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Effect of Sertraline on Ca^{2+} Fluxes in Rabbit Corneal Epithelial Cells

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Abstract

The effect of sertraline, a selective serotonin reuptake inhibitor (SSRI), on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in a rabbit corneal epithelial cell line (SIRC) is unclear. This study explored whether sertraline changed basal $[Ca^{2+}]_i$ levels in suspended SIRC cells by using fura-2 as a Ca^{2+}-sensitive fluorescent dye. Sertraline at concentrations between 10-100 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The Ca^{2+} signal was reduced by 23% by removing extracellular Ca^{2+}. Sertraline induced Mn^{2+} influx, leading to quench of fura-2 fluorescence, suggesting Ca^{2+} influx. This Ca^{2+} influx was inhibited by phospholipase A_2 inhibitor aristolochic acid, but not by store-operated Ca^{2+} channel blockers and protein kinase C/A modulators. In Ca^{2+}-free medium, pretreatment with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin, cyclopiazonic acid or 2,5-di-tert-butylhydroquinone greatly inhibited sertraline-induced Ca^{2+} release. Inhibition of phospholipase C with U73122 abolished sertraline-induced $[Ca^{2+}]_i$ rise. At concentrations of 5-50 μM, sertraline killed cells in a concentration-dependent manner. The cytotoxic effect of 25 μM sertraline was not reversed by prechelating cytosolic Ca^{2+} with BAPTA/AM. Collectively, in SIRC cells, sertraline induced $[Ca^{2+}]_i$ rises by causing phospholipase C-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx *via* phospholipase A_2-sensitive Ca^{2+} channels. Sertraline-caused cytotoxicity was mediated by Ca^{2+}-independent pathways.

Key Words: Ca^{2+}, corneal, sertraline, SIRC

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Introduction

Sertraline is an antidepressant that belongs to the category of serotonin reuptake inhibitors (SSRIs) (2). Recent evidence shows that sertraline possesses different biological activities that appear to be unrelated to its inhibition of serotonin reuptake. Previous studies showed that sertraline was proapoptotic to T cells (3) and toxic to human colon cancer cell lines and colorectal cancer-xenografted mice (12). Furthermore, sertraline was shown to have neuroprotective effects in human neuroblastoma cells (34), to inhibit insulin secretion and action in pancreatic β cells (18), to inhibit human osteoclast and osteoblast formation and function (16), and to cause mitochondrial dysfunction (24). At the channels level, sertraline was shown to inhibit cardiac ion channels (23), to increase intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in MG63 human osteosarcoma cells (25) but blocked Ca^{2+} entry in smooth muscle cells (6). Aldana and Sitges (1) show that sertraline inhibits pre-synaptic Na^+ channels in nerve endings. Despite various lines of evidence, the effect of sertraline on corneal epithelial cells is unclear.

An increase in $[Ca^{2+}]_i$ is a pivotal signal for diverse pathophysiological responses in cells (7). Inositol 1,4,5-trisphosphate (IP_3), derived from activation of phospholipase C, is a predominant messenger for releasing store Ca^{2+} from the endoplasmic reticulum (19). Mobilization of store Ca^{2+} may activate Ca^{2+} influx across the plasma membrane *via* store operated Ca^{2+} entry (30). An unregulated $[Ca^{2+}]_i$ rise can alter apoptosis, gene expression, protein dysfunction, proliferation, ion channel function *etc*. (7). However, whether sertraline could increase $[Ca^{2+}]_i$ and evoke death in corneal cells is unknown. SIRC rabbit corneal epithelial cell is a good model for research on corneal cells (13). Several reagents have been shown to cause a rise in $[Ca^{2+}]_i$ in SIRC cells, such as ketoconazole (26). Thus it is important to examine the effect of a reagent on cellular Ca^{2+} signaling in order to understand different aspects of its *in vitro* effect. The diverse biological activities of sertraline, with striking findings of a broad-spectrum cellular signaling and pronounced anticancer effect, have inspired us to assess its effect on Ca^{2+} mobilization and viability in corneal cells.

Using fura-2 as a fluorescent Ca^{2+}-sensitive dye, we found that sertraline induced concentration-dependent $[Ca^{2+}]_i$ rises both in the presence and absence of extracellular Ca^{2+} in SIRC cells. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response relationships were explored, and the pathways underlying sertraline-induced Ca^{2+} entry and Ca^{2+} release were evaluated. The effect of sertraline on cell viability was examined.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes® (Eugene, OR, USA). The other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

SIRC rabbit corneal epithelial 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

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 Ca^{2+}-containing medium except that Ca^{2+} was replaced with 3 mM EGTA and 2 mM $MgCl_2$. Sertraline was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other reagents 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

Trypsinized cells (10^6 per ml) were loaded with 2 μM fura-2/AM for 30 min at 25°C in culture medium. 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. 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-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described (27). Mn^{2+} quenching of fura-2 fluorescence was performed in Ca^{2+}-containing medium containing 50 μM $MnCl_2$. $MnCl_2$ was added to cell suspension in the cuvette 30 sec before the fluorescence recoding was started. Data were recorded at excitation signal at 360 nm (Ca^{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described previously (10).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increase in the intensity of color directly 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 10,000 cells/well in culture medium for 24 h in the presence of zero or different concentrations of sertraline. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after sertraline treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, 5 μM BAPTA/AM was added to cells for 1 h followed by washout with Ca^{2+}-containing medium. Then cells were incubated in a 37°C incubator overnight in the presence of 25 μM sertraline. Cells were washed again followed by incubation with WST-1 for 30 min at 37°C. The absorbance of samples (A_{450}) was determined using 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.

Statistics

Data were reported as means ± SEM of three separate experiments, and 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 Sertraline on $[Ca^{2+}]_i$

Fig. 1A shows that before addition of sertraline, the basal $[Ca^{2+}]_i$ level was 51 ± 2 nM. At concentrations between 10 and 100 μM, sertraline induced $[Ca^{2+}]_i$ rises in a concentration-dependent manner in Ca^{2+}-containing medium. The $[Ca^{2+}]_i$ rise induced by 100 μM sertraline attained to a net increase of 175 ± 2 nM followed by a sustained phase within the 250 s interval. The Ca^{2+} response saturated at 100 μM sertraline because at a concentration of 200 μM, sertraline induced a similar response as that induced by 100 μM sertraline. Experiments were performed to determine the relative contribution of extracellular Ca^{2+} influx and

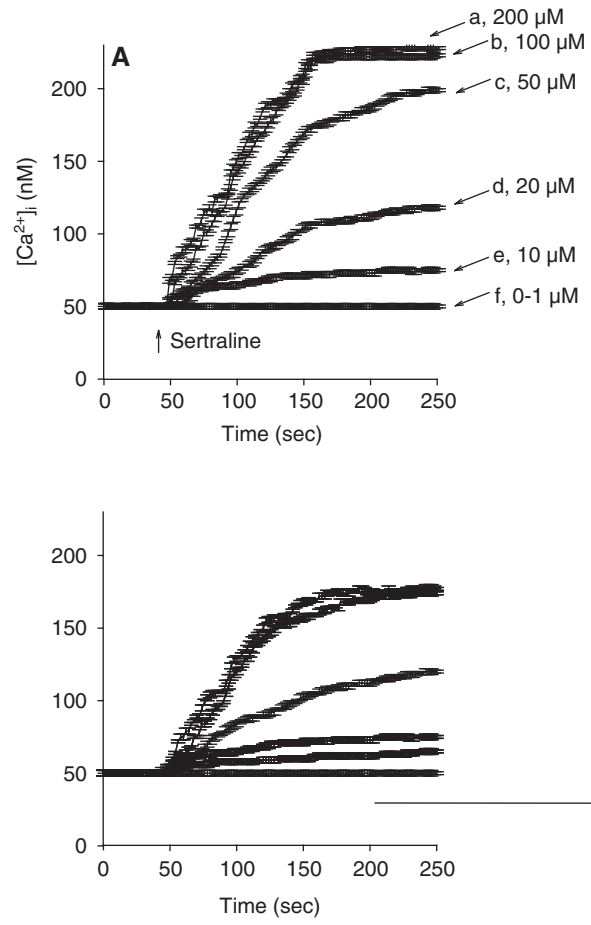

Fig. 1. Effect of sertraline on $[Ca^{2+}]_i$ in fura-2-loaded cells. (A) Sertraline was added at 30 sec. The concentration of sertraline was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Similar to (A) except that experiments were performed in Ca^{2+}-free medium. (C) A concentration-response plot of sertraline-induced $[Ca^{2+}]_i$ rises. Y axis is the percentage of the net (baseline subtracted) area under the curve (30-250 sec) of the $[Ca^{2+}]_i$ rise induced by 100 μM sertraline. Data are mean ± SEM of three separate experiments. $*P < 0.05$ compared to control.

intracellular Ca^{2+} release in sertraline-induced $[Ca^{2+}]_i$ rises. The data in Fig. 1B show that sertraline induced $[Ca^{2+}]_i$ rises in Ca^{2+}-free medium. Removal of extracellular Ca^{2+} did not alter baseline. Sertraline (100 μM) increased $[Ca^{2+}]_i$ by 120 ± 2 nM above baseline. The concentration-response curves of sertraline-induced $[Ca^{2+}]_i$ rises in both Ca^{2+}-containing medium and Ca^{2+}-free medium are shown in Fig. 1C. The EC_{50} values are 25 ± 2 μM or 25 ± 1 μM in Ca^{2+}-containing medium or Ca^{2+}-free medium, respectively.

Sertraline-Induced $[Ca^{2+}]_i$ Rise Involves Ca^{2+} Influx

Experiments were performed to exclude the possibility that the smaller sertraline-induced response in Ca^{2+}-free medium was caused by 0.3 mM EGTA-induced depletion of intracellular Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+} but quenches fura-2 fluorescence at all excitation wavelengths. Quenching of fura-2 fluorescence excited at the Ca^{2+}-insensitive excitation wavelength of 360 nm by Mn^{2+} implies Ca^{2+} influx. (10). Fig. 2 shows that 100 μM sertraline induced an immediate decrease in the 360 nm excitation signal (compared to control) by 80 ± 2 arbitrary units at 125 sec. This suggests that sertraline-induced $[Ca^{2+}]_i$ rise involved Ca^{2+} influx from extracellular space.

Regulation of Sertraline-Induced $[Ca^{2+}]_i$ Rise

Three store-operated Ca^{2+} influx inhibitors (1 μM nifedipine, 0.5 μM econczole, and 5 μM SK&F96365), the protein kinase C activator phorbol 12-myristate 13-acetate (1 nM PMA), the protein kinase C inhibitor (2 μM GF109203X), the protein kinase A inhibitor (1 μM H-89), the voltage-gated Ca^{2+} channel blocker (1 μM nimodipine) all failed to affect 100 μM sertraline-induced $[Ca^{2+}]_i$ rise in Ca^{2+}-containing medium (Fig. 3A). In contrast, 20 μM aristolochic acid (a phospholipase A_2 inhibitor) and U73122 (a phospholipase C inhibitor) inhibited the sertraline-induced Ca^{2+} response by 80 ± 2% and 79 ± 3, respectively ($P < 0.05$) in Ca^{2+}-containing medium (Fig. 3A). Fig. 3B shows the original traces of the effects of aristolochic acid on sertraline-induced Ca^{2+} signal. In Ca^{2+}-free medium, aristolochic acid abolished the sertraline-induced Ca^{2+} response (Fig. 3C) ($P < 0.05$). The concentrations of these modulators have been used to effectively inhibit Ca^{2+} entry through different Ca^{2+} channels in various cell types (14, 25, 31, 40). Furthermore, these modulators did not affect basal $[Ca^{2+}]_i$ level before addition of sertraline (data not shown).

Source of Sertraline-Induced Ca^{2+} Release

Previous reports showed that the endoplasmic reticulum was a major Ca^{2+} store in most cell types, including SIRC cells (26). Three inhibitors of endoplasmic reticulum Ca^{2+} pumps: thapsigargin (35), 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ) (29) and cyclopiazonic acid (CPA) (8) were used as selective tools to deplete the endoplasmic reticulum Ca^{2+} stores to see whether sertraline used this store to cause intracellular Ca^{2+} release. Fig. 4A shows that in Ca^{2+}-free medium, addition of 1 μM thapsigargin evoked a $[Ca^{2+}]_i$ rise of 130 ± 2 nM. Addition of 100 μM sertraline at 500 sec induced a tiny $[Ca^{2+}]_i$ rise that was smaller than the control sertraline-induced response

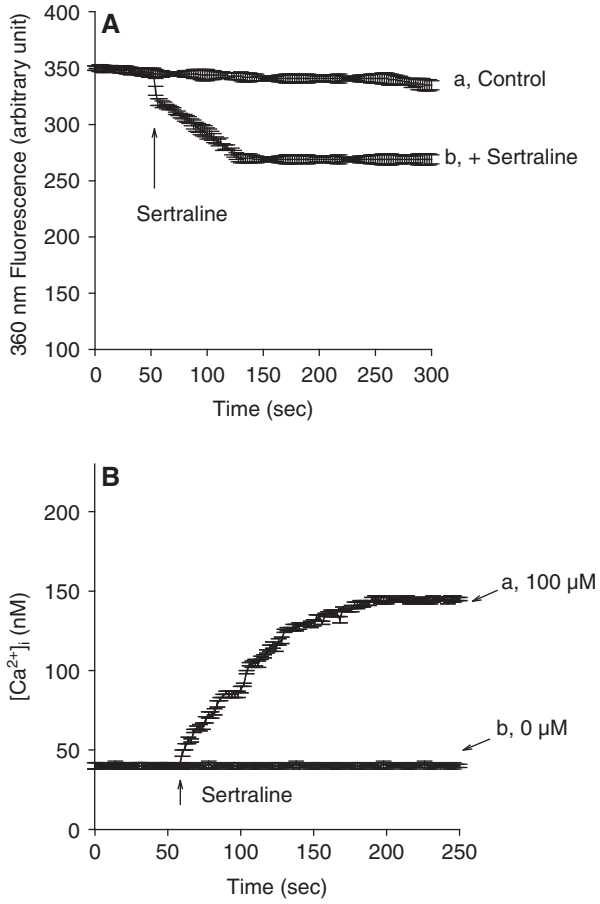

Fig. 2. Effect of sertraline on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. (A) Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without sertraline. Trace b: sertraline (100 μM) was added as indicated. Data are mean ± SEM of three separate experiments. (B) Sertraline was added at 30 sec. The concentration of sertraline was indicated. The experiments were performed in Ca^{2+}-containing medium. Data are mean ± SEM of three separate experiments.

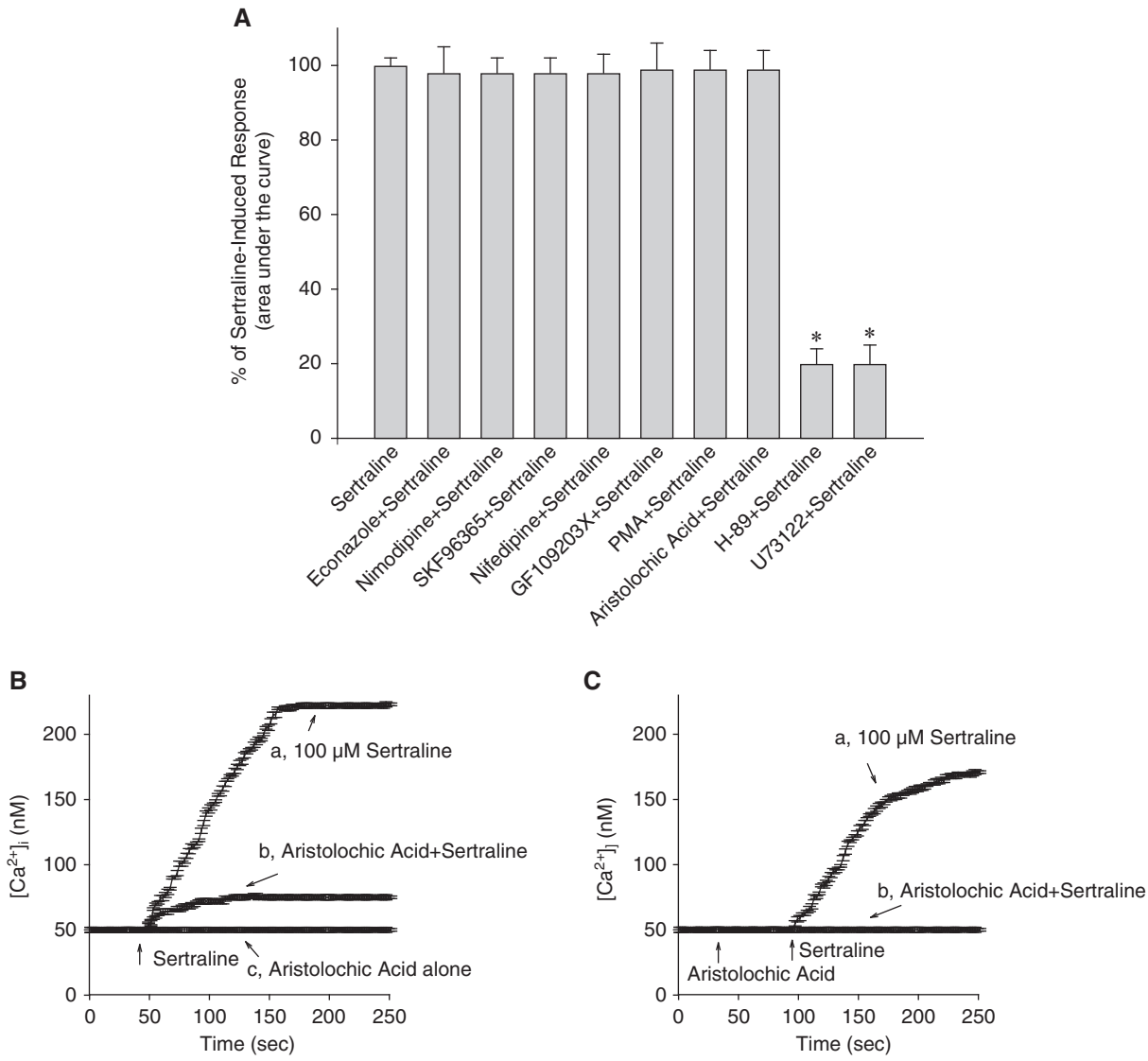

Fig. 3. Effect of Ca^{2+} channel modulators on sertraline-induced $[Ca^{2+}]_i$ rise. (A) The experiments were performed in Ca^{2+}-containing medium. In modulator-treated groups, the modulator was added 1 min before sertraline (100 μM). Data are expressed as the percentage of the area under the curve (25-250 sec) of 100 μM sertraline-induced $[Ca^{2+}]_i$ rise, and are mean ± SEM of three separate experiments. $*P < 0.05$ compared to the 1st column. (B) Aristolochic acid was added 1 min before setraline in Ca^{2+}-containing medium. (C) Aristolochic acid was added 1 min before 100 μM setraline in Ca^{2+}-free medium. Data are mean ± SEM of three separate experiments.

(Fig. 1B) by 93 ± 2% ($P < 0.05$). Fig. 4B shows that treatment with 50 μM CPA induced a $[Ca^{2+}]_i$ rise of 55 ± 1 nM. Addition of 100 μM sertraline at 500 sec failed to induce a $[Ca^{2+}]_i$ rise. Fig. 4C shows that addition of BHQ induced a $[Ca^{2+}]_i$ rise of 90 ± 1 nM. Subsequently added sertraline (100 μM) at 500 sec did not induce a $[Ca^{2+}]_i$ rise.

A Role of Phospholipase C in Sertraline-Induced $[Ca^{2+}]_i$ Rise

Phospholipase C-dependent formation of inositol 1,4,5-trisphosphate is a key step for releasing Ca^{2+} from the endoplasmic reticulum (7). Because sertraline was able to release Ca^{2+} from the endoplasmic reticulum, the role of phospholipase C in this release was examined. U73122, a phospholipase C inhibitor (36) was applied to see whether this enzyme was required for sertraline-induced Ca^{2+} release. Fig. 5A shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 130 ± 2 nM. ATP is a phospholipase C-dependent agonist of $[Ca^{2+}]_i$ rise in most cell types (11). Fig. 5B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ (second column) but abolished ATP-induced $[Ca^{2+}]_i$ rises (fourth column). This suggests that U73122 effectively suppressed phospholi-

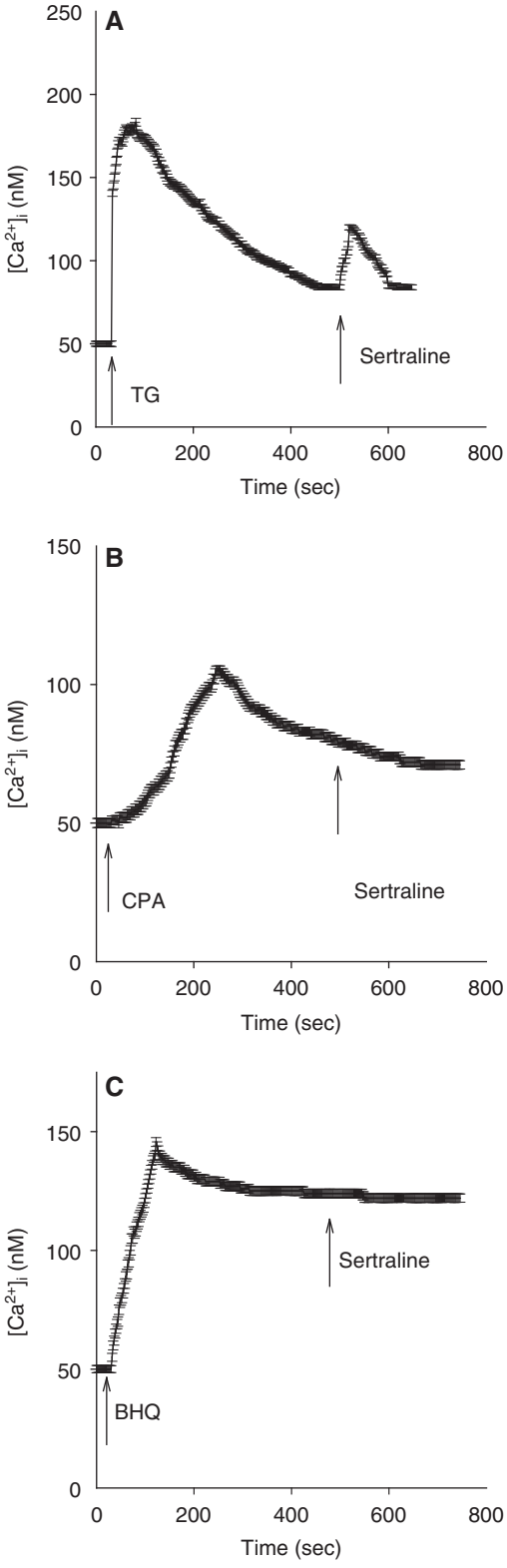

Fig. 4. Role of intracellular Ca^{2+} stores in sertraline-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) (B) (C) Thapsigargin (TG, 1 μM), sertraline (100 μM), cyclopiazonic acid (CPA; 50 μM), and 2,5-di-tert-butylhydroquinone (BHQ) (50 μM) were added at time points indicated. Data are mean ± SEM of three separate experiments.

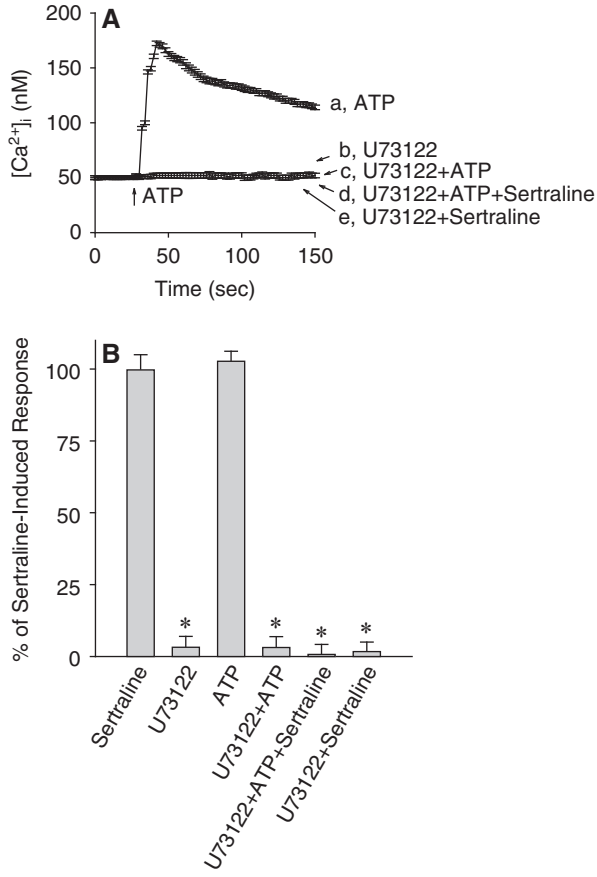

Fig. 5. Effect of U73122 on sertraline-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) Trace a: ATP (10 μM) was added at 25 sec. Trace b: U73122 (2 μM) was added at 25 sec. Trace c: ATP was added 60 sec after U73122 (25 sec). Trace d: sertraline was added 90 sec after ATP (60 sec) and U73122 (25 sec). Trace e: sertraline was added 60 sec after U73122 (25 sec). (B) First column is 100 μM sertraline-induced $[Ca^{2+}]_i$ rise. Second column shows that 2 μM U73122 did not alter basal $[Ca^{2+}]_i$. Third column shows ATP-induced $[Ca^{2+}]_i$ rise compared to sertraline control. Fourth column shows that U73122 pretreatment for 200 sec completely abolished ATP-induced $[Ca^{2+}]_i$ rise (*$P < 0.05$ compared to 3rd column). Fifth column shows that U73122 (incubation for 200 sec) and ATP (incubation for 50 sec) pretreatment totally inhibited 100 μM sertraline-induced $[Ca^{2+}]_i$ rise (*$P < 0.05$ compared to 3rd column). Sixth column shows that U73122 pretreatment for 200 sec completely abolished sertraline-induced $[Ca^{2+}]_i$ rise (*$P < 0.05$ compared to 3rd column). Data are mean ± SEM of three independent biological replicates.

pase C activity. Fig. 5B also shows that addition of 100 μM sertraline after U73122 and ATP treatments failed to cause a $[Ca^{2+}]_i$ rise (fifth column).

Effect of Sertraline on Cell Viability

Because unregulated $[Ca^{2+}]_i$ rise may change cell

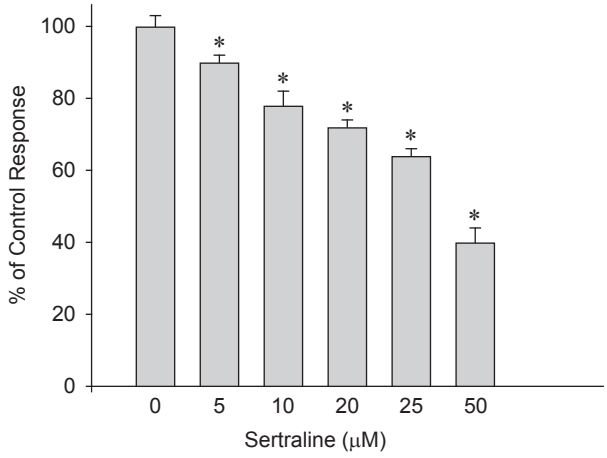

Fig. 6. Sertraline-induced cell death. Cells were treated with 0-50 μM sertraline for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in sertraline-free groups. Control had 10,556 ± 722 cells/well before experiments, and had 13,669 ± 755 cells/well after incubation for 24 h. *$P < 0.05$ compared to control.

viability (7), experiments were performed to examine the effect of sertraline on viability of SIRC cells. Cells were treated with 0-50 μM sertraline for 24 h, and the tetrazolium assay was performed. In the presence of 5-50 μM sertraline, cell viability decreased in a concentration-dependent manner (Fig. 6).

Lack of a Role of Ca^{2+} in Sertraline-Induced Cell Death

An important question was whether the sertraline-induced cytotoxicity was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (37) was used to prevent a $[Ca^{2+}]_i$ rise during sertraline treatment. Fig. 7A shows that 5 μM BAPTA/AM loading abolished 25 μM sertraline-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium. This suggests that BAPTA/AM effectively prevented a rise in $[Ca^{2+}]_i$. Fig. 7B shows that in the presence of 25 μM sertraline, BAPTA/AM loading did not reverse sertraline-induced cell death ($P > 0.05$ between columns 3 and 4).

Lack of a Role of PLA_2 or PLC Inhibitor in SIRC Cell Viability

In Figs. 3 and 5, the PLA_2 inhibitor aristolochic acid (20 μM) and the PLC inhibitor U73122 (2 μM) were shown to inhibit sertraline-induced $[Ca^{2+}]_i$ rises. Therefore, the role of PLA_2 or PLC activation in SIRC cell viability was examined. Cells were treated with aristolochic acid (20 μM) or U73122 (2 μM) for 24 h, and the tetrazolium assay was performed. Aristolochic acid or U73122 loading did not induce cell death (Fig. 8).

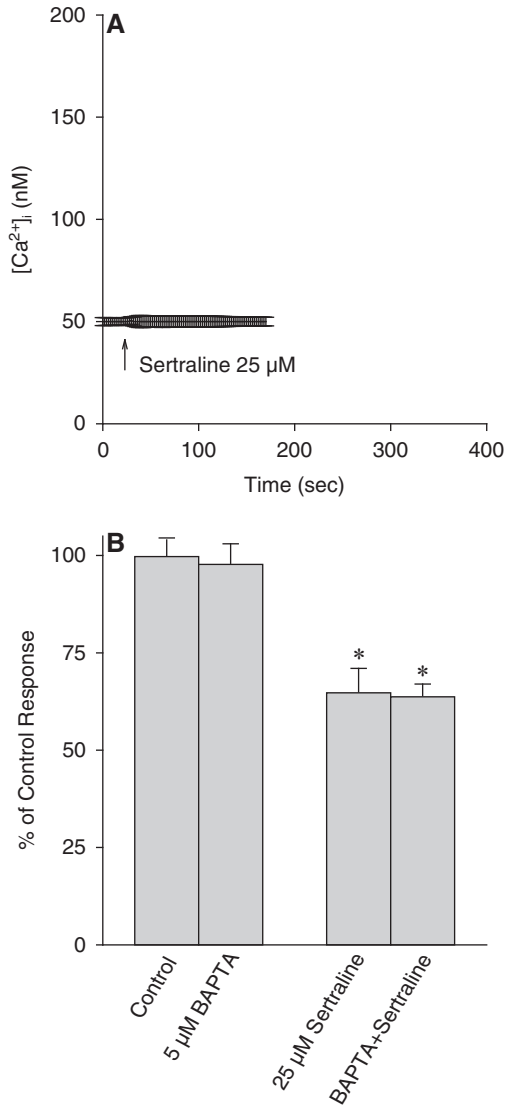

Fig. 7. Lack of a relationship between sertraline-induced $[Ca^{2+}]_i$ rise and cell death. (A) Following BAPTA/AM treatment, cells were incubated with fura-2/AM as described in Methods. Then $[Ca^{2+}]_i$ measurements were conducted in Ca^{2+}-containing medium. Sertraline (25 μM) was added as indicated. (B) Cells were treated with BAPTA/AM (0 or 5 μM) as described in Methods before cell viability was measured. Sertraline (0 or 25 μM) was added during the viability assay. Control had 10,556 ± 722 cells/well before experiments, and had 13,669 ± 755 cells/well after incubation for 24 h. *$P < 0.05$ compared with control. Data are mean ± SEM of three separate experiments.

Discussion

Selective serotonin reuptake inhibitors (SSRIs)

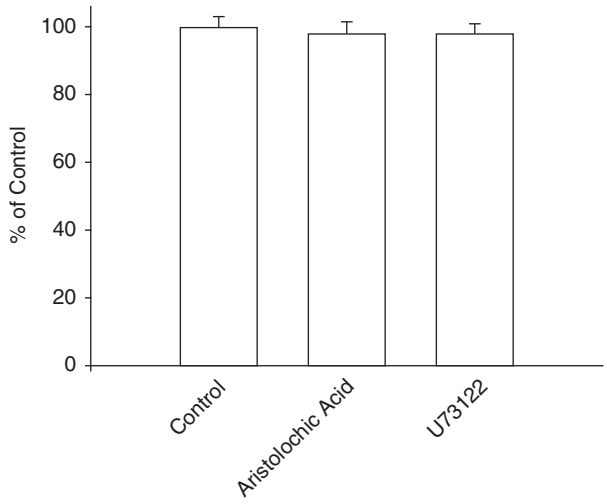

Fig. 8. Lack of a relationship between PLA_2 or PLC and cell death. Cells were treated with aristolochic acid (20 μM) or U73122 (2 μM) for 24 h, and cell viability assay was performed. Data are mean ± SEM of three separate experiments.

are a first-line treatment for depression. Evidence shows that SSRIs have various *in vitro* effects that are independent of serotonin. Ca^{2+} signaling plays a crucial role in the function of almost all cell types including corneal epithelial cell line (7, 21). Ca^{2+} is involved in corneal epithelial functions such as volume regulation and ion transport (21). ATP was shown to induce intracellular Ca^{2+} changes through gap-junctional communication (22). Platelet-activating factor was shown to induce cyclooxygenase-2 gene expression in corneal epithelium in a Ca^{2+}-dependent manner (5). The present study demonstrates that sertraline induced $[Ca^{2+}]_i$ rises and cell death in SIRC corneal epithelial cells. The data show that sertraline induced a concentration-dependent $[Ca^{2+}]_i$ rise between 10 μM and 100 μM. Sertraline induced a $[Ca^{2+}]_i$ rise by depleting intracellular Ca^{2+} stores and causing Ca^{2+} influx from extracellular milieu because removing extracellular Ca^{2+} reduced 100 μM sertraline-induced $[Ca^{2+}]_i$ rises by 23%. Similarly, sertraline induced a $[Ca^{2+}]_i$ rise by depleting intracellular Ca^{2+} stores and causing Ca^{2+} influx from extracellular milieu in MG63 cells (25). Because removal of extracellular Ca^{2+} reduced the sertraline-induced response throughout the measurement period, it suggests that Ca^{2+} influx occurred during the whole stimulation period. The ability of sertraline to induce Ca^{2+} influx was also independently demonstrated by sertraline-induced Mn^{2+} quench of fura-2 fluorescence.

The results suggest that sertraline did not cause Ca^{2+} influx *via* stimulating store-operated Ca^{2+} entry because nifedipine, econazole and SK&F96365 failed to inhibit the $[Ca^{2+}]_i$ rise. Recent evidence shows that nifedipine, econazole and SKF96365 block store-operated Ca^{2+} channels in different models (14, 31, 40). Our findings also show that aristolochic acid, a phospholipase A_2 inhibitor, significantly inhibited 100 μM sertraline-induced $[Ca^{2+}]_i$ rise. Yagami *et al.* (39) showed that L-type voltage-dependent Ca^{2+} channel is involved in the snake venom group IA secretory phospholipase A_2-induced neuronal apoptosis. Thus, Ca^{2+} signaling may be altered by phospholipase A_2 inhibitors in SIRC cells.

The sertraline-induced Ca^{2+} influx in SIRC cells seems to be mediated by non-L-type Ca^{2+} channels in a protein kinase C-independent manner. One possible Ca^{2+} entry pathway was *via* the transient receptor potential vanilloid 1 receptors which have been shown to induce inflammatory cytokine release in corneal epithelium through MAPK signaling (42). Isoforms of protein kinase C have been shown to specifically enhance store-operated Ca^{2+} entry in human corneal epithelial cells (41). Our data show that activation or inhibition of protein kinase C did not affect sertraline-induced Ca^{2+} signal. Another possible mechanism that might contribute to sertraline-induced $[Ca^{2+}]_i$ rise is that sertraline inhibited plasma membrane Ca^{2+} ATP pump so that Ca^{2+} could not be pumped out of the cells and $[Ca^{2+}]_i$ would rise *via* leaks in the plasma membrane.

Regarding the Ca^{2+} stores involved in sertraline-induced Ca^{2+} release, the thapsigargin-sensitive endoplasmic reticulum stores might be the main one because thapsigargin/CPA/BHQ pretreatment nearly abolished sertraline-induced $[Ca^{2+}]_i$ rise. Furthermore, it seems that phospholipase C-dependent pathways played a dominant role in sertraline-induced Ca^{2+} release, because the response was abolished when phospholipase C activity was suppressed by U73122. This is consistent with the data reported in human platelets (15) that sertraline activated phospholipase C.

Previous studies (6, 20) performed in muscle cells and myocytes showed that sertraline blocked Ca^{2+} entry *via* inhibiting L-type Ca^{2+} and transient outward K^+ currents. Thus, sertraline may alter Ca^{2+} signaling differently in nonexcitable and excitable cells. Sertraline has been shown to be toxic to several cell lines including melanoma cell (32), human colon cancer cell lines and colorectal cancer-xenografted mice (12). This study shows that sertraline was cytotoxic to corneal epithelial cells in a concentration-dependent manner. Ca^{2+} overloading is known to initiate processes leading to cell death (7). Whether sertraline is cytotoxic to SIRC cells in a Ca^{2+}-dependent manner is an important issue. Our data show that sertraline-induced cell death was not reversed by preventing sertraline-induced $[Ca^{2+}]_i$ rise. This implies that sertraline caused cell death *via* a Ca^{2+}-independent manner. In another study performed in Madin-Darby canine

renal tubular cells, Huang *et al.* (17) showed that Ca^{2+} influx was involved in sertraline-induced apoptosis. Emptying of intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} can modulate cell death in many cell types (33). However, Ca^{2+}-independent apoptosis could be found in some cell types such as pancreatic islet cells (4), *etc*. Furthermore, sertraline-induced $[Ca^{2+}]_i$ rise may lead to other lethal responses, and that cell death is induced by other signals. Evidence shows that mitochondrial matrix Ca^{2+} overload enhances generation of reactive oxygen species (ROS) (9). ROS could contribute to necrosis observed in human lens epithelial cells (38).

The reason that $[Ca^{2+}]_i$ measurements were performed at room temperature was that fura-2 would leak drastically from the cytosol into the extracellular solution and also the intracellular stores at 37°C in a short period of time. The fura-2 transport proteins are much more active at 37°C than at room temperature. Furthermore, we were only interested in the changes of cytosolic Ca^{2+} levels. If the $[Ca^{2+}]_i$ measurements were performed at 37°C, it will make the measurements unfeasible and the data questionable. Because our data suggest that chelation of cytosolic Ca^{2+} did not alter viability, there is no association between $[Ca^{2+}]_i$ and viability. Therefore, temperature differences do not play a role in the interpretation of the $[Ca^{2+}]_i$ data and viability data.

In a human study, 21 adults were treated with 25-150 mg of sertraline once a day for 30 days, and the plasma level of sertraline was found to be 8 nM-0.32 μM (28). Our data show that sertraline increased $[Ca^{2+}]_i$ at ~10 μM. Thus, in situations where sertraline is administered at higher doses, alteration in Ca^{2+} handling in corneal epithelial cells might have clinical significance.

In summary, the data suggest that sertraline induced Ca^{2+} release and Ca^{2+} influx, and evoked Ca^{2+}-independent cell death in SIRC cells. Because a rise in $[Ca^{2+}]_i$ can interfere with many cellular processes, caution should be exercised in using low concentrations of sertraline for other *in vitro* research, and it should be noted that sertraline at μM ranges may be cytotoxic to many cell types. The possible secondary effects induced by sertraline on the eyes are currently unknown. Furthermore, the results cast doubt on the selectivity of sertraline as an inhibitor of serotonin-reuptake.

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