**Communication**

**Ca\(^{2+}\) Pool Emptying Stimulates Ca\(^{2+}\) Entry Activated by S-Nitrosylation**

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The entry of Ca\(^{2+}\) following Ca\(^{2+}\) pool release is a major component of Ca\(^{2+}\) signals; yet despite intense study, how “store-operated” entry channels are activated is unresolved. Because S-nitrosylation has become recognized as an important regulatory modification of several key channel proteins, its role in Ca\(^{2+}\) entry was investigated. A novel class of lipophilic NO donors activated Ca\(^{2+}\) entry independent of the well defined NO target, guanylate cyclase. Strikingly similar entry of Ca\(^{2+}\) induced by cell permeant alkylators indicated that this Ca\(^{2+}\) entry process was activated through thiol modification. Significantly, Ca\(^{2+}\) entry activated by either NO donors or alkylators was highly stimulated by Ca\(^{2+}\) pool depletion, which increased both the rate of Ca\(^{2+}\) release and the sensitivity to thiol modifiers. The results indicate that S-nitrosylation underlies activation of an important store-operated Ca\(^{2+}\) entry mechanism.

Ca\(^{2+}\) signals in cells are complex events involving both intracellular Ca\(^{2+}\) pool release and extracellular Ca\(^{2+}\) entry. Emptying of intracellular Ca\(^{2+}\) pools is the major trigger for activation of Ca\(^{2+}\) entry during the generation of receptor-mediated Ca\(^{2+}\) signals (1–3). However, the mechanism by which Ca\(^{2+}\) pool depletion is coupled to activation of “store-operated” Ca\(^{2+}\) entry channels remains an important but unresolved question (1–5). Recently, several major channels have been shown to be regulated by thiol nitrosylation, a process becoming recognized as an important NO-mediated post-translational modification effecting control over a diverse array of signaling and regulatory proteins (6–9). 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 (10, 11). 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\(^{2+}\) and other ions including the N-methyl-d-aspartate receptor (12), cyclic nucleotide-gated cation channel (13, 14), Ca\(^{2+}\)-activated K\(^{+}\) channel (15), l-type Ca\(^{2+}\) channel (16), and most recently, the ryanodine receptor Ca\(^{2+}\) release channel (17). 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 (13–17). Because of the reactivity of thiols toward NO, the sphere of influence of NO can be highly restricted; hence, rather than being diffusion-dependent, NO (or an equivalent of the nitrosonium ion, NO\(^{+}\)) may be donated and exchanged between neighboring protein thiols by local transnitrosation events (6–9, 13, 14). Here, we have utilized a combination of membrane-permeant NO donors and alkylators to probe the role of S-nitrosylation in the process of Ca\(^{2+}\) entry and its relationship to Ca\(^{2+}\) pool depletion.

**EXPERIMENTAL PROCEDURES**

**Intracellular Calcium Measurements**—The DDT, MF-2 hamster smooth muscle and DC-3F Chinese hamster lung fibroblast lines were cultured as described previously (20, 21). Cells grown on coverslips for 1 day were loaded with fura-2/acetoxymethylester as described previously (22, 23). Fluorescence measurements (505 nm emission) are shown as 340/380 nm (excitation) ratios obtained from groups of 10–12 cells. Details of Ca\(^{2+}\) measurements were recently described for DDT, MF-2 (24) and DC-3F cells (21). Resting Ca\(^{2+}\) levels were approximately 60–90 nM in DDT, MF-2 cells and 25–50 nM in DC-3F cells; maximal activation by GEA3162 resulted in up to 600 nM Ca\(^{2+}\). Measurements shown are representative of at least three and, in most cases a larger number, of independent experiments.

**Materials and Miscellaneous Procedures**—GEA3162, GEA5024, and LY83583 were from Alexis Corp. (San Diego, CA). 2,5-Di-tert-butylhydroquinone (DBHQ), and 4-vinylpyridine (4-VP), were from Aldrich. Thapsigargin was from LC Services (Woburn, MA). Fura-2/acetoxymethylester was from Molecular Probes (Eugene, OR). 8-Br-cGMP was from Calbiochem (San Diego, CA). N-Ethylmaleimide (NEM) and all other compounds were from Sigma. Measurements of cGMP were made using the standard protocol of the NEN Life Science Products RIA kit.

**RESULTS AND DISCUSSION**

The action of different NO-donating molecules on Ca\(^{2+}\) entry was examined using intact fura-2-loaded cells (24) in which the coupling process between intracellular Ca\(^{2+}\) pools and Ca\(^{2+}\) entry channels itself remains functionally intact. Cells selected for study included the DDT, MF-2 smooth muscle and DC-3F lung fibroblast cell lines, which have been extensively used to study function and distribution of Ca\(^{2+}\) pools (18, 19, 22–25) and their relationship to Ca\(^{2+}\) entry (5, 20, 21, 26). A profound, dose-dependent increase in cytosolic Ca\(^{2+}\) was induced by application of the NO-donating oxatriazole derivative, GEA3162, as shown in Fig. 1A. An unusually lipophilic NO-releasing agent, GEA3162, was recently characterized as a highly effective NO donor *in vitro* and in mediating the actions of NO on intact cells (27, 28). Although lipophilic, this mesoionic 3-aryl-substituted oxatriazole-5-imine derivative is sufficiently amphipathic that it may preferentially localize to donate NO in close proximity to the membrane surface. The increase in Ca\(^{2+}\) after application of GEA3162 was preceded by a lag, which

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1 The abbreviations used are: GEA3162, (5-amino-3-(3, 4-dichlorophenyl)-1,2,3,4-oxatriazole-5-imine); GEA5024 (5-amino-3-chloro-2-methyl-4-oxatriazole-5-imine); DBHQ, 2,5-di-tert-butylhydroquinone; 8-Br-cGMP, 8-bromo-cyclic monophosphate.
Ca\(^{2+}\) Entry Activated by S-Nitrosylation

![Diagram](image1.png)

**FIG. 1.** Entry of Ca\(^{2+}\) into DDT\(\cdot\)MF-2 cells activated by the NO donor GEA3162. Cytosolic Ca\(^{2+}\) was measured in fura-2-loaded DDT\(\cdot\)MF-2 cells attached to glass coverslips as described (11). A, the NO donor GEA3162 was added at 10, 25, 50, or 100 \(\mu M\) at the time indicated by the arrow. B, 100 \(\mu M\) GEA3162 was added as indicated by the arrow shortly after replacing the bathing medium with nominally Ca\(^{2+}\)-free medium; Ca\(^{2+}\)-free conditions were maintained as shown by the bar, after which medium was replaced with standard (1 \(mM\)) Ca\(^{2+}\)-containing medium.

Ca\(^{2+}\) entry itself was dose-dependent and of at least 1 min in duration. The GEA3162-induced rise in Ca\(^{2+}\) was clearly due to entry; in the absence of extracellular Ca\(^{2+}\), 100 \(\mu M\) GEA3162 induced no change in cytosolic Ca\(^{2+}\) (Fig. 1B), indicating that no release from pools occurred. An immediate and large increase in cytosolic Ca\(^{2+}\) was observed upon Ca\(^{2+}\) readdition, indicating that entry had become fully activated. The action of GEA3162 was not attributable to any change in Ca\(^{2+}\) efflux because experiments (not shown) revealed no effect on the ability of the plasma membrane Ca\(^{2+}\) pump to pump down Ca\(^{2+}\) in the cells. As seen in Fig. 1 (A and B), the GEA3162-induced Ca\(^{2+}\) entry mechanism became deactivated with time; after reaching a maximum within a few minutes, the entry of Ca\(^{2+}\) always decreased. In other experiments, reapplication of 100 \(\mu M\) GEA3162 after deactivation caused no further increase in Ca\(^{2+}\); removal of GEA3162 for 5 min and subsequent readdition also did not cause reactivation of the entry process. Other structurally diverse NO donors activated similar Ca\(^{2+}\) entry; application of sodium nitroprusside (SNP) or sodium nitrite (NO\(_2^-\)) each induced increases in cytosolic Ca\(^{2+}\) (Fig. 2, A and C). In both cases, relatively high levels of the donors were required (likely due to lower efficiency at physiological pH), and the rise in Ca\(^{2+}\) was smaller and more variable than with GEA3162 but again occurred after a significant lag period. As with GEA3162, no significant changes in Ca\(^{2+}\) were observed with either SNP or NO\(_2^-\) in the absence of external Ca\(^{2+}\); however, Ca\(^{2+}\) entry again commenced immediately upon readdition of external Ca\(^{2+}\) (Fig. 2, B and D).

Crucial to investigate was the relationship between this NO donor-induced entry process and the operation of intracellular Ca\(^{2+}\) pools. The results shown in Fig. 3 reveal that pool emptying has a major stimulatory action on the Ca\(^{2+}\) entry pathway. Pools were emptied with either of two distinct intracellular Ca\(^{2+}\) pump blockers, thapsigargin (29) and DBHQ (30). As shown in Fig. 3A, 10 \(\mu M\) DBHQ caused a rapid release of pool Ca\(^{2+}\) followed by a later rise of Ca\(^{2+}\) representing store-operated Ca\(^{2+}\) entry (26). Upon application of GEA3162 there was a large and almost instantaneous rise in cytosolic Ca\(^{2+}\). Thus, pool emptying had completely eliminated the lag seen with normal pool-filled cells (Fig. 1A). The effect of pool emptying was even more profound at lower GEA3162 levels (Fig. 3, B and C). 10 \(\mu M\) GEA3162 had no effect on normal cells (Fig. 1A) but was able to induce a substantial and rapid effect after pool emptying (Fig. 3C). At 25 \(\mu M\) (Fig. 3B) the long (>4 min) delay in onset of Ca\(^{2+}\) entry was almost completely eliminated after pool emptying. Emptying of pools with either thapsigargin or the ionophore, ionomycin, gave identical stimulation of the GEA3162-induced influx. The enhancement of NO donor-induced Ca\(^{2+}\) entry after pool emptying was not due to increased cytosolic Ca\(^{2+}\); at longer times following pool emptying with DBHQ or thapsigargin (up to 3 h) at which time cytosolic Ca\(^{2+}\) had returned to a level indistinguishable from basal levels (yet pools remained completely empty), the sensitivity to and rapidity of action of GEA3162 were exactly as observed upon addition immediately following pool emptying. The potentiation of the effect of GEA by pool emptying was not a reflection of the inability of the intracellular Ca\(^{2+}\) pumps to buffer Ca\(^{2+}\) in the cytosol. Thus, as shown in Fig. 3A (inset), GEA3162-induced entry of Mn\(^{2+}\), monitored by quenching of fura-2 excited at its isosbestic wavelength, 360 nm, revealed identical kinetics and stimulation by pool emptying as seen for changes in cytosolic Ca\(^{2+}\) measured by ratio fluorimetry. Mn\(^{2+}\) is not a substrate for Ca\(^{2+}\) pumps and hence reliably reports influx without being pumped into organelles or out of the cell. When 100 \(\mu M\) GEA3162 was added in the presence of 1 \(mM\) Mn\(^{2+}\), a significant entry of Mn\(^{2+}\) occurred. However, the onset of Mn\(^{2+}\) entry was slow to develop, and it took almost 90 s before maximal entry was occurring. After the pools had been emptied with 1 \(\mu M\) thapsigargin, 100 \(\mu M\) GEA induced an immediate entry of Mn\(^{2+}\), which re-
mained at this rate for approximately 45 s before declining. Under this condition the contribution of endogenous store-operated entry without GEA was almost negligible. Thus, the kinetics of GEA-dependent Mn$^{2+}$ influx were almost identical to the kinetics of Ca$^{2+}$ entry induced by 100 $\mu$M GEA as shown in Figs. 1A and 3A. Experiments revealed almost identical NO donor-induced Ca$^{2+}$ entry in the unrelated DC-3F fibroblast cell line, which again was highly stimulated by the emptying of Ca$^{2+}$ pools. These results indicate operation of an important and potentially widespread NO donor-induced Ca$^{2+}$ entry mechanism that undergoes striking stimulation by pool emptying. Entry is activated at $\mu$M NO donor concentrations that may correspond to NO levels in the physiological nm range (27, 28).

A major target for NO is the heme group of the guanylyl cyclase enzyme, and many effects of NO are mediated through the ensuing increased cGMP levels (10, 11). However, no changes in Ca$^{2+}$ entry could be observed with application of 8-Br-cGMP over a broad range (10 $\mu$M to 1 mM). 8-Br-cGMP also did not modify NO donor-induced Ca$^{2+}$ influx. Additionally, the guanylyl cyclase inhibitor, LY83583 (31), had no effect on NO donor-induced Ca$^{2+}$ entry. Measurements of cGMP did not reveal any significant changes in cGMP levels associated with Ca$^{2+}$ entry activated by GEA3162. This latter result is significant in indicating that global NO elevation within the cells was not occurring and that the NO-donating activity of GEA3162 may be spatially restricted as a result of the lipophilic character of the molecule. Earlier studies suggested that NO-induced cGMP changes might mediate store-operated Ca$^{2+}$ entry and that pool emptying could activate synthesis of NO (32, 33). Subsequent work has suggested that such an effect may occur in certain cell types and that increased cGMP may be dependent on, rather than the cause of, increased Ca$^{2+}$ levels (34–36). In contrast, the action of NO donors on Ca$^{2+}$ entry described here appears to be entirely independent of cGMP, and instead may reflect an important direct action of NO. Recently, much attention has focused on S-nitrosylation events as major direct protein-modifying regulatory responses induced by NO that are independent of changes in cGMP (6–9). Indeed, as described above, several major channels for Ca$^{2+}$ and other ions are revealed to be activated by S-nitrosylation (13–17). In the present studies, the lack of involvement of cGMP in mediating the action of NO was consistent with a direct S-nitrosylation event mediating Ca$^{2+}$ entry but certainly not proof. The role of thiol modification could only be ascertained by comparing the actions of known sulphydryl-modifying reagents.

The results shown in Fig. 4 reveal that the actions of two quite different membrane-permeant alkylating agents, 4-VP and N-ethylmaleimide (NEM), were impressively similar to the effects of NO donors. Added to normal cells, 1 mM 4-VP induced a modest increase in cytosolic Ca$^{2+}$ but only after a delay of approximately 2 min (Fig. 4A). After pool emptying with the pump blocker, DBHQ, the action of 4-VP was greatly stimulated, inducing a rapid, large, and transient increase in Ca$^{2+}$ almost identical to the NO donor-induced response. Again, in the absence of extracellular Ca$^{2+}$, even at 10 mM, 4-VP induced no release of Ca$^{2+}$, but immediately upon Ca$^{2+}$ readdition, the increased level of cytosolic Ca$^{2+}$ reflected a large, transient entry of extracellular Ca$^{2+}$ (Fig. 4C). Significantly, following the complete response to 4-VP, the effect of 100 $\mu$M GEA3162 was entirely blocked (Fig. 4C), indicating that the alkylating agent and NO donor were activating the same Ca$^{2+}$ entry mechanism. Reversed addition of the agents (GEA3162 followed by 4-VP) resulted in blockade of the action of 4-VP. If submaximally effective 4-VP concentrations were used, subsequently added GEA3162 induced an effect that corresponded inversely in size with that induced by 4-VP. From these results it is concluded that there is a stoichiometric activation of a finite number of entry channels by either NO donors or alkylators and that pool emptying profoundly stimulates the same mechanism of Ca$^{2+}$ entry induced by either type of agent. The more powerful alkylator, NEM, at 10 $\mu$M induced effects that were very similar to 4-VP, activating a slight increase in Ca$^{2+}$ alone that was greatly stimulated by pool emptying, in this case with thapsigargin (Fig. 4B). Concentrations of NEM above 10 $\mu$M could not be used because they induced nonspecific modification of the Ca$^{2+}$ handling machinery of cells (especially Ca$^{2+}$ pool release) not seen with 4-VP. As with 4-VP the action of NEM was clearly on Ca$^{2+}$ entry and was again able to completely prevent the action of subsequently added GEA (Fig. 4D). The competition between the actions of either of the two alkylators and GEA3162 is interesting. 4-VP and NEM are both membrane permeant. Of many NO donors tested, GEA3162 and the close structural analogue, GEA5024 (27), were most effective in activating Ca$^{2+}$ entry. As mentioned above, these compounds differ from other NO donors in being lipophilic enough to penetrate the membrane; yet by virtue of weak charge on the oxatriazole ring, they may be sufficiently amphiphilic to selectively donate NO at the surface of the membrane in the vicinity of reactive thiols of the entry channel or an associated protein.

The results presented here reveal a novel and significant regulatory mechanism involved in the coupling of pool emptying to Ca$^{2+}$ entry. Nitrosylation of thiols is becoming recognized as a widespread post-translational protein modification that undergoes striking stimulation by pool emptying. Entry is activated at $\mu$M NO donor concentrations that may correspond to NO levels in the physiological nm range (27, 28).

![Ca$^{2+}$ Entry Activated by S-Nitrosylation](image-url)
controlling the activity of a spectrum of major regulatory proteins (6–9). The data indicate that Ca\textsuperscript{2+} entry is activated as a consequence of direct modification of one or more thiols either on the channel itself or a protein involved in its coupling to pool emptying. Importantly, activation via thiol nitrosylation provides a strong analogy with at least three other major Ca\textsuperscript{2+} channels, the ryanodine-sensitive Ca\textsuperscript{2+} release channel (17), the \textit{l}-type Ca\textsuperscript{2+} channel (16), and the cyclic nucleotide-gated channel (13, 14). In all cases, increased channel activity induced by NO-donors results from S-nitrosylation, and this stimulatory action is mimicked by modification of the presumed same thiol group (or groups) by alkylating agents. Whereas nitrosylation of the three other Ca\textsuperscript{2+} channels is of uncertain physiological role, in the present study it appears that the major physiological activating condition, namely emptying of pools, facilitates an increase in the susceptibility of the channel to activation by thiol modification. Such modification does not necessarily require a generalized increase in NO levels within the cytosol and indeed may reflect a localized transnitrosation event from a nearby donor nitrosothiol (7, 8); this does not necessarily require a generalized increase in NO levels.

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