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Calcium elevation elicited by reverse mode Na^+/Ca^{2+} exchange activity is facilitated by intracellular calcium stores in bovine chromaffin cells ☆

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Abstract

The Na^+/Ca^{2+} exchanger (NCX) in plasma membranes either moves Ca^{2+} out of (forward mode) or into (reverse mode) cells depending on the electrochemical gradient of these ions across the membrane. In this report, we characterize the sources responsible for the elevation in $[Ca^{2+}]_i$ elicited by reverse mode NCX activity. The elevation in $[Ca^{2+}]_i$ elicited by reverse mode NCX activity was significantly diminished by thapsigargin. KB-R7943 could only partially suppress the $[Ca^{2+}]_i$ change. Measurement of the $[Ca^{2+}]_i$ concurrent with reverse mode NCX current by perforated whole-cell patch showed that elevation in $[Ca^{2+}]_i$, but not the current, was inhibited by thapsigargin. The change in $[Ca^{2+}]_i$ response elicited by nicotinic acetylcholine receptor agonist was inhibited by thapsigargin. These suggest the importance of intracellular Ca^{2+} stores in facilitating the $[Ca^{2+}]_i$ elevation elicited by reverse mode NCX activity under physiological condition.

Keywords: Na^+/Ca^{2+} exchanger; Inositol (1,4,5)-trisphosphate receptor; Calcium-induced calcium release; Acetylcholine receptor; Electrophysiology

Intracellular Ca^{2+} stores can be considered either as a Ca^{2+} sink that helps maintain $[Ca^{2+}]_i$ homeostasis or as a Ca^{2+}-releasing source that is activated by a number of input signals. In recent years, increasing attention has been focused on the role these stores play in facilitating elevation of $[Ca^{2+}]_i$ caused by extracellular stimuli [1,2]. Using endoplasmic reticulum (ER)-targeted aequorin, it was shown that during depolarization, bovine chromaffin cells release Ca^{2+} from their intracellular ER stores [3]. Without these intracellular Ca^{2+} stores, the exocytosis level is unsustainable under repetitive membrane depolarizations, which allow Ca^{2+} influx through the voltage-gated Ca^{2+} channels [4].

In cardiac myocytes, the opening of a cluster of RyRs creates Ca^{2+} sparks in a specific subcellular localization; the summation of these sparks forms the entire intracellular Ca^{2+} transient [5,6]. During excitation–contraction coupling, a small amount of Ca^{2+} influx via the L-type Ca^{2+}-channels can initiate CICR by opening RyRs [7]. However, Ca^{2+} influx through the Na^+/Ca^{2+}-exchanger (NCX) on the plasma membrane also triggers CICR and induces Ca^{2+} sparks in cardiac myocytes [8,9].

The plasma membrane NCX utilizes the electrochemical gradient of the substrate ions as the driving force to translocate Ca^{2+} across the membrane; therefore, it can work bi-directionally. Physiologically, the primary function of the NCX is to move Ca^{2+} out of the cell, i.e., the so-called forward mode (fNCX). However, under certain conditions, the exchanger can reverse direction to move Ca^{2+} into the cell; this is called the reverse mode (rNCX). It is usually accepted that the fNCX is favored at the resting membrane

☆ *Abbreviations:* AchR, acetylcholine receptor; 2-APB, 2-aminoethoxydiphenyl borate; CICR, calcium-induced calcium release; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; IP_3, inositol (1,4,5)-trisphosphate; IP_3R, IP_3 receptor; nAchR, nicotinic AchR; NCX, Na^+/Ca^{2+} exchanger; NMG, *N*-methyl-D-glucamine; rNCX, reverse mode NCX; PLC, phospholipase C; RyR, ryanodine receptor; Tg, thapsigargin.

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potential and that the rNCX may operate when the cell is depolarized [10,11]. In bovine chromaffin cells, activation of the rNCX elevates the $[Ca^{2+}]_i$ and induces catecholamine release [12,13]. However, the ability of the rNCX-elicited elevation in $[Ca^{2+}]_i$ to release Ca^{2+} from intracellular Ca^{2+} stores is not clear.

To investigate the interaction between the rNCX and intracellular Ca^{2+} stores in bovine chromaffin cells, cell is pretreated with ouabain to increase the intracellular Na^+ concentration ($[Na^+]_i$) and the $[Ca^{2+}]_i$ is elevated by replacing the extracellular Na^+ with *N*-methyl-D-glucamine (NMG), which activates the rNCX activity. Our results showed that the rNCX-elicited elevation in $[Ca^{2+}]_i$ was slow at first but became more robust as time went on. The late elevation in $[Ca^{2+}]_i$ was greatly inhibited by pretreatment with Tg. KB-R7943, a blocker for K^+-independent rNCX, partially inhibited the elevation in $[Ca^{2+}]_i$. Both 2-aminoethoxydiphenyl borate (2-APB), or caffeine–ryanodine pretreatment inhibited most of the rNCX-elicited elevation in $[Ca^{2+}]_i$. 2-APB could further suppress the rNCX-elicited $[Ca^{2+}]_i$ elevation in caffeine–ryanodine pretreated cell to a level similar to that of Tg treatment. To simultaneously record the $[Ca^{2+}]_i$ and whole-cell current when the extracellular Na^+ was replaced with NMG, cell was loaded with 65 mM of Na^+ by perforated whole-cell patch technique. Elevation of $[Ca^{2+}]_i$, but not the outward current, elicited by rNCX, was inhibited in cells pretreated with Tg. These results support the notion that the intracellular Ca^{2+} stores are involved in the elevation of $[Ca^{2+}]_i$, induced by rNCX in bovine chromaffin cells.

Materials and methods

Chemicals. Ryanodine and caffeine were purchased from Sigma (St. Louis, MO, USA). Tg, U73122, and 2-APB were from CalBiochem (EMD Biosciences, Inc., San Diego, CA, USA). Fura 2 acetomethoxylmethyl ester (Fura 2 AM) was from TefLabs (Austin, TX, USA). KB-R7943 was from Tocris Cookson Ltd. (Avon, UK). All other chemicals were purchased from Sigma unless otherwise indicated.

Isolation and culture of bovine chromaffin cells. Bovine adrenal glands were supplied by a local slaughterhouse. The adrenal glands were immediately removed after the animals were killed and kept on ice. Chromaffin cells were isolated as described before [12]. Cells were plated at a density of 2×10^5 cells/ml. All experiments were carried out between 3 and 7 days after the cells were isolated. Each experiment was repeated using cells from different dishes of different batches.

$[Ca^{2+}]_i$ imaging. For measurement of $[Ca^{2+}]_i$, cells were incubated in bath buffer (150 mM NaCl, 5 mM glucose, 10 mM Hepes, 1 mM $MgCl_2$, 5 mM KCl, and 2.2 mM $CaCl_2$, pH 7.3) containing 5 μM fura 2 AM for 1 h at 37 °C. Cells were then washed three times with bath buffer and used for measurements. For fura 2 excitation, a Lamda DG4 system (Sutter Instrument Co., Novato, CA, USA) was used, which was controlled by Metafluor software (Molecular Devices Corp., Downingtown, PA, USA). Ratiometric calcium estimates were made using 10-nm-wide filters centered on 340 and 380 nm (Chroma Technology Corp., Rockingham, VT, USA), capturing the emitted light (485–540 nm) at each excitation wavelength for 300 ms through a 20× objective (Zeiss Axiovert 200 microscope, Carl Zeiss AG, Germany) and directing it to a cooled CCD camera (CoolsnapFx; Roper Scientific, Tucson, AZ, USA). The ratio within each cell was computed from images obtained at excitation wavelengths of 340 and 380 nm and by subtracting the appropriate background fluorescence at each wavelength. Ratios were computed every second. The calcium calibration buffer kit (Molecular Probes, Carlsbad, California) was used to transform the ratio into Ca^{2+} concentration according to manufacturer's manual.

Reverse mode NCX activity. Cells were incubated in bath buffer containing 0.1 mM ouabain to increase the $[Na^+]_i$. To elicit rNCX activity, a glass micropipette with an opening of about 3 μm loaded with NMG buffer (135 mM NMG, 5 mM glucose, 10 mM Hepes, 1 mM $MgCl_2$, 5 mM KCl, and 2.2 mM $CaCl_2$, pH 7.3) was positioned 20 μm from the recorded cell. The buffer was puffed onto the cell for 60 or 120 s as indicated in each figure at 5 psi under the control of a Picospritzer III (Parker Instrument, Parker Hannifin, Fairfield, NJ, USA).

Inhibition of intracellular Ca^{2+} stores. To characterize the roles of the intracellular Ca^{2+} stores, 1 μM of Tg was added to the bath buffer for 1 h to inhibit the Ca^{2+} pumps on the ER. 2-APB (10 μM) and U-73122 (100 μM) were added 15 min before the experiment to inhibit IP_3R and phospholipase C (PLC), respectively. To deplete the RyR-gated Ca^{2+} stores, cells were pretreated with caffeine (40 mM) in bath buffer with or without ryanodine (20 μM) for 1 min and then washed with bath buffer with or without ryanodine, respectively, for another minute three times. To understand the role of Tg-sensitive Ca^{2+} stores in modulating $[Ca^{2+}]_i$ elevation, Ca^{2+}-free bath buffer (the same constituents as those in bath buffer except without $CaCl_2$) with 10 μM of DMPP or Ca^{2+}-free high K^+ buffer (the NaCl in bath buffer was substituted with an equimolar amount of KCl and containing no $CaCl_2$) was used to stimulate the fura 2-loaded cells incubated in normal bath buffer for 10 s.

Electrophysiological recording. Fura 2-loaded chromaffin cell was used for perforated whole-cell patch with a pipette solution containing (in mM): 65 cesium aspartate, 65 sodium aspartate, 10 Hepes, 5 NaCl, and 0.5 TEA-Cl (pH 7.3). Amphotericin B (0.5 mg/mL) was added and sonicated in the pipette solution right before use. The whole-cell current was recorded by a HEKA EPC10 amplifier controlled by Pulse software (HEKA Instruments, Inc., Southborough, MT, USA). The experiment began when the series resistance was lowered below 20 MΩ. To elicit the rNCX activity, the cell was puffed with the NMG solution for 120 s; the whole-cell current and fura 2 ratio were simultaneously recorded. The current was sampled at 10 kHz and low-pass filtered at 500 Hz; the fura 2 ratio was recorded at an interval of 1 s.

Results

Elevation in $[Ca^{2+}]_i$ induced by rNCX depends on intracellular Ca^{2+} stores

To understand the relationship between Na^+ influx and the intracellular Ca^{2+} stores, the reverse mode Na^+/Ca^{2+} exchange activity was investigated. The $[Na^+]_i$ was elevated by ouabain treatment and the extracellular Na^+ was replaced by NMG to activate rNCX activity. Under this condition, the rNCX is able to transport Ca^{2+} into cytosol and elevate the $[Ca^{2+}]_i$ [12]. Fig. 1A shows representative $[Ca^{2+}]_i$ traces elicited by the rNCX. If the cell was puffed with bath buffer, there was no significant change in $[Ca^{2+}]_i$; however, when the extracellular Na^+ was replaced with NMG, the $[Ca^{2+}]_i$ was slowly elevated at the beginning but greatly enhanced later. Some cells showed quick elevation in $[Ca^{2+}]_i$ near the end of the NMG application and some were enhanced a few seconds right after the application (as shown in Figs. 2–5). Overall, the $[Ca^{2+}]_i$ usually continued to rise during the period of NMG application and slowly declined to resting level after the Na^+ replacement. The addition of KB-R7943, an inhibitor of K^+-independent rNCX activity, in the buffers only

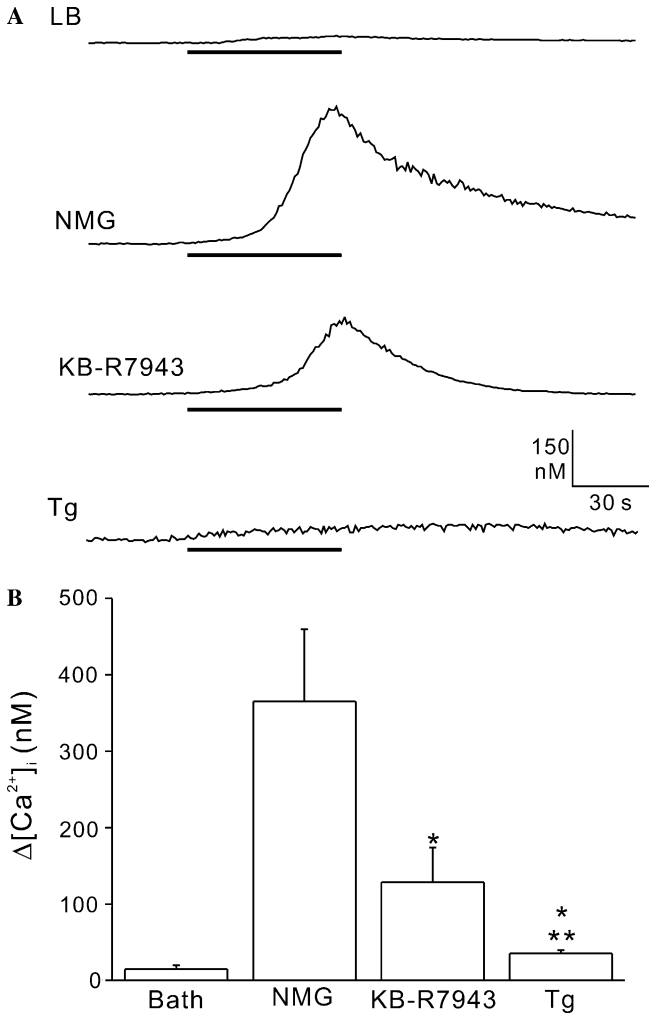

Fig. 1. Reverse mode Na^+/Ca^{2+} exchange activity elevates the $[Ca^{2+}]_i$. Cells were incubated in bath buffer containing 100 μM of ouabain and 5 μM of fura 2 AM for 1 h. Ouabain was contained in bath and all of the buffers used for stimulation. A single chromaffin cell was puffed with NMG buffer for 60 s from a micropipette. The elevation in $[Ca^{2+}]_i$ was recorded and calibrated as the fluorescence intensity ratio of fura 2 excited alternatively by 340 and 380 nm. (A) Representative recordings from cells puffed with bath buffer (Bath), NMG buffer (NMG), or NMG buffer containing KB-R7943 (KB-R7943), and Tg-pretreated cells puffed with NMG buffer (Tg) for 60 s. The black bar under each trace indicates the period of solution change. (B) Averaged maximal changes in $[Ca^{2+}]_i$ during the solution change of each treatment. Data are means ± SEM; numbers of samples for bath, NMG, KB-R7943, and Tg were 9, 21, 13, and 14, respectively. $^*p < 0.05$ by Student's t test when compared to the bath group; $^{**}p < 0.05$ when compared to the NMG group.

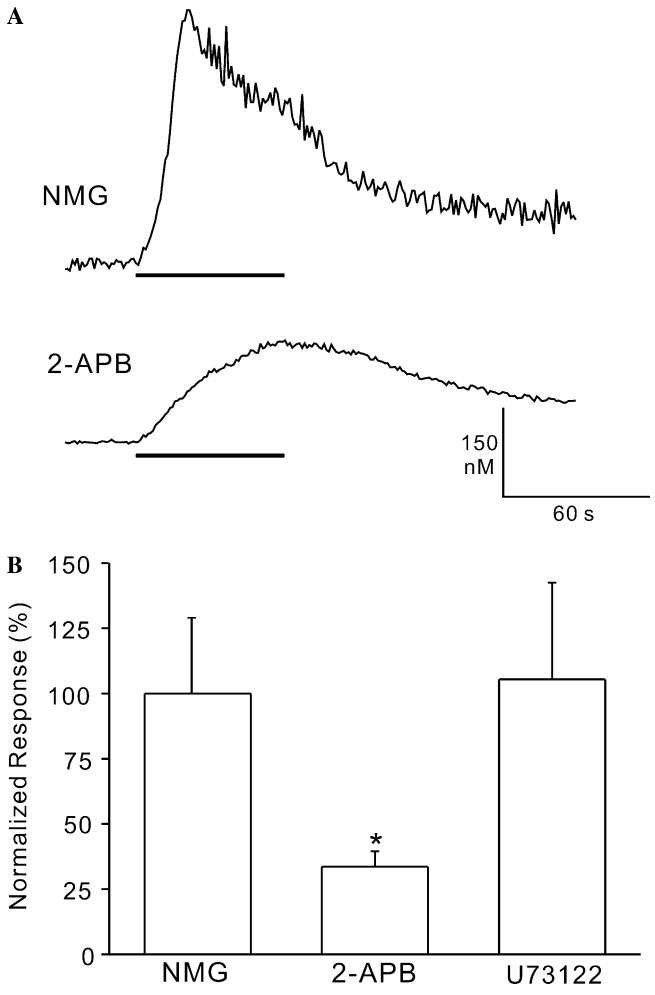

Fig. 2. 2-APB inhibits the rNCX-elicited elevation in $[Ca^{2+}]_i$. Chromaffin cells were pretreated with ouabain for 1 h, and then the extracellular Na^+ was replaced by NMG for 60 s as indicated by the black bar. 2-APB (10 μM) or U73122 (100 μM) was added to the bath 15 min before the experiment. (A) Representative $[Ca^{2+}]_i$ recordings from cells puffed with NMG buffer without inhibitors (NMG) or with 2-APB (2-APB). (B) Normalized $[Ca^{2+}]_i$ changes during the solution change for cells treated without blocker (NMG, $n = 12$), with 2-APB ($n = 16$), or with U73122 ($n = 7$). The changes in the elevation of $[Ca^{2+}]_i$ were normalized to the averaged response of cells treated with blocker. $^*p < 0.05$ by Student's t test when compared to the NMG group.

partially blocked the elevation of $[Ca^{2+}]_i$. However, when the intracellular Ca^{2+} stores were depleted by Tg, there was almost no elevation in $[Ca^{2+}]_i$ when stimulated with the NMG buffer. The averaged results (Fig. 1B) showed that NMG application elevated the $[Ca^{2+}]_i$ with a peak response of 365.2 ± 94.5 nM, significantly higher than that of cells puffed with bath buffer (14.8 ± 4.7 nM). KB-R7943 and Tg treatments significantly inhibited the changes in $[Ca^{2+}]_i$ to 128.5 ± 45.3 and 35.5 ± 4.1 nM, respectively. Tg and KB-R7943 did not have any effect on the resting $[Ca^{2+}]_i$, which were 50.3 ± 9.0 and 49.6 ± 3.1 nM, respectively, comparing to the cells in normal bath buffer (51.2 ± 7.4 nM). These results indicate the importance of intracellular Ca^{2+} stores in enhancing the rNCX-elicited elevation in $[Ca^{2+}]_i$ in bovine chromaffin cells. The partial inhibition effect of KB-R7943 suggests the possible involvement of both K^+-dependent and -independent rNCX activities in bovine chromaffin cells.

2-APB blocks elevation of $[Ca^{2+}]_i$

It has been reported that IP_3R and RyR are the two main channels responsible for Ca^{2+} release from intracellular Ca^{2+} stores [14]. To understand the functions of these

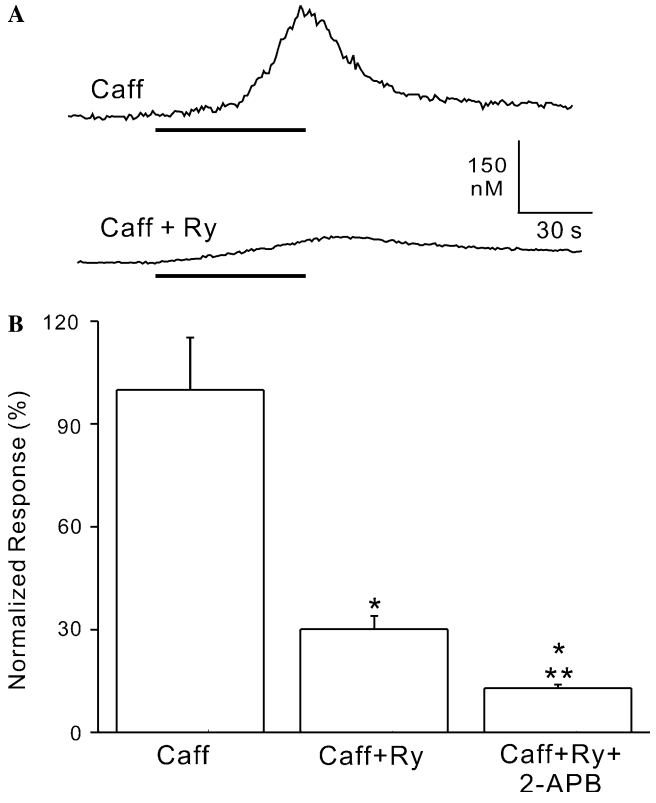

Fig. 3. Caffeine-sensitive stores are involved in rNCX-elicited elevation in $[Ca^{2+}]_i$. Before fura 2 loading, cells were pretreated with caffeine (40 mM) with or without ryanodine (20 μM) for 1 min, and then the caffeine was washed off; this was repeated three times. Cells were then treated with ouabain for 1 h and extracellular Na^+ was replaced by NMG buffer for 60 s to elicit rNCX activity. Some caffeine–ryanodine pretreated cells were treated with 2-APB (10 μM) 15 min before stimulation. (A) Representative $[Ca^{2+}]_i$ recordings from cells pretreated with caffeine only (Caff); caffeine and ryanodine (Caff + Ry). The period of solution change is indicated by the black bar under each trace. (B) Normalized maximal $[Ca^{2+}]_i$ changes during solution change for cells of different treatments. The changes in the elevation of $[Ca^{2+}]_i$ were normalized to the averaged response of cells pretreated with caffeine only. Some cells pretreated with caffeine and ryanodine were further treated with 2-APB (Caff + Ry + 2-APB). Data are means ± SEM; numbers of samples for Caff, Caff + Ry, and Caff + Ry + 2-APB are 38, 27, and 28, respectively. $^*p < 0.05$ by Student's t test when compared to the Caff group; $^{**}p < 0.05$ by Student's t test when compared to the Caff + Ry group.

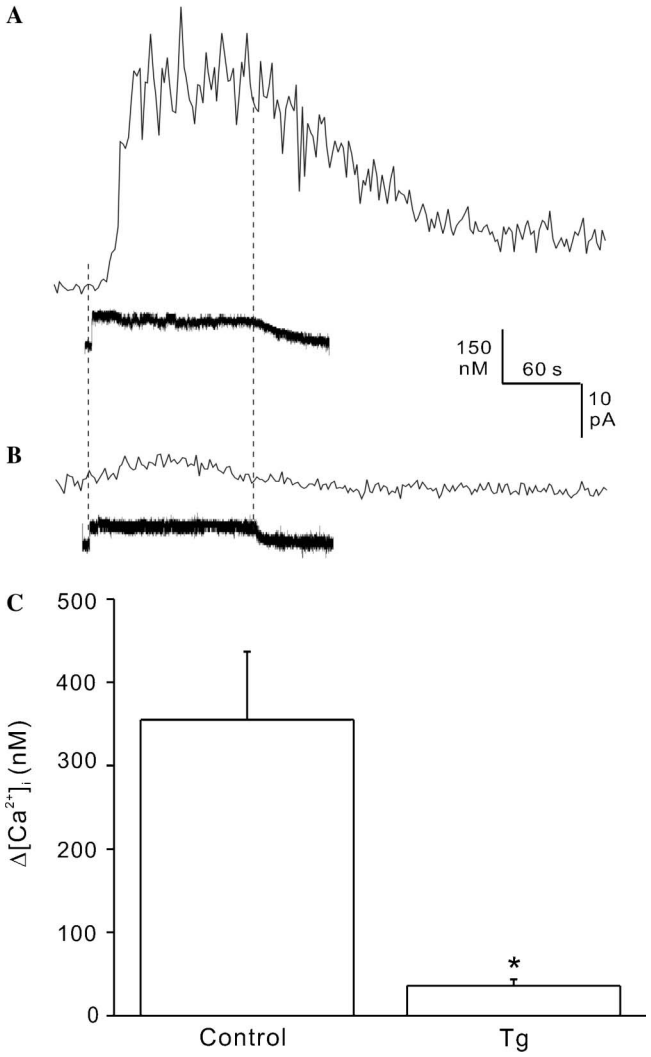

Fig. 4. The rNCX current can elicit $[Ca^{2+}]_i$ elevation. Cells were perforated whole-cell patched and voltage-clamped at −70 mV. The whole-cell current and $[Ca^{2+}]_i$ were simultaneously recorded. To elicit rNCX activity, cells were loaded with 65 mM Na^+ and 65 mM Cs^+ through the patch-pipette and then stimulated with NMG buffer for 120 s as indicated by the two vertical dashed lines. (A) Representative $[Ca^{2+}]_i$ (upper panel) and whole-cell current (lower panel) traces from a control cell. (B) Representative $[Ca^{2+}]_i$ (upper panel) and whole-cell current (lower panel) traces from a cell treated with Tg. (C) Averaged maximal changes in $[Ca^{2+}]_i$. Data are means ± SEM; numbers of samples for the Control and Tg groups were 15 and 10, respectively. $^*p < 0.05$ by Student's t test.

stores, we first characterized the roles of IP_3R-gated Ca^{2+} stores in rNCX-elicited $[Ca^{2+}]_i$ elevation. In bovine chromaffin cells, 10 μM of 2-APB and 100 μM of U73122 was able to efficiently block the elevation of $[Ca^{2+}]_i$ stimulated by histamine, which opened the IP_3R through the activation of PLC, in our laboratory (data not shown). Fig. 2 shows that 2-APB, which blocks the opening of IP_3R, slowed down the rNCX-elicited $[Ca^{2+}]_i$ elevation and partially inhibited the maximal $[Ca^{2+}]_i$ change. On the contrary, U73122, a PLC inhibitor, had no effects in blocking the rNCX-elicited $[Ca^{2+}]_i$ elevation. The normalized results showed that 2-APB blocked the maximal change in $[Ca^{2+}]_i$ to 29.9 ± 2.5% of that in control cells (261.3 ± 48.2 nM). U73122 had no effect on the rNCX-elicited elevation in $[Ca^{2+}]_i$ (112.7 ± 28.9%). Both 2-APB and U73122 did not cause any significant difference in the basal $[Ca^{2+}]_i$ comparing to that of control cells. These results suggest that IP_3R-gated Ca^{2+} store is very important in facilitating the rNCX-elicited elevation in $[Ca^{2+}]_i$, and that other Tg-sensitive stores may also be involved in facilitating this process.

Depletion of ryanodine-sensitive Ca^{2+} stores inhibits the elevation of $[Ca^{2+}]_i$

To further characterize the roles the intracellular Ca^{2+} stores play in the rNCX-elicited elevation of $[Ca^{2+}]_i$,

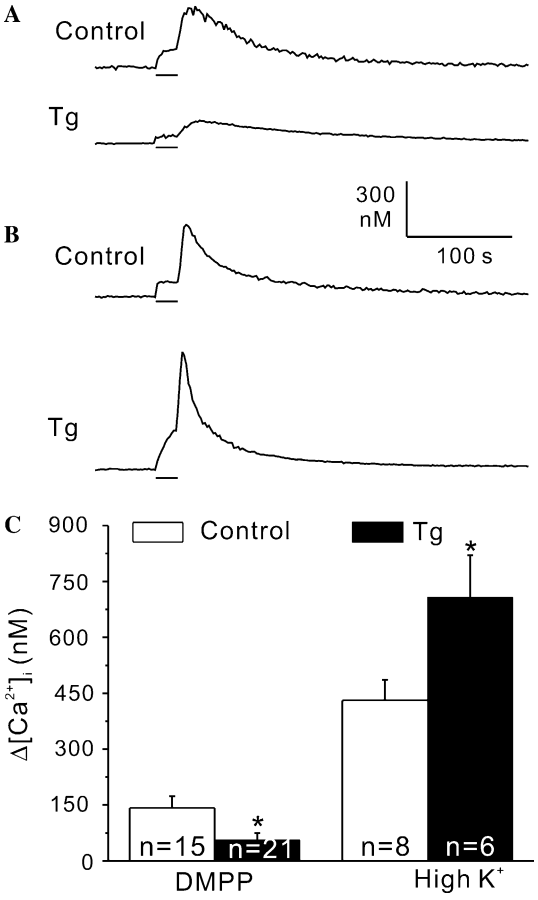

Fig. 5. Tg blocks DMPP-elicited elevation in $[Ca^{2+}]_i$. Fura 2-loaded bovine chromaffin cells were incubated in bath buffer and stimulated with Ca^{2+}-free solution puffed from a micropipette for 10 s. (A) Representative $[Ca^{2+}]_i$ responses stimulated by Ca^{2+}-free bath buffer containing DMPP (10 μM) with (lower panel, Tg) or without (upper panel, Control) Tg pretreatment. (B) $[Ca^{2+}]_i$ responses stimulated with Ca^{2+}-free high-K^+ buffer with (lower trace) or without (upper trace) Tg pretreatment. The black bar under each trace indicates the period of solution change. (C) Averaged maximal $[Ca^{2+}]_i$ changes stimulated by DMPP or high K^+ during the recording period treated with or without Tg. The sample number of each group was the digit in each column. Data are means ± SEM. $^*p < 0.05$ by Student's t test when compared to control cells.

Ca^{2+} released through the RyR was blocked by pretreating the cells with ryanodine and caffeine [3]. For cells pretreated with caffeine only, the rNCX-elicited elevation in $[Ca^{2+}]_i$ was smaller than that of untreated cells shown in the previous figures (Fig. 3). For cells pretreated with caffeine and ryanodine, the rNCX-elicited $[Ca^{2+}]_i$ elevation was slowed down and the maximal changes in $[Ca^{2+}]_i$ response were diminished. The rNCX-elicited $[Ca^{2+}]_i$ response in caffeine–ryanodine pretreated cells was further inhibited by 2-APB. The averaged results showed that rNCX-elicited elevation in $[Ca^{2+}]_i$ in cells pretreated with caffeine–ryanodine was significantly inhibited to $30.1 \pm 3.9\%$ of that of caffeine-treated cells. The changes in $[Ca^{2+}]_i$ from cells pretreated with caffeine–ryanodine were further suppressed to $13.0 \pm 0.9\%$ by 2-APB. These results suggest that the intracellular Ca^{2+} stores gated by IP_3R and RyR are both involved in facilitating the rNCX-elicited elevation in $[Ca^{2+}]_i$.

Thapsigargin blocks the rNCX-elicited elevation in $[Ca^{2+}]_i$ but not the outward current

Activation of the electrogenic rNCX causes an outward current. To understand whether the decrease in rNCX-elicited elevation in $[Ca^{2+}]_i$ by Tg was due to the depletion of intracellular Ca^{2+} stores or the decrease in the outward current, cells were loaded with Na^+ by perforated whole-cell patch. The elevation in $[Ca^{2+}]_i$ and whole-cell outward current elicited by the NMG buffer were simultaneously recorded. Fig. 4A shows representative traces of the fura-2 ratio and whole-cell current from single perforated-patched cells. The results showed that an outward current was elicited during the NMG buffer application when the cell was voltage clamped at −70 mV; the $[Ca^{2+}]_i$ was quickly elevated after the replacement of extracellular Na^+ with NMG. For cells pretreated with Tg (Fig. 4B), an outward current was still elicited by NMG buffer application, but the $[Ca^{2+}]_i$ response was greatly inhibited. The averaged results showed that there was no difference in the cell size (7.97 ± 0.44 vs 7.39 ± 0.34 pF) and outward current (4.69 ± 0.90 vs 4.44 ± 0.86 pA) of the cells tested without or with Tg treatment. The maximal change in $[Ca^{2+}]_i$ was 355.1 ± 81.8 nM in cells without Tg pretreatment; however, this change was significantly lowered to 35.9 ± 7.5 nM in cells treated with Tg. These results suggest that changing the Na^+ gradient without depolarizing the cell is enough to evoke rNCX activity in bovine chromaffin cells; the influx of Ca^{2+} through the exchanger may induce the release of Ca^{2+} from intracellular Ca^{2+} stores to enhance $[Ca^{2+}]_i$ elevation.

Thapsigargin treatment inhibits DMPP-elicited $[Ca^{2+}]_i$ elevation

It is known that Na^+ influx through the nAChRs depolarizes the cell and opens voltage-operated Ca^{2+} channels to elevate the $[Ca^{2+}]_i$ in bovine chromaffin cells. To characterize whether this Na^+ influx can elicit $[Ca^{2+}]_i$ elevation or not, cell was stimulated with DMPP, an nAchR agonist, in Ca^{2+}-free bath buffer to minimize the Ca^{2+} influx through the voltage-gated Ca^{2+} channels. Since DMPP can depolarize the cell, we used Ca^{2+}-free high-K^+ buffer to stimulate the cell in order to monitor the depolarization-elicited elevation in $[Ca^{2+}]_i$ without Na^+ influx. Fig. 5 shows that in the absence of external Ca^{2+}, DMPP elicited a small elevation in $[Ca^{2+}]_i$. After DMPP had diffused out and fresh Ca^{2+}-containing bath buffer was introduced, the increase in $[Ca^{2+}]_i$ continued. This late-phase rise in $[Ca^{2+}]_i$ was abolished when cells were pretreated with Tg. On the contrary, if cells were stimulated by Ca^{2+}-free high-K^+ buffer with the same protocol, Tg pretreatment enhanced the elevation in $[Ca^{2+}]_i$. Tg treatment has no significant effect on the resting $[Ca^{2+}]_i$ which were 51.7 ± 3.9 and

41.6 ± 5.9 nM in control and Tg-treated cells, respectively. The averaged results showed that the maximal changes in $[Ca^{2+}]_i$ elicited by DMPP were 142.1 ± 32.8 nM for control cells and decreased to 55.1 ± 3.9 nM by Tg pretreatment. Tg treatment did not affect the DMPP-elicited inward currents, which were 206.2 ± 57.7 and 204.4 ± 56.8 pA for control and Tg-treated cells, respectively. On the contrary, the high K^+-evoked $[Ca^{2+}]_i$ change was 431.5 ± 54.3 nM for control cells but significantly increased to 707.3 ± 113.4 nM by Tg pretreatment. These results suggest that in response to DMPP, the release of Ca^{2+} from intracellular Ca^{2+} stores is required to support the elevation in $[Ca^{2+}]_i$; whereas intracellular stores are required to buffer the high K^+-evoked elevation in $[Ca^{2+}]_i$. These hint the possible linkage between the Na^+ influx and elevation in $[Ca^{2+}]_i$; and this elevation is facilitated by the release of Ca^{2+} from intracellular Ca^{2+} stores.

Discussion

This is the first report suggesting that most of the $[Ca^{2+}]_i$ elevation elicited by rNCX is facilitated by Ca^{2+} released from the intracellular Ca^{2+} stores in bovine chromaffin cells. Our data suggest that both the IP_3R and RyR may work synergistically to enhance the rNCX-elicited elevation in $[Ca^{2+}]_i$. The elevation in $[Ca^{2+}]_i$ by activating Na^+ influx through the nAchR is also facilitated by the intracellular Ca^{2+} stores. It has been reported that activation of the rNCX in cardiac myocytes can trigger CICR [15]. Therefore, the connection between the rNCX and intracellular Ca^{2+} stores may be activated physiologically by the opening of the nAchR, which increases the $[Na^+]_i$ and depolarizes the membrane potential in bovine chromaffin cells [16].

If the intracellular Ca^{2+} stores work just as a sink for buffering the Ca^{2+} influx brought about by the rNCX in bovine chromaffin cells, the elevation in $[Ca^{2+}]_i$ would be higher if the Ca^{2+}-pumps on these stores were blocked by Tg. However, our results show that the elevation in $[Ca^{2+}]_i$ was unexpectedly diminished to a level slightly but significantly higher than that of control cells. Other treatments which block the release of Ca^{2+} from IP_3R- and RyR-gated Ca^{2+} stores all show similar effects in inhibiting the rNCX-elicited elevation in $[Ca^{2+}]_i$. These results support the proposal that the elevation in $[Ca^{2+}]_i$ elicited by rNCX is mostly responsible by the Ca^{2+} release from intracellular stores in bovine chromaffin cells.

The activity of NCX can be either K^+-dependent or -independent [11]. The stoichiometry for K^+-independent NCX is 3 Na^+ in exchange for 1 Ca^{2+}; for K^+-dependent NCX, the stoichiometry is 4 Na^+ in exchange for 1 Ca^{2+} and 1 K^+. The K^+-dependent NCX is usually named NCKX for discrepancy. The activities of both types of exchangers can be found within a single bovine chromaffin cell (unpublished results). In this report, both exchangers should have simultaneously been activated since K^+ was present in all of the buffers used. Even for the perforated patch, the pipette solution contained 70 mM Cs^+, which can substitute for K^+ in activating the NCKX (unpublished results). KB-R7943 has been shown to specifically block the activity of NCX, but not NCKX [17]. This is the reason why KB-R7943 can only partially inhibit the rNCX-elicited elevation in $[Ca^{2+}]_i$.

Recently, a group of calmodulin-like calcium binding proteins (CaBPs) was shown to be able to bind Ca^{2+} at different affinities [18]. Some of them can interact with the N-terminal of the IP_3R with a high affinity and open the gate of the channel in the absence of IP_3 [19]. This suggests that Ca^{2+} release from IP_3R-gated Ca^{2+} stores might not necessarily require the formation of IP_3, as the elevation in the $[Ca^{2+}]_i$ can trigger the binding of these CaBPs to the IP_3R. However, some other reports have shown opposite roles for CaBPs in modulating IP_3R's activities by imaging whole-cell changes in $[Ca^{2+}]_i$ [20,21]. Discrepancies among these reports might have originated from the methodologies used. The expression of CaBP1 can be detected in bovine chromaffin cells (data not shown) which hints at a possible mechanism responsible for the opening of the IP_3R. CaBP1 is one of the possible candidates for mediating this pathway, but this needs to be further characterized.

Though 2-APB was used to block the IP_3R in this report, some suggest that 2-APB can also block the TRP channels [22]. The basal $[Ca^{2+}]_i$ in Tg-treated cells is not different from that of control cells suggesting that the depletion of intracellular Ca^{2+} stores does not evoke significant Ca^{2+} influx through the capacitative calcium entry pathway. The concentration of 2-APB used in this report is lower than the IC_{50} for the most TRP channels but well enough for inhibiting histamine-elicited $[Ca^{2+}]_i$ elevation. Therefore, it is unlikely the effect of 2-APB in inhibiting the rNCX-elicited $[Ca^{2+}]_i$ elevation is due to its effect on store-operated Ca^{2+} influx.

Other than IP_3R, RyR is another channel for releasing Ca^{2+} into the cytosol from the intracellular stores. Caffeine has been shown to elicit CICR through the RyR in chromaffin cells [23]. It is not certain how much the overlapping in intracellular Ca^{2+} stores is responsible by RyR and IP_3R. When cells were pretreated with caffeine and ryanodine to deplete the RyR-gated intracellular Ca^{2+} stores [24], the rNCX-elicited elevation in $[Ca^{2+}]_i$ also significantly decreased suggesting the involvement of the RyR. Either 2-APB or caffeine–ryanodine pretreatment alone can block the $[Ca^{2+}]_i$ response to a level of about 30% of that of control cells. However, the application of both treatments can suppress the $[Ca^{2+}]_i$ elevation to the level similar to that of Tg treatment. These results suggest that the rNCX-elicited elevation in $[Ca^{2+}]_i$ is synergistically facilitated by both IP_3R and RyR.

Previous report showed that depletion of intracellular Ca^{2+} stores inhibits the DMPP-elicited exocytosis [25]. Our results show that Tg treatment blocked the DMPP- but enhanced the depolarization-elicited elevation in $[Ca^{2+}]_i$. The slight $[Ca^{2+}]_i$ elevation during the period of

stimulation was due to an influx of Ca^{2+} through the Ca^{2+} channels. The addition of EGTA (0.5 mM) to the stimulation buffers abolished this elevation (data not shown). Even so, the elevation in $[Ca^{2+}]_i$ induced by this influx through Ca^{2+} channels was not enhanced by Tg when cell was stimulated by DMPP. The main difference between these two stimulations was the influx of Na^+ during DMPP application. Therefore, it is possible that rNCX may be activated by the opening of nAchR under physiological stimulation, and the intracellular Ca^{2+} stores are involved in facilitating the rNCX-elicited elevation in $[Ca^{2+}]_i$ in bovine chromaffin cells.

Physiologically, when the nAchR is opened, the cell is depolarized. The Na^+ concentration outside the cell, especially in the intercellular region, may decrease to a certain level, while the $[Na^+]_i$ in the subplasmallema region is increased. Because of the electrogenic properties of the rNCX, depolarization favors the reverse mode activity [11]. Therefore, the rNCX can be activated when chromaffin cells are depolarized by the opening of the nAchR even the exposure to Ach is only a short period. The elevation in $[Ca^{2+}]_i$ induced by calcium channel opening is fast and transient; on the contrary, the rNCX-elicited elevation in $[Ca^{2+}]_i$ is slow but sustained because of the involvement of the intracellular Ca^{2+} stores. Therefore, the rNCX may play an important role in elongating the period of the elevation in $[Ca^{2+}]_i$ when the nAchR is activated in bovine chromaffin cells.

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