+ conductance in the papers just mentioned are direct, take place at comparatively low concentrations and are very marked. We find that fluoxetine exerts a triphasic effect on K^+ efflux (Fig. 3), with

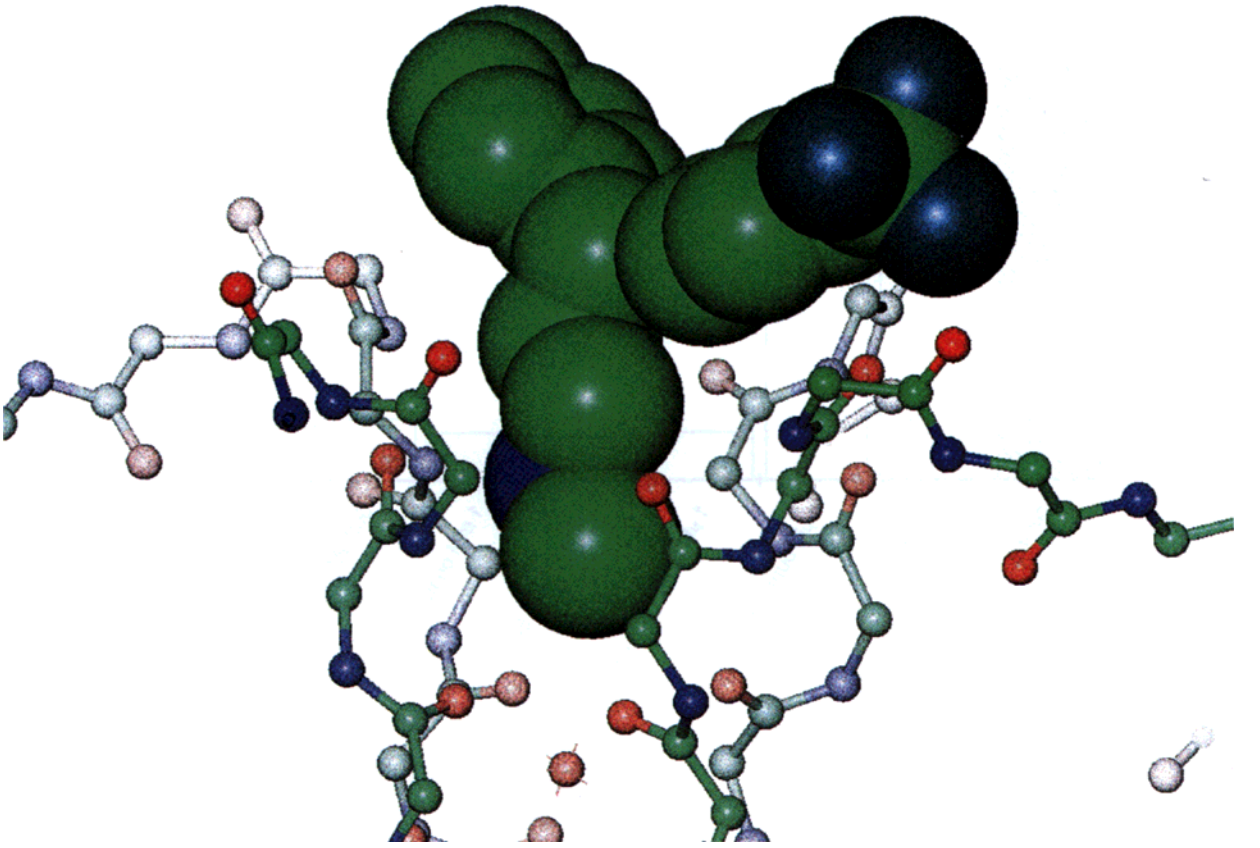

Fig. 6. Simulation of fluoxetine interaction with the outermost region of the selectivity filter of the Kcs A K^+ channel. Fluoxetine is shown in space-filling mode (no hydrogens). The K^+ channel is shown in ball and stick mode; only its backbone (and no hydrogens) are shown for clarity. Green: C; blue: N; red: O; dark green: F.

inhibitory effects up to 100 μM, followed by stimulatory effects up to 500 μM, and a reversion to inhibition at 1,000 μM. Our observations partially resemble those in Farrugia's prior report of a biphasic effect by fluoxetine in amphotericin-perforated patch whole cell recordings of intestinal smooth muscle cells (Farrugia, 1996) in that in both cases, progressive increases in [fluoxetine] first decreased and then increased K^+ efflux. Farrugia (1996) reported that low fluoxetine concentrations decreased the outwardly delayed rectifier potassium current, and higher concentrations activated calcium-activated potassium channels. That would explain part of our results since intermediate fluoxetine concentrations would have blocked the Na^+ pump with the consequent rise of $[Na^+]$ in the cell and inhibition of the Na^+-Ca^{2+} exchanger known to be present in these cells (Shepard & Rae, 1998). Fluoxetine concentrations above 500 μM could have then overwhelmed all different K^+ efflux pathways involved (Fig. 3, insert). In Farrugia's case, this pattern of effects occurred at 100× lower fluoxetine concentrations than in our case. We also find here that the K^+ efflux pathways inhibited at 50 μM fluoxetine are essential for RVD, which is consistent with the presence of volume activated K^+ channel activity in a variety of cells (Hallows & Knauf, 1994).

RVI

As in other tissues (Hallows & Knauf, 1994), we find that NKCC activity contributes to the RVI response; RVI in CBCEC can be partially inhibited by 1 μM bumetanide (Figs. 1 and 2), which is a fairly specific inhibitor of NKCC in many systems including the present one (Diecke et al., 1998). As expected, bumetanide (1 μM) also inhibited K^+ influx (Fig. 4*A*). This effect appeared specific for NKCC, since such decline in K^+ influx was the same as that measured upon Cl^--removal from the medium.

Interestingly, we found that 25 μM fluoxetine also inhibits K^+ influx (Fig. 4*B*, arrow) as much as either 1 μM bumetanide or Cl^--removal from the bathing solution (Fig. 4*A*, arrow). This equivalence suggests that 25 μM fluoxetine inhibits NKCC. Consistent with this, both 1 μM bumetanide and 20 μM fluoxetine partially inhibited RVI (Fig. 1). Moreover, at 50 μM, fluoxetine completely

inhibited RVI (Figs. 1 and 2) suggesting that at such concentration it could be directly or indirectly inhibiting other RVI-related pathways such as a Na^+ gradient-driven Na^+/H^+ exchanger-Cl^-/HCO_3^- exchanger tandem.

In keeping with this, the overall inhibitory effects of fluoxetine on K^+ influx were too large to be attributable only to NKCC inhibition. With 500 μM fluoxetine, the influx was only 13% of the control value (Fig. 4*B*, ● symbols). The inhibitory effects of fluoxetine were similar when NKCC activity was abolished in Cl^--free medium (Fig. 4*B*, ▲ symbols). It has been reported by us (Diecke et al., 1998) that the two main K^+ influx pathways into CBCEC are the NKC cotransporter and the Na^+ pump, each one accounting for approximately 50% of the total K^+ influx under the conditions of that study. In the present case, comparing the points in the two top curves in Fig. 4*B* at zero fluoxetine concentration (● and ▲ symbols), the decline in K^+ influx upon Cl^- removal indicates that NKCC activity accounts for about 30% of the total K^+ influx, which is of the same order as the inhibition seen in the prior study. Hence, since 500 μM fluoxetine inhibited K^+ influx by 87% (Fig. 4*B*), it follows that such a large inhibition can only be explained by fluoxetine also inhibiting the Na^+ pump.

In summary, the data in Fig. 4*B* reveals two lines of evidence that fluoxetine inhibits both the NKC cotransporter and the Na^+ pump over a similar concentration range. (i) fluoxetine inhibits K^+ influx ($IC_{50} = 108 \pm 26$ μM) in the absence of ambient Cl^-, when such influx is attributable to Na^+ pump activity. (ii) Subsequent to inhibition of the Na^+ pump by ouabain, fluoxetine inhibited ($IC_{50} = 121 \pm 59$ μM) the K^+ influx attributable to residual NKCC under those conditions. The IC_{50} values in both cases were not significantly different from one another.

Further evidence that fluoxetine (500 μM) inhibits Na^+ pump activity is summarized in Fig. 5. As shown in Fig. 4*A* (symbols ○ and △), 1 μM bumetanide is sufficient to fully inhibit NKCC activity based on the equivalence between its inhibitory effect on total K^+ influx in the presence and absence of Cl^- in the medium. As bumetanide is a selective inhibitor of NKCC, comparing the effect of bumetanide alone with that of bumetanide plus fluoxetine (Fig. 5), it is apparent that the larger suppressive effect in the latter case also reflects inhibition of Na^+ pump activity by fluoxetine. This suggestion is supported by the findings that ouabain by itself or in combination with 500 μM fluoxetine inhibited K^+ influx to the same extent as 500 μM fluoxetine alone.

Fluoxetine-insensitive Pathways

From our results, fluoxetine emerges as a "broad spectrum" inhibitor of ion transport mechanisms associated with RVD and RVI. As noted, fluoxetine dose-dependently inhibited RVD between 20 and 50 μM (Fig. 2), and this dose-dependent inhibition by fluoxetine corresponded with its potency to inhibit K^+ efflux (Fig. 3). Interestingly, 50 μM fluoxetine was sufficient to completely suppress RVD (Figs. 1 and 2) but 100 μM fluoxetine only inhibited K^+ efflux by 50% (Fig. 3). Such inhibition does not appear to be due to a nonspecific increase in K^+ permeability. First, two different cell viability tests indicated no loss of function; second, the declines in K^+ efflux at both 50 μM and 1 mM fluoxetine were similar. Hence, there appear to be fluoxetine insensitive K^+ efflux pathways (Fig. 3) which do not contribute to the RVD response.

Mechanisms of Action of Fluoxetine

The effects of fluoxetine on NKCC and the Na^+ pump we report here are novel. In principle, such effects could be direct, or could stem from an intracellular $[K^+]$ buildup secondary to fluoxetine-induced inhibition of K^+ channel-mediated efflux. However, our evidence suggests that these effects are direct, since we find that in the fluoxetine concentration range between 50 and 500 μM, it inhibited dose-dependently NKCC and the Na^+ pump (Fig. 4*B*), but did not consistently inhibit K^+ efflux (Fig. 3, insert). As a qualification, from this evidence we cannot exclude additional, more complex effects of fluoxetine on intracellular regulatory mechanisms.

Fluoxetine (Prozac®) is widely prescribed as an antidepressant, and its therapeutic action is frequently ascribed to selective inhibition of presynaptic serotonin reuptake (Fuller & Wong, 1977). However, fluoxetine has other effects. It inhibits K^+ permeability in the lens and the corneal epithelium (Rae et al., 1995), and our results (Fig. 3, insert) coupled with those of Farrugia (1996) show that such fluoxetine effects are complex. Lastly, we now describe here that fluoxetine (5–500 μM) also progressively inhibits NKCC activity and the Na^+ pump. It is unclear whether any of these other effects contribute to the clinical action of fluoxetine. Therapeutic doses of fluoxetine can result in a blood level of at least 1 μM (Altamura, Moro, & Percudani, 1994). Given variations in dosage, it is conceivable that fluoxetine plasma levels could approach the 5 μM level at which we begin to note effects on NKCC activity (Fig. 4*B*).

Possible Modes of Action of Fluoxetine

As mentioned above, the effects we report here on K^+ channel(s) appear direct. However, with regard to the NKC cotransporter and the Na^+ pump, the fluoxetine effects could be either direct or indirect (or both).

Regarding possible direct effects of fluoxetine, given the very high conservation of the K^+-recognition site sequences among all K^+ channels (Doyle et al.,

1998), the 3-D structures at these sites could be very similar. The simulation summarized in Fig. 6 for the Kcs A K^+ channel suggests that fluoxetine could block K^+ channels by binding to their K^+-recognition sites located at the extracellular opening of its pore. From our simulation, the key to the effect would be the amino group of fluoxetine taking the place of the outermost K^+ ion in the selectivity filter. Other compounds which include nitrogen in a short linear aliphatic chain would be expected to do likewise, and this is borne out by our observation of simulated TEA binding to the Kcs A pore entrance (*not shown*). If the structure of K^+ recognition sites in other proteins are similar to that of the Kcs A K^+ channel (Doyle et al., 1998), fluoxetine could conceivably block their K^+-recognition sites as well. Consistent with this, TEA has been recently reported to block the Na^+ pump in a voltage-dependent mode (Eckstein-Ludwig et al., 1998). Hence, fluoxetine could block putative K^+ recognition sites in other transport pathways such as the NKC cotransporter and the Na^+ pump. Admittedly, this is speculative, since in either case the location of such sites has not yet been determined.

On the other hand, the fluoxetine effects reported here could also be indirect, since it has been shown to inhibit kinase activities (Silver, Sigg, & Moyer, 1986; Vaitla et al., 1997) and oxidative metabolism (Souza et al., 1994). Such effects could contribute to the inhibitory effects of fluoxetine on corneal endothelial ion transport, since it is known that the activities of both the NKCC cotransporter (Diecke et al., 1998; Lytle, 1998) and the Na^+ pump (Blanco, Sanchez, & Mercer, 1998) are phosphorylation-dependent. Additional studies are needed to clarify which of these possibilities accounts for the fluoxetine effects described.

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