Characteristics of a store-operated calcium-permeable channel, SOCC:

Sarcoendoplasmic reticulum calcium pump function controls channel gating.

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Abstract

We examined the single channel properties and regulation of store-operated calcium channels (SOCC). In HSG cells, carbachol (CCh) induced flickery channel activity while thapsigargin (Tg) induced burst-like activity, with relatively lower open probability (NP_o) and longer mean open time. Tg- and CCh-activated channels were permeable to Na^+ and Ba^{2+}, but not to NMDG, in the absence of Ca^{2+}. The channels exhibited similar Ca^{2+}, Na^+, and Ba^{2+} conductances and were inhibited by 2-aminoethoxydiphenylborate, xestospongin C, Gd^{3+}, and La^{3+}. CCh-stimulated flickery activity changed to burst-like activity by (i) addition of Tg, (ii) using Na^+ instead of Ca^{2+}, (iii) using Ca^{2+}-free bath solution, or (iv) buffering [Ca^{2+}] with BAPTA-AM. Buffering [Ca^{2+}], induced a 2-fold increase in NP_o of Tg-stimulated SOCC. Reducing free [Ca^{2+}] in the ER with the divalent cation chelator, TPEN, induced burst-like channel activity similar to that seen with CCh+Tg. Thus, SOCC is activated by stimulation of muscarinic receptors, inhibition of SERCA, and lowering [Ca^{2+}] in the internal store. Importantly, SOCC activity depends on [Ca^{2+}]_i and the free [Ca^{2+}] in the internal store. These novel findings reveal that SERCA plays a major role in the gating of SOCC by (i) refilling the internal Ca^{2+} store(s) and (ii) decreasing the [Ca^{2+}]_i-dependent inhibition.
Introduction

Calcium influx into non-excitable cells is mediated via store-operated Ca\(^{2+}\) entry (SOCE) channel(s), SOCC, which are activated by the depletion of Ca\(^{2+}\) from intracellular calcium store(s) (1-3). Physiologically, Ca\(^{2+}\) store depletion is typically achieved following receptors-mediated activation of phosphatidylinositol 4,5, bisphosphate (PIP\(_2\)) hydrolysis, generation of inositol 1,4,5 trisphosphate (IP\(_3\)), and release of Ca\(^{2+}\) via the inositol trisphosphate receptor (IP\(_3\)R) by the binding of IP\(_3\) to the IP\(_3\)R. According to the capacitative Ca\(^{2+}\) entry hypothesis (1) SOCC inactivation occurs when the internal Ca\(^{2+}\) store(s) is refilled, primarily by the uptake of Ca\(^{2+}\) into the store(s) by the sarco-endoplasmic Ca\(^{2+}\) pump (SERCA). Although the general assumption is that inactivation of SOCC involves a reversal of the activation mechanism, this has not yet been established. Moreover, the precise molecular mechanism(s) that senses the status of the internal Ca\(^{2+}\) store to either activate (upon depletion), or inactivate (upon refill), SOCC is not known.

Three main mechanisms currently proposed for the activation of SOCC include involvement of a diffusible messenger, recruitment of intracellular vesicles, and a physical interaction between the SOCC and IP\(_3\)R (4, 5). According to the latter, i.e. the conformational coupling model (2), the IP\(_3\)R acts as the sensor of the internal Ca\(^{2+}\) store and conveys the signal to the SOCC via a conformational change that results in activating the plasma membrane channel. However, store-operated calcium influx is efficiently activated in a variety of cells by the intracellular Ca\(^{2+}\) pump (SERCA) inhibitor, thapsigargin, that depletes intracellular Ca\(^{2+}\) stores without changing cellular levels of IP\(_3\) (6). Thus, there has been much discussion about the direct involvement of PIP\(_2\) hydrolysis, IP\(_3\), or IP\(_3\)R in the regulation of SOCC (3,4,7,8).
Recently studies with the Trp family of putative Ca\(^{2+}\) channel proteins, which have been proposed as molecular components of the SOCC, have provided information regarding the possible involvement of IP\(_3\)R and PIP\(_2\) hydrolysis in store-operated Ca\(^{2+}\) influx (9, 10). For example, some Trps, such as Trp3 and Trp6, are activated by PIP\(_2\) hydrolysis, likely via an involvement of IP\(_3\), diacylglycerol, or PIP\(_2\) itself (9, 10). Other Trps, such as Trp1, can be activated by an agonist or by thapsigargin alone, i.e. without PIP\(_2\) hydrolysis (8, 11). Based on these studies, it can be suggested that agonists, which result both in an increase in IP\(_3\) and store-depletion activate a different SOCC channel than that activated by thapsigargin, which induces store-depletion without increase in IP\(_3\). Alternatively, it can be suggested that both reagents activate the same channel. Since there is little information regarding the single channel properties of the endogenous SOCC(s) in non-excitable cells, these possibilities have not yet been fully resolved. Previous studies using fura 2 to measure changes in [Ca\(^{2+}\)]\(_i\) have shown that although the rate of activation of store-operated Ca\(^{2+}\) influx by thapsigargin is slower, the final level and the characteristics of the Ca\(^{2+}\) influx are similar to that achieved with maximal concentrations of an agonist (3, 6, 12). Similar conclusions have been made based on electrophysiological studies, which have mainly involved measurements of whole cell currents (3, 13) and by noise analysis of whole cell currents (14, 15). In these studies the SOCC-associated current was activated by the inclusion of IP\(_3\) in the pipette solution. Thus, these studies suggest that SOCC activation might not be due to PIP\(_2\) hydrolysis per se but rather due to IP\(_3\), acting either directly on the SOCC or indirectly via an effect on the IP\(_3\)R. Other recent reports are consistent with the involvement of the IP\(_3\)R in the activation of SOCC (16, 17, 18, 19) although this mechanism has been recently questioned (20).
In the present study, we have examined the single channel characteristics of SOCC activated by the muscarinic agonist, carbachol, the SERCA inhibitor, thapsigargin, and the permeant divalent cation chelator, TPEN, in the human submandibular gland cell line (HSG) by using the cell-attached patch clamp technique. We have previously shown that both carbachol and thapsigargin strongly induce SOCE and the store-operated Ca$^{2+}$ current ($I_{SOC}$) in these cells (13, 21, 22). The present data demonstrate that carbachol, thapsigargin, and TPEN activate the same SOCC in HSG cells. Importantly, we show that the gating of this channel is regulated by the function of SERCA, which determines the [Ca$^{2+}$] in the internal Ca$^{2+}$ store and in the vicinity of SOCC.
Experimental Procedures:

**Cell Culture.** HSG cells were cultured in EMEM medium supplemented with 10 % FBS, 1% penicillin/streptomycin at 37 °C in 5 % CO₂. For electrophysiology and [Ca²⁺]ᵢ measurements, confluent cells were detached from tissue culture dishes and plated on glass cover slips. Measurements were done after 24 hours. In some experiments, cells were pre-incubated with 2APB, xestospongin, or BAPTA-am for 3 to 30 min, as indicated. CCh and Tg were perfused to the bath at rate of 5 ml/min.

**Electrophysiology.** Patch clamp experiments were performed in the cell-attached configuration with pipette solution containing 100 mM Na/HEPES and 2 mM CaCl₂, pH 7.2 (HCl). In some experiment 100 mM NMDG/HEPES was used. Total Cl⁻ ion concentration in the pipette was estimated to be about 6 mM. Cells were bathed in a standard bath solution containing (mM): 145 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES (pH 7.4, with NaOH). This solution was replaced by a high KCl solution containing (mM) 145 KCl, 5 NaCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES (pH 7.4, with KOH) after the seal was formed. For measurement of Ba²⁺ or Na⁺ permeabilities, pipette solution contained either 2 mM BaCl₂ or 2 mM NaCl, 100 mM NMDG/HEPES (pH 7.2, HCl), and 0.1 mM EGTA. In addition, 2 mM NMDGCl was added to the buffer during Na⁺ current measurements in order to maintain equal [Cl⁻]. Liquid junction potentials (8.0, 4.3 and 4.2 mV for Ca²⁺, Na⁺ and Ba²⁺ currents measurement, respectively) were calculated using P-Clamp7 software. Currents were recorded using an Axopatch 200A amplifier and digitized with Digidata 1200 (Axon instrument) at a rate of 4 kHz by filtering at 1 kHz. All experiments were done at room temperature.

**Data Analyses.** P-Clamp 7 (Axon instrument) and Origin 6 (Microcal) were used for analyses of data. The probability of the channel being open (P₀) was defined as the ratio of open channel area to the total area in all-point amplitude histogram. The mean
amplitudes of the single channel currents were determined from all-point amplitude histogram fit to a sum of Gaussian distributions and were used to calculate the slope conductance. Mean open times were determined from the total open time of the channel.

$\left[Ca^{2+}\right]_i$ measurements. Fura2 fluorescence in single cells was measured as described earlier (11, 22) by using an SLM 8000/DMX 100 spectrofluorimeter attached to an inverted Nikon Diaphot microscope with a Fluor 40x oil-immersion objective. Images were acquired using an enhanced CCD camera (CCD-72, MTI) and the Image-1 software (Universal Imaging Corporation, PA). Analog plots of the fluorescence ratio (340/380) in single cells are shown.
Results

Activation of SOCC in HSG cells by Carbachol and Thapsigargin

HSG cells did not display any significant spontaneous currents at membrane potentials between −80 to +80 mV (115/123 cells, Figure 1A shows representative traces in a cell clamped at −40 and +40 mV). However, stimulation of the cells with either CCh (1 mM) or Tg (2 μM) induced channel activity in 67/123 cells (about 54%). Most of the cells activated by CCh (31/35, 94%) displayed currents with a “flicker” mode, i.e. with relatively short openings (representative traces are shown in Figure 1B. Note that the traces in Figures 1A and B were recorded from the same cell). Closed “C” (indicating the 0 current level) and open “O” states are indicated in the current recordings at −40 and +40 mV (the pipette solution contained 100 mM Na-Hepes, pH 7.4, 2 mM Ca²⁺). The activity was quite stable for 3 to 5 minutes after which there was a gradual decay. The all-point amplitude histogram for the current at −40 mV (Figure 1E) revealed one major peak with unitary amplitude of about -1 pA. Occasional overlapping channel openings were seen corresponding to multiples of the unitary conductance level (about − 2 pA, see expanded trace in Figure 1B which shows simultaneous openings of multiple channels). Since the number of events in the second peak (>1 pA) was relatively low, data analysis was focused on the major peak. The amplitude–voltage (I-V) relationship (Figure 1D) of the CCh-induced major current peak (-1 pA), showed a linear fit, reversing at about 0 mV, with a slope conductance of 20 pS (each point was derived from 6 to 14 patches). The NP₀ of the channel with CCh was 19.6±2.6% (see Table 1). Consistent with our previous studies of SOCE and the store-operated current (I_{SOC}) in HSG cells, CCh-activation of the current (both inward and outward) was not observed when either Gd³⁺ (1 mM, 6/6...
cells, see Figure 1D), or La$^{3+}$ (1 mM, 6/6 cells, data not shown), was included in the pipette solution. Zn$^{2+}$ did not block channel activity (data not shown).

Store-depletion by inhibition of SERCA with Tg also stimulated single channel events in HSG cells (Figure 1C), although the channel characteristics were markedly different from those seen with CCh (compare traces in Figures 1B with those in Figure 1C). Majority of cells (26/32, 81%) stimulated with Tg displayed “burst-like” channel activity that displayed longer openings at one current level, than that stimulated by CCh. However, the major peak revealed in the all-point amplitude histogram (Figure 1F) with unitary current amplitude of about -1 pA, was similar to that in CCh-stimulated cells. Further, Tg also stimulated occasional events corresponding to multiples of this unitary conductance level. In general, the number of these events was less than that obtained in CCh-stimulated cells and could not be clearly resolved in the all-point amplitude histogram. The $N_p$ of channel activation with Tg (9.8± 0.8%) was significantly less than that with CCh (see Table 1). Thus, there is apparently less channel activity with Tg than with CCh. The I-V relationship of the major (-1 pA) current (Figure 1D) revealed a linear fit with a single channel conductance of 20 pS, which was not different from that obtained with CCh. Additionally, Tg-stimulated channel current, both inward and outward, was also blocked by inclusion of Gd$^{3+}$ (1 mM, n=5) or La$^{3+}$ (1 mM, n=6), but not Zn$^{2+}$, in the pipette solution (data not shown).

**Characteristics of CCh- and Tg-stimulated channel activity in HSG cells**

To further characterize the CCh- and Tg-stimulated channel activities, we measured the relative permeability of the respective channels to various cations. In the presence of 100 mM NMDG + 0.1 mM EGTA in the pipette, no inward currents were seen with
either CCh or Tg (Figure 2A and 2B, n=7). However, an outward current was seen between −40 mV and +80 mV. This outward current likely represents K⁺ efflux via SOCC, as it was insensitive to the Ca²⁺-activated K⁺ channel blocker, charybdotoxin (added to the pipette and bath solutions) but was blocked when Gd³⁺ or La³⁺ were added to the pipette solution (data not shown). When the pipette solution contained 100 mM NMDG + 2 mM Ca²⁺, both inward and outward currents were seen between −80 mV and +80 mV in CCh-stimulated cells (Figure 2B). The I-V curve displayed a linear fit and reversed at 0 mV. The amplitude of this current at the various voltages was similar to that seen when 100 mM Na⁺ + 2 mM Ca²⁺ was used in the pipette solution (see Figure 1F, 2B, and 2D). Increasing the [Ca²⁺] in the pipette solution to 20 mM, induced a linear current-voltage relationship, however, the reversal potential shifted to about +20 mV (Figure 2B). Similar results were obtained when Tg was used to stimulate SOCC (Figure 2A).

The permeability of the Tg- or CCh-stimulated channel to Na⁺ was measured by including 100 mM of Na-Hepes + 0.1 mM EGTA or 100 mM Na-Hepes + 2 mM Ca²⁺ in the pipette solution. The I-V plots of the currents stimulated by Tg is shown in Figure 2C and that by CCh in 2D. The calculated conductances for Na⁺ were 44 pS and 42 pS with CCh and Tg, respectively. When 2 mM Ca²⁺ was included in the pipette, the current was reduced. Importantly, similar currents were obtained with 2 mM Ca²⁺ + 100 mM Na⁺ or 2 mM Ca²⁺ + 100 mM NMDG⁺. The relative current amplitudes measured at −40 mV under these different conditions are shown in Figure 2G and 2H. These data suggest that under physiological conditions (i.e. below −10 mV, with 1 mM Ca²⁺ + 100 mM Na⁺ in the external medium) the channel activated by either CCh or Tg primarily permitted Ca²⁺ influx. Na⁺ did not contribute to this inward
current. Na$^+$ influx was seen only when Ca$^{2+}$ was removed from the external medium. Similar results have been reported for CRAC, a calcium-release activated Ca$^{2+}$ channel in T lymphocytes. It has been shown that cations currents via CRAC are increased by removing divalent cations (14, 15, 23), the conductance changes from 0.5 pS in a medium containing Na$^+$ + Ca$^{2+}$ + Mg$^{2+}$ to about 44 pS in a medium containing Na$^+$, without Ca$^{2+}$ and Mg$^{2+}$. Our data show that the channel conductance, with either CCh or Tg, is increased about 2-fold when Ca$^{2+}$ is removed from the pipette solution (note that in our experiments the pipette solution did not contain Mg$^{2+}$ but the bathing solution contained 1 mM Mg$^{2+}$). While this manuscript was in preparation, Yue et al have reported characteristics of the CaT1-induced cation channel activity (24). This channel also displayed similar Na$^+$ conductance (between 40-50 pS) in divalent cation free conditions. Furthermore, the relative increase in the Na$^+$ current upon removal of Ca$^{2+}$ and Mg$^{2+}$ was less than that seen with CRAC. Importantly, the Na$^+$ conductances of CRAC, CaT1, and the channels stimulated by either Tg or CCh in HSG cells appear to be similar. However, the cation selectivity and Ca$^{2+}$ conductance of CRAC is quite distinct from that seen for the channel activated in HSG cells.

Permeability of the channels to Ba$^{2+}$ was also examined by including 2 mM Ba$^{2+}$, instead of Ca$^{2+}$, with 100 mM NMDG in the pipette solution. Similar Ba$^{2+}$ currents were induced by Tg (Figure 2E) and CCh (Figure 2F). In either case, the Ba$^{2+}$ currents were slightly smaller than Ca$^{2+}$ currents (see Figure G for the relative amplitudes). The calculated Ba$^{2+}$ conductance was 15 pS with both CCh and Tg. In aggregate, the data presented in Figure 2 strongly indicate that CCh and Tg activate the same calcium-permeable cation channel in HSG cells. The activation of this channel by Tg suggests that it is a store-operated cation channel, SOCC. Another
interesting similarity in the channel behavior between CCh- and Tg-stimulated cells was that the $P_o$ of the channel (with 2 mM Ca$^{2+}$ in the pipette solution) increased at more negative membrane potentials (data not shown). This behavior is also similar to that recently reported for the CaT1 channel (12).

A major problem in identifying store-operated channels is the lack of specific pharmacological tools. As we have shown above, inorganic cations, such as La$^{3+}$, Ni$^{2+}$, Zn$^{2+}$, and Gd$^{3+}$, appear to have some selectivity in blocking various calcium influx pathways. Recently, there has been much focus on the effects of 2-aminoethoxydiphenylborate, 2APB (25), which was first reported to block store-operated calcium channels by intervening with IP$_3$–induced Ca$^{2+}$ release (19). However, as reported more recently, 2APB might directly block SOCC and some Trp channels (20, 26) but not other calcium channels; e.g. arachidonic acid-activated calcium influx. Irrespective of how 2APB inhibits store-operated calcium channels, Ma et al. (27) have suggested that this inhibitory effect represents an important functional similarity between invertebrate Trp channels, mammalian Trp channels, and mammalian store-operated channels. Thus, we tested the effect of 2APB on CCh- and Tg-stimulated $[\text{Ca}^{2+}]_i$ changes and channel activation. In control cells both CCh and Tg induced a transient increase in $[\text{Ca}^{2+}]_i$, due to internal Ca$^{2+}$ release in a Ca$^{2+}$-free media. After reintroduction of Ca$^{2+}$ into the bath $[\text{Ca}^{2+}]_i$ was significantly increased, demonstrating activation of Ca$^{2+}$ influx (Figure 3 A and E). However, when the cells were exposed to 2APB prior to reintroduction of Ca$^{2+}$, $[\text{Ca}^{2+}]_i$ increase due to store-operated influx was completely inhibited (Figure 3 B and F). This was further confirmed by measuring SOCC activity in cell-attached patches in HSG cells. Cells were pre-incubated with 2APB for 3 to 5 min before either CCh or Tg were added.
No currents were detected in either case at membrane potentials between +40 to –80 mV (Figure 3C and G show currents measured at –40 mV, n=6 in each case). We also tested the effects of xestospongin C (16, 19) on SOCC activation. As seen with 2APB, SOCC activation by either CCh or Tg was completely blocked by xestospongin C treatment (Figure 2D and H, n=6). Xestospongin C also blocked $[\text{Ca}^{2+}]_i$ increases due to Ca$^{2+}$ influx in Tg and CCh-stimulated cells (data not shown, number of cells imaged per experiment was more than 50, experiments were repeated three times). While xestospongin has been suggested as an inhibitor of the IP$_3$R, it appears to be structurally similar to a dimeric form of 2-APB (28) and thus could also have direct effects on SOCC. This possibility has to be further examined. In aggregate, the data described above demonstrate that the channels activated by Tg and CCh have similar pharmacological characteristics. These data provide further evidence that Tg and CCh activate the same SOCC in HSG cells.

**Effect of SERCA inhibition on CCh-stimulated channel activity**

Figure 1 illustrates a dramatic difference in the channel characteristics seen in Tg- and CCh-stimulated cells. Possible explanations for this are (i) activation of different channels by Tg and CCh, and (ii) lack of internal Ca$^{2+}$ accumulation in the Tg-stimulated cells. The data described above strongly suggest that CCh and Tg do not activate different cation channels in HSG cells. To further rule out this possibility and to test the role of SERCA, Tg was added to cells after the channel had been activated by CCh. Tg addition induced a dramatic change in the channel behavior (Figure 4A shows traces recorded from the same cell used in 1A and 1B. Tg was added to the cell after CCh-stimulated channel activity was recorded. 7/9 cells displayed this change in channel activity). The rapid and transient openings of the channel induced by CCh
were converted to more stable activity, with longer mean open times at the -1 pA current level (see the expanded traces). The resulting channel behavior was more like that in cells treated with Tg alone (compare with Figure 1C). Additionally, the major current level seen in the all-point histogram, -1 pA (Figure 4B), was similar to that seen in CCh- or Tg-stimulated cells. The I-V relationship of the -1 pA current (Figure 4C) revealed a linear fit with a single channel conductance of 20 pS, which was not different from that obtained in cells stimulated by either Tg or CCh. The mean open times calculated for CCh+Tg or Tg were significantly longer than that seen with CCh (p<0.05, n=6 for each group (see Table 1). NP₀ of the channel with CCh was significantly (p<0.05, n=6) higher than the channel with CCh+Tg or Tg alone. These data demonstrate that inhibition of SERCA activity in CCh-stimulated HSG cells induces a dramatic change in the activity of SOCC. We suggest that the “flicker” mode of SOCC seen with CCh is due to the following sequence of events: (i) activation by IP₃-dependent release of Ca²⁺ from the internal Ca²⁺ store, (ii) Ca²⁺-dependent feedback inhibition of the IP₃R and SOCC, (iii) SERCA-dependent uptake of Ca²⁺ into the store and (iv) refill-dependent inactivation of SOCC. Thus, inhibition of SERCA by the addition of Tg prevented the refill-dependent inactivation of SOCC and increased the open time.

To determine whether the flickery channel activity in CCh-stimulated cells is due to recycling of Ca²⁺ in the store, channel activity was examined with 2 mM Na⁺ and 100 mM NMDG in the pipette solution. In this condition, Na⁺ enters the cell via SOCC but is not be taken up into the stores. Thus, the channel should not be subject to effects due to recycling of Ca²⁺. Single channel activities in CCh- and Tg-stimulated cells are shown in Figure 5. Note that the current amplitude, as expected, is less than
that seen in Figure 2 where the pipette solution contained 100 mM Na\textsuperscript{+}. Importantly, channel activity in CCh-stimulated cells did not show a flickery behavior (Figure 5A), but rather displayed a burst-like pattern similar to that in seen in cells stimulated by Tg (Figure 5B). Thus, gating properties of the CCh-stimulated channel was dramatically altered when Na\textsuperscript{+} was used as the permeant cation. These data are consistent with our suggestion that the difference in the behavior of SOCC in Tg and CCh-stimulated cells is determined by the local release and uptake of Ca\textsuperscript{2+} in the store.

**Effect of Ca\textsuperscript{2+} store-refill on SOCC**

To further demonstrate that the effect of Tg on CCh-stimulated SOCC activity was due to inhibition of Ca\textsuperscript{2+} store-refilling, we altered store-refilling by decreasing the ambient [Ca\textsuperscript{2+}], using two different protocols. In the first, the cells were exposed to a Ca\textsuperscript{2+}-free bath solution, keeping the Ca\textsuperscript{2+} in the pipette solution as the only source of external Ca\textsuperscript{2+}. The typical “flicker” behavior of SOCC seen with CCh in the control condition (Figure 6A-1) was changed to one more like that seen with CCh+Tg (compare traces in Figure 6A-2 with Figures 1C, all recordings are at –40 mV). When Ca\textsuperscript{2+}-containing medium was reintroduced to the cells, the typical CCh-induced “flicker” current behavior was restored (Figure 6A-3, note that the three traces in Figure 5A were recorded from the same cell). Average mean open time for the CCh-induced channel in Ca\textsuperscript{2+}-free medium was 2.61±0.31 ms (total number of events ranged from 1322 to 1534), with an open probability of 19±1.8% (n=5). Thus, this maneuver altered CCh-stimulated channel activity; mean open time was increased, while NP\textsubscript{o} was not significantly changed.
In the second protocol, the cells were loaded with BAPTA-AM (50 µM for 30 min) to buffer [Ca^{2+}]_{i}. Importantly, the BAPTA-loaded cells did not display any spontaneous SOCC activity. However, [Ca^{2+}]_{i} buffering again resulted in a significant change in the single channel properties of SOCC. The mean open time of the CCh-stimulated channel activity (Figure 6B) increased from 1.7± 0.07 ms (in control cells) to 2.17±0.2 ms (n=6). However, there was no significant change in the NP_{o} (20.1±2.7% in BAPTA-loaded cells, vs 19.8±2.6% in control cells). Loading cells with BAPTA-AM also changed the characteristics of the Tg-induced SOCC (Figure 6C). While the characteristic longer open time of the Tg-activated channel was maintained (2.55± 0.4 ms, n=7), NP_{o} was significantly increased from 9.8±0.8% to 24.8±4.2%, and more openings were seen with the higher current level in BAPTA-loaded cells (see expanded trace in Figure 6C). In aggregate, these data demonstrate that a decrease in [Ca^{2+}]_{i} increases activation of SOCC by Tg. Thus, it can be suggested that the lower channel activity (lower NP_{o}) seen in Tg-stimulated cells is due to an inhibitory effect exerted by the relatively high [Ca^{2+}]_{i} in the subplasma membrane region resulting from inhibition of the SERCA. However, the channels that are active display longer open times since refilling of the internal Ca^{2+} store is prevented by Tg. Importantly, these data also show that NP_{o} of SOCC does not depend on IP_{3} or PIP_{2} hydrolysis. Thus, our data suggest at least two types of inhibitory mechanisms in the regulation of SOCC, one by the local [Ca^{2+}]_{i} and another by refilling of internal Ca^{2+} stores. In addition, the closing of the channel in BAPTA-loaded TG-treated cells demonstrates spontaneous channel inactivation. More detailed studies will be required to fully describe the events involved in the inactivation of SOCC.
SOCC activation by lowering the [Ca\(^{2+}\)] in the internal Ca\(^{2+}\) store

To exclude possible effects due to changes in [Ca\(^{2+}\)]\(_i\), we examined whether SOCC could be activated by directly decreasing the [Ca\(^{2+}\)] in the lumen of the internal Ca\(^{2+}\) store by using the cell permeant Ca\(^{2+}\) chelator, TPEN. Hofer et al. have previously reported (29) that TPEN induced activation of I\(_{\text{CRAC}}\) in RBL-1 cells. As shown in Figure 7A addition of 0.5 mM TPEN induced channel activity (6/9 cells). Addition of Tg or CCh to cells treated with TPEN did not change the pattern of channel activity (data not shown). The major current amplitude, about –1 pA (Figure 7B), the I-V relationship (Figure 7C), and Ca\(^{2+}\) conductance, 22 pS, were similar to that seen in cells stimulated by either Tg or CCh. The pattern of the single channel activity was also comparable to that seen in cells treated with CCh+Tg with NP\(_o\)=16.1±1.9% and mean open time= 2.75±0.8 ms. These data suggest that the same SOCC is activated by TPEN, Tg, and CCh. It is important to note that the NP\(_o\) of SOCC activation with TPEN was higher than that with Tg and similar to that seen with CCh.

The ability of TPEN to attenuate release of calcium from internal stores was examined using Fura 2 (Figure 7D). In the absence of external Ca\(^{2+}\), TPEN induced a very small transient increase in the fura 2 fluorescence ratio. Addition of Tg or CCh after this increase, did not induce further release of Ca\(^{2+}\) from internal stores. Note that when either Tg or CCh were added to HSG cells in a nominally Ca\(^{2+}\)-free medium, a transient increase in fluorescence was seen with substantially higher peak fluorescence ratios (between 3.0 and 5.0, see Figure 3). Thus, it is highly unlikely that the TPEN-induced small increase in [Ca\(^{2+}\)]\(_i\) represents any substantial depletion of the internal store. Importantly, when TPEN was added to cells in the presence of external Ca\(^{2+}\), there was a significant increase in fura 2 fluorescence (Figure 7E shows average
fluorescence values 5 minutes after TPEN addition), demonstrating an increase in Ca\(^{2+}\) influx. CCh or Tg did not induce additional increase in fluorescence in TPEN-treated cells.
Discussion

This study describes the characteristics and regulation of an endogenous SOCC in HSG cells. Store-operated calcium channels have been identified in almost all non-excitatory cells and in some excitable cells. However, there is relatively little information regarding the single channel properties of this type of calcium channel or the mechanisms that regulate its gating. According to the capacitative Ca\(^{2+}\) entry hypothesis, SOCC is activated when internal Ca\(^{2+}\) stores are depleted and inactivated when these stores are refilled (1,6). In addition, SOCC activity is decreased by elevations of intracellular [Ca\(^{2+}\)], although the exact mechanism by which Ca\(^{2+}\) induces this feed-back inhibition is not yet known (3). The data presented above demonstrate that gating of SOCC is determined by the [Ca\(^{2+}\)] in the ER lumen and in the region of the cell where Ca\(^{2+}\) influx occurs. Importantly, inhibition of SERCA function dramatically alters the single channel properties of SOCC. Since SERCA-mediated uptake of Ca\(^{2+}\) into the ER regulates [Ca\(^{2+}\)] in the ER and in the sub-plasma membrane region near the site of SOCC, we suggest that SERCA function controls the gating of SOCC.

We report that calcium-permeable cation channel activity is stimulated in HSG cells when internal Ca\(^{2+}\) stores are depleted either in the absence, by using Tg, or in the presence, by using CCh, of an increase in IP\(_3\). Based on the similarities in their pharmacological characteristics, cation conductances, and relative cation permeabilities, we conclude that the same SOCC is activated by CCh and Tg. In the presence of physiological levels of external Na\(^+\) and Ca\(^{2+}\) and at negative membrane potentials the channels activated by Tg and CCh primarily allow Ca\(^{2+}\) influx. In the
absence of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} in the pipette solution, the channel is permeable to Na\textsuperscript{+} or Ba\textsuperscript{2+}, but not to NMDG. The respective conductances of the channel for Ca\textsuperscript{2+}, Na\textsuperscript{+} or Ba\textsuperscript{2+} in CCh-stimulated cells are similar to those in Tg-stimulated cells. The relative current amplitudes are in the order Na\textsuperscript{+}>Ca\textsuperscript{2+}=Ba\textsuperscript{2+} and is similar in CCh- and Tg-stimulated cells. The Na\textsuperscript{+} conductances of the internal Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel, CRAC, in T lymphocytes (15), and the more recently reported CaT1 (24), are similar to what we have reported here. Furthermore, external Ca\textsuperscript{2+} also blocks influx of monovalent cations via CRAC and CaT1 (15, 23, 24). However, several other properties of CRAC and SOCC appear to be quite distinct from each other (3, 14). Unlike SOCC, CRAC displays a very small Ca\textsuperscript{2+} conductance, is relatively selective for Ca\textsuperscript{2+}, and is blocked by Zn\textsuperscript{2+}. We have shown here, and earlier (13), that in HSG cells SOCC is blocked by Gd\textsuperscript{3+} and La\textsuperscript{3+} but not by Zn\textsuperscript{2+}. This divalent cation sensitivity appears to be more similar to those described for Trp1, Trp3 (11, 30, 31), and CaT1 (32). Another evidence that the channels activated by CCh and Tg are store-operated is their inhibition by 2APB and xestospongin C, which have been suggested to be relatively specific for SOCC and also possibly for some Trp channels (20, 26, 27).

A significant, and novel, observation made in this study is that SOCC displays markedly distinct single channel properties depending on how it is activated. When stimulated by CCh, it displays a “flickery” activity, with a relatively higher P\textsubscript{o} and shorter mean open time, while in Tg-stimulated cells it displays “burst-like” activity with relatively longer mean open time, although with a lower P\textsubscript{o}. However, when Na\textsuperscript{+} is used as the permeant cation instead of Ca\textsuperscript{2+}, (i) CCh-stimulated channel does not display flickery activity, and (ii) the pattern of CCh- and Tg-stimulated activities are
similar. Thus, the “flickery” channel activity seen in CCh-stimulated cells represents a unique gating characteristic of this channel, which is related to Ca\(^{2+}\). We suggest that this flickery activity is due to rapid activation and inactivation of SOCC by the following sequence of events; Ca\(^{2+}\) release from the internal store, Ca\(^{2+}\)-dependent inactivation of IP\(_3\)R, and re-accumulation of Ca\(^{2+}\) into the store. Consistent with this, we have shown that inhibition of SERCA activity in CCh-stimulated cells by Tg converts the “flickery” SOCC activity to more stable openings, with longer mean open times. A similar effect on CCh-stimulated SOCC activity is also seen when Ca\(^{2+}\) store-refilling is decreased by BAPTA-loading of the cell or Ca\(^{2+}\) is removed from the bath solution. SOCC is also activated when [Ca\(^{2+}\)] in the ER lumen is lowered by treating cells with the permeant divalent cation chelator, TPEN. Importantly, it displays a similar burst-like pattern of activity as seen with Tg. In aggregate, these findings clearly demonstrate that internal Ca\(^{2+}\)store-refilling, i.e. the Ca\(^{2+}\) content of the internal store regulates the gating of SOCC. The present findings can also explain previous reports (13, 33, 34) showing that intracellular [Ca\(^{2+}\)]\(_i\) buffering is required to detect agonist-stimulation of the store-operated Ca\(^{2+}\) current, whereas Na\(^+\) currents can be more readily measured. It is likely that in the absence of intracellular [Ca\(^{2+}\)]\(_i\) buffering there is recycling of the internal store Ca\(^{2+}\) due to the function of IP\(_3\)R and SERCA which allows some degree of internal store refilling, thus preventing full activation of the current.

Another important factor that influences the gating of SOCC appears to be the local [Ca\(^{2+}\)] in the vicinity of calcium influx. We have shown that when SOCC is stimulated by CCh or CCh+Tg, it displays a higher NP\(_o\) than when stimulated by Tg. This suggests that conditions leading to PIP\(_2\) hydrolysis and elevation of IP\(_3\) might be more
efficient in the activation of SOCC. However, we have shown that when $[\text{Ca}^{2+}]_i$ is buffered in cells by loading with BAPTA-AM, $P_o$ of SOCC stimulated by Tg is significantly increased and is similar to that of SOCC stimulated by CCh. Importantly, BAPTA-AM loading of HSG cells, under the conditions described above, does not, by itself, activate SOCC. Consistent with these findings, $P_o$ of SOCC in CCh-stimulated cells was decreased by addition of Tg, although it was not affected by buffering $[\text{Ca}^{2+}]_i$. These data strongly suggest that the $P_o$ of SOCC following internal $\text{Ca}^{2+}$ store depletion appears to be determined by store depletion and by $[\text{Ca}^{2+}]_i$ rather than the increase in IP$_3$ or PIP$_2$ hydrolysis. We have reported earlier that SERCA inhibition by Tg induces an increase in the $[\text{Ca}^{2+}]_i$ in the sub-plasma membrane region in HSG cells (13, 21). We suggest that this increase in the local $[\text{Ca}^{2+}]_i$ exerts an inhibitory effect on SOCC. This accounts for the low $P_o$ of SOCC seen in Tg-treated cells and for the Tg-induced decrease in SOCC activity in CCh-stimulated cells. Feed-back inhibitory effects of $[\text{Ca}^{2+}]_i$ on store-operated calcium entry in HSG (13) and other cells (3) has been previously reported. This negative effect of $\text{Ca}^{2+}$ on SOCC might be exerted directly on the SOCC protein itself, either via binding of $\text{Ca}^{2+}$ to the channel protein or mediated via a $\text{Ca}^{2+}$-dependent protein such as calmodulin. Irrespective of the mode of inhibition, these data suggest that for maximal SOCC activity, $[\text{Ca}^{2+}]_i$ in the sub-plasma membrane region where $\text{Ca}^{2+}$ influx occurs must be maintained at a low level.

The present and our previous studies (13, 21) demonstrate that SERCA has a major role in the regulation of $[\text{Ca}^{2+}]_i$ in the subplasma membrane region of the cell. Based on the data discussed above, we suggest that SERCA function is critical in the gating of SOCC. Furthermore, following agonist-stimulated internal $\text{Ca}^{2+}$ store depletion, at
which time SOCC is activated, SERCA activity is increased as a result of a decrease in lumenal \([Ca^{2+}]_l\) (35) and other regulatory mechanisms such as phosphorylation (36).

Thus, it appears that cells modulate the activity of SERCA to accommodate for the \(Ca^{2+}\) entering the cells via SOCC. This ensures that (i) \([Ca^{2+}]_i\) near the SOCC is maintained at low levels and (ii) internal \(Ca^{2+}\) store(s) are not fully depleted. The latter is likely an important protective mechanism for the cell since the cell undergoes a “stress” response when internal \(Ca^{2+}\) store are depleted for extended time periods (37). Interestingly, it appears that in agonist-stimulated cells, incomplete depletion of the store which prevents full activation of SOCC is compensated by keeping \([Ca^{2+}]_i\) low, which increases the \(N_{Po}\) of SOCC. Thus, SOCC is regulated in a complex way by the \([Ca^{2+}]\) in two areas of the cell, in the lumen of the internal \(Ca^{2+}\) store and in the sub-plasma membrane region at the site of \(Ca^{2+}\) influx. Importantly, \([Ca^{2+}]\) in both these areas of the cell are primarily regulated by the function of SERCA. The present data do not exclude an additional role for the plasma membrane calcium pump (PMCA) or mitochondria in the regulation of \([Ca^{2+}]\) near the plasma membrane (38, 39). In fact, local \([Ca^{2+}]_i\) buffering by mitochondria has been suggested to be involved in regulating the gating of \(I_{CRAC}\) in lymphocytes (39).

In conclusion, we have described the single channel properties of SOCC in HSG cells. As predicted by the capacitative \(Ca^{2+}\) entry hypothesis this channel is activated by store depletion and its activity is determined by refilling of internal \(Ca^{2+}\) store(s). In addition, SOCC activity is also inhibited by increases in subplasma-membrane \([Ca^{2+}]_i\).

Based on our present and previous data, we conclude that SERCA, by regulating both internal \(Ca^{2+}\) store-refilling and \([Ca^{2+}]_i\) increase, controls the gating of SOCC. We have previously reported that \(Ca^{2+}\) entering HSG cells is rapidly taken up into the
internal store with minimal diffusion in the sub-plasma membrane region (21). Thus, it is reasonable to hypothesize that some region of the ER is localized very close to the plasma membrane, which facilitates the regulation of $[\text{Ca}^{2+}]_i$ near the SOCC by SERCA-mediated uptake of $\text{Ca}^{2+}$ into the lumen of the ER. This juxtaposition of ER and the plasma membrane could also enable relaying the signal from the store lumen to the plasma membrane to either activate or inactivate SOCC. Further studies will be required to elucidate the nature of this critical mechanism.
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Figure Legends

Figure 1. **Store-operated channel currents in HSG cell.** The cell was clamped at membrane potentials –40 or +40 mV and perfused with bath solution only (A) or (B) bath solution containing CCh (1 mM) or (C) Tg (1 mM). Traces in A and B were recorded from the same cell and those in C were from a different cell. These traces show representative recordings (n=8 cells for A and B, n=6 cells for C). Traces of the recording at –40 mV on expanded time scales are showed at the bottom of B and C. Note that C and O indicate the closed (0 current level) and open states of the channel. The all-point amplitude histograms corresponding to the currents seen at –40 mV in B and C are showed in D and E, respectively. F shows the I-V curve of CCh, and Tg induced current with a calculated slope conductance of 20 pS for each experimental condition. +Gd$^{3+}$ indicates the CCh-induced current in the presence of 1 mM Gd$^{3+}$ in the pipette solution. Gd$^{3+}$ induced similar block of Tg-activated current (data not shown).

Figure 2. **Cation selectivity of CCh- and Tg-activated channel:** Channel activity was recorded using using cell attached patches with the pipette solutions as indicated in the Figure, in cells stimulated with either Tg (A,C,E) or CCh (B,D,F). Relative current amplitudes at –40 mV are given in G. The third bar in each group represents the amplitude of the current with NMDG, which was not detectable at –40 mV.

Figure 3: **Inhibition of CCh-and Tg-stimulated channel activity by 2-APB and xestospongin C.** [Ca$^{2+}$]$_i$ was monitored using fura-2 fluorescence and is represented as the 340/380 nm ratio. CCh (A, B) or Tg (E, F) was administrated to the bath in Ca$^{2+}$-free
medium and then in medium with mM Ca$^{2+}$. 2-APB was applied to the cells (B and F) by inclusion in the bath solution, shown by the bar. These are the representative traces from 50-60 cells in each case. CCh- (C, D) and Tg- (G, H) activated SOCC was recorded at –40 mV in the cells pre-incubated with 2-APB (75 µM in the bath solution) for 3 to 5 min or xestospongin C (xestC, 25 µM) for 20 min after which either CCh or Tg was introduced into the bath (traces show recordings after addition of either CCh or Tg). These are the representative traces from more than six cells for each group.

**Figure 4. Effect of Tg on CCh-stimulated channel activity in HSG cells:** A. shows single channel activity following addition of Tg to CCh-stimulated cells. Note that the traces shown here were recorded from the same cell used for recording the activity prior to stimulation (Figure 1A) and following CCh addition (Figure 1B). An expanded trace is shown at the bottom of Figure 4A. An all point histogram is shown in B and I-V curve is shown in C. The pipette solution used in these measurements is indicated in Figure 4C. All other experimental conditions were similar to those described for Figure 1.

**Figure 5. Single channel properties of CCh- and Tg-activated SOCC with Na$^+$ as the permeant cation.** Single channel activities were recorded in cells clamped at –40 mV with 2 mM Na$^+$-Hepes and 100 mM NMDG-Hepes (pH 7.4) in the pipette solution. Cells were stimulated either with CCh (A) or Tg (B). Open (O) and closed (C, 0 current level) states of the channel are indicated in the Figure.

**Figure 6. Effect of decreasing external Ca$^{2+}$ and increasing intracellular Ca$^{2+}$ buffer on the kinetics of CCh-induced SOCC:** The cell was stimulated with CCh in
a Ca\textsuperscript{2+}-containing bath solution (A-1), after which the solution was replaced with a Ca\textsuperscript{2+}-free solution and the current was recorded after 30 s (A-1) and then Ca\textsuperscript{2+} was reintroduced into the bath for 3 min (A-3). This is a representative trace from five cells. B and C show SOCC activity in BAPTA-loaded HSG cells. Cells were loaded with BAPTA-am (50 µM) for 30 to 60 min and the channel activities were recorded after stimulation with CCh- (B) or Tg (C). These are the representative traces from at least five cells in each group. An expanded partial trace from C is shown in Figure 3C.

**Figure 7. Activation of SOCC by TPEN:** A. Current recording from a cell, clamped at –40 mV, before (top trace) and after introduction of 0.5 mM TPEN into the bath solution (lower two traces). B. All-point histogram of events stimulated by TPEN. C. I-V curve of current measured (pipette solution contained 10 mM Na-Hepes, 2 mM Ca\textsuperscript{2+}). D. Fura-2 fluorescence changes in cells after TPEN addition, [Ca\textsuperscript{2+}] in external medium is indicated in figure. E. Average ratio seen 5 minutes after TPEN addition in each case.
Table 1. Single channel characteristics of SOCC in HSG cells.

All measurements were made with 100 mM Na-Hepes and 2 mM Ca\(^{2+}\) in the pipette solution. All activating reagents were added to the bath solution (100 mM KCl, 2 mM CaCl\(_2\), 1 mM Mg\(^{2+}\)).

| Activation                  | NP\(_o\) (%) | MOT (ms) |
|-----------------------------|-------------|----------|
| CCh                         | 19.6 ± 2.6  | 1.7 ± 0.1|
| CCh+Calcium-free bath       | 19.0 ± 1.8  | 2.6 ± 0.3|
| CCh+BAPTA-loading           | 20.1 ± 2.7  | 2.1 ± 0.3|
| CCh+Tg                      | 13.8 ± 1.2  | 2.2 ± 0.6|
| Tg                          | 9.8 ± 0.8   | 2.5 ± 0.5|
| Tg+BAPTA-loading            | 24.8 ± 4.2  | 2.5 ± 0.4|
| TPEN                        | 16.1 ± 1.9  | 2.7 ± 0.8|
A: CCh+Tg

- 40 mv
- 1s
- 200 ms
- 0
- -1

B

C

100 mM Na^+ 2 mM Ca^{2+}

40 mv

-40 mv

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A-1

\[ \text{CCh} + \text{Ca}^{2+} \]

A-2

\[ \text{CCh} + \text{Ca}^{2+} \text{ free} \]

A-3

\[ \text{CCh} + \text{Ca}^{2+} \]

\[ \frac{2 \text{ pA}}{1 \text{ s}} \]

B

\[ \text{CCh} + \text{BAPTA-am} \]

C

\[ \text{Tg} + \text{BAPTA-am} \]

60 ms
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