Antisense-Inhibition of Plasma Membrane Ca$^{2+}$ Pump Induces Apoptosis in Vascular Smooth Muscle Cells

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ABSTRACT—The effect of antisense oligodeoxynucleotides (ODNs) of plasma membrane Ca$^{2+}$-pumping ATPase (PMCA) on rat aortic vascular smooth muscle cells (VSMCs) in primary culture was examined. More than 80% of the PMCA expressed in cultured VSMCs was the PMCA-1B subtype. Exposed to antisense ODNs against PMCA-1, not only the expression of the PMCA protein but also mRNA of PMCA-1B was diminished in a concentration-dependent manner. Extracellular Na$^+$-independent $^{45}$Ca$^{2+}$ efflux catalyzed via PMCA was inhibited with antisense ODNs. Both the resting and ionomycin- or ATP-stimulated levels of intracellular Ca$^{2+}$ were increased by antisense ODNs. Furthermore, prolonged treatment with antisense ODNs caused apoptosis in VSMCs. The occurrence of apoptosis was inhibited by FK506, a potent immunosuppressant. These results demonstrate that the PMCA was specifically inhibited by antisense ODNs and suggest that PMCA plays an important role in regulation of intracellular Ca$^{2+}$ concentrations, especially at the resting condition to prevent an occurrence of apoptosis that may be induced through the activation of calcineurin.

Keywords: Plasma membrane calcium pump, Vascular smooth muscle cell, Antisense oligodeoxynucleotide, Apoptosis, Calcineurin

The contractile state of vascular smooth muscle cells (VSMCs) is dependent on availability of Ca$^{2+}$ for activation of their contractile systems. Because the localization and the integrated intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$^i$) contain specific information, it is strictly regulated by influx and efflux across the plasma membrane as well as uptake into and release from the intracellular stores, such as the sarcoplasmic reticulum (1). In VSMCs, there are two Ca$^{2+}$ extrusion systems: the plasma membrane Ca$^{2+}$-ATPase pump (PMCA: extracellular Na$^+$ (Na$^+$)$_o$-independent) and the Na$^+$/Ca$^{2+}$ exchanger (NCX: Na$^+$$_i$-dependent system). An important difference between the two systems is the affinity for Ca$^{2+}$. In the physiological range of [Ca$^{2+}$]$_o$(≤1 μM), the PMCA plays a more important role than the NCX in Ca$^{2+}$ extrusion (2). Thus, a change in the activity of the PMCA would cause a significant change in [Ca$^{2+}$]$_i$, which could subsequently influence the contractile state of VSMCs.

Most of the studies on the physiological role of the PMCA were performed on erythrocytes lacking internal organelles. The PMCA in erythrocytes is the only enzyme that extrudes Ca$^{2+}$. The PMCA has been purified from a number of tissues (3 – 6) and the biochemical properties of the PMCA purified from bovine aorta are similar to those of the human erythrocyte enzyme (6). There have been several studies with intact VSMCs (2, 7, 8). The precise physiological role of the PMCA in VSMCs, however, has been difficult to elucidate because there is no selective inhibitor of the PMCA. In sarcoplasmic reticulum, thapsigargin, a selective inhibitor of sarcoplasmic reticulum Ca$^{2+}$-ATPase, has been useful for determining the definite role of the system in the contraction of myocytes (9) and of the other systems. The availability of a specific inhibitor of the PMCA might be especially useful for investigating the function of the PMCA under physiological conditions.

To overcome the problem, the PMCA isoforms had been overexpressed in COS-1 cells (10), CHO cells (11), and vascular endothelial cells (12). The long-term overexpression of the PMCA in these experiments, however, might have had an effect on the down-regulation of the sarcoplasmic reticulum Ca$^{2+}$ pump (SERCA) and the other

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Ca\textsuperscript{2+} regulation systems, so the physiological role of the PMCA is still unclear.

In the present study, we report on the use of antisense phosphorothioated ODNs that selectively interfere with the expression of the PMCA and attempt to establish the physiological role of the PMCA in primary cultured rat VSMCs. Antisense ODNs may block translation and/or processing of specific mRNAs by forming RNA-DNA duplexes (13). These RNA-DNA duplexes may have a direct sterical effect by blocking translation or by preventing translocation of the mRNA along the ribosome, or they may activate RNaseH-dependent degradation of mRNA. We show that antisense ODNs is a useful tool to investigate the physiological role of the PMCA.

MATERIALS AND METHODS

Cell culture

VSMCs were isolated from rat aorta (250 – 300 g, male) by enzymatic dispersion as described by Chamley et al. (14). The resulting cells were cultured for 4 – 5 days in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. After reaching confluence, cells were cultured in serum-free medium (Cosmedium 001) for an additional 24 h to enhance redifferentiation (15).

Western blot

The protein samples were separated on 7% polyacrylamide gels that contained 0.1% SDS according to Laemmli (16) and transferred to Immobilon PVDF membranes in buffer containing 25 mM Tris, 0.7 M glycine and 0.02% SDS at 12V for 3 h. Non-specific binding was blocked by 5% non-fat dry milk overnight at 4°C. Then the membranes were incubated for 4 h with the anti-PMCA monoclonal antibody clone 5F10 (diluted 1:1000 in Tris buffered saline 5% non-fat dry milk). For staining, goat anti-mouse IgG-coupled alkaline phosphatase conjugate was used. The reaction was developed with CSPD using a chemiluminescence detection of chemiluminescence on film and densitometry for an additional 24 h to enhance redifferentiation (15).

RNA preparation

Total RNA was isolated from VSMCs using the Quick Prep Total RNA Extraction kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s procedure. The RNA was dissolved in water treated with diethyl pyrocarbonate and used for cDNA synthesis. The integrity of the prepared RNA was verified by electrophoresis on agarose-formaldehyde gels.

Reverse transcription-polymerase chain reaction (RT-PCR)

First strand cDNA synthesis was carried out using the RT-PCR high in a total reaction volume of 40 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\textsubscript{2}, 10 mM dithiothreitol (DTT), 50 pmol random hexamers, 1 mM each dNTP, 0.5 units/μl RNase inhibitor and 2.5 units/μl Moloney murine leukemia virus reverse transcriptase (RNase minus). The reaction mixtures were incubated for 10 min at 30°C followed by 20 min at 42°C. The reaction was terminated by incubation for 5 min at 99°C followed by 5 min at 4°C.

The constructions of primers for PMCA isoforms were carried out according to Keeton et al. (7). PCR was performed in a total reaction volume of 25 μl containing 0.5 μl of the cDNA mixture, 1.25 units of recombinant Thermus aquaticus DNA polymerase (rTaq), 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl\textsubscript{2}, and 5 pmol of each primer. The reaction mixtures were incubated in a PTC-100 programmable thermal cycler (MJ Research, Watertown, MA, USA) for 150 s at 94°C and then the following cycle was repeated at appropriate times: 30 s at 94°C (denaturation), 30 s at 65°C (annealing) and 30 s at 72°C (extension). For relative quantitative RT-PCR analysis, PCR was performed for cycles in which the amount of PCR product increases exponentially (17). After this program, 10 μl of each reaction mixture was subjected to electrophoresis on 2% agarose gels to size fractionate the PCR products. The gels were stained with ethidium bromide or SYBR Green I, visualized on an ultraviolet transilluminator, and photographed. The density of each DNA band was measured, and the value for PMCA was expressed as a ratio to the value for G3PDH amplified from an aliquot of the same RT reaction. Primers for SERCA2b and NCX1 were as follows: the left primer for SERCA2b was AAGCTATGGGAGTGGTGGTG and the right one was CACTTCCAGCTTGGTGTGT, respectively. Left primer and right primers for NCX1 were TCTTCAGAAGTCTCGGAAGAT and CACTTCCAGCTTGGTGTGT, respectively.

Sequencings of PMCA fragments

Appropriate PCR products were extracted from agarose gels using a NB-7000 pen-touch recovery (Nippon Eido, Tokyo), and sequenced with the ABI PRISM Dye Terminator Cycler Sequencing Ready Reaction Kit using an Applied Biosystems model 373A automated sequencer.

Design of ODNs for PMCA

Phosphorothioate ODNs for PMCA were designed to target for the presumed translation initiation site (position from –6 to 12). The ODNs were purchased from Hokkaido System Science Tokyo. The ODNs had the following sequences: antisense, 5’-CATGTCGCCCATTACAAG-3’;
sense 5'-CTTGTAAATGGGCCCATG-3'; mismatch, 5'-ATCCAGATCTGCGCATCC-3'. Sense and mismatch ODNs were used as controls. The ODNs were purified by high-performance liquid chromatography, lyophilized to dryness, resuspended as stock solutions in sterile water, and stored at −20°C. In these experiments, VSMCs were incubated in serum-free medium for 48 h with or without ODNs, and the medium was replaced every 24 h.

**Measurement of \(^{45}\text{Ca}^{2+}\) efflux**

The time course of \(^{45}\text{Ca}^{2+}\) efflux from cells cultured in a plastic sheet was measured at 37°C according to Furukawa et al. (2). After cells were incubated in 0.5 ml of balanced salt solution (BSS: 146 mM NaCl, 4 mM KCl, 2 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 10 mM glucose, 0.1% bovine serum albumin, 10 mM HEPES/Tris, pH 7.4) containing 5 μCi of \(^{45}\text{Ca}^{2+}\) for 4 h at 37°C, they were rinsed with a modified BSS containing no added CaCl\(_2\) and NaCl but containing 146 mM choline chloride (Na\(^+\)- and Ca\(^{2+}\)-free BSS) for 2 min. After this washing, the time course of \(^{45}\text{Ca}^{2+}\) efflux was followed by transferring the plastic sheet to a new medium every 10 s throughout the experiments. Twenty seconds after the start of \(^{45}\text{Ca}^{2+}\) efflux measurement, the medium was switched to ones containing ionomycin, which increased [Ca\(^{2+}\)], transiently (18). The amount of \(^{45}\text{Ca}^{2+}\) lost from cells during each 10-s interval was measured by liquid scintillation counting. The protein content of cells was determined using a modified Bradford method (19).

**Measurement of intracellular \(\text{Ca}^{2+}\) concentrations**

Using the fluorescent \(\text{Ca}^{2+}\) indicator dye fura 2, intracellular Ca\(^{2+}\) concentration of VSMCs was monitored. Smooth muscle cells attached to glass cover slips were loaded with fura 2 by incubating them with 4 μM fura 2-acetoxymethylester for 40 min at 37°C in BSS. Fura 2-loaded cells were washed 3 times and then incubated with fresh BSS for an additional 20 min at 37°C to completely hydrolyze the entrapped ester. The glass cover slip was fixed to a holder which was inserted into a cuvette diagonally. The fluorescence signal was monitored at 510 nm with excitation wavelengths alternating between 340 and 380 nm using a F2000 spectrofluorometer equipped with a built-in stirrer (Hitachi, Tokyo). A high pass cut-off filter (cut off below 430 nm) was placed in front of the emission monochrometer to reduce the background signal. After each measurement, Mn\(^{2+}\) was added to a cuvette to a final concentration of 10 mM to evaluate fura 2 leakages from cells. To estimate autofluorescence from cells at 340 and 380 nm, 10 μM ionomycin was then added to completely quench the fura 2 fluorescence. The concentration of intracellular Ca\(^{2+}\) was calculated as described by Grynkiewicz et al. (20) after correction for fluorescence from extracellular fura 2, and autofluorescence was determined as described above.

**Flowcytometric analysis of cellular DNA**

VSMCs cultured in serum-free medium (Cosmedium 001) to enhance redifferentiation were further incubated with or without ODNs. Cells were harvested by trypsinization following by centrifugation. The cells were suspended in PBS containing 1 μg/ml propidium iodide and fluorescence was measured on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Reagents**

The sources of materials used in this work were as follows: Dulbecco’s modified Eagle’s medium was from Nissui Pharmaceuticals, Tokyo; Cosmedium 001 was from Cosmo Bio Inc., Tokyo; fetal calf serum was from JRH Biosciences, Lenexa, KS, USA; Immobilon PVDF membrane was from Millipore Co., Bedford, MA, USA; monoclonal antibody clone 5F10 was from Affinity Bioreagents, Neshanic Station, NJ, USA; goat anti-mouse IgG and CSPD were from Bio-Rad, Hercules, CA, USA; RT-PCR high and rTaq DNA polymerase were from Toyobo, Osaka; ABI PRISM Dye Terminator Cycler Sequencing Ready Reaction Kit was from PE Biosystems, Foster City, CA, USA; bovine serum albumin (fatty acid-free) was from Sigma-Aldrich Co., St. Louis, MO, USA; ionomycin was from Calbiochem-Novabiochem Co., San Diego, CA, USA; fura 2-acetoxymethylester was from Dojindo Laboratories, Kumamoto; \(^{45}\text{CaCl}_2\) was from Du Pont-New England Nuclear, Boston, MA, USA. FK506 was generously provided by Fujisawa Pharmaceutical Co. Ltd., Osaka. All other chemicals were of analytical grade.

**Statistics**

The values given in figures are mean ± S.E.M., unless otherwise stated, and the statistical analyses were performed with ANOVA and Student’s t-test. A value of \(P<0.05\) was considered statistically significant. The n value indicates the number of animals.

**RESULTS**

**Effect of antisense ODNs on the expression of PMCA protein**

In several cases, the most effective antisense ODNs were targeted to the translation initiation site of mRNA. For this reason, we have chosen a sequence in the translation initiation site of the PMCA. The sequences of the antisense, the sense (reverse 5’ to 3’ orientation compared antisense), and the mismatched ODNs are shown in Materials and Methods. The primary cultured rat VSMCs were treated in parallel with or without ODNs. In order to examine the effect of antisense ODNs on PMCA, we detected PMCA protein by
Western blot analysis using 5F10 that was able to detect PMCA in rat VSMCs (20). The expression of the PMCA protein was inhibited by antisense ODNs in a concentration-dependent manner (Fig. 1). Incubation time longer than 48 h was required for sufficient inhibition. Up to 75% of the control (without ODNs) was inhibited by anti PMCA1 ODNs (20 μM) after 48-h incubation. However, the treatment with ODNs did not affect the expression of Na⁺/K⁺-ATPase protein. Furthermore, there was no significant difference in PMCA protein expression between cells treated with control and sense or mismatch ODNs (Fig. 2). In addition, all the ODNs did not seem to have an effect on cell morphology, as observed by phase-contrast microscopy and total protein content of cells (data not shown).

Effect of antisense ODNs on the expression of PMCA mRNA

Antisense ODNs may bind to and block the translation of mRNAs by several putative mechanisms including simply blocking ribosomal reading or by activating RNaseH, an endogenous enzyme that selectively degrades mRNA. So,
we examined whether antisense ODNs would block the expression of the PMCA mRNA. The RT-PCR analyses using isoform specific primers have confirmed that PMCA1b, 4a and 4b are present and PMCA2 and PMCA3 are absent in rat VSMCs in primary culture, as previously reported (21). Quantitative RT-PCR analysis was carried out to quantify each PMCA isoform in VSMCs. Appropriate cycles of PCR were determined in the exponential phase for amplification of each cDNA from the cells (17). The quantity of each PMCA isoform was expressed as a ratio to the quantity of house keeping gene (G3PDH) amplified from an aliquot of the same RT reaction products. Quantitative RT-PCR analyses showed that the ratio of PMCA isoforms (PMCA1b, 4a, 4b) expressed in VSMCs were 76:5:19, confirming that PMCA1b is the major isoform of PMCA (Fig. 3). Antisense ODNs decreased only PMCA1b mRNA to 30% of the level in control cells (Fig. 4).

Effects of antisense ODNs on the \([\text{Ca}^{2+}]\) mobilization

We also investigated the effects of ODNs on other regulatory mechanisms for \([\text{Ca}^{2+}]\). Major isoforms of SERCA and NCX were type 2b and type 1, respectively (data not shown). However, neither SERCA type 2b nor NCX type 1 changed their mRNA expression by the treatment with the antisense ODNs, suggesting that anti-PMCA antisense ODNs had blocked specifically not only the expression of the PMCA protein but also that of mRNA (Fig. 4). Figure 5 shows the effect of antisense ODNs on \(\text{Na}^{+}\)-independent \(^{45}\text{Ca}^{2+}\) efflux from rat VSMCs in primary culture. The time course of ionomycin (final concentration of 0.5 \(\mu\)M) induced \(^{45}\text{Ca}^{2+}\) efflux was consistently lower in antisense ODNs treated cells than in sense ODNs or control cells. Furthermore, unstimulated \(^{45}\text{Ca}^{2+}\) efflux (basal efflux) was significantly lower in antisense ODNs treated cells. Modification of \(\text{Ca}^{2+}\) efflux must have had an influence on...
Apoptosis Induced by Ca^{2+}-Pump Antisense

Table 1. Effect of ODNAs on intracellular Ca^{2+} concentrations of primary cultured VSMCs

| ODNs          | Intracellular Ca^{2+} concentration (nM) |
|---------------|-----------------------------------------|
|               | resting | ionomycin-stimulated | ATP-stimulated |
| Control       | 83 ± 21 | 201 ± 56             | 463 ± 97       |
| Antisense, 20 μM | 149 ± 33* | 568 ± 87*           | 865 ± 114*     |
| Mismatch, 20 μM | 88 ± 45   | 239 ± 52             | 447 ± 132      |
| Sense, 20 μM  | 92 ± 22  | 196 ± 39             | 423 ± 86       |

Control or ODNs-treated VSMCs were loaded with fura 2 as described in “Materials and Methods”. Then [Ca^{2+}], was measured at the resting state and ionomycin (0.5 μM)- or ATP (30 μM)-stimulated state. Resting and stimulated levels indicated the average value during 1 min just before the addition of ionomycin (0.5 μM) or ATP (30 μM) and the peak value of Ca^{2+} transient induced by the addition of ionomycin or ATP, respectively. Data are the mean ± S.E.M. *P<0.05 (n = 5), different from the control.

Fig. 6. Flowcytometric analysis of cell cycle progression in rat aortic smooth muscle cells stained with propidium iodide. VSMCs were harvested by trypsinization and stained with propidium iodide, and then DNA content and cell cycle of VSMCs were analyzed with flowcytometer on the fluorescence intensity of propidium iodide. They were treated with 20 μM antisense (B), sense (C) or mismatch-sense (D) ODNs for PMCA for 96 h before the harvesting procedures. Forty-thousand cells were used for each assay.

<Diagram of flow cytometry showing cell cycle progression in VSMCs treated with different ODNs">

[Ca^{2+}]. Fura 2 was loaded onto VSMCs and [Ca^{2+}], was measured at resting and ionomycin- or ATP-stimulated states (Table 1). Resting level of [Ca^{2+}] in VSMCs treated with antisense ODNs increased significantly compared with cells treated with sense or mismatch ODNs and control cells. Significant difference at ionomycin- or ATP-stimulated states was also observed between them. Antisense-treatment induced apoptosis of VSMCs [Ca^{2+}], plays a central role in apoptotic processes (8, 22 – 24). Effects of PMCA inhibition by antisense ODNs on viability of VSMCs were investigated. Cellular DNA of VSMCs was stained with propidium iodide and then the content of DNA was measured using a FACScan flowcytometer. Figure 5 shows representative DNA histograms recorded after staining of VSMCs with propidium iodide.
Control cells had two peaks and transit between them corresponding to cell cycle phases, G2/M, G0/G1 and S, respectively (Fig. 6A). VSMCs treated with sense or mismatch ODNs had the same profile (Fig. 6C and D). However, antisense ODNs treated cells had an additional peak with lower fluorescence intensity (Fig. 6B). This sub G1 peak corresponds to apoptotic cells (25). This peak was significantly reduced by the treatment of VSMCs with 10 nM FK506 for 24 h (Fig. 7).

DISCUSSION

A critical problem in assessing the physiological role of the PMCA has been the absence of a selective and specific inhibitor. We therefore explored the possibility that antisense ODNs might selectively and specifically suppress the PMCA expression. The results in this study demonstrate that the designed antisense ODNs can sufficiently inhibit the expression of the PMCA in rat VSMCs in primary culture. This approach therefore enables us to resolve and identify the cellular function of the PMCA.

We used VSMCs isolated from rat aorta in primary culture. It has been reported that VSMCs undergo a spontaneous modulation of phenotype from a contractile state to a synthetic one (26), and electrical responses to several agonists alter during culturing (27). When attempting to examine the various physiological functions of VSMCs, it is desirable to use the cell cultures that approximate in vivo conditions as closely as possible. VSMCs in primary culture closely maintain the natural character of smooth muscle cells in their in vivo state.

Antisense ODNs inhibited the expression of the PMCA protein in VSMCs, whereas another cation-transport system in the plasma membrane, \( \text{Na}^+ / \text{K}^+ \) ATPase, was not affected by the antisense treatment. PMCA protein was not affected by either sense or mismatch ODNs. Furthermore, all the ODNs did not seem to have an effect on cell morphology, as observed by phase-contrast microscopy and total protein content of cells (S. Sasamura et al., unpublished data). Antisense ODNs also reduced the amount of PMCA mRNA but not SERCA-2b or NCX1 expressed in VSMCs. Antisense ODNs exert their inhibitory effects on mRNA function by a variety of mechanisms, including inhibition of RNA-splicing, inhibition of protein translation by disrupting ribosome assembly, and RNase-H-mediated cleavage of target mRNA. Because both mRNA and protein of PMCA were reduced, it is suggested that RNase-H recognized duplexes of mRNA-antisense ODNs and selectively cleaved the hybridized mRNA, resulting in the reduction of PMCA protein expression. Antisense ODNs also have an advantage over the use of the overexpression of the PMCA which induces down-regulation of SERCA and the other \( \text{Ca}^{2+} \) regulatory systems (10–12).

In the experiment of Fig. 5, we studied the effect of antisense on \( \text{Na}^+ \)-independent \( \text{Ca}^{2+} \) efflux from VSMCs under the condition of having blocked the expression of the PMCA. \( \text{Ca}^{2+} \) efflux from cultured aortic smooth muscle cells is effected mainly by two distinct extrusion systems, the \( \text{Na}^+ / \text{Ca}^{2+} \) exchanger and PMCA, and \( \text{Na}^+ \)-independent \( \text{Ca}^{2+} \) efflux from VSMCs is catalyzed by PMCA (2, 28, 29). In the absence of \( \text{Na}^+ \), stimulated and unstimulated \( 45 \text{Ca}^{2+} \) efflux from VSMCs exposed to antisense ODNs compared to control cells; i.e., sense ODNs treated or untreated cells. On the other hand, \( [\text{Ca}^{2+}]_i \) of antisense-treated cells in both stimulated and unstimulated states was markedly increased compared to that in control cells. These results suggest that PMCA plays an important role in regulation of \( [\text{Ca}^{2+}]_i \) of VSMCs not only at the resting state but also during \( \text{Ca}^{2+} \) mobilization.

Increase in \( [\text{Ca}^{2+}]_i \) induces apoptosis and \( \text{Ca}^{2+} \) channel blockers such as verapamil prevent apoptosis (22, 23),

![Fig. 7. Effect of FK506 on the occurrence of apoptosis in VSMCs.](image-url)
suggesting an important role of Ca\textsuperscript{2+} in the induction of apoptosis. Leszczynski et al. have suggested that apoptosis of the VSMCs may be regulated by several pathways with or without DNA fragmentation (30). Calcineurin, a serine/threonine protein phosphatase, participates in signaling cascades essential for various biological functions including apoptosis (31). It is specifically activated by sustained elevation of [Ca\textsuperscript{2+}], and inhibited by FK506, a potent immunosuppressant (32). Prolonged treatment of VSMCs with antisense ODNs induced the appearance of a population of cells in sub G1 phase having low fluorescence intensity of propidium iodide. The sub G1 phase was significantly reduced by pretreatment of VSMCs with FK506. It is therefore suggested that the inhibition of PMCA with antisense ODNs causes apoptosis of VSMCs with DNA fragmentation via Ca\textsuperscript{2+}-dependent calcineurin signaling cascades. However, the details of the mechanism of apoptosis induced by the inhibition of PMCA remains to be elucidated.

The data presented in this study demonstrates that the effects of antisense ODNs are the result of decreases in the expression of PMCA protein and mRNA. The antisense ODNs did not affect the expression of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase protein, SERCA-2b mRNA and NCX1 mRNA. Designed antisense ODNs can be effectively used to knock down the PMCA function in VSMCs. On the other hand, sense and mismatch ODNs did not affect the expression of the PMCA protein. Thus, antisense ODNs directed against the PMCA may provide a very useful and specific tool to further elucidate its physiological role in VSMCs as well as in various cell types relying on cellular Ca\textsuperscript{2+} signaling mechanisms (33).

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