Ca\textsuperscript{2+} Entry Activated by S-Nitrosylation

RELATIONSHIP TO STORE-OPERATED Ca\textsuperscript{2+} ENTRY*

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The coupling between Ca\textsuperscript{2+} pools and store-operated Ca\textsuperscript{2+} entry channels (SOCs) remains an unresolved question. Recently, we revealed that Ca\textsuperscript{2+} entry could be activated in response to S-nitrosylation and that this process was stimulated by Ca\textsuperscript{2+} pool emptying (Favre, C. J., Ufret-Vincenty, C. A., Stone, M. R., Ma, H-T., and Gill, D. L. (1998) J. Biol. Chem. 273, 30855–30858). In DDTMF-2 smooth muscle cells and DC-3F fibroblasts, Ca\textsuperscript{2+} entry activated by the lipophilic NO donor, GEA3162 (5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatrizolium), or the alkylation, N-ethylmaleimide, was observed to be strongly activated by transient external Ca\textsuperscript{2+} removal, closely resembling activation of SOC activity in the same cells. The nonadditivity of SOC and NO donor-activated Ca\textsuperscript{2+} entry suggested a single entry mechanism. Calycinulin A-induced reorganization of the actin cytoskeleton prevented SOC but had no effect on GEA3162-induced Ca\textsuperscript{2+} entry. However, a single entry mechanism could account for both SOC and NO donor-activated entry if the latter reflected direct modification of the entry channel by S-nitrosylation, bypassing the normal coupling process between channels and pools. Small differences between SOC and GEA3162-activated Ba\textsuperscript{2+} entry and sensitivity to blockade by La\textsuperscript{3+} were observed, and in HEK293 cells SOC activity was observed without a response to thiol modification. It is concluded that in some cells, S-nitrosylation modifies an entry mechanism closely related to SOC and/or part of the regulatory machinery for SOC-mediated Ca\textsuperscript{2+} entry.

Cytosolic Ca\textsuperscript{2+} signals control a vast array of cellular functions ranging from short term responses such as contraction and secretion to longer term regulation of cell growth and proliferation (1). The generation of receptor-induced cytosolic Ca\textsuperscript{2+} signals is complex, involving two closely coupled components: rapid, transient release of Ca\textsuperscript{2+} stored in the endoplasmic reticulum (ER), followed by slowly developing intracellular Ca\textsuperscript{2+} entry (1–5). G protein-coupled receptors and tyrosine kinase receptors, through activation of phospholipase C, generate the second messenger, inositol 1,4,5-trisphosphate. This chemical message diffuses rapidly within the cytosol to interact with inositol 1,4,5-trisphosphate receptors located on the ER, which serve as Ca\textsuperscript{2+} channels to release luminal stored Ca\textsuperscript{2+} and generate the initial Ca\textsuperscript{2+} signal phase (1, 3). The resulting depletion of Ca\textsuperscript{2+} stored within the ER lumen serves as the primary trigger for a message that is returned to the plasma membrane, resulting in the slow activation of "store-operated" Ca\textsuperscript{2+} entry channels (2, 4–6). This second Ca\textsuperscript{2+} entry phase of Ca\textsuperscript{2+} signals serves to mediate longer term cytosolic Ca\textsuperscript{2+} elevations and provides a means to replenish intracellular stores (2, 4). Whereas receptor-induced generation of inositol 1,4,5-trisphosphate and the function of Ca\textsuperscript{2+} release channels to mediate the initial Ca\textsuperscript{2+}-signaling phase is well understood, the mechanism for coupling ER Ca\textsuperscript{2+} store depletion with Ca\textsuperscript{2+} entry remains a crucial but unresolved question (4–6).

Recently, several major channels have been shown to be regulated by thiol nitrosylation, a process becoming recognized as an important nitric oxide (NO)-mediated posttranslational modification affecting control over a diverse array of signaling and regulatory systems (7–12). Such S-nitrosylation-mediated effects are direct and independent of activation of guanylyl cyclase, which is a major target for NO and a frequent mediator of the actions of NO (13, 14). Studies have revealed that nitrosothiol formation underlies the direct modifying action of NO on a number of important plasma membrane and intracellular channels for Ca\textsuperscript{2+} and other ions including the N-methyl-D-aspartate receptor (8), cyclic nucleotide-gated cation channel (15, 16), Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel (17), L-type Ca\textsuperscript{2+} channel (18), and the ryanodine receptor Ca\textsuperscript{2+} release channel (19). For several of these channels, NO donor-induced S-nitrosylation results in channel activation, and this activation is mimicked by alkylation of the same thiol groups (15–19). Because of the reactivity of thiols toward NO, the sphere of influence of NO can be highly restricted and, rather than diffusion-dependent, NO (or an equivalent of the nitrosonium ion, NO\textsuperscript{+}) may be donated and exchanged between neighboring protein thiols by local transnitrosation events (7, 9–11, 15, 16).

We recently utilized a combination of membrane-permeant NO donors and alkylators to probe the role of S-nitrosylation in the process of Ca\textsuperscript{2+} entry and its relationship to Ca\textsuperscript{2+} pool depletion (20). A novel class of lipophilic NO donors, including the oxatriazole-5-imine derivative, GEA3162, activated Ca\textsuperscript{2+} entry independent of the well defined NO target, guanylyl cyclase. Strikingly similar Ca\textsuperscript{2+} entry induced by cell permeant alkylators indicated that this Ca\textsuperscript{2+} entry process was activated through thiol modification. Significantly, Ca\textsuperscript{2+} entry activated by NO donors or alkylators was stimulated by Ca\textsuperscript{2+} pool depletion, which increased the rate and size of the Ca\textsuperscript{2+} response and the sensitivity to thiol modifiers. These results led us to postulate that S-nitrosylation may underlie activation of an

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1 The abbreviations used are: ER, endoplasmic reticulum; NO, nitric oxide; fura-2/AM, fura-2 acetoxymethylester; GEA3162, 5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatrazolium; NEM, N-ethylmaleimide.

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important store-operated Ca\textsuperscript{2+} entry mechanism. Here we have examined the relationship between store-operated Ca\textsuperscript{2+} entry occurring independently of S-nitrosylation and Ca\textsuperscript{2+} entry activated in response to S-nitrosylation.

**EXPERIMENTAL PROCEDURES**

*Culture of Cells—*DDT, MF-2 smooth muscle cells derived from hamster vas deferens were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2.5% calf serum as described previously (21, 22); DC-3F Chinese hamster lung fibroblasts were cultured in α-modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum as described previously (23, 24).

*Measurement of Intracellular Calcium—*Cells grown on coverslips for 1 day were transferred to Hepes-buffered Krebs medium (107 mM NaCl, 6 mM KCl, 1.2 mM MgSO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 11.5 mM glucose, 0.1% bovine serum albumin, 20 mM Hepes-KOH, pH 7.4) and loaded with fura-2/AM (2 μM) for 10 min at 20 °C. Cells were washed, and the dye was allowed to deesterify for a minimum of 15 min at 20 °C. Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (25, 26). Fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; Ca\textsuperscript{2+} measurements are shown as 340/380-nm ratios obtained from a group of 10–12 cells. Details of these Ca\textsuperscript{2+} measurements were recently described for DDT MF-2 (27) and DC-3F cells (24). Resting Ca\textsuperscript{2+} levels in DDT, MF-2 cells were approximately 60–90 nm and 25–50 nm in DC-3F cells; maximal activation by GEA3162 resulted in up to 600 nm Ca\textsuperscript{2+}. All measurements shown are representative of a minimum of three, and in most cases, a much larger number of independent experiments.

*Materials and Miscellaneous Procedures—*GEA3162 was from Alexis Corp., San Diego, CA. 2,5-di-tet-butylhydroquinone and 4-vinylpyridine were from Aldrich. Thapsigargin was from LC Services, Woburn, MA. Fura-2/acetoxymethylester was from Molecular Probes, Eugene, OR. N-Ethylmaleimide (NEM) and all other compounds were from Sigma.

**RESULTS AND DISCUSSION**

Our previous results revealed that NO donors including nitroprusside, nitrite, and the lipophilic donor, GEA3162, were effective in directly inducing Ca\textsuperscript{2+} entry (20). A highly similar entry of Ca\textsuperscript{2+} was induced with alkylators including NEM and 4-vinylpyridine, indicating that the activation of Ca\textsuperscript{2+} entry resulted from thiol modification, either nitrosylation or alkylation (20). The Ca\textsuperscript{2+} entry observed with NO donors or with alkylators, in both cases, was substantially enhanced by emptying Ca\textsuperscript{2+} pools before administration of the activator. Pool emptying increased three parameters of thiol modifier-induced Ca\textsuperscript{2+} entry: the time-dependence of entry, the size of the Ca\textsuperscript{2+} entry response, and the sensitivity to thiol modifier (20). The most prominent of these effects was the time dependence. Thus, in normal cells with filled pools, there was a pronounced lag in the Ca\textsuperscript{2+} entry response to thiol modifiers of at least 1 min; after pool emptying, the Ca\textsuperscript{2+} entry response was extremely rapid, suggesting that pool emptying had allowed the Ca\textsuperscript{2+} entry channel to alter its configuration to expose a thiol group that was important in modifying channel activity (20). The question of whether this putative entry channel was indeed the store-operated Ca\textsuperscript{2+} channel is important to address.

Store-operated Ca\textsuperscript{2+} entry channels display a further important characteristic. In many cells, the entry of Ca\textsuperscript{2+}, activated after pool depletion, becomes deactivated with time, and transient removal and readdition of extracellular Ca\textsuperscript{2+} is a well-described means for reactivating the entry mechanism (24, 28–31). The effect is frequently referred to as the Ca\textsuperscript{2+} “over-shoot” response, since it results in a transiently high reactivation of Ca\textsuperscript{2+} entry, which then deactivates once again with time (24, 28). The results in Fig. 1 reveal that transient removal of external Ca\textsuperscript{2+} has a dramatic enhancing effect on the operation of Ca\textsuperscript{2+} entry activated by GEA3162. Untreated DDT, MF-2 cells exposed briefly to nominally Ca\textsuperscript{2+}-free medium then returned to medium containing normal Ca\textsuperscript{2+} showed no change in cytosolic Ca\textsuperscript{2+} (Fig. 1A). The addition of GEA3162 at 25 μM, a submaximal concentration under normal conditions (20), in the continuous presence of external Ca\textsuperscript{2+} induced a modest rise of Ca\textsuperscript{2+} after a lag of approximately 2 min. If external Ca\textsuperscript{2+} was removed before the addition of 25 μM GEA3162 (Fig. 1B), no significant change in Ca\textsuperscript{2+} occurred for several minutes, confirming the lack of any effect of the NO donor on release of internal Ca\textsuperscript{2+}. However, upon readdition of external Ca\textsuperscript{2+}, a rapid entry of Ca\textsuperscript{2+} occurred, resulting in a considerably larger peak of Ca\textsuperscript{2+} (Fig. 1B) than that observed in the continuous presence of external Ca\textsuperscript{2+} (Fig. 1A). Thus the removal and readdition of Ca\textsuperscript{2+} considerably enhanced the effectiveness of GEA3162. Control experiments revealed that prolonged (10 min) removal of external Ca\textsuperscript{2+} did not cause any release of Ca\textsuperscript{2+} from pools and that following such prolonged external Ca\textsuperscript{2+} removal, no entry of Ca\textsuperscript{2+} was observed upon the readdition of Ca\textsuperscript{2+} in the absence of GEA3162. The stimulatory effect of transient Ca\textsuperscript{2+} removal on the action of GEA3162 was further characterized as shown in Fig. 2. In this experiment external Ca\textsuperscript{2+} was transiently removed after the addition of different GEA3162 concentrations. After adding GEA3162 at 1 μM, a 3-min period of external Ca\textsuperscript{2+} removal resulted in only a very slight entry of Ca\textsuperscript{2+} (Fig. 2A). However, after the addition of 10 μM GEA3162, the transient removal of external Ca\textsuperscript{2+} triggered a much more significant and rapid increase in Ca\textsuperscript{2+} following Ca\textsuperscript{2+} readdition (Fig. 2B). Under normal conditions of external Ca\textsuperscript{2+}, GEA3162 at 10 μM was below its effective threshold and induced almost no Ca\textsuperscript{2+} entry (20). Therefore, the entry of Ca\textsuperscript{2+} observed after the brief removal of external Ca\textsuperscript{2+} represents a real potentiation of the effect of GEA3162. The resultant increase in Ca\textsuperscript{2+} was rapid but transient and began to decline after 1 min. Removal of Ca\textsuperscript{2+} prevented any further entry of Ca\textsuperscript{2+}, and the Ca\textsuperscript{2+} level fell rapidly. After a further 3 min, readdition of Ca\textsuperscript{2+} resulted again in a rapid and transient entry of Ca\textsuperscript{2+}. The entry of Ca\textsuperscript{2+} could be repeatedly reactivated by transient removal of Ca\textsuperscript{2+} (Fig. 2B). Although the peak size of the response to 10 μM GEA3162 after the initial transient Ca\textsuperscript{2+} depletion was smaller, the peaks following subsequent brief periods of Ca\textsuperscript{2+} removal were larger and ap-
proached the maximal size attainable. Thus, external Ca\(^{2+}\) removal followed by the readaddition in the presence of 25 \(\mu\)M GEA3162 (Fig. 2C) resulted in a rapid and maximal activation of Ca\(^{2+}\) entry. Again, the activation rapidly deactivated with time, and cycling of reactivation of Ca\(^{2+}\) entry in response to transient Ca\(^{2+}\) removal could be repeated several times in succession.

This pattern of deactivation and reactivation by transient removal of Ca\(^{2+}\) is highly similar to the operation of store-operated Ca\(^{2+}\) entry channels. As shown in Fig. 3A, after thapsigargin-induced pool emptying in the absence of external Ca\(^{2+}\), readaddition of Ca\(^{2+}\) caused a large increase in cytosolic Ca\(^{2+}\), reflecting a high level of store-operated Ca\(^{2+}\) entry. The entry of Ca\(^{2+}\) rapidly deactivated with time, and subsequent removal of external Ca\(^{2+}\) prevented any further Ca\(^{2+}\) entry. Upon the readaddition of external Ca\(^{2+}\), maximal store-operated Ca\(^{2+}\) entry was restored. This overshoot response pattern classically reflects the operation of store-operated Ca\(^{2+}\) entry and is believed to represent the function of Ca\(^{2+}\)-binding sites, which negatively control store-operated Ca\(^{2+}\) entry (24, 28, 29, 31). According to such a model, as Ca\(^{2+}\) increases in the cytosol, binding of Ca\(^{2+}\) to such regulatory sites inhibits entry; transient external Ca\(^{2+}\) removal prevents Ca\(^{2+}\) entry, allowing cytosolic Ca\(^{2+}\) to fall rapidly as Ca\(^{2+}\) is pumped out of the cell. As a result, Ca\(^{2+}\) dissociates from the regulatory site, permitting the channel to become fully reactivated; upon readaddition of Ca\(^{2+}\), a high level of Ca\(^{2+}\) entry is again observed. As shown in Fig. 3A, this process could be repeated many times. However, it is important to reiterate that the entry observed was completely dependent on pool depletion. Thus, transient removal of Ca\(^{2+}\) at the beginning of the trace before pools were emptied induced no entry of Ca\(^{2+}\). Indeed, in experiments with normal, pool-filled cells, repeated transient removal and readaddition of Ca\(^{2+}\) over a period of 30 min induced no change in cytosolic Ca\(^{2+}\) (not shown). The means of activation, the appearance, and the size of the overshoot responses after pool emptying were all remarkably similar to those described above, activated in response to the NO donor. Yet in the case of the NO donor, pools were not emptied. This point is reinforced from the data shown in Fig. 3B. Thus addition of 15 \(\mu\)M GEA3162 before thapsigargin had no effect on the size of the Ca\(^{2+}\) pool released by subsequent addition of thapsigargin. Moreover, in this experiment the size of overshoots induced after application of both GEA3162 and thapsigargin was not measurably different from that induced by each agent alone. Also, addition of 15 \(\mu\)M GEA3162 to cells after pool depletion with thapsigargin resulted in little significant change in the size of overshoots induced by repeated transient Ca\(^{2+}\) removal (Fig. 3A). Thus, thapsigargin and NO donor induced similar shaped and sized overshoots that did not appear to be additive. This suggested they were activating either the same or a closely coupled entry mechanism.

As described above and earlier (20), thiol modification by either nitrosylation or alkylation activated a very similar entry of Ca\(^{2+}\). We therefore examined whether the stimulatory action of transient Ca\(^{2+}\) removal also activated Ca\(^{2+}\) entry induced by alkylators. Experiments utilized the DC-3F fibroblast cell line in which responses to NO donors and alkylators were similar to DDT, MF-2 cells. As shown in Fig. 4A, the addition of the alkylator, NEM, at 10 \(\mu\)M induced only a slight increase in cytosolic Ca\(^{2+}\) (Fig. 4A). However, if extracellular Ca\(^{2+}\) was transiently removed for just a short (2 min) period, a substantial entry of Ca\(^{2+}\) immediately followed the readaddition of Ca\(^{2+}\) (Fig. 4B). As with DDT, MF-2 cells, transient Ca\(^{2+}\) removal without alkylator or NO donor present had no effect on cytosolic Ca\(^{2+}\) in DC-3F cells (24). The shape and time dependence of the transient Ca\(^{2+}\) removal-induced entry response seen after NEM treatment (Fig. 4B) and after pool emptying (24) were impressively similar in the DC-3F cells. NEM-induced entry of Ca\(^{2+}\) into DDT, MF-2 cells was similarly potentiated by transient removal of Ca\(^{2+}\) (data not shown).
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**Fig. 4.** Transient Ca^{2+} depletion induces a large potentiation of N-ethylmaleimide-activated Ca^{2+} entry in DC-3F cells. A, 10 μM NEM was added under standard external conditions. B, 10 μM NEM addition was followed by replacement of medium with nominally Ca^{2+}-free medium for 2 min as shown by the bar (no Ca^{2+}) followed by return of standard external Ca^{2+} medium. NEM was maintained after the addition.

**Fig. 5.** Calyculin A treatment blocks store-operated Ca^{2+} entry in response to thapsigargin-induced pool emptying in DDT_MF-2 cells but does not block Ca^{2+} entry activated by the NO donor, GEA3162. Bars indicate times of replacement of medium with nominally Ca^{2+}-free medium (no Ca^{2+}). A, 2 μM thapsigargin (TG) and 100 μM GEA3162 (GEA) were added at the times shown. B, cells were initially treated with the phosphatase inhibitor calyculin A (CalyA) at 100 nM for 10 min before the addition of 2 μM thapsigargin and 100 μM GEA3162 at the times indicated by the respective arrows. Each of the agents was maintained in medium after addition throughout successive changes of medium with or without Ca^{2+}.

GEA3162 was activating the same Ca^{2+} entry pathway as pool emptying, then this result would suggest that GEA3162 was able to reverse the deactivation process occurring as a result of Ca^{2+} inhibition.

Clearly, the picture was very different in the presence of 100 nM calyculin A. The experiment shown in Fig. 5B included a 10-min pretreatment with the phosphatase inhibitor that was sufficient to rearrange cortical actin into a tight band closely associated with the plasma membrane (32) and prevent store-operated Ca^{2+} entry. As seen in this experiment, whereas the size of the release peak of Ca^{2+} in response to thapsigargin was unchanged, almost no Ca^{2+} entry followed, and hence the second peak was eliminated. A very slight level of Ca^{2+} entry appeared to remain, as seen by the removal of external Ca^{2+}; however, subsequent readition of Ca^{2+} caused only a small reactivation of Ca^{2+} entry (that is, almost no overshoot), conclusively demonstrating that activation of store-operated entry had been blocked. Whereas the overshoot response was blocked, application of 100 μM GEA3162 gave an immediate and large activation of Ca^{2+} entry. The effects of applying lower GEA3162 concentrations was also unaltered by calyculin A treatment (not shown); thus, although the duration and peak sizes of the responses were smaller than with 100 μM GEA3162, they were not different to the responses observed without calyculin A. Therefore, on the basis of sensitivity to modification by calyculin A, it might be concluded that the entry activated by pool emptying was quite distinct from that activated by S-nitrosylation.

However, this interpretation is not necessarily valid. Thus, we concluded from our recent work that the coupling mechanism between pools and store-operated Ca^{2+} entry channels is through a trafficking event involving physical movement of some component of the ER toward the plasma membrane (32). In this model, the channels may be presynaptic within the plasma membrane, but activation may be elicited by close approach of the ER membrane. Indeed, recent evidence suggests that for one type of Ca^{2+} entry channel, the TRP3 channel, activation may occur via a reversible interaction between the ER-located inositol 1,4,5-trisphosphate receptor and the TRP3 channel itself or a closely related component within the
plasma membrane (33). Although there is some uncertainty about whether pool emptying is necessary for activation of this particular channel (33, 34), there is certainly precedent for believing that the activation of store-operated Ca\(^{2+}\) entry channels may involve interactions between the ER and plasma membrane (32). The action of calyculin A on preventing store-operated Ca\(^{2+}\) entry is believed to result from physical interruption of the coupling process that occurs between the ER and plasma membrane as a result of reorganization of F-actin into a tight cortical layer beneath the plasma membrane (32). Evidence for this action of calyculin A was based on the morphological redistribution of actin observed. In addition, the inhibitory action of calyculin A on store-operated Ca\(^{2+}\) entry by cytochalasin D could be reversed by cytochalasin D. Thus, depolymerization of actin with cytochalasin D after calyculin A caused the cortical actin barrier to be removed and the coupling between ER and the plasma membrane to be reestablished, permitting activation of store-operated Ca\(^{2+}\) entry (32). Since this action of calyculin A is not considered to be a direct effect on the channel itself but rather a prevention of the interaction with ER, it appeared to us that the lack of effect of calyculin A on GEA3162-induced Ca\(^{2+}\) with ER, it appeared to us that the lack of effect of calyculin A on the channel itself but rather a prevention of the interaction between ER and the plasma membrane to be reestablished, permitting activation of store-operated Ca\(^{2+}\) entry (32). Since this action of calyculin A is not considered to be a direct effect on the channel itself but rather a prevention of the interaction with ER, it appeared to us that the lack of effect of calyculin A on GEA3162-induced Ca\(^{2+}\) entry (32). Since this action of calyculin A is not considered to be a direct effect on the channel itself but rather a prevention of the interaction with ER, it appeared to us that the lack of effect of calyculin A on GEA3162-induced Ca\(^{2+}\) entry might reflect a direct action of the NO donor on the entry channel, perhaps circumventing or even mimicking the activation that results from interaction with ER.

Therefore, we sought to determine other parameters defining operation of the entry mechanisms to examine any differences between store-operated and GEA3162-activated entry. Our attention turned toward examination of cation sensitivity and specificity of the entry processes. We investigated the passage of different divalent alkaline-earth cations and of the blocking action of La\(^{3+}\). The passage of Sr\(^{2+}\) and Ba\(^{2+}\) ions could be assessed directly by fura-2 ratio-fluorimetry. Experiments revealed that both store-operated entry and entry activated by GEA3162 allowed passage of Sr\(^{2+}\) ions (Fig. 6). The addition of 25 \(\mu M\) GEA3162 to DDT,MF-2 cells in medium containing 1 mM Sr\(^{2+}\) in place of 1 mM Ca\(^{2+}\) activated entry of Sr\(^{2+}\) with kinetics similar to that of Ca\(^{2+}\) (Fig. 6A). When using cells in which pools had been pool-depleted in the absence of external Ca\(^{2+}\) (Fig. 6B), the addition of Sr\(^{2+}\) caused a rapid entry of Sr\(^{2+}\) through store-operated Ca\(^{2+}\) entry channels. The kinetics of deactivation of Sr\(^{2+}\) entry were not dissimilar from those for Ca\(^{2+}\) entry (compare with Fig. 5A), although there did not appear to be a residual of Sr\(^{2+}\) entry as appeared for Ca\(^{2+}\). This might suggest that a second entry channel was activated by store depletion. Application of 25 \(\mu M\) GEA3162 also clearly induced Sr\(^{2+}\) entry (Fig. 6B), and clearly, the emptying of pools activated both the rate and extent of entry, consistent with the results on Ca\(^{2+}\) entry (20). The slightly slower deactivation of Sr\(^{2+}\) entry may result from less efficient pumping of Sr\(^{2+}\) out of the cell and/or a difference in the relative ability of Sr\(^{2+}\) to effect deactivation of entry channels. We did not observe any significant difference in the Sr\(^{2+}\) concentration dependence of entry activated by pool emptying as opposed to GEA3162 (not shown). However, this result was significantly different from results obtained with Ba\(^{2+}\) (Fig. 7). In this experiment, the concentration dependence of externally applied Ba\(^{2+}\) entry activated by pool emptying was compared with that for Ba\(^{2+}\) entry activated by GEA3162. In cells in which pools were emptied, removal followed by the readoption of Ca\(^{2+}\) caused the familiar overshoot of Ca\(^{2+}\) entry (Fig. 7). Further removal of Ba\(^{2+}\) followed by the addition instead of Ba\(^{2+}\) resulted in Ba\(^{2+}\) entry, which, as for Sr\(^{2+}\), was also detectable by fura-2 ratio-fluorimetry. The entry appeared long-lasting, since Ba\(^{2+}\) is a poor Ca\(^{2+}\) pump substrate (31) and, hence, was not removed from cells by the plasma membrane pump even if the entry mechanism did become activated (compare with Sr\(^{2+}\) and Ca\(^{2+}\) in Figs. 5A and 6B, respectively). Under these conditions, only very slow entry of Ba\(^{2+}\) was observed at 0.1 mM Ba\(^{2+}\), although entry was larger and more rapid with higher levels of Ba\(^{2+}\) (Fig. 7A). If the experiment was undertaken with pool-filled cells in the absence of Ca\(^{2+}\), the addition of 25 \(\mu M\) GEA3162...
activated entry when Ba\(^{2+}\) was subsequently added (Fig. 7B). However, the Ba\(^{2+}\) dependence of entry appeared measurably different. Even now, 0.02 mM Ba\(^{2+}\) resulted in significant entry. From analyses from several experiments using ranges of Ba\(^{2+}\), it appeared that the rate of store-operated Ba\(^{2+}\) entry was half-maximal at approximately 1 mM Ba\(^{2+}\), whereas the rate of GEA3162-dependent Ba\(^{2+}\) entry was activated half-maximally with approximately 0.1 mM Ba\(^{2+}\).

The results with Ba\(^{2+}\) indicated a significant difference in the apparent selectivity for passage of cations activated by store emptying as opposed to GEA3162. In other studies on Ca\(^{2+}\) entry, criteria for defining differences between putative entry channels have rested on the effectiveness of La\(^{3+}\) before transient removal and readdition of Ca\(^{2+}\); during the absence of Ca\(^{2+}\), La\(^{3+}\) at the indicated \(1 \mu M\) concentrations was added (arrow) and maintained after the readdition of Ca\(^{2+}\). Normal pool-filled cells in Ca\(^{2+}\)-free medium were treated with 25 \(\mu M\) GEA3162/GEA; first arrow) followed by the addition of the indicated \(1 \mu M\) concentrations of La\(^{3+}\) (second arrow), which were maintained after subsequent readdition of Ca\(^{2+}\).

**Fig. 8.** La\(^{3+}\) blockade of Ca\(^{2+}\) entry into DDT, MF-2 cells in response to thapsigargin-induced Ca\(^{2+}\) pool emptying (A) or Ca\(^{2+}\) entry activated by the NO donor, GEA3162. Bars indicate times of replacement of medium with nominally Ca\(^{2+}\)-free medium (no Ca\(^{2+}\)). A, Ca\(^{2+}\) pools were emptied by pretreating the cells with Ca\(^{2+}\)-pump blocker, thapsigargin (2 \(\mu M\), for 10 min in the presence of Ca\(^{2+}\) before transient removal and readdition of Ca\(^{2+}\); during the absence of Ca\(^{2+}\), La\(^{3+}\) at the indicated \(1 \mu M\) concentrations was added (arrow) and maintained after the readdition of Ca\(^{2+}\). B, normal pool-filled cells in Ca\(^{2+}\)-free medium were treated with 25 \(\mu M\) GEA3162/GEA; first arrow) followed by the addition of the indicated \(1 \mu M\) concentrations of La\(^{3+}\) (second arrow), which were maintained after subsequent readdition of Ca\(^{2+}\).

Overall, our conclusions concerning the nature and relationship of the two modes of activating Ca\(^{2+}\) entry present an interesting picture. The S-nitrosylation-activated entry is stimulated by both of the two conditions that define the operation of store-activated Ca\(^{2+}\) entry, Ca\(^{2+}\) pool emptying and transient external Ca\(^{2+}\) removal. Moreover, when both mechanisms are simultaneously activated by external Ca\(^{2+}\) addition, their effects can appear nonadditive. Whereas the effects of cytoskeletal reorganization by calyculin A indicate that the mode of activation of the entry mechanisms may be quite distinct, we could reconcile such a difference by considering a possible direct action of S-nitrosylation on the channel, which might circumvent the process of pool-emptying. If this were the case, then the action of pool emptying might help to change the conformation of the channel to increase the availability of a reactive thiol toward nitrosylation or alkylation. In addition, transient Ca\(^{2+}\) removal may also cause reconfiguration of the channel into a more susceptible conformation. However, careful analysis of divergent cation selectivity and La\(^{3+}\) blockade does reveal a significant difference between the two modes of Ca\(^{2+}\) entry. A difference in ion conductivity is difficult to reconcile with the premise that both activities result from activation of a single channel, even though recent evidence for the “slip-mode” operation of Na\(^{+}\) channels (36) provides some precedent for such changes. At this stage, we might conclude that the two entry mechanisms are distinct but related. This conclusion may derive strength from other observations. Thus, we have observed that in human embryonic kidney HEK293 cells, in which store-operated Ca\(^{2+}\) entry can be activated, the entry of Ca\(^{2+}\) is not stimulated by GEA3162 or alkylators.2 This may underscore an impression that has been previously suggested (2, 4, 6) that store-operated Ca\(^{2+}\) entry differs significantly between cells and may represent a family of distinct channel proteins and/or different association with regulatory proteins. Interest in the TRP family of channels as potential members of the store-operated family has revealed at least six different gene products that vary significantly with respect to their ion selectivity and, more importantly, their possible relationship to pool emptying (33, 34, 37, 38). We utilized the HEK293 cell lines stably transfected by Zhu et al. (35) to express the TRP-3 channel protein.2 In these cells as well as the parent and control-transfected cells, the lack of action of either GEA3162 or alkylators indicated that the TRP-3 channel was not a likely target for activation of Ca\(^{2+}\) entry by S-nitrosylation. These results notwithstanding, it is interesting to consider that very significant differences in both the conductance properties and the ability to couple to store depletion were noted by Xu et al. (39) for operation of Drosophila-derived transient receptor potential (TRP) and TRP-like (TRPL) proteins cotransfected into mammalian cells. Thus, these experiments suggested that the coassembly of channel monomers into multimeric channels could confer quite different properties related to the relative makeup of the distinct subunits within the channel assemblies. Thus, it is possible that differences in physiological operation (for example, pool coupling and desensitization by Ca\(^{2+}\)), susceptibility to S-nitrosylation, as well as ion selectivity among different cells may all be related to the relative expression of an extended family of related but distinct channel proteins.

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