Calmodulin Modulates the Delay Period between Release of Calcium from Internal Stores and Activation of Calcium Influx via Endogenous TRP1 Channels*

Received for publication, May 8, 2002, and in revised form, August 22, 2002
Published, JBC Papers in Press, August 23, 2002, DOI 10.1074/jbc.M204531200

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In the present study we have explored the role of calmodulin (CaM) and inositol 1,4,5-trisphosphate receptor (IP3R) in the communication process activated after the release of calcium from the endoplasmic reticulum (ER) and the activation of calcium influx via endogenous TRP1 channels from Chinese hamster ovary cells. Experiments using combined rapid confocal calcium and electrophysiology measurements uncovered a consistent delay of around 900 ms between the first detectable calcium released from the ER and the activation of the calcium current. This delay was evident with two different methods used to release calcium from the ER: either the blockade of the microsomal calcium ATPase with thapsigargin or activation of bradykinin receptors linked to the IP3 cascade. Direct application of IP3 or a peptide from the NH2-terminal region of the IP3R activated store operated calcium, reducing the delay period. Introduction of CaM into the cell via the patch pipette increased the delay period from 900 ms to 10 ± 2.1 s (n = 18). Furthermore, the use of selective CaM antagonists W7 and trifluoperazine maleate resulted in a substantial reduction of the delay period to 200 ± 100 ms with 5 μM trifluoperazine maleate (n = 16) and 150 ± 50 ms with 500 nM W7 (n = 22). CaM reduced also the current density activated by thapsigargin or bradykinin to about 60% from control. The CaM antagonists did not affect significantly the current density. The results presented here are consistent with an antagonistic effect of IP3R and CaM for the activation of store operated calcium after depletion of the ER. The functional competition between the activating effect of IP3R and the inhibiting effect of CaM may modulate the delay period between the release of calcium from the ER and the activation of calcium influx observed in different cells, as well as the amount of current activated after depletion of the ER.

In a wide variety of nonexcitable and in many excitable cells, activation of G-protein-coupled receptors initiates a linear sequence of events leading to depletion of intracellular calcium storage compartments (the endoplasmic reticulum, ER) via the production of inositol 1,4,5-trisphosphate (IP3) by phospholipase C, and the subsequent induction of calcium influx from the extracellular space (1).

Depletion of the ER appears to be a prerequisite for the activation of calcium influx, because several experimental maneuvers that induce depletion of the ER, such as the introduction of IP3 into the cell or blockade of the microsomal calcium ATPase with thapsigargin (TG) and other selective blockers, are equally effective activators of calcium influx (1).

The activation of calcium influx after store depletion has been termed store-operated calcium entry (SOCE) and appears to be a well preserved mechanism from insects to humans (2). The finding that the transient receptor potential protein (TRP) from the Drosophila photoreceptor encodes a calcium-permeable channel activated after depletion of intracellular calcium stores, provided the first evidence for the identification of the molecular entity responsible for SOCE (3). An intense search in mammalian tissues led to the isolation of several mammalian TRP homologues, some of which are activated after depletion of the ER (4).

The diverse TRP superfamily of channels has been divided in three subfamilies (TRPC, TRPV, and TRPM) based on structural motifs (5). Members from the TRP superfamily are found in a wide range of organisms, from yeast to humans (6). One of the most prominent features in this superfamily is the wealth of regulatory mechanisms responsible for channel activation, including changes in osmolarity (TRPV4), ligands such as vanilloid (TRPV1), cold and menthol (TRPM8), store depletion and diacylglycerol (TRPC subfamily) (for review see Ref. 6).

Although the search for new TRPC homologues in a wide variety of organisms has proven very fruitful, the identification of the mechanism communicating the depleted state of the ER to the plasmalemmal channel remains elusive. Several models have been proposed to explain the communication between the ER and store operated channels (SOC), which can be divided into two main classes: one involving a physical coupling between the ER and the plasmalemmal channel and another suggesting the presence of a diffusible messenger responsible for communicating the depleted state of the ER to the plasma membrane (7).

Recent evidence favoring the physical coupling model shows direct and functional interactions between the IP3 receptor

* This work was supported in part by equipment from the Alexander von Humboldt Foundation (to L. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org

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(\textit{IP}_3 \text{R}) and human TRP3 channel (8). Furthermore, a common binding site for calmodulin (CaM) and \textit{IP}_3 \text{R} has been identified at the carboxyl termini of TRP channels (9). Interestingly, we have previously shown that the carboxyl-terminal domain of the \textit{Drosophila} TRP channel confers the thapsigargin sensitivity to TRPL, a channel that is not originally activated after depletion of the ER (10). We have shown also that CaM inhibits SOCE in vascular endothelial cell (11).

The modulation of channel activity by CaM appears to be a common feature found in several TRP members. In the \textit{Drosophila} photoreceptor, CaM mediates termination of the light response \textit{in vivo} via modulation of TRP and TRPL channels (12). CaT1, a TRP member recently identified, is modulated by a competitive interaction between CaM and protein kinase C (13). A CaM binding site has been identified in human TRP1 and TRP3 and mouse TRP4–TRP7 (9).

In the present study, we have characterized the role of CaM and \textit{IP}_3 \text{R} in the communication between the ER and endogenous TRP1 channels from Chinese hamster ovary (CHO) cells using rapid confocal microscopy and electrophysiology. The results presented here show: 1) a consistent delay of several hundreds of milliseconds between the first detectable release of calcium from the ER and the activation of TRP1 channels; 2) CaM antagonists significantly reduced the delay period; 3) introduction of CaM into the cell via the patch pipette significantly increased the delay period; 4) single channel measurements revealed a competitive interaction between CaM and \textit{IP}_3 in the modulation of TRP1 single channel activity; 5) a peptide from the \textit{IP}_3 \text{R} restored channel activity in excised inside-out patches; and 6) introduction of dsRNA from a segment of the TRP1 sequence resulted in very potent and specific reduction of TRP1 protein and SOCE.

All of these results show that a competitive interaction between CaM and \textit{IP}_3 \text{R} is sufficient to explain the activation of TRP1 channels and SOCE, after depletion of the ER in CHO cells. Our results show also that CaM has a dominant effect on TRP1 channel activity, which may play a role in preventing calcium influx under resting conditions.

MATERIALS AND METHODS

Reagents and Solutions—All salts were used as analytical grade purchased from Sigma. The PBS solution contained (mM): 140 Na-aspartate, 2 MgCl\textsubscript{2}, 10 HEPES, 2 Na-HPO\textsubscript{4}, and was adjusted to pH 7.2 with NaOH. The solution NA contained (mM): 140 Na-aspartate, 2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 HEPES, and was adjusted to pH 7.2 with NaOH. As HEPES, pH 7.2, with NaOH. In all whole cell experiments, the pipette (NA) solution contained in addition 2 mM ATP and 1 mM GTP from Sigma. As indicated, the calcium in PBS and NA solutions was adjusted to either 1 or 100 nM with EGTA as previously described (14). Bovine brain calmodulin, the 100-kDa calcium-binding protein from bovine brain, and the calmodulin antagonists W7 and trifluoperazine dimaleate (TFP) were purchased from Calbiochem (San Diego, CA).

Cell Culture—CHO cells were purchased from American Type Culture Collection (ATCC) and maintained in culture using Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 1% antibiotics, 2 mM L-glutamine, and 10% fetal bovine serum. Cells were grown on plastic Petri dishes and maintained with 5% carbon dioxide in a humidity controlled incubator (NUAIRE, Plymouth, MN). Cells were grown on plastic Petri dishes and maintained with 5% carbon dioxide in a humidity controlled incubator (NUAIRE, Plymouth, MN). The solution NA contained in addition 2 mM ATP and 1 mM GTP from Sigma. As indicated, the calcium in PBS and NA solutions was adjusted to either 1 or 100 nM with EGTA as previously described (14). Bovine brain calmodulin, the 100-kDa calcium-binding protein from bovine brain, and the calmodulin antagonists W7 and trifluoperazine dimaleate (TFP) were purchased from Calbiochem (San Diego, CA).

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Cloning of Partial cDNA Encoding TRP1—Degenerate oligonucleotide primers from TRP-conserved regions were 5’-TGGGCCATCCGCATCTCAAGAGGAACCA-3’ and 5’-TGTCATAGGCAAAGGGAAGCCAGACGAG-3’ synthesized by Life Technologies (Karlsruhe, Germany). The first primer was designed according to Okada et al. (15); the second primer was designed based on the highly TRP conserved amino acid sequence EWFKAR. mRNA from CHO cultures was isolated following standard procedures and cDNA was synthesized in vitro. A PCR was performed for 19 cycles and the products were analyzed in a polyacrylamide gel. Over five independent PCR reactions from different cultures produced similar results, a single product of 450 bp. The PCR product was cloned in the pCRII vector (Invitrogen, Carlsbad, CA). After sequencing the fragment, the nucleotide sequence was introduced into the pEGTA program (National Center for Biotechnology Information) to identify homologues. The search indicated that the cloned sequence was over 93.7% identical to rat TRP1 (accession number AF061266). The partial clone from CHO-TRP1 has been submitted to GenBank (accession number AF484942).

Stable Expression of the Human Bradykinin Type 2 Receptor in CHO Cells—The full-length cDNA encoding the human bradykinin type 2 receptor (Bk2R) was cloned into the EcoRI site of the pcDNA3 vector (Invitrogen) using standard ligation techniques. 50% confluent CHO cells were transfected with 5 \mu M of the construct mixed with LipofectAMINE Plus (Life Technologies). Transfected CHO cells were maintained in G418 (Life Technologies) for 1 month, and antibiotic-resistant colonies were isolated. The G418-resistant cells were maintained in culture and used in the studies described. Double-stranded RNA Interference—The entire 450-bp TRP1 DNA fragment isolated by PCR was cloned in pBluescript (Stratagene, La Jolla, CA) and linearized with BamHI to produce single stranded RNA using the MEGAscript kit (AMBION, Austin, TX) with the T3 polymerase. In a separate experiment the vector with insert was linearized with EcoRV to produce the complementary RNA strand with the T7 polymerase. Equal amounts of both single stranded RNA products were mixed and heated at 60 °C and cooled down slowly for 30 min until room temperature was reached. The product obtained was compared by non-denaturing electrophoresis with both single stranded RNA products to confirm that the double stranded RNA of dsRNA were mixed with LipofectAMINE Plus (Life Technologies) and the mixture was placed on 60-mm petri dishes containing 50% confluent CHO cells. 48 and 72 h later the same cells were transfected again with 5 \mu g of dsRNA each time. Calcium measurements were performed 24–48 h after the third transfection. Control cells were exposed to LipofectAMINE alone using the same protocol. For Bk2R RNAi experiments a fragment from nucleotides 420 to 762 was isolated by PCR and cloned in pBluescript. The dsRNA was prepared as described above for TRP1. CHO cells were transfected three times with the dsRNA using a similar procedure to the one described above.

Western Blotting Analysis—Total membrane extracts from control cells and RNAi-treated cells were resolved by SDS-PAGE and transferred to nitrocellulose paper as previously reported (16). To ensure equal amounts of protein loading, the amount of total protein was assessed using the MicroBCA protein assay kit (Pierce) and albumin as control. 10 \mu g of protein was loaded for every experimental condition. Antibody anti-TRPC1 (ALAMONE Labs, number ACC-010) was diluted 1:1000 in buffer A containing (in mM): 50 Tris, 150 NaCl, pH 7.4, and 2% bovine serum albumin (Sigma). After 30 min incubation at 37 °C, the nitrocellulose sheets were washed 5 times for 15 min each time with buffer A and incubated for 30 min with peroxidase-labeled mouse anti-rabbit antibody diluted 1:5000 (Sigma). After a second washing period, bound antibody was visualized with the Supersignal Ultra chemiluminescent substrate (Pierce). A detection kit (Pierce) for the human type Bk2 receptor was detected with antibody AS 346-2 (a generous gift from Dr. Müller-Esterl, Johannes Gutenberg University at Mainz) as previously described (16).

Free Calcium Determinations—The free calcium reported in the different solutions was adjusted by the addition of EGTA purchased from Sigma. To determine the amount of EGTA needed to obtain the free calcium for each experimental condition, we used the program “Bound and Determined” (BAD 4.3.5) as previously described (14). Free calcium concentrations reported in the text were corroborated using FURA-2 tetrapotassium salt (Molecular Probes, Eugene OR) and an Aminco-Bowman Series II spectrophotometer.

Synthetic Peptides from \textit{IP}_3 \text{R}—The peptide IP\textsubscript{R}-P1 (EYLYSIY-SEEYEWLTWTWD) was purchased from ResGen (Carlsbad, CA). This peptide has been previously reported to bind several TRP channels and compete with CaM for a common binding site (9). The scrambled peptide IP\textsubscript{R}-P2 (TWLSTDEVLEIYEWEYSE) was used as control. This peptide contains the same amino acids as IP\textsubscript{R}-P1 but in random order. Peptides were introduced into the cell via the patch pipette or perfused on isolated patches (single channel experiments) via a Picospritzer II (General Valve, Fairfield, NJ).

Measurements of Free Cytosolic Calcium in Cell Populations—Free calcium in CHO cells was monitored as previously described (16). CHO cells were incubated in physiological solution containing (mM): 120 NaCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.8 MgSO\textsubscript{4}, 4.75 KCl, 1.2 CaCl\textsubscript{2}, 10 HEPES, 0.05% bovine serum albumin, and 5 \mu M fura-2 AM (Molecular Probes). Following a 30-min incubation at room temperature (22 °C), cells were washed twice with physiological solution and treated for 2 min with 0.25% trypsin. Cells were later dispersed with a plastic pipette and...
subjected to centrifugation, and resuspended in PBS. Fluorescence was measured using an Amino-Bowman Series II spectrophotometer. Excitation wavelength alternated between 340 and 380 nm, and fluorescence intensity was monitored at an emission wavelength of 510 nm. Calibration of the fura-2 associated with the cells was accomplished using Triton lysis in the presence of a saturating concentration of calcium followed by addition of EGTA (pH 8.5). Free calcium was calculated as previously described (16).

**Combined Confocal Calcium and Electrophysiology Measurements**—CHO cells were loaded with 5 μM Fluo-4-AM using the loading procedure described above for FURA-2-AM. Cells were placed on glass coverslips and allowed to attach to the glass surface. Combined patch clamp and confocal microscopy measurements were performed using an EPC9 amplifier (Heka Instruments, Bonn, Germany) and a real-time confocal system (Noran Instruments, Oxon, England). The EPC9 amplifier was synchronized with the confocal acquisition unit via a TTL pulse. The microscope objective used was Nikon 60X Plan-Apo 1.40 oil immersion. The confocal acquisition window was set to 300 × 300 pixels to improve acquisition speed. At this resolution the system acquired one image every 10 ms. Five images were averaged and the result was saved, producing one averaged image every 50 ms. All images were stored on the hard disk of an Indigo (Silicon Graphics, Mountain View, CA) for off-line analysis. CHO cells showed diameters in the range of 15–20 μm, the focal point was moved to approximately the middle of the cell, using a focal resolution of 10 μm. Conventional whole cell experiments and perforated patch mode were performed on Fluo-4-AM-loaded CHO cells. The voltage protocol consisted of a ramp from −100 to +100 mV delivered continuously every 50 ms for the duration of the experiment. Patch pipette resistance ranged from 6 to 10 MΩ. To compare among different cells, the amount of current from each cell was corrected by the cell capacitance and reported throughout the text as pA/pF. TG and Bk were applied to the cell via a Picospritzer II (General Valve). Drug applications were synchronized with electrophysiology and confocal acquisition via a TTL pulse delivered by a DG535 Digital Delay/Pulse Generator (Stanford Research Systems, Sunnyvale, CA). Whole cell data was recorded and stored on a PC computer hard disk for off-line analysis using Igor Pro 4 software (Wavemetrics, Lake Oswego, OR). Fluorescence intensity was measured with procedures written in-house with Igor Pro.

**Single Channel Experiments**—For cell-attached and inside-out experiments, the pipette contained the PBS solution with the calcium reported, or each condition, as indicated in the figure legends. Data was stored on FM tape (VETTER PCM recorder) for off-line analysis. Single channel recordings were digitized at 10 kHz using a Digidata 1200 (Axon Instruments, Union City, CA) and filtered at 5 kHz during analysis using pClamp software (Axon Instruments). An in-house designed multibarreled microperfusion system was used to apply the different concentrations of CaM and IP₃.

**RESULTS**

**Simultaneous Measurements of Confocal Calcium and Whole Cell Currents**—Fluo-4-loaded single CHO cells stably transfected with the human Bk2R responded to Bk with elevation of intracellular calcium in a dose-dependent manner, showing a maximum response to 75 nM Bk (data not shown). Over 90% of the cells studied showed this response. These results are consistent with the affinity of this receptor for Bk measured in different expression systems (16). Nontransfected CHO cells did not respond to Bk at the concentrations tested in this study (up to 1 μM).

Combined whole cell patch clamp and rapid confocal intracellular calcium measurements with Fluo-4-loaded single CHO cells uncovered a consistent delay between the first detectable increment of intracellular calcium and the activation of calcium influx current. Approximately 85% of the cells studied (43 of 50) responded to a saturating Bk concentration (100 nM) with a rapid elevation in intracellular calcium observed within the first 50 ms after Bk application, followed 900 ± 100 ms later by an increment in inward current (Fig. 1). In these experiments the extracellular solution was PBS and the intracellular solution was the NA solution with 100 nM free calcium ("Materials and Methods"). Under these conditions the inward current activated by Bk showed a reversal potential of +45 ± 5 mV (n = 40), only a current carried primarily by calcium ions would show such a positive reversal potential given the solutions used. Replacing the bath solution for the NMDG-Cl solution with 2 mM calcium ("Materials and Methods") did not result in a significant change of the reversal potential (+48 ± 4 mV, n = 17), further supporting the calcium selectivity of the Bk-activated inward current.

The delay between the first detectable increment of intracellular calcium and the activation of calcium influx current was slightly longer in cells studied in the perforated patch mode using nystatin in the patch pipette (800 ± 50 ms, n = 35). The differences between the whole cell and the perforated patch mode were not statistically significant, probably because of the fact that whole cell experiments were completed within the first minute after disrupting the plasma membrane. Under these conditions, the dilution of intracellular components was not favorable.

The initial fluorescence increment (IFI) obtained after Bk stimulation was localized to well defined spots in the cells (Fig. 1). Because no inward current was measured in this initial time periods, the IFI most likely reflects the release of calcium from intracellular storage compartments (ER). By the time the inward current was activated, the elevation in fluorescence intensity was observed throughout the cytosol, part of which most likely reflects the influx of calcium from the extracellular space.

**CaM Increases the Delay Period between Initial Release from ER and Activation of SOC**—Fig. 2A illustrates representative experiments using combined rapid confocal microscopy and whole cell current measurements. Under control conditions, after Bk stimulation, a typical delay of 900 ± 100 ms between IFI and activation of inward current was observed. Introduction of two CaM selective antagonists, W7 and TFp, in the patch pipette significantly reduced the delay period between IFI and inward current activation, when compared with control experiments (Fig. 2). Under these conditions, the delay period observed was 200 ± 100 ms with 5 μM TFp (n = 16) and 150 ± 50 ms with 500 nM W7 (n = 22). This reduction represents 1/4 of the control value. Increasing TFp to 20 μM or W7 to 5 μM did not result in further reductions of the delay period (data not shown) therefore, the remaining studies were carried out with these CaM antagonists concentrations.

Introduction of CaM in the patch pipette resulted in a significant increment of the delay period between IFI and the activation of inward current. In experiments where 10 μM CaM was introduced in the patch pipette, the delay period increased to 10 ± 2.1 s, a value around 10 times greater than control (n = 18, Fig. 2B). The CaM effects were observed with 100 nM free intracellular calcium (in the patch pipette) adjusted with EGTA ("Materials and Methods"). Buffering calcium in the patch pipette to 1 nM prevented the effects of CaM on the delay period between IFI and activation of inward current (data not shown). With 1 nM free intracellular calcium the delay period observed in the presence of 10 μM CaM was 950 ± 150 ms (n = 7), a value not significantly different from control (p > 0.1). These results suggest that the CaM-calcium complex modulates the delay period between the initial release of calcium from the ER and the activation of SOC. CaM blockade reduces significantly the delay period, and SOCE initiates faster.

CaM and the CaM antagonists affected also the time to reach the maximum current. In control conditions, the whole cell current density reached its peak with a time constant of 15 ± 4.1 s, to a value of 6.12 ± 0.13 pA/pF at −100 mV (n = 25, Fig. 2C). With 10 μM CaM in the pipette, the maximum current density was reached with a time constant of 25 ± 5.2 s to a value of 3.65 ± 0.34 pA/pF (n = 18, Fig. 2C). The CaM antag-
onists reduced the time to reach the maximum current density to 6.2 ± 1.8 s with TFP (n = 16, Fig. 2C) and 6.8 ± 1.5 s with W7 (n = 22, Fig. 2C). The maximum current density obtained with CaM was about 60% from control (Fig. 2B). Both CaM antagonists did not alter significantly the maximum current density compared with control (6.16 ± 0.43 and 6.12 ± 0.32 pA/pF for W7 and TFP, respectively, Fig. 2B). These results resemble the effects of CaM on the TG-induced calcium influx current from vascular endothelial cells (11). To determine whether the effects observed with CaM were the result of calcium buffering by this calcium-binding protein, we performed similar experiments to those indicated above but replaced CaM with 20 μM S-100 protein from bovine brain, a calcium-binding protein with some structural similarity to CaM.

With 20 μM S-100 in the patch pipette, the delay period between IFI and activation of inward current was 850 ± 120 ms (n = 8), a value not significantly different from the value obtained in the absence of S-100 (910 ± 140 ms, n = 6). These results strongly suggest that the effects observed with CaM are not the result of buffering the calcium in the cell.

Because any maneuver that alters the rate of release of calcium from the ER may affect also the time of activation of SOC (17), we designed experiments to explore the effect of incubating cells with W7 and TFP on the rate of calcium release from the ER. To explore this we took two avenues: first we explored the increments in intracellular calcium in CHO cells incubated for 5 min with the CaM antagonist and compared the time course of calcium increments with control cells (not exposed to CaM antagonists). To ensure that the CaM antagonists were permeating adequately into the cells, we performed additional experiments in which the CaM antagonists were introduced in the cell via the patch pipette, as we have done in previous experiments. With both experimental procedures the extracellular (PBS) solution contained less than 1 nM free calcium, to minimize the effects of calcium influx. Under these experimental conditions, fluorescence increments would reflect primarily the release of calcium from the ER.

Fig. 3A illustrates representative experiments of the increment in single cell fluorescence after application of 100 nM Bk in a cell under control conditions and a cell incubated for 5 min with W7. Fig. 3B shows the time course of the increments in fluorescence in response to Bk in control cells (CON, n = 16) and W7-treated cells (n = 14). Fig. 3C shows representative examples in an expanded time scale to illustrate the time course of the rise in average cell fluorescence in a control cell (CON) and a cell exposed to TFP or W7 for 5 min. The time course of the rapid increment in intracellular calcium was well fitted by a sigmoidal function, providing half-activation constants of 340 ± 20 ms for the control condition, 325 ± 25 ms with W7, and 320 ± 20 ms with TFP. Similar time constants were obtained in cells in which the CaM antagonists were introduced via the patch pipette (data not shown). Under these conditions, half-activation constants were 350 ± 22 ms for control cells (n = 8), 318 ± 21 ms for W7 (n = 12), and 321 ± 25 ms for TFP (n = 14).

As indicated by these results, neither of the CaM antagonists altered significantly the time course of average cell fluorescence increments in response to Bk. Because these experiments were performed in low extracellular calcium (<1 mM), the increment in fluorescence reflects essentially the release of calcium from ER. Therefore, these results strongly suggest that
the effects of CaM and the CaM antagonists on the inward current are not the result of altering the time course of calcium release from ER, but rather because of the modulation of the communication between release of ER and SOC activation.

Identification of the Channels Activated after Depletion of the ER

In the next series of experiments we explored the ionic selectivity of the Bk-activated inward current. With the PBS extracellular solution containing 2 mM calcium, the current-voltage relationship presented a reversal potential of 40 ± 5 mV (n = 22). A similar inward current was activated also with 200 nM of the calcium ATPase inhibitor, TG. Fig. 4A illustrates a representative experiment with the TG-induced inward current. The current reversal potential obtained from the current-voltage relationship was 46 ± 6 mV (n = 15), a value not significantly different from the reversal potential obtained for the Bk-activated current.

The effects of CaM and the CaM antagonists on the inward current are not the result of altering the time course of calcium release from ER, but rather because of the modulation of the communication between release of ER and SOC activation.
Reducing the extracellular calcium to less than 1 nM ("Materials and Methods") resulted in a shift of the reversal potential to $-1 \pm 4$ and $0 \pm 3$ mV for the Bk ($n = 12$) and the TG ($n = 9$)-induced currents, and a linear current-voltage relationship (Fig. 4A). These results indicate that, in the absence of extracellular calcium, the TG-Bk-induced current permeates primarily monovalent cations. The switch in selectivity from divalent to monovalent cations upon removal of extracellular calcium has been previously observed for other depletion-activated channels (18, 19).

Application of 100 nM Bk in the cell-attached mode produced the activation of single channel currents in 22 of 35 attempts; the remaining patches did not show channel activity upon agonist stimulation. 200 nM TG induced similar single channel currents in 15 of 22 attempts. Fig. 4B illustrates representative experiments showing the channel activity induced by Bk or TG. In the majority of the experiments, both agonists induced the activity of 2–4 channels, suggesting some form of channel clustering.

Channel activity was induced in the cell-attached mode and then explored at different voltages in the inside-out configuration. For these experiments we used either 2 or 1 nM extracellular free calcium ("Materials and Methods"). Examples of channel activity in the inside-out configuration are illustrated in Fig. 4C. With 2 mM calcium, the channel reversal potential was $55 \pm 5$ and $0 \pm 3$ mV with 1 nM calcium (Fig. 4D). The current-voltage relationships for the TG and the Bk-induced channel activity were indistinguishable (Fig. 4D). The single channel conductance measured from the slope obtained between $-100$ and $+100$ mV in low extracellular calcium (1 nM) was 10 and 2.5 pS with 2 mM extracellular calcium (Fig. 4D).

Excising the patches of membrane from previously stimulated cells to form the inside-out configuration resulted in rapid run-down of channel activity as illustrated in Fig. 5. Channel activity in the inside-out mode could be recovered by the application of 10 μM IP$_3$ (Fig. 5A). The effect of IP$_3$ was inhibited when the patch of membrane was simultaneously exposed to 1 μM CaM (Fig. 5B). Fig. 5C illustrates the number of channels multiplied by the open probability (NP$_o$) measured over time for the representative experiment shown in Fig. 5A, and Fig. 5D shows the NP$_o$ plot for the experiment in B.

We characterized further the antagonistic effects of CaM and IP$_3$ on single channel activity. Fig. 6A shows the recovery of channel activity with three different IP$_3$ concentrations. As illustrated in the figure, the maximum activity was obtained with 10 μM IP$_3$. However, even with 100 μM IP$_3$ the effect of CaM was evident (Fig. 6A). Fig. 6B shows the $P_o$ plot for the experiment illustrated in Fig. 6A. Fig. 6C shows the pooled data from 15 independent single channel experiments in which three different concentrations of CaM and IP$_3$ were explored. As indicated in the figure, the inhibitory effect of CaM prevailed, even at 100 μM IP$_3$, which was the concentration that induced the maximum increment in channel $P_o$. With 100 μM IP$_3$, the $P_o$ decreased from 0.8 ± 0.1 with 0.1 CaM to 0.4 ± 0.09 with 1 μM CaM, to 0.2 ± 0.05 with 10 μM CaM (Fig. 6C). The dose-response curves were well fitted by a Hill equation, providing Hill coefficients of 1.8 for 0.1 CaM, 3 for 1 μM CaM, and 2.8 for 10 μM CaM. These results indicate that at least two CaM may be required for the inhibition of channel activity.

These results indicate that the inhibitory effect of CaM on channel activity prevailed even at saturating concentrations of IP$_3$, suggesting a dominant effect of CaM on channel activity. The consequences of this finding in the modulation of SOCE will be discussed later in this article.

The effect of IP$_3$ on channel activity can have at least two possible explanations. One is that IP$_3$ may be interacting directly with the channel. The second possibility is that IP$_3$ may activate the IP$_3$R, which in turn might modulate channel activity at the plasma membrane. For the second hypothesis to be true, the excised patches of membrane must retain fragments of the ER with functional IP$_3$Rs.
Modulation of TRP channel activity by peptides from the type 3 IP$_3$R has been previously shown in excised patches from cells expressing hTRPC3 (20). Furthermore, IP$_3$R and CaM share a common binding site in several TRPC channels (21).

To explore if the effects of IP$_3$ observed in the present study were mediated by IP$_3$R, a series of experiments were designed to study the effect of a synthetic peptide from the type 3 IP$_3$R on whole cell currents and single channel activity. Fig. 7A illustrates the effect of introducing 50 μM of the NH$_2$-terminal peptide from the type 3 IP$_3$R (IP$_3$R-P1) into the cell via the patch pipette. This peptide has been previously shown to induce hTRPC3 channel activity in excised patches and bind to several TRP channels, competing with CaM for this common binding site (21).

Introducing the IP$_3$R-P1 peptide resulted in activation of inward current in the absence of TG or Bk stimulation in 12 of 14 cells explored (Fig. 7A). Application of 200 nM TG after the IP$_3$R-P1-induced inward current reached steady state resulted only in a minor increment in current amplitude. Cells not exposed to the IP$_3$R-P1 or cells exposed to 50 μM of the scrambled peptide IP$_3$R-P2 (used as control) did not produce inward current until the application of 200 nM TG, which resulted in typical inward current activation. The amount and time course of inward current induced by TG in the presence of IP$_3$R-P2 was indistinguishable from the current activated in the absence of both peptides (Fig. 7A, CON).

These results indicate that TG cannot increase further the current activated by IP3R-P1, suggesting that this peptide and TG activate the same single channel conductance. On the contrary, the IP$_3$R-P2 peptide failed to activate inward current.

Fig. 7B illustrates a dose-response curve for IP$_3$R-P1 showing that the maximum amount of current is obtained with 50 μM of this peptide. Fig. 7C shows the effect of IP$_3$R-P1 on single channel activity from excised inside-out patches. As illustrated in the figure, IP$_3$R-P1 increased channel $P_o$ from 0.12 ± 0.1 to 0.71 ± 0.11 (n = 6). Addition of 100 μM IP$_3$ in the continuous presence of IP$_3$R-P1 resulted only in a minor (not statistically significant) increment of channel $P_o$ to 0.76 ± 0.13 (n = 6; p > 0.1). Addition of 100 μM CaM, in the continuous presence of IP$_3$R-P1, resulted in rapid reduction of channel $P_o$ from 0.76 ± 0.13 to 0.21 ± 0.09 (n = 6).

Similarly to the whole cell experiments, IP$_3$R-P2 failed to increase channel activity in inside-out patches as illustrated in Fig. 7D (control = 0.15 ± 0.12, 50 μM IP$_3$R-P2 = 0.16 ± 0.12, n = 6). However, in the presence of IP$_3$R-P2, 100 μM IP$_3$ increased channel activity to 0.69 ± 0.13 in 5 of 6 patches tested. Under these conditions, 10 μM CaM produced a typical $P_o$ channel reduction from 0.7 ± 0.11 to 0.26 ± 0.13 (n = 6). These results strongly suggest that part of the effects observed with IP$_3$ on SOC channel activity (Fig. 6) is mediated by the IP$_3$R. Furthermore, similarly to the results obtained with IP$_3$, the inhibitory effect of 10 μM CaM prevailed even in the presence of 50 μM IP$_3$R-P1 peptide.

**TRP1 Is an Essential Component of SOCE in CHO Cells**—In an attempt to determine the TRP channels present in CHO cells and responsible for the inward current activated after depletion of the ER, we performed PCR experiments using degenerated oligonucleotides that ensure the identification of several of the TRPC members (“Materials and Methods”). We tested these oligonucleotides with a cDNA library from rat retina and found PCR amplification of TRP1, TRP4, TRP5, and TRP6. However, in five independent PCR reactions using cDNA from CHO cells, we found exclusively the amplification
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of a single product. The product obtained under these conditions was sequenced and compared with the data base at the National Center for Biotechnology Information using the Blast program. The results indicate that the product obtained was over 93% identical to rat TRP1 at the nucleotide level (accession number AF081266) and 99% identical at the amino acid level. These results show the presence of a TRP1 homologue in CHO cells, confirming previously published results showing expression of TRP1 and TRP2 in this cell line (22).

Although these results provide evidence indicating that CHO cells possess a TRP1 homologue, a direct correlation between this channel and SOCE was necessary. To accomplish this, we took advantage of the fact that introduction of dsRNA into a wide variety of cells and organisms results in potent and highly specific gene silencing (23). This phenomenon has been termed RNA interference or RNAi (24).

Experiments were carried out to evaluate the role of TRP1 in the Bk- and TG-induced SOCE. For this purpose, dsRNA from TRP1 was synthesized by standard techniques and introduced into CHO cells using LipofectAMINE ("Materials and Methods"). A repetitive transfection protocol was designed to ensure the transfection of the majority of cells ("Materials and Methods"). Calcium measurements with cell populations from TRP1-dsRNA-treated CHO cells showed that TG-induced calcium influx was markedly reduced in these cells, without any significant effect on the release of calcium from the ER (Fig. 8A).

The integral of calcium influx from control cells in response to TG was 360 ± 80 nM s⁻¹ (Fig. 8C, n = 32). In TRP1-dsRNA-treated CHO cells the integral calcium influx was significantly reduced to 36 ± 28 nM s⁻¹ (n = 23). Similarly to the TG experiments, the calcium influx induced by Bk in TRP1-dsRNA-treated CHO cells was also greatly reduced. The integral of calcium influx in response to Bk in control cells was 440 ± 51 nM s⁻¹ (Fig. 8D, n = 24). This value was reduced to 50 ± 26 nM s⁻¹ (n = 24) in TRP1-dsRNA-treated CHO cells.

The reduction of TRP1 protein in dsRNA-treated CHO cells was confirmed by Western blotting analysis. As illustrated in Fig. 8E, CHO cells stably transfected with the Bk2R react with the specific anti-Bk2R antibody showing a single band (Fig. 8E, lane 1). The same cells exposed to the specific anti-TRP1 antibody also recognize a single product (Fig. 8E, lane 1). Cells not transfected with Bk2R do not show a specific band for this receptor; however, under these experimental conditions, a single product is recognized by the specific anti-TRP1 antibody (Fig. 8E, lane 2).

The use of dsRNA for the Bk2R significantly reduced the amount of Bk2R protein as illustrated in Fig. 8E, lane 3. This treatment did not affect the expression of TRP1 (Fig. 8E, lane 3). On the other hand, CHO cells transfected with Bk2R and treated with dsRNA for TRP1 did not alter the expression of Bk2R but greatly reduced the amount of TRP1 protein (Fig. 8E, lane 4). These results indicate that the RNAi is specific for the sequence used. Interfering with the expression of Bk2R did not alter the expression of TRP1 protein and vice versa.

These observations are consistent with the calcium experiments, where introduction of dsRNA for TRP1 reduced significantly the Bk- and TG-induced calcium influx, but not the release from the ER (Fig. 8, A–D). Similarly, introduction of dsRNA for Bk2R eliminated the Bk-induced calcium mobilizations (both release from the ER and calcium influx) without affecting the TG-induced calcium release from the ER and calcium influx (data not shown). The lack of effect of Bk stimulation in Bk2R-dsRNA-treated cells may represent the interference of the dsRNA with the synthesis of Bk2 receptors in these cells, because the IP₃ cascade appears to be unaffected as indicated by experiments in which the stimulation of endoge-

![Fig. 8. TRP1 is an essential component of SOCE in CHO cells.](image)

Changes in intracellular calcium monitored in Fura-2-loaded cell populations. Each experiment was performed with 1.5–2 million cells in suspension. Experiments were initially performed with 1 nM free extracellular calcium obtained by the addition of EGTA as indicated under "Materials and Methods" (Rel) and later 2 mM calcium was added to the bath solution (Inf). Data shows the mean ± S.D. from several independent measurements. A, control cells, and B, cells treated with dsRNA from TRP1 ("Materials and Methods"). The area under the curve (gray boxes) was integrated for each experimental condition representing the release (Rel) and calcium influx (Inf) and plotted in C for TG- and, D for BK-stimulated cells. Notice that the release was not affected by treatment with dsRNA from TRP1 but the influx activated by TG and BK (B-Inf) was significantly reduced. E, Western blotting experiments illustrating the effect of transfecting cells with dsRNA on protein concentration. Plasma membranes from control and dsRNA-treated cells were isolated as indicated under "Materials and Methods." Ten micrograms of protein were loaded in each lane and exposed to specific anti-Bk2R and anti-TRP1 antibodies. Lane 1, cells exposed to anti-Bk2R; lane 2, cells exposed to anti-TRP1; lane 3, cells treated with dsRNA for Bk2R and exposed to both antibodies; lane 4, cells treated with dsRNA for TRP1 and exposed to both antibodies. Notice that in lane 3 the signal for Bk2R was significantly reduced. In lane 4 the signal for TRP1 was also significantly reduced by treatment with dsRNA from this channel. F, representative whole cell currents obtained under control conditions (before the addition of thapsigargin in a control cell, −TG) and after addition of 200 nM TG in a control cell (+TG) and a cell transfected to dsRNA for TRP1 in the presence of 200 nM TG (−TRP1). G, mean ± S.D. of the whole cell current density (WCI) obtained at −100 mV in control cells exposed to 100 nM Bk, cells exposed to dsRNA for Bk2R, and current induced with 200 nM TG (TG-Bk2R), cells exposed to dsRNA for TRP1 and stimulated with 100 nM Bk (Bk-TRP1) or 200 nM TG (TG-TRP1). Notice the significant reduction in current density in response to Bk and TG cells exposed to dsRNA for TRP1. The sequence used for dsRNA from Bk2R was nucleotides 420–762, and the sequence for TRP1 was the entire PCR fragment (450 bp; GenBank™ accession number AF448492).
loration, where control cells showed current densities of 6.3 ± 0.82 pA/\(\mu F\) (n = 12, Fig. 8G, Bk) and TRP1 dsRNA-treated cells of 0.17 ± 0.5 pA/\(\mu F\) (n = 19, Fig. 8G, Bk-TRP1). Similarly to the results obtained with calcium measurements, the TG-induced activation of the inward current was not affected in CHO cells exposed to dsRNA from the Bk2R (Fig. 8I). This result is in agreement with previously published data showing that TRP1 and other TRP homologues (TRP2, TRP3, etc.), then the dsRNA from TRP1 could have interfered with these other channels, making difficult the interpretation about the relative contribution of TRP1 to SOCE.

We have used combined rapid confocal calcium and electrophysiological measurements to explore the correlation between the first measurable release of calcium from the ER and the activation of calcium current. Although this technique cannot detect the first release of calcium from the entire ER, because we are scanning from a section of the cell 10-\(\mu m\) thick, what we could measure was the first release of calcium in that particular focal plane.

Using these techniques we have observed consistently a delay of around 900 ms between the first measurable intracellular calcium increment and the activation of calcium current induced by Bk. What this result indicates is that at least 900 ms separate the first detectable release from the ER from the activation of calcium influx. A delay between depletion of the ER and activation of SOC has been observed also for \(I_{\text{calc}}\) in different cells (29, 30). The mechanism underlying this phenomenon has not been satisfactorily explained yet.

Using combined confocal microscopy and the patch clamp technique we have studied the contribution of CaM to the delay period between the initial release of intracellular calcium from internal stores and the activation of calcium current. In our previous work we have shown that CaM inhibits the TG-activated calcium current in vascular endothelium in a concentration dependent manner (11). In the present study we have explored in more detail the mechanism by which CaM modulates SOC.

Our results show that CaM increases the delay period between the first measurable intracellular calcium increment and the activation of calcium current, whereas CaM antagonists significantly reduced the delay period. In the presence of CaM the amount of calcium current was 60% from control, whereas CaM antagonists did not affect the current amplitude. The effects of CaM were not observed when intracellular calcium was maintained at 1 nM by the addition of EGTA. These results indicate that CaM not only increases the period between release of intracellular calcium and activation of calcium influx, but also modulates the amount of calcium current activated after depletion of the ER. CaM requires free calcium above 1 nM to exert its effects on TRP1. We have previously shown that the inhibition of SOC by CaM in vascular endothelium requires also free calcium above 1 nM (11). In Drosophila photoreceptors, the CaM-calcium complex mediates termination of the light response in vivo via modulation of TRP and TRPL channels (12). TRP possesses one CaM binding domain whereas TRPL has two, in both channels these CaM binding domains are at their carboxyl-terminal ends. In mammalian TRP channels at least one CaM binding site has been recently identified also at the COOH-terminal region (9). All these results suggest that CaM may play a conserved role as an inhibitor of TRP channel activity. This conclusion is further supported by the single channel experiments reported here, showing that the CaM-calcium complex reduces drastically single channel open probability in a concentration-dependent manner.

Single channel activity induced by either TG or Bk runs down rapidly in excised patches, the addition of \(I_{\text{calc}}\) recovered the activity. There are at least two possible explanations for the effect of \(I_{\text{calc}}\). One is that \(I_{\text{calc}}\) may directly bind to the TRP1 protein; the second possibility is that \(I_{\text{calc}}\) may bind to a regulatory protein associated to TRP1 and responsible for channel activation.

DISCUSSION

Activation of calcium influx after depletion of intracellular calcium storage compartments is a phenomenon observed in practically all cells explored from a wide variety of organisms (1). For many years the molecular identity of the SOC responsible for the influx of calcium after depletion of intracellular stores remained elusive. However, recently several candidates have been proposed.

Our initial findings showed that the TRP from Drosophila melanogaster encoded a thapsigargin-activated, calcium-permeable channel (3). Because of this study, several TRP homologues have been identified in a wide variety of organisms from yeast to humans (4, 6).

The best characterized SOC is the calcium release-activated calcium current (\(I_{\text{calc}}\)) (25). The recent finding showing that the expression of CaT1 (a calcium channel related to the TRP superfamily) produces channels that are indistinguishable from \(I_{\text{calc}}\) further supports the hypothesis that some members from this large family of cationic channels may be responsible for SOCE.

Although the identification of new TRP homologues has been very successful, the clear demonstration that any of the members from this new family of channels may be responsible for SOCE has not been so fruitful (7). Expression of some TRP homologues results in augmented SOCE, but these findings are not consistent from cell to cell or from group to group (for review see Ref. 2). An explanation for this inconsistency has not been found to the present time.

One possible explanation might rely on the assumption that introduction of foreign channels in a given cell may not necessarily result in a physiological interaction between the TRP channel and the mechanisms modulating SOCE. To complicate things further, several TRP homologues aggregate to form functional heteromultimers with novel properties. In particular, it has been shown that TRP1 and TRP5 form novel cationic channels in the mouse brain (27), while co-expression of Drosophila TRP and TRPL result in SOC channels with novel properties (28).

To avoid this complication we decided to study endogenous TRP1 channels from CHO cells, a cell line that has been frequently used to express TRP homologues and to study SOCE. Our results show the presence of a TRP1 homologue in this cell line. We consistently isolated a fragment of TRP1 using degenerated oligonucleotides from conserved regions of several TRP mammalian homologues (“Materials and Methods”). This result is in agreement with previously published data showing endogenous expression of TRP1 and TRP2 in CHO cells (22).

The RNAi technique allowed us to study the contribution of endogenous TRP1 channel to SOCE in CHO cells. We have shown here that introduction of dsRNA from TRP1 results in specific reduction in the amount of TRP1 protein and potent inhibition of SOCE. These results strongly suggest that TRP1 is a major component of SOCE in CHO cells. Most importantly, no sequence homology was observed between the TRP1 fragment from CHO cells used in the RNAi experiments and TRP homologues other than TRP1. This is important because, if substantial sequence similarities were to be found between TRP1 and other TRP homologues (TRP2, TRP3, etc.), then the dsRNA from TRP1 could have interfered with these other channels, making difficult the interpretation about the relative contribution of TRP1 to SOCE.
An interesting candidate for the second hypothesis is IP₃R. It is possible that the excised patch of membrane may contain pieces of ER with IP₃Rs. In fact, functional interactions between human TRP3 and IP₃R have been previously studied in this patch clamp configuration (31). It has been shown that several members of the TRPC subfamily (TRPC1–TRPC7) have a common binding site for CaM and peptides from the IP₃R (9). This common binding site is located at the COOH-terminal region of TRP channels, in particular amino acids 720–749 from human TRP1. This region is perfectly conserved in mouse, rat, and *Xenopus laevis* TRP1 proteins. Although we have not isolated yet the full-length cDNA from CHO-TRP1, this amino acid region is most likely present also in CHO-TRP1, which is over 93% identical to rat TRP1. Interestingly, it has been reported that amino acids 664–793 from human TRP1 are involved in the modulation of SOCE, by exerting an inhibitory effect (32).

A recent study shows activation of human TRP3 by peptides from the IP₃R through displacement of inhibitory calmodulin from a common binding domain (21). We have used a synthetic peptide (IP₃R-P1) containing this sequence from the type 3 IP₃R, which has been previously shown to interact with TRP channels displacing CaM from a common binding domain. The results presented here show that introduction of the IP₃R-P1 peptide into the cell via the patch pipette activates the calcium current in CHO cells in the absence of any stimulation with TG or Bk. After current activation with the peptide, addition of TG induced only a minor increment in current amplitude, indicating that TG and IP₃R-P1 activate the same channel. The scrambled peptide IP₃R-P2, which contains the same amino acids found in IP₃R-P1 but in random order, failed to activate calcium current, however, the subsequent addition of TG produced typical current activation.

In excised inside-out patches, the IP₃R-P1 peptide induced channel activity with similar conductance to the channel activity induced by TG and Bk. Subsequent addition of 100 μM IP₃ failed to increase channel activity induced by the IP₃R-P1 peptide. These results show that the IP₃R-P1 peptide mimics the effect of IP₃ in terms of recovering channel activity after run-down, strongly suggesting that at least part of the effects produced by IP₃ in single channels is mediated by the IP₃R.

The results presented here are consistent with a previous report (21) showing an antagonistic effect of IP₃ and CaM for the activation of TRP3 channel activity. However, we show here that this competitive interaction between IP₃ and CaM is sufficient to explain the activation of TRP1 channels and SOCE, after depletion of the ER in CHO cells. Our results also show that the inhibitory effect of 10 μM CaM on TRP1 channel activity prevails even at saturating IP₃ concentrations or with 50 μM IP₃-P1 peptide. These results suggest that CaM has a dominant effect on channel activity, which may play a role in preventing calcium influx under nonstimulated resting conditions.

Whereas the competitive interaction between IP₃ and CaM may be sufficient to explain the mechanism by which agonist stimulation leads to SOCE activation, less clear is the mechanism induced by calcium ATPase inhibitors. We found that TG and Bk activate similar calcium currents and single channel conductance, and that this conductance is eliminated by the introduction of dsRNA from TRP1. Furthermore, introduction of IP₃R-P1 peptide into the cell produced current activation, under these conditions; subsequent addition of TG resulted in only a minor increment in current amplitude. All these results strongly suggest that the mechanism by which TG induces SOC activity involves the IP₃R. One possible explanation may involve changes in ER intraluminal or cytosolic calcium induced by TG, which may result in changes in IP₃R activity. Changes in IP₃R activity may modulate protein-protein interactions between IP₃R and TRP1, as proposed in a recent review (33).

One way of testing this hypothesis would be monitoring the simultaneous activity of IP₃R and TRP1 channels in response to TG and Bk. Finding a correlate between IP₃R and TRP1 channel activities has proven very difficult so far. We are currently working in the design of experimental procedures, which may allow us to monitor the simultaneous activity of these two channels in real time.

**Acknowledgments**—We thank Dr. Müller-Esterl for providing the anti-Bk2R antibody used in this study. The services from the Molecular Biology and Microscopy units and the Library at the Institute of Cellular Physiology are greatly appreciated.

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