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Original Article

Role of SERCA2b in mobilization of nuclear Ca^{2+} in HeLa cells

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Ca^{2+} liberation from the endoplasmic reticulum activates sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to return Ca^{2+} to storage. We explored the role of SERCA in dynamic changes of intranuclear Ca^{2+} in single HeLa cells. Application of forskolin, as an activator of SERCA, caused the phosphorylation of SERCA2b but not SERCA3 on serine residues, which increased the rate of Ca^{2+} uptake. Forskolin also induced the changes of Ca^{2+} movement pattern in the nucleus when cells were stimulated with the Ca^{2+}-releasing agents, histamine or A23187. Immunofluorescence staining showed that SERCA2b was densely populated on *special parts* of the nuclear envelope, but SERCA3 only existed in endoplasmic reticulum. Injection of an anti-SERCA2 antibody into the cytoplasm blocked the rise in the nuclear Ca^{2+} concentration ($[Ca^{2+}]_n$). However, injection of an anti-SERCA3 antibody did not affect the initiation of Ca^{2+} oscillations in the nucleus. Our data suggest that the activated-SERCA2b elevates the rate of uptake of free Ca^{2+} into stores along the nuclear envelope, which might support and maintain the nuclear Ca^{2+} homeostasis.

Key words: SERCA2b, nuclear Ca^{2+}, forskolin, *special parts* of nuclear envelope, HeLa cell

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Abbreviations: SERCA: sarco-endoplasmic reticulum Ca^{2+}-ATPase, $[Ca^{2+}]_n$: nuclear Ca^{2+} concentration, $[Ca^{2+}]_c$: cytoplasmic Ca^{2+} concentration, DBHQ: 2,5-di-ter-butyl-1,4-benzohydrorequinoe, BAPTA: bis-(o-aminophenoxy)-N,N,N′,N′-tetra-acetic acid, $DiOC_6(3)$:3,3′-dihexiloxadicarbocyanine

Introduction

Ca^{2+} liberation from stores in the endoplasmic reticulum activates the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to return free Ca^{2+} to storage. The SERCA family in the non-excitable cells contains SERCA2b and SERCA3. Lee and his colleagues[1] indicated that SERCA3 is distributed in the basal pole of pancreatic and salivary gland, whereas SERCA2b is expressed in the nuclear envelope and the lumenal pole of salivary gland cells. Previous studies have demonstrated a wide range of functions of SERCA both in physiology and pathology, like cell growth control[2-4]. Furthermore, the function of SERCA2b and SERCA3 are different. SERCA3 is closely associated with hypertension and heart failure[5-7]. It is more resistant to peroxide stimulation than SERCA2b[8], and it can be suppressed by nitric oxide in smooth muscle cells[9-10]. In contrast, overexpression of SERCA2b induces repetitive Ca^{2+} oscillations. It can be modulated by calreticulin and calnexin within the endoplasmic reticulum and the nuclear envelope[11-13]. In addition, the levels in mRNA and protein of SERCA2b are increased upon endoplasmic reticulum-stress treatments[14].

Free Ca^{2+} gradients across the nuclear membrane were firstly found by Williams and his co-workers[15]. Subsequently, many studies have shown that such gra-

dients were present not only in resting cells, but also in agonist-stimulated cells[16-21]. Recently, several sources of nuclear Ca^{2+} have been suggested[22-26]. There may come from (i) a primary Ca^{2+} signal originating *from* the lumen of the nuclear envelope, (ii) a secondary Ca^{2+} signal originating *from* the cytoplasm or the immediate perinuclear vicinity of the nuclear envelope. These signals pass though nuclear pore by passive diffusion of a Ca^{2+} waves and/or puffs. However, until now, it is unclear whether the role of SERCA involves in the mobilization of nuclear Ca^{2+}. In order to clarify this question, we explored the dynamic change of intranuclear Ca^{2+} in single HeLa cells using forkolin as an activator of SERCA. We also observed the distribution of SERCA in the nucleus, including special parts of the nuclear envelope.

Materials and Methods

Cell culture

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (BIGCOBRL, Grand Island, NY) supplemented with 10% (V/V) heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS) and were maintained in a 5% CO_2 incubator at 37°C. For Ca^{2+} image studies, cells were seeded at a low density on glass-bottom 35-mm diameter culture dishes for one day.

Solutions and materials

Experiments were performed with the Tyrode's solution (pH 7.4) containing 135 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 0.50 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5.0 mM HEPES, 5.55 mM Glucose, and 0.1% bovine serum albumin. Fluo-3-AM, BAPTA-AM, A23187 and $DiOC_6(3)$ were obtained from Molecular Probes (Eugene, OR). Histamine and forskolin were from Wako Pure Chemical Industries Inc. (Osaka, Japan). DBHQ, anti-SERCA3b (clone No. PL/IM 430) and anti-SERCA2 (clone No. IID8) mouse monoclonal antibodies were purchased from BIOMOL Research Lab. Inc. (Plymouth Meeting, PA). Anti-phosphoserine antibody and FITC-conjugated ConA was from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) or ethanol was used as the solvent.

Confocal calcium imaging experiments

Cells were loaded with fluo-3-AM (1-2 μM) for 30 min to 45 min at 37°C. When cells were co-loaded with BAPTA-AM (2 μM), a chelator was added at the same time. After cells were washed three times with Tyrode's solution and incubated for 10 min, the changes in intracellular Ca^{2+} distribution were observed using a confocal laser scanning microscope system (Anchored Cell Analysis and Sorting System, model ACAS570, Meridian Instruments Inc., Okemos, MI) with an objective lens (Dapo 1 00 UV / 1.30 oil, Olympus). Fluo-3 was excited with an argon laser at 488 nm. The scan frame of fluorescence images was sized at 50×50 pixels on 0.7 μm-thick sections allowing a scan time of 5.4-5.7 sec/frame. In some experiments, a two-step-program on scan time (10.0 sec and 5.7 sec) was used in one continuous measurement. To improve the signal-to-noise ratio, we averaged more than 40 consecutive video frames to obtain each image. After the measurement was completed, the locations of the cytoplasm and nucleus were immediately differentiated according to the distribution of $DiOC_6(3)$ staining. For analysis of nuclear and cytoplasmic calcium kinetics, cells were divided into the nucleus and the cytoplasm based on the distribution of $DiOC_6(3)$ staining.

Western blotting and Immunoprecipitation

For immunoprecipitation, cells were scraped and were mixed with an equal volume of lysis buffer (RIPA buffer: 150 mM NaCl, 1.0% NP-40, 1% sodium deoxychloate, 0.1% SDS, 0.1 mM DTT, 0.05 mM PMSF, 0.002 mg/ml aprotinin, 0.002 mg/ml leupeptin). Aliquots of the listed material were incubated at 4°C overnight with protein-A plus protein-G agarose beads, which were precoated with SERCA2 or SERCA3 antibody, in the TBS-T buffer including 5% lipid-free skim milk (pH 7.6, 20 mM tris base, 137 mM NaCl, 0.1% Tween 20 and 5% SKIM milk). The beads were removed by centrifugation in a microtube, washed with PBS, and digested in SDS sample buffer. For western blotting, the sample was subjected to SDS-PAGE on a 7.5% mini gels and then electrobloted to nitrocellulose membranes. The nitrocellulose membranes were incubated with monoclonal anti-phosphoserine antibody (dilution 1:500), and then incubated with a peroxidase-labeled anti-mouse IgG secondary antibody (dilution 1:1000). The immunoreactive antibodies were visualized using the ECL detection system from Amersham.

Immunofluorescence staining

The cells were plated at low densit on glass-bottom 35-mm diameter culture dishes for one day. Cells were fixed in sucrose-cacodylate buffer (0.1 M sodium

cacodylate, 0.1 M sucrose, 0.25% glutaraldehyde) for 15 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS). The cells were blocked with 1% bovine serum albumin in PBS for 30 min at room temperature. To detect SERCA2b, SERCA3, cells were reacted with anti-SERCA2, anti-SERCA3 mouse monoclonal antibodies for 2 hrs, and then reacted with FITC-labeled goat anti-mouse IgG, TRITC-labeled goat anti-mouse IgG secondary antibodies for 60 min in a dark room. The cells were visualized using a laser scanning confocal microscope (ZEISS, LSM510).

Microinjection

The microinjection system consists of an Eppendorf micromanipulator 5171, a microinjector 5246 and glass micropipettes (Femtotis II, Eppendorf) with an inner tip diameter of about 0.5 μm. The holding pressure was 10 hPa, the injection pressure was 500 hPa, and the injecton time was 0.5 sec. Anti-SERCA2 (diluted 1/10) or anti-SERCA3b (diluted 1/10) mouse monoclonal antibodies, buffered with HEPES (10 mM, pH 7.2), were used in glass capillaries. After microinjection, the HeLa cells were placed in an incubator for 1 to 2 hrs and then Fluo-3-AM was loaded for calcium recording.

Results

Forskolin stimulates SERCA activity

The first step in this study was to examine the effect of forskolin on the Ca^{2+} uptake of SERCA in HeLa cells. In single cells, histamine (0.3 μM) evoked Ca^{2+} oscillations (Fig. 1A). However, in the presence of forskolin (10 μM) the oscillations induced by histamine were

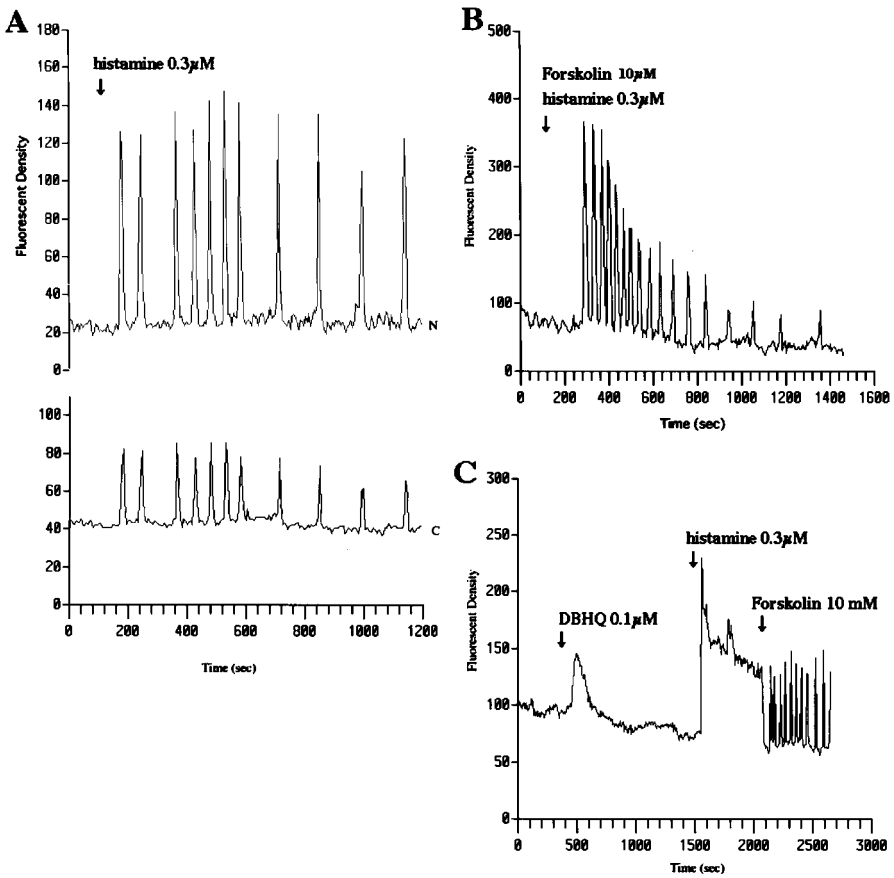

Fig. 1. Effects of forskolin and DBHQ on the change of Ca^{2+} signaling. *A: Representative tracing of cytoplasmic and nuclear Ca^{2+} response evoked by histamine (0.3 μM) in fluo-3-loaded HeLa cells in the Tyrode's solution (n=14). Tracing N or tracing C shows the response in the nucleus or in the cytoplasm. B: Intracellular Ca^{2+} response in the single HeLa cells that* were exposed to both 0.3 μM histamine and 10 μM forskolin as indicated (n=18). C: After the activity of SERCA was partly blocked by 0.1 μM 2,5-di-ter-butyl-1,4-benzohydrorequinoe (DBHQ), cells were stimulated with 0.3 μM histamine at the time indicated (see middle of the tracing). Subsequently, initiation of Ca^{2+} oscillations (see right of the tracing) occurred with addition of forskolin at much higher concentration (10 mM) (n=4).

gradually declined (Fig. 1B). This change in Ca^{2+} signal suggests that the forskolin activates the Ca^{2+} uptake of SERCA. To confirm the role of forskolin on activity of SERCA, DBHQ, an inhibitor of SERCA, was used at low concentration (0.1 μM) to partly block SERCA (Fig. 1C, left). As a result, histamine induced a continuous elevation rather than oscillations in the Ca^{2+} signal (Fig. 1C, middle). However, a high concentration of forskolin (10 mM) initiated the switching from a sustained Ca^{2+} elevation to Ca^{2+} oscillations (Fig. 1C, right).

To confirm that forskolin promotes phosphorylation of SERCA and thereby increases the activity of SERCA, we detecred serine-phosphorylation of SERCA. As shown in the Fig. 2, stimulation with forskolin (100 μM) for 5 min clearly enhanced the serine-phosphorylation of SERCA2b compare with control. When the cells were treated with DBHQ (1 μM) for 30 min prior to forskolin (100 μM) for 5 min, serine-phosphorylation in SERCA2b was inhibited, but was not completely abolished. In contrast, forskolin did not induce serine-phosphorylation of SERCA3 (see the lower panel in Fig. 2). This showed that forskolin strengthens the serine-phosphorylation of SERCA2b.

Effect of forskolin on unclear Ca^{2+} ($[Ca^{2+}]_n$) elevation.

Next, we investigated whether forskolin affects the changes in nuclear Ca^{2+} concentration ($[Ca^{2+}]_n$) by using an enhancer of Ca^{2+} leakage, A23187. In control cells (no treatment with forskolin), A23187 did not lead any Ca^{2+} oscillations either in the nucleus or in the cytoplasm (Fig. 3A tracing 1 to 4). In contrast, in the forskolin-treated cells, A23187 (5 mM) induced Ca^{2+} oscillations in the nucleus (see Fig. 3B tracing 1), which were not observed in the cytoplasm (see Fig. 3B tracing 2 to 4).

Then, we used BAPTA-AM, an inhibitor of Ca^{2+} mobilization. As shown in *Fig. 1A* when the cells were exposed to histamine (0.3 μM) without BAPTA, Ca^{2+} oscillation was observed in both nucleus and cytoplasm. Under the condition of BAPTA the treatment of cells with histamine (0.3 μM) caused a transient elevation in nuclear Ca^{2+} concentration ($[Ca^{2+}]_n$) (Fig. 4A, tracing 1), accompanied by a decrease in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) (see Fig. 4A, tracing 2 to 4). Furthermore, under the same condition the cells were pretreated with forskolin at low concentration (10 μM) for 5 min, histamine caused the prolongation of elevation in $[Ca^{2+}]_n$ (Fig. 4B, tracing 1) and decrease in $[Ca^{2+}]_c$ (Fig. 4B, tracing 2 to 5).

SERCA2b is populated on the *special parts* of nuclear envelope

Then, we carried out immunofluorescence staining using anti-SERCA2 (clone No. IID8) and anti-SERCA3 (clone No. PL/IM430) antibodies. As shown by confocal scanning microscopy (Fig. 5A), s*pecial intranuclear parts* of the nuclear envelope in HeLa cells were clearly stained with the fluorescence of anti-SERCA2 antibody. The fluorescence on the *special intranuclear parts* was stronger than that on the endoplasmic reticulum. However, the fluorescence of anti-SERCA3 was not present on the *special intranuclear parts* of the nuclear envelope (Fig. 5B). The immunofluorescence of SERCA2b was associated with that of FITC-conjugated ConA (a letin that binds mannose residues of nuclear envelope glycoproteins, data not shown). This finding proves that the SERCA2b seems to be a major component involved in regulating Ca^{2+} mobilization in the nucleus.

Anti-SERCA2b antibody blocks mobilization of the nuclear Ca^{2+}

In Order to compare properies of the two SERCAs with respect to regulating nuclear Ca^{2+} homeostasis, we injected the two anti-SERCA antibodies into the cytoplasm and incubated the cells for 1 to 2 hours to allow the antibodies to diffuse. After the stimulation with histamine (0.3 μM), different patterns of Ca^{2+} dynamics were observed in the anti-SERCA2 (IID8) and anti-SERCA3 (PL/IM430) antibody-injected cells (Fig. 6). Anti-SERCA3 (PL/IM430)-injected cells displayed decaying oscillations (Fig. 6A), while anti-SERCA2b (IID8)-injected cells showed a simple and dull rise in $[Ca^{2+}]_c$ (Fig. 6B). At the same time, fluo-3 fluorescence intensity in the nucleus was too low to measure changes in $[Ca^{2+}]_n$ (Fig. 6B). These results confirm that SERCA2b is involved in maintaining $[Ca^{2+}]_n$ and initiates Ca^{2+} oscillations, whereas SERCA3 propagates Ca^{2+} oscillations.

Discussion

SERCA in the endoplasmic reticulum-nuclear envelope network is essential to maintain the uptake of Ca^{2+}. Cyclic AMP (cAMP) enhances the frequency and the peak amplitude of $InsP_3$-induced Ca^{2+} oscillations in single rat hepatocytes[27]. However, high levels of cAMP or PKA abolish Ca^{2+} oscillations[28]. Because forskolin activates the catalytic subunit of adenylate cyclase and promotes the transport of Ca^{2+} by SERCA2 into the

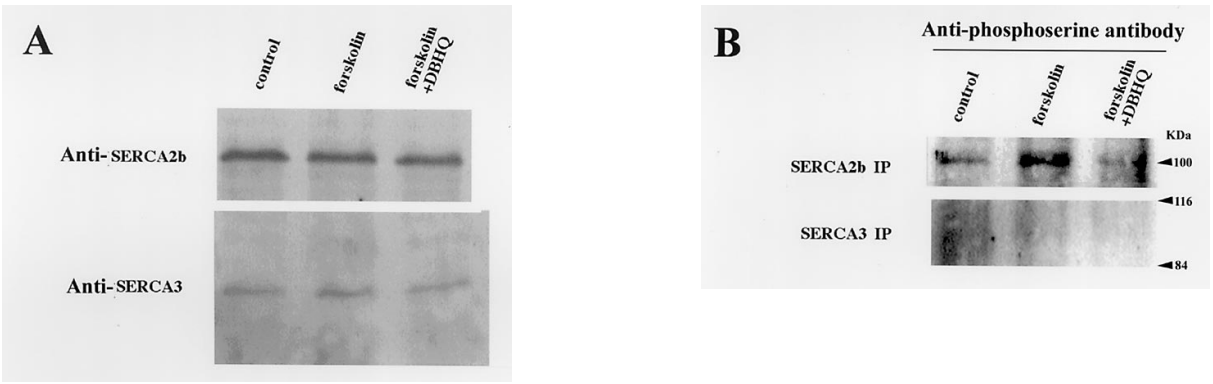

Fig. 2. Effect of forskolin on serine-phosphorylation of SERCA. To detect serine-phosphorylation of SERCA, a specific monoclonal anti-phosphoserine antibody was used to perform a Western blotting analysis. Cells were pretreated with or without 1 μM DBHQ for 30 min and then 100 μM forskolin was added into the medium and cultured for another 5 min. Immunoprecipitation and Western blotting was carried out as described in Materials and Methods. *A: Anti-SERCA2 or anti-SERCA3 antibodies were used in the western blotting as controls. B:* The upper panel shows the serine-phosphorylation of SERCA2b (100 KDa), while no serine-phosphorylation was observed for SERCA3 (97 KDa) in the lower panel.

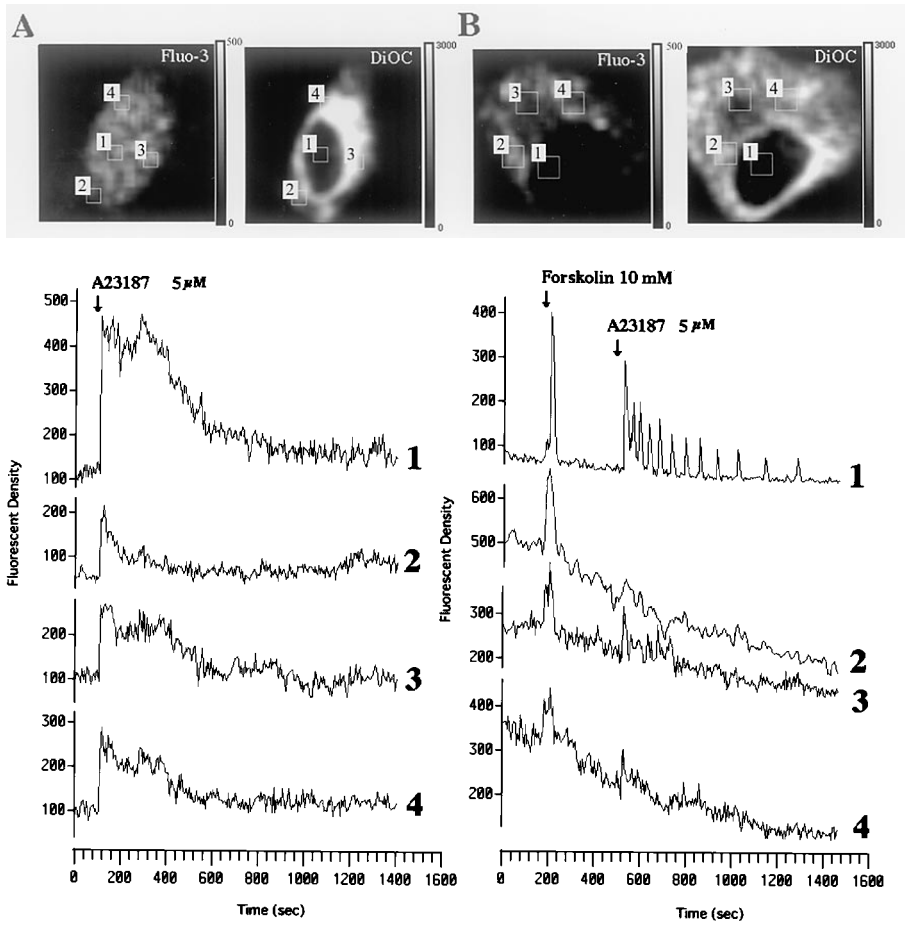

Fig. 3. Forskolin activates the switching from transient to oscillations in nuclear Ca^{2+} signaling. After fluo-3-AM loading as described in Materials and Methods, cells were treated with (B) or without (A) 10 mM forskolin for 5 min, then they were stimulated with 5 mM A23187 at the time indicated. After measurement was completed, the regions of cytoplasm and nucleus were immediately separated regarding the distribution of $DiOC_6$ (3) staining. Tracing 1 shows the response in the nucleus, while tracing 2 to 4 shows responses in different areas of the cytoplasm.

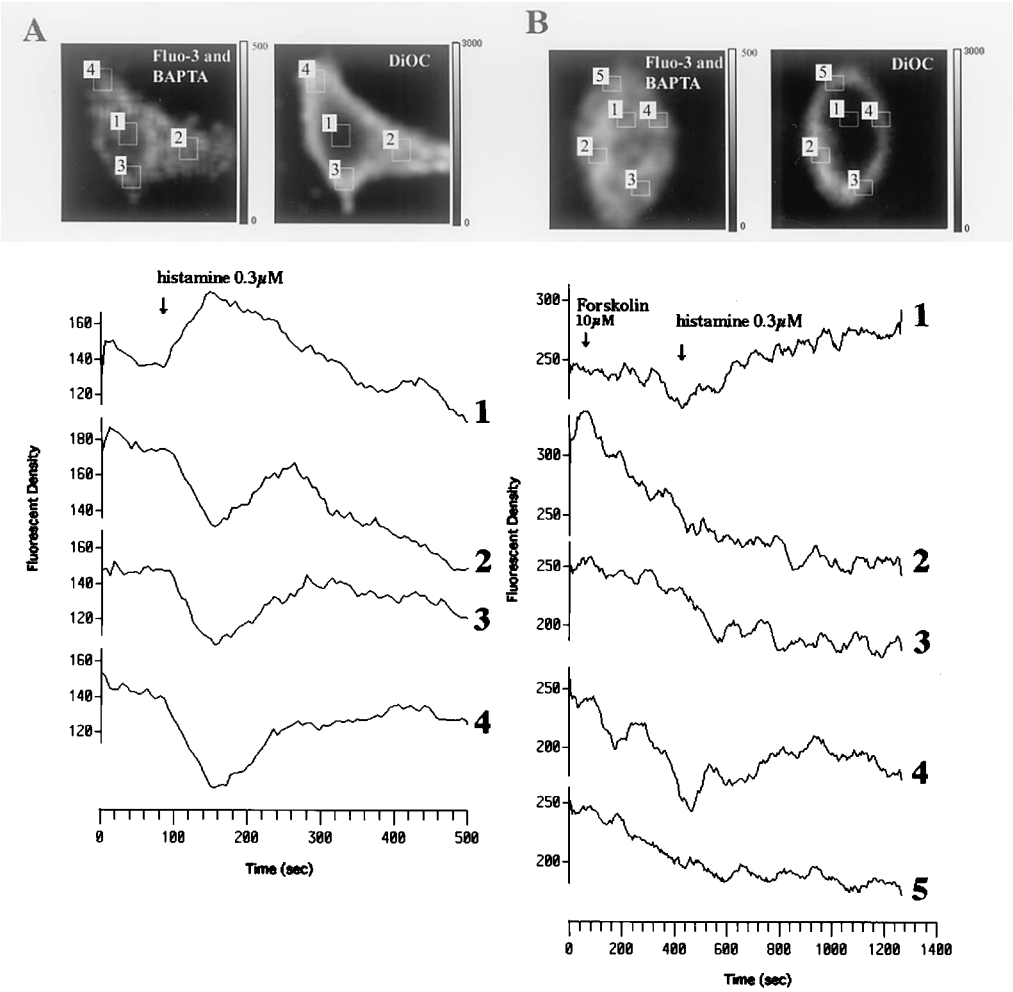

Fig. 4. Forskolin prolonged the histamine-induced Ca^{2+} elevations in the nucleus. After HeLa cells were co-loaded with BAPTA-AM and fluo-3-AM, they were treated with (B) or without (A) 10 μM forskolin. Then cells were stimulated with histamine (0.3 μM) in Tyrode's solution. After measurement was completed, the regions of cytoplasm and nucleus were immediately separated regarding distribution of $DiOC_6$ (3) staining. Tracing 1 showed a response in the nucleus, and tracing 2 to 4 or 5 showed responses in different areas of the cytoplasm.

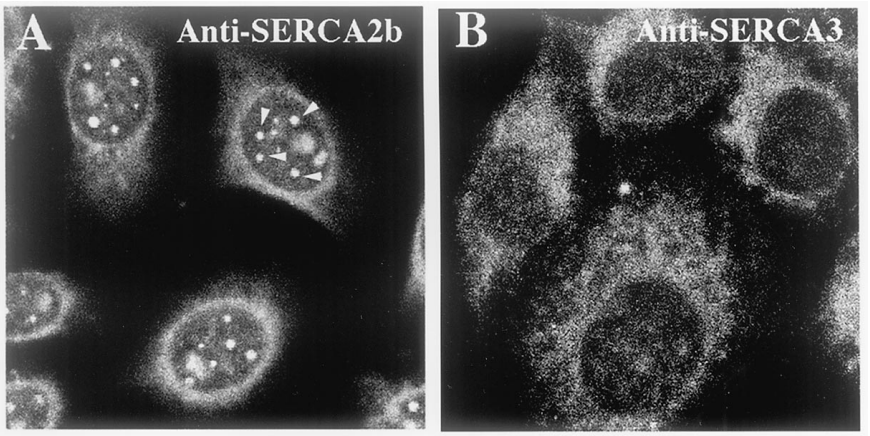

Fig. 5. SERCA2b was densely distributed on the *special parts* of the nuclear envelope of HeLa cells. Cells were fixed and were incubated with anti-SERCA2 or anti-SERCA3 monoclonal antibodies, and then were stained with FITC-labeled goat anti-mouse, TRITC-labeled goat anti-mouse, or FITC-labeled goat anti-rabbit secondary antibodies. Bar=10 μM.

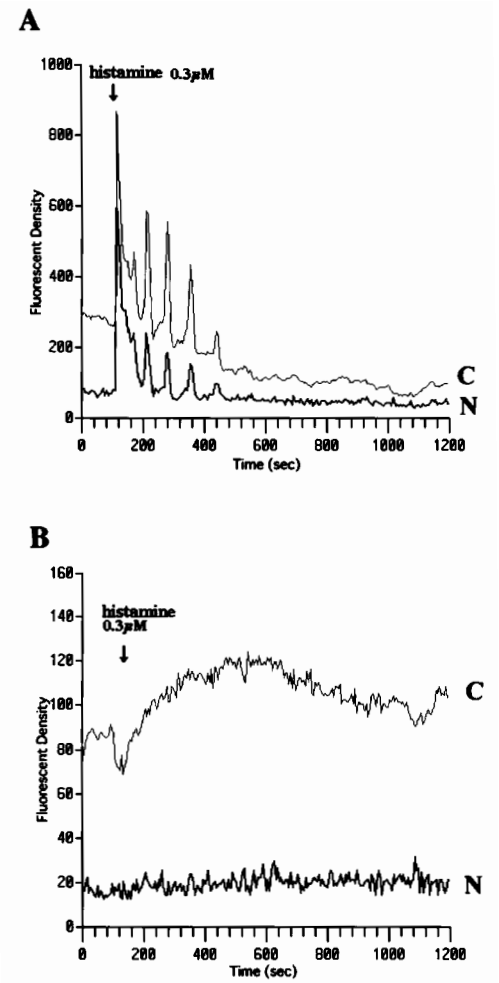

Fig. 6. Interaction between aspects of nuclear Ca^{2+} and inhibition of SERCA injected by anti-SERCA antibodies. Anti-SERCA3 (A) or anti-SERCA2 (B) antibodies were injected into the cytoplasm, and the cells were incubated for 1 to 2 hrs, and then were stimulated with 0.3 μM histamine. After measurement was completed, the regions of the cytoplasm and the nucleus were immediately separated regarding the distribution of $DiOC_6$ (3) staining. Similar results were obtained from the cells injected with the anti-SERCA3 antibody (A, n=5) or in the cells injected with the anti-SERCA2 antibody (B, n=5). Tracing N or tracing C shows the response in the nucleus or in the cytoplasm, respectively.

stores, our data also demonstrated that forskolin results in the suppression of the peak amplitude (Fig. 1B), or turns on the switching from a sustained elevation to oscillations in the Ca^{2+} signal (Fig. 1C). *These results also demonstrated that Ca^{2+} oscillations depend on the balance between Ca^{2+} release from and Ca^{2+} uptake into the stores*. The activation of SERCA2b by forskolin is due to the serine-phosphorylation of SERCA2b in HeLa cells (Fig. 2). This suggests that SERCA2 was phosphorylated at the serine site by a cAMP-dependent protein kinase (PKA). *Previously studies reported that SERCA3 was hardly activated by PKA[29], and we also observed that SERCA3 was hardly phosphorylated at the serine site by PKA (Fig. 2)*. Moreover, SERCA2b, or a Ca^{2+} pump named nuclear Ca^{2+} -ATPase, was identified on the nuclear envelope[1,30-31]. SERCA2 in HeLa cells may be involved in DBHQ-insensitive Ca^{2+} pools (Fig. 1C and Fig. 2), *because DBHQ was not able to block ATP-dependent Ca^{2+} transport into nucleus and ATP-induced rise in $[Ca^{2+}]_n$[32-34]*. The present results show that SERCA2b was densely destributed on special intranuclear parts, probably the invaginations of unclear envelope (Fig. 5). These invaginations are dynamic and tubular membrane-bound[35-36]. The density of immunofluorescence about SERCA2b on the invaginations was stronger than that on the endoplasmic reticulum, suggesting that SERCA2b has an important role in controlling the mobilization of nuclear Ca^{2+}.

The nuclear Ca^{2+} signal has two distinct sources: a primary signal—generating from the nuclear Ca^{2+} stores along the nuclear envelope, and a secondary signal—passing through the nuclear pores into the nucleus. The secondary Ca^{2+} signal has clearly been observed[37-38]. However, the primary Ca^{2+} signal in the nucleus has yet to be directly investigated. Related studies have shown that (i) InsP3R and ryanodine receptor as well as CD38/ADP-ribosyl cyclase locate on the nuclear inner membrane[33,34,39,40], (ii) nuclear lipid signaling (such as $InsP_3$ pathway) is independent of cytoplasm[41], and (iii) PLCβ_1 and PLCδ_4 is intranuclear components[42-44]. The result that SERCA2b was densely distributed on the invaginations of the nuclear envelope raises the possibility that the primary nuclear Ca^{2+} signal comes from the stores along the nuclear envelope. *In order to identify the role of SERCA and to avoid the interference of $InsP_3$ receptor and plasma membrane receptor during usage of histamine, we selected A23187, an enhancer of Ca^{2+} leakage from Ca^{2+} stores*[45-47]. Forskolin switched the sustained release by A23187-induced Ca^{2+} leakage to Ca^{2+} oscillations only in the nucleus (Fig. 3B). Likewise, application of forskolin also prolonged the rise in $[Ca^{2+}]_n$ and the decrease in $[Ca^{2+}]_c$ in BAPTA-loaded HeLa cells (Fig. 4B). A possible explanation is that serine-phosphorylation of SERCA2b increases the activity of this pump and replenishes free Ca^{2+} into lumen of stores along the nuclear envelope.

In the microinjection experiments, we found further evidence that nuclear Ca^{2+} pools was depleted by anti-SERCA2 antibody, therefore, SERCA2b is involved

with mobilization of the nuclear Ca^{2+} and with Ca^{2+} oscillations (Fig. 6B), while the SERCA3-coupled pools did not associate with initiation of nuclear Ca^{2+} movement (Fig. 6A).

We believe that a primary nuclear Ca^{2+} signal exists in the nucleus. The cell cycle[48] and some endogenous agents, e.g., arachidonate, prostaglandin, and sphingolipids[49-51], may regulate intranuclear Ca^{2+} liberation, because their receptors, such as prostaglandin E2 receptors, exist on the nuclear envelope.

In conclusion, the activation of SERCA2b, not but SERCA3, elevates the rate of uptake of free Ca^{2+} into stores along the nuclear envelope, and the process is involved to support and maintain the nuclear Ca^{2+} homeostasis.

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