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Paroxetine-Induced Ca^{2+} Movement and Death in OC2 Human Oral Cancer Cells

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Abstract

The effect of the antidepressant paroxetine on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in OC2 human oral cancer cells is unclear. This study explored whether paroxetine changed basal $[Ca^{2+}]_i$ levels in suspended OC2 cells by using fura-2 as a Ca^{2+}-sensitive fluorescent dye. Paroxetine at concentrations between 100-1,000 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The Ca^{2+} signal was reduced by 50% by removing extracellular Ca^{2+}. Paroxetine-induced Ca^{2+} influx was inhibited by the store-operated Ca^{2+} channel blockers nifedipine, econazole and SK&F96365, and protein kinase C modulators. In Ca^{2+}-free medium, pretreatment with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin abolished paroxetine–induced $[Ca^{2+}]_i$ rise. Inhibition of phospholipase C with U73122 did not alter paroxetine-induced $[Ca^{2+}]_i$ rise. Paroxetine at 10-50 μM induced cell death in a concentration-dependent manner. The death was not reversed when cytosolic Ca^{2+} was chelated with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. Propidium iodide staining suggests that apoptosis plays a role in the death. Collectively, in OC2 cells, paroxetine induced $[Ca^{2+}]_i$ rise by causing phospholipase C-independent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx *via* store-operated Ca^{2+} channels in a manner regulated by protein kinase C and phospholipase A2. Paroxetine (up to 50 μM) induced cell death in a Ca^{2+}-independent manner.

Key Words: Ca^{2+}, OC2, oral cancer, paroxetine

Introduction

Paroxetine is the most potent selective serotonin re-uptake inhibitor (SSRI), which is available in immediate release and controlled release formulations (30). SSRIs show antidepressant properties in many patients with a diagnosis of depression (16). However, at the cellular level, paroxetine appears to have many

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effects that are dissociated from its effect in the brain. Paroxetine was shown to kill human cancer cells (10, 29) and to induce apoptosis in glioma and neuroblastoma cells (21). Paroxetine also directly alters ion channel activities such as blocking of neuronal Na^+ currents (9, 39) and suppression of purinergic receptors (26) and G protein-activated K^+ channels (17). Paroxetine is thought to evoke cell death *via* apoptosis in human osteosarcoma cells by activation of p38 MAP kinase and caspase-3 pathways (5) and to cause cell death and intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) rise in renal tubular cells (4).

Ca^{2+} ions play a pivotal role in various biological events. A rise in $[Ca^{2+}]_i$ is a crucial trigger for numerous pathophysiological responses in cells (3). However, an abnormal $[Ca^{2+}]_i$ rise often causes interference of ion flux, dysfunction of proteins, apoptosis, proliferation and other processes (7). In this regard, paroxetine was thought to induce $[Ca^{2+}]_i$ rise in human prostate cancer PC3 cells (5) and renal tubular cells (4); but the mechanism was not completely elucidated.

Cyclic anti-depressants have been shown to alter oral conditions in patients, and individually related dental prophylactic program has been recommended (32). Ulceration of the oral mucosa was shown to occur in patients treated with sertraline, a SSRI similar to paroxetine (2). However, the effect of paroxetine on $[Ca^{2+}]_i$ and viability in human oral cells has not been examined. The present study investigated the effect of this compound on $[Ca^{2+}]_i$ in OC2 human cancer cells. The OC2 cell line is a useful model for oral cancer research. It has been shown that in this cell line, $[Ca^{2+}]_i$ can increase in response to the stimulation of various ligands such as thimerosal (19), tamoxifen (6), fendiline (12) and safrole (13). The Ca^{2+} signal comprises of Ca^{2+} influx and stores Ca^{2+} release (6, 12, 13, 19).

In this study, fura-2 was used as a fluorescent Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$ changes. We show that paroxetine induced concentration-dependent $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+} in OC2 cells. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots in the presence and absence of extracellular Ca^{2+} were established, and the pathways underlying paroxetine-evoked Ca^{2+} entry and Ca^{2+} release were explored. The effect of paroxetine on cell viability and the involvement of apoptosis were also examined.

Materials and Methods

Cell Culture

OC2 cells obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes and 5 mM glucose. Ca^{2+}-free medium contained similar components as the Ca^{2+}-containing medium except that $CaCl_2$ was omitted and replaced with 2 mM $MgCl_2$ and 0.3 mM EGTA. Paroxetine was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other agents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter viability or basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

Confluent cells grown on 6-cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6 cells/ml. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed twice with Ca^{2+}-containing medium and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7 cells/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer 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. Chemicals such as nifedipine, econazole, SK&F96365, PMA, GF109203X, U73122 and thapsigargin were added at the time points indicated in the figures. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 and 5 mM $CaCl_2$ 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. $[Ca^{2+}]_i$ was calculated as previously described (11). Mn^{2+} quench of fura-2 fluorescence was performed in Ca^{2+}-containing medium contained 50 mM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 1 min before starting the fluorescence recoding. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as de-

scribed previously (25).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Assays were performed according to the manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at 10,000 cells/well in culture medium for 24 h in the presence of 0-50 μM paroxetine. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1; 10 μM pure solution) was added to the samples after paroxetine treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA/AM) to chelate cytosolic Ca^{2+}, 5 μM BAPTA/AM was added to cells for 1 h followed by a washout with Ca^{2+}-containing medium. Then, cells were incubated in a 37°C incubator overnight in the presence of paroxetine. Cells were washed followed by incubation with WST-1 for 30 min at 37°C. The absorbance of samples (A_{450}) was determined by using an enzyme-linked immunosorbent assay reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

Measurements of Subdiploidy Nuclei by Flow Cytometry

Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium. After treatment with 0-50 μM paroxetine for 24 h, cells were collected from the media, and were washed twice with ice-cold Ca^{2+}-containing medium before being resuspended in 3 ml of 70% ethanol at -20°C. The cells were centrifuged for 5 min at 200 ×g. Ethanol was decanted and the cell pellet was washed twice with ice-cold medium and cells were suspended in 1 ml propidium iodide solution (1% Triton X-100, 20 μg propidium iodide, 0.1 mg/ml RNase). The cells were incubated in the dark for 30 min at room temperature. Cell fluorescence was measured in the FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the MODFIT software.

Chemicals

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Paroxetine was a gift from Glaxosmithkine; and other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Statistics

Data are reported as representative or means ± SEM of three experiments. Data were analyzed by two-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 significant difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Fig. 1A shows that the basal $[Ca^{2+}]_i$ level was approximately 50 nM. At concentrations between 100 and 1,000 μM, paroxetine evoked $[Ca^{2+}]_i$ rises in a concentration-dependent manner in the Ca^{2+}-containing medium. At 50 μM, paroxetine did not cause a $[Ca^{2+}]_i$ rise (data not shown). The $[Ca^{2+}]_i$ rise induced by 500 μM paroxetine attained 65 ± 2 nM (n = 3) followed by a slow decay. The Ca^{2+} response saturated at 1,000 μM paroxetine because at a concentration of 1,500 μM, paroxetine induced a similar response as that induced by 1,000 μM. Fig. 1C (filled circles) shows the concentration-response plot of paroxetine-induced response.

Two possible sources of a Ca^{2+} signal are extracellular medium and intracellular Ca^{2+} stores. Further experiments were performed to determine the relative contribution of extracellular Ca^{2+} entry and intracellular Ca^{2+} release in paroxetine-induced $[Ca^{2+}]_i$ rises. The $[Ca^{2+}]_i$ rises evoked by 100, 500 and 1,000 μM paroxetine in Ca^{2+}-free medium are shown in Fig. 1B. Removal of extracellular Ca^{2+} did not change the baseline suggesting that the amount of leaked fura-2 from the cells was insignificant. At a concentration of 500 μM, paroxetine evoked a $[Ca^{2+}]_i$ rise by 24 ± 2 nM (n = 3) above the baseline followed by a gradual decay. The concentration-response plot of paroxetine-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-free medium is shown in Fig. 1C (open circles). The EC_{50} value is approximately 1,000 μM.

Experiments were performed to confirm that paroxetine-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths (25). Thus, quench of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} suggests Ca^{2+} entry. Fig. 2 shows that 500 μM paroxetine evoked an immediate decrease in the 360 nm excitation signal (compared to the upper trace). This implies that paroxetine-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} entry. The decrease attained to a maximum of 55 ± 2 units (n = 3) at the time point of 75 sec.

Experiments were further conducted to explore

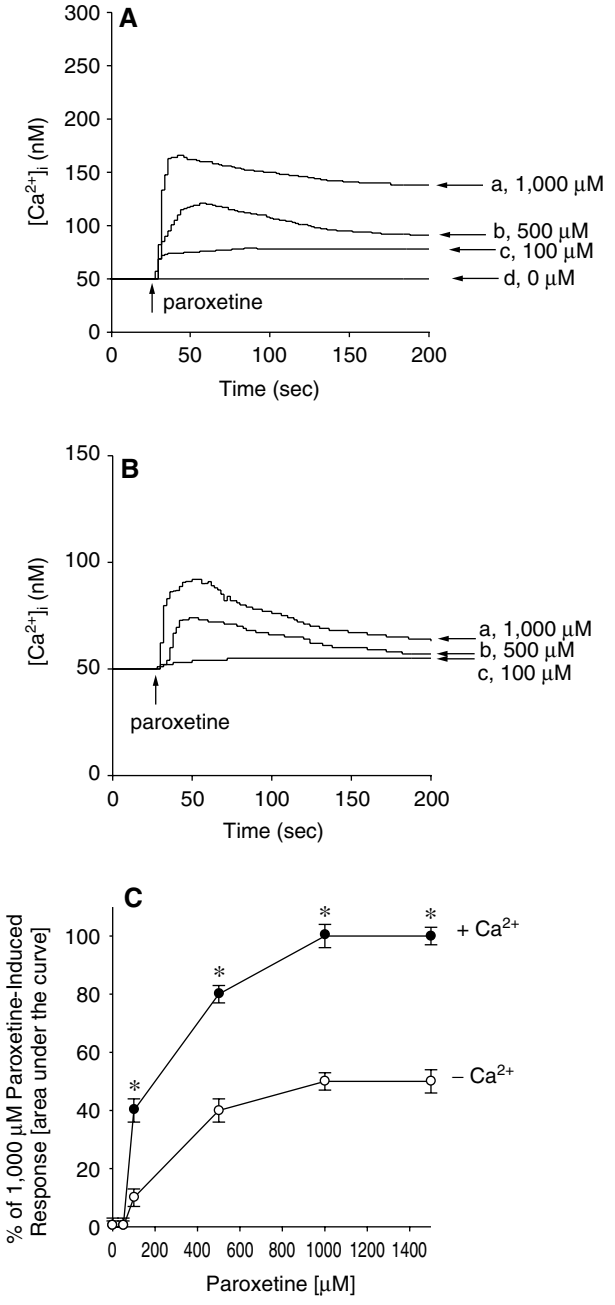

Fig. 1. A. Effect of paroxetine on $[Ca^{2+}]_i$ in fura-2-loaded OC2 cells. Paroxetine was added at 25 sec. The concentration of paroxetine was indicated. The experiments were performed in the Ca^{2+}-containing medium. B. Effect of removal of Ca^{2+} on paroxetine-induced $[Ca^{2+}]_i$ rise. Experiments were performed in the Ca^{2+}-free medium (Ca^{2+} was replaced with 0.3 mM EGTA). In A & B, data are representative of three experiments. C. Concentration-response plots of paroxetine-induced $[Ca^{2+}]_i$ rise in the presence (filled circles) or absence (open circles) of extracellular Ca^{2+}. Y axis is the percentage of control which is the net (baseline subtracted) area under the curve (25-200 sec) of the $[Ca^{2+}]_i$ rise induced by 1,000 μM paroxetine in Ca^{2+}-containing medium. Data are means ± SEM of three experiments. $^*P < 0.05$ compared with open circles.

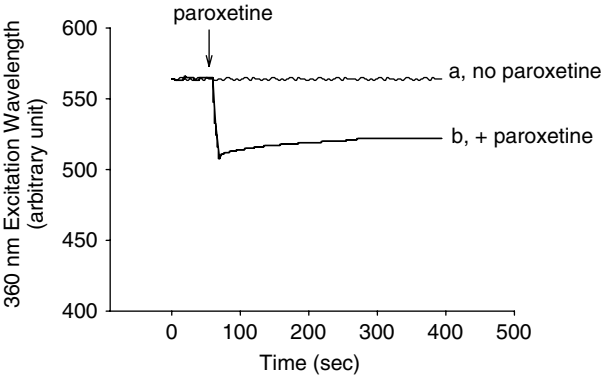

Fig. 2. Effect of paroxetine on Ca^{2+} influx by measuring Mn^{2+} quench of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Upper trace: no paroxetine was present. Lower trace: 500 μM paroxetine was added as indicated. Data are representative of three experiments.

the Ca^{2+} entry pathway of the paroxetine-induced response. The store-operated Ca^{2+} influx inhibitors: nifedipine (1 μM), econazole (0.5 μM) and SK&F96365 (5 μM); phorbol 12-myristate 13 acetate (PMA; a protein kinase C activator) and GF109203X (a protein kinase C inhibitor), all inhibited 500 μM paroxetine-induced $[Ca^{2+}]_i$ rise (Fig. 3A). These chemicals did not affect the basal level of $[Ca^{2+}]_i$ in the absence of paroxetine. The effects of these chemicals on paroxetine-induced $[Ca^{2+}]_i$ rise in the absence of external Ca^{2+} were also explored and the data are shown in Fig. 3B.

Previous studies have shown that the endoplasmic reticulum is the major Ca^{2+} store in OC2 cells (6, 12, 13, 19). Fig. 4A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca^{2+} pumps (34), after 1,000 μM paroxetine-induced $[Ca^{2+}]_i$ rise failed to induce a $[Ca^{2+}]_i$ rise. Fig. 4B shows that addition of thapsigargin induced a $[Ca^{2+}]_i$ rise of 85 ± 3 nM (n = 3), and subsequently added paroxetine did not induce a $[Ca^{2+}]_i$ rise.

Phospholipase C-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca^{2+} from the endoplasmic reticulum (3). Because paroxetine released Ca^{2+} from the endoplasmic reticulum, the role of phospholipase C in this event was examined. U73122, a phospholipase C inhibitor (35), was used to investigate whether this chemical affected paroxetine-induced Ca^{2+} release. Fig. 5 shows that incubation with 2 μM U73122 did not change the basal $[Ca^{2+}]_i$ nor affect 1,000 μM paroxetine-induced $[Ca^{2+}]_i$ rises.

Given that acute incubation with paroxetine

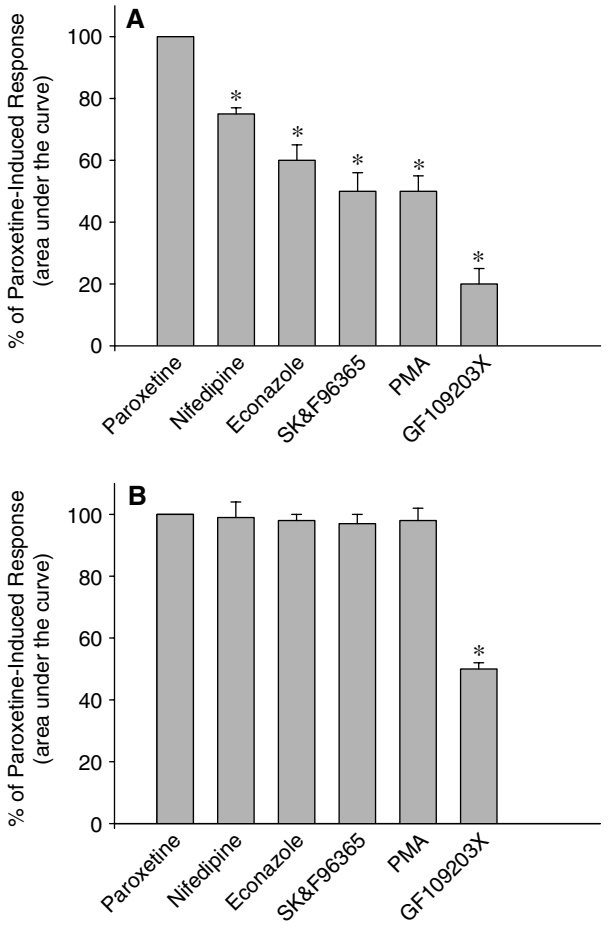

Fig. 3. Effect of Ca^{2+} channel blockers on paroxetine-induced $[Ca^{2+}]_i$ rise. Experiments were performed in the Ca^{2+}-containing (A) or Ca^{2+}-free medium (B). The $[Ca^{2+}]_i$ rise induced by 500 μM paroxetine was taken as the control. In blocker- or modulator-treated groups, the reagent was added 1 min before paroxetine. The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SK&F96365, 10 nM for phorbol 12-myristate 13-acetate (PMA) and 2 μM for GF109203X. Data are expressed as the percentage of control (1st column) that is the maximum value of 500 μM paroxetine-induced $[Ca^{2+}]_i$ rise, and are means ± SEM of three experiments. $*P <$ 0.05 compared to the control.

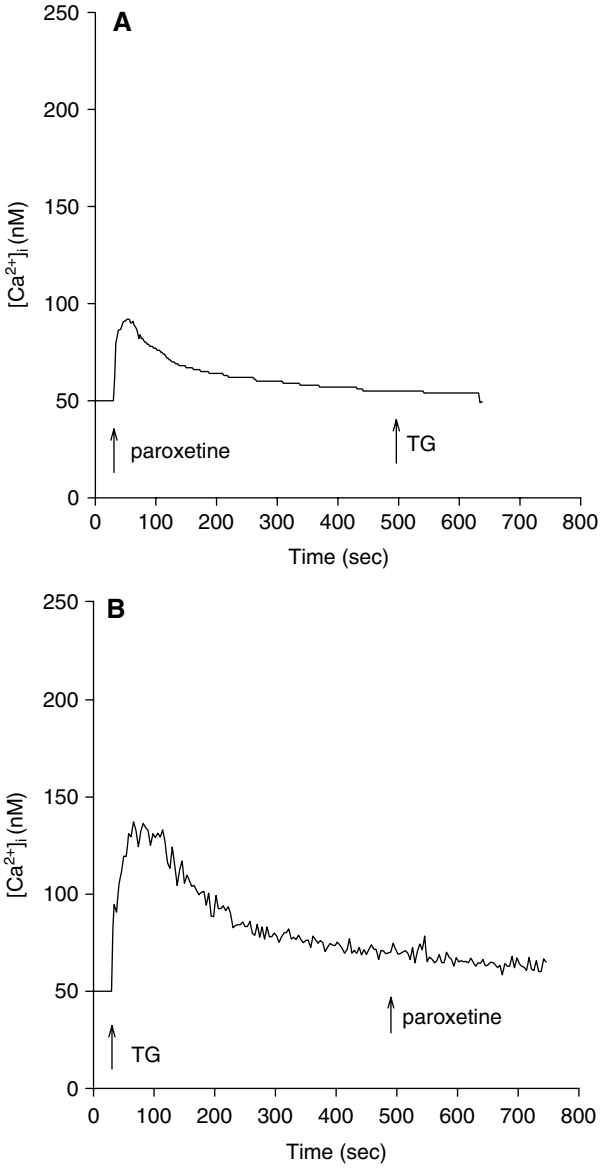

Fig. 4. Intracellular Ca^{2+} stores of paroxetine-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. Paroxetine (1,000 μM) and thapsigargin (1 μM) were added at time points indicated. Data are representative of three experiments.

induced a substantial and lasting $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rise often alters cell viability (7), experiments were performed to examine the effect of paroxetine on viability of OC2 cells. Cells were treated with 0-50 μM paroxetine for 24 h, and the tetrazolium assay was performed. In the presence of 10, 20 and 50 μM paroxetine, cell viability decreased in a concentration-dependent manner (Fig. 6).

The next issue was whether the paroxetine-induced cytotoxicity was related to a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (36) was used to prevent a $[Ca^{2+}]_i$ rise during paroxetine pre-treatment. Fig. 6 also shows that 5 μM BAPTA/AM loading did not significantly alter viability of the control cells. In the presence of 10 μM or 20 μM paroxetine, BAPTA loading further enhanced paroxetine-induced cell death (n = 3; $P < 0.05$).

Propidium iodide staining was exercised to explore the role of apoptosis in paroxetine-induced cell death. The percentage of cells that underwent apoptosis was analyzed by flow cytometry *via* measuring subdiploidy nuclei, a hallmark of apoptosis, after cells were treated with 0, 10, 20, or 50 μM paroxetine for 24 h. As shown in Fig. 7, apoptosis was observed in the 10-50 μM paroxetine-treated groups.

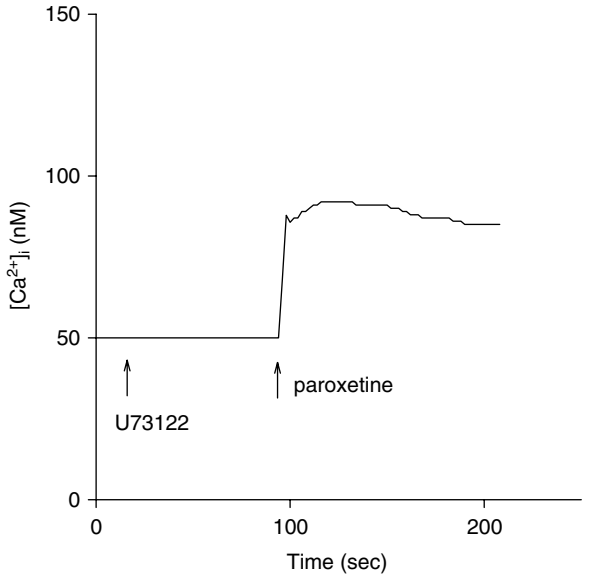

Fig. 5. Lack of effect of U73122 on paroxetine-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. U73122 (2 μM) and paroxetine (1,000 μM) were added as indicated. Data are typical of three experiments.

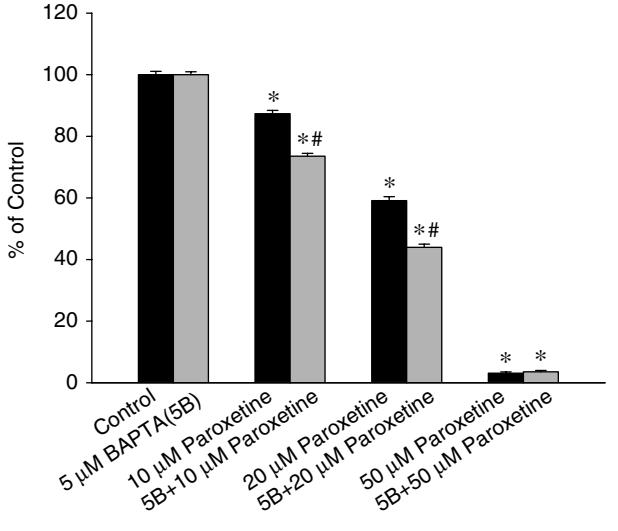

Fig. 6. Cytotoxic effect of paroxetine on OC2 cells. Cells were treated with 0-50 μM paroxetine for 24 h, and the cell viability assay was performed. Data are means ± SEM of three experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control response that is the increase in cell numbers in paroxetine-free groups. Control had 10,997 ± 774 cells/well before the experiments, and had 13,589 ± 745 cells/well after incubation for 24 h. $^{*}P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with paroxetine in Ca^{2+}-containing medium. Cell viability assay was subsequently performed. $^{\#}P < 0.05$ compared to the pairing group.

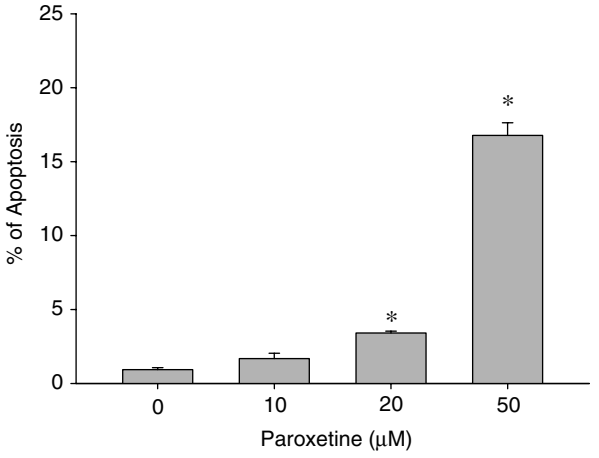

Fig. 7. Effect of paroxetine on apoptosis of OC2 cells. Cells were treated with 0-50 μM paroxetine for 24 h. Experiments were performed in the Ca^{2+}-containing medium. Apoptosis assays were performed with propidium iodide staining and results were analyzed with cytofluorimetry. Data are presented as percentage of apoptotic cells and are means ± SEM of five experiments. $^{*}P < 0.05$ compared to control.

Discussion

Ca^{2+} signaling plays a crucial role in the function of almost all cell types including oral cancer cells. For instance, berberine induces apoptosis in human HSC-3 oral cancer cells *via* simultaneous activation of the death receptor-mediated and mitochondrial pathway that are accompanied by a rise in $[Ca^{2+}]_i$ (22). Our study is the first to show that paroxetine induced $[Ca^{2+}]_i$ rise in human OC2 cells and we also examined the underlying mechanisms. Our data show that paroxetine induced a concentration-dependent $[Ca^{2+}]_i$ rise between 100-1,000 μM. The concentration range of paroxetine for increasing $[Ca^{2+}]_i$ was different between OC2 cells and MDCK renal tubular cells (4). In MDCK cells, the concentration range of paroxetine was 15-200 μM. The data suggest that paroxetine increased $[Ca^{2+}]_i$ by depleting intracellular Ca^{2+} stores and causing Ca^{2+} influx from extracellular milieu because removing extracellular Ca^{2+} reduced 50% of paroxetine-induced $[Ca^{2+}]_i$ rise. Removal of extracellular Ca^{2+} reduced the paroxetine-induced response throughout the measurement period suggesting that Ca^{2+} influx occurred during the whole stimulation period. The ability of paroxetine to induce Ca^{2+} influx was also independently demonstrated by paroxetine-induced Mn^{2+} quench of fura-2 fluorescence.

The mechanism of paroxetine-induced Ca^{2+} influx was examined. The results suggest that paroxetine might cause Ca^{2+} influx *via* stimulating store-operated Ca^{2+} entry which is induced by depletion of intracellular Ca^{2+} stores (27) based on the inhibition

of paroxetine-induced $[Ca^{2+}]_i$ rise by nifedipine, econazole and SK&F96365. These three compounds have been widely applied as blockers of store-operated Ca^{2+} entry in different cell types (14, 15, 28, 33). Because activation of phospholipase C produces IP_3 and diacylglycerol, which stimulates protein kinase C, the effect of regulation of protein kinase C activity on paroxetine-induced $[Ca^{2+}]_i$ rise was examined. Both activation and inhibition of protein kinase C inhibited paroxetine-induced $[Ca^{2+}]_i$ rise. Activation or inhibition of protein kinase C has been shown to regulate store-operated Ca^{2+} channels in different preparations such as vascular smooth muscle (20), vascular myocytes (31), vein myocytes (1), HL60 cells (18) and glomerular mesangial cells (23). After removal of external Ca^{2+}, only GF109203X inhibited Ca^{2+} release. This suggests that nifedipine, econazole, SK&F96365 and PMA only inhibited paroxetine-induced Ca^{2+} influx, whereas GF109203X inhibited both Ca^{2+} influx and Ca^{2+} release. Another possible mechanism that might evoke paroxetine-induced $[Ca^{2+}]_i$ rise was that paroxetine inhibited plasma membrane Ca^{2+} ATP pump so that cytosolic Ca^{2+} could not be pumped out of the cells and $[Ca^{2+}]_i$ would rise *via* passive leaks in the plasma membrane.

Regarding the Ca^{2+} stores involved in paroxetine-induced Ca^{2+} release, the thapsigargin-sensitive endoplasmic reticulum stores might be the dominant store because thapsigargin pretreatment abolished paroxetine-induced $[Ca^{2+}]_i$ rise. Conversely, paroxetine pretreatment abolished thgapsigargin-induced $[Ca^{2+}]_i$ rise. Furthermore, it seems that phospholipase C-dependent pathways did not have a role in paroxetine-induced Ca^{2+} release since the maximum value of paroxetine-induced response was not altered under U73122 stimulation. However, U73122 did slightly slow the decay kinetics of paroxetine-induced $[Ca^{2+}]_i$ response. This may be because phospholipase C activity is involved in regulation of the kinetics of paroxetine-induced $[Ca^{2+}]_i$ rise.

Paroxetine has been suggested to kill human cancer cells (10, 29) and canine renal tubular cells (4), and to induce apoptosis in glioma, neuroblastoma cells (21) and human osteosarcoma cells (5). In this study, we show that paroxetine was cytotoxic to OC2 human oral cancer cells in a concentration-dependent manner. Ca^{2+} overloading is known to initiate processes leading to alteration in cell viability (7). Because paroxetine induced $[Ca^{2+}]_i$ rises and cell death, it would be interesting to know whether the death occurred in a Ca^{2+}-dependent manner. Our data show that the paroxetine-induced cell death was slightly enhanced when cytosolic Ca^{2+} was chelated. This implies that in this case, Ca^{2+} overload played a protective role in paroxetine-induced cell death. Emptying of intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} can modulate cell viability in many cell types (7). However, Ca^{2+}-independent cell death could be observed in some cell types such as thymic lymphoma cells (24) and neutrophils (8). Furthermore, paroxetine-induced cell death was found to involve apoptosis based on propidium iodide staining, which is consistent with the apoptotic effect of paroxetine observed in other cell lines.

Note that the Ca^{2+} measurements were made in less than 10 min while the viability assays were made after overnight incubation with paroxetine. Thus, these two sets of experiments were performed under totally different conditions. This was because our instrument could only measure $[Ca^{2+}]_i$ online, and that cells did not die after acute (tens of min) treatment with 1,000 μM paroxetine.

In healthy volunteers administered paroxetine 30 mg/day, a maximum plasma concentration of 0.17 μM was reached after 6 h. In elderly individuals, the plasma concentration was increased, and in patients with renal impairment, the plasma concentration was 4-fold that of healthy volunteers (37). In a suicidal attempt, a patient ingesting 3.6 g paroxetine was found to have a plasma concentration of 80 μM (38). Thus, our data that paroxetine at concentrations higher than 10-50 μM could cause death in oral cancer cells may be of clinical significance. Furthermore, the data that 500-1,000 μM paroxetine induced $[Ca^{2+}]_i$ rises may not be clinically relevant.

Collectively, the results show that in OC2 human oral cancer cells, paroxetine induced Ca^{2+} release from endoplasmic reticulum in a phospholipase C-independent manner and also caused Ca^{2+} influx *via* phospholipase A2-dependent store-operated Ca^{2+} entry. Paroxetine also evoked cell death that involved apoptosis. Because a rise in $[Ca^{2+}]_i$ can alter many cellular responses, caution should be applied in using paroxetine in other *in vitro* studies.

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