Inhibitory Mechanism of Store-operated Ca\(^{2+}\) Channels by Zinc*

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Capacitative calcium influx plays an important role in shaping the Ca\(^{2+}\) response of various tissues and cell types. Inhibition by heavy metals is a hallmark of store-operated calcium channel (SOCC) activity. Paradoxically, although zinc is the only potentially physiological relevant ion, it is the least investigated in terms of inhibitory mechanism. In the present study, we characterize the inhibitory mechanism of the SOCC by Zn\(^{2+}\) in the human salivary cell line, HSY, and rat salivary submandibular ducts and acini by monitoring SOCC activity using fluorescence imaging. Analysis of Zn\(^{2+}\) inhibition indicated that Zn\(^{2+}\) acts as a competitive inhibitor of Ca\(^{2+}\) influx but does not permeate through the SOCC, suggesting that Zn\(^{2+}\) interacts with an extracellular site of SOCC. Application of the reducing agents, dithiothreitol (DTT) and \(\beta\)-mercaptoethanol, totally eliminated Zn\(^{2+}\) and Cd\(^{2+}\) inhibition of SOCC, suggesting that cysteines are part of the Zn\(^{2+}\) and Cd\(^{2+}\) binding site. Interestingly, reducing conditions failed to eliminate the inhibition of SOCC by La\(^{3+}\) and Gd\(^{3+}\), indicating that the Zn\(^{2+}\) and lanthanides binding sites are distinct. Finally, we show that changes in redox potential and Zn\(^{2+}\) are regulating, via SOCC activity, the agonist-induced Ca\(^{2+}\) response in salivary ducts. The presence of a specific Zn\(^{2+}\) site, responsive to physiological Zn\(^{2+}\) and redox potential, may not only be instrumental for future structural studies of various SOCC candidates but may also reveal novel physiological aspects of the interaction between zinc, redox potential, and cellular Ca\(^{2+}\) homeostasis.

The release of Ca\(^{2+}\) from inositol 1,4,5-trisphosphate (InsP\(_3\))-sensitive intracellular Ca\(^{2+}\) stores is followed in many cell types by the opening of store-operated Ca\(^{2+}\) channels (SOCC)\(^3\) that refill the Ca\(^{2+}\) stores (1–5). The genes linked to SOCC activity have not been clearly identified. However, an accumulation of body evidence indicates that members of the family of membrane proteins, Trp, first discovered in Drosophila but with many mammalian homologues, are mediating the store-operated Ca\(^{2+}\) flux (6). The cation influx mediated by SOCC is highly Ca\(^{2+}\)-specific. Other divalent cations such as Ba\(^{2+}\) and Sr\(^{2+}\) are also permeable albeit at a much slower rate (3, 7). In the absence of divalent cations, it has been reported that the SOCC becomes permeable to Na\(^{+}\) (8, 9). However, recent studies have indicated that this mono-valent cation influx is not mediated by the SOCC but rather by MIC channels (10).

Calcium influx mediated by the SOCC participates in refill of internal Ca\(^{2+}\) stores in addition to modulating the Ca\(^{2+}\) response triggered by various agonists (2). Such regulation of the Ca\(^{2+}\) response has been suggested to control a wide range of processes including secretion, cell growth, and proliferation (11). The opening of the SOCC under metabolic stress, it should be added, results in a prolonged rise of intracellular Ca\(^{2+}\), an important element in the signaling cascade leading to cell death (12, 13).

Trivalent cations such as La\(^{3+}\) and Gd\(^{3+}\) and the divalent cations Cd\(^{2+}\) and Zn\(^{2+}\) have been employed previously as inhibitors of the SOCC and have contributed to understanding of this entry route for cations (14–16). Paradoxically, La\(^{3+}\), Gd\(^{3+}\), and Cd\(^{2+}\), which are not physiologically relevant, have been the most commonly studied cations, whereas zinc, which is highly relevant physiologically, is the least understood with respect to the mechanism by which it affects inhibition (1). Endogenous Zn\(^{2+}\), by virtue of its interactions with specific Zn\(^{2+}\) binding sites, modulates a number of ion channels, including NMDA and GABA receptors, as well as regulating transporters such as the dopamine transporter (17–20). Zinc inhibition of Ca\(^{2+}\) influx, mediated by SOCC, has been shown to modulate intracellular Ca\(^{2+}\) signals, thereby modifying cellular functions (14). The majority of zinc ions are complexed to metalloproteins such as enzymes, metallothioneins, and transcription factors. It is, therefore, unclear whether the minute concentrations of free zinc, found in most organs, are sufficient to block SOCC-mediated Ca\(^{2+}\) influx. It is also not known whether zinc binding sites are allosteric or whether they are associated with the cation permeation pathway. In the present study, we describe the basic characteristics of the Zn\(^{2+}\) inhibitory site on the SOCC. Our results indicate that Zn\(^{2+}\), at physiological concentrations, competitively blocks the Ca\(^{2+}\) flux via SOCC, without permeating through this pathway. We further show that the SOCC inhibition by zinc, but not by lanthanides, is completely eliminated by an increase in redox potential, indicating that cysteines are part of the Zn\(^{2+}\) inhibitory site that is distinct from the lanthanide site. Such interplay between zinc and redox, shown previously with regard to sequestration and release of zinc ions by cysteine-rich metallothioneins (21), may have important physiological implications with respect to the regulation of the Ca\(^{2+}\) response in salivary submandibular acini and duct cells.

EXPERIMENTAL PROCEDURES

Cell and Tissue Preparation—HSY (human salivary gland cell line) were grown in Dulbecco’s modified Eagle’s medium as described previously (22). Min-6 cells (a mouse insulinoma cell line) were grown in

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§ The abbreviations used are: SOCC, store-operated calcium channel(s); LTCC, L-type calcium channel(s); DTT, dithiothreitol; GABA, \(\gamma\)-aminobutyric acid, Type A; NMDA, N-methyl-D-aspartate; TG, thapsigargin.

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Fig. 1. The experimental paradigm of SOCC opening and its inhibition by heavy metals. In a, SOCC was activated by emptying intracellular Ca\(^{2+}\) pools, in nominally Ca\(^{2+}\)-free Ringer’s solution in the presence of 0.2 μM thapsigargin followed by the addition of 1 mM CaCl\(_2\). Changes in intracellular Ca\(^{2+}\) concentrations were monitored using Fura-2 fluorescence, allowing estimation of SOCC activity. In b, Ca\(^{2+}\) permeation through the SOCC was determined as described in a. Calcium (1 mM) was added in the presence or absence of the indicated metals (20 μM). All three metals strongly inhibited Ca\(^{2+}\) permeation through the SOCC.

Dulbecco’s modified Eagle’s medium with 1% penicillin-streptomycin, 1% l-glutamine, 71.5 μM β-mercaptoethanol, and 15% calf serum bovine donor (23). Rat submandibular ducts and acini were prepared essentially as described previously (24). The preparations were mounted on glass coverslips that were coated with Celletak (BD Biosciences), according to the manufacturer’s instructions, 30 min prior to the Fura-2 loading.

Fluorescent Calcium Imaging—Cells grown on coverslips were loaded for 30 min at room temperature with 5 μM Fura-2 AM (1 mM stock in Me\(_2\)SO, Tef Labs, Austin, TX) in Ringer’s solution composed of (in mM): 120 NaCl; 5.4 KCl; 1.8 CaCl\(_2\); 0.8 MgCl\(_2\); 10 HEPES; 10 glucose; pH 7.4, containing 0.1% bovine serum albumin. Ducts and acini were loaded by 2.5 μM Fura-2 AM in the presence of 0.025% pluronic F-127 (Molecular Probes). Fura-2-loaded cells were washed twice in Ringer’s solution containing 0.1% bovine serum albumin and incubated for an additional 15 min at room temperature to facilitate the hydrolysis of the dye. Coverslips were then mounted in a perfusion chamber placed on the microscope stage, and Ca\(^{2+}\) imaging was carried out as described previously (25). The zinc-dependent Ca\(^{2+}\) rise triggered by ZnR (25) was eliminated by pretreating HSY cells with 80 μM zinc for 30 min and then thoroughly washing with zinc-free Ringer’s. All the results shown are the average of at least 120 cells, from at least three independent experiments. For the sake of clarity, only every 4–5th data point is shown in the graphs.

RESULTS

Zinc Acts as a Competitive Inhibitor of Ca\(^{2+}\) Permeation through SOCC—The opening of the SOCC following depletion of intracellular stores plays an important role in the physiology of exocrine glands (14, 26). To activate and monitor SOCC in HSY cells, loaded with the Ca\(^{2+}\)-sensitive dye Fura-2, we depleted intracellular calcium stores using 200 nM thapsigargin (TG, Alomone Labs) in nominally calcium-free Ringer’s solution. Application of TG was followed by a slow rise of [Ca\(^{2+}\)]
, leaking from the intracellular pools, and recovery of Ca\(^{2+}\) to resting levels. The subsequent addition of Ca\(^{2+}\) to the perfusing solution was followed by a massive calcium influx (Fig. 1a), which was totally blocked by application of 20 μM Gd\(^{3+}\) or La\(^{3+}\) (Fig. 1b), indicating that it is mediated by SOCC (1). Application of 20 μM Zn\(^{2+}\) had a similar inhibitory effect (Fig. 1b). The free Zn\(^{2+}\) concentration in extracellular fluids is estimated to be in the range of 10\(^{-7}\)–10\(^{-5}\) molar (27). Therefore, to assess the physiological significance, it was important to determine the concentration range at which Zn\(^{2+}\) strongly inhibits the SOCC. Varying concentrations of Zn\(^{2+}\) were applied, and the maximal initial calcium influx rate was determined at different Ca\(^{2+}\) concentrations. To quantify the inhibitory effect of Zn\(^{2+}\), the differential calcium influx rate (d[Ca\(^{2+}\)]/dt) was plotted against the Zn\(^{2+}\) concentration in the perfusing solution (Fig. 2a). Fitting the data with a Michaelis-Menten equation yielded apparent K\(_{i}\) values for each calcium concentration. The K\(_{i}\) values of Zn\(^{2+}\) inhibition of SOCC suggest that Zn\(^{2+}\) ions have the potential to inhibit SOCC activity at concentrations found not only in exocrine glands but also in many other tissues, including pancreatic islets of Langerhans (28), brain (29), colon (30), and the male reproductive system (27).

Zinc modulates the activity of channels and transporters such as the dopamine transporter, GABA, and NMDA channels by interacting with allosteric sites (31, 32). On other proteins such as the glycine receptor, CIC chloride channel, and the L-type Ca\(^{2+}\) channel, zinc acts as a competitive inhibitor of ion permeation (33–35). To distinguish these two modes of inhibition, the K\(_{i}\), for Zn\(^{2+}\) inhibition of Ca\(^{2+}\) influx through the SOCC was plotted versus extracellular Ca\(^{2+}\) concentration (Fig. 2b). The linear dependence indicates that Zn\(^{2+}\) acts as a competitive inhibitor of Ca\(^{2+}\) influx through the SOCC.

Zn\(^{2+}\) Does Not Permeate through the SOCC—The permeation of Zn\(^{2+}\) through several types of cationic channels, most
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notably glutamate channels, purinergic P2X receptor, and the L-type Ca²⁺ channels, has important physiological and pathophysiological implications (36, 37). In contrast to these, Zn²⁺ does not permeate through, but rather modulates channels such as the GABA receptor and the dopamine transporter (31, 38, 39). To determine whether Zn²⁺ modulation of the SOCC also involves permeation through the SOCC, we used a similar experimental paradigm to that described in Fig. 1a but replaced the Ca²⁺ applied following the depletion of the stores with 80 μM Zn²⁺ (Fig. 3a). Because Fura-2 is ~100-fold more sensitive to Zn²⁺ than to Ca²⁺, it can be effectively used to determine whether Zn²⁺ permeates into cells (40, 41). As shown in Fig. 3a, no change in Fura-2 fluorescence was observed following the opening of the SOCC in the presence of zinc, suggesting that the SOCC is impermeable to Zn²⁺. To confirm that this result in HSY cells did not stem from insufficient sensitivity, we monitored Zn²⁺ permeation in Min-6 insulinoma cells that functionally express both SOCC and LTCC (23, 42, 43). As shown in Fig. 3b, depolarization that triggers the opening of LTCC was followed by a robust Zn²⁺ influx. Using the SOCC opening paradigm, no change in the fluorescent signal was observed when Zn²⁺ was applied (Fig. 3b), although Ca²⁺ permeation is monitored. Our results, therefore, indicate that the SOCC is impermeable to Zn²⁺.

The Zn²⁺ Inhibitory Site—The amino acid residues that are involved in the Zn²⁺ inhibitory sites on the SOCC are unknown. Previous work on several membrane proteins including the purinergic receptors subunits P2X2 and P2X4 (44), glycine receptor (45) NMDA and GABA receptors, and the CIC chloride channel (17, 35), implicate cysteine residues as part of the Zn²⁺ binding site. The role of cysteines in Zn²⁺ inhibition of SOCC was assessed, therefore, under reducing and non-reducing conditions. As shown in Fig. 4a, application of Zn²⁺ (50 μM) or Cd²⁺ (500 μM) inhibited the Ca²⁺ influx (×, ○), β-mercaptoethanol (β-Me, 5 mM) treatment together with the heavy metals (□-Zn²⁺, ■-Cd²⁺) eliminated the inhibition of the SOCC. Control cells were superfused only with Ca²⁺-containing Ringer’s solution. In b, following the opening of SOCC, cells were perfused with Zn²⁺ (■, 50 μM), or Cd²⁺ (○, 500 μM), in the presence of Ca²⁺ (1.5 mM). Subsequently, DTT (60 μM or 4 mM respectively) was added. The application of the reducing agent totally reversed the block of SOCC. When cells were perfused with DTT and Zn²⁺ without Ca²⁺ (●), no change in fluorescence was monitored, indicating that DTT does not change the permeability of SOCC to Zn²⁺.

FIG. 3. SOCC is impermeable to Zn²⁺. In a, SOCC was activated by depleting the stores with 100 μM UTP and 0.2 μM TG. Cells were then perfused with Ca²⁺-free Ringer’s solution with Zn²⁺ (80 μM), and finally, Zn²⁺ was removed, and Ca²⁺ (1.5 mM) added. A fluorescent rise is monitored following the addition of Ca²⁺, indicating that SOCC was indeed opened. Although Fura-2 is highly sensitive to zinc, no change in fluorescence was monitored following its addition. Thus, zinc is a reversible blocker of SOCC that does not permeate through this channel. In b, to determine the sensitivity of this assay, permeation of zinc through LTCC was monitored in Min-6 cells, loaded with Fura-2 and treated with UTP and TG as in a. Cells were depolarized by Ca²⁺-free Ringer’s solution containing 50 mM K⁺ in the presence of 100 μM zinc. The rapid reduction of the signal by N,N,N’,N’-tetakis(2-pyridylmethyl)ethylenediamine (TPEN, 50 μM), a membrane-permeable heavy metal chelator, indicates that the rise in fluorescence is related to zinc. To determine whether Zn²⁺ permeates via the SOCC, in Min-6 cells, SOCC was activated by emptying intracellular calcium stores, as above (not shown on graph). Cells were then perfused with Ca²⁺-free Ringer’s solution with Zn²⁺ (100 μM) or 1.5 mM Ca²⁺ (ions added at the time indicated by *). No change in fluorescence was monitored following Zn²⁺ application, whereas Ca²⁺ permeation via SOCC, not affected by TPEN, is clearly monitored. Taken together, the data indicate that in Min-6 cells, as in HSY cells, zinc does not permeate through this pathway.

FIG. 4. Reducing agents prevent blocking of SOCC by Zn²⁺: SOCC activity was monitored following the depletion of intracellular Ca²⁺ stores using UTP (100 μM) and TG (0.2 μM) in Ca²⁺-free Ringer’s solution. In a, although application of Zn²⁺ (50 μM) or Cd²⁺ (500 μM) inhibited the Ca²⁺ influx (×, ○), β-mercaptoethanol (β-Me, 5 mM) treatment together with the heavy metals (□-Zn²⁺, ■-Cd²⁺) eliminated the inhibition of the SOCC. Control cells were superfused only with Ca²⁺-containing Ringer’s solution. In b, following the opening of SOCC, cells were perfused with Zn²⁺ (■, 50 μM), or Cd²⁺ (○, 500 μM), in the presence of Ca²⁺ (1.5 mM). Subsequently, DTT (60 μM or 4 mM respectively) was added. The application of the reducing agent totally reversed the block of SOCC. When cells were perfused with DTT and Zn²⁺ without Ca²⁺ (●), no change in fluorescence was monitored, indicating that DTT does not change the permeability of SOCC to Zn²⁺.
Changes in Redox Potential Have a Physiological Effect on Agonist-induced Ca\textsuperscript{2+} Response by Altering the Zn\textsuperscript{2+} Sensitivity of SOCC—Changes in redox potential play a fundamental physiological and pathophysiological role in salivary secretion (48). We hypothesized that redox potential is involved in shaping the agonist-induced calcium response by modulating the zinc inhibitory effect of the SOCC. To test this, we first monitored the Ca\textsuperscript{2+} response in Fura-2-loaded rat submandibular salivary ducts attached to glass coverslips (Fig. 6a) following depletion of intracellular stores using TG, in the presence and absence of Zn\textsuperscript{2+} (50 \textmu M). A similar inhibition of SOCC was apparent when stores were depleted using TG or carbachol in ducts (Fig. 6a and b). This experimental paradigm was also applied to acini and yielded similar results (not shown). We then determined the \textit{K}_i for the inhibitory effects of Zn\textsuperscript{2+} in both acini and ducts, by monitoring Ca\textsuperscript{2+} influx following the depletion of intracellular Ca\textsuperscript{2+} stores using TG in the presence of varying zinc concentrations (Fig. 6c). The apparent \textit{K}_i of both acini and duct was similar. The physiological effect on agonist-induced Ca\textsuperscript{2+} response in the presence of 50 \textmu M Zn\textsuperscript{2+}, a concentration sufficient to inhibit the SOCC, was next determined. Ducts were superfused with Ca\textsuperscript{2+} Ringer’s solution, and SOCC opening was induced using carbachol (100 \textmu M). As shown in Fig. 6d, application of zinc was followed by a rapid return of Ca\textsuperscript{2+} to resting levels within 1 min and eliminated the elevated plateau triggered by SOCC. Similar results were obtained using 20 \textmu M La\textsuperscript{3+} (not shown). In contrast, the addition of zinc in the presence of DTT significantly prolonged the Ca\textsuperscript{2+} response (to about 3 min) and re-established the elevated plateau, completely reversing the inhibitory effect of zinc. The application of DTT in the absence of zinc did not significantly change the Ca\textsuperscript{2+} response, indicating that it is not the DTT which modulates the Ca\textsuperscript{2+} response. These results show that under conditions of increased redox potential, the zinc inhibition of SOCC is totally eliminated, thereby dynamically regulating the profile of the Ca\textsuperscript{2+} response. Interestingly, the duration of the Ca\textsuperscript{2+} response in the presence of DTT (in the absence or presence of zinc) was even slightly longer than in the control, suggesting that endogenous zinc is present in a concentration sufficient to partially inhibit the SOCC.

**DISCUSSION**

The main goal of the present study was to characterize the mechanism by which Zn\textsuperscript{2+} blocks SOCC. Because of the prominent role of SOCC in shaping the Ca\textsuperscript{2+} response in exocrine cells following stimulation, we focused on the rat salivary ductal and acinar cells as well as the HSY cell line. We found that Zn\textsuperscript{2+} inhibits Ca\textsuperscript{2+} influx through SOCC with an apparent \textit{K}_i of 5.0–2 ± 0.1 \textmu M in salivary gland and HSY cells, which is well within the concentration of extracellular Zn\textsuperscript{2+} (27). Our results indicate that although Zn\textsuperscript{2+} acts as a competitive inhibitor of Ca\textsuperscript{2+} influx, it does not permeate through this pathway. An important finding of this work is that Zn\textsuperscript{2+} inhibition is eliminated by reducing conditions, whereas the inhibition by the lanthanides is not affected, indicating that the SOCC harbors a redox-sensitive Zn\textsuperscript{2+} inhibitory site that is distinct from the lanthanides site. Zinc and cadmium share similar physico-chemical characteristics, the most notable of which is the interaction with thiolate

**Fig. 5. Reducing agents do not prevent the blocking of SOCC by lanthanides.** In a, SOCC was opened by UTP (100 \textmu M) followed by application of the SOCC inhibitors La\textsuperscript{3+} or Gd\textsuperscript{3+} (20 \textmu M) in the presence of the reducing agent DTT (4 mM) and Ca\textsuperscript{2+} (1.5 mM). In contrast to its effect on zinc, DTT did not eliminate the inhibition by La\textsuperscript{3+} or Gd\textsuperscript{3+}, indicating that the lanthanide and zinc inhibitory sites are distinct. In b, SOCC inhibition by La\textsuperscript{3+} was also not eliminated at a much lower concentration of 2 \textmu M. However, zinc inhibition of SOCC was still eliminated in the presence of high concentration of Zn\textsuperscript{2+} (1 mM).

monitored by Fura-2 fluorescence, and its rate was similar when Cd\textsuperscript{2+} was applied following the opening of SOCC (Fig. 4a) or when Cd\textsuperscript{2+} was applied prior to the opening of SOCC (not shown). This Cd\textsuperscript{2+} flux is known to be mediated by the maito-toxin-sensitive pathway, which is distinct from the SOCC (46). We next asked whether an increase in redox potential may reverse the zinc-mediated block of the SOCC. This is of potential physiological importance, considering the changes in redox potential that are monitored in multiple cell types (47). Following the block of SOCC by Zn\textsuperscript{2+} and Cd\textsuperscript{2+}, application of DTT restored Ca\textsuperscript{2+} permeation (Fig. 4b). Using this experimental paradigm, we found that as little as 60 \textmu M DTT was sufficient to totally eliminate zinc inhibition of the SOCC.

Although Ca\textsuperscript{2+} permeates strongly in the presence of DTT, no apparent increase in Fura-2 fluorescence was observed when Ca\textsuperscript{2+} was replaced with zinc, suggesting that DTT treatment does not render SOCC more permeable to zinc (Fig. 4b). Our results, therefore, suggest that the zinc inhibitory site precedes the cation selectivity region on the SOCC.

To determine whether redox potential will have an effect on SOCC inhibition by lanthanides, we determined their inhibitory potency in the presence and absence of reducing agents. In contrast to the striking effects that reducing conditions had on Zn\textsuperscript{2+} inhibition, both DTT (Fig. 5a) and \textbeta-mercaptoethanol (data not shown) failed to eliminate the inhibition of SOCC by 20 \textmu M La\textsuperscript{3+} or Gd\textsuperscript{3+}. To address the possibility that DTT is acting by chelating the metal ions, thereby affecting SOCC inhibition, we determined the inhibitory effect of 2 \textmu M La\textsuperscript{3+} or 1 mM zinc in the presence of 4 mM DTT. As shown in Fig. 5b, La\textsuperscript{3+} at a minute concentration (2 \textmu M) was still effective in inhibiting SOCC, and the elimination of zinc inhibition by DTT persisted even in the presence of 1 mM Zn\textsuperscript{2+}. Thus, our results indicate that it was the change in the redox potential induced by DTT and \textbeta-mercaptoethanol and not the chelation of zinc that eliminated the inhibition of SOCC. The effect of DTT on zinc inhibition of SOCC suggests that the Zn\textsuperscript{2+} inhibitory site may involve cysteines. Furthermore, the lack of effect on lanthanide inhibition of SOCC following the change in redox potential suggests that the inhibitory sites for Zn\textsuperscript{2+} and lanthanides are distinct.
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In a, ducts were treated with TG (0.2 μM) in calcium-free Ringer’s solution to deplete the intracellular stores. Subsequently, Ca2+ (1.5 mM) was added in the presence or absence of Zn2+ (50 μM). Calcium permeation through the SOCC was only apparent following TG treatment (not shown). In b, the same experimental paradigm was applied using carbachol (CCh, 100 μM), instead of TG, to deplete the intracellular stores. In c, a dose-response analysis of Zn2+ inhibition of SOCC performed on ducts and acini, using the Michaelis-Menten equation, yielded calculated Ki values of 0.48 ± 0.05 and 0.49 ± 0.05 μM, respectively. In d, ducts were perfused with Ca2+-containing Ringer’s solution with carbachol (100 μM). The calcium response was monitored in the presence or absence of 50 μM zinc. In the presence of zinc, the duration of the Ca2+ response was drastically reduced, and the elevated plateau phase was eliminated, indicating that both effects are mediated by zinc inhibition of Ca2+ permeation via the SOCC. The same paradigm was used while applying both Zn2+ (50 μM) and DTT (4 mM). The reducing agent restored the full duration of the Ca2+ response and the elevated plateau phase following carbachol application. As shown, application of DTT did not change the calcium response profile as compared with control, indicating that DTT does not affect SOCC activity.

First, although DTT may also chelate La3+ and Cd2+, under similar reducing conditions, its affinity for these metal ions is significantly lower than for Zn2+. Thus, DTT does not affect SOCC activity. Second, Zn2+ acts as a competitive inhibitor of Ca2+ permeation, indicating that the Zn2+ inhibitory site of SOCC is at, or near, the permeation pathway and is not an allosteric site. In contrast to the LTCC, studies by this laboratory (Fig. 3d) and others (1) indicate that SOCC is insensitive to Zn2+. Zinc blocking of SOCC regulates agonist-induced Ca2+ response of salivary gland ducts. In a, ducts were treated with TG (0.2 μM) in calcium-free Ringer’s solution to deplete the intracellular stores. Subsequently, Ca2+ (1.5 mM) was added in the presence or absence of Zn2+ (50 μM). Calcium permeation through the SOCC was only apparent following TG treatment (not shown). In b, the same experimental paradigm was applied using carbachol (CCh, 100 μM), instead of TG, to deplete the intracellular stores. In c, a dose-response analysis of Zn2+ inhibition of SOCC performed on ducts and acini, using the Michaelis-Menten equation, yielded calculated Ki values of 0.48 ± 0.05 and 0.49 ± 0.05 μM, respectively. In d, ducts were perfused with Ca2+-containing Ringer’s solution with carbachol (100 μM). The calcium response was monitored in the presence or absence of 50 μM zinc. In the presence of zinc, the duration of the Ca2+ response was drastically reduced, and the elevated plateau phase was eliminated, indicating that both effects are mediated by zinc inhibition of Ca2+ permeation via the SOCC. The same paradigm was used while applying both Zn2+ (50 μM) and DTT (4 mM). The reducing agent restored the full duration of the Ca2+ response and the elevated plateau phase following carbachol application. As shown, application of DTT did not change the calcium response profile as compared with control, indicating that DTT does not affect SOCC activity.

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Third, application of zinc at widely varying concentrations (i.e. 10–1000 μM), which would have dramatically changed its free concentration, did not attenuate the capacity of DTT to eliminate Zn2+ inhibition (Figs. 4–5). Also, a range of La3+ concentrations (2–20 μM) in the presence of DTT did not change its effectiveness vis-à-vis SOCC inhibition (Fig. 5). Thus, we suggest that the effect of the reducing agents on zinc inhibition is related to a change in redox and is not mediated by zinc chelation.

Both characterization of Zn2+ binding sites and insertion of an artificial Zn2+ site into the sequence of membrane proteins are powerful approaches available today to those studying their structure-function aspects (31, 52). This may be particularly useful for SOCC since very little is known about domains or residues playing a functional role in ion permeation through this pathway (53). To this end, our results can provide a working model, illustrated in Fig. 7, on the nature of the Zn2+ inhibitory site of SOCC. According to our results, Zn2+ acts as a competitive inhibitor of Ca2+ permeation, indicating that the Zn2+ inhibitory site is at, or near, the permeation pathway and is not an allosteric site. In contrast to the LTCC, studies by this laboratory (Fig. 3b) and others (1) indicate that SOCC is insensitive to Zn2+. Thus, we propose that Zn2+ interacts with an extracellular site, perhaps at a vestibule preceding the cation selectivity domain of SOCC. Zinc is often coordinated in...
its binding site by cysteines, histidines, or both. Our data, supporting a role for cysteines in the Zn$^{2+}$ inhibition site (Figs. 4–5), do not exclude the participation of other residues such as histidines. The properties of the zinc inhibitory site on the SOCC and the CIC chloride channel are similar. On the latter, zinc ions interact with 3 cysteines on the extracellular site of the ion permeation pathway (35). It will be interesting to determine whether there are common structural motives on CIC channel and Trps, related to the zinc inhibitory site. Future studies, combining chemical modification and site-directed mutagenesis of susceptible cysteines, will be required for the identification of the residue coordinating Zn$^{2+}$ to its inhibitory site in the various Trp proteins. Such analysis may be helpful not only in identifying the Zn$^{2+}$ binding site but also in providing a clue to the specific role played by various Trps in SOCC activity, a question that is still hotly debated (54).

The redox-dependent regulation of the SOCC by zinc is a novel functional aspect of this channel. Such regulation may be of particular physiological interest in salivary glands because of the dramatic effects that changes in redox potential trigger there, e.g. after exposure to secretagogues such as isoproterenol (55, 56). This, together with the pivotal role played by sustained calcium rise in regulating ion content and pH of salivary secretions (57, 58), points toward the potential importance of this mode of regulation. Interestingly, although duct and HSG cells express similar Trp proteins, they exhibit altered sensitivity to zinc; the mechanism for this striking difference is not understood (26, 59). A mechanism involving redox potential may provide a plausible explanation.

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FIG. 7. Schematic model for the zinc inhibitory site on SOCC. Zinc interacts with cysteines and may also coordinate with other residues at an extracellular domain along the SOCC permeation pathway, thereby inhibiting Ca$^{2+}$ permeation into the cells.
Inhibitory Mechanism of Store-operated Ca\textsuperscript{2+} Channels by Zinc
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