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Short communication

Inhibition by antisense oligonucleotides of plasma membrane Ca^{2+} ATPase in vascular endothelial cells

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Abstract

Antisense oligodeoxynucleotides were used to knock down plasma membrane Ca^{2+} ATPase, and the role of plasma membrane Ca^{2+} ATPase was investigated in human aortic endothelial (HAE) cells. The peak of thapsigargin-evoked intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was higher in antisense-treated than in untreated cells, but the declining time course was unaffected by the antisense treatment. The declining time was prolonged in both antisense-treated and untreated cells by reducing external Na^+, but the prolongation was more marked in the antisense-treated cells. These results provide the evidence of a functional role of plasma membrane Ca^{2+} ATPase, although other mechanisms including Na^+/Ca^{2+} exchange may play the primary role in regulating $[Ca^{2+}]_i$. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Antisense oligodeoxynucleotide; Na^+/Ca^{2+} exchange; Ca^{2+} pump; Ca^{2+} concentration, Intracellular; Thapsigargin

1. Introduction

In vascular endothelial cells, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in response to various vasoactive substances and mechanical stimulation, causing synthesis and release of endothelial active molecules (Davies, 1995; Inagami et al., 1995). The elevated $[Ca^{2+}]_i$ is in turn reduced by the re-uptake into the internal stores and/or by the extrusion via the plasma membrane (Dolor et al., 1992; Marin et al., 1999). We have already suggested that both Na^+/Ca^{2+} exchange and plasma membrane Ca^{2+} ATPase participate in reducing $[Ca^{2+}]_i$ in human aortic endothelial (HAE) cells (Goto et al., 1996). However, it is still unclear up to what extent $[Ca^{2+}]_i$ can be regulated by each mechanism. In this study, HAE cells were treated with antisense oligodeoxynucleotides targeting the 5′ translation start site of the human plasma membrane Ca^{2+} ATPase isoform 1 (Verma et al., 1988). Using this, we have investigated the physiological role of plasma membrane Ca^{2+} ATPase in regulating $[Ca^{2+}]_i$ in HAE cells.

2. Materials and methods

2.1. Endothelial cell culture with antisense oligodeoxynucleotides

The sequence of antisense oligodeoxynucleotides consisted of 18 mer (5′-CATGTCGCCCATTACAAG-3′), which was designed to target the 5′ translation start site of isoform 1 of human plasma membrane Ca^{2+} ATPase (Verma et al., 1988). The specificity of antisense oligomers was confirmed by comparison with all other sequences in GenBank with the use of the Basic Local Alignment Search Tool (BLAST). HAE cells, purchased from Clonetics (San Diego, CA, USA), were seeded on coverslips (9×9 mm) with a cell density of approximately 5000 cells/cm^2, and incubated in endothelial basal medium (Clonetics) for 24 h. The cells were divided into two groups; i.e., control and antisense-treated cells. In control cells, the medium was exchanged every 48 h for 4 days. In the antisense-treated cells, the culture medium containing 20 μM antisense oligodeoxynucleotides was exchanged every 12 h for 4 days. The measurement of $[Ca^{2+}]_i$ and the Western blot analysis were carried out on the fifth culture day.

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2.2. Western blot analysis

Samples containing approximately 10^5 cells were loaded on a 7% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Blots were first incubated overnight with non-fat milk, and then incubated with anti-plasma membrane Ca^{2+} ATPase (5F10, Sigma, USA) and anti-Na^+/K^+ ATPase. The antigen–antibody complex was detected by incubation with alkaline phosphatase-labeled anti-IgG antibody (Bio-Rad Laboratories, CA, USA). After photodetection of chemiluminescence on film (ECL, Amersham, USA), densitometry was performed. To quantify the amount of plasma membrane Ca^{2+} ATPase expressed in each sample, the ratio of photodensity for anti-plasma membrane Ca^{2+} ATPase to photodensity for anti-Na^+/K^+ ATPase was calculated.

2.3. $[Ca^{2+}]_i$ measurement using fura 2

The methods for measuring endothelial $[Ca^{2+}]_i$ have been described in detail (Goto et al., 1996; Nakao et al., 1999). In brief, the cells on coverslips were loaded with 5 μM fura 2 acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan) and $[Ca^{2+}]_i$ measurement was performed in a single cell. The excitation wavelength was alternated at 400 Hz between 340 and 380 nm and the emission fluorescence at 500 nm was measured using a spectro-photofluorimeter (CAM-230, Japan Spectroscopic, Tokyo, Japan). The emission intensities at 340 and 380 nm excitation (F_{340} and F_{380}) were sampled onto the hard disc of the computer running the Superscope software (GW instruments, MA, USA). $[Ca^{2+}]_i$ was calculated from the following equation described by Grynkiewicz et al. (1985). $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$, where K_d is the dissociation constant (224 nM for fura 2 at 37°C), R is F_{340}/F_{380} (the ratio of relative fluorescence), R_{min} and R_{max} are the F_{340}/F_{380} values measured in Ca^{2+}-free (4 mM EGTA) HEPES-buffered saline (HBS) and by the addition of 10 μM ionomycin to HBS, respectively, and S_{f2}/S_{b2} is the ratio of fluorescence intensities measured at excitation wavelength 380 nm in the Ca^{2+}-free and ionomycin-containing solutions.

The results were expressed as the means ± S.E.M. The statistical analysis was performed using Student's non-paired *t*-test, and values of $P < 0.05$ were considered statistically significant.

2.4. Solution and drugs

HBS contained (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, glucose 11.1 and HEPES 5.0 (pH = 7.4). The Ca^{2+}-free solution was made by simply omitting $CaCl_2$ from HBS. In Na^+-free solution, NaCl in HBS was totally replaced with equimolar *N*-methyl-D-glucamine plus HCl (pH = 7.4). The bath solution was warmed by the use of a water jacket around the perfusing tube, so that the temperature of the chamber was kept at 37°C. The flow rate of the bath perfusion was 2–3 ml/min and the solution exchange was completed within a few seconds.

3. Results

3.1. Immunological evidence for knock down of Ca^{2+} ATPase

Fig. 1A shows that both plasma membrane Ca^{2+} ATPase and Na^+/K^+ ATPase are present in cultured HAE cells. Antisense-treated cells exhibited a significant decrease in plasma membrane Ca^{2+} ATPase expression but not in Na^+/K^+ ATPase. No significant change in the Na^+/K^+ ATPase expression was confirmed by measuring photodensity for anti-Na^+/K^+ ATPase. The normalized value was 100 ± 7.1% in control and 119 ± 3.9% in antisense-treated cells ($N = 4$, NS). The amount of plasma membrane Ca^{2+} ATPase expressed in each sample was evaluated by calculating the ratio of photodensity for anti-plasma membrane Ca^{2+} ATPase to photodensity for anti-Na^+/K^+. On average of four experiments, the antisense treatment inhibited the expression of plasma membrane Ca^{2+} ATPase by 80.5 ± 4.2%.

3.2. Resting $[Ca^{2+}]_i$ under inhibition of Ca^{2+} ATPase activity

The resting $[Ca^{2+}]_i$ was measured when the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was changed from 1 to 0 mM. At 1 mM $[Ca^{2+}]_o$, the resting $[Ca^{2+}]_i$ was 35.1 ± 1.7 nM ($n = 21$) and 33.5 ± 1.7 nM ($n = 13$) in the control and antisense-treated cells, respectively (NS). At 0 mM $[Ca^{2+}]_o$, the $[Ca^{2+}]_i$ was 35.8 ± 1.6 nM ($n = 24$) and 32.2 ± 1.7 nM ($n = 16$) in the control and antisense-treated cells, respectively (NS). The results were summarized in Table 1.

3.3. Thapsigargin-induced $[Ca^{2+}]_i$ transient in antisense-treated cells

The role of plasma membrane Ca^{2+} ATPase in the declining phase of $[Ca^{2+}]_i$ elevated by 100 nM thapsigargin was examined. Thapsigargin has been shown to inhibit the endoplasmic reticulum Ca^{2+} ATPase without affecting the formation of inositol triphosphate (Takemura et al., 1989; Thastrup et al., 1990). To eliminate the influence of the Ca^{2+} entry from extracellular space, Ca^{2+} was removed from the extracellular solution. Under this condition, HAE cells exhibit a transient response of $[Ca^{2+}]_i$ to thapsigargin (Hosoki and Iijima, 1995; Goto et al., 1996), just like the one demonstrated in Fig. 1Ba. Qualitatively, similar response was recorded in the antisense-treated cells (Fig. 1Bb). The quantitative analysis was made by measur-

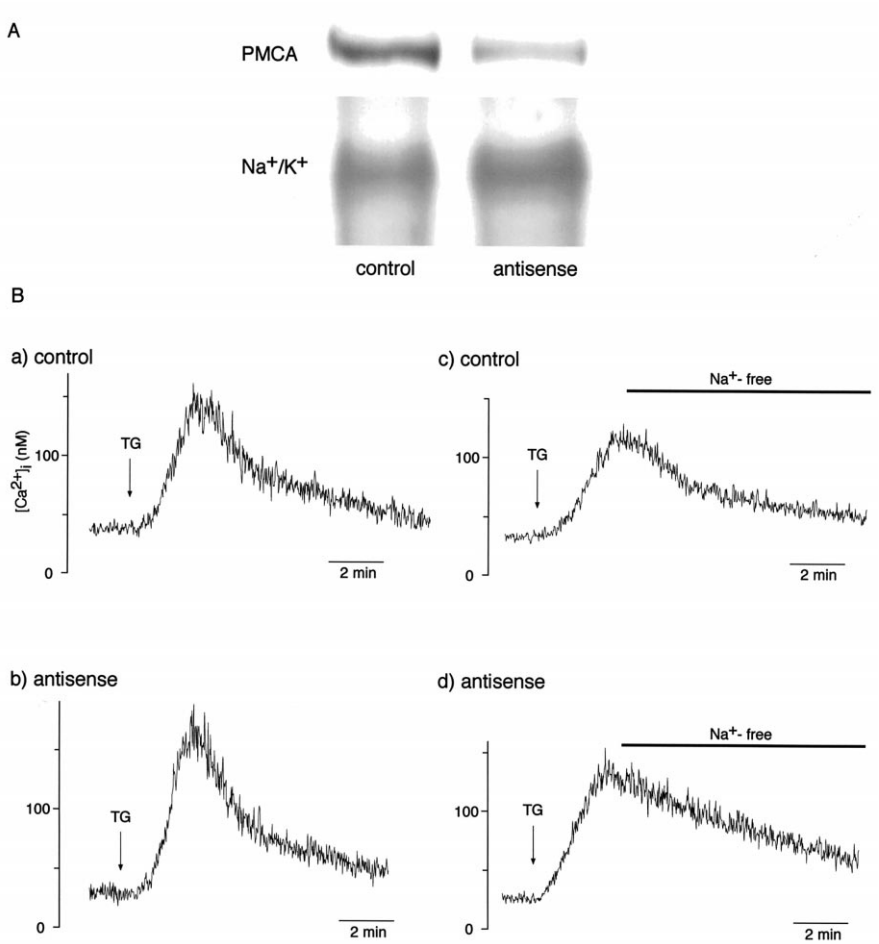

Fig. 1. (A) Western blot analysis of plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/K^+ ATPase proteins (Na^+/K^+) in HAE cells. Left band: control. Right: cells treated with antisense oligodeoxynucleotides. (B) The $[Ca^{2+}]_i$ response to 100 nM thapsigargin (TG) recorded in control (a) and antisense-treated cells (b). The external solution is Ca^{2+} free. Effect of Na^+-free solution on the control cell (c) and antisense-treated cells (d). Na^+-free solution was added immediately after the peak of thapsigargin-induced Ca^{2+} transient.

ing the 50% increasing time, the peak $[Ca^{2+}]_i$ and the 50% declining time. On the average, the 50% increasing time was 86.4 ± 6.9 s ($n = 12$) and 82.6 ± 8.9 s ($n = 6$) in the control and antisense-treated cells (NS). The peak $[Ca^{2+}]_i$ was 139.9 ± 6.2 nM ($n = 24$) and 165.2 ± 9.4 nM ($n = 16$) in the control and antisense-treated cells, respectively ($P < 0.05$). The 50% declining time was 127.2 ± 12.3 s ($n = 12$) and 119.0 ± 13.0 s ($n = 6$) in the control and antisense-treated cells, respectively (NS). These results suggest that plasma membrane Ca^{2+} ATPase functions only at a higher $[Ca^{2+}]_i$ and has little role during the declining phase of $[Ca^{2+}]_i$.

3.4. Effect of Na^+-free solution on thapsigargin-induced declining phase of $[Ca^{2+}]_i$

The declining phase of $[Ca^{2+}]_i$ should be influenced not only by plasma membrane Ca^{2+} ATPase but also

Table 1
Effect of antisense oligodeoxynucleotides on the resting $[Ca^{2+}]_i$, the peak $[Ca^{2+}]_i$ and 50% declining time during 100 nM thapsigargin
$[Na^+]_o$: external Na^+ concentration. Values are means ± S.E.M. of each experiment. The number of cells is indicated in parentheses.

	Resting $[Ca^{2+}]_i$ (nM)		Peak $[Ca^{2+}]_i$ (nM)	50% Declining time (s)	
	1 mM $[Ca^{2+}]_o$	0 mM $[Ca^{2+}]_o$		Normal $[Na^+]_o$	Na^+ free
Control	35.1 ± 1.7 (21)	35.8 ± 1.6 (24)	139.9 ± 6.2 (24)	127.2 ± 12.3 (12)	186.6 ± 28.3 (6)
Antisense	33.5 ± 1.7 (13)	32.2 ± 1.7 (16)	165.2 ± 9.4 (16)[a]	119.0 ± 13.0 (6)	323.8 ± 12.1 (5)[a]

[a] $P < 0.05$ denotes significant difference from control.

Na^+/Ca^{2+} exchange (Goto et al., 1996). To exclude Na^+/Ca^{2+} exchange, the Na^+-free solution was applied on top of the thapsigargin-induced $[Ca^{2+}]_i$ response. In support of the role of Na^+/Ca^{2+} exchange, the declining phase was significantly prolonged by reducing external Na^+ both in control and antisense-treated cells (Fig. 1Bc and Bd). Furthermore, a more marked prolongation was observed in the antisense-treated cells (Fig. 1Bd). The time required for 50% relaxation was 186.6 ± 28.3 s ($n = 6$) in control cells and 323.8 ± 12.1 s ($n = 5$) in antisense-treated cells. The results clearly show that the role of plasma membrane Ca^{2+} ATPase isoform 1 to the declining phase becomes obvious when Na^+/Ca^{2+} exchange was suppressed.

4. Discussion

4.1. Regulation of $[Ca^{2+}]_i$ at resting condition in HAE cells

Plasma membrane Ca^{2+} ATPase is a multigene family formed by at least four gene products (Marin et al., 1999). Among the four isoforms, isoform 1 is known to exist ubiquitously in most tissues in contrast to the other isoforms — isoform 2, isoform 3 and isoform 4, which are present in a more restricted expression pattern (Marin et al., 1999). The antibody used in the present study is targeted to amino acids 724 to 783 of the sequence of human plasma membrane Ca^{2+} ATPase isoform 1. Since the sequence of this region is conserved among four isoforms, the Western blot analysis should represent the total amount of various plasma membrane Ca^{2+} ATPase in HAE cells. Thus, the experimental finding that the treatment of antisense oligodeoxynucleotides resulted in 80% inhibition of Ca^{2+} ATPase may indicate that isoform 1 is a major component of plasma membrane Ca^{2+} ATPase in HAE cells.

In spite of the marked inhibition of plasma membrane Ca^{2+} ATPase, however, the resting $[Ca^{2+}]_i$ was not affected by the treatment of antisense oligodeoxynucleotides. The result is in sharp contrast to the previous finding that the resting $[Ca^{2+}]_i$ was markedly increased in vascular smooth muscle cells when plasma membrane Ca^{2+} ATPase isoform 1 was knocked down by using antisense oligodeoxynucleotides (Furukawa and Ohizumi, 1995). We consider that, since the background Ca^{2+} influx is negligibly small in HAE cells (Ono et al., 1998), $[Ca^{2+}]_i$ might have remained changed a little even if plasma membrane Ca^{2+} ATPase was inhibited by antisense oligodeoxynucleotides. If the inhibition of plasma membrane Ca^{2+} ATPase could cause a slight $[Ca^{2+}]_i$ elevation, such an excess of Ca^{2+} might have been taken up by intracellular organelles and/or extruded through the plasma membrane by mechanism(s) other than plasma membrane Ca^{2+} ATPase. This idea is supported by the previous finding that 2 μM vanadate, which inhibits plasma membrane Ca^{2+} ATPase, caused an increase in $[Ca^{2+}]_i$ for 10 min but thereafter returned to the basal level (Goto et al., 1996). Alternatively, different isoforms of plasma membrane Ca^{2+} ATPase might exist and function as a physiological Ca^{2+} extrusion system in endothelial cells.

4.2. Extrusion mechanism of $[Ca^{2+}]_i$ elevated by thapsigargin

It has been reported that Na^+/Ca^{2+} exchange exists in vascular endothelial cells (Sage et al., 1991; Li and van Breemen, 1995) and contributes to the sequestration of $[Ca^{2+}]_i$ elevated by thapsigargin or ATP (Goto et al., 1996). In the present study, the antisense-treatment caused little difference in the declining phase of the thapsigargin-evoked Ca^{2+} transient, and the reduction of $[Na^+]_o$ prolonged the Ca^{2+} transient both in control and antisense-treated cells. The findings indicate that the major mechanism for extruding Ca^{2+} is not plasma membrane Ca^{2+} ATPase, but Na^+/Ca^{2+} exchange. This view is in line with the recent report that, in the absence of extracellular Na^+, ATP evoked a sustained $[Ca^{2+}]_i$ rise by cyclopiazonic acid in rat brain endothelial cells (Dömötör et al., 1999). But we never deny the possibility that plasma membrane Ca^{2+} ATPase isoform 1 can also contribute to the extrusion of Ca^{2+} during thapsigargin stimulation in HAE cells. In fact, the peak $[Ca^{2+}]_i$ in response to thapsigargin significantly increased in antisense-treated cells as compared to control cells. Furthermore, the reduction of $[Na^+]_o$ resulted in a more marked prolongation of the Ca^{2+} transient in the antisense-treated cells. It should also be noted that we only measured the global levels of Ca^{2+} within the endothelial cells in this study. It is certainly possible that changes in plasma membrane Ca^{2+} ATPase could affect sub-sarcolemma levels of Ca^{2+}, which might not be detected using fura 2 fluorescence. The present results indicate that, under the physiological condition, both plasma membrane Ca^{2+} ATPase and Na^+/Ca^{2+} exchange are functional and plasma membrane Ca^{2+} ATPase may play a supplemental role for regulating $[Ca^{2+}]_i$, and that an increase in Ca^{2+} is likely to be extruded primarily by Na^+/Ca^{2+} exchange.

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