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TRPC4 Does Not Participate in the Store-Operated Ca^{2+} Entry in Bovine Adrenocortical Fasciculate Cells

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ABSTRACT

Store-operated Ca^{2+} entry (SOCE) plays an important role in the regulation of diverse nonexcitable cell functions. However, a SOCE channel has not been identified. One candidate is transient receptor potential protein C (TRPC). Because TRPC4 has been reported in bovine adrenocortical fasciculate cells (BAFCs), we used fura-2-loaded BAFCs to examine whether TRPC4 participates in SOCE. Both 10 μM uridine triphosphate and 100 μM cyclopiazonic acid increased fura-2 fluorescence intensity by 2 phases in the presence of the extracellcular Ca^{2+}. The first phase was Ca^{2+} release from the endoplasmic reticulum, and the second phase was Ca^{2+} influx from the extracellular space. When the extracellular Ca^{2+} was exchanged for Sr^{2+} and Ba^{2+}, the second phase induced by uridine triphosphate or cyclopiazonic acid disappeared. The putative SOCE channel is highly Ca^{2+}-selective, but TRPC4 is not. Both Sr^{2+} and Ba^{2+}, in addition to Ca^{2+}, can permeate via TRPC4. Therefore, our results suggest that TRPC4 is not an SOCE channel in BAFCs. (Jikeikai Med J 2008 ; 55 : 25-31)

Key words : adrenal cortex, calcium, uridine trisphosphate, transient receptor potential protein C

INTRODUCTION

Store-operated calcium entry (SOCE) plays an important role in many nonexcitable cell functions, including several pathophysiolgical outcomes[1]. SOCE is initiated by the depletion of luminal Ca^{2+} of the endoplasmic reticulum (ER) by inositol-3,4,5-trisphosphate (IP_3) via stimulation of $G_{q/11}$-coupled receptors or the treatment of cells with sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitors (i.e., thapsigargin and cyclopiazonic acid [CPA])[2,3].

Although several hypotheses for the mechanism of SOCE activation have been proposed in the last decade, the precise mechanism remains controversial. However, the 3 predominant hypotheses are : 1) conformational coupling and secretion-like coupling, 2) vesicular fusion, and 3) a diffusible messenger[1].

These hypotheses postulate the involvement of unidentified Ca^{2+} channels in SOCE. One candidate is the transient receptor potential protein (TRP) superfamily[4,5]. Mammalian TRPs include 7 families : TRPC, TRPM, TRPT, TRPA, TRPP, TRPML, and TRPN[6]. Of these TRP families, the most predominant and widespread is TRPC[6]. TRPC is subdivided into 7 subfamilies from TRPC1 to TRPC7[6].

We have previously shown that the conformational coupling mechanism might participate in SOCE in bovine adrenocortical fasciculate cells

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(BAFCs)[7]. Philipp et al.[8] have reported that TRPC4 develops in bovine adrenocortical cells. These reports suggest the possible conformational coupling of TRPC4 in the plasma membrane (PM) and IP_3 receptors in the ER.

TRPCs are nonselective cation channels that can allow not only Ca^{2+} but also Sr^{2+} and Ba^{2+} to cross them[9,10]. However, putative SOCE channels are highly Ca^{2+}-selective[11,12]. Therefore, we examined the expression of TRPC4 in BAFCs and examined the indirectly whether TRPC4 participates in SOCE through the use of Sr^{2+} and Ba^{2+} as an extracellular divalent cation pool in fura-2-loaded BAFCs, because Sr^{2+} and Ba^{2+} bind to fura-2 and increase its fluorescence intensity in the same manner as Ca^{2+} [13].

MATERIALS AND METHODS

1. Primary culture of BAFCs

BAFCs were isolated aseptically with 0.1% collagenase and 0.005% deoxyribonuclease I (DNase I) in Krebs-Ringer bicarbonate buffer (123.4 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mg/ml glucose, and 3 mg/ml bovine serum albumin ; pH 7.4) as previously described[14]. The isolated cells were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum, and antibiotics on collagen-coated cover slips at 37°C under 5% CO_2 in the air as a gas phase[14]. The cells grown for 2 to 3 days in primary monolayer culture were used for the subsequent experiments.

2. Reverse-transcription polymerase chain reaction of TRPC4 messenger RNA from BAFCs

BAFCs were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted with the acid guanidium-pheno-chloroform method with DNase I treatment (Takara Bio, Otsu). Complementary (c) DNA was synthesized from 5 μM of total RNA with the Super Script III First-strand Synthesis System for the reverse-transcription polymerase chain reaction (RT-PCR) (Invitrogen). Then, RT-PCR of TRPC4 messenger (m) RNA was performed with a specific primer for TRPC4 (Gen Bank accession No. NM-174478 ; forward, 5′-CTCTGGGAAGAATGCTCCTG-3′ ; reverse, 5′-TATATCCGCATGGTCAGCAA-3′ ; annealing temperature, 63°C ; PCR cycles, 35) designed with the Primer 3 Web site (version 0.4.0, http:/www-genome.wi.mit.edn/cgi-bin/primer/primer3-www.cgi) using Bioneer Accu Power PCR PreMix (Bioneer. Daejeon. Republic of Korea). The PCR products were separated with 2% agarose gel electrophoresis, and the intensity of ethidium bromide staining was detected under ultraviolet light.

3. Measurement of fura-2 fluorescent intensity in cells with the fluorescence imaging system

The cells on cover slips were incubated with 5 μM acetoxymethylester of fura-2 (fura-2/AM) for 90 minutes at 37°C in cremophor EL (0.02%) containing Krebs-Ringer HEPES buffer (123.4 mM NaCl, 5.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM HEPES, 2 mg/ml glucose, and 2 mg/ml bovine serum albumin ; pH 7.4 [Ca^{2+}-buffer]). After incubation, the cover slip was set up on a chamber of the microscope. The cells were continuously perfused with the buffer, to which agents were then added. Buffers used were Ca^{2+}-buffer, Sr^{2+}-buffer (1.2 mM Ca^{2+} replaced with 1.2 mM Sr^{2+}), and Ba^{2+}-buffer (1.2 mM Ca^{2+} replaced with 1.2 mM Ba^{2+}). The cells were observed with an inverted epifluorescent microscope (TE300, Nikon, Tokyo) with an objective lens of 20 to 40 × magnification. The fluorescence images were obtained with a cooled charge-coupled device camera (C-6790, Hamamatsu Photonics, Hamamatsu). The emission wavelength was 510 nm, and the excitation wavelength alternated every 0.5 second between 340 nm and 380 nm. Fluorescence images were analyzed with the Aquacosmos program (Hamamatsu Photonics).

The increase in the concentration of intracellular divalent cation (Ca^{2+}, Sr^{2+}, and Ba^{2+}) is expressed as the ratio of the fluorescence intensity at 340 nm to that at 380 nm (I 340/I 380).

4. Statistical analysis

The statistical analysis was performed with Student's t-test. Statistical significance was assumed at $^*p < 0.05$.

5. Materials

The materials were purchased from the following companies: fura-2/AM from Dojindo (Kumamoto); collagenase from Funakoshi (Tokyo), Ham F-10 medium, DNase I, and CPA from Sigma-Aldrich (St. Louis, MO, USA); uridine triphosphate (UTP) from Yamasa Co. (Chiba). All the other chemicals were of reagent grade.

RESULTS

1. TRPCs mRNA in BAFCs

The mRNA of TRPC4 was detected in BAFCs after 3 days of primary culture (Fig. 1).

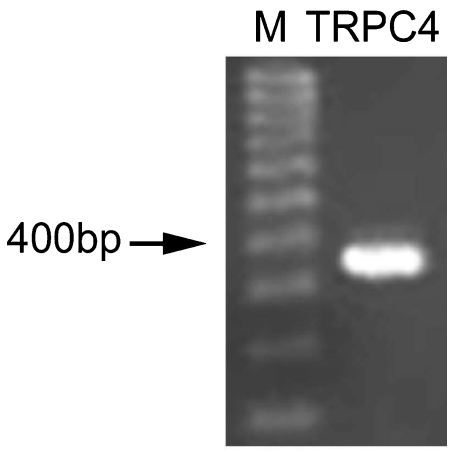

Fig. 1. RT-PCR for TRPC4 in BAFCs
The specific primers of TRPC4 are: forward, 5′-CTCTGGGAAGAATGCTCCTG-3′, and reverse, 5′-TATATCCGCATGGTCAGCAA-3′. The annealing temperature was 63°C, and 35 cycles were performed PCR. The expected length of the amplified fragment from mTRPC4 is 365 base pairs.
M: marker; TRPC4: mRNA for TRPC4

2. Effect of UTP on the fura-2 fluorescence intensity in BAFCs in the Ca^{2+}-buffer, the Sr^{2+}-buffer and the Ba^{2+}-buffer

The fura-2 fluorescence intensity was increased by 10 μM UTP in 2 phases in the Ca^{2+}-buffer (Fig. 2 [A]). The first phase was fast and transient and was followed by a sustained second phase. We have previously reported that UTP enhances IP_3 production via $G_{q/11}$ protein-coupled $P2Y_2$ receptors in BAFCs[15]. The increased IP_3 binds to IP_3 receptors in the ER membrane and releases the luminal Ca^{2+} to deplete Ca^{2+} in the ER. The depletion of Ca^{2+} in the ER triggers Ca^{2+} influx from an extracellular pool by the activation of SOCE channels[7]. Therefore, the first phase by UTP was induced by Ca^{2+} release from ER, and the second phase was sustained by Ca^{2+} influx from an extracellular space via SOCE channels in BAFCs[7].

Then, we examined the effect of UTP on the fura-2 fluorescence intensity in BAFCs in the presence of extracellular Sr^{2+} (1.2 mM) (Sr^{2+}-buffer) or Ba^{2+} (1.2 mM) (Ba^{2+}-buffer) instead of Ca^{2+}. Both with the Sr^{2+}-buffer and with the Ba^{2+}-buffer, 10 μM UTP induced a fast and transient increase in fura-2 fluorescence intensity (Fig. 2). However, the second sustained phase by UTP was not observed in BAFCs. The results suggest that the first phase was induced by Ca^{2+} release from ER and was not followed by the influx of Sr^{2+} or Ba^{2+} from the extracellular pool. The fluorescence intensity 10 minutes after the addition of UTP differed significantly between Ca^{2+}-buffer and Sr^{2+}-buffer or Ba^{2+}-buffer ($^*p < 0.05$).

3. Effect of CPA on the fura-2 fluorescence intensity in BAFCs in the presence of extracellular Ca^{2+}, Sr^{2+}, and Ba^{2+}

To investigate the above possibility, we used CPA, an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase[16], to activate SOCE in BAFCs. CPA inhibits the uptake of the released Ca^{2+} in the ER, depletes the luminal Ca^{2+}, and increases cytoplasmic Ca^{2+}. The depletion of Ca^{2+} in the ER activates SOCE channels.

Therefore, the rapid increase in fura-2 fluorescence intensity by CPA was observed by Ca^{2+} release from the ER, and the sustained phase was caused by Ca^{2+} influx from the extracellular space.

As shown in Fig. 3 (A), 100 μM CPA caused a rapid increase in the fura-2 fluorescence intensity in Ca^{2+}-buffer. The increase in fura-2 fluorescence intensity was sustained in Ca^{2+}-buffer. However, the sustained phase decreased rapidly after Ca^{2+}-buffer was replaced with Sr^{2+}-buffer (A) or Ba^{2+}-buffer (B).

In Sr^{2+}-buffer, treatment with 100 μM CPA produced only the first rapid increase in fura-2 fluo-

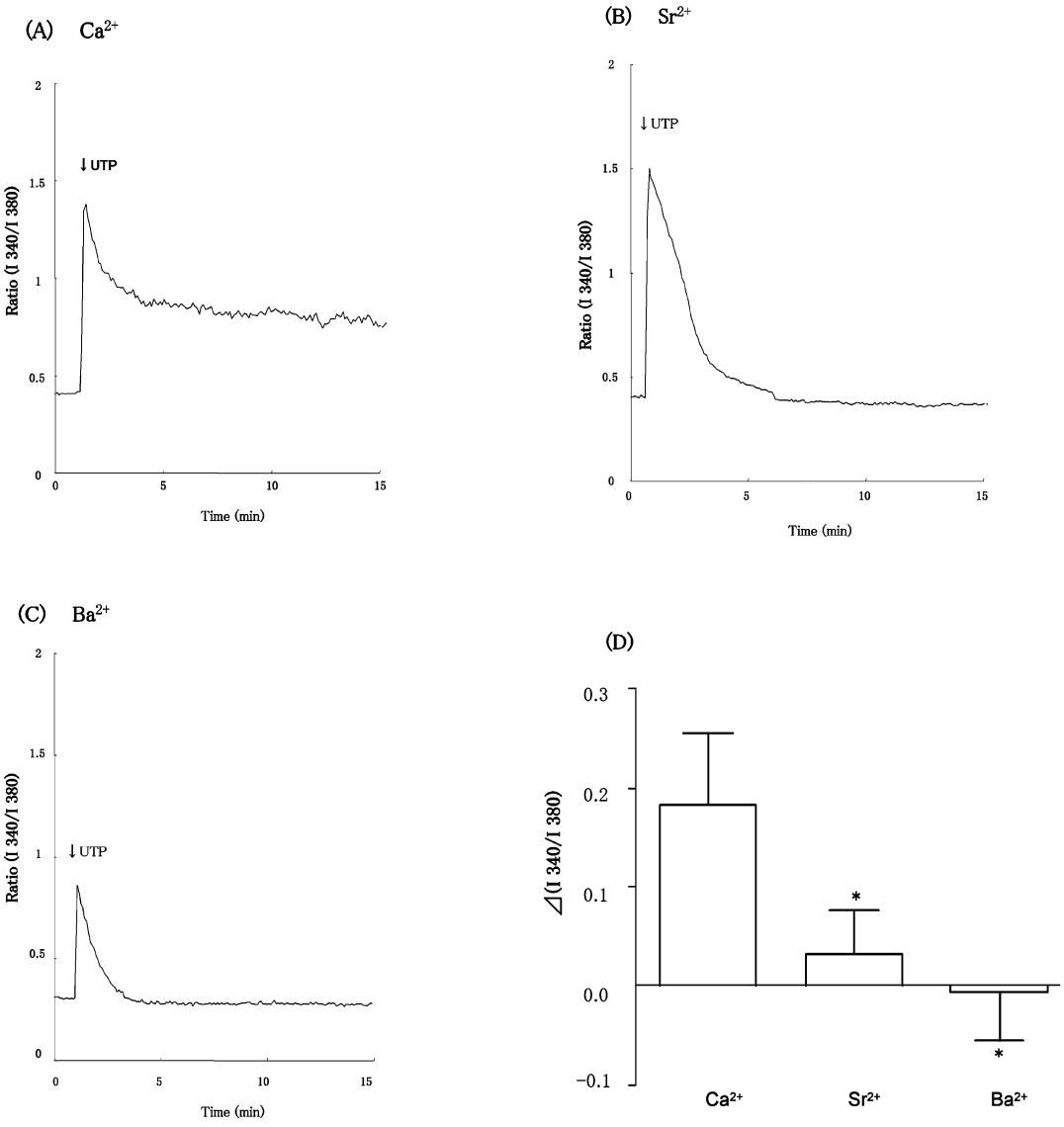

Fig. 2. Effect of UTP on the fura-2 fluorescence intensity in Ca^{2+}-buffer, Sr^{2+}-buffer, and Ba^{2+}-buffer in BAFCs
BAFCs on a cover slip were perfused with Ca^{2+}-buffer, Sr^{2+}-buffer, or Ba^{2+}-buffer. UTP (10 μM) was added 50 seconds after the start of fluorescence determination in Ca^{2+}-buffer (A), Sr^{2+}-buffer (B), and Ba^{2+}-buffer (C). Each trace is a typical trace from the 4 experiments.
(D) The ratio of the fluorescence intensity 10 minutes after the start of fluorescence determination. *Statistically different from the value in Ca^{2+}-buffer ($p < 0.01$). Each value represents the mean ± SE. $n = 4$.

rescence intensity, and the second sustained phase was not observed. However, after Sr^{2+}-buffer was replaced with Ca^{2+}-buffer, the fura-2 fluorescence intensity increased rapidly and was sustained (Fig. 4).

These results indicate Sr^{2+} could not enter BAFCs from the extracellular pool through CPA-induced SOCE.

DISCUSSION

SOCE plays an important role in the regulation of intracellular Ca^{2+} mobilization in nonexcitable cells[1]. However, the precise mechanism of SOCE activation has not been established.

We have reported UTP-induced SOCE in BAFCs[7]. We have also reported that SOCE in

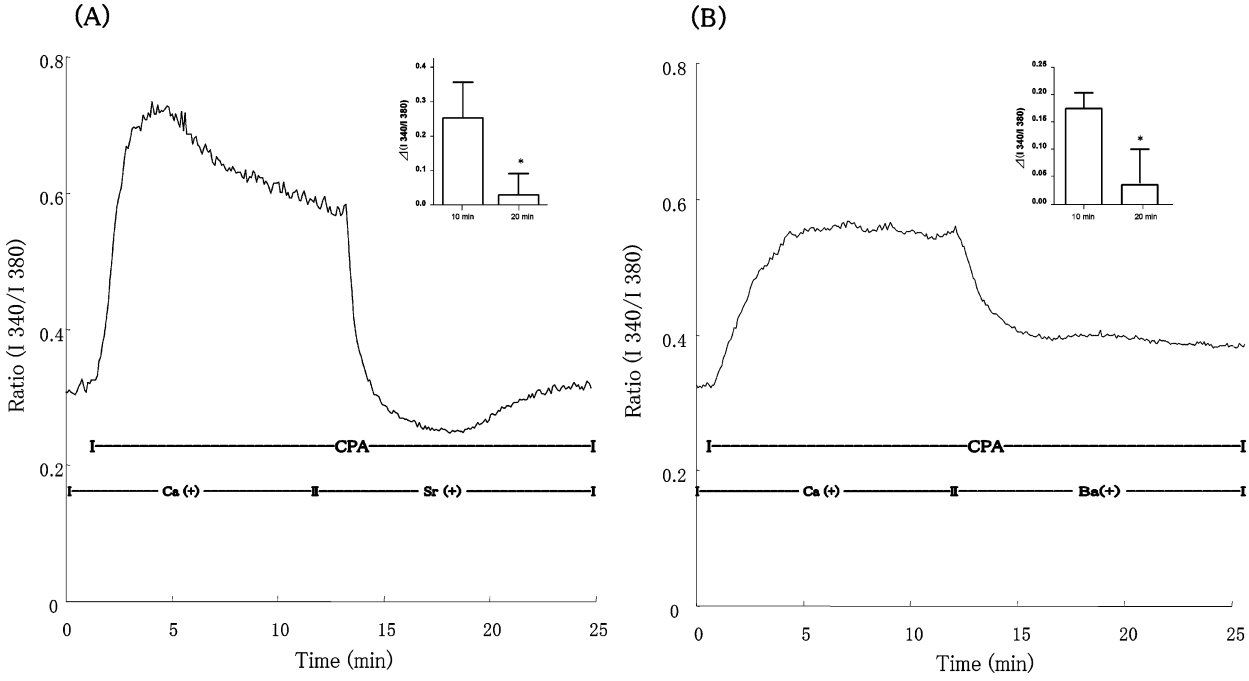

Fig. 3. Effect of CPA on the fura-2 fluorescence intensity in Ca^{2+}-buffer, Sr^{2+}-buffer, and Ba^{2+}-buffer in BAFCs
CPA (100 μM) was added 50 seconds after the start of fluorescence determination in Ca^{2+}-buffer. At the plateau phase of fluorescence intensity by CPA, Ca^{2+}-buffer was replaced with Sr^{2+}-buffer (A) and Ba^{2+}-buffer (B) containing CPA. Each trace is a typical trace from the 4 experiments. $n=4$.
(C) The ratio of the fura-2 fluorescence intensity 10 minutes and 25 minutes after the start of fluorescence determination. *Statistically different from the value 10 minutes after the start of fluorescence determination ($p<0.01$). Each value represents the mean±SE. $n=4$.

BAFCs is closely related to glucocorticoid production[17,18]. Therefore, the activation mechanisms of SOCE in BAFCs are necessary to clarify the effect of Ca^{2+} on glucocorticoid synthesis.

Recently, the mechanisms of SOCE activation have been narrowed down to 3 hypotheses: 1) the conformational coupling model and secretion-like model, 2) the vesicular fusion model, and 3) the diffusible messenger (the Ca^{2+} influx factor) model[1]. In the conformational coupling and the secretion-like coupling model, IP_3 receptors in the ER are loosely attached to the SOCE channels in the PM, and IP_3 receptor-SOCE channel interaction may occur after Ca^{2+} store depletion to activate SOCE[19]. In the secretion-like coupling model, IP_3 receptor-SOCE channel complexes do not form, but peripheral ER containing IP_3 receptors travel to the PM after Ca^{2+} store depletion[20]. The vesicular fusion model suggests that SOCE channels are not in the PM. There are SOCE channels containing vesicles in the cytosol, and the vesicles are inserted into the PM by an exocytic-like mechanism upon Ca^{2+} store depletion[21]. In these models, the intracellular dynamics of actin filaments plays an important role. The diffusible messenger model proposed by Takemura et al.[22] suggests that a SOCE channel-activating factor is released from the ER by Ca^{2+} store depletion and that the factor diffuses to the PM to activate SOCE channels.

We have previously shown that a myosin-light chain kinase inhibitor (ML-9), calmodulin inhibitors (W-7 and E6 berbamine), and cytochalasin D inhibits UTP- and CPA-induced SOCE concomitant with the depolymerization of actin filaments in BAFCs[8]. On the basis of these results, we have suggested that the conformational coupling and secretion-like model was predominant in BAFCs[8]. This model advocates that SOCE channels in the PM and IP_3 receptors in the ER form SOCE units or that IP_3 receptors are close to the PM, and once the luminal Ca^{2+} in the ER is depleted, the SOCE channels and IP_3 receptors inter-

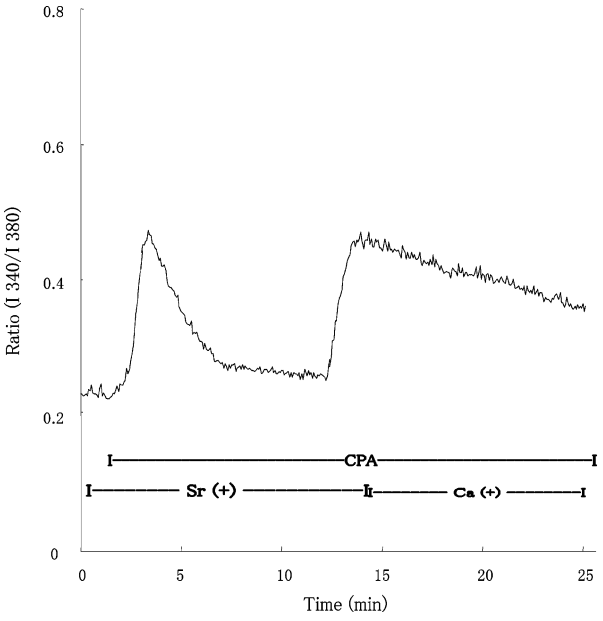

Fig. 4. Effect of CPA on the fura-2 fluorescence intensity in Sr^{2+}-buffer followed by Ca^{2+}-buffer in BAFCs
BAFCs on a cover slip were perfused with Sr^{2+}-buffer. CPA (100 μM) was added 50 seconds after the start of fluorescence determination. When the first phase of the fluorescence intensity by CPA decreased, the buffer was replaced with Ca^{2+}-buffer containing CPA (100 μM). The trace is a typical trace from the 5 experiments.

act with each other, after which SOCE is activated[1]. However, because the applicable model differs with cell types and cell lines, our proposed model may not be universally applicable. Although these models have been proposed on the basis of evidence obtained with different types of cells, the SOCE channels in the PM have not been established.

A candidate for the SOCE channel is a member of the TRP superfamily[4,5]. One of the 7 TRP families, the TRPC family is expressed in many types of cells and cell lines. The TRPCs are reported to be cation channels involved in SOCE channels[4-6]. Philipp et al.[8] have reported that TRPC4 is abundantly expressed in bovine adrenocortical cells and that TRPC1 is expressed at a traceable level. They did not detect any other TRPC subtypes. We have also shown that TRPC4 is expressed in BAFCs. Philipp et al. also suggested that the character of TRPC4 channels resemble calcium release-activated Ca^{2+} channels, a type of SOCE channel[8]. SOCE channels are highly Ca^{2+}-selective[11], but TRPC4 is not[9]. SOCE channels are highly permeable to Ca^{2+}, but Sr^{2+} and Ba^{2+} are poorly permeable divalent cations[11]. However, TRPC4 is permeable to not only Ca^{2+} but also Sr^{2+} and Ba^{2+} [9]. Both Sr^{2+} and Ba^{2+} reportedly bind to fura-2 to increase the fura-2 fluorescence intensity in the same manner as does Ca^{2+} [13]. Therefore, if extracellular Sr^{2+} and Ba^{2+} enter the cells, the fura-2 fluorescence intensity will increase.

Under our experimental conditions, UTP and CPA will induce Ca^{2+} influx after the depletion of luminal Ca^{2+} in the ER. However, when Ca^{2+} in the perfusing buffer was replaced by Sr^{2+} or Ba^{2+}, the second increase in fura-2 fluorescence intensity was not observed. Therefore, treatment with UTP and CPA could not make BAFCs permeable to either cation. These results suggest that TRPC4 does not act as SOCE channels in BAFCs. Experimental evidence in HEK 293 cells[23-25], CHO cells[26], and rat glial cells[10] also suggest that TRPCs are cation channels not involved in SOCE. Our results and these reports suggest that TRPC4 do serve as SOCE channels in BAFCs.

More recently, promising candidates for an SOCE activating system have been proposed. One is Orai1 in the PM as a SOCE channel in HEK 293 cells[27], and the other is STIM1 in the ER membrane as a Ca^{2+} sensor in T lymphocytes[28,29]. According to one hypothesis, STIM1 has an EF-hand on the lumen side and detects the luminal Ca^{2+} depletion. Then STIM1 polymerizes in the ER membrane and moves to the PM, after which Orai1 is activated[30]. The possible involvement of Orai1 and STIM1 in the SOCE system should be studied in BAFCs.

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