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Original article

Down-regulation of voltage-gated Ca^{2+} channels in Ca^{2+} store-depleted rat insulinoma RINm5F cells

Yuk M. Leung [a,*], Kar L. Wong [b], Shiao W. Chen [c], Dah Y. Lu [a], Chang S. Kuo [a], Yun R. Chen [a], Ya W. Chen [d], Tzu H. Cheng [c,**]

[a] Graduate Institute of Neural and Cognitive Sciences, China Medical University, Taichung 40402, Taiwan
[b] Department of Anesthesia, China Medical University, Taichung 40402, Taiwan
[c] Department of Biological Sciences and Technology, China Medical University, Taichung 40402, Taiwan
[d] Department of Physiology, China Medical University, Taichung 40402, Taiwan

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ABSTRACT

Background: Glucose-stimulated insulin secretion in pancreatic islet β-cells is initiated by ATP-induced closure of ATP-sensitive potassium channels (K_{ATP} channels), subsequent depolarization and opening of voltage-gated Ca^{2+} channels (VGCC), eventually leading to insulin exocytosis. A variety of natural and environmental toxins have been known to cause Ca^{2+} store depletion and consequently death in many cell types, but the impact of sustained Ca^{2+} store depletion on β-cell plasmalemmal ion channels is unknown.

Purpose: This report examined whether sustained Ca^{2+} store depletion induced by cyclopiazonic acid (CPA) could affect voltage-gated ion channels and K_{ATP} channels in rat insulinoma RINm5F cells.

Methods: Microfluorimetric Ca^{2+} imaging and patch-clamping experiments were employed in this study.

Results: Glucose- and KCl-stimulated Ca^{2+} signals were substantially attenuated after a 24-h CPA treatment. Consistently, patch clamp experiments also demonstrated that VGCC currents were much reduced after a 24-h CPA treatment. Quantitative RT-PCR experiments showed that gene expression of α-1A and α-1C was reduced, suggesting that expression of P/Q- and L-type VGCC was down-regulated. Voltage-gated K^+ channels, K_{ATP} channels and store-operated Ca^{2+} entry were, however, unaffected. Inhibition of extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) could not rescue the KCl-stimulted Ca^{2+} signal attenuated by sustained Ca^{2+} store depletion.

Conclusion: Our work shows, for the first time, that sustained Ca^{2+} store depletion in insulinoma RINm5F cells leads to selective down-regulation of VGCC possibly via pathways other than ERK or JNK.

* *Corresponding author*. Graduate Institute of Neural and Cognitive Sciences, China Medical University, Taichung 40402, Taiwan.
** *Corresponding author*. Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan.
E-mail addresses: ymleung@mail.cmu.edu.tw (Y.M. Leung), thcheng@mail.cmu.edu.tw (T.H. Cheng).

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1. Introduction

An elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) depends on two major pathways; one is Ca^{2+} entry from the extracellular milieu while the other is Ca^{2+} mobilization from the intracellular Ca^{2+} stores [1]. Intracellular Ca^{2+} stores are found mainly in the endoplasmic/sarcoplasmic reticulum. The functional integrity of intracellular Ca^{2+} stores is essential to cell proliferation. For instance, depletion of the intracellular Ca^{2+} store by thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), induces growth arrest in DDT1MF-2 smooth muscle cells [2], and apoptosis in human prostate cancer LNCaP cells [3]. Similarly, thapsigargin induces Ca^{2+} store depletion and subsequently death in mast cells, PC12 cells and hippocampal neurons [4–6]. Depletion of the intracellular Ca^{2+} store by the ryanodine receptor agonists caffeine and ryanodine has also been demonstrated to induce apoptosis in Chinese hamster ovary cells [7].

Toxins from animals and fungi have also been known to inhibit SERCA (thus causing store depletion): for example, myotoxin from snake venom [8], the mycotoxins cyclopiazonic acid and paxilline [9,10], palytoxin from marine animals [11] and mastoparan from wasp venom [12]. In addition, albeit with the exact mechanisms unclear, a wide spectrum of substances such as the anticancer drug tamoxifen [13], β-amyloid and prion particles [14], and the environmental toxicant nonylphenol [15] have been shown to cause Ca^{2+} store depletion and cell death.

Pancreatic β-cells play an important role in glucose homeostasis by secreting insulin. When glucose is low, the resting membrane potential of β-cells is maintained mainly by ATP-sensitive K^+ (K_{ATP}) channels [16,17]. When β-cells are exposed to high glucose, the ATP/ADP ratio rises as a result of glucose metabolism. The raised ATP/ADP ratio causes K_{ATP} channel closure; cessation of K^+ outflow, together with a continuous depolarizing current of yet uncertain nature, causes depolarization of β-cells [16,17]. Voltage-gated Ca^{2+} channels (VGCCs) then open, leading to Ca^{2+} entry, which culminates in insulin exocytosis. Depolarization also activates voltage-gated K^+ (Kv) channels; the latter permits the K^+ efflux needed to repolarize the cell membrane, thus dampening excitability [18,19].

Not much is known about the toxic effects of sustained Ca^{2+} store depletion on β-cell functions. In one report, after primary rat β-cells and INS-1E β-cells had been challenged with cyclopiazonic acid (CPA), gene expression of calbindin, calreticulin, Grp94, Rab3a and HNF-1α was modulated [20]. In particular, insulin-1 and -2 mRNAs were severely degraded [20]. The effects of sustained Ca^{2+} store depletion on β-cell plasma membrane ionic channels are hitherto unknown. In the present work, CPA was used to induce sustained Ca^{2+} store depletion and cytotoxicity in rat insulinoma RINm5F cells. Our results showed that there was a selective down-regulation of VGCCs, but not Kv and K_{ATP} channels, in RINm5F cells whose Ca^{2+} stores had been chronically emptied.

2. Materials and methods

2.1. Chemicals and cell culture

CPA, SP600125, PD98059 and SK&F 96365 were obtained from Calbiochem (San Diego, CA). Diazoxide was purchased from Sigma (St. Louis, MO). Fura-2 AM was from Invitrogen (Carlsbad, CA). Tetrodotoxin (TTX) was purchased from Alomone (Jerusalem, Israel). Rat insulinoma RINm5F cells were cultured at 37 °C in 5% CO_2 in Roswell Park Memorial Institute 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen; Carlsbad, CA) and penicillin-streptomycin (100 units/mL, 100 μg/mL) (Invitrogen).

2.2. Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide (MTT) method. Cells were cultured in a 96-well plate at a density of 1.5×10^4/well, and were then treated with either 0.1% dimethyl sulfoxide (DMSO) or 30 μM CPA for 3 or 24 hours. MTT (final concentration at 0.5 mg/mL) was subsequently added to each well and then further incubated for 4 hours. The culture medium was then removed and 100 μl of DMSO was added to each well for 15 minutes (with shaking) to dissolve the cells. The absorbance at 595 nm was measured using an enzyme-linked immunosorbent assay reader and was used as an indicator of cell viability.

2.3. Electrophysiology

Electrophysiological experiments were performed as described in a previous report [21]. RINm5F cells were voltage-clamped using the whole-cell configuration. Borosilicate glass tubes (o.d. 1.5 mm, i.d. 1.10 mm, Sutter Instrument, Novato, CA) were prepared with a micropipette puller (P-87, Sutter Instrument), and then fire-polished by a microforge (Narishige Instruments, Inc., Sarasota, FL). For measurements of Kv channel currents, intracellular solution contained: 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, and 5 mM MgATP (pH 7.25 adjusted with KOH). For measurement of ATP-sensitive K^+ currents, the concentration of ATP was reduced to 1 mM in the above-mentioned intracellular solution. For measurements of Ca^{2+} currents, intracellular solution contained: 120 mM CsCl, 20 mM TEA-Cl, 8 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, and 5 mM MgATP (pH 7.25 adjusted with CsOH). For measurements of K^+ currents, the bath solution contained: 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (pH 7.4 adjusted with NaOH). When Ca^{2+} currents were measured, 1 μM TTX was added to the above-mentioned bath solution to block Na^+ channels. The currents were recorded using an EPC-10 amplifier with Pulse 8.60 acquisition software and analyzed by Pulsefit 8.60 software (HEKA Electronik, Lambrecht, Germany). Data were filtered at 2 kHz and sampled at 10 kHz. After the establishment of a whole-cell configuration, the cells were held at −70 mV and subject to various protocols as

described in the text and the figure legends. All experiments were carried out at room temperature (~25 °C).

2.4. Microfluorimetric measurement of cytosolic Ca^{2+}

Microfluorimetric measurement of cytosolic Ca^{2+} concentration was performed using fura-2 as the Ca^{2+}-sensitive fluorescent dye as described previously [22]. Briefly, cells were incubated with 5 μM fura-2 AM for 1 hour at 37 °C and then washed. The bath solution contained: 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4 adjusted with NaOH). When Ca^{2+}-free bath solution was used, Ca^{2+} was omitted from the above bath solution and 20 μM ethylene glycol tetraacetic acid (EGTA) was supplemented. Cells were alternately excited with 340 nm and 380 nm using an optical filter changer (Lambda 10-2, Sutter Instruments). Emission was collected at 500 nm and images were captured using a CCD camera (CoolSnap HQ2, Photometrics, Tucson, AZ) connected to an inverted Nikon TE 2000-U microscope. Images were analyzed with MAG Biosystems Software (Sante Fe, MN). All imaging experiments were conducted at room temperature (~25 °C).

2.5. Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted using a TRIzol kit (MDBio, MD). The reverse transcription (RT) reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using the oligo(dT) primer. Quantitative real-time polymerase chain reaction (PCR) using SYBR Green I Master Mix was analyzed with a model 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). After pre-incubation at 50 °C for 2 minutes and 95 °C for 10 minutes, the PCR was performed as 40 cycles at 95 °C for 10 seconds and 60 °C for 1 minute. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T). The oligonucleotide primers for P/Q-type (α-$_{1A}$), N-type (α-$_{1B}$) and L-type (α-$_{1C}$ and α-$_{1D}$) VGCCs, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as reported in previous publications [23,24]: α-$_{1A}$Forward: CAA AGG TAC CAC CAA CGC CReverse: TGG TCA TGC TCA GAT CTG TCCα-$_{1B}$Forward: GCA CTG GAG ATC AAG CTT GCReverse: CCC ACT GTC ATC TCG TCA GGα-$_{1C}$ Forward: 5′-CCGGAAGCCAGTGCATTTT-3′;Reverse: 5′-TGGT GAAGATCGTGTCATTGACA-3′α-$_{1D}$Forward: 5′-GAAGAGGACG AGCCTGAGGTT-3′;Reverse: 5′-TTTTCTCCTTCATGTTCAAC TCTGA-3′GAPDHForward: 5′-ACCACAGTCCATGCCATCAC-3′;Reverse: 5′-TCCACCACCCTGTTGCTGTA-3′

2.6. Statistical analysis

Data are presented as mean ± SEM. The unpaired or paired Student *t* test was employed where appropriate. Analysis of variance (ANOVA) was used to compare multiple groups, followed by the Tukey's honestly significant difference (HSD) *post-hoc* test. A p value of <0.05 was considered to have statistical significance.

3. Results

The effect of CPA on cell viability was first examined. While a 3-hour incubation with CPA did not significantly affect cell viability, a 24-hour challenge with CPA caused substantial cell death (65%) as shown in the MTT assay (Fig. 1). This suggests that chronic Ca^{2+} store depletion had a cytotoxic effect on RINm5F cells. We were interested in the ion channel properties of the cells after 3-hour CPA treatment and those viable cells after a 24-hour CPA challenge. In electrophysiological and Ca^{2+} imaging experiments, the cells were rigorously washed before the experiments and only viable cells remained attached to the substratum. Trypan blue exclusion test showed that 98.4 ± 1.0 % of the attached cells were viable.

We examined whether sustained CPA-induced Ca^{2+} store depletion would affect intracellular Ca^{2+} release and store-operated Ca^{2+} entry. The cells were treated with CPA for 3 or 24 hours. The cells were then washed extensively in Ca^{2+}-containing bath solution (to remove CPA and to replenish the Ca^{2+} pool). Before the experiments, the cells were bathed in nominally Ca^{2+}-free bath solution containing 20 μM EGTA. For 3-hour (Fig. 2A–C) and 24-hour treatments (Fig. 2D–F), the basal $[Ca^{2+}]_i$ and Ca^{2+} release upon CPA re-challenge were comparable in the control groups and the CPA-challenged groups. This suggests that the Ca^{2+} stores and their Ca^{2+} release channels were still fully functional even when the Ca^{2+} stores had been discharged by CPA for 24 hours. Ca^{2+} entry after Ca^{2+} store depletion [store-operated Ca^{2+} entry (SOCE)] in the CPA-treated groups (3 and 24 hours) was similar in magnitude to that in the control groups, suggesting that CPA-induced chronic store depletion did not adversely affect SOCE.

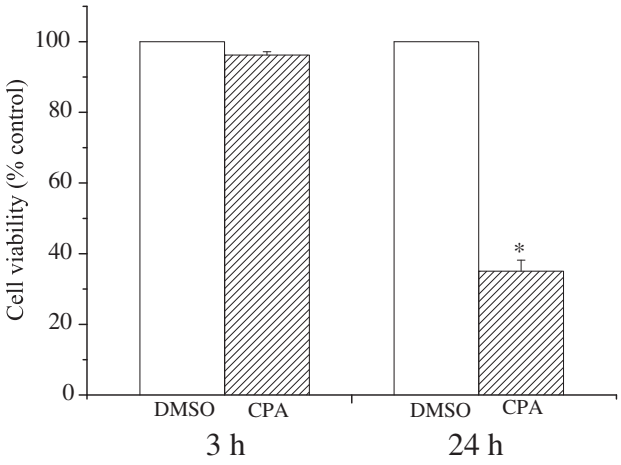

Fig. 1 – CPA-induced Ca^{2+} store depletion for 24 hours caused cell death. RINm5F cells were incubated with 0.1% DMSO or 30 μM CPA for 3 or 24 h before the MTT assay. Substantial cell death (65%) was observed in cells treated with CPA for 24 hours. Results are displayed as the mean ± SEM of three separate experiments. * $p < 0.05$. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide.

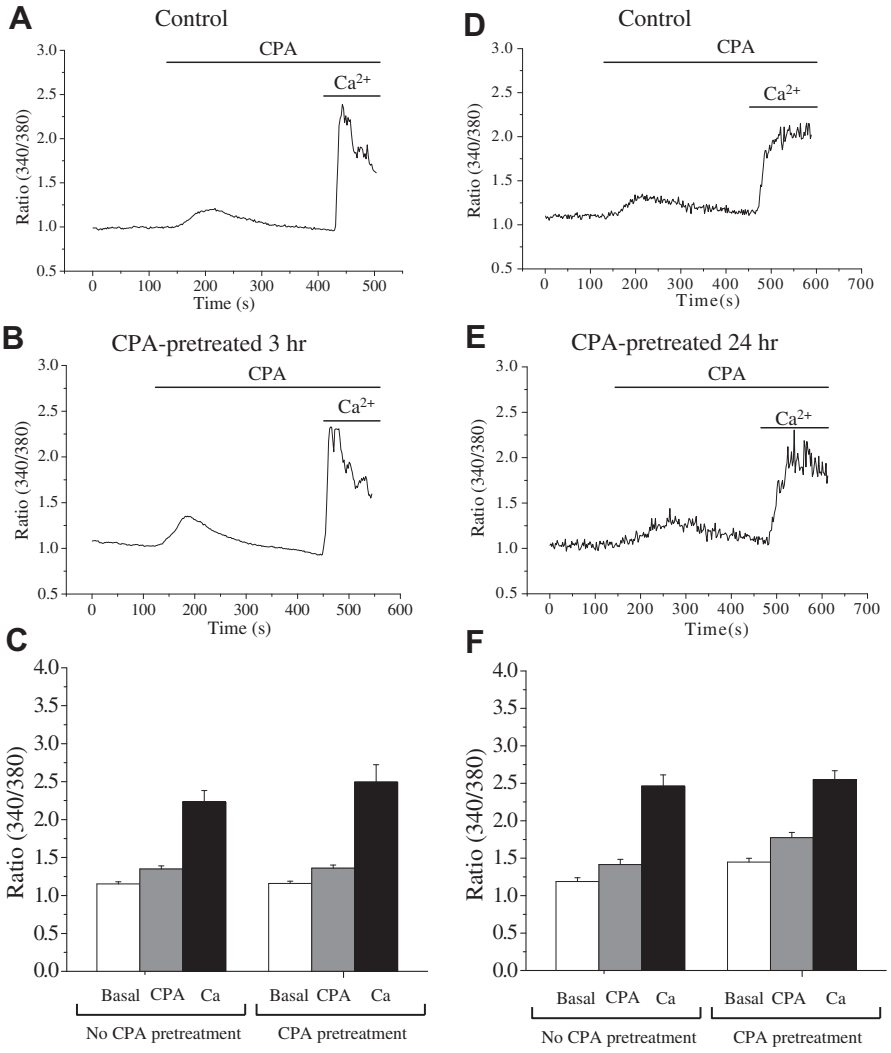

Fig. 2 – Sustained Ca^{2+} store depletion did not affect intracellular Ca^{2+} release and SOCE. After RINm5F cells were treated with 0.1% DMSO (control) or 30 μM of CPA for (A,B) 3 hours or (D,E) 24 hours, they were washed extensively in Ca^{2+}-containing bath solution, bathed in Ca^{2+}-free bath solution and then assayed for $[Ca^{2+}]_i$. The cells were first challenged with 30 μM CPA to cause store depletion before replenishment of 2 mM bath Ca^{2+}. (C) Quantitative results of the 3-hour group. (F) Quantitative results of the 24-hour group. Results are displayed as the mean ± SEM of 31–60 cells. There is no significant difference between the control and CPA-treated groups. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide; SOCE = store-operated Ca^{2+} entry.

We then examined whether glucose-stimulated $[Ca^{2+}]_i$ elevation would be affected by chronic store depletion. The cells were rigorously washed and finally bathed in Ca^{2+}-containing bath solution. While there was no significant difference in glucose-stimulated $[Ca^{2+}]_i$ elevation between the control group and CPA group at the 3-hour time point (Fig. 3A–C), a 24-hour Ca^{2+} store depletion by CPA did attenuate glucose-induced Ca^{2+} signaling (Fig. 3D–F).

As the glucose-triggered Ca^{2+} signal most likely involves opening of VGCCs, we proceeded to test whether KCl-stimulated VGCC opening was affected. Application of 30 mM KCl causes depolarization, which directly opened VGCCs. A 3-hour Ca^{2+} store depletion did not suffice to affect KCl-triggered VGCC opening (Fig. 4A–C); attenuation of VGCC activity required a 24-hour CPA pretreatment (Fig. 4D, E, and G). To distinguish whether this attenuation of VGCC activity was due to store depletion *per se* or due to any possible deleterious effects of continuous SOCE during the 24-hour time period, in one group the cells were co-treated with CPA and SK&F 96365 (SOCE inhibitor; 10 μM). SK&F 96365 did not prevent or relieve the attenuation of VGCC activity in cells treated with CPA for 24 hours at all (Fig. 4D–G), suggesting that the decreased VGCC activity was unlikely to be related to the continuous SOCE.

Patch clamp experiments were performed to confirm that the reduced glucose- and KCl-stimulated Ca^{2+} signals were due to decreased currents through VGCCs. Serial depolarizing pulses triggered Ca^{2+} currents in RINm5F cells; and in cells treated with CPA for 24 hours, substantial reduction of currents was observed (Fig. 5A and B). SK&F 96365 co-treatment did not

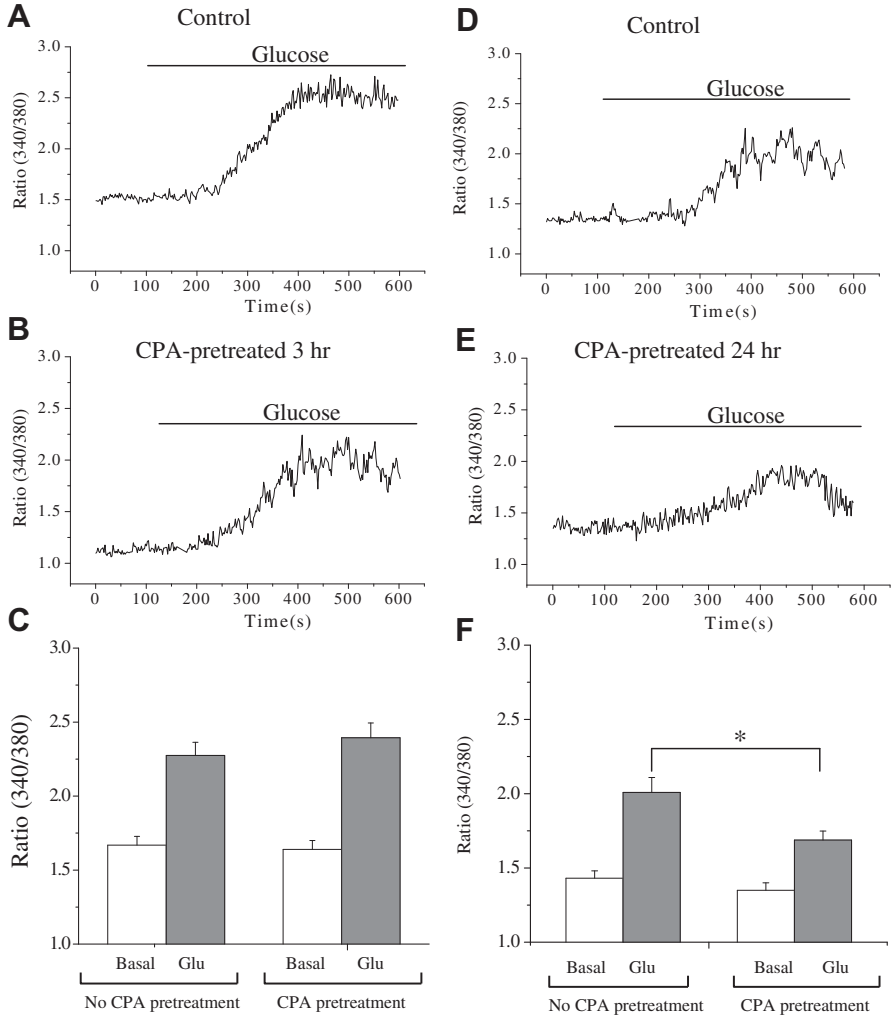

Fig. 3 – Sustained Ca^{2+} store depletion for 24-hour inhibited glucose-induced Ca^{2+} signal. After RINm5F cells were treated with 0.1% DMSO (control) or 30 μM CPA for (A,B) 3 h or (D,E) 24 h, they were washed extensively in and bathed in Ca^{2+}-containing bath solution, and then assayed for $[Ca^{2+}]_i$. The cells were treated with 20 mM glucose. (C) Quantitative results of the 3-hour group. (F) Quantitative results of the 24-hour group. Results are displayed as the mean ± SEM of 9–60 cells. * Indicates significant ($p < 0.05$) difference from the glucose-stimulated control group. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide.

prevent or relieve the reduction in currents in cells treated with CPA for 24 hours. We pondered whether the decreased VGCC activity after a 24-hour Ca^{2+} store depletion was due to reduced VGCC gene expression. Multiple types of VGCCs are expressed in RINm5F cells, including P/Q-, N- and L-types [25,26]. We then used quantitative RT-PCR to investigate which VGCC types were susceptible to gene down-regulation. It was found that α_{1A} and α_{1C} gene expression were selectively suppressed, while that of α_{1B} and α_{1D} was relatively unaffected (Fig. 5C). These results suggest that chronic Ca^{2+} store depletion selectively down-regulated expression of P/Q- and L-type VGCCs in RINm5F cells.

K_{ATP} channels are an essential component in the metabolism-excitation coupling of β-cells; their closure by ATP results in depolarization [16,17]. Kv channels are important in repolarizing β-cells and curbing excitability [18,19]. Unexpectedly, Ca^{2+} store depletion for 3 or 24 hours did not cause any reduction in Kv and K_{ATP} channel currents (Fig. 6). In fact, a 3-hour Ca^{2+} store depletion caused a slight but significant enhancement of Kv channel currents (Fig. 6A). We also examined whether Ca^{2+} store depletion would affect membrane potential. As measured by current clamp, the membrane potentials in control and CPA-treated cells for 24 hours were -68.7 ± 1.1 and -71.7 ± 1.8 mV, respectively ($p > 0.05$). In the control group, cell capacitance was 12.6 ± 1.6 pF and pipette series resistance was 17.8 ± 1.3 MΩ ($n = 7$), while in the CPA group, cell capacitance was 11.1 ± 1.5 pF and pipette series resistance was 13 ± 2.5 MΩ ($n = 6$). The data suggest Ca^{2+} store depletion for 24 hours did not affect membrane potential.

Extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) have been known to be activated during endoplasmic reticulum (ER) stress [27–30]. Since chronic Ca^{2+} store depletion inevitably leads to ER stress,

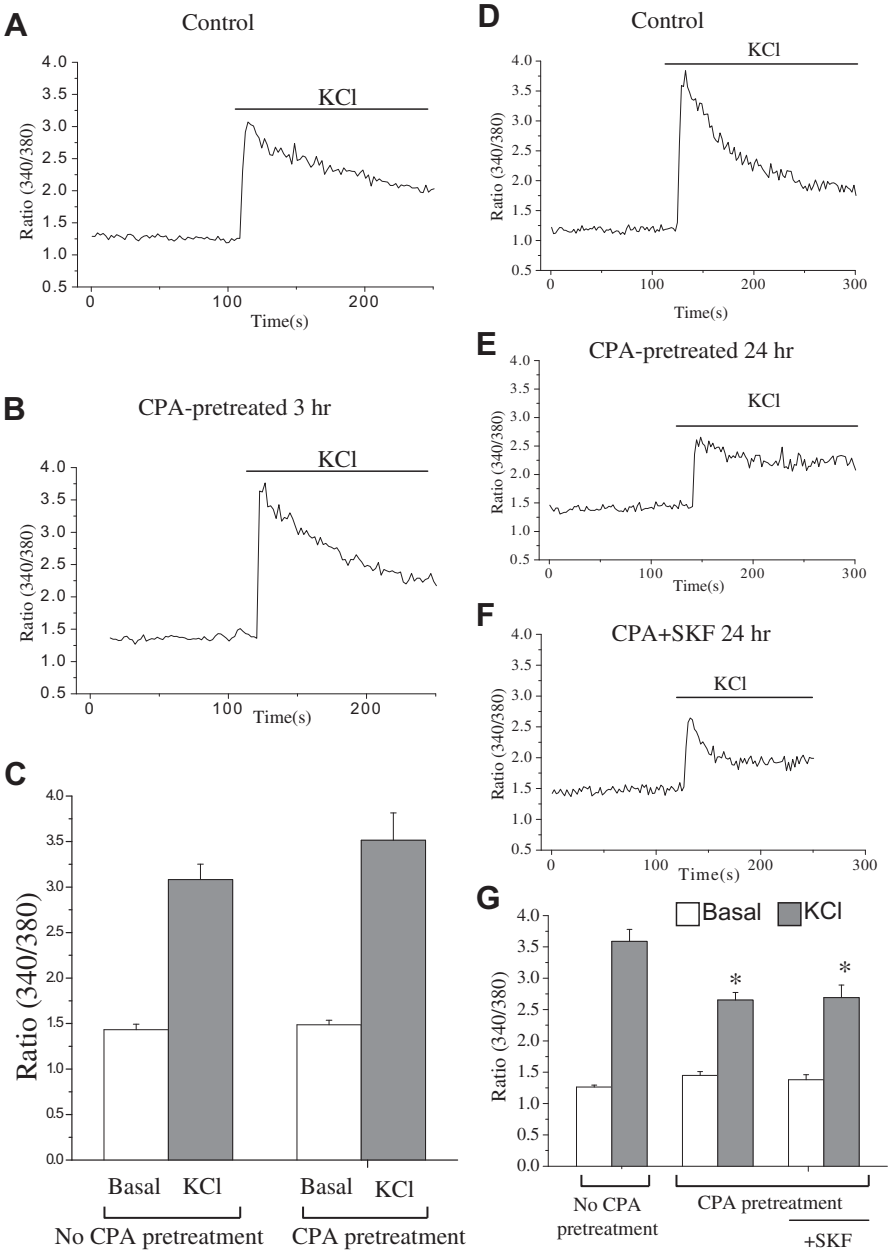

Fig. 4 – Sustained Ca^{2+} store depletion for 24 hours inhibited KCl-induced Ca^{2+} signal. After RINm5F cells were treated with 0.1% DMSO (control), 30 μM CPA or 30 μM CPA plus 10 μM SK&F 96365 for (A,B) 3 hours or (D–F) 24 h, they were washed extensively in and bathed in Ca^{2+}-containing bath solution, and then assayed for $[Ca^{2+}]_i$. The cells were exposed to 30 mM KCl. (C) Quantitative results of the 3-hour group. (G) Quantitative results of the 24-hour group. Results are displayed as the mean ± SEM of 11–66 cells. * Indicates significant ($p < 0.05$) difference from the KCl-stimulated control group. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide.

we examined whether ERK and JNK pathways were involved in mediating the down-regulation of VGCCs. SP600125 and PD98059, inhibitors of JNK and ERK respectively, were employed. As shown in Fig. 7, KCl-triggered $[Ca^{2+}]_i$ elevation, which was indicative of VGCC opening, was attenuated by a 24-hour CPA-induced Ca^{2+} store depletion. The down-regulation could not be reversed or relieved by co-treatments with SP600125 or PD98059, or a combination of these two inhibitors. These data suggest that JNK and ERK pathways were possibly not involved in the VGCC down-regulation caused by sustained Ca^{2+} store depletion.

4. Discussion

Intracellular Ca^{2+} store depletion caused by pharmacological manipulation of SERCA or Ca^{2+} release channels has been

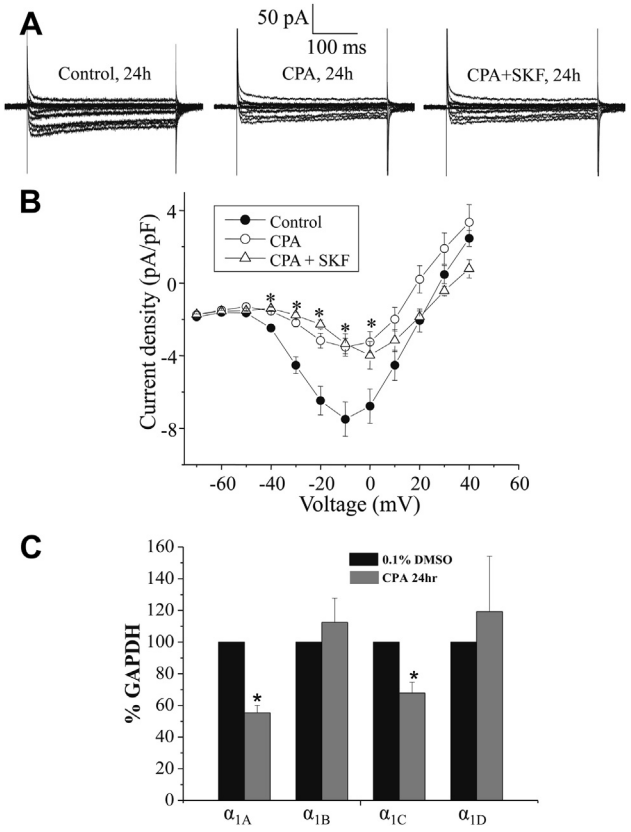

Fig. 5 – Sustained Ca^{2+} store depletion for 24 h caused reduction in Ca^{2+} currents and expression of VGCC. (A) After RINm5F cells were treated with 0.1% DMSO (control), 30 μM CPA or 30 μM CPA plus 10 μM SK&F 96365 for 24 hours, they were washed extensively in and bathed in Ca^{2+}-containing bath solution, and then assayed for Ca^{2+} currents. The cells were held at −70 mV and then stimulated with increasing depolarizing pulses up to + 70 mV at 10 mV increments. (B) Current density is plotted against voltages. Results are displayed as the mean ± SEM of 12–16 cells. Control group: cell capacitance was 8.6 ± 0.9 pF and pipette series resistance was 14.1 ± 2 MΩ (n = 16); CPA group: cell capacitance was 7.3 ± 0.6 pF and pipette series resistance was 20.6 ± 2.7 MΩ (n = 13); CPA + SKF group: cell capacitance was 6.7 ± 0.4 pF and pipette series resistance was 21.3 ± 2.2 MΩ (n = 12). (C) After RINm5F cells were treated with 0.1% DMSO (control) or 30 μM CPA for 24 h, total RNA was extracted and VGCC mRNA was quantified by real time PCR. Results are displayed as the mean ± SEM from three separate experiments. * Indicates significant ($p < 0.05$) difference of the CPA and CPA + SKF group from the control group. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide; PCR = polymerase chain reaction; VGCC = voltage-gated calcium channel.

shown to induce cell growth arrest or cell death [2,3,7]. A variety of natural toxins from animals and fungi have also been shown to inhibit SERCA and would thus induce Ca^{2+} store depletion and cell death [8–12]. A wide spectrum of substances, such as the anticancer drug tamoxifen [13], β-amyloid and prion particles [14], and the environmental toxicant nonylphenol [15] have been shown to cause Ca^{2+} store depletion and cell death. Not much is known about the effects of sustained Ca^{2+} store depletion on β-cell functions and gene expression. In one study, β-cell gene expression was extensively studied after CPA-induced Ca^{2+} store depletion; while expression of *Bip*, *calreticulin*, *Grp94*, *ATF4*, *caspase 12* and *Sec61* genes was up-regulated, that of *Pcsk1*, *Pcsk2*, *Rab3a*, *Pdx-1*, *HNF-1α* and *HNF-3α* was down-regulated [20]. Of note, the degradation of insulin-1 and -2 mRNAs was particularly severe [20]. It is unknown whether sustained Ca^{2+} store depletion affects β-cell plasma membrane ionic channels. VGCCs, and Kv and K_{ATP} channels are essential regulatory components in metabolism–excitation coupling and hence insulin secretion of β-cells. In this work, we reported for the first time that sustained Ca^{2+} store depletion caused cell death and a selective down-regulation of VGCCs in a β-cell line (RINm5F cells).

We showed that persistent (24 hour) Ca^{2+} store depletion by CPA resulted in marked cell death (Fig. 1). It is of interest to know the electrophysiological properties of the remaining viable cells. Results in Fig. 2 suggest that cells treated with CPA even for 24 hours (followed by extensive washing and Ca^{2+} store replenishment) still had functionally intact stores to be discharged again by a CPA re-challenge, suggesting that a prolonged Ca^{2+} store emptying did not appear to affect the Ca^{2+}-handling molecules of the stores (SERCA, intracellular Ca^{2+} release-channels such as leak channels of unknown nature, inositol-1,4,5-trisphosphate receptors and ryanodine receptors). Indeed, the latter two receptor-channels, by allowing Ca^{2+} to flow out of the store, account partly for β-cell apoptosis after SERCA inhibition [31]. SOCE is important in Ca^{2+} signaling triggered by hormones and neurotransmitters in β-cells [32]. SOCE has been found to be composed of STIM (Ca^{2+} sensor of the store) and ORAI (plasmalemmal channel) [33]. SOCE was not affected even after a 24-hour Ca^{2+} store depletion. Also found to be resistant to a 24-hour Ca^{2+} store depletion were Kv and K_{ATP} channels (Fig. 6), responsible for repolarization and maintenance of resting potential, respectively. Of note, a 3-hour Ca^{2+} store depletion modestly enhanced Kv channel density. The cause of this phenomenon is not understood, but could be related to an enhanced surfacing of Kv channels destined for initiating cellular apoptosis [34,35]. The observations that SOCE, and Kv and K_{ATP} channels were not significantly affected argue against the theory that VGCC down-regulation reflects a general protein synthesis shut-down.

Results in microfluorimetric imaging experiments showed a reduction in glucose- and KCl-triggered Ca^{2+} signaling after a 24-hour CPA treatment (Figs. 3 and 4). This is supported by the findings in patch-clamp experiments that decreased VGCC activities were observed after 24-hour Ca^{2+} store depletion (Fig. 5). L-, N- and P/Q-type VGCCs have been observed in RINm5F cells [25,26]. Quantitative RT-PCR data revealed that P/Q- and L-type VGCC gene expression was selectively down-regulated (Fig. 5).

Ca^{2+} store depletion triggers SOCE. The latter causes sustained and elevated cytosolic Ca^{2+} level which might exert deleterious effects such as activation of nucleases and proteases. To distinguish the effect of Ca^{2+} store depletion

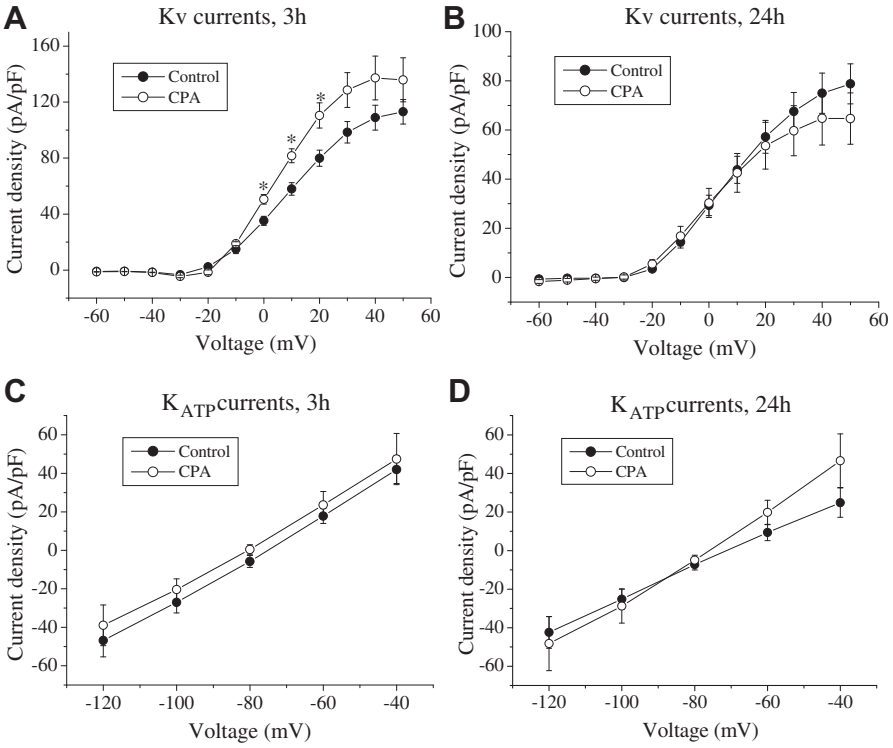

Fig. 6 – Sustained Ca^{2+} store depletion did not affect Kv and K_{ATP} channel currents. After RINm5F cells were treated with 0.1% DMSO (control) or 30 μM CPA for (A,C) 3 hours or (B,D) 24 hours, they were washed extensively in and bathed in Ca^{2+}-containing bath solution, and then examined for Kv or K_{ATP} currents. When Kv currents were measured, the cells were held at −70 mV and then stimulated with increasing depolarizing pulses up to +50 mV at 10 mV increments. When K_{ATP} currents were measured, the cells were held at −70 mV and then stimulated with −120 mV hyperpolarizing pulses at 10-s intervals. Diazoxide (300 μM) was added to open K_{ATP} channels and when the K_{ATP} current magnitude reached equilibrium, the cells were stimulated by serial depolarizing pulses up to −40 mV at 20-mV increments to obtain the I–V relation. Results are displayed as the mean ± SEM of 8–15 cells. For Kv channel current measurements, in the control group at 3 hours, cell capacitance was 9.8 ± 0.6 pF and pipette series resistance was 19 ± 3 MΩ ($n = 8$). In the control group at 24 hours, cell capacitance was 11.2 ± 0.6 pF and pipette series resistance was 16.6 ± 1.7 MΩ ($n = 8$). In the CPA group at 3 h, cell capacitance was 8.5 ± 0.6 pF and pipette series resistance was 15.5 ± 2.1 MΩ ($n = 9$). In the CPA group at 24 hours, cell capacitance was 6.6 ± 0.6 pF and pipette series resistance was 18 ± 2.3 MΩ ($n = 8$). For K_{ATP} current measurements, in the control group at 3 hours, cell capacitance was 10 ± 0.6 pF and pipette series resistance was 17 ± 2.2 MΩ ($n = 15$). In the control group at 24 hours, cell capacitance was 10.5 ± 0.7 pF and pipette series resistance was 19 ± 1.5 MΩ ($n = 14$). In the CPA group at 3 hours, cell capacitance was 7.9 ± 0.5 pF and pipette series resistance was 17.1 ± 2.9 MΩ ($n = 8$). In the CPA group at 24 hours, cell capacitance was 9.1 ± 0.7 pF and pipette series resistance was 15.7 ± 4 MΩ ($n = 9$). * Indicates significant ($p < 0.05$) difference from the control group. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide; K_{ATP} = ATP-sensitive K^+; Kv = voltage-gated K^+.

per se and its consequent SOCE, the SOCE inhibitor SK&F 96365 was used. Since this drug did not prevent the effect of CPA (Figs. 4 and 5), we attribute the down-regulation of VGCCs to Ca^{2+} store depletion *per se*, but not the resultant SOCE.

ER stress has been known to activate the ERK and JNK pathways in a variety of cell types, including β-cells [27–30,36]. Since sustained Ca^{2+} store depletion inevitably leads to ER stress, we asked if these two kinases were involved in the down-regulation of VGCCs by CPA-induced Ca^{2+} store depletion in RINm5F cells. However, using two drugs to inhibit ERK and JNK, we did not observe any prevention or relief of CPA effects on VGCC activities (Fig. 7). The pathway(s) by which sustained Ca^{2+} store depletion affected VGCC expression is yet to be determined.

Our data on the effects of Ca^{2+} store depletion on rat insulinoma RINm5F cells may have relevance to diabetes mellitus. ER stress has been implicated in the malfunctions and apoptosis of β-cells in both type 1 and 2 diabetes mellitus [37]. This pathology arises either due to Ca^{2+} store depletion induced by nitric oxide (type 1) or excessive production of insulin (type 2) [37,38]. Of note, L-type VGCCs are down-regulated in β-cells of Zucker diabetic fatty rats, which are animal models for type 2 diabetes mellitus [39]. The β-cells of these rats also exhibit reduced Ca^{2+} signaling in response to glucose and KCl. It is also noteworthy that anomalies could also arise not due to defective VGCCs *per se*, but due to deranged coupling of VGCC activities with exocytotic machineries. For example, a recent report showed that there is

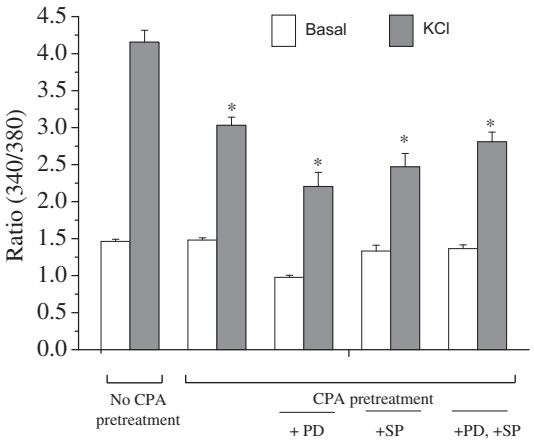

Fig. 7 – Reduction in KCl-triggered Ca^{2+} signal by sustained Ca^{2+} store depletion was not reversed by inhibition of JNK and ERK. After RINm5F cells were treated with 0.1% DMSO (control), 30 μM CPA or 30 μM CPA plus other drugs (PD, 30 μM PD98059; SP, 30 μM SP600125) for 24 hours, they were washed extensively in and bathed in Ca^{2+}-containing bath solution, and then assayed for $[Ca^{2+}]_i$. The cells were stimulated with 30 mM KCl. Results are displayed as the mean ± SEM of 14–92 cells. * Indicates significant ($p < 0.05$) difference from the KCl-stimulated control group. CPA = cyclopiazonic acid; DMSO = dimethyl sulfoxide; ERK = extracellular signal-regulated kinase; JNK = c-Jun N-terminal kinase.

a functional uncoupling between VGCCs and secretory vesicles in β-cells of mice suffering from high-fat-diet-induced diabetes [40].

In conclusion, CPA-induced Ca^{2+} store depletion in RINm5F cells caused cell death and selectively down-regulated VGCCs via pathway(s) other than ERK and JNK. VGCC down-regulation may be one of the consequences of β-cell toxicity after exposure to toxicants which cause sustained Ca^{2+} store depletion.

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