

# Effect of Nortriptyline on $\text{Ca}^{2+}$ Handling in SIRC Rabbit Corneal Epithelial Cells

Kai-Hsien Chang<sup>1</sup>, Hung-Pin Tan<sup>1</sup>, Chun-Chi Kuo<sup>2</sup>, Daih-Huang Kuo<sup>3</sup>, Pochuen Shieh<sup>3</sup>,  
Fu-An Chen<sup>3</sup>, and Chung-Ren Jan<sup>4</sup>

<sup>1</sup>Department of Psychiatry, Yung-Kang Veterans Hospital, Tainan 71051, Taiwan, ROC

<sup>2</sup>Department of Pharmacy, Tajen University, Pingtung 90741

<sup>3</sup>Department of Nursing, Tzu Hui Institute of Technology, Pingtung 92641

and

<sup>4</sup>Department of Medical Education and Research, Kaohsiung Veterans General Hospital,  
Kaohsiung 81362, Taiwan, Republic of China

## Abstract

To explore the effect of nortriptyline, a tricyclic antidepressant, on cytosolic free  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in corneal epithelial cells,  $[\text{Ca}^{2+}]_i$  levels in suspended SIRC rabbit corneal epithelial cells were measured by using fura-2 as a  $\text{Ca}^{2+}$ -sensitive fluorescent dye. Nortriptyline at concentrations between 20-200  $\mu\text{M}$  increased  $[\text{Ca}^{2+}]_i$  in a concentration-dependent manner. The  $\text{Ca}^{2+}$  signal was reduced partly by removing extracellular  $\text{Ca}^{2+}$ . Nortriptyline-induced  $\text{Ca}^{2+}$  influx was inhibited by the store-operated  $\text{Ca}^{2+}$  channel blockers econazole and SK&F96365, the phospholipase A2 inhibitor aristolochic acid, and alteration of activity of protein kinase C. In  $\text{Ca}^{2+}$ -free medium, 200  $\mu\text{M}$  nortriptyline pretreatment greatly inhibited the rise of  $[\text{Ca}^{2+}]_i$  induced by the endoplasmic reticulum  $\text{Ca}^{2+}$  pump inhibitor thapsigargin. Conversely, pretreatment with thapsigargin or 2,5-di-tert-butylhydroquinone (BHQ; another endoplasmic reticulum  $\text{Ca}^{2+}$  pump inhibitor) nearly abolished nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise. Inhibition of phospholipase C with U73122 decreased nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise by 75%. Taken together, nortriptyline induced  $[\text{Ca}^{2+}]_i$  rises in SIRC cells by causing phospholipase C-dependent  $\text{Ca}^{2+}$  release from the endoplasmic reticulum and  $\text{Ca}^{2+}$  influx *via* store-operated  $\text{Ca}^{2+}$  channels.

**Key Words:**  $\text{Ca}^{2+}$ , corneal epithelial cells, nortriptyline, SIRC cells

## Introduction

Nortriptyline is a classic tricyclic antidepressant that is used in treatment of various psychological disorders such as migraine (26), smoking cessation (15), Parkinson's disease (32) and depression (30). In *in vitro* studies, most of the effects exerted by nortriptyline are inhibitory, including inhibition of opioid receptors (42),  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel (39), priming of human neutrophils (36), human cytochrome P450 enzymes (33), human neutrophil phagocytosis and oxidative burst (25), and astroglial inwardly rectifying Kir4.1 channels (37).

A regulated rise in cytosolic free  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) is a pivotal messenger in all cell types and can trigger many physio-pathological processes (5). However, an abnormal elevation in  $[\text{Ca}^{2+}]_i$  is often cytotoxic (8). Thus, it is important to examine the effect of an agent on cellular  $\text{Ca}^{2+}$  signaling in order to understand its *in vitro* effect. Nortriptyline has been shown to increase  $[\text{Ca}^{2+}]_i$  and decrease viability in MG63 human osteosarcoma cells (13) and renal tubular cells (6). The effect of nortriptyline on  $[\text{Ca}^{2+}]_i$  in corneal cells is unknown. SIRC rabbit corneal epithelial cells were applied in this study. SIRC cells have characteristics similar to human

Corresponding author: Dr. Chung-Ren Jan, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362, Taiwan, R.O.C. Fax: +886-7-3468056, E-mail: crjan@isca.vghks.gov.tw

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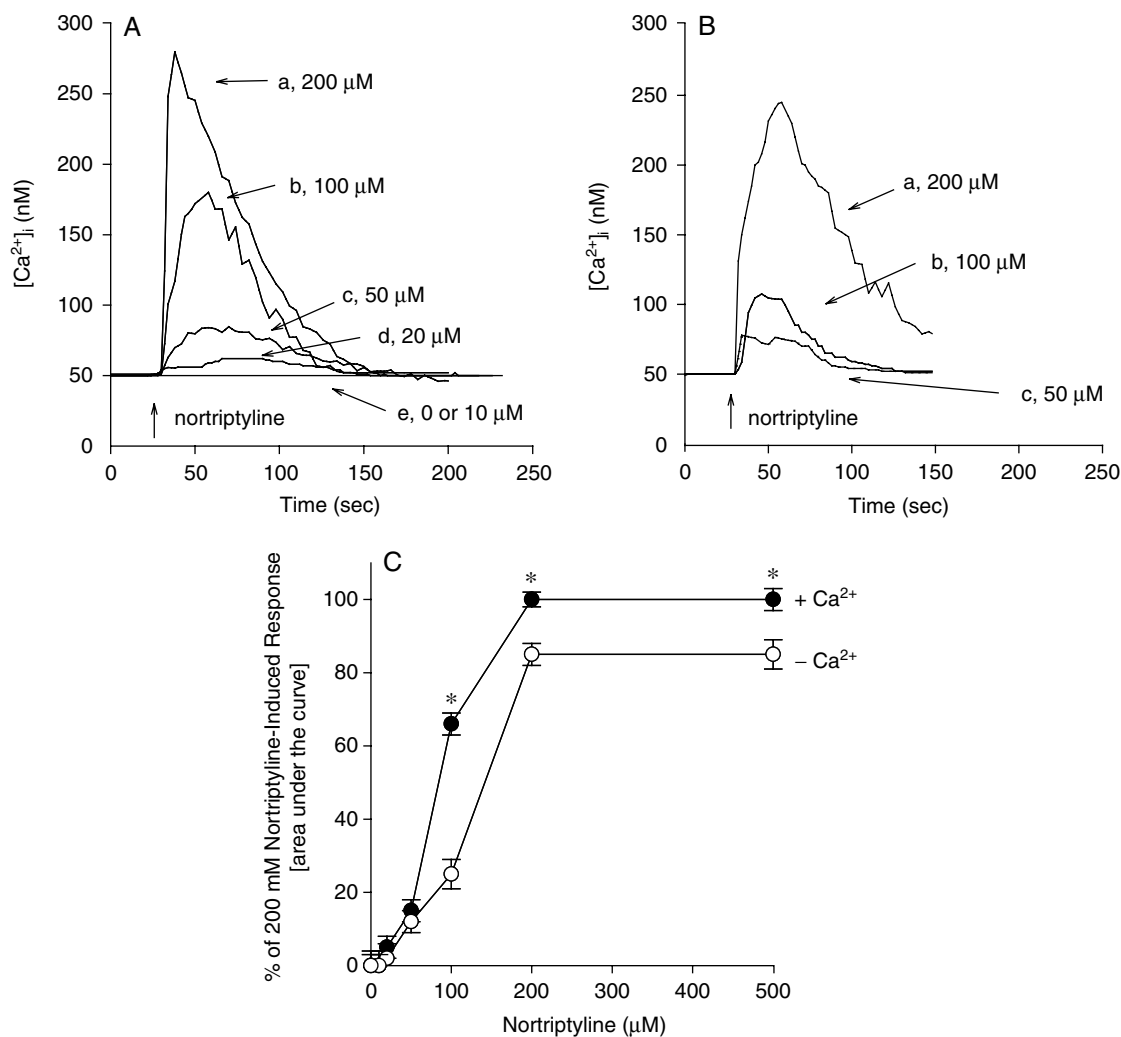


Fig. 1. A. Effect of nortriptyline on  $[Ca^{2+}]_i$  in fura-2-loaded SIRC cells. Nortriptyline was added at 25 sec. The concentration of nortriptyline was indicated. The experiments were performed in a  $Ca^{2+}$ -containing medium. B. Effect of removal of  $Ca^{2+}$  on nortriptyline-induced  $[Ca^{2+}]_i$  rise. Experiments were performed in a  $Ca^{2+}$ -free medium ( $Ca^{2+}$  was replaced with 0.3 mM EGTA). In A & B, data shown are representatives of three experiments. C. Concentration-response plots of nortriptyline-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 200  $\mu$ M nortriptyline in  $Ca^{2+}$ -containing medium. Data are means  $\pm$  SEM of three experiments. \* $P < 0.05$  compared with open circles.

corneal epithelial cells (2, 29), and, due to active responsiveness under experimental conditions, have been used as a model for corneal epithelial cell research. Several agents, such as tamoxifen (14), ketoconazole (21), fendiline (22) and econazole (7), have been found to stimulate SIRC cells by causing a  $[Ca^{2+}]_i$  increase. The inositol 1,4,5-trisphosphate (IP3)-sensitive  $Ca^{2+}$  store is the major  $Ca^{2+}$  store that releases  $Ca^{2+}$  into the cytosol when cells are stimulated by endogenous agents such as ATP (14). But exogenous agents can release  $Ca^{2+}$  from IP3-insensitive stores (7, 21, 22). The  $Ca^{2+}$  release may induce  $Ca^{2+}$  influx across the plasma membrane *via* the process of store-operated  $Ca^{2+}$  entry (27).

Using fura-2 as a fluorescent  $Ca^{2+}$  indicator,

this study shows that nortriptyline induced a significant  $[Ca^{2+}]_i$  increase in SIRC cells. The time course and the concentration-response relationship, the  $Ca^{2+}$  sources of the  $Ca^{2+}$  signal and the role of phospholipase C in the signal have been explored.

## Materials and Methods

### Chemicals

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

### Cell Culture

SIRC rabbit corneal epithelia 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 at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### Solutions Used in [Ca<sup>2+</sup>]<sub>i</sub> Measurements

Ca<sup>2+</sup>-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM Hepes, 5 mM glucose. The experimental 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 basal [Ca<sup>2+</sup>]<sub>i</sub>.

### [Ca<sup>2+</sup>]<sub>i</sub> Measurements

Confluent cells grown on 6-cm dishes were trypsinized and made into a suspension in culture medium at a density of 10<sup>6</sup> 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 with Ca<sup>2+</sup>-containing medium twice and were made into a suspension in Ca<sup>2+</sup>-containing medium at a density of 10<sup>7</sup> 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. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca<sup>2+</sup>-containing or Ca<sup>2+</sup>-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. For calibration of [Ca<sup>2+</sup>]<sub>i</sub>, after completion of the experiments, the detergent Triton X-100 and 5 mM CaCl<sub>2</sub> were added to the cuvette to obtain the maximal fura-2 fluorescence. The Ca<sup>2+</sup> chelator EGTA (10 mM) was subsequently added to chelate Ca<sup>2+</sup> in the cuvette to obtain the minimal fura-2 fluorescence. [Ca<sup>2+</sup>]<sub>i</sub> was calculated as previously described (12).

### Statistics

Data are reported as representative of means ± SEM of three experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS<sup>®</sup>, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between

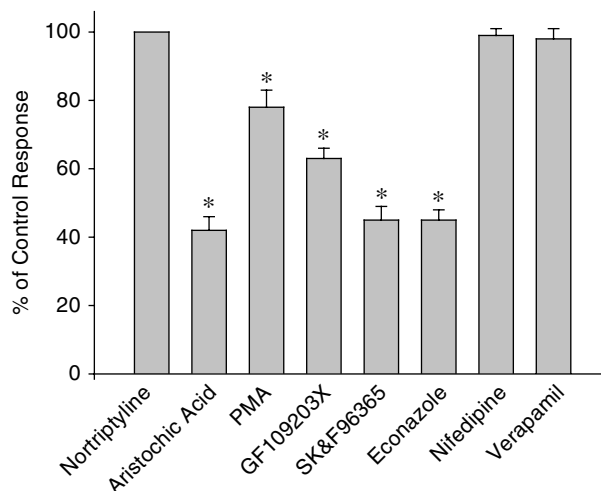


Fig. 2. Effect of Ca<sup>2+</sup> channel blockers and phospholipase A2 inhibitor on nortriptyline-induced [Ca<sup>2+</sup>]<sub>i</sub> rise. The [Ca<sup>2+</sup>]<sub>i</sub> rise induced by 200 µM nortriptyline was taken as the control. In blocker- or modulator-treated groups, the reagent was added 1 min before nortriptyline. The concentration was 1 µM for nifedipine and verapamil; 0.5 µM for econazole; 5 µM for SK&F96365; 20 µM for aristolochic acid, 10 nM for phorbol 12-myristate 13-acetate (PMA) and 2 µM for GF109203X. Data are expressed as the percentage of control (1<sup>st</sup> column from the left) that is the maximum value of 200 µM nortriptyline-induced [Ca<sup>2+</sup>]<sub>i</sub> rise, and are means ± SEM of three experiments. \**P* < 0.05 compared to the control.

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<sup>2+</sup>]<sub>i</sub> level was approximately 50 nM. At concentrations between 20 and 200 µM, nortriptyline evoked [Ca<sup>2+</sup>]<sub>i</sub> rises in a concentration-dependent manner in a Ca<sup>2+</sup>-containing medium. At 10 µM, nortriptyline did not cause a [Ca<sup>2+</sup>]<sub>i</sub> rise (data not shown). The [Ca<sup>2+</sup>]<sub>i</sub> rise induced by 200 µM nortriptyline attained to 225 ± 2 nM (n = 3) followed by a slow decay. The Ca<sup>2+</sup> response saturated at 200 µM nortriptyline because at a concentration of 200 µM, nortriptyline induced a similar response as that induced by 500 µM. Fig. 1C (filled circles) shows the concentration-response plot of nortriptyline-induced response.

Two possible sources of a Ca<sup>2+</sup> signal are extracellular medium and intracellular Ca<sup>2+</sup> stores. Further experiments were performed to determine the relative contribution of extracellular Ca<sup>2+</sup> entry and intracellular Ca<sup>2+</sup> release in nortriptyline-induced [Ca<sup>2+</sup>]<sub>i</sub> rises. The [Ca<sup>2+</sup>]<sub>i</sub> rises evoked by 20, 50, 100 and 200

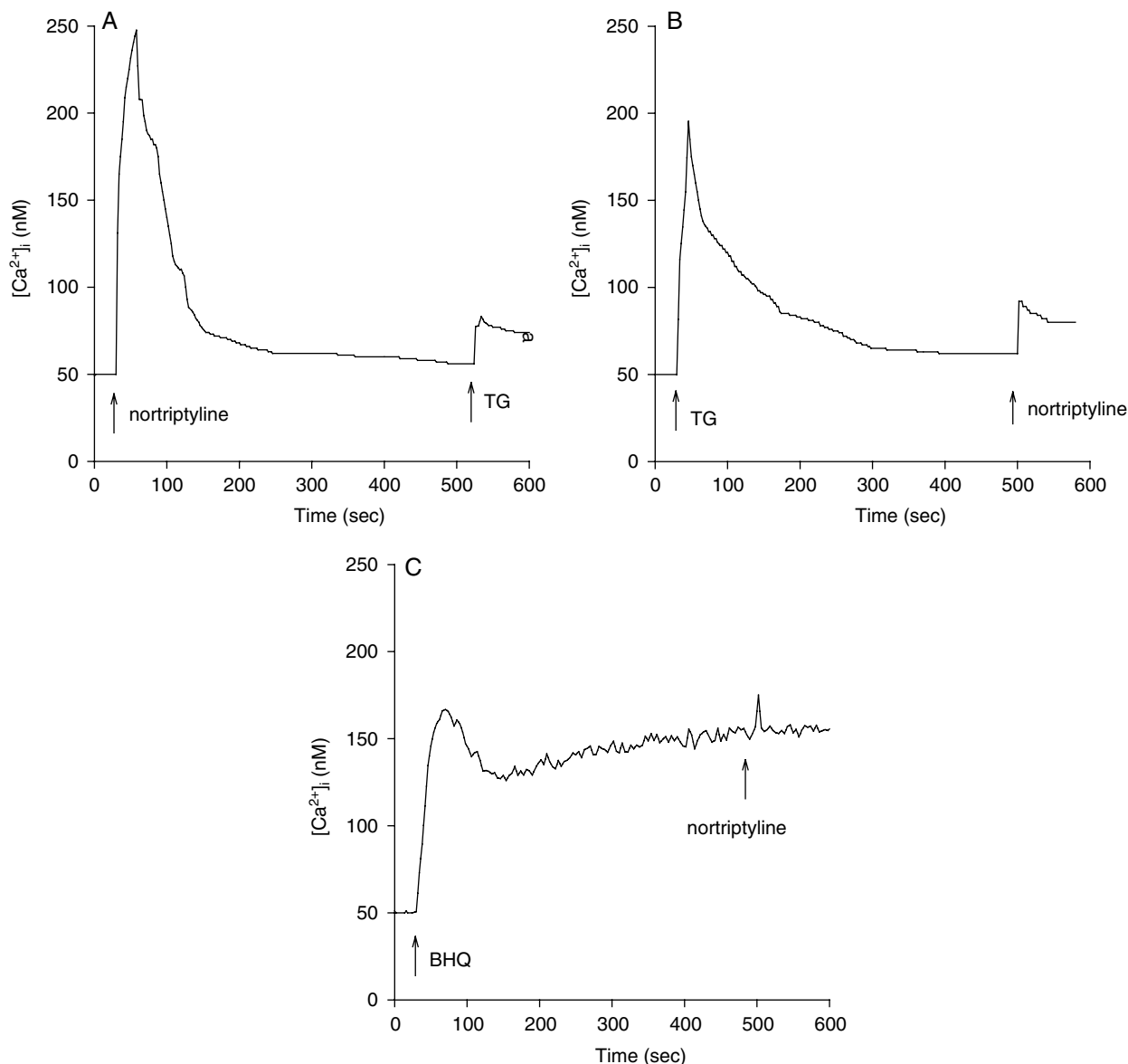


Fig. 3. Intracellular  $\text{Ca}^{2+}$  stores of nortriptyline-induced  $\text{Ca}^{2+}$  release. Experiments were performed in a  $\text{Ca}^{2+}$ -free medium. Nortriptyline (200  $\mu\text{M}$ ), thapsigargin (TG, 1  $\mu\text{M}$ ) and BHQ (50  $\mu\text{M}$ ) were added at the time points indicated. Data are representative of three experiments.

$\mu\text{M}$  nortriptyline in  $\text{Ca}^{2+}$ -free medium are shown in Fig. 1B. Removal of extracellular  $\text{Ca}^{2+}$  did not change the baseline suggesting that the amount of leaked fura-2 from the cells was insignificant. At a concentration of 200  $\mu\text{M}$ , nortriptyline evoked a  $[\text{Ca}^{2+}]_i$  rise by  $200 \pm 2$  nM ( $n = 3$ ) above the baseline followed by a gradual decay. The concentration-response plot of nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rises in  $\text{Ca}^{2+}$ -free medium is shown in Fig. 1C (open circles). The  $\text{EC}_{50}$  value is approximately 75  $\mu\text{M}$ .

Experiments were further conducted to explore the  $\text{Ca}^{2+}$  entry pathway of the nortriptyline-induced response. The store-operated  $\text{Ca}^{2+}$  influx inhibitors econazole (0.5  $\mu\text{M}$ ) and SK&F96365 (5  $\mu\text{M}$ ), aris-

tolochic acid [20  $\mu\text{M}$ ; a phospholipase A2 (PLA2) inhibitor], phorbol 12-myristate 13 acetate [PMA; a protein kinase C (PKC) activator] and GF109203X (a PKC inhibitor) partly inhibited 200  $\mu\text{M}$  nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise. In contrast, the L-type  $\text{Ca}^{2+}$  channel blockers nifedipine and verapamil had no effect on nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise (Fig. 3).

Previous studies have shown that the endoplasmic reticulum is the major  $\text{Ca}^{2+}$  store in SIRC cells (7, 14, 21, 22). Fig. 3A shows that in a  $\text{Ca}^{2+}$ -free medium, after pretreatment with 200  $\mu\text{M}$  nortriptyline, addition of 1  $\mu\text{M}$  thapsigargin (TG), an inhibitor of endoplasmic reticulum  $\text{Ca}^{2+}$  pumps (40), evoked a  $[\text{Ca}^{2+}]_i$  rise of  $20 \pm 2$  nM ( $n = 3$ ). Fig. 3B shows that

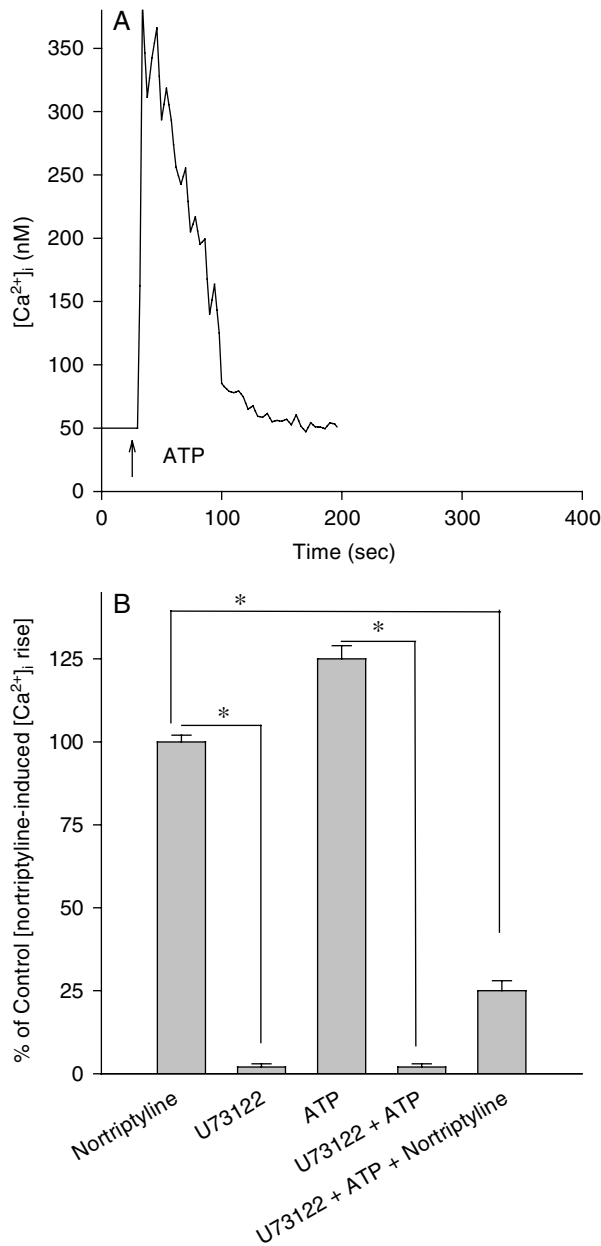


Fig. 4. Effect of U73122 on nortriptyline-induced  $Ca^{2+}$  release. Experiments were performed in  $Ca^{2+}$ -free medium. (A) ATP ( $10 \mu M$ ) was added as indicated. Data are representative of three experiments. (B) Experiments were performed by adding U73122 ( $2 \mu M$ ; for 1 min), ATP ( $10 \mu M$ ; for 100 sec) and nortriptyline ( $200 \mu M$ ; for 100 sec) sequentially. U73122 did not increase  $[Ca^{2+}]_i$  while abolished ATP-induced  $[Ca^{2+}]_i$  rise. Subsequently added nortriptyline induced a  $[Ca^{2+}]_i$  rise smaller than control response shown in Fig. 1B. Data are means  $\pm$  SEM of three experiments.  $*P < 0.05$ .

addition of TG induced a  $[Ca^{2+}]_i$  rise of  $147 \pm 2$  nM. Subsequently added nortriptyline ( $200 \mu M$ ) at 500 sec induced a  $[Ca^{2+}]_i$  rise of  $21 \pm 2$  nM, which was smaller than the control nortriptyline (Fig. 1B) by 90% ( $n = 3$ ). Furthermore, Figs. 3A and 3B suggest that nortrip-

tyline pretreatment decreased TG-induced  $[Ca^{2+}]_i$  rise by  $86 \pm 2\%$  ( $n = 3$ ). Another inhibitor of endoplasmic reticulum  $Ca^{2+}$  pump, 2,5-di-tert-butylhydroquinone (BHQ) (43), induced a  $[Ca^{2+}]_i$  rise of  $120 \pm 3$  nM ( $n = 3$ ) followed by a gradual decline and a gradual rise. Similarly, subsequently added nortriptyline did not induce a  $[Ca^{2+}]_i$  rise.

Phospholipase C (PLC)-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing  $Ca^{2+}$  from the endoplasmic reticulum (5, 8). Because nortriptyline released  $Ca^{2+}$  from the endoplasmic reticulum, the role of PLC in this event was examined. U73122, a PLC inhibitor (41), was used to investigate whether this enzyme was stimulated during nortriptyline-induced  $Ca^{2+}$  release. Fig. 4A shows that ATP ( $10 \mu M$ ) induced a transient  $[Ca^{2+}]_i$  rise of  $320 \pm 2$  nM ( $n = 3$ ). ATP is a PLC-dependent agonist of  $[Ca^{2+}]_i$  rise in most cell types (10). Fig. 4B shows that incubation with  $2 \mu M$  U73122 did not change the basal  $[Ca^{2+}]_i$  but abolished ATP-induced  $[Ca^{2+}]_i$  rises. This suggests that U73122 effectively suppressed PLC activity. Fig. 4B also shows that addition of  $200 \mu M$  nortriptyline after U73122 and ATP treatments caused a  $[Ca^{2+}]_i$  rise smaller than the control (1st column) by  $75 \pm 3\%$  ( $n = 3$ ).

## Discussion

$Ca^{2+}$  signaling plays a crucial role in the function of almost all cell types including corneal epithelial cells. Evidence shows that in human corneal epithelial cells, asialoganglioside ganglioside ceramide induces  $Ca^{2+}$  influx through L-type voltage-dependent  $Ca^{2+}$  channels. This leads to P2Y receptor stimulation along with membrane depolarization resulting from increases in ATP release into the medium. Intracellular  $Ca^{2+}$  transients led to time-dependent extracellular signal-regulated kinase (ERK) MAPK pathway stimulation, followed by an increase in IL-8 release (9). Kimura et al (18) have shown that ATP and its analogs increase  $[Ca^{2+}]_i$  in rabbit corneal epithelium. In bovine corneal epithelial cells,  $Ca^{2+}$  signaling-coupled muscarinic receptor subtypes have been characterized (35). Grant and Acosta (11) suggest that intracellular pH and  $[Ca^{2+}]_i$  are closely associated in rabbit corneal epithelial cells.

Our study is the first to show that nortriptyline-induced  $[Ca^{2+}]_i$  rise in SIRC human corneal epithelial cells, and the underlying mechanisms were examined. The data show that nortriptyline induced a concentration-dependent  $[Ca^{2+}]_i$  rise in SIRC cells between  $20 \mu M$  and  $200 \mu M$ . The results suggest that nortriptyline increased  $[Ca^{2+}]_i$  by depleting intracellular  $Ca^{2+}$  stores and causing  $Ca^{2+}$  influx from extracellular milieu because removing extracellular  $Ca^{2+}$  reduced nortriptyline-induced  $[Ca^{2+}]_i$  rise. Removal



of extracellular  $\text{Ca}^{2+}$  reduced the nortriptyline-induced response throughout the measurement period suggesting that  $\text{Ca}^{2+}$  influx occurred during the whole stimulation period.

The mechanism of nortriptyline-induced  $\text{Ca}^{2+}$  influx was examined. The results suggest that nortriptyline might cause  $\text{Ca}^{2+}$  influx *via* stimulating store-operated  $\text{Ca}^{2+}$  entry which is induced by depletion of intracellular  $\text{Ca}^{2+}$  stores, based on the inhibition of nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise by econazole and SK&F96365. Econazole and SK&F96365 have been used as blockers of store-operated  $\text{Ca}^{2+}$  entry in different cell types (16, 17, 28). Furthermore, aristolochic acid, a PLA2 inhibitor, significantly inhibited nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise. PLA2 activity is thought to be associated with  $\text{Ca}^{2+}$  movement. Tedesco *et al.* (38) show that snake PLA2 neurotoxins evoked  $\text{Ca}^{2+}$  overload in nerve terminals of cultured neurons. Lupescu *et al.* (23) suggest that human parvovirus B19 capsid protein VP1-induced  $\text{Ca}^{2+}$  entry was suppressed if PLA2 activity was inhibited. Most importantly, recent evidence has shown that PLA2 controls endothelial store-operated  $\text{Ca}^{2+}$  entry and vascular tone in intact aorta (3) and enhances store-operated  $\text{Ca}^{2+}$  entry in dystrophic skeletal muscle fibers (4). Singaravelu *et al.* (34) showed that PLA2 mediated store-operated  $\text{Ca}^{2+}$  entry in rat cerebellar granule cells. Therefore, these studies are in agreement with our results that PLA2 activity was necessary for nortriptyline-evoked  $\text{Ca}^{2+}$  signal in SIRC cells. Furthermore, activation or inhibition of PKC inhibited nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise. Because activation of PLC produces IP3 and diacylglycerol, which stimulates PKC, the effect of regulation of PKC activity on nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise was examined. Both activation and inhibition of PKC inhibited nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise. Activation or inhibition of PKC has been shown to regulate store-operated  $\text{Ca}^{2+}$  channels in different preparations such as vascular smooth muscle (20), vascular myocytes (31), vein myocytes (1), HL60 cells (19) and glomerular mesangial cells (24). Another possible mechanism that might cause nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise was that nortriptyline inhibited plasma membrane  $\text{Ca}^{2+}$  ATP pump so that cytosolic  $\text{Ca}^{2+}$  could not be pumped out of the cells and  $[\text{Ca}^{2+}]_i$  would rise *via* leaks in the plasma membrane.

Regarding the  $\text{Ca}^{2+}$  stores involved in nortriptyline-induced  $\text{Ca}^{2+}$  release, the TG/2,5-di-tert-butylhydroquinone (BHQ)-sensitive endoplasmic reticulum stores might be the main store because TG/BHQ pretreatment both nearly abolished nortriptyline-induced  $[\text{Ca}^{2+}]_i$  rise; conversely, nortriptyline pretreatment decreased a major part of TG-induced  $[\text{Ca}^{2+}]_i$  rise. Furthermore, it seems that PLC-dependent pathways played a significant role in nortriptyline-

induced  $\text{Ca}^{2+}$  release since the response was inhibited by 75% when PLC activity was suppressed by U73122.

Taken together, the data show that nortriptyline induced  $\text{Ca}^{2+}$  release from the endoplasmic reticulum and also caused  $\text{Ca}^{2+}$  influx *via* store-operated  $\text{Ca}^{2+}$  entry in a PLC- and PLA2-dependent manner. Because a rise in  $[\text{Ca}^{2+}]_i$  can alter many cellular responses, caution should be applied in using nortriptyline in other *in vitro* studies.

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