Ca\textsuperscript{2+}-dependent Inactivation of a Store-operated Ca\textsuperscript{2+} Current in Human Submandibular Gland Cells

ROLE OF A STAUROSPORINE-SENSITIVE PROTEIN KINASE AND THE INTRACELLULAR Ca\textsuperscript{2+} PUMP*

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Stimulation of human submandibular gland cells with carbachol, inositol trisphosphate (IP\textsubscript{3}), thapsigargin, or tert-butylhydroxyquinone induced an inward current that was sensitive to external Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and was also carried by external Na\textsuperscript{+} or Ba\textsuperscript{2+} (in a Ca\textsuperscript{2+}-free medium) with amplitudes in the order Ca\textsuperscript{2+} > Ba\textsuperscript{2+} > Na\textsuperscript{+}. All cation currents were blocked by La\textsuperscript{3+} and Gd\textsuperscript{3+} but not by Zn\textsuperscript{2+}. The IP\textsubscript{3}-stimulated current with 10 \textmu M 3-deoxy-3-fluoro-D-myo-inositol 1,4,5-triphosphate and 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid in the pipette solution, showed 50% inactivation in <5 min and >5 min with 10 and 1 mM [Ca\textsuperscript{2+}]\textsubscript{i}, respectively. The Na\textsuperscript{+} current was not inactivated, whereas the Ba\textsuperscript{2+} current inactivated at a slower rate. The protein kinase inhibitor, staurosporine, delayed the inactivation and increased the amplitude of the current, whereas the protein Ser/Thr phosphatase inhibitor, calyculin A, reduced the current. Thapsigargin- and tert-butylhydroxyquinone-stimulated Ca\textsuperscript{2+} currents inactivated faster. Importantly, these agents accelerated the inactivation of the IP\textsubscript{3}-stimulated current. The data demonstrate that internal Ca\textsuperscript{2+} store depletion-activated Ca\textsuperscript{2+} current (I\textsubscript{SOC}) in this salivary cell line is regulated by a Ca\textsuperscript{2+}-dependent feedback mechanism involving a staurosporine-sensitive protein kinase and the intracellular Ca\textsuperscript{2+} pump. We suggest that the Ca\textsuperscript{2+} pump modulates I\textsubscript{SOC} by regulating [Ca\textsuperscript{2+}]\textsubscript{i}, in the region of Ca\textsuperscript{2+} influx.

In non-excitable cells, such as salivary gland cells, activation of Ca\textsuperscript{2+}-mobilizing receptors, e.g. muscarinic-cholinergic, on the cell surface produces a biphasic increase in cytosolic [Ca\textsuperscript{2+}] (1–3),

\[ ([Ca^{2+}]_i) \text{,} \]

with an initial phase due to Ca\textsuperscript{2+} release from internal Ca\textsuperscript{2+} stores via IP\textsubscript{3}-dependent Ca\textsuperscript{2+} channels in the intracellular membrane and a second phase due to Ca\textsuperscript{2+} influx across the plasma membrane. Although the molecule(s) that mediates Ca\textsuperscript{2+} influx across the plasma membrane has not yet been identified, there is conclusive evidence to demonstrate that it is regulated by the [Ca\textsuperscript{2+}]\textsubscript{i} in the intracellular Ca\textsuperscript{2+} store(s). This Ca\textsuperscript{2+} influx has been termed capacitative, or store-operated Ca\textsuperscript{2+} entry (SOCE) (1–4). Recently, methods have been developed to measure directly the Ca\textsuperscript{2+} influx current by using the whole cell patch clamp technique in the presence of high external [Ca\textsuperscript{2+}] and high internal [Ca\textsuperscript{2+}] buffer (5–9). Activation of inward Ca\textsuperscript{2+} currents by store depletion has been reported in a number of different cell types (4, 5), and it has been suggested that this current is mediated by a channel. However, the current appears to vary in different cell types. The most well studied of these currents is I\textsubscript{CRAC} (Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} current) which has some unique characteristics, including very low single channel conductance, high Ca\textsuperscript{2+} selectivity, inward rectification, reversal at very positive potentials, and Ca\textsuperscript{2+}-dependent inactivation (5–9).

The precise mechanism(s) that activates and inactivates SOCE is not yet known. Two main mechanisms have been proposed for the activation. (i) The status of the internal Ca\textsuperscript{2+} store is related to the plasma membrane via a physical interaction between the Ca\textsuperscript{2+} store membrane and the plasma membrane. (ii) A diffusible messenger either released from the store during depletion or activated in response to depletion transmits the signal from ER to the plasma membrane. Two different inactivation mechanisms have been described that are either dependent on the refilling internal Ca\textsuperscript{2+} store or independent of store refilling. We have previously shown that refilling of internal Ca\textsuperscript{2+} stores in salivary gland cells inactivates agonist-stimulated Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx (10). Ca\textsuperscript{2+}-dependent inactivation of Ca\textsuperscript{2+} influx which is independent of internal Ca\textsuperscript{2+} store has also been described by us and others in rat parotid acinar cells (11–13) and also for I\textsubscript{CRAC} in T-lymphocytes (8, 9). We and others (12, 14) have reported that in rat parotid acinar cells Ca\textsuperscript{2+} influx is inactivated by the protein Ser/Thr phosphatase inhibitors, okadaic acid and calyculin A, and pre-treatment of cells with staurosporine, a relatively nonspecific protein kinase inhibitor, prevents the inactivation. We have recently reported (14) that the staurosporine-sensitive protein kinase is involved in the Ca\textsuperscript{2+}-induced feedback inhibition of Ca\textsuperscript{2+} influx in rat parotid acinar cells. Importantly, this kinase does not appear to be involved in the refill-dependent inactivation of SOCE, i.e. refill-dependent inactivation was not prevented by staurosporine (14). Thus, we had hypothesized that Ca\textsuperscript{2+}, via a protein kinase, modulates SOCE in store-depleted rat parotid acinar cells.

HSG cells, a human submandibular gland cell line, have been used to study muscarinic receptor-activated Ca\textsuperscript{2+} signaling and Ca\textsuperscript{2+} influx (15–17). We have demonstrated that emptying of the Ca\textsuperscript{2+} store by CCh, IP\textsubscript{3}, or the Ca\textsuperscript{2+} pump inhibitor TG (or BHQ) activates an SOCE-type Ca\textsuperscript{2+} influx mechanism.

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† The abbreviations used are: BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; BHQ, tert-butylhydroxyquinone; CCh, carbachol; [Ca\textsuperscript{2+}]\textsubscript{i}, intracellular calcium concentration; [Ca\textsuperscript{2+}]\textsubscript{e}, extracellular calcium concentration; ER, endoplasmic reticulum; HSG, human submandibular gland; I\textsubscript{CRAC}, Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} current; I\textsubscript{SOC}, Ca\textsuperscript{2+} store depletion-activated Ca\textsuperscript{2+} current; IP\textsubscript{3}, inositol 1,4,5-triphosphate; F-IP\textsubscript{3}, 3-deoxy-3-fluoro-D-myo-inositol 1,4,5-triphosphate; K\textsubscript{Ca}, Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel; PMCA, plasma membrane Ca\textsuperscript{2+} pump; SERCA, sarcoendoplasmic reticulum Ca\textsuperscript{2+} pump; SOCE, store-operated Ca\textsuperscript{2+} current; Tg, thapsigargin.

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in these cells (17), which was previously monitored by measuring fura2 fluorescence or the Ca\textsuperscript{2+} dependent K* current (I\textsubscript{KCa}) (16, 17). To understand further the mechanism of Ca\textsuperscript{2+} influx in HSG cells, we have now directly measured the store-operated Ca\textsuperscript{2+} current. We report here the activation of an inward Ca\textsuperscript{2+} current by internal Ca\textsuperscript{2+} store depletion (I\textsubscript{SOCE}) in HSG cells. This is the first report of a Ca\textsuperscript{2+} influx current in a salivary gland cell. I\textsubscript{SOCE} is Ca\textsuperscript{2+}-selective and is regulated by a Ca\textsuperscript{2+}-dependent feedback inhibition, which is determined by the [Ca\textsuperscript{2+}]\textsubscript{i}, in the region of the channel and a protein kinase activity. By using the K\textsubscript{Ca} current as a readout for Ca\textsuperscript{2+} influx, we had previously reported that in HSG cells, [Ca\textsuperscript{2+}]\textsubscript{i} at the site of Ca\textsuperscript{2+} influx is maintained at a very low level (i.e. below that required for activation of K\textsubscript{Ca}) by the intracellular Ca\textsuperscript{2+} pump (SERCA) activity. The present data demonstrate that SERCA modulates the Ca\textsuperscript{2+}-dependent feedback inhibition of the store-operated Ca\textsuperscript{2+} influx channel.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HSG cells were cultured as described earlier (15–17). The cells were detached from the tissue culture dish with 0.25% trypsin, 1.0 mM EDTA (Biofluids). A single cell suspension was seeded on coverslips, kept in a 35-mm culture dish (Corning, NY), and used after 24 h. All reagents were of the highest available grade.

**Patch Clamp Experiments**—Patch clamp in a whole cell configuration was performed on single HSG cells attached to coverslips (17, 18). Patch electrodes were made from 1.0-mm borosilicate glass tubing with filament (BF-100–50-10, Sutter Instrument Co., Novato, CA). The resistance of the pipette was typically between 3 and 6 MΩ when filled. The chamber was connected with an Ag-AgCl pellet through a 150 mM NaCl-containing agar bridge. Cell membrane and pipette capacitative transients were subtracted from the records by the amplifier circuitry before sampling. Voltages have not been compensated for liquid junction potentials. Membrane currents were measured with an Axopatch 200A amplifier in conjunction with pClamp 6.1 software and a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA). The currents were filtered at 2 kHz (low-pass bessel filter) and sampled at an interval of 10 ms. In the step protocol, the cell was held at 0 mV and stepped from −100 mV to −40 mV in 20 mV steps. The cells were held at each step for 200 ms and at the holding potential for 312 ms. In the ramp protocol, the holding potential was +50 mV, and during the ramp the membrane potential was changed from −120 mV to +60 mV within 500 ms. The leakage subtraction function was used with either ramp or step protocol to minimize the leak currents. The currents were digitized and recorded directly onto the hard drive of a Dell Pentium computer. The peak currents measured in the ramp, step, and gap-free protocol were used for all calculations. The I-V relationship was calculated using the peak amplitude of the current during the ramp protocol and exported to the Origin 4.1 (Microcal Software, Inc., Northampton, MA) for further analysis.

Coverslips with cells were cut to an approximately 0.5 × 0.5-mm piece that was placed in a chamber (Warner Instrument Corp., Hamden, CT) that was perfused at a rate of approximately 5 ml/min while the bath solution was continuously removed by a vacuum line. Complete solution changes were achieved within 10 s. The standard extracellular solution contained (mM): 135 sodium glutamate, 1 MgCl\textsubscript{2}, 10 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES, pH 7.4 (NaOH). The pipette was filled with (mM): 135 NMDG glutamate, 10 CaCl\textsubscript{2}, 10 BAPTA, 1 MgCl\textsubscript{2}, 1 ATP, 10 HEPES, pH 7.2 (CsOH). 10 μM inositol 1,4,5,trisphosphate (IP\textsubscript{3}), or its non-metabolizable analog F-IP\textsubscript{3}, was included in the pipette solution. All experiments shown here were performed with F-IP\textsubscript{3}. 10 mM CaCl\textsubscript{2} was replaced with 1 mM Ca\textsubscript{2+}EGTA, 10 mM BaCl\textsubscript{2}, or Ca\textsuperscript{2+}-free medium as indicated in some experiments. Tg, BHQ, or CCh was continuously administered in the cell medium by perfusion. All experiments were performed at room temperature.

**Ca\textsuperscript{2+} Measurements—**[Ca\textsuperscript{2+}]\textsubscript{i}, in HSG cells was determined by measuring fura2 fluorescence as described previously (12, 14, 16). All other experimental conditions are described in the text.

**Data Analysis**—All the values given below are the mean ± S.E. of the indicated number of experiments. The Student’s t test or analysis of variance test, as indicated, was used for statistical analysis of the data.

**RESULTS**

**Ca\textsuperscript{2+} Store Depletion-activated Inward Ca\textsuperscript{2+} Current in HSG Cells**—To measure directly SOCE in HSG cells, we have used the whole cell patch clamp protocol described by Hoth and Penner (6), with some modifications (see “Experimental Procedures”). The fast Ca\textsuperscript{2+} chelator BAPTA (10 mM) was included in the patch pipette and high [Ca\textsuperscript{2+}] (10 mM) in the external medium. The inclusion of BAPTA into the cell has been shown to prevent intracellular Ca\textsuperscript{2+}-dependent feedback inhibition of the inward Ca\textsuperscript{2+} current, and thus facilitate detection of this current (6, 7). This was also true for HSG cells. The inward current was not detected when BAPTA was omitted from the pipette solution or low concentrations of EGTA, 0.5–1.0 mM, were used (data not shown).

Stimulation of HSG cells by CCh, or direct activation of internal Ca\textsuperscript{2+} release by IP\textsubscript{3}, caused activation of an inward current (Fig. 1, which shows a continuous recording of the current at 0 mV using the gap-free configuration of pClamp 6.0. The current recording was initiated as soon as the whole cell configuration was established. Consistent with previous I\textsubscript{RAC} measurements (6, 7), the IP\textsubscript{3}-induced current had a shorter onset (15.3 ± 5.4 s, n = 7) of activation as compared with that of the CCh-stimulated current (30.6 ± 7.3 s, n = 4, including the perfusion time, <10 s). We have shown previously that in rat parotid acinar cells peak [Ca\textsuperscript{2+}]\textsubscript{i} due to internal Ca\textsuperscript{2+} release is reached within 6 s after CCh stimulation of cells, whereas Ca\textsuperscript{2+} influx is detected after a lag of about 10 s (19). Similar lag times have also been reported in pancreatic acinar cell (20). IP\textsubscript{3}-stimulated inward currents were detected in 70.2% (52/74) of the cells tested while CCh-stimulated currents were detected in 53.8% (7/13) of the cells. The maximum steady state current activated by IP\textsubscript{3} at 0 mV in the continuous recording protocol had an average amplitude of 14.5 ± 5.2 pA (n = 7). A similar pattern of current was seen with 1,4,5-IP\textsubscript{3} and its non-metabolizable analog, F-IP\textsubscript{3}. All the studies described here, including the results in Fig. 1, were carried out with F-IP\textsubscript{3} to rule out possible effects due to hydrolysis or metabolism of IP\textsubscript{3}. For convenience we refer to this analog as IP\textsubscript{3}.

Note that control cells (i.e. loaded with solution that did not contain IP\textsubscript{3}) displayed currents in 20% of the cells (4/20) with an average amplitude of 5 ± 2 pA and an onset time of 312 ± 50 s.

The ramp protocol was also used to record the IP\textsubscript{3}-induced inward current and to determine the I-V curve (Fig. 2). The IP\textsubscript{3}-induced inward Ca\textsuperscript{2+} current increased at negative membrane potentials, up to −120 mV, and showed inward rectification with a reversal potential more positive than +10 mV. Tg, or BHQ, also activated a similar inward current although
A 60 mV
35 mV
-120 mV
Ca2+
Na+
Ba2+

pA
200 ms

Fig. 2. Ca2+ selectivity of the inward current. The inward cation currents activated by IP3 (A) and their I-V curves (B) are shown. The currents were recorded using a 500-ms ramp protocol from -120 to +60 mV and holding potential of +35 mV. Na+ (150 mM) and Ba2+ (10 mM) currents were recorded in a Ca2+-free medium, whereas the currents recorded in the presence of La3+ (1 mM) was obtained in Ca2+ (10 mM)-containing medium.

with a different temporal profile (further discussed below), suggesting that depletion of internal Ca2+ stores activates a cation channel in HSG cells. In some cells stimulated by IP3, CCh, or Tg, but not in unstimulated cells, a small inward current was detected at higher (+40 mV to +60 mV) membrane potentials (Fig. 2, A and B). The nature of this inward current is not presently clear. It should be noted that the internal and external solutions used blocked the Ca2+-activated K+ current, which has been previously described in these cells (17). Furthermore, Ca2+-activated Cl− currents are not detected in HSG cells, but only a volume-regulated outwardly rectifying Cl− current, which has been previously described in these cells (21).

To determine the permeability of the store depletion-activated channel to other cations, IP3-induced inward current was measured in cells perfused with Ca2+-free medium, i.e. with Na+ as the only cation in the medium. This condition decreased the amplitude of the inward current by 41% (n = 4, Fig. 2A). Under these conditions, the estimated permeability of Na+, relative to that of Ca2+, was 0.59 and 0.66 at -120 and 0 mV, respectively. However, when [Ca2+]i was replaced by Ba2+, the inward current was only slightly less than that obtained with 10 mM Ca2+. Furthermore, when 1 mM Ca2+ was used (see Fig. 4A), the peak amplitude of the current (53 ± 5.1 pA) was significantly less (p < 0.05, n = 3 to 5) than that measured with 10 mM Ca2+ (127 ± 10.8 pA), 10 mM Ba2+ (109 ± 7.9 pA), or Na+(75 ± 5.6 pA) in the medium. Thus, the store depletion-activated cation channel appeared to conduct Ba2+ and Na+ when Ca2+ ion was not present in the external medium and demonstrated cation permeability in the order Ca2+ > Ba2+ > Na+. Importantly, these data suggest that with a normal physiological external solution, i.e. containing 1 mM [Ca2+], and about 140 mM Na+, the channel is relatively more permeant to Ca2+ than Na+. This is further illustrated by the data in Fig. 2B showing the IV curves of the Ca2+, Ba2+, and Na+ currents. The IV curve of the IP3-induced Ca2+ current was significantly different from that of the Na+ current (p < 0.05, analysis of variance analysis). Additionally, in the presence of La3+, the Ca2+ current was almost completely inhibited and did not show any voltage sensitivity.

Previously we have shown that Ca2+ channel antagonists La3+ and Gd3+, but not Zn2+, blocked SOCE in HSG cells (17). Consistent with these results, IP3 failed to evoke inward currents in the presence of 1 mM La3+ (Fig. 2B). Fig. 3 shows continuous recordings of the inward current (at 0 mV and 10 mM Ca2+). The IP3-activated current was greatly reduced by perfusion of La3+ into the medium, and the reduction was reversed when La3+ was removed (Fig. 3A). A similar inhibition was also seen with Gd3+ (Fig. 3B) but not with Zn2+ (data not shown). Furthermore, La3+ (1 mM) also reversibly blocked IP3-induced activation of Na+ and Ba2+ (n = 5). In aggregate the data in Fig. 2 suggest that Ba2+, Na+, and Ca2+ pass through the same store-operated Ca2+-permeable cation channel in HSG cells. Furthermore, the divalent cation sensitivity of the inward Ca2+ current is similar to that of SOCE which we have previously reported (17). These data demonstrate the activation of a store-operated Ca2+-permeable cation channel in HSG cells. Al-

FIG. 3. Effects of La3+ and Gd3+ on the IP3-induced Ca2+ current. The inward current stimulated by IP3 was continuously recorded as described for Fig. 1. 1 mM La3+ (A) or 1 mM Gd3+ (B) was included in the perfusion medium which contained 10 mM Ca2+ for the period shown by the bar. These traces are representative of recordings from seven cells.

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through this current exhibits several characteristics similar to those of $I_{\text{CRAC}}$, it is not inhibited by Zn$^{2+}$ (5). Thus, we have referred to this current as $I_{\text{SOC}}$.

$Ca^{2+}$-dependent Inactivation of $I_{\text{SOC}}$—A ramp protocol was used to record $I_{\text{SOC}}$ with 1 mM $Ca^{2+}$ in the external medium and then repeated to record another trace in the same cell after $[Ca^{2+}]_e$ was increased to 10 mM (Fig. 4A). The average peak amplitude of the current with 1 mM $Ca^{2+}$ was 40.3 ± 10.5% ($n = 3$) of that with 10 mM $Ca^{2+}$ in the same cell. Similar effects were seen in a continuous recording of $I_{\text{SOC}}$ at 0 mV (Fig. 4B). The amplitude of the IP$_3$-stimulated inward current decreased significantly with reduction of $[Ca^{2+}]_e$ concentration from 10 to 1 mM and increased again when $[Ca^{2+}]_e$ was changed back to 10 mM. However, with subsequent shifts in $[Ca^{2+}]_e$ from 10 to 1 mM and vice versa, the amplitude of the current with either 10 or 1 mM $Ca^{2+}$ gradually decreased, and after the third exposure to 10 mM $Ca^{2+}$, the current at 1 mM $Ca^{2+}$ was not detectable above basal levels. The current seen with 10 mM $Ca^{2+}$ was decreased by about 50%. These data demonstrate that the amplitude of $I_{\text{SOC}}$ is dependent on $[Ca^{2+}]_e$, as was previously observed for $Ca^{2+}$ influx (17, 22, 23). These data also show that $I_{\text{SOC}}$ inactivates, either as a function of time after activation or due to exposure of cells to high $[Ca^{2+}]_e$. However, the inhibition did not appear to be dependent on the presence of high $[Ca^{2+}]_e$ per se, since it was observed even after the 10 mM $Ca^{2+}$-containing medium was replaced with 1 mM $Ca^{2+}$ medium.

To examine further the apparent inhibition of $I_{\text{SOC}}$ by $Ca^{2+}$ in HSG cells, we used a step protocol to measure IP$_3$-generated $I_{\text{SOC}}$ at various times after the whole cell configuration was established (<30 s and 1–5 min). During the first sequence (Fig. 5A), at each holding potential from −100 to −40 mV, the inward current evoked by IP$_3$, via internal $Ca^{2+}$ release, was biphasic, with a rapid initial increase followed by a relatively slower decay. However, the amplitude of the initial current at each holding potential gradually decreased in the subsequent sequences. Two to three minutes after stimulation (Fig. 5, C and D), the peak current at any membrane potential was reduced by about 50%, and 5 min after stimulation the current could not be detected even at −100 mV (Fig. 5E).

A similar time-dependent inhibition of the inward $Ca^{2+}$ current was observed using repeated ramp protocols (<30 s and 1, 3, 5, and 10 min, see Fig. 6). The IP$_3$-induced inward $Ca^{2+}$ current (with 10 mM $Ca^{2+}$) was significantly reduced at 3 and 5 min (traces II and III, respectively) after stimulation as compared with the initial response (trace I). Five minutes after stimulation, the inward current was reduced to 47 ± 3.5% ($n = 3$) of the initial current, while 10 min after stimulation, the current was barely detectable. However, the inactivation of the current was significantly delayed when the cell was in a low, more physiological, $[Ca^{2+}]_e$, (1 mM, Fig. 6B). The peak amplitudes of the inward current at 5 (trace II) and 10 (trace III) min after stimulation were 64 ± 6.7 and 54.5 ± 7.3% ($n = 4$), respectively, of the initial current. In aggregate these data indicate that the $Ca^{2+}$-dependent feedback inhibition of $I_{\text{SOC}}$ depends on $[Ca^{2+}]_e$, and although it is detected at physiological $Ca^{2+}$ levels, it occurs more rapidly at higher $[Ca^{2+}]_e$. Further-
more, the data in Fig. 4B strongly indicate that this inhibition is not due to \([Ca^{2+}]_o\) per se.

We hypothesized that the inactivation of \(I_{SOC}\) was determined by the rate (and thus the amount) of \(Ca^{2+}\) entering the cell, i.e. when cells are pulsed at more negative membrane potentials in the presence of high \([Ca^{2+}]_o\), \(Ca^{2+}\) influx is greater and thus the rate of inactivation is also higher than seen with lower \([Ca^{2+}]_o\), and membrane potentials closer to 0 mV. To demonstrate this, we used the continuous recording protocol and stimulated the cells with IP\(_3\) at a holding potential of \(-80\) mV, instead of 0 mV. Whereas the amplitude of the initial current at \(-80\) mV was higher than that at 0 mV, the current was transient and inactivated rapidly (in <20 s) to a level close to the resting current (Fig. 7, compare with trace in Fig. 1). This result demonstrates that the driving force for \(Ca^{2+}\), thus the amount of \(Ca^{2+}\) entering the cell, modulates the inactivation of \(I_{SOC}\). The inactivation of \(I_{SOC}\) did not show any rebound phenomena.

**Role of a Staurosporine-sensitive Protein Kinase in the \(Ca^{2+}\)-dependent Inactivation of SOCE**—Based on our previous studies, we have suggested that the \(Ca^{2+}\)-dependent feedback inhibition of SOCE in rat parotid gland cells is mediated via a staurosporine-sensitive protein kinase (12, 14). We have also observed that other protein kinase inhibitors are ineffective in preventing the \(Ca^{2+}\)-induced inhibition of \(Ca^{2+}\) influx in these cells.2

To determine whether SOCE in HSG cells is also affected by modulators of protein phosphorylation, we studied the effects of okadaic acid and calyculin A on thapsigargin-stimulated \([Ca^{2+}]_o\) changes in fura-loaded HSG cells. Similar to our results with rat parotid acinar cells, both these inhibitors induced a significant inhibition of thapsigargin-stimulated \(Ca^{2+}\) influx but not internal \(Ca^{2+}\) release (Table I). We also examined the inhibition of \(Ca^{2+}\) influx with 5 mM \([Ca^{2+}]_o\), since the patch clamp protocol uses high \([Ca^{2+}]_o\). Calyculin A was more effective in blocking \(Ca^{2+}\) influx at this higher \([Ca^{2+}]_o\). The inhibition by either phosphatase inhibitor was prevented when cells were pretreated with staurosporine (data not shown). The effect of staurosporine and calyculin A was further tested on \(I_{SOC}\) in HSG cells. Consistent with the data shown in Table I, calyculin A induced a partial, but reversible, inhibition of the IP\(_3\)-induced \(Ca^{2+}\) current (Fig. 8A), whereas staurosporine (500 nM) enhanced this current in an irreversible manner (Fig. 8B). Thus, the response of SOCE in HSG cells and rat parotid gland cells to modulators of protein phosphorylation is similar. Furthermore, these data also demonstrate that the effects of these agents on SOCE are not indirectly induced via effects on the membrane potential of the cell as has been suggested (5).

Fig. 9A shows the effect of staurosporine on the time-dependent inactivation of \(I_{SOC}\) in HSG cells. Cells were preincubated with the protein kinase inhibitor staurosporine (500 nM) for 10 min prior to the current measurement. Trace I was the initial recording (<30 s after whole cell configuration was established) and Trace II was recorded immediately after whole cell configuration was achieved, i.e. dialysis of cells with IP\(_3\). The traces of B, C, D, and E were recorded at 1, 2, 3, and 5 min after. These data are representative of currents obtained in three cells.
Effects of phosphatase inhibitors on Tg-stimulated Ca\textsuperscript{2+} influx in HSG cells

HSG cells were treated with 100 nM calyculin A for 10 min or with 1 μM okadaic acid for 30 min. Tg-stimulated increase in [Ca\textsuperscript{2+}]\textsubscript{i} (initial peak), measured in a medium containing 1.5 mM Ca\textsuperscript{2+}, is due to intracellular Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx. The phosphatase inhibitors only block the influx component of this increase. Tg-stimulated peak increase in Ca\textsuperscript{2+}-free medium (i.e. due to internal Ca\textsuperscript{2+} release) is between 80 and 100 nM, and this is not different from the value obtained in phosphatase inhibitor-treated cells, either in the presence or absence of external Ca\textsuperscript{2+}. 300 seconds after Tg addition, 5 mM Ca\textsuperscript{2+} was added to the external medium, and the increase in [Ca\textsuperscript{2+}]\textsubscript{i} was noted. The values given represent [Ca\textsuperscript{2+}]\textsubscript{i} (over basal which was between 75 and 90 nM and not altered by the incubations).

| Change in [Ca\textsuperscript{2+}]\textsubscript{i} (μM) | Initial peak | 5 mM Ca\textsuperscript{2+} addition |
|--------------------------------------------------|--------------|---------------------------------|
| Control cells                                   | 166±5.4      | 356±12                          |
| +Calyculin A                                     | 73.3±12.7\textsuperscript{a} | 124±17.4\textsuperscript{a}     |
| +Okadaic acid                                    | 116±10.4\textsuperscript{a} | 205±17.8\textsuperscript{a}     |

\textsuperscript{a} Significantly different from the values obtained in control cells (p < 0.005, n = 5, Student’s t test).

Table I

Significantly different from the values obtained in control cells (p < 0.005, n = 5, Student’s t test).

To determine whether the inactivation of I\textsubscript{SOC} is Ca\textsuperscript{2+}-specific, the rate of inactivation was examined under several different conditions. The experimental protocol was similar to that described for Fig. 9A, and the amplitudes of the peak currents determined in the different experiments have been plotted in Fig. 9B. The highest rate of inactivation of I\textsubscript{SOC} was seen with 10 mM [Ca\textsuperscript{2+}], in the medium; with 40% decrease in the current by 5 min and >90% by 10 min. The Na\textsuperscript{+} currents, measured in the absence of external Ca\textsuperscript{2+}, did not show significant inactivation, and >80% of the initial current was still present 15 min after the cell was dialyzed with IP\textsubscript{3}. Ba\textsuperscript{2+} currents showed a slower rate of inactivation initially (at 5 min) than that seen with either 1 or 10 mM Ca\textsuperscript{2+} in the medium. After 15 min, the Ba\textsuperscript{2+} current was inhibited by about 50%, similar to that seen with 1 mM [Ca\textsuperscript{2+}]. When cells were first treated with staurosporine, despite the presence of 10 mM external Ca\textsuperscript{2+}, there was <20% decrease in the current at 5 min and about 40% decrease at 15 min. Furthermore, increasing the [BAPTA] in the internal solution to 20 mM significantly reduced the rate of inactivation of I\textsubscript{SOC}. Finally, decreasing the frequency of the step protocol from 1 per min to 1 per 5 min decreased the rate of inactivation of I\textsubscript{SOC} by 50% of the current was seen 15 min after stimulation. In aggregate, these data demonstrate that the inactivation of I\textsubscript{SOC} is Ca\textsuperscript{2+}-specific and is determined by the amount of Ca\textsuperscript{2+} entering the cell as well as by the buffering of [Ca\textsuperscript{2+}].

TG- and BHQ-induced I\textsubscript{SOC} and the Effect on the IP\textsubscript{3}-induced I\textsubscript{SOC}—The data described above suggest that the increase in [Ca\textsuperscript{2+}]\textsubscript{i} in the region of Ca\textsuperscript{2+} influx likely determines the inactivation rate of I\textsubscript{SOC}, as has been reported for I\textsubscript{CRAC} in T-lymphocytes (8, 9). However, since 10 or 20 mM BAPTA was used in the pipette solution to buffer [Ca\textsuperscript{2+}], in these experiments, this might appear to be an unlikely possibility. One possible explanation is that there is a limited amount of BAPTA in this region of the cell, which saturates as more Ca\textsuperscript{2+} enters the cell (see “Addendum”). Furthermore, BAPTA from this region might not diffuse out readily. Our recent studies with HSG cells have suggested that the SERCA in the ER and the site of SOCE in the plasma membrane are in close proximity since the Ca\textsuperscript{2+} which enters the cell via this pathway is rapidly transported into the ER without any increase in [Ca\textsuperscript{2+}]\textsubscript{i}, in the region of Ca\textsuperscript{2+} influx, as indicated by the lack of activation of K\textsubscript{ca} (17). Similar data were also reported by Mogami et al. (24, 25). These studies are consistent with earlier reports showing that [Ca\textsuperscript{2+}]\textsubscript{i} (measured using fura2) remains close to resting levels when Ca\textsuperscript{2+} influx occurs during refilling of inter-
nal Ca\(^{2+}\) stores (3, 10, 25, 26). Additionally, internal Ca\(^{2+}\) stores are refilled even in the presence of BAPTA, although at a somewhat slower rate (19, 26). Thus, we hypothesized that in our present experimental system, \([\text{Ca}^{2+}]_i\) at the site of SOCE was determined by the following: (i) the amount of Ca\(^{2+}\) entering the cell within a given time, (ii) the buffering capacity of BAPTA, and (iii) the SERCA activity. We have presented evidence for the first two points above.

To demonstrate a role for SERCA activity in the regulation of ISOC, we used the SERCA inhibitors, Tg and BHQ, which, by blocking the uptake of Ca\(^{2+}\) into intracellular stores, allow Ca\(^{2+}\) to “leak” out of the ER, thus activating SOCE (27). Fig. 10A shows the activation of ISOC by Tg. Additionally, when cells were preincubated with Tg (10 \(\mu\)M) for 5–10 min, IP\(_3\) failed to induce any further inward currents in HSG cells (\(n = 5\), data not shown). These data are important in that they demonstrate that IP\(_3\) and thapsigargin activate the same pathway. However, the Tg-induced inward current (measured at 0 mM and 10 mM Ca\(^{2+}\)) was relatively transient as compared with that activated by IP\(_3\) or CCh and was inactivated within 2 min after stimulation (similar results were obtained with BHQ, these data are not shown). It should be noted that a few cells treated with Tg or BHQ showed longer activation of ISOC (up to 3 min), whereas about half of the cells did not display any inward current (15/27). Tg-induced Ca\(^{2+}\) current recorded using the ramp protocol also inactivated rapidly (Fig. 9B, compare traces I and II). These data suggest that inhibition of SERCA activity increases the rate of inactivation of the ISOC.

A possible explanation of these results is that when the SERCA activity is inhibited, there is an increase in the ambient \([\text{Ca}^{2+}]_i\) in the region of the SOCE since Ca\(^{2+}\) uptake into intracellular Ca\(^{2+}\) stores is prevented. This would increase the inactivation rate of the SOCE. To test this, we examined the effect of Tg on IP\(_3\)-induced Ca\(^{2+}\) currents. Fig. 11A shows that addition of Tg to cells displaying IP\(_3\)-activated ISOC (continuous recording at 0 mM and 10 mM external Ca\(^{2+}\)) resulted in a rapid inactivation of the current. The Tg-induced inhibition of ISOC was attenuated when the cells were loaded with 20 mM BAPTA instead of 10 mM BAPTA (\(n = 4\), data not shown). The effect of thapsigargin on ISOC was further examined by using the ramp protocol (Fig. 11B). ISOC was first activated by dialysis of IP\(_3\) into the cell (trace I); the cell was then perfused with medium containing Tg for 1–2 min, and another trace (trace II) was recorded. ISOC appeared to be completely inactivated at this...
DISCUSSION

The data presented above demonstrate the activation of an inward Ca\textsuperscript{2+} current in HSG cells by conditions resulting in the depletion of the internal Ca\textsuperscript{2+} store(s). This is the first report of this current in salivary gland cells, a widely used cell type for studying mechanisms of Ca\textsuperscript{2+} signaling and Ca\textsuperscript{2+} influx (2, 3, 10–17). Inward currents activated in response to store depletion have been measured in a number of cell types (4, 5). The best characterized of these currents is the I\textsubscript{CRAC}, which has been detected in mast cells, RBL cells, and T-lymphocytes. These previous studies suggest that I\textsubscript{CRAC} is mediated by a Ca\textsuperscript{2+} channel, which is gated by the depletion of the internal Ca\textsuperscript{2+} store(s) (5). We have referred to the internal Ca\textsuperscript{2+} store depletion-activated inward Ca\textsuperscript{2+} current in HSG cells as I\textsubscript{SOC} since several characteristics were noted that distinguished it from I\textsubscript{CRAC}.

Unlike I\textsubscript{CRAC}, I\textsubscript{SOC} in HSG cells was not sensitive to Zn\textsuperscript{2+}, but was inhibited by Gd\textsuperscript{3+}, consistent with our previous studies with HSG cells. Furthermore, okadaic acid and calyculin A inhibited I\textsubscript{SOC} in HSG cells. Conversely, okadaic acid, but not calyculin A, prevented refill-dependent inactivation of I\textsubscript{CRAC} in T-lymphocytes. We have also shown that staurosporine increased I\textsubscript{SOC} in HSG cells and prevented the Ca\textsuperscript{2+}-induced feedback inhibition. We have described similar effects of staurosporine on Ca\textsuperscript{2+} influx in HSG cells (this report) and rat parotid acinar cells (12, 14). Importantly, these data demonstrate that the characteristics of I\textsubscript{SOC} and SOCE in HSG cells are similar (17) which indicates that they represent the same mechanism. Additionally, several characteristics of I\textsubscript{SOC}, e.g. sensitivity to phosphatase inhibitors and staurosporine, are similar to those of SOCE in rat parotid acinar cells. However, like I\textsubscript{CRAC}, SOCE in rat parotid acinar cells is inhibited by low concentrations of Zn\textsuperscript{2+} (5, 28). Furthermore, a Ca\textsuperscript{2+}-conducting nonspecific cation channel activated by store depletion in rat pancreatic acinar cells is not inhibited by La\textsuperscript{3+} or Gd\textsuperscript{3+} (29). In aggregate, these data demonstrate marked differences between the SOCE and store-operated cation currents in different cell types in their sensitivity to divalent and trivalent cations and effects of modulators of protein phosphorylation. These characteristics could provide a useful tool to identify different “isoforms” of this putative Ca\textsuperscript{2+} channel.

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**Fig. 10.** Tg-induced inward currents in HSG cells. A, 10 \muM Tg-containing medium (with 10 mM Ca\textsuperscript{2+}) was continuously perfused, indicated by horizontal bar. Current was recorded with the holding potential at 0 mV. B, the ramp protocol was used to record Tg-induced inward currents at the beginning (trace I) and 2 min (trace II) after whole cell model was established. [Ca\textsuperscript{2+}] in the medium was 10 mM. These represent recordings from four cells.

**Fig. 11.** Tg increases the rate of inactivation of I\textsubscript{SOC} in IP\textsubscript{3}-stimulated cells. A, a continuous recording of IP\textsubscript{3}-induced inward current at a holding potential of 0 mV and 10 mM Ca\textsuperscript{2+}. Tg was added to the perfusion medium where indicated. B, a ramp protocol was used to measure IP\textsubscript{3}-induced Ca\textsuperscript{2+} currents. Trace I shows the initial current stimulated by IP\textsubscript{3}. The cell was then perfused with Tg (10 \muM) for 2 min, and the current was recorded (trace II). The data represent results from four cells.
Ca\textsuperscript{2+} on I\textsubscript{SOC} in this study. However, we have examined the inactivation of I\textsubscript{SOC} that was detected in the continued presence of a non-metabolizable analog of IP\textsubscript{3}, using three different experimental protocols. In the step protocol, a biphasic current was stimulated, with an initial peak increase followed by a steep inactivation. The initial amplitude of the current and the subsequent inactivation rate was greater at more negative membrane potentials and higher [Ca\textsubscript{2+}]\textsubscript{i}. In the ramp protocol, the current appeared to inactivate rapidly as the membrane potential was shifted from negative to positive potentials. It can be suggested that this decrease in the current amplitude reflects combined effects of the decrease in driving force for Ca\textsuperscript{2+} influx and possible fast inactivation mechanism(s) as has been reported for I\textsubscript{CRAC} in lymphocytes (8, 9). Notably, pretreatment of HSG cells with staurosporine did not affect the pattern of I\textsubscript{SOC} during the first ramp (compare trace 1 in Figs. 2A and 9A). Thus, we do not believe that the Ca\textsuperscript{2+}-dependent inactivation mechanism we have described above contributes to the decrease in the amplitude of the current during the first ramp. However, it is likely that this inactivation mechanism accounts for the somewhat exaggerated curvature of the I-V curve (for the Ca\textsuperscript{2+} current, see Fig. 2B) at the more negative membrane potentials. Similar observations were previously made by Hoth and Penner (6). Importantly, the present data clearly demonstrate a slower, time-dependent, inactivation of I\textsubscript{SOC} in HSG cells (seen in both the ramp as well as step protocols) that is induced by Ca\textsuperscript{2+} and to a lesser extent by Ba\textsuperscript{2+}, but not by Na\textsuperscript{+}. In the continuous recording protocol, a rapid run down of the current was seen with high [Ca\textsubscript{2+}]\textsubscript{i} at more negative membrane potentials. Lewis and coworkers (8, 9) have described Ca\textsuperscript{2+}-dependent slow and fast inactivation mechanisms of I\textsubscript{CRAC} in T-lymphocytes. The fast inactivation was attributed to the [Ca\textsuperscript{2+}] near the mouth of the channel and the slow inactivation to the Ca\textsuperscript{2+}-dependent activation of a phosphatase enzyme, which was sensitive to okadaic acid but not calyculin A. However, our data presently do not establish that the fast and slow inactivation mechanisms of I\textsubscript{SOC} in HSG cells are mutually distinct. Importantly, in HSG cells both phosphatase inhibitors inactivate I\textsubscript{SOC}, whereas the protein kinase inhibitor, staurosporine, prevents the inactivation. Thus I\textsubscript{CRAC} and I\textsubscript{SOC} may be fundamentally different, with respect to the regulation of their activity and to nature of the molecular components.

We have shown that I\textsubscript{SOC} inactivates within 5 or 10 min in cells subjected to repeated step or ramp protocols, respectively. The possibility that high external [Ca\textsuperscript{2+}] \textit{per se} inhibits Ca\textsuperscript{2+} influx can be ruled out since IP\textsubscript{3}-induced I\textsubscript{SOC} measured with 10 mM Ca\textsuperscript{2+} in the medium was inactivated when the holding potential was -80 mV but not 0 mV. Thus, the rate of inactivation of I\textsubscript{SOC} depends on the driving force for Ca\textsuperscript{2+} influx, i.e. on the amount of Ca\textsuperscript{2+} entering the cell. In aggregate these data are consistent with the suggestion that an increase in the [Ca\textsuperscript{2+}]\textsubscript{i}, in the region of the SOCE induces a feedback inhibition of Ca\textsuperscript{2+} influx (14, 19). Furthermore, the data show that the inactivation is attenuated by pretreatment of the cells with staurosporine, suggesting the involvement of a protein kinase in the inactivation. There are two possible explanations for these results. (i) Ca\textsuperscript{2+} directly activates the protein kinase or inhibits the protein phosphatase, which in either case would result in an increase in the protein phosphorylation level. (ii) Alternatively, the effect of Ca\textsuperscript{2+} and phosphorylation are independent, and the Ca\textsuperscript{2+}-dependent inactivation cannot be exerted when the protein is dephosphorylated. The present data do not distinguish between these two possibilities.

Our present and previous data (17) and the studies reported by Mogami et al. (24, 25) suggest that the driving force for Ca\textsuperscript{2+} influx and the SERCA activity regulate the ambient [Ca\textsuperscript{2+}]\textsubscript{i}, in the region of the putative store-operated Ca\textsuperscript{2+} channel (SOC). Such a suggestion might seem unlikely in the present study, since the cells were dialyzed with a solution containing 10 mM BAPTA, which tightly buffers changes in [Ca\textsuperscript{2+}]\textsubscript{i}. However, as discussed above, there may be a limited amount of BAPTA in this subplasma membrane region of the cell, which would tend to saturate as more Ca\textsuperscript{2+} enters the cell (see “Addendum”). It is reasonable to predict that there is an equilibrium between free BAPTA, [Ca\textsuperscript{2+}]\textsubscript{i}, and Ca\textsuperscript{2+}-bound BAPTA in the region of the cell near the SOC (see Fig. 12 and discussion below). Our data demonstrate that despite the presence of BAPTA, this equilibrium is affected by the activity of the SERCA pump and the rate of Ca\textsuperscript{2+} entering the cell. In the ramp and step protocols, repeated application of a high driving force, although for a short time, could drive sufficient amounts of Ca\textsuperscript{2+} into the cell to alter the BAPTA-Ca\textsuperscript{2+} equilibrium in the region of the SOC. This suggestion is consistent with our observations that (i) increasing intracellular [BAPTA] to 20 mM (Fig. 2B) and (ii) increasing the time interval between the step sequences (data not shown) decreased the rate of inactivation of I\textsubscript{SOC}.

BAPTA has been shown to effectively clamp [Ca\textsuperscript{2+}]\textsubscript{i} and block global increases in [Ca\textsuperscript{2+}]\textsubscript{i} (21, 26). The subplasma membrane [Ca\textsuperscript{2+}]\textsubscript{i}, has also been reported to be clamped by BAPTA since the activation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} or K\textsuperscript{+} currents is prevented in BAPTA-loaded cells, including HSG cells (2, 17). However, it is possible that significant changes in [Ca\textsuperscript{2+}]\textsubscript{i} can occur in localized subplasma membrane regions of BAPTA-loaded cells, i.e. in the region of Ca\textsuperscript{2+} influx. As mentioned above, such changes depend on the amount of Ca\textsuperscript{2+} introduced into the region and the BAPTA concentration. That BAPTA in fact reaches this region of the cell is inferred from previous studies showing that it takes longer for the internal Ca\textsuperscript{2+} stores to be refilled in BAPTA-loaded cells and that the rate of refilling can be increased at higher [Ca\textsuperscript{2+}]\textsubscript{i} (19, 22, 26). This is consistent with our previous data showing that SERCA has a very high affinity for Ca\textsuperscript{2+} and has a high pump rate at resting [Ca\textsuperscript{2+}]. On the other hand the plasma membrane Ca\textsuperscript{2+} pump (PMCA) is relatively less active at resting [Ca\textsuperscript{2+}] (20, 30, 31). Thus, it is not clear what contribution, if any, its activity has in the regulation of [Ca\textsuperscript{2+}]\textsubscript{i} in localized subplasma membrane regions in BAPTA-loaded cells. However, most likely, under physiological conditions, when BAPTA is not present,
the PMCA activity would also have a role in decreasing the [Ca\(^{2+}\)] in this region.

We have recently shown (17) that Ca\(^{2+}\) entering the cell is rapidly accumulated into the internal Ca\(^{2+}\) store by the SERCA activity thus preventing significant diffusion of Ca\(^{2+}\) in the subplasma membrane region. According to the model we have proposed, in cells where the IP\(_3\)-sensitive channel has been activated, Ca\(^{2+}\) which enters via SOC is taken up into the internal Ca\(^{2+}\) stores and then released at localized regions of the cell by the IP\(_3\)-sensitive Ca\(^{2+}\) channel. Mogami et al. (24) have proposed a similar model and have further suggested that when internal Ca\(^{2+}\) stores are depleted, the SERCA activity is increased. This is consistent with our present data since such an increase in SERCA activity would facilitate the rapid uptake of Ca\(^{2+}\) into the store, either in the presence or absence of BAPTA in the cytosol. Thus, the increase in the rate of inactivation of ISOC that was induced by Tg and BHQ directly demonstrates a functional association between SERCA and the mechanism against uncontrolled Ca\(^{2+}\) influx. Parekh has suggested that there may be localized regions in the cell where the buffer is saturated. However, inconsistent with our findings, I\(_{\text{CRAC}}\) induced by thapsigargin or by IP\(_3\) in RBL cells showed similar rates of slow inactivation, although in T-lymphocytes thapsigargin decreased the rate of inactivation of I\(_{\text{CRAC}}\) (9, 10). Parekh has suggested that the contribution of SERCA activity to the measurement of I\(_{\text{SOC}}\) might differ in different cell types.

In summary, our data demonstrate directly that the SERCA activity is involved in regulating the rate of inactivation of ISOC. These data could also offer an explanation for the observation in a number of cells, including in HSG cells (shown above), that thapsigargin is not very effective in stimulating inward Ca\(^{2+}\) currents. According to our present results this could be due to an accelerated inactivation of the Ca\(^{2+}\) channel. However, such a functional interaction between SERCA and SOC might not be present in all types of cells. Alternatively, the current might be more sensitive to other mechanisms of regulation, i.e. protein phosphorylation. In this case, the detection of the current would depend on the presence of such regulatory mechanisms and the rate at which they are activated.

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