Ca\(^{2+}\) pathway involved in the refilling of store sites in rat adrenal medullary cells

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Matsuoka H, Harada K, Ikeda T, Uetsuki K, Sata T, Warashina A, Inoue M. Ca\(^{2+}\) pathway involved in the refilling of store sites in rat adrenal medullary cells. Am J Physiol Cell Physiol 296: C889–C899, 2009. First published January 28, 2009; doi:10.1152/ajpcell.00439.2008.—It has been suggested that store-operated Ca\(^{2+}\) entry (SOC) facilitates catecholamine secretion and synthesis in bovine adrenal medullary (AM) cells. However, there has been no experimental result clearly showing that cation channel activity is enhanced by store Ca\(^{2+}\) depletion. Thus the present experiments were undertaken to address the issue of whether rat AM cells have SOC channels. Inhibition of the sarco(endo)plasmic reticulum Ca\(^{2+}\) (SERCA) pump resulted in a sustained increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) in rat AM cells. This increase was completely suppressed by 2 mM Ni\(^{2+}\) but not by 100 μM D600. A bath application of Ni\(^{2+}\), but not D600, produced an outward current at −60 mV in rat AM cells, whereas exposure to a SERCA pump inhibitor did not affect either the whole cell current level or the Ni\(^{2+}\)-induced outward current. The refilling of intracellular store sites was suppressed by the addition of Ni\(^{2+}\) to the perfusate. RT-PCR revealed that transcripts for transient receptor potential channels 1 (TRPC1) and 5 (TRPC5) were present in rat adrenal medullas. Immunocytochemistry showed that TRPC1 channels, which have been implicated in SOC in certain types of cells, were mainly localized in the endoplasmic reticulum (ER) and not in the plasma membrane, and that STIM1, a Ca\(^{2+}\) sensor in the ER, was not expressed in rat AM cells. On the basis of these results, we conclude that rat AM cells lack the SOC mechanism.

Ca\(^{2+}\) store sites; transient receptor potential channel; endoplasmic reticulum

SINCE THE DEPLETION OF INTRACELLULAR Ca\(^{2+}\) store sites was first reported to result in an increase in Ca\(^{2+}\) influx in nonexcitable cells (20, 21), similar results have been observed in many types of excitable as well as nonexcitable cells (38). It has been suggested that store-operated Ca\(^{2+}\) entry (SOC) is involved in various cellular functions, such as modulation of gene transcription (7), muscle contraction (52), and hormone secretion (39) channels play a pivotal role in SOC has been accumulating.

MATERIALS AND METHODS

Male Wistar rats (200–400 g; n = 66) were used in the experiments. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

Whole cell recording. The animals were killed by cervical dislocation, and the adrenal glands were excised and immediately put into an ice-cold Ca\(^{2+}\)-deficient balanced salt solution in which 1.8 mM CaCl\(_2\) was simply omitted from standard saline. The standard saline contained 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.53 mM NaH\(_2\)PO\(_4\), 5 mM D-glucose, 5 mM HEPES, and 4 mM NaOH (pH 7.4). After the adrenal gland was cut in half with microscissors, the adrenal cortex was completely removed from the adrenal medulla using microscissors and forceps under stereoscopic observation. The adrenal medulla was cut into two or three pieces and then incubated in a 0.3–0.5% collagenase-containing Ca\(^{2+}\)-free solution at 36°C for 30 min. To facilitate the enzyme action, the preparations were gently stirred with 100% O\(_2\) gas. After that, the tissues were washed several times and kept in the Ca\(^{2+}\)-free solution at 5°C. One piece of tissue was put in a bath apparatus, which was placed on an inverted microscope, and the AM cells were dissociated mechanically with fine needles and allowed to adhere to the bottom for a few minutes before the bath apparatus was perfused with saline at a rate of 1 ml/min. The whole cell current in an isolated rat AM cell was recorded using the nystatin perforated patch method, as described elsewhere (23). The standard pipette solution contained 120 mM potassium isethionate, 20 mM KCl, 10 mM NaCl, 10 mM HEPES, and 2.6 mM KOH (pH 7.2); in a 10 mM Cl\(^{-}\) pipette solution, 20 mM KCl was equimolarly replaced with potassium isethionate. On the day of the experiment, nystatin dissolved in dimethyl sulfoxide (5 mg in 100 μl) was added to the pipette solution at a final concentration of 100 μg/ml while the solution was being vortex-mixed. All chemicals were bath applied. The membrane potential was corrected for a liquid junction potential of −3 mV between the standard pipette solution and CA secretion. Hence, whether rat AM cells have SOC mechanisms or not remains obscure. The absence of SOC mechanisms may not affect the endocrine function of AM cells, since they develop various voltage-dependent Ca\(^{2+}\) channels (14), which may allow the cells to accumulate sufficient amounts of Ca\(^{2+}\).

Recently, evidence for the notion that STIM1 (41, 57) and transient receptor potential (TRPC) (22, 29, 55) or Orai1 (10, 39) channels play a pivotal role in SOC has been accumulating. The aims of the present experiments were to elucidate whether SOC channel activity occurs in rat AM cells and to clarify whether STIM1 and TRPC channels are expressed at the mRNA and protein levels.

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Perfusion experiment. The rat adrenal glands were perfused retrogradely, as described elsewhere (51). Briefly, the adrenal glands were removed from the rats under pentobarbital (60 mg/kg ip) anesthesia and then perfused retrogradely via the adrenal vein with saline at a rate of 0.45 ml/min. The glands were subjected to a 40-min recurrent saline perfusion with 1 ml of saline containing 10 μM fluo-4 AM or fura-2 AM and 0.2% Pluronic F-127, and then part of the adrenal cortex covering the medulla was carefully removed with microscissors. The adrenal gland was transferred to a chamber with the naked medulla on the glass bottom and placed between one pair of silver circles for electrical stimulation with 60-V pulses of 1.5-ms duration at 10 Hz for 30 s. For ratiometric analysis, the chamber with the adrenal gland was mounted on the stage of an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan). Two infusion pumps (STC-523; Terumo, Tokyo, Japan) were used to perfuse the adrenal medulla and to apply the chemicals. The perfusates were switched with a miniature rotary valve (type 86729; Hamilton, Reno, NV) located at the inlet of the chamber, and the outflow was sucked out by a roller pump. Changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were measured ratiometrically. Light from a xenon lamp (XBO 100W; Osram, Munich, Germany) was transmitted by band-pass filters (342 and 380 nm) mounted on an RS232C-controlled chopper wheel. The light was introduced into the microscope through a liquid light guide (77568; Oriel, Darmstadt, Germany) and was reflected with a 400-nm dichroic mirror through a 495-nm cutoff filter. The light was focused through a ×20 objective lens onto a portion (~200 μm in diameter) of the adrenal medulla. The adrenal medulla was alternatively irradiated with 342- and 380-nm light for 0.5 s each, and the intensity of fura-2 fluorescence was measured with a photomultiplier. The ratio of the fluorescence intensity excited at 342 nm to that excited at 380 nm, R(342/380), was calculated at 4-s intervals as an index of changes in [Ca\(^{2+}\)]\(_i\). To compare the relative amplitudes of changes in [Ca\(^{2+}\)]\(_i\), we normalized the ratio (R(342/380)) value to its initial value obtained under the resting conditions and denoted this as NR(342/380). In some of the experiments, secertory responsese were amperometrically measured with changes in [Ca\(^{2+}\)]\(_i\). An electrode made of a bundle of carbon fibers (T300-1000; Toray, Tokyo, Japan) was placed near the preparation, and the oxidation current at the tip of the electrode to which 500 mV were applied was measured and converted to a rate (pmol/min) of CA secretion based on a calibration obtained with 1 μM epinephrine.

For imaging analysis, the chamber was mounted on the stage of a confocal laser scanning microscope (LSM410; Carl Zeiss, Tokyo, Japan) and was reflected with a 400-nm dichroic mirror at 45°. An electrode made of a bundle of carbon fibers (T300-1000; Toray, Tokyo, Japan) was placed near the preparation, and the oxidation current at the tip of the electrode to which 500 mV were applied was measured and converted to a rate (pmol/min) of CA secretion based on a calibration obtained with 1 μM epinephrine.

For imaging analysis, the chamber was mounted on the stage of a confocal laser scanning microscope (LSM410; Carl Zeiss, Oberkochen, Germany), and the adrenal gland was continuously perfused at a rate of 0.45 ml/min at 25–28°C. Illumination with 488-nm light was provided by an argon laser. Emission above 510 nm was monitored, and fluorescence images were acquired every 10 s. The extent of photobleaching was estimated by a curve fitting of the intensities of responsive areas at the resting state with a polynomial function \((a t^2 + b t + c)\), where \(a\), \(b\), and \(c\) are constants and \(t\) is time. The intensity in each frame was then corrected for photobleaching and expressed as a fraction of its value obtained under the resting conditions.

Immunoprecipitation and immunoblot. The brain, adrenal cortex, and adrenal medulla were minced and homogenized in 10 volumes of a solution containing 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, and a protease inhibitor cocktail [1 mM M-[2-(4-aminoethyl)benzene sulfonyl fluoride, 0.3 μM aprotinin, 2 μM E-64, 1 mM EDTA, and 2 μM leupeptin (Calbiochem, San Diego, CA)]. The homogenates were centrifuged at 500 g for 10 min at 4°C to remove the cell debris, and then the supernatants were mixed with equal volumes of a SDS buffer containing 25 mM Tris·HCl (pH 6.8), 4% SDS, and 20% glycerol. Protein concentrations in the samples were determined using a BSA protein assay kit (Pierce, Rockford, IL). Since CA was found to interfere with the assay system, the proteins in the adrenal medulla samples were precipitated with acetone and the precipitate dissolved in the Laemmli buffer was subjected to the protein measurement. After the addition of 5% (vol/vol) 2-mercaptoethanol and 1% (vol/vol) bromphenol blue to the sample, the proteins were separated by 10% (wt/vol) SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% (wt/vol) fat-free powdered milk dissolved in PBS-T solution, which contained 2 mM NaH₂PO₄, 8 mM NaHPO₄, 145 mM NaCl, and 0.1% Tween 20. The PVDF membrane was incubated with rabbit or mouse antibody (Ab). The immunoreaction was detected by incubating the membrane with the respective secondary Ab linked to horseradish peroxidase (Amersham, Little Chartfont, UK) and then with ECL-Plus (Amersham). Immunoblotting was repeated at least three times for each Ab. The immunoprecipitation assay was performed in a manner similar to that described elsewhere (28). Briefly, cell lysates were incubated with anti-TRPC1 Ab coupled with protein G-Sepharose at 4°C for 3 h. The washed beads were subjected to immunoblot analysis with anti-TRPC1 Ab.

Immunocytochemistry. After collagenase treatment, one or two pieces of adrenal medulla tissue were placed in a glass-bottom dish (P35GC-0-14-C; MatTek, Ashland, MA) and then dissociated using fine needles. The dissociated AM cells were fixed in 2% paraformaldehyde in PBS (pH 7.2) for 1 h and then preincubated in PBS with 5% fetal bovine serum and 0.3% Triton-X for 30 min. For indirect immunofluorescence studies, the cells were treated with primary Abs. After incubation, the cells were washed three times in PBS and then treated with a secondary Ab conjugated with Alexa 488 or 546 (Molecular Probes, Eugene, OR). The fluorescence was observed using a confocal laser scanning microscope. The objective lens was an oil-immersion lens with a magnification of ×63 and a numerical aperture of 1.4. For Alexa 488, a 488-nm laser was used and 510- to 560-nm emission was observed, whereas for Alexa 546, a 543-nm laser was used and emission >570 nm was observed. Fluorescence was observed with a full width at a half-maximum of 0.7 μm. To examine the specificity for the immunoreaction, we treated the preparation with a non-immune serum instead of a primary Ab, and almost no immunoreactivity was observed under the same conditions as used for the primary Ab.

Human embryonic kidney (HEK)-293T cells or PC12 cells were plated onto collagen-coated glass coverslips, maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine, and transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two days after the transfection, they were homogenized or fixed with 4% paraformaldehyde in PBS for 30 min at room temperature.

RT-PCR. To isolate poly(A)\(^+\) RNA from the rat brain, adrenal medulla, and adrenal cortex, we used a Micro-fast kit (Invitrogen) according to the manufacturer’s instructions. Oligo(dt) primer was utilized for the RT reaction to obtain cDNAs. PCR was carried out with 0.5 μl of DNA template, 4 pmol of primer, 2 mM dNTPs, 0.5 units of Taq (Takara, Otsu, Japan), and PCR buffers supplied with the kit in a final volume of 25 μl. Table 1 shows the nucleotide sequences of the gene-specific primers used. The PCR protocol used started with an initial 2-min denaturation step at 94°C, followed by 30–40 cycles of the profile consisting of 30 s of denaturation at 94°C, 30 s of annealing at 54–60°C, and 30 s of extension at 72°C. To obtain the maximum fidelity, we used a hot-start procedure. In each PCR reaction, a 198-bp PCR product of β-actin mRNA was amplified and used as a control. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Sources of agents. Thapsigargin, cyclopiazonic acid (CPA), and chlorobutanol were obtained from Wako Pure Chemical (Osaka, Japan). Muscarine chloride was obtained from Sigma-Aldrich (St. Louis, MO), and methoxyverapamil (D600) was obtained from Knoll Pharmaceutical (Mt. Olive, NJ). Fura-2 AM and fluo-4 AM were obtained from Dojindo (Kumamoto, Japan), and collagenase was...
obtained from Yakult (Tokyo, Japan). Rabbit anti-TRPC1 Ab (ACC-010), rabbit anti-TRPC5 Ab (ACC-020), and rabbit anti-TRPC6 Ab (ACC-017) were obtained from Alomone Labs (Jerusalem, Israel). Rabbit anti-TRPC1 Ab (SC-20110), rabbit anti-TRPC5 Ab (ACC-020), and rabbit anti-TRPC6 Ab (ACC-010) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-green fluorescent protein (GFP) Ab (A6455) was obtained from Invitrogen, and rabbit anti-calnexin Ab (SPA-860) was obtained from StressGen (Victoria, BC, Canada). BODIPY-FL-thapsigargin and secondary Abs were obtained from Chemicon (Temecula, CA), and anti-actin MAb (MAB1501R) was obtained from BD Bioscience (San Jose, CA), and anti-actin MAb (MAB1501R) was obtained from Chemicon (Temecula, CA).

RESULTS

SOC is generally thought to be responsible for a sustained increase in [Ca\(^{2+}\)] in response to sarco(endo)plasmic reticulum Ca\(^{2+}\) (SERCA) pump inhibition. Thus we investigated whether rat AM cells exhibited such a Ca\(^{2+}\) response. Figure 1, A and B, shows the Ca\(^{2+}\) response of rat AM cells. As shown in Fig. 2, A and B, an increase in [Ca\(^{2+}\)], in response to 1 μM thapsigargin, an irreversible inhibitor of the SERCA pump (47), was suppressed completely by 2 mM Ni\(^{2+}\) but only marginally by 100 μM D600, a voltage-dependent Ca\(^{2+}\) channel blocker (6, 11, 31). These properties of the Ca\(^{2+}\) influx pathway were contrasted with those of the voltage-dependent Ca\(^{2+}\) channels activated by 40 mM K\(^{+}\) (Fig. 2, C and D): the high K\(^{+}\)-induced increase in [Ca\(^{2+}\)], diminished by 50 and 80% in the presence of 2 mM Ni\(^{2+}\) and 100 μM D600, respectively. These results indicate that the thapsigargin-induced increase in [Ca\(^{2+}\)], was due to Ca\(^{2+}\) influx through a pathway other than voltage-dependent Ca\(^{2+}\) channels, as noted in other types of cells (42, 48, 57).

The foregoing results led us to examine the effects of Ni\(^{2+}\) and D600 on the whole cell current in isolated rat AM cells (Fig. 3). Bath application of 1 mM Ni\(^{2+}\) induced an outward current (1.3 ± 0.2 pA, n = 5) at −70 mV, whereas 100 μM D600 did not (n = 4) (Fig. 3A). The Ni\(^{2+}\)-induced current diminished successively as the membrane potential was hyperpolarized from −50 to −80 mV (Fig. 3, B and D). This voltage dependence was not altered by a decrease in pipette Cl\(^{−}\) concentration from 30 mM (E\(_{Cl}\) = −41 mV) to 10 mM (E\(_{Cl}\) = −69 mV) (Fig. 3C), indicating that Cl\(^{−}\) channels were not

Table 1. Nucleotide sequences of gene-specific primers

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<td>5'-ggagcaatcttctctc-3'</td>
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TRPC, transient receptor potential channels.

Fig. 1. Application of cyclopiazonic acid (CPA) induces an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in rat adrenal medullary (AM) cells. A: fluorescence images of change in Ca\(^{2+}\) signal in rat AM cells in response to splanchic nerve stimulation (STI) and 10 μM CPA. B: relative intensities of fluo-4 fluorescence in the area marked in A plotted against time. Intensities expressed in arbitrary units were averaged per pixel, corrected for photobleaching, and then expressed as a fraction of the resting level (see MATERIALS AND METHODS). Adrenal medullas, which were loaded with fluo-4 AM, were continuously perfused retrogradely through the adrenal vein. The medullas were transsynaptically stimulated at 10 Hz with a 1.5-ms pulse of 60 V and exposed to 10 μM CPA during the indicated periods (STI and CPA). a, b, and c correspond to the same letters in A. C: summary of increases of relative intensity in response to STI and CPA (n = 6). #P < 0.001.
involved. The effect of 1 mM Ni\(^{2+}\) was not mimicked by 0.1 mM Cd\(^{2+}\) \((n = 3)\), 0.1 mM Zn\(^{2+}\) \((n = 3)\), or 0.1 mM La\(^{3+}\) \((n = 3)\). Similarly, bath application of 3 \(\mu\)M ruthenium red, a nonspecific inhibitor of cation channels \((17, 18, 33, 37)\), did not induce any current in the AM cells \((n = 3)\), which produced outward currents in response to 1 mM Ni\(^{2+}\).

Next, we investigated whether Ni\(^{2+}\)-sensitive channels are involved in the refilling of Ca\(^{2+}\) store sites. Bath application of 10 mM caffeine at intervals of 4 min reproducibly induced outward currents through mobilizing intercellular Ca\(^{2+}\) \((24)\). When 1 mM Ni\(^{2+}\) was added to the perfusate during the intervals between applications of caffeine, the amplitude of caffeine-induced outward currents significantly diminished \((Fig. 4B)\), suggesting that a Ni\(^{2+}\)-sensitive pathway is involved at least in part in the refilling of Ca\(^{2+}\) store sites. These results led us to examine whether the Ni\(^{2+}\)-sensitive current was altered by the depletion of Ca\(^{2+}\) store sites. As shown in Fig. 4C, bath application of 10 \(\mu\)M CPA did not induce any current at −60 mV for at least 3 min \(\text{[change in whole cell current was 0.04 ± 0.02 pA \(n = 7\) at 3 min, and exposure to CPA produced no effect on 1 mM Ni\(^{2+}\)-induced outward currents \[amplitude of the Ni\(^{2+}\)-induced current 3 min after application of CPA was 100.4 ± 3.4% \(n = 7\) of the current level before CPA]}. Furthermore, the whole cell current level at −40 mV did not depend on the Ca\(^{2+}\) contents in the store sites \(\text{[Fig. 4, D and E]}\). The whole cell was restored to the original level after the washout of 10 mM caffeine, irrespective of the duration of intervals between caffeine applications and whether Ca\(^{2+}\) mobilization occurred in response to caffeine. These results were consistently observed in three AM cells.

Fig. 3. Effects of Ni\(^{2+}\) and D600 on whole cell current in rat AM cells. A: generation of an outward current in response to 1 mM Ni\(^{2+}\) but not to 100 \(\mu\)M D600. The whole cell current was recorded in a dissociated rat AM cell at −60 mV with the perforated patch-clamp technique \(\text{[see MATERIALS AND METHODS]}\). Ni\(^{2+}\) \((1 \text{ mM})\) or D600 \((100 \text{ \(\mu\)M})\) was added to perfusate during the indicated periods. B and C: Ni\(^{2+}\)-induced outward currents at various holding potentials in the presence of 30 and 10 mM Cl\(^{-}\) in the pipette solution, respectively. The membrane potential was held at the indicated levels. D: summary of voltage dependence of Ni\(^{2+}\)-induced outward currents \((n = 3–11 \text{ for each point})\). There was no difference in voltage dependence between 30 and 10 mM Cl\(^{-}\). Thus the data obtained under the 2 conditions were combined.

Fig. 2. Inhibition of thapsigargin (TG)-induced increase in Ca\(^{2+}\) signal by Ni\(^{2+}\) but not by methoxyverapamil (D600). A: TG-induced increase in Ca\(^{2+}\) signal is sensitive to 2 mM Ni\(^{2+}\) but not to 100 \(\mu\)M D600. B: summary of inhibition of TG-induced increase in Ca\(^{2+}\) signal by Ni\(^{2+}\) and D600 \((n = 6)\). Fura-2 AM was loaded in rat adrenal medullas, and the ratio of the fluorescence intensity excited at 342 nm to that excited at 380 nm was calculated, normalized to its initial value obtained under the resting condition, and denoted as NR(342/380) \(\text{[see MATERIALS AND METHODS]}\). C: 40 mM K\(^{+}\)-induced increase in Ca\(^{2+}\) signal was more sensitive to D600 than to Ni\(^{2+}\). Na\(^{+}\) \((34.6 \text{ mM})\) in standard saline was equimolarly replaced with K\(^{+}\) in 40 mM K solution. D: summary of inhibitions of 40 mM K\(^{+}\)-induced increase in Ca\(^{2+}\) signal by Ni\(^{2+}\) and D600 \((n = 6)\). \#P < 0.001.
suggest that the depletion of Ca\textsuperscript{2+} in store sites does not result in the development of an inward cation current.

No effects of Ca\textsuperscript{2+} store depletion on CA secretion. Since Ca\textsuperscript{2+} store depletion was reported to facilitate Ca\textsuperscript{2+} influx with the consequent increase in CA secretion in bovine AM cells (12) and PC12 cells (27), we investigated whether SERCA pump inhibition results in an increase in CA secretion in rat AM cells. The effects of CPA and high K\textsuperscript{+} on Ca\textsuperscript{2+} signal and CA secretion were studied in the same perfused adrenal medulla (Fig. 6). This result suggests that the Ca\textsuperscript{2+} influx pathway(s) unveiled by the inhibition of SERCA pumps is not efficiently coupled with the secretory machinery, which differs from voltage-dependent Ca\textsuperscript{2+} channels (14).

Expression of TRPC channels. Accumulating evidence indicates that TRPC channels are involved in SOC (22, 29, 55). Therefore, we examined the expression of TRPC channels at the mRNA and protein levels. As shown in Fig. 6A, a PCR product for each of the seven subtypes whose size was equal to the estimated product was detected clearly in brain cDNAs. On the other hand, PCR products for TRPC1 and TRPC5 were clearly detected. This pattern of expression of mRNA and protein levels suggests that TRPC channels are involved in SOC (22, 29, 55).

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Fig. 4. Whole cell current analysis of the effects of Ca\textsuperscript{2+} store depletion in rat AM cells. A: exposure to 1 mM Ni\textsuperscript{2+} inhibits refilling of Ca\textsuperscript{2+} store sites. Ni\textsuperscript{2+} (1 mM) was added to the perfusate during the indicated period (dashed horizontal bar) between applications of 10 mM caffeine (solid horizontal bar). Caffeine at 10 mM was added to Ca\textsuperscript{2+}-free saline where 1.8 mM Ca\textsuperscript{2+} in standard saline was replaced with 3.6 mM Mg\textsuperscript{2+}. The whole cell current was recorded at −40 mV with the nystatin method. B: summary of caffeine-induced outward currents in the presence and absence of 1 mM Ni\textsuperscript{2+} in the interval between caffeine applications. Peak amplitude of caffeine-induced outward current (caffeine I) after perfusion of saline with (Ni) (n = 12) or without 1 mM Ni\textsuperscript{2+} (Con) (n = 7) was expressed as a fraction of that before perfusion. C: no effect of 10 \mu M CPA on 1 mM Ni\textsuperscript{2+}-induced outward current. The whole cell current was recorded at −60 mV, and 1 mM Ni\textsuperscript{2+} and/or 10 \mu M CPA was added to the perfusate during the indicated periods (shaded horizontal bars for Ni\textsuperscript{2+} and dashed horizontal bar for CPA). D: no effect of Ca\textsuperscript{2+} store depletion on the whole cell current level. Caffeine (10 mM) was added to the perfusate during the indicated periods (solid horizontal bars). The whole cell current was recorded at −40 mV. E: summary of holding current levels just before application of caffeine. Current levels were measured with reference to the initial level of the experiment. I\textsuperscript{(+)} (n = 5) and I\textsuperscript{−} (n = 4) represent current levels before application of caffeine with and without generation of outward current, respectively; ns, not statistically significant.
shows that the SC anti-TRPC1 Ab detected a band of \( \sim 120 \) kDa in homogenates of HEK-293T cells transfected with a TRPC1-GFP construct but not with a mock vector; the same band was also recognized by an anti-GFP Ab. These results indicate that the band represents a TRPC-GFP fusion protein and that the SC Ab is immunoreactive against TRPC1 channels. This notion was further confirmed immunocytochemically. Figure 7B clearly shows that only the HEK-293T cell transfected with the construct was stained with the SC Ab.

These results led us to examine the expression of TRPC1 channels with this Ab. Immunoblotting of the homogenates of rat brain, adrenal cortex, or adrenal medulla did not result in clear detection of any bands of 80–100 kDa, probably due to either the low sensitivity of the Ab or the low expression levels of the channels. Thus immunoprecipitation and immunoblotting were combined for better detection. Figure 7C
clearly shows that 83-kDa bands were detected in all homogenates of rat brain, adrenal cortex, and adrenal medulla. Next, the distribution of TRPC1 channels was immunocytochemically examined in dissociated AM cells. As shown in Fig. 7D, the TRPC1 channel-like immunoreactivity (IR) was distributed mainly in the cytoplasm in a punctate manner and hardly at all in the plasma membrane (n = 12). Similarly, the anti-TRPC1 Ab made by Alomone (AL) was used to detect TRPC1 channels in immunoblotting and immunocytochemistry. The AL Ab also failed to detect any bands of 80–100 kDa in immunoblotting of rat brain, adrenal cortex, and adrenal medulla but recognized the 83-kDa band in immunocomplexes precipitated from the preparations with the SC anti-TRPC1 Ab (not shown). The AL Ab, however, produced less punctate staining in the cytoplasm than did the SC Ab (not shown; n = 7).

Since the punctate distribution of TRPC1 channel-like IR resembles that of the endoplasmic reticulum (ER), AM cells were double stained with the anti-TRPC1 Ab and BODIPY-FL-thapsigargin, a chemical that selectively binds to the ER (9, 25). Figure 7D clearly shows that the majority of TRPC1 channel-like IR coincided with BODIPY-FL thapsigargin binding (n = 12), suggesting that TRPC1 channels are present in the ER in AM cells. This notion was further examined by transfection of PC12 cells with the TRPC1-GFP construct. The TRPC1-GFP protein was distributed in a punctate manner in the cytoplasm and not in the plasma membrane (n = 5), and this distribution was not affected at all by exposure to 10 μM CPA (not shown; n = 5). On the other hand, the TRPC5-GFP protein expressed in PC12 cells was clearly present in the plasma membrane as well as in the cytoplasm (Fig. 8A, n = 5). To explore the notion that TRPC1-GFP proteins are present in the ER, we stained the transfected PC12 cells with an Ab against calnexin, a marker protein for the ER (Fig. 8B). The majority of TRPC1-GFP fluorescence was consistent with calnexin-like IR, which was visible as rhodamine-like fluorescence (n = 9).

Absence of STIM1. The foregoing results suggest that rat AM cells lack mechanisms for SOC. If that is the case, STIM1, a protein that functions as a Ca\(^{2+}\) sensor in the ER (41, 57), would be expected to be absent in AM cells. This possibility was examined using immunoblotting and immunocytochemistry. Immunoblotting detected STIM1 of 84 kDa in homogenates of rat brain and adrenal cortex but not of the adrenal medulla (Fig. 9, A and B). The absence of STIM1 in the AM cells was also immunocytochemically confirmed (Fig. 9, C and D). The STIM-like IR materials were found in the cytoplasm in the adrenal cortical cells, whereas the AM cells barely exhibited any STIM1-like IR. The intensity (11.6 ± 1.3, n = 5) of IR in the cytoplasm of the AM cells was equal to that (11.6 ± 1.4, n = 9) in the nucleus of the adrenal cortical cells.

**DISCUSSION**

**Absence of SOC.** The main finding of the present work was that STIM1, which plays an essential role in transmitting a
decrease in store Ca\(^{2+}\) contents to the plasma membrane, was
not detected in rat AM cells at the mRNA and protein levels.
On the other hand, STIM1 was expressed in adrenal cortex
cells, where SOC has been implicated in the production of
steroid hormones (8, 44). The lack of STIM1 expression
strongly suggests that SOC is absent in the rat AM cells. This
notion is consistent with the electrophysiological findings that
exposure to CPA, which produced an increase in Ca\(^{2+}\) signal
in the presence of external Ca\(^{2+}\) ions, did not result in the
development of an inward current at \(-60\) mV, unlike the
membrane response in mouse smooth muscle cells (53). Fur-
thermore, the whole cell current level was not altered by a
change in store Ca\(^{2+}\) contents in response to caffeine. Bath
application of caffeine induced a transient outward current
through mobilizing Ca\(^{2+}\) and then an inward current. This
inward current may have nothing to do with SOC channels.
First, the refilling of Ca\(^{2+}\) store sites was strictly dependent on
the duration of the interval between caffeine applications, not
on the generation of the inward current. Second, the current
was terminated rapidly after the washout of the caffeine. We

![Fig. 8. Transfection of PC12 cells with TRPC1-GFP construct and TRPC5-GFP construct. A: confocal images of a PC12 cell expressing TRPC1-GFP or TRPC5-GFP. GFP was visible as FITC-like fluorescence. B: confocal images of TRPC1-GFP fluorescence and calnexin-like IR visible as rhodamine-like fluorescence. Image at right represents an overlay image of TRPC1-GFP fluorescence and rhodamine-like fluorescence of calnexin-like IR. Coincidence of both types of fluorescence is shown in yellow. PC12 cells transfected with TRPC1-GFP construct were treated with an anti-calnexin Ab (1:200) overnight and then with Alexa 546-conjugated anti-rabbit IgG. Asterisks indicate the nucleus.](image1)

![Fig. 9. Lack of expression of STIM1 in rat AM cells. A: immunoblotting for STIM1 of lysates of rat B, AC, and AM. STIM1 of 84 kDa was detected in B and AC but not in AM. The same amount of protein (6 \(\mu\)g) was loaded in each lane. B: summary of amounts of STIM1 in B, AC, and AM. The amount of protein in AC and AM is expressed relative to that in B (\(n = 5\)). C: confocal images of immunostaining (top) and DIC images (bottom). Immunostaining in AC and AM cells was performed with anti-STIM1 Ab under the same conditions. Asterisks indicate the nucleus. D: summary of staining intensities in 10 AC and 5 AM cells. The intensity per pixel, expressed in arbitrary units, was measured using NIH Image software. \#P < 0.001.](image2)
did not investigate the ionic mechanism of the sustained membrane current response to caffeine. In frog sympathetic neurons, caffeine has been reported to induce inward currents through the inhibition of M-type K⁺ channels (1). Our conclusion differs from Fomina and Nowicky's findings (12) that store depletion in bovine AM cells resulted in a gradual development of an inward current, which diminished over 4 or 5 min after reaching a peak. This transient current was suppressed by millimolar concentrations of Zn²⁺. One possible explanation of the difference is that our preparation was acutely dissociated rat AM cells, whereas Fomina and Nowicky used cultured bovine AM cells. We recently reported that rat AM cells do not express M-type K⁺ channels and that muscarinic receptor stimulation induces depolarization through inhibiting TASK channels (26). On the other hand, in cultured bovine AM cells, histamine has been shown to induce depolarization with inhibition of M-type K⁺ channels (49).

Recent experiments have clearly indicated that the activity of TRPC channels is regulated not only by receptors coupled with phospholipase C but also by the Ca²⁺ content of store sites (2, 35). RT-PCR and immunoblotting clearly indicated that rat AM cells expressed TRPC1 and TRPC5 channels. To our surprise, TRPC1 channels were localized in the cytoplasm and not in the plasma membrane in rat AM cells, in contrast with the distribution of TRPC1 channels in the human submandibular gland cell line (29) and rat mesangial cells (15). Double staining with BODIPY-thapsigargin suggested that TRPC1 channels were present in the ER and not in vesicles. On the other hand, TRPC3 (43), TRPC4 (36), TRPC5 (4), and TRPC6 (45) channels have been shown to be present in the vesicles that fuse to the membrane upon receptor stimulation. The distribution of TRPC1 channels in rat AM cells was consistent with the finding that exogenous expression of TRPC-GFP proteins in the rat AM cell line, PC12 cells, resulted in the main localization of the fusion protein in the ER and not in the plasma membrane. This localization was in marked contrast with that of TRPC5-GFP proteins, which were clearly expressed in the plasma membrane. These results suggest that rat AM cells and PC12 cells may lack the trafficking mechanism of TRPC1 channels to the plasma membrane. The interaction of certain channels and receptors with scaffold proteins (30), such as PDZ (PSD95/DLG/ZO-1) domain-containing proteins and Homers (54), is known to play an essential role in their trafficking to the plasma membrane. Homer 1b inhibits surface expression of the metabotropic glutamate receptor mGluR5 in heterologous cells, causing mGluR5 to be retained with the ER (40). On the other hand, the kainate receptor subunit KA2 is able to exist away from the ER when it forms a heteromer with GluR6, a kainate receptor subunit (34).

Ca²⁺ influx pathway in refilling Ca²⁺ store sites. The thapsigargin-induced increase in [Ca²⁺], was completely inhibited by 2 mM Ni²⁺ but only marginally by 100 μM D600. These results suggest that voltage-dependent Ca²⁺ channels are not involved in the increase in [Ca²⁺], as noted previously. Bath addition of 1 mM Ni²⁺ apparently induced an outward current, whereas 100 μM D600 did not. The Ni²⁺-induced outward current diminished as the holding potential was hyperpolarized, and this voltage dependence was not affected by a change in the concentration of pipette Cl⁻. These results suggest that the Ni²⁺-sensitive current is due to either the activation of K⁺ channels or the inhibition of cation channels. The present results did not definitively allow us to elucidate the ionic mechanism for the Ni²⁺-induced outward current. The finding that the addition of 1 mM Ni²⁺ in the interval between caffeine applications resulted in no or diminished generation of an outward current in response to caffeine may be consistent with the notion that 1 mM Ni²⁺ inhibits Ca²⁺-permeable cation channels, which are active in resting conditions. Because exposure to CPA had no effect on the Ni²⁺-induced outward current, this putative Ca²⁺-permeable channel is not sensitive to the Ca²⁺ content in store sites. The T-type Ca²⁺ channel could pass Ca²⁺ as a window current at −60 mV (5); therefore, T-type channels might be responsible for the Ni²⁺-sensitive current. The voltage dependence of the Ni²⁺-sensitive current might also be consistent with the involvement of T-type channels. However, the effect of 1 mM Ni²⁺ could not be mimicked by 0.1 mM Cd²⁺, which was expected to inhibit T-type Ca²⁺ channels significantly (13). Further study is necessary to elucidate the ionic mechanism for the Ca²⁺ efflux pathway unveiled by exposure to a SERCA pump inhibitor.

There are two possible mechanisms that could explain the increase in [Ca²⁺], in response to SERCA pump inhibition. One is an increase in Ca²⁺ influx; the other is a decrease in Ca²⁺ efflux. The former may be negated by the present results. Thus the latter would be feasible. We have elucidated that the α²β2 isoform of Na⁺/K⁺-ATPase is selectively localized in the plasma membrane in the vicinity of the peripheral ER (28). Thus Ca²⁺ ions leaking from the peripheral ER are possibly extruded efficiently by nearby Na⁺/Ca²⁺ exchangers, which are driven by a large Na⁺ gradient due to the α²β2 isoform. When Ca²⁺ uptake into the ER is blocked by SERCA pump inhibition, this putative mechanism of Ca²⁺ extrusion may be impaired with a consequent increase in [Ca²⁺]. This hypothesis may be explored by measurement of the effects of CPA on [Ca²⁺], under the conditions where the ER in the vicinity of the plasma membrane is retracted toward the cell center by microtubule depolymerization (46).

In conclusion, on the basis of the absence of the development of an inward current in response to SERCA pump inhibition and the lack of expression of proteins involved in SOC, we conclude that rat AM cells lack the SOC mechanism.

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REFERENCES


