Ca^{2+}-pumps and Na^+-Ca^{2+}-exchangers in coronary artery endothelium versus smooth muscle

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Abstract

Vascular endothelial cells (EC) and smooth muscle cells (SMC) require a decrease in cytoplasmic Ca^{2+} concentration after activation. This can be achieved by Ca^{2+} sequestration by the sarcoplasmic reticulum Ca^{2+} pumps (SERCA) and Ca^{2+} extrusion by plasma membrane Ca^{2+} pumps (PMCA) and Na^+-Ca^{2+}-exchangers (NCX). Since the two cell types differ in their structure and function, we compared the activities of PMCA, NCX and SERCA in pig coronary artery EC and SMC, the types of isoforms expressed using RT-PCR, and their protein abundance using Western blots. The activity of NCX is higher in EC than in SMC but those of PMCA and SERCA is lower. Consistently, the protein abundance for NCX protein is higher in EC than in SMC and those of PMCA and SERCA is lower. Based on RT-PCR experiments, the types of RNA present are as follows: EC for PMCA1 while SMC for PMCA4 and PMCA1; EC for SERCA2 and SERCA3 and SMC for SERCA2. Both EC and SMC express NCX1 (mainly NCX1.3). PMCA, SERCA and NCX differ in their affinities for Ca^{2+} and regulation. Based on these observations and the literature, we conclude that the tightly regulated Ca^{2+} removal systems in SMC are consistent with the cyclical control of contractility of the filaments and those in EC are consistent with Ca^{2+} regulation of the endothelial nitric oxide synthase near the cell surface. The differences between EC and SMC should be considered in therapeutic interventions of cardiovascular diseases.

Keywords: PMCA • SERCA • sodium–calcium exchanger • coronary artery

Introduction

Coronary tone is a finely tuned parameter as blood supply to the heart is necessary for survival and its dysregulation may lead to heart failure or other cardiovascular diseases. Several intrinsic factors and agents control the coronary tone by acting via endothelial cells (EC) or directly on smooth muscle cells (SMC). Changes in cytosolic Ca^{2+} concentration ([Ca^{2+}]_i) in SMC and EC are pivotal to regulation of coronary tone. An increase in [Ca^{2+}]_i in EC and SMC has opposite effects on vascular contractility. In EC, an increase in [Ca^{2+}]_i activates nitric oxide synthase (eNOS) with a consequent increase in production of NO, which causes SMC to relax by activating guanylate cyclase [12, 32]. In EC eNOS is localized on the plasma membrane (PM) and its activation has a paracrine function. In contrast, in SMC [Ca^{2+}]_i increases transiently and then decreases in contractile cycle.
The increased \([\text{Ca}^{2+}]\) activates calcium calmodulin-dependent myosin light chain kinase, leading to increased phosphorylation of the 20 kD myosin light chains, cross-bridge formation between the myosin heads and the actin filaments and eventually SMC contraction [20, 32]. The contractile filaments are not just located at the surface but may also be deeply embedded in the cell. Since, EC and SMC are vastly different in their structure and function, it is logical that they would require different pathways to lower \([\text{Ca}^{2+}]\).

\([\text{Ca}^{2+}]\) is maintained in EC and SMC at low levels: it increases during cell excitation and is then restored to normal levels. The excess \([\text{Ca}^{2+}]\); following stimulation may be resequestered by the sarco-/endoplasmic reticulum \(\text{Ca}^{2+}\) ATPases (SERCA) into the reticulum or expelled from the cell by the plasma membrane \(\text{Ca}^{2+}\) ATPases (PMCA) and the \(\text{Na}^{+}–\text{Ca}^{2+}\) exchangers (NCX) [2, 3, 32, 33, 38]. Each of these transporters is encoded by different genes whose transcripts may be alternatively spliced thus giving a large milieu of pathways for lowering \([\text{Ca}^{2+}]\). Each pathway differs in its role and modes of regulation [4, 10, 11, 22, 29, 32, 34, 36, 39, 42]. SERCA are encoded by three genes (SERCA1–3) with the SERCA2 splice SERCA2b being most widely distributed [29]. SERCA3 lacks the site for phospholamban regulation present in SERCA2. PMCA are encoded by four genes (PMCA1–4), however, alternative splicing gives over 30 possible isoforms that differ in regulation by protein kinases and calmodulin and to some degree in \(\text{Ca}^{2+}\) kinetics [4, 10, 11, 22, 32, 37–39]. PMCA1 and 4 are the most ubiquitously expressed isoforms. NCX are encoded by the genes NCX1–3 of which NCX1 is widely expressed and shows extensive alternative splicing [21, 25, 33, 34]. Although both PMCA and NCX can expel \(\text{Ca}^{2+}\), they differ in this role. PMCA has a higher affinity for \(\text{Ca}^{2+}\) than does NCX. In contrast, it is not clear that the higher affinity enables PMCA to remove more cytosolic \(\text{Ca}^{2+}\) than does NCX [24, 30, 33, 40]. PMCA works only as a \(\text{Ca}^{2+}\) extrusion system but NCX may also allow \(\text{Ca}^{2+}\) entry depending on electrochemical gradients of \(\text{Na}^{+}\) and \(\text{Ca}^{2+}\). Studies in cardiac function on \(\text{Ca}^{2+}\) transporters have been useful in developing appropriate therapeutic strategies in cardiovascular diseases. Here, we compare EC and SMC from pig coronary artery for activities, protein abundance and isoform expression of PMCA, SERCA and NCX. Based on the comparison, we present a composite picture for the roles of PMCA, NCX and SERCA in the function of EC and SMC. To our knowledge, such a comparison has not been made previously.

Materials and methods

Membrane isolation

Pig heart and brain were obtained from local abattoirs. PM-enriched fractions were isolated by differential centrifugation followed by sucrose density gradient centrifugation as described previously [17]. Alternatively, coronary artery EC and SMC were cultured and then used for PM isolation [16]. Aliquots were stored at \(-80^\circ\text{C}\).

RNA isolation and RT-PCR

In most experiments, SMC and EC cultured from pig coronary artery were used [16]. The cultured EC were tested using immunocytochemistry and found to be positive for von Willebrand factor and eNOS and negative for \(\alpha\)-actin. SMC were positive for \(\alpha\)-actin and negative for von Willebrand factor and eNOS. RNA was isolated using a total RNA isolation kit (Qiagen, Missisauga, ON, USA). The isolated RNA was DNase I digested and reverse transcribed using the ThermoScript RT-PCR system (Invitrogen, Burlington, ON), following instructions of the manufacturer. In initial experiments, the conditions for PCR were optimized for each primer set and template. Routinely, PCR was carried out with AmpliTaq (Applied Biosystems, Foster City, CA, USA) at 2.5 mM MgCl2. Primers are given in Table 1. A typical PCR cycle consisted of denaturation at 94°C for 40 sec, annealing at 58°C (PMCA), 60°C (NCX), 66°C (phospholamban) for 40 sec, and extension at 72°C for 50 sec. After 30 cycles, the reaction at 72°C was continued for another 5 min.

Western blot analysis

Different amounts of protein (between 1 and 50 \(\mu\)g) of the PM-enriched fraction were electrophoresed on 7.5% SDS-polyacrylamide gels and then electroblotted onto a nitrocellulose membrane in transfer buffer containing 0.05% SDS. The membrane was treated with a blocking solution containing 5% (w/v) non-fat dry milk in a tris buffer [in mM: 10 mM Tris, 140 mM NaCl, and 0.1% (v/v) polyoxyethylene 20 sorbitan monostearate, pH = 7.4] overnight at 4°C. The blots
were incubated with primary antibodies diluted in the tris buffer and 2% (w/v) non-fat dry milk at 22–24°C for 1 hr. Mouse monoclonal anti-PMCA antibody (5F10) and rabbit polyclonal anti-PMCA1 antibody were from Affinity-BioReagents (Golden, CO, USA); mouse monoclonal anti-PMCA4 (JA9) were from Sigma-Aldrich (Oakville, ON) and mouse monoclonal anti-NCX (R3F1) was from Swant (Bellinzona, Switzerland). After incubation, membrane was washed in the tris-buffer and then incubated with horseradish peroxidase-conjugated secondary antibody diluted in the tris buffer for 1.5 hr. Bands were visualized using an Enchanced Chemiluminiscence Kit (GE Healthcare BioSciences Inc., Baie d’Urfé, Quebec, Canada) following instructions of the manufacturer.

### Biochemical assays

Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase assays for PMCA were performed on the PM-enriched fractions in a coupled enzyme assay that monitored the disappearance of fluorescence of NADH, as described previously [5]. Thapsigargin, ouabain and Na-azide were included in these assays to inhibit other ATPases. The difference between the total ATPase and the basal Mg\(^{2+}\)-ATPase was the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase. SERCA-mediated \(^{45}\)Ca\(^{2+}\) uptake was examined in saponin-permeabilized EC and SMC as described previously in solutions containing an ATP-regenerating system, Mg\(^{2+}\), oxalate (to increase retention of Ca\(^{2+}\) in the sarco-/endoplasmic reticulum, Na-azide (to inhibit mitochondrial uptake) in the absence or presence of 2 \(\mu\)M of the SERCA inhibitor thapsigargin [35]. The difference in the uptake with and without thapsigargin was designated as the SERCA pump activity. NCX-mediated \(^{45}\)Ca\(^{2+}\) uptake was examined using Na\(^+-\)loaded cells in media containing Na\(^+\) or N-methylglucamine to substitute for Na\(^+\), as previously described [8]. The difference in Ca\(^{2+}\) uptake in the two solutions was defined as the NCX-mediated uptake.

### Data analysis

Band intensities of ethidium bromide-stained gels and Western blots were analyzed using Kodak 1D Image Analysis Software. All the values are mean ± S.E.M. of 3–6 replicates. Statistical significance was determined using Student’s t-test, and values of \(P < 0.05\) were considered to be significant.
Results

Phenotypes of the cultured EC and SMC used have been reported previously [7, 9, 16]. Cultured EC react positively to the endothelial markers anti-eNOS and anti-von-Willebrand factor, but not to the smooth muscle marker anti-α-actin. Similarly, SMC react positively to anti-α-actin but not to anti-eNOS and anti-von-Willebrand factor.

PMCA in EC and SMC

RT-PCR was used to determine the PMCA genes expressed in EC and SMC (Fig. 1). All the primers flanked the cryptic splice site C that lies within PMCA regulatory domain [39]. Sequences of the primers are given in Table 1. The heart and brain cDNA was used to optimize PCR conditions. PCR products for PMCA2 and 3 were not observed in EC or SMC. Expected sizes for PMCA1b, PMCA4a and PMCA4b were 429, 902 and 727 bp, respectively. Although RT-PCR using RNA from EC gave bands for both PMCA1b and 4b, in co-PCR experiments PMCA1b band dominated. In contrast, bands for both PMCA1 and PMCA4 were observed in co-PCR experiments using different dilutions of the reverse transcripts prepared from SMC (Fig. 1). These results are consistent with those previously reported using freshly isolated EC and SMC from pig aorta [7, 31]. Although not shown, the following controls gave no PCR products: no reverse transcriptase and no cDNA template. Identities of PMCA1b and PMCA4b bands were also confirmed by the sequencing of gel-purified PCR products. Specificity of the PMCA4a was confirmed by RT-PCR with PMCA4a specific primers (Table 1).

The relative abundance of PMCA proteins was determined using three different antibodies: one that recognized all PMCA isoforms (5F10), anti-PMCA4 (JA9) and a polyclonal anti-PMCA1 antibody. Figure 2A shows the results of the Western blot using 5F10 in one experiment. Both SMC and EC gave a band at 140 kDa corresponding to PMCA. Thus, the PMCA protein abundance based on Figure 2B in EC was 22 ± 2% of that in SMC. In similar experiments using the anti-PMCA1 antibody, the abundance in EC was 27 ± 4% of that in SMC. In contrast, PMCA4 in EC was much smaller (8 ± 1% of the SMC value). These results are consistent with those of the PCR experiments in Fig. 1 in that EC express mainly PMCA1.

PMCA activity was measured in PM-enriched fractions isolated from SMC and EC as Ca²⁺-stimulated hydrolysis of ATP in a coupled enzyme assay containing calmodulin and the following inhibitors of other ATPases: sodium azide (mitochondrial Ca²⁺-ATPase), ouabain (Na⁺-K⁺-ATPase) and thapsigargin (SERCA). PMCA activity was lower in EC (0.3 ± 0.1 nmol/min/mg protein) than in SMC (1.1 ± 0.2 nmol/min/mg protein). Thus, PMCA activity in PM-enriched EC fractions was 27 ± 9% of that in SMC (Fig. 2C). These PMCA activity results are consistent with the values of abundance of total PMCA obtained in Western blots (Fig. 2C).

SERCA in EC and SMC

Time course of the ⁴⁵Ca²⁺ uptake by permeabilized EC and SMC with and without thapsigargin is shown in Fig. 3A. In the presence of thapsigargin, both EC and SMC showed only small ⁴⁵Ca²⁺ uptake but the uptake was much greater in the absence of thapsigargin. The thapsigargin-sensitive component of the uptake is shown in
Fig. 3B. The SERCA activity in EC ($0.15 \pm 0.05$ nmol/min/mg protein) was also lower than in SMC ($1.5 \pm 0.3$ nmol/min/mg protein). Thus, the SERCA activity in EC was only $10 \pm 3\%$ of that in SMC.

NCX in EC and SMC

NCX1 contains six cryptic exons that can result in alternative splicing [21, 25, 33, 34]. Primers flanking the cryptic exons were used to determine the NCX1 isoforms expressed in EC and SMC (Table 1). One major (309 bp) and a slightly larger minor band was observed in RT-PCR products of EC and SMC RNA (Fig. 4A). The two bands were sequenced. Translation of the sequences showed that the 309-bp band corresponded to NCX1.3 and the larger one to NCX1.7. A PM protein, phospholemman, inhibits NCX1 [42]. Therefore, we also determined if EC and SMC contain mRNA for this protein. RT-PCR product for phospholemman mRNA (277 bp) was detected in SMC but not in EC. The identity of this band was confirmed by sequencing.

NCX protein abundance was examined in Western blots using the antibody R3F1 that cross-reacts with all the isoforms. PM-enriched fractions from EC and SMC were used in this experiment. PM-enriched fraction from EC (20 g protein) gave an intense doublet at 116 and 112 kD in Western blots but SMC membranes showed very faint bands even with 50 g protein and none with 20 g (Fig. 4B). Thus, NCX was more abundant in EC than in SMC.

NCX activity was determined as $^{45}$Ca$^{2+}$ entry into Na$^+$-loaded cells in Na$^+$-free solutions. Na$^+$-loaded cells
(EC or SMC) were placed in normal Na+ or in Na+ substituted with N-methylglucamine (Fig. 5A), and the difference in the 45Ca2+ entry between them was defined as NCX activity (Fig. 5B). Time course of the uptake in Figure 5B shows that the NCX-dependent 45Ca2+ accumulation was linear for 5 min and that the rate of this accumulation was significantly greater in EC than in SMC. This uptake was inhibited by KB-R 7943 and monensin. When examined in several experiments, the NCX activity in EC (0.89 ± 0.09 nmol/min/mg protein) was greater than in SMC (0.2 ± 0.01 nmol/min/mg protein). The NCX activity in EC was 447 ± 43% of that in SMC. The greater activity of NCX in EC than in SMC is consistent with the greater NCX1 protein abundance observed in Western blots in Fig. 4.

SERCA, PMCA and NCX activities in EC and SMC

The activities of SERCA, PMCA and NCX were determined using different preparations and hence the absolute activities of the three transporters should not be compared to each other. Therefore, activities of PMCA, SERCA, and NCX in EC are presented as percent of the corresponding values in SMC in Fig. 6. As shown in Fig. 6, the PMCA activity was lower in EC than in SMC. SERCA activity in EC was also lower than in SMC. In contrast, NCX activity was much higher in EC than in SMC. Thus, activities of the two Ca2+ pumps were lower in EC than in SMC but the NCX activity was greater in EC.

Discussion

Pig coronary artery EC and SMC differ in PMCA, SERCA and NCX whether one considers their activities, protein abundance or types of mRNA. Here, we will compare our results with observations of others in the literature and examine how properties and abundance of these transporters may relate to differences in function between EC and SMC.

This is the first report comparing the activity levels of PMCA, SERCA and NCX in EC and SMC (Fig. 6). Relative protein abundance of PMCA and NCX in the tissues has also not been reported previously. RT-
PCR has been used to examine the types of mRNA expressed for PMCA and NCX in EC and SMC [1, 19, 25, 28, 34]. Here, we have reported that coronary artery SMC contain mRNA encoding PMCA4a, 4b and 1b. Expression of PMCA isoforms in vascular SMC has been reported but there is only limited literature on the coronary artery [1, 19, 28, 31]. In some studies, mRNA for PMCA1b and PMCA4b were reported in SMC and in others PMCA4a mRNA was also reported to be present. There are no reports on the presence of PMCA2 or 3 in vascular SMC. We observed that EC contain predominantly PMCA1 mRNA. This was also consistent with a very low abundance of PMCA4 protein in EC in Western blots. There are no reports on Western blots using vascular EC, but with RT-PCR they have been shown to contain mRNA encoding PMCA1b and PMCA4b [19, 31]. NCX1 mRNA expression has been reported in vascular EC and SMC although the exact phenotypes of the cells and proportions of different isoforms are not given [25, 34, 41]. The reports are consistent with our observations that they both contain mRNA encoding NCX1.3 and NCX1.7. Thus, these data are consistent with the literature.

This section focuses on the relationship between the type of Ca\textsuperscript{2+} transporters present in EC and SMC and the structure and function of the two cell types. To facilitate this discussion, key properties and our observations of SERCA, PMCA and NCX are summarized in Table 2. SERCA pump activity is lower in EC than in SMC. This is consistent with the previous observations that the SERCA inhibitor cyclopiazonic acid causes a smaller increase in [Ca\textsuperscript{2+}]i in EC than in SMC. SERCA3 pump that is expressed in EC has a lower affinity for Ca\textsuperscript{2+} than SERCA2b, which is the only isoform in SMC (Table 2). PMCA also have high affinity for Ca\textsuperscript{2+} and their activity is lower in EC than in SMC. In contrast, NCX has a much lower affinity for Ca\textsuperscript{2+} and is present in higher levels in EC than in SMC. The lower activity of NCX in SMC is in contradiction with its ability to remove large amounts of cytosolic Ca\textsuperscript{2+}. The larger abundance of SERCA2b in SMC is also consistent with the regulation of Ca\textsuperscript{2+} from around the contractile filaments that may be near cell surface and deeper in the cell. The regulation of [Ca\textsuperscript{2+}]i in SMC occurs mostly by high affinity pathways that are more tightly regulated to provide the needed [Ca\textsuperscript{2+}]i transients. The tighter regulation is fostered by: (1) presence of larger amounts of higher Ca\textsuperscript{2+} affinity systems, (2) better control of these systems: PMCA activity can be regulated by calmodulin and protein kinases, SERCA2b activity can be regulated by phospholamban and calmodulin-dependent kinase, NCX can be regulated by phosp-
holemman, and (3) presence of limited diffusional spaces by cytoskeletal elements [24, 26, 30, 40]. In contrast, EC are paracrine cells that secrete vasoactive agents and contain Ca\(^{2+}\)-stimulated eNOS on PM or caveoli [6,12]. EC have higher activity and abundance of NCX, which has low affinity for Ca\(^{2+}\), and they do not contain phospholemman that can regulate NCX. EC contain SERCA3 that also has a low affinity for Ca\(^{2+}\) and does not contain sites for phospholamban binding or regulation by calmodulin-dependent protein kinase. We conclude that the tightly regulated Ca\(^{2+}\) removal systems in SMC are consistent with the cyclical control of contractility of the filaments and those in EC are consistent with Ca\(^{2+}\) regulation of eNOS near the cell surface [13, 24, 40].

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| Table 2 Comparison of properties of SERCA, PMCA and NCX in vascular EC and SMC [4, 14–16, 18, 23, 27, 29, 35] |
|---------------------------------------------------------------|
| **SERCA**                                                     |
| Activity level                                               | µmol/g protein/min: EC: 0.1–0.2 ≪ SMC: 1–2                   |
| Isoforms expressed                                           | SERCA2b in SMC                                               |
|                                                               | SERCA3 and 2b in EC                                           |
| Hill coefficient for Ca\(^{2+}\)                            | SERCA2b: 2                                                   |
|                                                               | SERCA3: 2                                                   |
| Km for Ca\(^{2+}\)                                           | SERCA2b: 0.27 ± 0.03 µM                                       |
|                                                               | SERCA3: 1.1 ± 0.1 µM                                         |
| pH optimum                                                    | SERCA2b: 6.8                                                 |
|                                                               | SERCA3: 7.2                                                 |
| Phospholamban inhibition                                     | SERCA2b: Yes                                                 |
|                                                               | SERCA3: No                                                  |
| Calmodulin-dependent kinase II activation                     | SERCA2b: Yes                                                 |
|                                                               | SERCA3: Not known                                            |
| ROS susceptibility                                           | Peroxide, superoxide or peroxynitrite: SMC ≫ EC              |

| **PMCA**                                                     |
| Activity level                                               | µmol/g protein/min: EC: 0.3 < SMC: 1.1                       |
| Isoforms expressed                                           | mainly PMCA1 in EC, PMCA4 and PMCA1 in SMC                  |
| Hill coefficient for Ca\(^{2+}\)                            | 1                                                          |
| Km for Ca\(^{2+}\)                                           | <0.5 µM for PMCA4, not known                                 |

| **NCX**                                                      |
| Activity level                                               | µmol/g protein/min: EC:1–3 ≫ SMC: 0.2–0.3                    |
| NCX/SERCA activity ratio                                     | Activity ratio: EC: 6–20 ≫ SMC: 0.14–0.2                     |
| Isoforms expressed                                           | NCX1.3 in EC and SMC and lower levels of NCX1.7             |
| Hill coefficient for Ca\(^{2+}\)                            | 1                                                          |
| Km for Ca\(^{2+}\)                                           | 2.6–7 µM                                                   |
| Hill coefficient for Na\(^{+}\)                              | 3                                                          |
| Km for Na\(^{+}\)                                            | 25–45 mM                                                   |
| Phospholemman mRNA                                           | SMC ≫ EC                                                   |
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