Effect of Fluoxetine on \([\text{Ca}^{2+}]_i\) and Cell Viability in OC2 Human Oral Cancer Cells

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Abstract

Fluoxetine is a serotonin-specific reuptake inhibitor that has been used as an antidepressant. This study examined the effect of fluoxetine on cytosolic free \([\text{Ca}^{2+}]_i\) and viability in OC2 human oral cancer cells. The \([\text{Ca}^{2+}]_i\)-sensitive fluorescent dye fura-2 was used to measure \([\text{Ca}^{2+}]_i\), and the water soluble tetrazolium (WST-1) regent was used to measure viability. Fluoxetine-induced \([\text{Ca}^{2+}]_i\) rises concentration-dependently. The response was reduced by half by removing extracellular \text{Ca}^{2+}. Fluoxetine-induced \([\text{Ca}^{2+}]_i\) entry was enhanced by activation of protein kinase C (PKC) with phorbol 12-myristate 13 acetate (PMA) but was inhibited by inhibition of the enzyme with GF109203X. In \text{Ca}^{2+}-free medium, treatment with the endoplasmic reticulum \text{Ca}^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) or thapsigargin abolished fluoxetine-evoked \([\text{Ca}^{2+}]_i\) rise. Conversely, treatment with fluoxetine inhibited BHQ/thapsigargin-evoked \([\text{Ca}^{2+}]_i\) rise. Inhibition of phospholipase C (PLC) with U73122 abolished fluoxetine-induced \([\text{Ca}^{2+}]_i\) rise. At 20-80 \(\mu\text{M}\), fluoxetine decreased cell viability concentration-dependently, which was not altered by chelating cytosolic \text{Ca}^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM). At 20-60 \(\mu\text{M}\), fluoxetine induced apoptosis as detected by annexin V/propidium iodide (PI) staining. Together, in OC2 cells, fluoxetine induced \([\text{Ca}^{2+}]_i\) rises by evoking PLC-dependent \text{Ca}^{2+} release from the endoplasmic reticulum and \text{Ca}^{2+} entry via PKC-regulated mechanisms. Fluoxetine also caused \text{Ca}^{2+}-independent apoptosis.

Key Words: apoptosis, \text{Ca}^{2+}, fluoxetine, human oral cancer cells, OC2

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Introduction

Fluoxetine is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class. Fluoxetine is designated (±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyloxy)propylamine hydrochloride and has the empirical formula of C\textsubscript{17}H\textsubscript{18}F\textsubscript{3}NO\textsubscript{2}HCL (36). Fluoxetine was first documented in 1974 (36), and has been approved for the treatment of major depression including pediatric depression, obsessive-compulsive disorder, bulimia nervosa, panic disorder and premenstrual dysphoric disorder (22). Fluoxetine’s mechanism of action is primarily that of an SSRI although it may produce some of its effects through 5-HT\textsubscript{2C} antagonism in a manner similar to the novel antidepressant agomelatine (11). Furthermore, a net decrease in the signalization pathway of 5-HT(4) receptors occurs after chronic selective serotonin reuptake inhibitor fluoxetine treatment. Therefore, long-term treatment with fluoxetine induces desensitization of 5-HT4 receptor-dependent signalling and functionality in rat brain (35).

It has been shown that fluoxetine is not solely effective by the instant inhibition of the serotonin transporter but also by its influence on mitotic and/or apoptotic processes. For example, fluoxetine induces apoptosis in maturing neuronal cells (24) and drug-resistant Burkitt’s lymphoma through Ca\textsuperscript{2+} responses (8). Despite various lines of evidence, the effect of fluoxetine on oral cells is unclear.

Ca\textsuperscript{2+} ions have a key role in different biological responses. A rise in cytosolic free Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) can induce many pathophysiological cellular events (1). Inositol 1,4,5-trisphosphate (IP\textsubscript{3}), derived from activation of phospholipase C (PLC), is a predominant messenger for releasing store Ca\textsuperscript{2+} from the endoplasmic reticulum (7). Mobilization of store Ca\textsuperscript{2+} may activate Ca\textsuperscript{2+} influx across the plasma membrane via store operated Ca\textsuperscript{2+} entry (7). However, an uncontrolled [Ca\textsuperscript{2+}], rise may induce ion movement, dysfunction of enzymes, apoptosis, and proliferation, etc. (7). Fluoxetine has been shown to induce [Ca\textsuperscript{2+}], rises in bladder cancer cells (28) and renal tubular cells (27). However, the in vitro and in vivo effects of fluoxetine on oral cancer cells are unknown.

In order to explore the effect of fluoxetine on [Ca\textsuperscript{2+}], in human oral cancer cells, the OC2 cell line was used because it produces measurable [Ca\textsuperscript{2+}]; rises upon pharmacological stimulation. The OC2 cell is commonly applied for oral cancer studies. It has been shown that in this cell, [Ca\textsuperscript{2+}], rises can be induced in response to the stimulation of various compounds such as carvacrol (19) and safrole (13).

The goal of this study was to explore the effect of fluoxetine on [Ca\textsuperscript{2+}]; viability in OC2 cells. Fura-2 was used as a Ca\textsuperscript{2+}-sensitive dye to measure [Ca\textsuperscript{2+}]. The [Ca\textsuperscript{2+}], rises were characterized, the concentration-response plots were established, the mechanisms underlying fluoxetine-evoked Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} release was examined. The effect of fluoxetine on viability and apoptosis was assessed by using water soluble tetrazolium (WST-1) regent and annexin V/propidium iodide (PI) fluorescent dye, respectively.

Materials and Methods

Chemicals

The chemicals for cell culture were from Gibco® (Gaithersburg, MD, USA). The other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

OC2 human oral cancer cells purchased from Bioresource Collection and Research Center (Taiwan, ROC) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in [Ca\textsuperscript{2+}], Measurements

Ca\textsuperscript{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 10 mM Hepes, and 5 mM glucose. Ca\textsuperscript{2+}-free medium contained similar chemicals as Ca\textsuperscript{2+}-containing medium except that CaCl\textsubscript{2} was replaced with 0.3 mM EGTA and 2 mM MgCl\textsubscript{2}. Fluoxetine was dissolved in dimethyl sulfoxide as a 0.1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability, apoptosis or basal [Ca\textsuperscript{2+}].

[Ca\textsuperscript{2+}], Measurements

The [Ca\textsuperscript{2+}], was measured as previously described (13, 19). Confluent cells grown on 6 cm dishes were trypsinized and suspended in culture medium at a density of 10\textsuperscript{6}/ml. Cell viability was assessed by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension). The viability was greater than 95% after the treatment. Cells were subsequently incubated with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca\textsuperscript{2+}-containing medium twice and was suspended in Ca\textsuperscript{2+}-containing medium at a density of 10\textsuperscript{7}/ml. Fura-2 fluorescence measurements were conducted in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was recorded with
a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. To calibrate [Ca\(^{2+}\)], after completion of the experiments, the detergent Triton X-100 (0.1%) and CaCl\(_2\) (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. The Ca\(^{2+}\) chelator EGTA (10 mM) was subsequently added to chelate Ca\(^{2+}\) in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells incubated in a cuvette had a viability of 95% after 20 min of fluorescence measurements. [Ca\(^{2+}\)]\(_i\) was calculated as previously described (3-5, 12).

**Cell Viability Analyses**

Viability was assessed as previously described (3-5). The measurement of viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Changes in color intensity correlated with the number of live cells. Assays were performed according to manufacturer’s instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of fluoxetine. The cell viability testing reagent 4-[3-[4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after fluoxetine treatment. The cell viability detecting reagent solution (Probes Invitrogen, Eugene, Oregon, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wavelength was at 488 nm and the emitted green fluorescence of annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 nm and 575 nm band pass filters, respectively. A total of 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of early apoptosis and late apoptosis/necrosis were determined, respectively, as the percentage of annexin V\(^{+/−}\)/PI- or annexin V\(^{+/−}\)/PI\(^{+}\) cells. Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software). X and Y coordinates refer to the intensity of fluorescence of annexin and PI, respectively.

**Statistics**

Data are reported as mean ± SEM of three separate experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS\(^{®}\), SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by post-hoc analysis using the Tukey’s HSD (honestly significantly difference) procedure. A P-value less than 0.05 was considered significant.

**Results**

**Effect of Fluoxetine on [Ca\(^{2+}\)]\(_i\)**

The basal [Ca\(^{2+}\)] level was 50 ± 1 nM (Fig. 1A). In Ca\(^{2+}\)-containing medium, fluoxetine induced a [Ca\(^{2+}\)]\(_i\) rise in a concentration-dependent manner at concentrations between 25 and 150 μM. At a concentration of 100 μM, fluoxetine evoked a [Ca\(^{2+}\)]\(_i\) rise that reached a net increase of 95 ± 2 nM (n = 3) followed by a slow decay. The Ca\(^{2+}\) response saturated at 150 μM fluoxetine because 200 μM fluoxetine did not evoke a greater response. Fig. 1B shows that in Ca\(^{2+}\)-free medium, 25-150 μM fluoxetine induced concentration-dependent increase in [Ca\(^{2+}\)]. Fig. 1C shows the concentration-response plots of fluoxetine-induced [Ca\(^{2+}\)]\(_i\) rises. The EC\(_{50}\) value was 100 ± 1 μM in both Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium by fitting to a Hill equation.
Regulation of Fluoxetine-Induced \([\text{Ca}^{2+}]_i\) Rise

Phorbol 12-myristate 13 acetate [PMA; 1 nM; a protein kinase C (PKC) activator] or GF109203X (2 \(\mu\)M; a PKC inhibitor) was applied 1 min before fluoxetine (150 \(\mu\)M). The concentration was 10 nM for phorbol 12-myristate 13-acetate (PMA), and 2 \(\mu\)M for GF109203X. Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 150 \(\mu\)M fluoxetine-induced \([\text{Ca}^{2+}]_i\) rise in \(\text{Ca}^{2+}\)-containing medium, and are mean \(\pm\) SEM of three separate experiments. \(*P < 0.05\) compared to the 1st column. \(^{#}P < 0.05\) compared to the 3rd column.

Source of Fluoxetine-Induced \([\text{Ca}^{2+}]_i\) Release

In most cell types including OC2 cells, the endoplasmic reticulum has been shown to be a main \(\text{Ca}^{2+}\) store (3-5). Thus the role of endoplasmic reticulum in fluoxetine-evoked \(\text{Ca}^{2+}\) release in OC2 cells was explored. The experiments were conducted in \(\text{Ca}^{2+}\)-free medium to exclude the involvement of \(\text{Ca}^{2+}\) influx. Fig. 3A shows that addition of 50 \(\mu\)M 2,5-di-tert-butylhydroquinone (BHQ), an endoplasmic reticulum \(\text{Ca}^{2+}\) pump inhibitor (34), induced a \([\text{Ca}^{2+}]_i\) rise of 20 \(\pm\) 3 nM. Subsequently added 150 \(\mu\)M fluoxetine failed to induce a \([\text{Ca}^{2+}]_i\) rise. Fig. 3B shows that after fluoxetine-induced \([\text{Ca}^{2+}]_i\) rise had decayed to baseline, addition of 50 \(\mu\)M BHQ induced a \([\text{Ca}^{2+}]_i\) rise of 10 \(\pm\) 2 nM, which was smaller than the BHQ response in Fig. 3A by 50%. Another endoplasmic reticulum \(\text{Ca}^{2+}\) pump inhibitor thapsigargin (30) was applied for similar purposes. Fig. 3C shows that thapsigargin (1 \(\mu\)M) induced a \([\text{Ca}^{2+}]_i\) rise of 51 \(\pm\) 1 nM.
Fluoxetine (150 μM) added afterwards failed to induce a [Ca^{2+}]_i rise. Conversely, Fig. 3D shows that after fluoxetine treatment for 470 sec, thapsigargin also failed to evoke a [Ca^{2+}]_i rise.

A Role of PLC in Fluoxetine-Induced [Ca^{2+}]_i Rise

PLC is one of the pivotal proteins that regulate the releasing of Ca^{2+} from the endoplasmic reticulum. Because fluoxetine released Ca^{2+} from the endoplasmic reticulum, the role of PLC in this process was explored. U73122 (31), a PLC inhibitor, was applied to explore if the activation of this enzyme was required for fluoxetine-induced Ca^{2+} release. Fig. 4A shows that ATP (10 μM) induced a [Ca^{2+}]_i rise of 51 ± 2 nM. ATP is a PLC-dependent agonist of [Ca^{2+}]_i rise in most cell types (10). Fig. 4B shows that incubation with 2 μM U73122 did not change basal [Ca^{2+}]_i, but abolished ATP-induced [Ca^{2+}]_i rise. This suggests that U73122 effectively suppressed PLC activity. The data also show that incubation with 2 μM U73122 did not alter basal [Ca^{2+}]_i, but abolished 150 μM fluoxetine-induced [Ca^{2+}]_i rise. U73343 (2 μM), a U73122 analogue, failed to have an inhibition (data not shown).

Effect of Fluoxetine on Cell Viability

Cells were treated with 0-80 μM fluoxetine for 24 h, and the tetrazolium assay was performed. In the presence of 20-80 μM fluoxetine, cell viability decreased in a concentration-dependent manner (Fig. 5). Fig. 5 also shows that 5 μM BAPTA/AM (32) loading did not change the control value of cell viability. In the presence of fluoxetine, BAPTA loading did not
Effect of Fluoxetine on Oral Cancer Cells

Since apoptosis plays a key role in cell death, the next issue was whether the fluoxetine-induced cytotoxicity was through apoptosis. Annexin V/PI staining was applied to detect apoptotic cells after fluoxetine treatment. Fig. 6 show that treatment with 20-60 μM fluoxetine induced an increase in percentage of apoptotic cells by 6.2 ± 2%, 9.7 ± 2% or 13.2 ± 2%, respectively (P < 0.05).
Our study shows that fluoxetine increased \([\text{Ca}^{2+}]_i\), and also caused apoptosis in an independent manner in OC2 human oral cancer cells. The \([\text{Ca}^{2+}]_i\) signal was composed of \([\text{Ca}^{2+}]_i\) entry and \([\text{Ca}^{2+}]_i\) release because the signal was reduced by half by removing extracellular \([\text{Ca}^{2+}]_i\). Although fluoxetine has also been shown to increase \([\text{Ca}^{2+}]_i\) in other cell types such as human frontal cortex (14), human bladder carcinoma cells (28), and renal tubular cells (27), it was also shown to inhibit \([\text{Ca}^{2+}]_i\)-activated currents of salamander rod photoreceptor somata and presynaptic terminals via inhibition of intracellular \([\text{Ca}^{2+}]_i\) dynamics (26), and to inhibit ATP-induced \([\text{Ca}^{2+}]_i\) increase in PC12 cells by inhibiting both extracellular \([\text{Ca}^{2+}]_i\) influx and \([\text{Ca}^{2+}]_i\) release from intracellular stores (16). Furthermore, fluoxetine was shown to decrease arteriolar myogenic tone by reducing smooth muscle \([\text{Ca}^{2+}]_i\) (33) and to inhibit synaptosomal \([\text{Ca}^{2+}]_i\) influx via possible \([\text{Ca}^{2+}]_i\) channel inhibition (25). Thus, the effect of fluoxetine on \([\text{Ca}^{2+}]_i\) signaling appears to vary among cell types.

Previous evidence shows that in OC2 cells the dominant \([\text{Ca}^{2+}]_i\) entry pathway is the store-operated \([\text{Ca}^{2+}]_i\) channels (6). The activity of many protein kinases is known to associate with \([\text{Ca}^{2+}]_i\) homeostasis (17, 20). Our data show that fluoxetine-evoked \([\text{Ca}^{2+}]_i\) rise was dramatically increased when PKC was activated and was inhibited when PKC was suppressed. The interactive relationship between PKC and \([\text{Ca}^{2+}]_i\) signaling has been well established. Mukherjee et al. (21) showed that \([\text{Ca}^{2+}]_i\) oscillations, \([\text{Ca}^{2+}]_i\) sensitization, and contraction were activated by PKC in small airway smooth muscle. In contrast, Bynagari-Settipalli et al. (2) showed that PKC negatively regulated ADP-induced \([\text{Ca}^{2+}]_i\) mobilization and thromboxane generation in platelets.

Regarding the \([\text{Ca}^{2+}]_i\) stores involved in fluoxetine-evoked \([\text{Ca}^{2+}]_i\) release, the BHQ/thapsigargin-sensitive endoplasmic reticulum store seemed to be the dominant one. The data further show that the \([\text{Ca}^{2+}]_i\) release was via a PLC-dependent mechanism, given the release was abolished when PLC activity was inhibited.

Cellular activation by many agonists results in the stimulation of PLC and the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to \(\text{IP}_3\) and diacylglycerol (DAG) (7). Each of these two molecules exerts a specific effect on the cell. The increased DAG concentration leads to the activation of PKC while \(\text{IP}_3\) binds to the \(\text{IP}_3\) receptor (\(\text{IP}_3\R\)), an intracellular \([\text{Ca}^{2+}]_i\)-release channel located in the endoplasmic reticulum, thereby inducing \([\text{Ca}^{2+}]_i\) release from internal stores (7). Therefore, it is suggested that fluoxetine induced \([\text{Ca}^{2+}]_i\), rises by evoking PLC-dependent \([\text{Ca}^{2+}]_i\) release from the endoplasmic reticulum and \([\text{Ca}^{2+}]_i\) entry via PKC-regulated \(\text{IP}_3\) signaling pathway.

Cell viability could be altered in a \([\text{Ca}^{2+}]_i\)-dependent or -independent manner (9, 23). Our data show that fluoxetine induced cell death in a concentration-dependent manner at ranges that induced \([\text{Ca}^{2+}]_i\), rises. However, fluoxetine-induced cell death did not appear to be caused by a preceding \([\text{Ca}^{2+}]_i\), rise. Because the data show that treatment with 20-60 \(\mu\text{M}\) fluoxetine induced an increase in the percentage of apoptotic cells, it is suggested that fluoxetine-induced cytotoxicity involved apoptosis. Although 60 \(\mu\text{M}\) fluoxetine caused cell death by 85% in viability experiments, the same concentration of fluoxetine only induced apoptosis in 13% of cells. Thus it is possible that the significant
loss of cell viability is through other pathways such as necrosis or autophagy. Evidence also suggests that fluoxetine (10-100 μM) induced apoptosis in other cell types such as colon cancer cells (15), Burkitt’s lymphoma cells (8), and ovarian carcinoma cells (18). Similarly, our data show that in OC2 cells, fluoxetine (20-60 μM) induced apoptosis. Therefore, fluoxetine may serve as an effective adjunctive reagent for the treatment of tumor.

A previous study explored the plasma concentration of fluoxetine after oral administration (29). The doses administered were 20 mg/day in healthy adults. No BioResponse (BR) fluoxetine-related adverse effects were reported at 20 mg. A single 20 mg dose of BR-fluoxetine resulted in a maximum plasma concentration (C_{max}) of ~ 3 μM after 12 h. A single 20 mg dose of BR-fluoxetine resulted in a mean C_{max} of ~ 5 μM after 24 h. BR-fluoxetine was well tolerated at single doses of up to 20 mg (29). However, in depression patients, the plasma concentration of fluoxetine after oral administration might be 10-fold higher than in healthy adults. Thus, our study may have clinical relevance and serve as a promising therapeutic agent for human oral cancer in the future.

Collectively, the results show that, via a PKC-sensitive pathway, fluoxetine induced Ca^{2+} release from endoplasmic reticulum in a PLC-dependent manner and also caused Ca^{2+} influx in OC2 human oral cancer cells. Fluoxetine also induced apoptosis in a Ca^{2+}-independent manner.

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