Functional linkage of Na^+ - Ca^{2+} -exchanger to sarco/endoplasmic reticulum Ca^{2+} pump in coronary artery: comparison of smooth muscle and endothelial cells

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Received: May 6, 2008; Accepted: August 12, 2008

Abstract

An increase in cytosolic Ca^{2+} concentration in coronary artery smooth muscle causes a contraction but in endothelium it causes relaxation. Na^+ - Ca^{2+} -exchanger (NCX) may play a role in Ca^{2+} dynamics in both the cell types. Here, the NCX-mediated $^{45}\text{Ca}^{2+}$ uptake was compared in Na^+ -loaded pig coronary artery smooth muscle and endothelial cells. In both the cell types, this uptake was inhibited by KB-R7943, SEA 0400 and by monensin, but not by cariporide. Prior loading of the cells with the Ca^{2+} chelator BAPTA increased the NCX-mediated $^{45}\text{Ca}^{2+}$ uptake in smooth muscle but not in endothelial cells. In the presence or absence of BAPTA loading, the Na^+ -mediated $^{45}\text{Ca}^{2+}$ uptake was greater in endothelial than in smooth muscle cells. In smooth muscle cells without BAPTA loading, thapsigargin diminished the NCX-mediated $^{45}\text{Ca}^{2+}$ entry. This effect was not observed in endothelial cells or in either cell type after BAPTA loading. The results in the smooth muscle cells are consistent with a limited diffusional space model in which the NCX-mediated $^{45}\text{Ca}^{2+}$ uptake was enhanced by chelation of cytosolic Ca^{2+} or by its sequestration by the sarco/endoplasmic reticulum Ca^{2+} pump (SERCA). They suggest a functional linkage between NCX and SERCA in the smooth muscle but not in the endothelial cells. The concept of a linkage between NCX and SERCA in smooth muscle was also confirmed by similar distribution of NCX and SERCA2 proteins when detergent-treated microsomes were fractionated by flotation on sucrose density gradients. Thus, the coronary artery smooth muscle and endothelial cells differ not only in the relative activities of NCX but also in its functional linkage to SERCA.

Keywords: NCX • SERCA • coupling • lipid rafts • BAPTA • thapsigargin

Introduction

The increase in cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) occurs by activation of extracellular Ca^{2+} entry via Ca^{2+} channels and Na^+ - Ca^{2+} -exchanger (NCX, $3\text{Na}^+ : 1\text{Ca}^{2+}$), and by Ca^{2+} release from intracellular sources [1–4]. The main pathways to lower $[\text{Ca}^{2+}]_i$ are the sarco/endoplasmic reticulum (SER) Ca^{2+} pump (SERCA), plasma membrane Ca^{2+} pump (PMCA) and NCX [2–7]. SERCA pumps have high affinity for Ca^{2+} and can sequester it into the SER [6–8]. PMCA extrude Ca^{2+} from cells with a high affinity [9–11]. NCX has

a lower affinity for Ca^{2+} and is often referred to as a high capacity low affinity Ca^{2+} mobilizing system [4, 8, 12, 13]. KNCX that exchange 4Na^+ for $1\text{Ca}^{2+} + 1\text{K}^+$ have also been reported [14]. NCX (and KNCX) is non-vectorial and depending on Na^+ and Ca^{2+} electrochemical gradients, it may expel Ca^{2+} from cells (forward mode) or allow its entry (reverse mode). NCX are encoded by three genes: NCX1, 2 and 3. NCX1 is expressed in most tissues, NCX2 in many tissues, and NCX3 mainly in skeletal muscle and brain [3, 4, 15]. Various NCX gene products perform similar functions but may differ in their regulation.

Coronary tone is regulated by changes in $[\text{Ca}^{2+}]_i$. In coronary artery, an increased $[\text{Ca}^{2+}]_i$ in smooth muscle cells (SMC) causes a contraction but an increase in $[\text{Ca}^{2+}]_i$ in the endothelial cells (EC) may cause relaxation. Expression and function of PMCA and SERCA in the coronary artery SMC and EC are well documented

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[16–18]. SMC express PMCA isoforms 1 and 4 and the SERCA isoform 2b. Coronary artery EC express much lower levels of PMCA1, and very low levels of SERCA2b and SERCA3 [16, 18]. Based on RT-PCR experiments, the coronary artery SMC and EC express mRNA for NCX1: mainly the splice variant NCX1.3 and smaller amounts of NCX1.7 [16]. This is in contrast to cardiac myocytes, which express mainly NCX1.1 [3, 4, 15]. The expression of mRNA for the NCX regulatory protein phospholemman occurs in coronary artery SMC but not in EC [16, 19–22]. Based on determination of changes in the $[Ca^{2+}]_i$ levels, it has been suggested that NCX plays a role in smooth muscle contractility [12, 13, 23–30]. Results of some of these experiments are ambiguous since the actual Ca^{2+} movement was not examined. Here, we compare properties of NCX-mediated $^{45}Ca^{2+}$ uptake in Na^+ -loaded SMC and EC. We show that in SMC there is a functional and possibly a spatial linkage between NCX and SERCA. The functional linkage was not observed in EC.

Methods

Cell cultures

Pig coronary artery SMC and EC were cultured as previously described [31]. Confluent SMC cultures from passage 3 were divided, cultured again and used on day 7 of growth and cells from passage 4 were split for EC. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with: 0.5 mM 4-(2-hydroxyethyl-1-piperazine ethane sulfonate) (HEPES) pH 7.4, glutamine (2 mM), gentamicin (50 mg/l), amphotericin B (0.125 mg/l) and 10% foetal bovine serum.

$^{45}Ca^{2+}$ -uptake measurement

Cells in 60 mm plates were washed twice in the MOPS (3-(N-Morpholino)-propanesulfonic acid) buffer (in mM: 20 MOPS, 140 NaCl, 2 $MgCl_2$, pH 7.4 at 37°C and 10 μM nitrendipine) and then Na^+ -loaded in the MOPS buffer containing 1 mM ouabain, 25 μM nystatin and 10 μM nitrendipine for 20 min. at 37°C with gentle shaking. The cells were then quickly washed twice in 2 ml of the above mentioned MOPS buffer with only nitrendipine and then placed in $^{45}Ca^{2+}$ solutions at pH 7.4 at 37°C with either 130 mM Na^+ or 130 NMG^+ (N-methylglucamine-HCl, pH 7.4 at 37°C for Na^+ substitution) with various additives. The cells were washed 6 times with ice-cold MOPS-tris (pH 7.4 determined at 37°C) with $MgCl_2$ and $LaCl_3$ to remove extracellular radioactivity. One millilitre of cold 0.1% Triton X-100 with 1 mM EGTA (ethyleneglycol bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid) was added to each plate. The cells were then scraped, placed into scintillation vials; 100 μl of the suspension was saved for protein estimation and the remainder used for scintillation counting. In some experiments, the cells were loaded with BAPTA prior to Na^+ -loading. BAPTA/AM (20 μM , 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)), was added to cells in the culture medium and placed for 2 hrs in the incubator at 37°C. The cells were then Na^+ -loaded and used as before. Any other inhibitors were included only in the $^{45}Ca^{2+}$ accumulation solution at specified concentrations. When the

additives were dissolved in solvents other than water, the solvents were also used in the vehicle controls.

Subfractionation of detergent-treated microsomes and Western blots

Smooth muscle from coronary arteries of 60 pig hearts (Maple Leaf Meats, Burlington, Canada) was dissected and used for isolating microsomes as described previously [32]. The microsomes were washed two times in 8% sucrose (weight/weight) and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at pH 7.0 and then suspended in 300 μl of the same solution. The microsomal suspension was mixed with 60 μl of 1 M NaCl and 40 μl of 10% Triton X-100. The suspension was kept on ice with occasional mixing for 15 min. after which 800 μl of 60% sucrose (w/w) was added to obtain a uniform mixture [33]. This suspension (600 μl) was pipetted into the bottom of a centrifuge tube for TLS-55 rotor and different concentrations of sucrose were layered on the top. The tubes were centrifuged at 160,000 g for 20 hrs at 4°C. Fractions (230 μl) were collected from the top, aliquoted and saved at -20°C for the analysis.

The primary antibodies were purchased from the following sources: anti-SERCA antibody IID8 (Affinity Bioreagents, Golden, MO, USA), anti-NCX1 antibody R3F1 (Swant Swiss Antibodies, Bellinzona, Switzerland), anti-caveolin-1 and anti-flotillin-2/ESA (BD Biosciences, Mississauga, Canada), anti-prion protein antibody 6H4 (Prionics AG, Switzerland). Western blots were treated with the primary antibodies and horseradish peroxidase conjugated anti-mouse IgG (GE Healthcare, Baie d'Urfe, QC, Canada) at a 20,000 $\times$ dilution was used. The peroxidase activity was visualized with a femto-kit (Pierce Chemical Company, Rockford, IL, USA) and a LAS3000 mini Luminiscent Image Analyzer (Fujifilm Life Science, Stamford, CT, USA). Ganglioside GM were detected in dot blots using horseradish peroxidase conjugated cholera toxin B subunit (Calbiochem, San Diego, CA, USA). Cholesterol determination was carried out using an Amplex red kit following instructions of the manufacturer (Invitrogen, Burlington, Canada).

Data analysis

Values presented are mean $\pm$ S.E.M. of the specified number of replicates. Student's *t*-test was used to test null hypotheses and *P*-values < 0.05 were considered to negate them. The results were also verified with a one-way ANOVA test. Each experiment was repeated two to four times with the specified number of replicates per experiment.

Results

In Western blots, the SMC reacted positively with anti-smooth muscle α -actin and SERCA2b-selective antibody, IID8, but not with selective antibodies against SERCA2a, endothelial NO synthase (eNOS) or von Willebrand factor as described previously [31, 34]. In Western blots, the EC reacted positively with antibodies against eNOS or von Willebrand factor as described previously and negatively with anti-smooth muscle α -actin [31, 34].

Fig. 1 Effect of BAPTA on the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC. Ca^{2+} uptake was carried out using Na^{+} -loaded cells in media containing extracellular Na^{+} or NMG^{+} . NCX-mediated Ca^{2+} uptake is the difference between the two groups. The data were normalized to total cell protein. Analysis by ANOVA followed by Tukey–Kramer multiple comparison test showed that the NCX-dependent Ca^{2+} uptake was greater in the BAPTA-loaded cells at 1 min. ($P < 0.05$, $q = 4.681$) and 5 min. ($P < 0.05$, $q = 4.371$) than in the cells without BAPTA loading ($P < 0.05$, $F = 22.982$, $df = 17$).

Time course of NCX-mediated $^{45}\text{Ca}^{2+}$ -entry

Na^{+} -loaded SMC were incubated in media containing extracellular Na^{+} or NMG^{+} to examine the accumulation of $^{45}\text{Ca}^{2+}$. Difference between uptake in the two solutions was defined as the NCX-mediated uptake. In an initial experiment, we observed that the value of the NCX-mediated uptake was very small compared to the uptake in the Na^{+} -containing solution (Fig. 1). Therefore, we considered preloading the cells with the Ca^{2+} -chelator BAPTA to increase the NCX-mediated accumulation of $^{45}\text{Ca}^{2+}$. SMC were preloaded with BAPTA for 2 hrs prior to Na^{+} -loading and then used to examine the uptake. Preloading with BAPTA caused an increase in the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake (Fig. 1). Thus, lowering $[\text{Ca}^{2+}]_i$ with BAPTA caused an increase in the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake.

Time course of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in Na^{+} - and BAPTA-loaded SMC and EC is in Fig. 2. $^{45}\text{Ca}^{2+}$ -accumulation in Na^{+} - and BAPTA-loaded cells in Na^{+} -containing and NMG^{+} -containing solutions are shown. The difference between the uptake in the two solutions is shown as NCX in the insets. The slope of the NCX-mediated $^{45}\text{Ca}^{2+}$ -accumulation over 5 min. did not differ significantly from that over 2 min. ($P > 0.05$) but the slope in 5–10 min. was significantly smaller ($P < 0.05$). Thus, in both SMC and EC, the NCX-mediated component of the uptake was linear with time for up to 5 min. Hence the uptake in 5 min. was examined in the subsequent experiments.

The $^{45}\text{Ca}^{2+}$ -accumulation in Na^{+} - and BAPTA-loaded cells in NMG^{+} -containing solutions and in those containing NMG^{+} plus K^{+} was compared to determine if there was any KNCX-activity

Fig. 2 Time course of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in BAPTA-loaded SMC and EC. The cells were loaded with BAPTA and then with Na^{+} and then $^{45}\text{Ca}^{2+}$ -uptake was examined in solutions containing Na^{+} or NMG^{+} . The difference in the uptake between the two solutions is shown in the insets as NCX. The data are mean $\pm$ S.E.M. of 6 replicates for each time point. The slope of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake over 5 min. did not differ significantly from the slope over 2 min. ($P > 0.05$), but the slope in 5–10 min. was significantly smaller ($P < 0.05$).

(Table 1). The values of the uptake in the two solutions did not differ significantly indicating that there was no detectable component of the KNCX-mediated $^{45}\text{Ca}^{2+}$ -accumulation. This experiment was repeated several times with and without BAPTA loading (not shown) but there was no detectable KNCX.

Effects of NCX inhibitors on the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake

We had defined the NCX-mediated $^{45}\text{Ca}^{2+}$ uptake by Na^{+} -loaded SMC and EC simply as the difference between the values in the

Table 1 NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC

	$^{45}\text{Ca}^{2+}$ -uptake (nmol/mg protein)	
	SMC	EC
NMG+	0.95 ± 0.11	5.05 ± 0.08
K+ plus NMG+	0.90 ± 0.09	5.12 ± 0.13
NCX	0.06	0.08

Note: For $^{45}\text{Ca}^{2+}$ -uptake, the Na⁺- and BAPTA-loaded SMC or EC were incubated in a solution containing NMG+ alone or NMG+ plus K+ for 5 min. The data are mean $\pm$ S.E.M. of 6 replicates. The presence of K+ did not alter the magnitude of the $^{45}\text{Ca}^{2+}$ -accumulation in SMC or EC ($P > 0.05$).

Na⁺ and NMG⁺-containing solutions. To validate this definition, we used several inhibitors (Table 2). The NCX inhibitors KB-R7943 and SEA 0400 caused nearly complete inhibition of the NCX-mediated uptake. Since the NCX activity depends on the Na⁺-gradient across the plasma membrane (PM), the Na⁺ ionophore monensin is expected to collapse this gradient and hence inhibit the NCX-mediated uptake. Monensin inhibited the NCX-mediated uptake in both SMC and EC. Low extracellular or high intracellular Na⁺-concentrations may also inhibit the Na⁺-H⁺-exchanger. Therefore, we also tested the effects of the Na⁺-H⁺-exchanger inhibitor cariporide. Cariporide had no effect on the NCX-mediated uptake in SMC or EC. Together, the use of these inhibitors validated our method to monitor the NCX-mediated $^{45}\text{Ca}^{2+}$ uptake.

Comparing NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake and the effect of BAPTA in SMC and EC

Two major observations emerged upon comparing the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC. The first observation was that SMC and EC differed in the effects of BAPTA loading. In three paired experiments, the NCX-mediated uptake was compared in SMC with and without BAPTA loading. Each time, the BAPTA-loaded cells showed greater NCX-mediated uptake (data not shown). In comparison, BAPTA caused a marginal but not significant increase in EC ($P > 0.05$). This discrepancy cannot be attributed to an inability of EC to absorb BAPTA-AM and digest it since they have been shown to respond to BAPTA loading in ascorbate release experiments [34]. The values of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake were also compared in several unpaired experiments conducted on different days (Fig. 3). The NCX-mediated uptake was 0.71 ± 0.08 nmol/mg protein in 15 experiments using SMC with no BAPTA loading and 1.95 ± 0.08 nmol/mg protein in 16 experiments using BAPTA-loaded SMC. Thus BAPTA loading caused a significant increase in the NCX-mediated uptake in SMC. An increase was not observed in EC in similar unpaired experiments. The second observation was that the NCX-mediated

Table 2 Inhibition of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC

	Percentage inhibition	
	SMC	EC
KB-R7943		
3 μM	56 ± 10	48 ± 10
10 μM	65 ± 10	67 ± 3
30 μM	91 ± 6	99 ± 2
SEA 0400		
0.3 μM	66 ± 5	92 ± 1
3 μM	100 ± 5	98 ± 2
Monensin		
20 μM	74 ± 8	91 ± 6
Cariporide		
2 μM	-8 ± 14	1 ± 12
10 μM	0 ± 8	-5 ± 14

Note: The NCX-mediated component was computed as the difference between the uptake in solutions with Na⁺ or NMG⁺. The inhibitors were included in the $^{45}\text{Ca}^{2+}$ -uptake solutions. The experiments with each inhibitor were conducted on separate days. In each experiment, the value at each concentration of the inhibitor was computed as percentage of the mean value in the absence of the inhibitor. Values in each experiment are mean $\pm$ S.E.M. from 6 to 12 replicates. KB-R7943, SEA 0400 and monensin caused significant inhibition ($P < 0.05$) at the concentrations used but cariporide did not cause a significant inhibition ($P > 0.05$). The experiments with each inhibitor were repeated three times and similar results were obtained.

$^{45}\text{Ca}^{2+}$ -uptake was significantly greater in EC than in SMC, with or without BAPTA loading (Fig. 3).

Comparing the effect of thapsigargin on NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC

We examined the effects of thapsigargin on the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake by SMC and EC with and without BAPTA loading (Fig. 4). In each of the 5 experiments, when SMC were not BAPTA-loaded, the addition of 1 μM thapsigargin to the uptake solution decreased the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake. This inhibition was $65 \pm 10\%$ in the 5 experiments (Fig. 4). However, thapsigargin did not cause a significant inhibition in the NCX-mediated uptake when BAPTA loaded SMC were used. In EC, it caused a small but statistically insignificant decrease in the NCX-mediated uptake with or without BAPTA loading (Fig. 4).

Fig. 3 Comparison of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC in 5 min. with and without BAPTA loading. The mean value of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake from 5 to 6 replicates on a single day was taken as one replicate. The values are mean $\pm$ S.E.M. from 15 experiments for group A (SMC without BAPTA loading), 7 for group B (EC without BAPTA loading), 17 for group C (SMC with BAPTA loading) and 15 for group D (EC with BAPTA loading). One way ANOVA followed by a multiple comparison with Tukey–Kramer test showed that there were significant differences between groups A and B ($Q = 5.885$, $P < 0.001$), A and C ($Q = 2.305$, $P < 0.05$), C and D ($Q = 6.959$, $P < 0.001$) but not B and D ($Q = 0.6919$, $P > 0.05$).

NCX and SERCA2 distribution in detergent-treated smooth muscle microsomes

Since there was evidence for a functional linkage between NCX and SERCA in SMC, an experiment was conducted to search for a potential spatial linkage. Microsomal membranes from the smooth muscle tissue of pig coronary artery were treated with Triton X-100 and subjected to isopycnic centrifugal flotation on a sucrose density gradient. The distribution of NCX, SERCA2 and several markers was examined (Fig. 5). The fractions with the lowest density had the highest cholesterol to protein ratio and those with the highest density were poor in cholesterol. The distribution for caveolin and the lipid raft markers ganglioside GM1, flotillin and prion proteins were also shown in Fig. 5. Quantification and further analysis of the various markers showed that their distributions were similar but not identical to that of cholesterol. The values of the correlation coefficient (r^2) for these markers were flotillin (0.8929), ganglioside GM1 (0.8399), prion protein (0.7175) and caveolin (0.5650). A major band at the expected molecular weight was obtained for NCX (116 kD) and for SERCA2 (110 kD). The distributions of NCX and SERCA2 correlated with each other with an r^2 value of 0.5249 ($P = 0.0077$). Repeating this experiment also gave similar distribution for NCX and SERCA2. Thus, there may be

Fig. 4 Thapsigargin inhibition of the NCX-mediated $^{45}\text{Ca}^{2+}$ -uptake in SMC and EC in 5 min. with and without BAPTA loading. The mean value of the inhibition from 5 to 6 replicates on a single day was taken as one replicate. The values are mean $\pm$ S.E.M. of the uptake from 8 days for group A (SMC without BAPTA loading), 6 days for group B (EC without BAPTA loading), 3 days for group C (SMC with BAPTA loading), and 3 days for group D (EC with BAPTA loading). One way ANOVA followed by a multiple comparison with Tukey–Kramer test showed that there were significant differences between groups A and B ($Q = 5.014$, $P < 0.05$), A and C ($Q = 5.284$, $P < 0.01$) but not for B and D ($Q = 1.072$, $P > 0.05$) or C and D ($Q = 0.1368$, $P > 0.05$).

a spatial co-localization of NCX and SERCA2 in the pig coronary artery smooth muscle. The distributions of NCX ($r^2 = 0.3745$, $P = 0.0344$) and SERCA2 ($r^2 = 0.8233$, $P < 0.0001$) also correlated significantly with the caveolin distribution.

It would have been ideal to conduct a similar experiment to test for a linkage between NCX and SERCA in EC. However, the abundance of SERCA protein in EC is very low [16]. Therefore, this experiment is not feasible.

Discussion

The results indicated that the NCX-mediated Ca^{2+} entry was greater in EC than in SMC and that this entry was increased by BAPTA loading only in SMC. Thapsigargin inhibited this NCX activity in SMC not loaded with BAPTA. NCX and SERCA2 showed similar distribution upon isopycnic flotation of detergent-treated microsomes from SMC. The Discussion will focus on the validity of the methods, comparison of these observations with the literature, and potential physiological and pathophysiological implications of the results.

Validation of the methods used

NCX-mediated Ca^{2+} entry in vascular smooth muscle has been examined in the literature using electrophysiology, monitoring

Fig. 5 Distribution of NCX and SERCA2 in fractions obtained from isopycnic flotation of the detergent-treated microsomes obtained from the coronary artery smooth muscle tissue. Fraction numbers are given on the top. Locations of protein markers with molecular weights in kD are shown on the right. In each lane, 2 μ g protein (5 μ g for NCX1) of the specified fraction 1–12 was used for Western blots. Flot. = flotillin, PrP = prion protein, Gang. = ganglioside. Ganglioside amounts were determined in dot blots using 1 μ g protein/fraction. The graph shows protein and cholesterol amounts and sucrose gradients in these fractions. Relative amounts of protein concentration/fraction were determined taking the value in fraction 12 as 100%. Relative amounts of cholesterol/ μ g protein were determined taking the value in fraction 1 as 100%.

[Ca²⁺]_i using fluorescence probes, examination of vascular contractility or as ⁴⁵Ca²⁺ uptake in Na⁺-loaded cells [2, 13, 23, 25–27, 29, 35, 36]. Each method has its own advantages and pitfalls. Here, we examined the NCX-mediated ⁴⁵Ca²⁺ uptake in the Na⁺-loaded SMC and EC. The main advantage of this method is that it gives a direct measure of the ⁴⁵Ca²⁺ taken up by the cells. One of its pitfalls is that it does not distinguish between ⁴⁵Ca²⁺-⁴³Ca²⁺ exchange and the actual ⁴⁵Ca²⁺ entry. However, this hurdle was overcome pharmacologically. The NCX-mediated ⁴⁵Ca²⁺ uptake in the Na⁺-loaded cells was defined as the difference in the uptake between the cells placed in solutions that contained Na⁺ or those in which Na⁺ was substituted with NMG⁺. This component of the uptake was inhibited nearly completely in both SMC and EC

by the NCX inhibitors KB-R7943 and SEA 0400 [37–39]. KB-R7943 may also inhibit L-type VOCC but this was not an issue since the uptake solutions contained 10 μ M nitrendipine, which is in excess of the nanomolar concentrations needed to block the L-type VOCC. The inhibition of Ca²⁺ uptake with SEA 0400, which is more selective for NCX than KB-R7943, further validates the role of NCX. It is also pointed out that SEA 0400 is more selective for NCX1, which is the NCX isoform expressed in SMC and EC [16, 37]. The inhibition with the Na⁺-ionophore monensin established the need for a Na⁺-gradient. However, alterations in Na⁺-gradients may also alter the Na⁺-pump activity but this possibility was eliminated using ouabain. The possibility that the observed activity of NCX was due to Na⁺-H⁺-exchanger was eliminated since

cariporide did not have any effect. Although these experiments validated the definition of the NCX-mediated Ca^{2+} entry used in this work the possibility of a partial contribution of passive entry *via* unspecified Ca^{2+} channels cannot be completely ruled out.

Linkage between NCX and SERCA in SMC

Loading SMC with BAPTA increased the NCX-mediated Ca^{2+} uptake. Thapsigargin inhibited the uptake when they were not loaded with BAPTA but it had no significant effect when they were loaded with BAPTA. These results are consistent with a hypothesis of a functional linkage between NCX and SERCA: Ca^{2+} that enters *via* NCX can be sequestered into SER by SERCA [25]. The inhibition of SERCA would block this pathway and decrease the entry. Chelation of the entered Ca^{2+} by BAPTA would allow for maintenance of the Ca^{2+} gradient and hence eliminate the need for the functional linkage with the SERCA pump. However, one cannot rule out the possibility that thapsigargin may open store depletion-dependent cation channels which are less selective for Ca^{2+} and may play a role [1, 36].

NCX and SERCA2 showed similar distribution when the Triton X-100-treated smooth muscle microsomes were fractionated by density gradient flotation. Caveolae are specialized lipid raft rich membranes containing the protein caveolin [23, 33, 40–42]. Centrifugation of non-ionic detergent-treated membranes have been used to separate lipid raft rich PM from other membranes with and without caveolin [33, 43]. However, the distributions of various lipid raft markers were similar but not identical to that of cholesterol with the cholesterol to caveolin distribution correlation being the weakest. These correlations are consistent with the lipid raft membrane being heterogenous and only part of it forming caveolae. The correlation between the distributions of NCX and SERCA2 in these fractions was statistically significant. These distributions also correlated significantly with that of caveolin. The data are consistent with a spatial linkage between SERCA2, caveolin and NCX in a model proposed based on electron microscopy studies [13]. Literature has also used high pH treatment along with sonication for similar membrane fractionation [44–46]. In preliminary experiments, this treatment was shown to cause oligomerization of the SERCA2 protein (data not shown). SERCA2 protein has also been shown to be polymerized by reactive oxygen species [47].

Although this is the first report on a functional and possibly a spatial linkage between NCX and SERCA in coronary artery SMC, the literature on various tissues supports this concept [1, 2, 12, 13, 23, 25–27, 36, 40–42]. The actions of NCX and SERCA have been proposed to be linked through a limited junctional cytoplasmic space model [13, 25–27]. Immunofluorescence studies on cultured arterial myocytes suggest that the exchanger molecules are organized in reticular patterns over the cell surfaces [48]. In a study based on $[\text{Ca}^{2+}]_i$ measurements, when NCX was arrested by removing both external Na^+ and Ca^{2+} , Ca^{2+} released from the SER was resequenced. However, when both NCX and SERCA

were blocked, the Ca^{2+} released from the SER was extruded from the cells by PMCA [27]. It has been suggested that localization of NCX in caveolae in conjunction with other Ca^{2+} transporters may lead to specialized regulation pockets [1, 2, 12, 13, 23, 25–27, 36, 40–43, 49, 50].

Differences in NCX-mediated Ca^{2+} uptake in EC and SMC

The NCX-mediated Ca^{2+} uptake in EC was greater than in SMC with and without prior loading with BAPTA. This result is consistent with the previously reported higher levels of NCX expression in Western blots in EC than in SMC [16]. Phospholemman mRNA abundance is greater in SMC than in EC [16]. Phosphorylated phospholemman inhibits NCX but its role in SMC remains to be determined [20, 21]. Another interesting difference is that thapsigargin inhibited the NCX-mediated Ca^{2+} entry in SMC but not in EC. A possible explanation for the lack of an effect of thapsigargin in EC is a very small SERCA to NCX activity ratio. The SERCA activity in EC is approximately one tenth of that in SMC and the NCX-mediated Ca^{2+} entry was approximately five-fold higher [16]. Therefore, the hypothesis of a functional NCX-SERCA linkage is consistent with the observation that the effects of thapsigargin and BAPTA were minimal in EC. Furthermore, in SMC, most of the SERCA protein is SERCA2 while EC contain SERCA3 and some SERCA2. Whether or not the SERCA isoform difference plays a role in this functional linkage remains to be shown.

Physiological and pathophysiological implications

The NCX-mediated $^{45}\text{Ca}^{2+}$ uptake was used here as an experimental tool but it does occur in physiological situations. First, this mode of NCX may play a role in refilling the SER in smooth muscle [13, 25–27]. The NCX-mediated Ca^{2+} uptake may also contribute to maintain a longer and stronger contraction. The NCX-mediated Ca^{2+} uptake has also been found in pathological situations when ion distributions are deregulated [3, 19, 51–53]. A potentially harmful effect of NCX in this mode is the subsequent Ca^{2+} overloading in the cell. Inhibition of this entry by agents such as SEA 0400 and KB-R7943 may be therapeutically useful [37]. In the coronary artery, Ca^{2+} deregulation can cause a dysfunction in coronary tone, and may lead to pathological states such as hypertension, ischaemia, and oxidative stress [54]. The difference in NCX activity between SMC and EC may be related to the unique Ca^{2+} handling requirements of the two cell types. In SMC, the tight $[\text{Ca}^{2+}]_i$ regulation may be performed by low capacity high affinity transporters such as PMCA. Furthermore, SMC contain a large amount of SER and SERCA, the SER restricts the mobility of Ca^{2+} throughout the cell by sequestering large amounts of Ca^{2+} . Therefore the presence of large PMCA and SERCA activity may replace the need for high capacity Ca^{2+} removal systems such as NCX. NCX-SERCA linkage may also be damaged during

pathophysiological states which may in turn, amplify the disturbance. SERCA2b is more susceptible to oxidative stress than SERCA3 [31, 47]. It remains to be demonstrated how this damage to SERCA2b affects NCX activity and/or the linkage between NCX and SERCA.

Evidence implicating the role of NCX-SERCA functional linkage in endothelium is rare although it was found that NCX activity is involved in the NO-induced depletion of Ca^{2+} in the SER [35, 55]. Thus, in some types of endothelial cells, the SER may have a functional NCX-SERCA linkage. The NCX-SERCA linkage may not be as important in coronary artery EC since the SERCA pump content is very small and present as two isoforms (SERCA3 and SERCA2). A different model based on a direct relationship between eNOS and NCX may be applicable in EC where high $[\text{Ca}^{2+}]_i$ may stimulate

NO synthesis. The removal of high $[\text{Ca}^{2+}]_i$ by NCX can stop or decrease NO synthesis to aid in sustaining the coronary tension. The presence of NO can also stimulate NCX-mediated Ca^{2+} extrusion through direct or indirect mechanisms, thus expelling more Ca^{2+} or the same amount of Ca^{2+} faster [56, 57]. This may be a feedback mechanism to decrease high levels of NO, especially under conditions of stress.

Acknowledgements

We thank Dr. P. K. Rangachari for his suggestions. This work was supported by a Grant-in-Aid (T6168) from the Heart & Stroke Foundation of Ontario.

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