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# Fluoxetine inhibits ATP-induced $[Ca^{2+}]_i$ increase in PC12 cells by inhibiting both extracellular $Ca^{2+}$ influx and $Ca^{2+}$ release from intracellular stores

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**Abstract**

Fluoxetine, a widely used antidepressant, has additional effects, including the blocking of voltage-gated ion channels. We examined whether fluoxetine affects ATP-induced calcium signaling in PC12 cells using fura-2-based digital calcium imaging, an assay for [$^3$H]-inositol phosphates (IPs) and whole-cell patch clamping.

Treatment with ATP (100 μM) for 2 min induced increases in intracellular free $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$). Treatment with fluoxetine (100 nM to 30 μM) for 5 min inhibited the ATP-induced $[Ca^{2+}]_i$ increases in a concentration-dependent manner ($IC_{50} = 1.85$ μM). Treatment with fluoxetine (1.85 μM) for 5 min significantly inhibited the ATP-induced responses following the removal of extracellular $Ca^{2+}$ or depletion of intracellular $Ca^{2+}$ stores. Whereas treatment for 10 min with nimodipine (1 μM) significantly inhibited the ATP-induced $[Ca^{2+}]_i$ increase, treatment with fluoxetine further inhibited the ATP-induced response. Treatment with fluoxetine significantly inhibited $[Ca^{2+}]_i$ increases induced by 50 mM $K^+$. In addition, treatment with fluoxetine markedly inhibited ATP-induced inward currents in a concentration-dependent manner. However, treatment with fluoxetine did not inhibit ATP-induced [$^3$H]-IPs formation.

Therefore, we conclude that fluoxetine inhibits ATP-induced $[Ca^{2+}]_i$ increases in PC12 cells by inhibiting both the influx of extracellular $Ca^{2+}$ and the release of $Ca^{2+}$ from intracellular stores without affecting IPs formation.


*Keywords:* ATP; Fluoxetine; Inositol phosphates; PC12 cells; Voltage-gated $Ca^{2+}$ channels

## 1. Introduction

Fluoxetine is a widely used antidepressant, the action of which is primarily attributed to the inhibition of serotonin reuptake into the synaptic clefts of the central nervous system (Wong et al., 1974, 1995). Fluoxetine has additional blocking effects on voltage-gated $K^+$ channels (Rae et al., 1995; Farrugia, 1996; Tytgat et al., 1997; Choi et al., 1999; Hahn et al., 1999;

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Choi et al., 2003a), $Na^+$ channels (Rae et al., 1995; Pancrazio et al., 1998; Hahn et al., 1999) and voltage-gated $Ca^{2+}$ channels (Hahn et al., 1999; Deak et al., 2000; Pacher et al., 2000; Wang et al., 2003). Moreover, fluoxetine inhibits ligand-gated ion channels, such as the 5-$HT_3$-receptor-gated channel (Fan, 1994a,b; Choi et al., 2003b; Eisensamer et al., 2003), the nicotinic-acetylcholine-receptor-gated channel (Garcia-Colunga et al., 1997), and calcium-activated potassium channels (Farrugia, 1996; Terstappen et al., 2003).

ATP induces an increase in the concentration of intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) in PC12 cells by activating P2X-receptor-regulated non-selective cation channels (Fasolato et al., 1990; Nakazawa et al., 1990; Reber et al., 1992) and P2Y-receptor-induced activation of phospholipase C (PLC) (Murrin and Boarder, 1992; Park et al., 1997). Moreover, the ATP-induced activation of non-selective cation channels depolarizes the cell membrane, which causes an increase in $[Ca^{2+}]_i$ through the activation of voltage-gated L-type $Ca^{2+}$ channels (Hur et al., 2001). ATP-induced $[Ca^{2+}]_i$ increases in PC12 cells may be involved in the release of monoamines (Majid et al., 1992; Nakazawa and Inoue, 1992; Suh et al., 1995), which play important roles in the pathogenesis of depression. However, fluoxetine has not been shown to affect ATP-induced calcium signaling via P2X receptors or P2Y receptors in neuronal cells.

We examined the effects of fluoxetine on ATP-induced calcium signaling in PC12 cells using fura-2-based digital calcium imaging, an assay for [$^3$H]-inositol phosphates (IPs), and whole-cell patch clamping. The finding shows that fluoxetine inhibits ATP-induced $[Ca^{2+}]_i$ increases in PC12 cells by inhibiting both the influx of extracellular $Ca^{2+}$ and the release of $Ca^{2+}$ from intracellular stores without affecting IPs formation.

## 2. Methods

### 2.1. Materials

Materials were purchased as follows: fura-2 acetoxymethyl ester (AM) from Molecular Probes (Eugene, OR, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS, heat-inactivated) and horse serum (HS, heat-inactivated) from Invitrogen (Carlsbad, CA, USA); ATP disodium salt and all other reagents from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture

Rat pheochromocytoma (PC12) cells were grown in DMEM with 5% FBS, 5% HS, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. To measure $[Ca^{2+}]_i$, cells from the stock culture were plated onto glass coverslips (25 mm round) at a density of $3 \times 10^4$ cells/coverslip. Cells were used for experiments 2–3 days after plating.

### 2.3. Digital calcium imaging

Digital calcium imaging was performed as described by Rhie et al. (2003). The cells were loaded with 2 μM fura-2 AM in HEPES-buffered Hank's salt solution (HEPES–HBSS) containing 0.5% bovine serum albumin for 45 min at 37 °C. The HEPES–HBSS was composed of the following: 20 mM HEPES; 137 mM NaCl; 1.26 mM $CaCl_2$; 0.4 mM $MgSO_4$; 0.5 mM $MgCl_2$; 5 mM KCl; 0.4 mM $KH_2PO_4$; 0.6 mM $Na_2HPO_4$; 3 mM $NaHCO_3$; and 5 mM glucose. To elicit depolarization-induced activation of the voltage-gated $Ca^{2+}$ channels, we used 50 mM $K^+$ HEPES–HBSS, in which 137 mM NaCl and 5 mM KCl were replaced with 92.3 mM NaCl and 50 mM KCl, respectively. The loading was terminated by washing with HEPES–HBSS for 15 min before starting the experiment. The coverslip was then mounted in a flow-through chamber, which was superfused at 2 ml/min. Solutions were selected with a multi-port valve coupled to several reservoirs. The chamber containing the fura-2-loaded cells was mounted on the stage of an inverted microscope (Nikon TE300, Tokyo, Japan) and excited alternately at 340 nm and 380 nm by rapidly switching filters (10 nm band-pass) mounted on a computer-controlled wheel (Lambda 10-2; Sutter Instruments, Novato, CA, USA) placed between a 100 W xenon arc lamp and the epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm) through a ×20 objective (Nikon; N.A. 0.5). Digital fluorescence images (510 nm, 40 nm band-pass) were collected with a cooled CCD camera (Photometrics; 1280 × 1035 binned to 256 × 207 pixels). Image pairs were collected every 6–20 s using an Axon Imaging Work Bench 2.2 (Axon Instruments, Foster City, CA, USA); exposure to excitation light was 120 ms per image.

$[Ca^{2+}]_i$ was calculated from the ratio of the background-subtracted digital images. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380 nm image. Background images were collected at the beginning of each experiment after removing cells from another area to the coverslip. Autofluorescence from cells not loaded with the dye was less than 5% and thus not corrected. Ratio values were converted to free $[Ca^{2+}]_i$ by the equation $[Ca^{2+}]_i = K_d(\beta R - R_{min})/(R_{max} - R)$, in which $R$ is the 340/380 nm fluorescence emission ratio, and $K_d = 224$ nM is the dissociation constant for fura-2. The maximum ratio ($R_{max} = 10.773$), the minimum ratio ($R_{min} = 0.311$), and the constant $\beta$ (the ratio of the fluorescence measured at 380 nm in $Ca^{2+}$-free and saturating solutions) were determined by treating cells