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Effect of Protriptyline on $[Ca^{2+}]_i$ and Viability in MDCK Renal Tubular Cells

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Abstract

Protriptyline has been used as an antidepressant. Clinically it has been prescribed in the auxiliary treatment of cancer patients. However, its effect on Ca^{2+} signaling and related physiology is unknown in renal cells. This study examined the effect of protriptyline on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability in MDCK renal tubular cells. Protriptyline induced $[Ca^{2+}]_i$ rises concentration-dependently. The response was reduced by 20% by removing extracellular Ca^{2+}. Protriptyline-induced Ca^{2+} entry was not altered by protein kinase C (PKC) activity but was inhibited by 20% by three modulators of store-operated Ca^{2+} channels: nifedipine, econazole and SKF96365. In Ca^{2+}-free medium, treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) or thapsigargin partially inhibited protriptyline-evoked $[Ca^{2+}]_i$ rises. Conversely, treatment with protriptyline inhibited partially BHQ or thapsigargin-evoked $[Ca^{2+}]_i$ rises. Inhibition of phospholipase C (PLC) with U73122 did not change protriptyline-induced $[Ca^{2+}]_i$ rises. Protriptyline at 5-200 μM decreased cell viability, which was not reversed by pretreatment with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM). Together, in MDCK cells, protriptyline induced $[Ca^{2+}]_i$ rises by evoking PLC-independent Ca^{2+} release from the endoplasmic reticulum and other unknown stores, and Ca^{2+} entry *via* PKC-insensitive store-operated Ca^{2+} entry. Protriptyline also caused Ca^{2+}-independent cell death.

Key Words: Ca^{2+}, MDCK cells, protriptyline, renal tubular cells

Introduction

Protriptyline is one of the tricyclic antidepressants (TCA) (22, 30, 42). It has been used to treat mood disorders such as obstructive sleep apnoea (21) and is used as a local anesthetic (34). Protriptyline acts

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by decreasing the reuptake of norepinephrine and to a lesser extent serotonin in the brain (13, 21, 34). Protriptyline increases the concentration of norepinephrine and serotonin (both chemicals stimulate nerve cells) and, to a lesser extent, blocks the action of another brain chemical, acetylcholine (13, 21, 34). Greenblatt *et al*. (13) reported a case of multiple complications and death following protriptyline overdose. So far, few studies have been performed to explore the *in vitro* effect of protriptyline. Protriptyline was shown to block the human ether-à-go-go-related gene (HERG) potassium channels (19) and potassium currents in rat isolated sympathetic neurons (43). Although previous studies have shown that TCA had cytotoxic effects on various cancer cells such as multiple myeloma cells (44) or malignant glioma cells (16), the effect of protriptyline on physiology in renal tubular cells is largely unknown.

Ca^{2+} ions have a key role in different biological responses. Rises in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) can induce many pathophysiological cellular events (3, 9). Inositol 1,4,5-trisphosphate (IP_3), derived from activation of phospholipase C (PLC), is a predominant messenger for releasing store Ca^{2+} from the endoplasmic reticulum (3, 9). Mobilization of store Ca^{2+} may activate Ca^{2+} influx across the plasma membrane *via* store-operated Ca^{2+} entry (10). However, uncontrolled $[Ca^{2+}]_i$ rises may induce ion movement, dysfunction of enzymes, apoptosis, and proliferation, *etc*. (3, 9). The kidney handles Ca^{2+} by filtration and reabsorption. In the proximal tubule, the reabsorption is passive and paracellular, but in the distal tubule is active and transcellular. Thus, renal tubular cells are exposed to very high concentrations of Ca^{2+} in both the extracellular and the intracellular compartments. Extracellular Ca^{2+} signaling is transmitted by the Ca^{2+} sensing receptor, located both in the luminal and basolateral sides of tubular cells. This receptor is able to control levels of extracellular Ca^{2+} and acts in consequence to maintain Ca^{2+} homeostasis. Furthermore, renal tubular cells possess several Ca^{2+} channels that regulate some of the cell functions (4). Previous studies have shown that protriptyline in the blood requires active transport into the liver, it is less metabolized by the cytochrome P450 (CYP) family, but exhibits more active renal excretion in adults. Therefore, protriptyline might have been excreted through kidney (30, 34). However, the effect of prortriptyline on $[Ca^{2+}]_i$ is unknown in renal tubular cells. Thus the effect of protriptyline on Ca^{2+} signaling in renal cells is worthy to be studied.

The aim of this study was to explore the effect of protriptyline on $[Ca^{2+}]_i$ in renal tubular cells. The MDCK cell line was used because it produces measurable $[Ca^{2+}]_i$ rises upon pharmacological stimulation. The MDCK cell is commonly applied for renal studies. It has been shown that in this cell, $[Ca^{2+}]_i$ rises can be induced in response to the stimulation of various compounds such as thymol (6), diindolylmethane (11) and angiotensin II (23).

Fura-2 was used as a Ca^{2+}-sensitive dye to measure $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ rises were characterized, the concentration-response plots were established, the mechanisms underlying protriptyline-evoked Ca^{2+} entry and Ca^{2+} release were examined. The effect of protriptyline on viability was also explored.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Aminopolycarboxylic acid/acetoxy methyl (Fura-2/AM) and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM) were from Molecular Probes® (Eugene, OR, USA). All other reagents were from Sigma-Aldrich® (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

MDCK cells purchased from Bioresource Collection and Research Center (Taiwan, ROC) were cultured in MEM 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) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 mM glucose. Ca^{2+}-free medium contained similar chemicals as Ca^{2+}-containing medium except that $CaCl_2$ was replaced with 0.3 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM $MgCl_2$. Protriptyline was dissolved in absolute ethanol as a 0.1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide (DMSO). The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability or basal $[Ca^{2+}]_i$.

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

The $[Ca^{2+}]_i$ was measured as previously described (6, 8, 11, 23, 32). Confluent cells grown on 6 cm dishes were trypsinized and suspended in culture medium at a density of 10^6/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^{2+}-containing medium twice and was suspended in Ca^{2+}-containing medium at a concentration of 10^7/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 $[Ca^{2+}]_i$, 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 (14).

Mn^{2+} Measurements

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 of 360 nm (Ca^{2+}-insensitive) and emission signal of 510 nm at 1-sec intervals as described previously (25).

Cell Viability Analyses

Viability was assessed as previously described (6, 8, 11). The measurement of viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increase in the amount of developed color correlated proportionally 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 protriptyline. 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 protriptyline treatment, and cells were incubated for 30 min in a humidified atmosphere. The cells were incubated with/without protriptyline for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with protriptyline. The cells were washed once with Ca^{2+}-containing medium and incubated with/without puerarin for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) 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 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 Protriptyline on $[Ca^{2+}]_i$

The basal $[Ca^{2+}]_i$ level was 50 ± 1 nM (Fig. 1A). In Ca^{2+}-containing medium, protriptyline induced $[Ca^{2+}]_i$ rises in a concentration-dependent manner at concentrations between 50 and 200 μM. At a concentration of 200 μM, protriptyline evoked $[Ca^{2+}]_i$ rises that reached a net increase of 251 ± 2 nM (n = 3) followed by a sustained phase. The Ca^{2+} response saturated at 200 μM protriptyline because 250 μM protriptyline did not evoke a greater response. Fig. 1B shows that in Ca^{2+}-free medium, 50-200 μM protriptyline induced concentration-dependent rises in $[Ca^{2+}]_i$. Fig. 1C shows the concentration-response plots of protriptyline-induced $[Ca^{2+}]_i$ rises. The EC_{50} value was 100 ± 1 μM and 125 ± 2 μM in Ca^{2+}-containing and Ca^{2+}-free medium, respectively by fitting to a Hill equation.

Protriptyline-Induced $[Ca^{2+}]_i$ Rises Involve Ca^{2+} Influx

Experiments were performed to exclude the possibility that the smaller protriptyline-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 (25). Fig. 2 shows that 200 μM protriptyline induced an immediate decrease in the 360 nm excitation signal (compared to

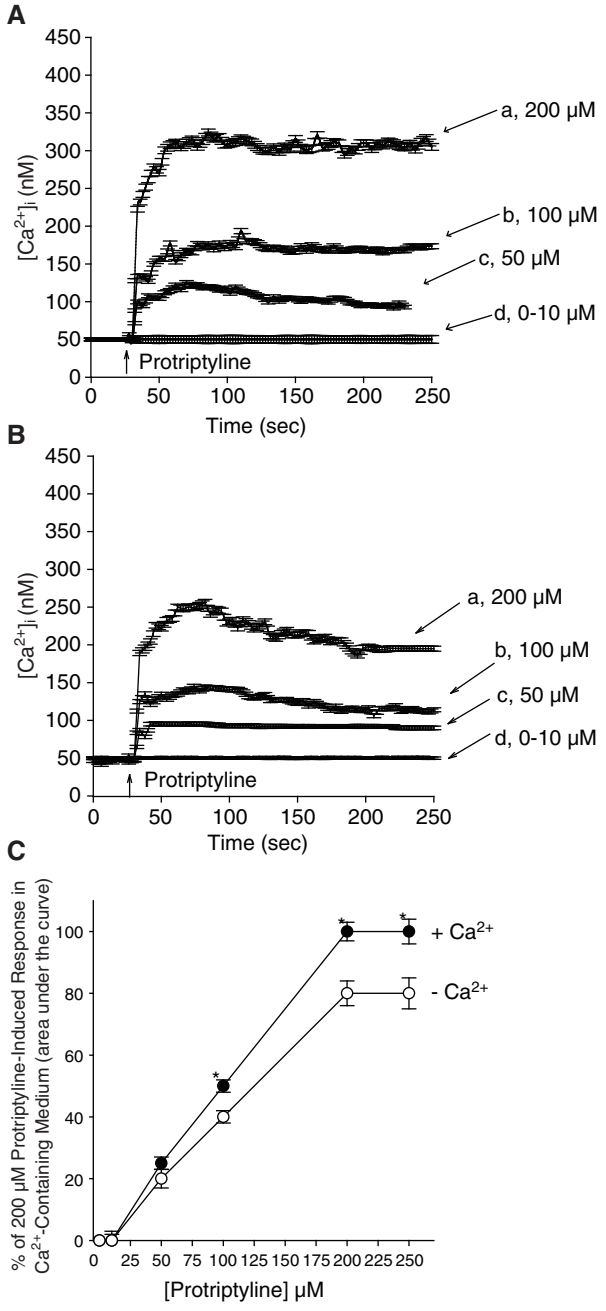

Fig. 1. Effect of protriptyline on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. (A) Protriptyline was added at 25 sec. The concentration of protriptyline was indicated. The experiments were performed in Ca^{2+}-containing medium. Y axis is the $[Ca^{2+}]_i$ induced by protriptyline in Ca^{2+}-containing medium. (B) Effect of protriptyline on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Protriptyline was added at 25 sec in Ca^{2+}-free medium. Y axis is the $[Ca^{2+}]_i$ rises induced by protriptyline in Ca^{2+}-free medium. (C) Concentration-response plots of protriptyline-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-250 sec) of the $[Ca^{2+}]_i$ rises induced by 200 μM protriptyline in Ca^{2+}-containing medium. Data are mean ± SEM of three experiments. *$P < 0.05$ compared to open circles.

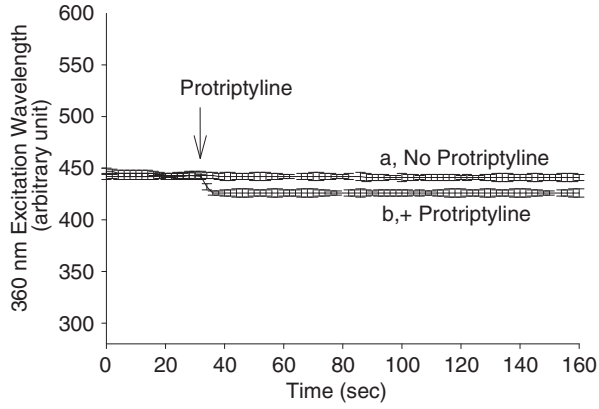

Fig. 2. Effect of protriptyline on Ca^{2+} influx by measuring Mn^{2+} quenching of fura-2 fluorescence. Experiments were performed in Ca^{2+}-containing medium. $MnCl_2$ (50 μM) was added to cells 1 min before fluorescence measurements. The y axis is fluorescence intensity (in arbitrary units) measured at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the emission wavelength of 510 nm. Trace a: control, without protriptyline. Trace b: protriptyline (200 μM) was added as indicated. Data are mean ± SEM of three separate experiments.

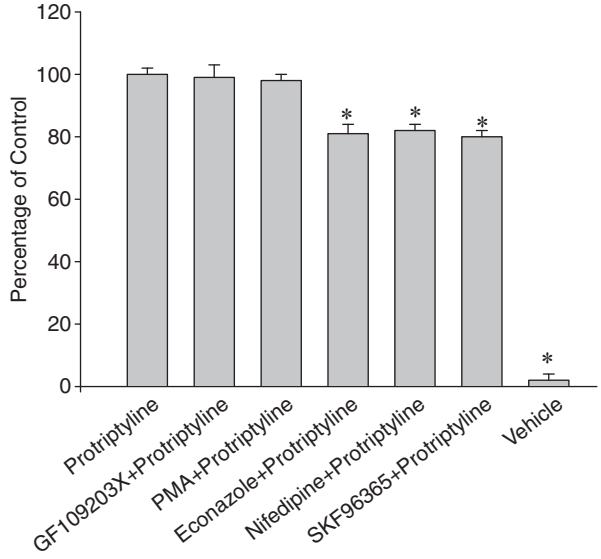

Fig. 3. Effect of Ca^{2+} channel modulators on protriptyline-induced $[Ca^{2+}]_i$ rises. In modulator-treated group, the modulator was added 1 min before protriptyline (200 μM). The concentration was 2 μM for GF109203X, 10 nM for phorbol 12-myristate 13-acetate (PMA), 0.5 μM for econazole, 1 μM for nifedipine, and 5 μM for SKF96365. Negative control (cells without exposure to protriptyline at all, 0.1% DMSO). Data are expressed as the percentage of control (1st column) that is the area under the curve (25-200 sec) of 200 μM protriptyline-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium, and are mean ± SEM of three separate experiments. *$P < 0.05$ compared to 1st column.

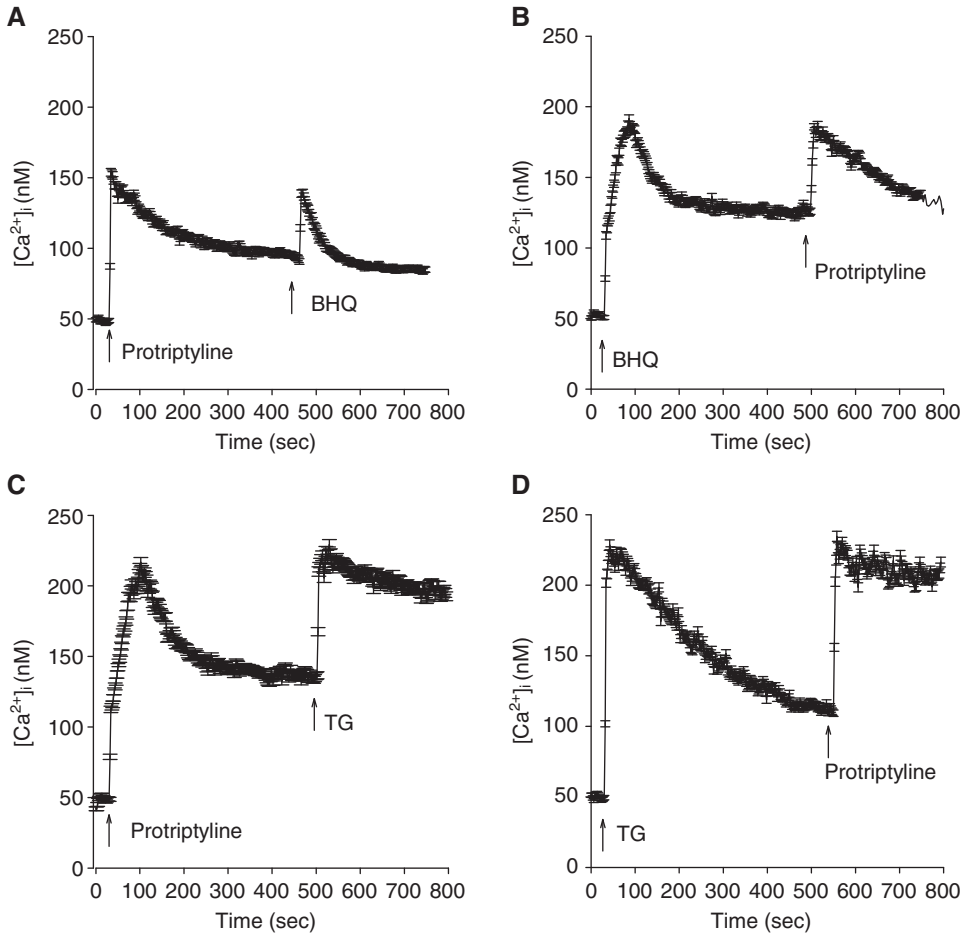

Fig. 4. Effect of BHQ/thapsigargin on protriptyline-induced Ca^{2+} release. (A-D) BHQ (50 μM), thapsigargin (TG, 1 μM) and protriptyline (200 μM) were added at time points indicated. Experiments were performed in Ca^{2+}-free medium. Data are mean ± SEM of three separate experiments.

control) by 10 ± 2 arbitrary units at 125 sec ($P < 0.05$). This suggests that protriptyline-induced $[Ca^{2+}]_i$ rises involved Ca^{2+} influx from extracellular space.

Regulation of Protriptyline-Induced $[Ca^{2+}]_i$ Rises

Phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C, PKC activator), GF109203X (2 μM; a PKC inhibitor), econzaole (0.5 μM), nifedipine (1 μM), or SKF96365 (5 μM) was applied 1 min before protriptyline (200 μM), in Ca^{2+}-containing medium. PMA and GF109203X failed to alter protriptyline-induced $[Ca^{2+}]_i$ rises. However, econazole, nifedipine and SKF96365 inhibited protriptyline-induced $[Ca^{2+}]_i$ rises by approximately 20% ($P < 0.05$).

Sources of Protriptyline-Induced Ca^{2+} Release

In most cell types including MDCK cells, the endoplasmic reticulum has been shown to be the main Ca^{2+} store (3, 9). Thus the role of endoplasmic reticulum in protriptyline-evoked Ca^{2+} release in MDCK cells was explored. The experiments were conducted in Ca^{2+}-free medium to exclude the involvement of Ca^{2+} influx. Fig. 4A shows that addition of 50 μM 2,5-di-tert-butylhydroquinone (BHQ), an endoplasmic reticulum Ca^{2+} pump inhibitor (39), after 200 μM protriptyline-induced $[Ca^{2+}]_i$ rises induced $[Ca^{2+}]_i$ rises of 50 ± 3 μM. Fig. 4B shows that BHQ alone induced $[Ca^{2+}]_i$ rises of 140 ± 2 nM. Protriptyline (200 μM) added afterwards induced $[Ca^{2+}]_i$ rises of 70 ± 2 μM, which were smaller than the protriptyline-induced response (Fig. 4A) by 30%. Another endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (36) was applied for similar purposes. Fig. 4C shows that addition of 1 μM thapsigargin after protriptyline-induced Ca^{2+} response induced $[Ca^{2+}]_i$ rises of 100 ± 2 nM. Fig. 4D shows that thapsigargin alone induced $[Ca^{2+}]_i$ rises of 175 ± 2 nM. Subsequently added protriptyline induced $[Ca^{2+}]_i$ rises of 120 ± 2 nM which were smaller than the protriptyline-induced $[Ca^{2+}]_i$ rises (Fig. 4C) by 33%. This suggests that protriptyline induced $[Ca^{2+}]_i$ rises by Ca^{2+} release from the endoplasmic reticulum and other unknown stores.

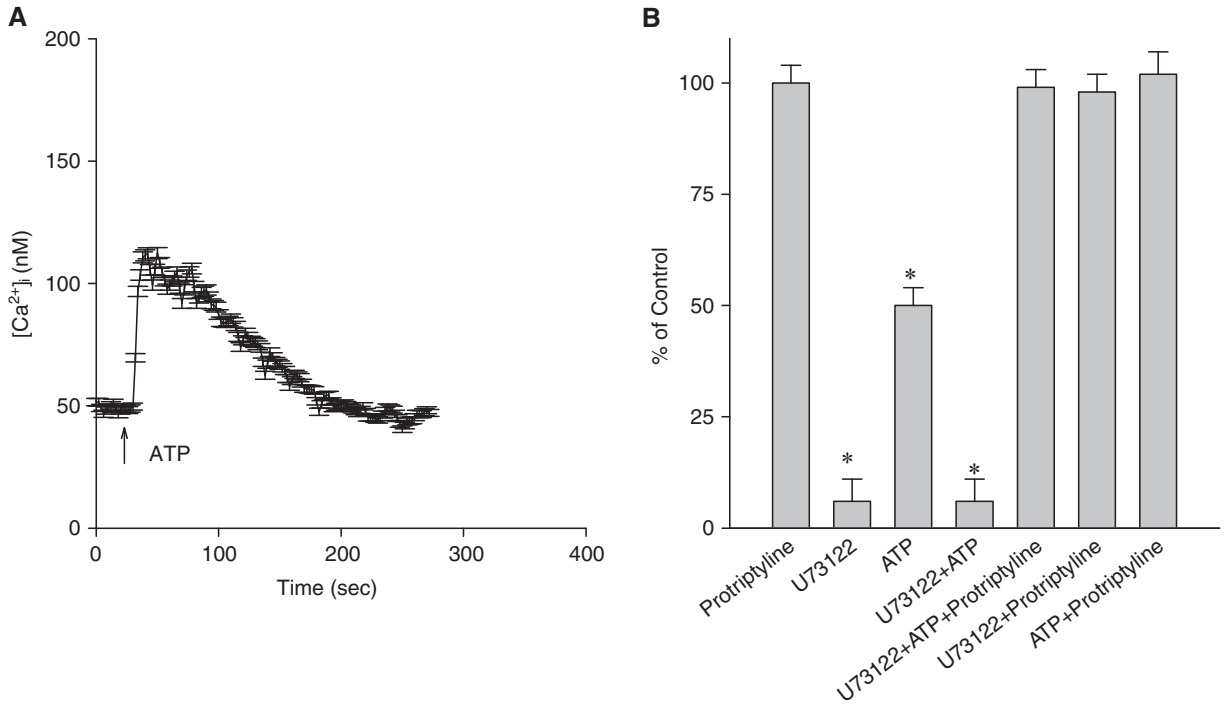

Fig. 5. Effect of U73122 on protriptyline-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added at 25 sec. (B) First column is 200 μM protriptyline-induced $[Ca^{2+}]_i$ rises. Second column shows that 2 μM U73122 did not alter basal $[Ca^{2+}]_i$. Third column shows ATP-induced $[Ca^{2+}]_i$ rises. Fourth column shows that U73122 pretreatment for 200 sec completely abolished ATP-induced $[Ca^{2+}]_i$ rises (*$P < 0.05$ compared to 3rd column). Fifth column shows that U73122 (incubation for 200 sec) and ATP (incubation for 50 sec) pretreatment did not inhibit 200 μM protriptyline-induced $[Ca^{2+}]_i$ rises. Sixth column shows that addition of U73122 did not alter protriptyline-induced $[Ca^{2+}]_i$ rises. Seventh column shows that addition of ATP did not alter protriptyline-induced $[Ca^{2+}]_i$ rises. Data are mean ± SEM of three experiments.

Lack of a Role of Phospholipase C (PLC) in Protriptyline-Induced $[Ca^{2+}]_i$ Rises

PLC is one of the pivotal proteins that regulate the releasing of Ca^{2+} from the endoplasmic reticulum. Because protriptyline released Ca^{2+} from the endoplasmic reticulum, the role of PLC in this process was explored. U73122 (37), a PLC inhibitor, was applied to explore if the activation of this enzyme was required for protriptyline-induced Ca^{2+} release. Fig. 5A shows that ATP (10 μM) induced $[Ca^{2+}]_i$ rises of 61 ± 2 nM. ATP is a PLC-dependent agonist of $[Ca^{2+}]_i$ rises in most cell types (3, 9, 12). Fig. 5B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rises. This suggests that U73122 effectively suppressed PLC activity. The data also show that incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ and 200 μM protriptyline-induced $[Ca^{2+}]_i$ rises. U73343 (2 μM), a U73122 analogue, failed to have an inhibition on ATP-induced $[Ca^{2+}]_i$ rises (not shown). The data suggest that U73122 inhibited ATP-induced $[Ca^{2+}]_i$ rises. Although U73122 is generally deemed as a selective inhibitor of PLC, the possibility that U73122 acted by PLC-independent action needs to be excluded. Thus U73343 was used as a negative control. U73343 is structurally very similar to U73122 while lacks inhibitory effect on PLC. Our results show that U73343 failed to alter ATP-induced $[Ca^{2+}]_i$ rises, suggesting that U73122 most likely suppressed ATP-induced $[Ca^{2+}]_i$ rises *via* inhibiting PLC activity.

Effect of Protriptyline on Viability in MDCK Cells

Because acute incubation with protriptyline induced substantial $[Ca^{2+}]_i$ rises, and that unregulated $[Ca^{2+}]_i$ rises may change cell viability (3, 9), experiments were performed to examine the effect of protriptyline on viability of cells. Cells were treated with 0-200 μM protriptyline for 24 h, and the tetrazolium assay was performed. In the presence of 5-200 μM protriptyline, cell viability decreased in a concentration-dependent manner (Fig. 6A). The intracellular Ca^{2+} chelator BAPTA/AM (38) was applied to prevent $[Ca^{2+}]_i$ rises during protriptyline pretreatment, in order to explore the role of Ca^{2+} in protriptyline-induced cell death. Fig. 6A also shows that 5 μM BAPTA/AM loading did not change the control value of cell viability. Protriptyline (5-200 μM) did not evoke $[Ca^{2+}]_i$ rises in BAPTA/AM-treated cells in both Ca^{2+}-containing and Ca^{2+}-free

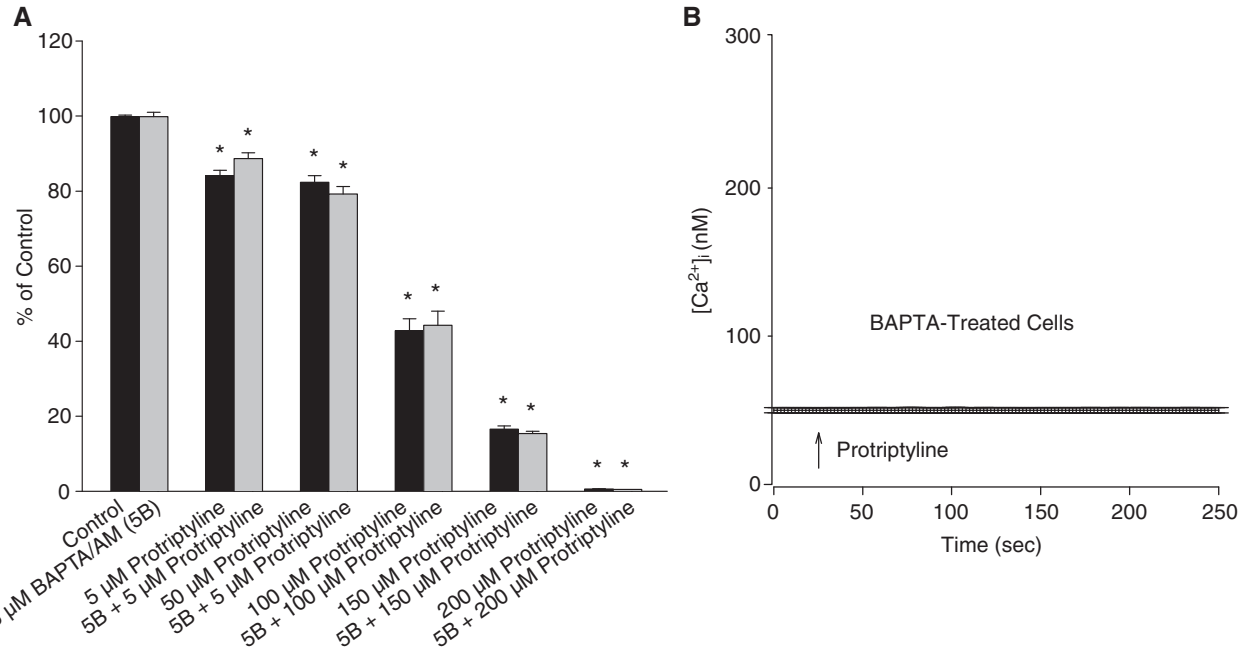

Fig. 6. (A) Effect of protriptyline on cell viability. Cells were treated with 0-200 μM protriptyline for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control response that is the increase in cell numbers in protriptyline-free groups. Control had 11,425 ± 121 cells/well before experiments, and had 11,767 ± 331 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 fura-2-loaded cells followed by treatment with protriptyline in Ca^{2+}-containing medium. (B) 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. Protriptyline (5-200 μM) was added as indicated. Data are mean ± SEM of three experiments.

solutions (Fig. 6B). This suggests that BAPTA loading for 25 h still effectively chelated cytosolic Ca^{2+}. In the presence of 5-200 μM protriptyline, BAPTA loading failed to reverse protriptyline-induced cell death.

Discussion

Protriptyline, a type of TCA, was used primarily in the clinical treatment of mood disorders in cancer patients, but the effect of protriptyline on physiology in kidney is unknown. Previous studies showed that protriptyline affected Ca^{2+} signaling in various cell models such as PC3 prostate cancer cells (7) and HepG2 human hepatoma cells (40). Our study shows that protriptyline increased $[Ca^{2+}]_i$ in MDCK renal tubular cells. This is the first demonstration that protriptyline induced $[Ca^{2+}]_i$ rises in MDCK cells. The Ca^{2+} signal was composed of Ca^{2+} entry and Ca^{2+} release because the signal was reduced by 20% by removing extracellular Ca^{2+}. The Mn^{2+} quenching data also suggest that Ca^{2+} influx occurred during protriptyline incubation.

Previous evidence showed that in MDCK cells the dominant Ca^{2+} entry pathway is the store-operated Ca^{2+} channels (6, 10). This study shows that protriptyline-evoked $[Ca^{2+}]_i$ rises were inhibited by 20% by econazole, nifedipine, and SKF96365. These three compounds have been used to inhibit store-operated Ca^{2+} entry, although there are so far no selective inhibitors for this entry (17, 18, 29, 31). Because 20% of protriptyline-induced $[Ca^{2+}]_i$ rises was *via* Ca^{2+} influx, this influx appears to be totally contributed by store-operated Ca^{2+} entry.

The activity of many protein kinases is known to associate with Ca^{2+} homeostasis (20, 24). The interactive relationship between PKC and Ca^{2+} signaling has been well established (28, 41). Mukherjee *et al.* (26) showed that Ca^{2+} oscillations, Ca^{2+} sensitization, and contraction were activated by PKC in small airway smooth muscle. In contrast, Bynagari-Settipalli *et al.* (5) showed that PKC negatively regulated ADP-induced Ca^{2+} mobilization and thromboxane generation in platelets. However, our data show that protriptyline-evoked $[Ca^{2+}]_i$ rises were not affected by PKC activity. Therefore, protriptyline might cause PKC-insensitive Ca^{2+} influx in MDCK cells. Regarding the Ca^{2+} stores involved in protriptyline-evoked Ca^{2+} release, the BHQ/thapsigargin-sensitive endoplasmic reticulum store seemed to play a role. Because BHQ/thapsigargin did not abolish protriptyline-induced Ca^{2+} release (BHQ inhibited the protriptyline-induced Ca^{2+} release by 30%, while thapsigargin inhibited this response by 33%), other possible Ca^{2+} stores include mitochondria, Golgi bodies,

nuclei, *etc*. (3, 9). However there are no selective inhibitors for these stores. The data further show that the Ca^{2+} release was *via* a PLC-independent mechanism, given the release was not altered when PLC activity was inhibited. The PLC-independent release could be due to other mechanisms such as phospholipase A_2/NADPH oxidase pathways (15, 35). Therefore the pathways underlying protriptyline-induced Ca^{2+} release in MDCK cells deserve further assessment.

Cell viability could be altered in a Ca^{2+}-dependent or -independent manner (27, 33). Our data show that protriptyline induced a Ca^{2+}-independent cell death in a concentration-dependent manner. Although protriptyline-induced Ca^{2+} signal did not cause cell death, it might interfere with numerous downstream Ca^{2+}-sensitive processes that integrate to alter physiology of MDCK cells (3, 9). $[Ca^{2+}]_i$ measurements and viability were two totally different assays. $[Ca^{2+}]_i$ measurements were conducted online and terminated within 4-15 min. After 20 min incubation with protriptyline or other Ca^{2+} channel inhibitors/modulators (nifedipine, econazole, SKF96365, PMA, GF109203X, BHQ, TG, *etc*.), cell viability was still > 95%. In contrast, in viability assays, cells were treated with protriptyline overnight in order to obtain measurable changes in viability.

Previous studies showed that protriptyline induced $[Ca^{2+}]_i$ rises through different pathways in PC3 cells (7) and HepG2 cells (40). In PC3 cells, 50-150 μM protriptyline induced $[Ca^{2+}]_i$ rises by inducing Ca^{2+} release partially from the endoplasmic reticulum and Ca^{2+} entry *via* PKC-sensitive store-operated Ca^{2+} channels (7). In HepG2 cells, 50-150 μM protriptyline induced $[Ca^{2+}]_i$ rises by inducing PLC-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry *via* nifedipine-sensitive store-operated Ca^{2+} channels (40). Our present data show that 50-200 μM protriptyline induced $[Ca^{2+}]_i$ rises by inducing PLC- and PKC-independent Ca^{2+} release from the endoplasmic reticulum (and other unknown stores) and Ca^{2+} entry *via* store-operated Ca^{2+} channels in MDCK cells. Therefore, it appears that the mechanisms of the effect of protriptyline on $[Ca^{2+}]_i$ was different among PC3, HepG2 and MDCK cells. Furthermore, protriptyline at a concentration of 50-200 μM caused cytotoxicity in PC3 cells (7) and MDCK cells, but at a lower concentration range of 20-40 μM in HepG2 cells (40). Because various cancer cell types derived from different origins may have different mechanisms of cytotoxicity, depending on the physiological function of this particular cell, therefore, the effect of protriptyline on cytotoxicity may depend on cell types and concentrations.

The plasma concentration of protriptyline after oral administration has been explored (1, 2). A single 20 mg dose of BR-protriptyline resulted in a mean C_{max} of ~10 μM after 24 h (1, 2). However, in depression patients, the plasma concentration of protriptyline after oral administration might be 10-fold higher than in healthy adults (1, 2). Our data show that protriptyline at a concentration of 50 μM induced $[Ca^{2+}]_i$ rises and cell death. Therefore, our study may have clinical relevance in some groups of patients. The potential use of protriptyline or its derivatives to cope with human kidney diseases needs further exploration in the future.

Collectively, the results show that protriptyline induced Ca^{2+} influx *via* PKC-insensitive store-operated Ca^{2+} entry and also Ca^{2+} release from endoplasmic reticulum (and other unknown stores) in a PLC-independent manner in MDCK cells. Protriptyline also caused cell death that was not triggered by a Ca^{2+} signal. Because rises in $[Ca^{2+}]_i$ can interfere with many cellular processes, caution should be exercised in using low concentrations of protriptyline for other *in vitro* research, and it should be noted that protriptyline at μM ranges may be cytotoxic to cancer cells.

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The authors declare no conflicts of interest.

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