

Ca²⁺ Entry Mediated by Store Depletion, S-Nitrosylation, and TRP3 Channels

COMPARISON OF COUPLING AND FUNCTION*

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The mechanism for coupling between Ca²⁺ stores and store-operated channels (SOCs) is an important but unresolved question. SOC-mediated Ca²⁺ entry is complex and may reflect more than one type of channel and coupling mechanism. To assess such possible divergence the function and coupling of SOCs was compared with two other distinct yet related Ca²⁺ entry mechanisms. SOC coupling in DDT₁MF-2 smooth muscle cells was prevented by the permeant inositol 1,4,5-trisphosphate (InsP₃) receptor blockers, 2-aminoethoxydiphenyl borate (2-APB) and xestospongins C. In contrast, Ca²⁺ entry induced by S-nitrosylation and potentiated by store depletion (Ma, H.-T., Favre, C. J., Patterson, R. L., Stone, M. R., and Gill, D. L. (1999) *J. Biol. Chem.* 274, 35318–35324) was unaffected by 2-APB, suggesting that this entry mechanism is independent of InsP₃ receptors. The cycloalkyl lactamamide, MDL-12,330A (MDL), prevented SOC activation (IC₅₀ 10 μM) and similarly completely blocked S-nitrosylation-mediated Ca²⁺ entry. Ca²⁺ entry mediated by the TRP3 channel stably expressed in HEK293 cells was activated by phospholipase C-coupled receptors but independent of Ca²⁺ store depletion (Ma, H.-T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) *Science* 287, 1647–1651). Receptor-induced TRP3 activation was 2-APB-sensitive and fully blocked by MDL. Direct stimulation of TRP3 channels by the permeant diacylglycerol derivative, 1-oleoyl-2-acetyl-*sn*-glycerol, was not blocked by 2-APB, but was again prevented by MDL. The results indicate that although the activation and coupling processes for each of the three entry mechanisms are distinct, sensitivity to MDL is a feature shared by all three mechanisms, suggesting there may be a common structural feature in the channels themselves or an associated regulatory component.

Ca²⁺ signals control many cellular functions ranging from short term responses such as contraction and secretion to long term regulation of cell growth and proliferation (1). Receptor-induced cytosolic Ca²⁺ signals are complex involving two

closely coupled components: rapid, transient release of Ca²⁺ stored in the endoplasmic reticulum (ER),¹ followed by slowly developing extracellular Ca²⁺ 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 (InsP₃), which diffuses rapidly within the cytosol to interact with InsP₃ receptors on the ER that serve as Ca²⁺ channels to release luminal stored Ca²⁺ and generate the initial Ca²⁺ signal phase (1, 3). The resulting depletion of Ca²⁺ stored within the ER lumen serves as the primary trigger for a message, which is returned to the plasma membrane resulting in the slow activation of “store-operated” Ca²⁺ entry channels (2, 4–6). This second Ca²⁺ entry phase of Ca²⁺ signals serves to mediate longer term cytosolic Ca²⁺ elevations and provides a means to replenish intracellular stores (2, 4). Whereas receptor-induced generation of InsP₃ and the function of Ca²⁺ release channels to mediate the initial Ca²⁺ signaling phase is well understood, the mechanism for coupling ER Ca²⁺ store depletion with Ca²⁺ entry remains a crucial but unresolved question (4–6).

Direct coupling between ER and plasma membrane has been hypothesized (7, 8), and evidence indicates that physical docking of ER with the PM is involved in SOC activation (9–12). The mammalian TRP family of receptor-operated ion channels appear to share some operational parameters with SOCs (13, 14). Kiselyov *et al.* (15, 16) provided evidence that activation of the human TRP3 channel can result from an interaction with InsP₃ receptors. Indeed, it appears that activation and maintenance of endogenous store-operated channels requires the InsP₃ receptor (17). However, whereas members of the TRP family of proteins appear widely expressed (18, 19), their involvement in physiological store-operated Ca²⁺ entry remains uncertain (17, 20).

One complexity that hinders a unified understanding of store-operated Ca²⁺ entry is the heterogeneous nature of the process among different cell types. Although the Ca²⁺-selective current, I_{CRAC}, is operational in some cells, a number of distinct store-operated currents of varying divalent cation selectivity have been described (4, 5). Also, significant differences in the activation and deactivation of store-operated Ca²⁺ entry have been observed among different cells (4, 21–24). In recent work (23, 24), we characterized a Ca²⁺ entry process activated by

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¹ The abbreviations used are: ER, endoplasmic reticulum; InsP₃, inositol 1,4,5-trisphosphate; NO, nitric oxide; fura-2/AM, fura-2 acetoxy-methyl ester; GEA3162, (5-amino-3-(3,4-dichlorophenyl)1,2,3,4-oxatriazolium); MDL-12,330A, *cis-N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine; 2-APB, 2-aminoethoxydiphenyl borate; DDA, 2',5'-dideoxyadenosine; PLC, phospholipase C; DAG, diacylglycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; SKF96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole; TG, thapsigargin.

S-nitrosylation that can be stimulated by depletion of stores. In this report, we have compared the function and coupling of store-operated Ca^{2+} entry with operation of TRP3 channels and the *S*-nitrosylation-activated entry mechanism. The results indicate that whereas clear differences exist in the activation and coupling of these different entry mechanisms, there may nevertheless be underlying structural and functional similarities.

EXPERIMENTAL PROCEDURES

Culture of Cells—DDT₁MF-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 (25, 26); 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 (27, 28). The T3-65 clonal line of HEK293 cells were cultured as described recently (17).

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_4 , 1 mM CaCl_2 , 1.2 mM KH_2PO_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 dye 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 (29, 30). Fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; Ca^{2+} measurements are shown as 340/380 nm ratios obtained from a groups of 10–12 cells. Details of these Ca^{2+} measurements were described for DDT₁MF-2 and (31) DC-3F cells (28) and the T3-65 clone of HEK293 cells (17). Resting Ca^{2+} levels in DDT₁MF-2 cells were approximately 60–90 nM, 25–50 nM in DC-3F cells, and 50–100 nM in HEK293 cells. All measurements shown are representative of a minimum of three, and in most cases, a larger number of independent experiments.

Materials and Miscellaneous Procedures—GEA3162 was from Alexis Corporation, San Diego, CA. Thapsigargin (TG) was from LC Services, Woburn, MA. Fura-2/acetoxymethyl ester was from Molecular Probes, Eugene, OR. Xestospongin C was from I. Pessah, University of California, Davis, CA, and 2-APB was from K. Mikoshiba, University of Tokyo. MDL-12,330A was from Calbiochem. OAG and other compounds were from Sigma.

RESULTS AND DISCUSSION

We recently revealed that the activation of store-operated Ca^{2+} channels in the plasma membrane likely requires a physical interaction between the plasma membrane and endoplasmic reticulum membrane (9). In addition, evidence indicates that the InsP_3 receptor is an essential component mediating the coupling between store emptying and the activation of store-operated channels (17). However, the activation of store-operated entry has proven an elusive process and the functional properties of store-operated channels can differ significantly between cell types. We determined recently that in some but not all cells, Ca^{2+} entry can be directly activated through a process mediated by *S*-nitrosylation (23). Since store depletion potentiates this process (23, 24), it was important to assess further its relationship to store-operated Ca^{2+} entry.

A useful tool in elucidating the coupling mechanism for SOC activation has been the cell-permeant InsP_3 receptor blocker, 2-aminoethoxydiphenyl borate (2-APB) (17, 32, 33). In HEK293 cells 2-APB not only blocks receptor-induced Ca^{2+} release from stores but also blocks store-operated Ca^{2+} entry activated in response to depletion of pools with either receptor agonists or sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump inhibitors. In addition, 2-APB blocks receptor-induced activation of TRP3 channels in HEK293 cells stably transfected to express TRP3 channels (17). To investigate the role of InsP_3 receptors in *S*-nitrosylation-mediated Ca^{2+} entry, experiments were first undertaken to assess the action of 2-APB on Ca^{2+} regulation in the DDT₁MF-2 cell line in which there is a large Ca^{2+} entry response to *S*-nitrosylating conditions (23, 24). The action of 2-APB on store-operated Ca^{2+} entry in fura-2-loaded

DDT₁MF-2 cells is revealed in Fig. 1. Application of 1 μM thapsigargin induced a rapid release of Ca^{2+} from stores observed as a sharp rise in cytosolic Ca^{2+} , followed by a second more slowly developing peak of cytosolic Ca^{2+} (Fig. 1A). The latter peak represents Ca^{2+} entry and was eliminated when the medium was replaced with nominally Ca^{2+} -free external medium (Fig. 1B). Upon readdition of external Ca^{2+} , entry of Ca^{2+} was transiently very large, representing the familiar "overshoot" response for store-operated Ca^{2+} channels (22, 28). If 75 μM 2-APB was added together with thapsigargin, it had little effect on the Ca^{2+} release component but completely blocked the Ca^{2+} entry component of the cytosolic Ca^{2+} signal response to thapsigargin (Fig. 1C). When 2-APB was added during the entry phase, it rapidly blocked entry (Fig. 1D). 2-APB is an effective cell-permeant inhibitor of the InsP_3 receptor (17, 32, 33), and the blockade of store-operated Ca^{2+} entry caused by 2-APB appears to reflect the involvement of the InsP_3 receptor in mediating and maintaining store-operated Ca^{2+} entry in DDT₁MF-2 cells as in HEK293 cells (17). Another InsP_3 receptor blocker, the natural product, xestospongin C, also blocks InsP_3 receptor function (34) and similarly blocked Ca^{2+} entry in DDT₁MF-2 cells (Fig. 1E). Although xestospongin C completely blocked entry, its action was much slower than 2-APB and required 20 min of treatment for a full effect. Both xestospongin C and 2-APB appear to have a similar mode of action. Evidence indicates that both molecules block the InsP_3 receptor but do not interact directly with the InsP_3 binding site (32, 34). Certainly, the similarity of action of the two molecules on Ca^{2+} entry provides good evidence that the action of both agents is related to their interaction with the InsP_3 receptor. However, the similarity of action of two molecules with apparently rather different structures was curious (see Fig. 1). We undertook mass spectral analysis of 2-APB and determined that the predominant species of 2-APB under the conditions used appears to be a dimeric structure of molecular mass 450 Da, most likely involving the coordinate covalent bond shown in Fig. 1.² This is a significantly more hydrophobic species than the monomer and likely accounts for the extremely rapid action of 2-APB in blocking InsP_3 receptors (17). Also, the dimeric form of 2-APB does share some distant structural similarity to the xestospongin C molecule and may explain the similarity in their mode of action.

The question of the relationship between *S*-nitrosylation-induced Ca^{2+} entry and store-operated Ca^{2+} entry was investigated using 2-APB. The typical operation of Ca^{2+} entry activated by these two processes is shown in Fig. 2A. Release and entry in response to thapsigargin application are shown, followed by induction of a Ca^{2+} overshoot by subsequent transient Ca^{2+} removal. The overshoot of Ca^{2+} entry rapidly diminished due to desensitization of the channel (9, 24). However, application of the lipophilic NO donor, GEA3162, induced a rapid and large further increase in Ca^{2+} . This response is typical of the action of a number of different NO donors and alkylators (23) and is believed to reflect a Ca^{2+} entry mechanism activated through *S*-nitrosylation (23, 24). The Ca^{2+} entry response to GEA3162 is considerably potentiated by store depletion (23) and also by transient removal and readdition of extracellular Ca^{2+} (24). As seen in Fig. 2B, a large and rapid entry of Ca^{2+} occurs upon removal and readdition of Ca^{2+} in the presence of GEA3162 even though stores had not been emptied. The stimulation of NO donor-induced Ca^{2+} entry by both pool emptying and transient Ca^{2+} removal has suggested an intriguing similarity with store-operated Ca^{2+} channels (23, 24). However, there is an important difference between the two

² D. L. Gill and H.-Y. Kim, unpublished observations.

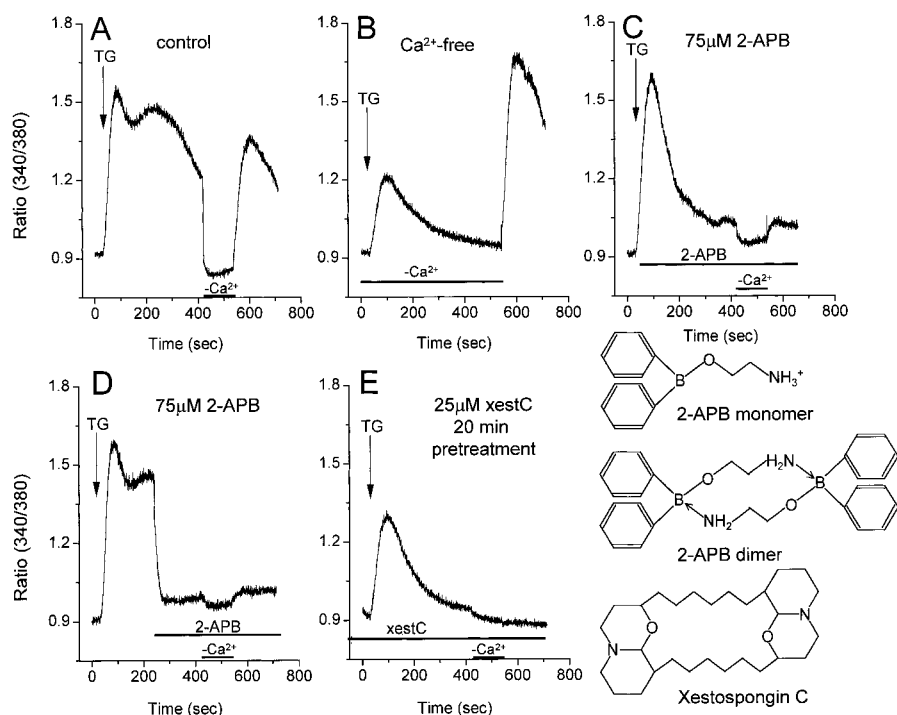


FIG. 1. Store-operated Ca^{2+} entry into DDT₁MF-2 cells is inhibited by 2-APB and xestospongion C. Cytosolic Ca^{2+} was measured in fura-2-loaded DDT₁MF-2 cells as described under "Experimental Procedures." Standard conditions included 1 mM Ca^{2+} in the external medium; medium was replaced with nominally Ca^{2+} -free medium ($-\text{Ca}^{2+}$) for the times shown (bars). *A*, Ca^{2+} pools were depleted by adding the Ca^{2+} pump blocker, thapsigargin (TG) at 1 μM (arrow) followed by transient Ca^{2+} removal. *B*, 1 μM TG was added (arrow) in the absence of Ca^{2+} , followed by the readdition of standard Ca^{2+} . *C*, 1 μM TG (arrow) was added together with 75 μM 2-APB (bar) maintained through Ca^{2+} removal and readdition. *D*, Ca^{2+} pools were released with 1 μM TG, and 75 μM 2-APB was added later (bar). *E*, cells were pretreated for 20 min with 25 μM xestospongion C (xestC) in the presence of Ca^{2+} followed by 1 μM TG addition (arrow) and removal/readdition of Ca^{2+} . Inset, three structures are presented: the monomer form of 2-APB (upper), the spectrally determined dimerized form of 2-APB (middle), and xestospongion C (lower).

entry mechanisms. Thus, while the InsP_3 receptor antagonist, 2-APB, completely blocks store-operated entry, it has little effect on the action of GEA3162 (Fig. 2C). In this experiment, 2-APB blocked the Ca^{2+} entry in response to store emptying with thapsigargin and the subsequent overshoot of Ca^{2+} entry, however, application of GEA3162 still gave a rapid and large Ca^{2+} entry response in the continued presence of 2-APB (Fig. 2C). Moreover, 2-APB did not diminish the GEA3162-dependent entry of Ca^{2+} following removal and readdition of Ca^{2+} (Fig. 2D).

These results suggested a fundamental difference between the function of SOCs and the NO donor-induced entry mechanism. Activation of SOCs appears to require the InsP_3 receptor as a coupling intermediary. In contrast, the action of GEA3162 might be consistent with the NO donor having a direct effect on a channel in the membrane. The results are also consistent with the effects of calyculin A-induced rearrangement of cortical actin, which prevents SOC activation by separating ER and plasma membrane (9) but does not prevent the action of GEA3162 (24). We considered it important to assess whether GEA3162 works directly to modify a channel or whether other intermediaries or second messengers might be involved. Our previous work indicated that the NO donor was functioning independently of cyclic GMP (23). In evaluating the possible involvement of cyclic AMP we utilized a number of different adenylyl cyclase modifiers including the cycloalkyl lactamamide, MDL-12330A, which has been utilized widely as an irreversible adenylyl cyclase inhibitor (35–37). Surprisingly, as shown in Fig. 3, this agent was effective in blocking store-operated Ca^{2+} entry in a number of different cell types. In DDT₁MF-2 cells, 100 μM MDL almost completely blocked both the entry phase following thapsigargin treatment and the subsequent overshoot response (Fig. 3, A–C). Very similar results

were obtained in the DC-3F fibroblast line (Fig. 3, D–F) and in HEK293 cells (not shown). In the A7r5 smooth muscle line in which entry of Ca^{2+} is observed as a larger and more sustained increase in cytosolic Ca^{2+} following store emptying, the action of MDL was again to completely block Ca^{2+} entry (Fig. 3, G–I). In each cell line, the action of MDL appeared to be specifically on Ca^{2+} entry with little effect on basal Ca^{2+} levels or the size of releasable pools.

To evaluate the effect of MDL on store-operated Ca^{2+} entry we wished to know whether its actions were related to adenylyl cyclase activity. We therefore assessed the effects of another permeant adenylyl cyclase inhibitor, 2',5'-dideoxyadenosine (DDA). At 100 μM , DDA had no significant effect on store-operated Ca^{2+} entry (Fig. 4A). In other experiments (not shown), addition of the permeant cyclic AMP analogues, 8-bromo-cyclic AMP or 8-CPT-cyclic AMP, at concentrations up to 1 mM, either alone or in combination with MDL, had no significant effect on Ca^{2+} entry. Also, the adenylyl cyclase activator, forskolin, alone or in combination with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, either with or without MDL, did not influence Ca^{2+} entry. The effects of MDL on adenylyl cyclase activity are reported to be irreversible (35). However, experiments revealed that the blocking action of MDL on Ca^{2+} entry could be readily reversed. When cells were treated with 100 μM MDL for 5 min followed by a wash in MDL-free medium for 10 min, store-operated Ca^{2+} entry returned (data not shown). After blocking Ca^{2+} entry induced by thapsigargin with MDL, Ca^{2+} entry returned within 3 min of washing away MDL (Fig. 4B). In this experiment, 100 μM MDL was added acutely with thapsigargin, and the entry phase of the Ca^{2+} response was eliminated. Both MDL and Ca^{2+} were removed for a 3-min period; upon Ca^{2+} readdition, a normal overshoot response was obtained. In combination, these results

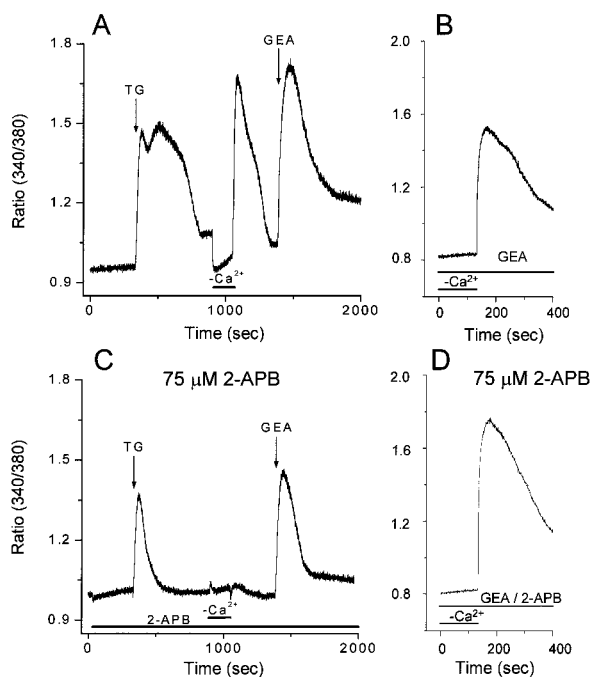


FIG. 2. Store-operated Ca^{2+} entry is blocked by 2-APB, while Ca^{2+} entry induced by the NO donor, GEA3162, is insensitive to 2-APB. Experiments utilized DDT₁MF-2 cells as described in the legend to Fig. 1. Bars indicate times of replacement of medium with nominally Ca^{2+} -free medium ($-\text{Ca}^{2+}$). A, 1 μM TG was added (arrow), followed by transient external Ca^{2+} removal and addition of 100 μM GEA3162 (GEA) as shown by bars. B, cells were treated with 100 μM GEA3162 in nominally Ca^{2+} -free medium for 3 min, and 1 mM Ca^{2+} was added in the continued presence of GEA3162. C, cells in nominally Ca^{2+} -free medium were treated and maintained with 75 μM 2-APB; after 3 min 1 μM TG was added followed by Ca^{2+} removal/readdition and addition of 100 μM MDL as shown by bars. D, cells in nominally Ca^{2+} -free medium were treated and maintained with 100 μM GEA3162 together with and 75 μM 2-APB; after 3 min, Ca^{2+} -containing medium was added.

indicate that the inhibition of store-operated Ca^{2+} entry with MDL did not appear related to effects on either adenylyl cyclase activity or the levels of cyclic AMP in cells.

The above actions of MDL were to block Ca^{2+} entry in response to store emptying with the Ca^{2+} pump blocker thapsigargin. Physiologically, store emptying does not occur through pump blockade. Therefore, it was important to assess the actions of MDL on Ca^{2+} entry in response to agonist-induced Ca^{2+} release. Generation of InsP_3 as a result of phospholipase C-coupled receptor activation results in a rapid release of Ca^{2+} from stores. Efficient receptor desensitization results in short-lived InsP_3 increases, hence store emptying and subsequent Ca^{2+} entry can be very transient (9, 17). If agonist treatment is undertaken in the absence of external Ca^{2+} , entry is prevented and stores remain at least partially empty (9). As shown in Fig. 4C, addition of bradykinin in the absence of external Ca^{2+} induced a rapid release of Ca^{2+} . Readdition of Ca^{2+} resulted in store-operated Ca^{2+} entry albeit smaller than that observed following the irreversible pump blockade induced by thapsigargin. Addition of MDL 1 min prior to bradykinin addition completely blocked this entry response (Fig. 4D). Significantly, MDL had no effect on agonist-induced Ca^{2+} release, indicating that it does not inhibit receptor-activated phospholipase C activation nor does it alter the function of InsP_3 receptors to release Ca^{2+} . Moreover, since MDL does not change basal levels of Ca^{2+} in cells or the release of Ca^{2+} in response to agonists or thapsigargin, this indicates that it does not alter cellular Ca^{2+} homeostasis maintained by Ca^{2+} pumps or the size or operation of Ca^{2+} stores.

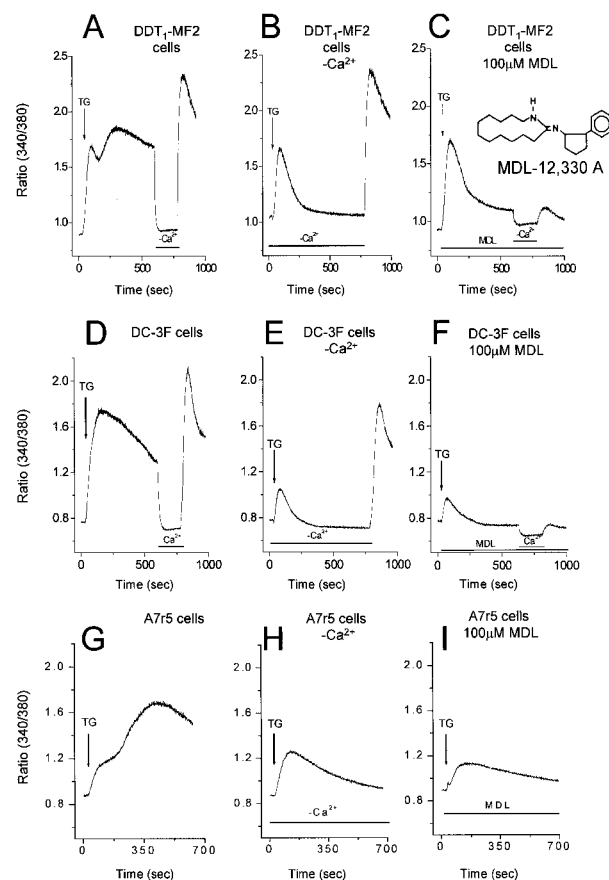


FIG. 3. Inhibition of store-operated Ca^{2+} entry by MDL-12,330A in different cell types. Experiments were undertaken as described in the legend to Fig. 1; bars indicate times of replacement of medium with nominally Ca^{2+} -free medium ($-\text{Ca}^{2+}$). A–C, using DDT₁MF-2 cells, Ca^{2+} levels were monitored after TG addition (arrow) for the times shown in the absence (A, B) or presence (C) of 100 μM MDL-12,330A (MDL) maintained through the experiment (bar). D–F, as for A–C except using DC-3F cells. G–I, as for A–C except using A7r5 smooth muscle cells. Inset, structure of MDL-12,330A.

We examined the kinetics of MDL's inhibitory effect on store-operated Ca^{2+} entry in more detail. Ca^{2+} entry following thapsigargin addition was compared in the presence of different MDL levels added 5 min before thapsigargin. The responses in the presence of 0, 10, and 100 μM MDL are shown in Fig. 5, A, B, and C, respectively. The concentration dependence of the action of MDL on the component of Ca^{2+} entry induced by thapsigargin is shown in Fig. 5D. Significant inhibition was observable with 1 μM MDL, and its effects were half-maximal at approximately 10 μM . This may be significantly different to the sensitivity of adenylyl cyclase to MDL, the IC_{50} of which is reported as 250 μM (35). The time dependence for the action of MDL was assessed by examining the overshoot response which occurs almost instantaneously after Ca^{2+} readdition (Fig. 5E). The effect of 100 μM MDL added simultaneously with Ca^{2+} was not observable until approximately 1 min after addition (Fig. 5F). Added 1 min prior to Ca^{2+} readdition, the action of MDL was substantial but not full (Fig. 5G). The action of MDL was almost complete when added for 10 min before Ca^{2+} readdition (Fig. 5H). Therefore, the effect of MDL is slightly different from that of 2-APB, which blocks store-operated Ca^{2+} entry extremely rapidly (Fig. 1C).

Returning to the question of Ca^{2+} entry activated in response to S-nitrosylation, MDL is clearly an effective blocker of this entry response as well. Following store emptying with thapsigargin, the large increment in Ca^{2+} entry induced by

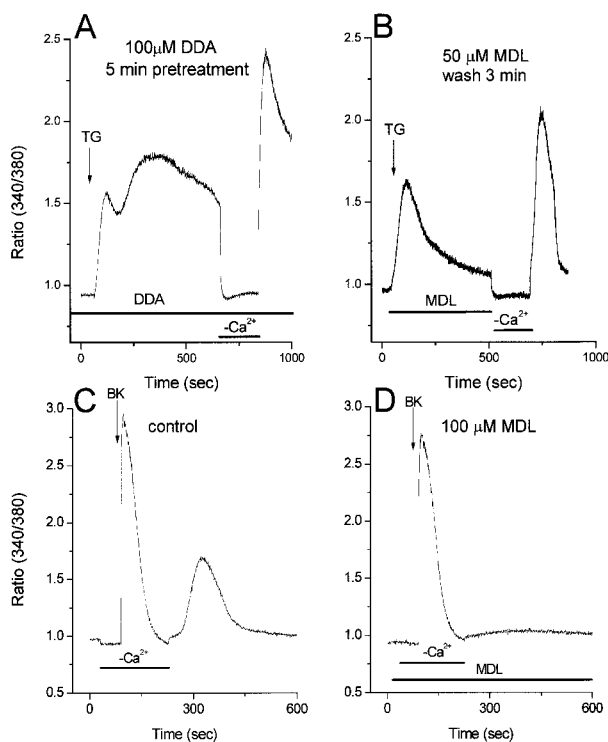


FIG. 4. The inhibitory action of MDL-12,330A on store-operated Ca^{2+} entry is independent of adenylyl cyclase, reversible, and downstream of PLC-coupled receptor-induced Ca^{2+} release. Ca^{2+} changes in DDT₁MF-2 cells were measured as described in the legend to Fig. 1 in nominally Ca^{2+} -free medium for the times shown (bars). *A*, Ca^{2+} stores were depleted with 1 μ M thapsigargin (TG) in the presence of the adenylyl cyclase inhibitor, 100 μ M DDA, added 5 min prior to TG. *B*, 1 μ M thapsigargin (TG) (arrow) was added concurrently with 50 μ M MDL (bar); MDL was removed simultaneously with replacement of medium with nominally Ca^{2+} -free medium. *C*, 1 μ M bradykinin was added in the absence of external Ca^{2+} , followed by the readdition of 1 mM Ca^{2+} medium. *D*, as for *C* except with 100 μ M MDL throughout.

GEA3162 (Fig. 6A) was completely blocked by the inclusion of 100 μ M MDL added at the same time as thapsigargin (Fig. 6B). In this experiment, MDL had also almost completely blocked store-operated Ca^{2+} entry. If, after pool emptying, MDL was added simultaneously with GEA3162, the increase in entry was rapidly attenuated, and only a small transient increase in entry was seen (Fig. 6C). If, after pool emptying, MDL was added without GEA3162, the store-operated entry was inhibited with a similar time dependence (Fig. 6D). In normal store-filled cells, the entry of Ca^{2+} observed following GEA3162 addition without external Ca^{2+} and subsequent readdition of Ca^{2+} (Fig. 6E) was completely blocked by inclusion of MDL in the medium (Fig. 6F). The concentration dependence of the blocking action of MDL on GEA3162-induced entry (not shown) revealed a very similar sensitivity to that of store-operated Ca^{2+} entry, with an IC_{50} of approximately 10 μ M.

These results indicate that the blocking action of MDL on store-operated Ca^{2+} entry and entry activated in response to the NO donor, GEA3162, is very similar. We and others (13–15, 17) have recently determined that some functional parameters of store-operated Ca^{2+} channels are shared with members of the TRP family of proteins. Receptor-induced activation of the human TRP3 channel stably expressed in HEK293 cells appears to have a requirement for the $InsP_3$ receptor (17), in agreement with earlier data (15). This requirement for the $InsP_3$ receptor appears to be the same for store-operated channels (17). However, the two channels differ with respect to one important parameter, that is, their relationship to store emptying. Thus, we and others (17, 18, 38–40) did not observe any

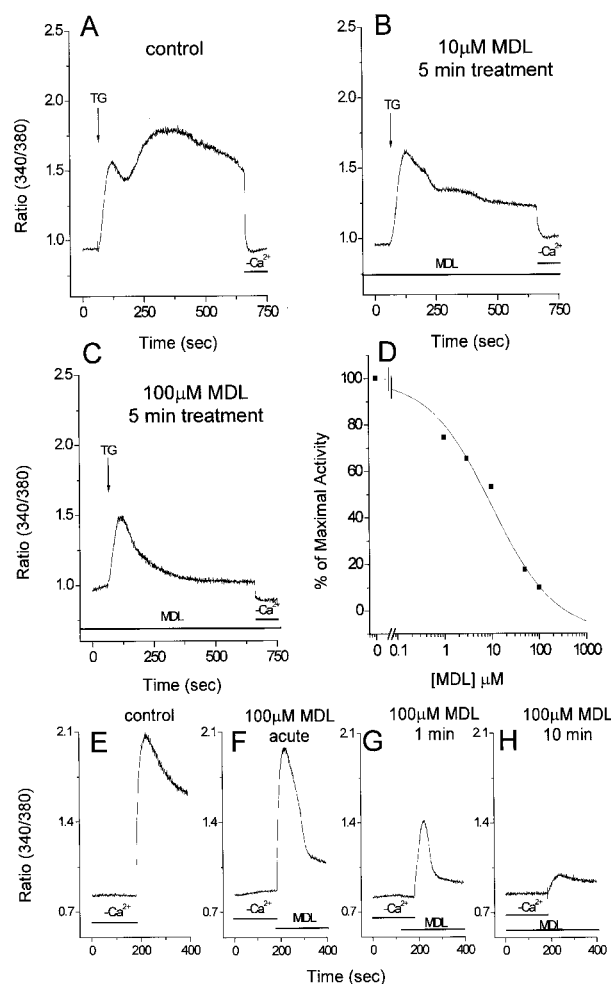


FIG. 5. Sensitivity and reversibility of MDL-12,330A-induced inhibition of store-operated Ca^{2+} entry. Ca^{2+} changes in DDT₁MF-2 cells were measured as described in the legend to Fig. 1 in nominally Ca^{2+} -free medium for the times shown (bars). *A*, 1 μ M thapsigargin (TG) was added at the time shown (arrow). *B*, cells were treated with 10 μ M MDL for 5 min prior to TG addition. *C*, cells were treated with 100 μ M MDL for 5 min prior to TG addition. *D*, semi-log plot of the concentration dependence of action of MDL on store-operated Ca^{2+} entry determined as in *C* and *D*; entry values were obtained from the area under the curve in the presence of MDL relative to a similar experiment undertaken in nominally Ca^{2+} -free medium. *E–H*, cells depleted of stores with 1 μ M TG in nominally Ca^{2+} -free medium were supplied with medium containing 1 mM Ca^{2+} and 100 μ M MDL (bars) at the times shown; *E*, without MDL; *F*, MDL added simultaneously with Ca^{2+} ; *G*, MDL added 1 min prior to Ca^{2+} ; *H*, MDL added 10 min prior to Ca^{2+} .

correlation between the operation of TRP3 channels and the emptying of stores, indicating that activation of the TRP3 channel, although requiring the $InsP_3$ receptor, occurs through a more direct coupling process (17). In view of this difference, assessing any pharmacological relationship between the action of TRP3 channels and SOCs was important. As shown in Fig. 7, MDL clearly blocked TRP3 activity. In the T3–65 clonal HEK293 line stably expressing TRP3 channels, the operation of these channels could be specifically observed by examining Sr^{2+} entry (17). Thus, although the cells also contain SOCs, the latter do not allow passage of Sr^{2+} under these conditions. Therefore, Sr^{2+} entry following PLC-coupled receptor agonist activation was a reliable means for independently assessing only function of TRP3 channels (17). As shown in Fig. 7A, application of carbachol in these cells induced a transient release of Ca^{2+} from stores in the absence of external Ca^{2+} . Application of external Sr^{2+} resulted in a rapid entry of Sr^{2+}

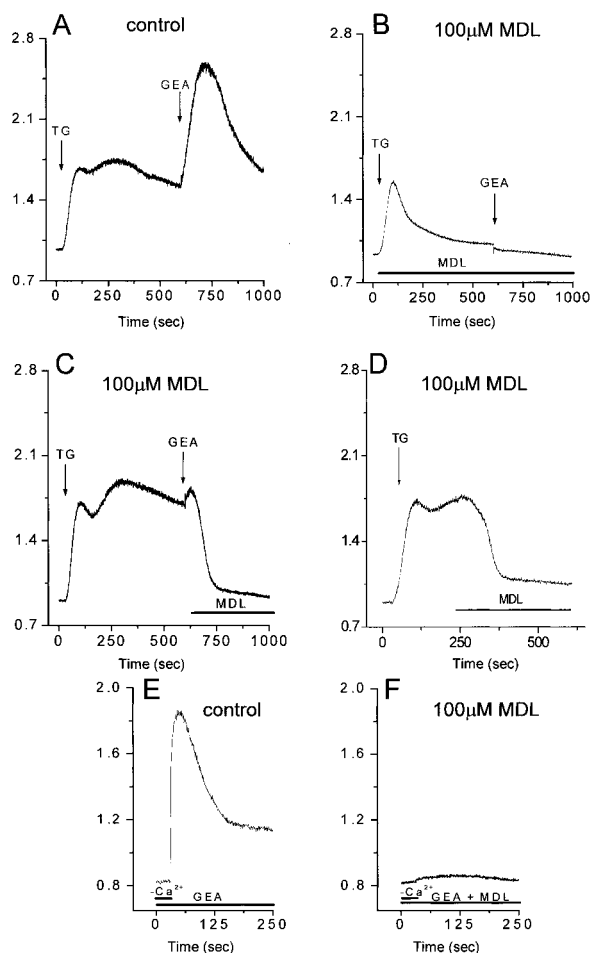


FIG. 6. MDL inhibits Ca^{2+} entry induced by S-nitrosylation in DDT₁MF-2 cells. Ca^{2+} changes were measured as in Fig. 1. Bars indicate presence of 100 μ M MDL or nominally Ca^{2+} -free medium for the times shown. A, Ca^{2+} stores were depleted with 1 μ M thapsigargin (TG) followed by addition of 100 μ M GEA3162 (GEA) (arrow). B, as for A but in the presence of 100 μ M MDL. C, as in B, but MDL was added simultaneously with GEA. D, 100 μ M MDL was added alone. E, Ca^{2+} entry stimulated by 100 μ M GEA3162 (added in the absence of external Ca^{2+}) in normal, store-filled cells, after return of medium containing 1 mM Ca^{2+} . F, as for E, in the presence of 100 μ M MDL.

mediated by the TRP3 channel. If MDL was applied at the start of the trace, the agonist-induced release of Ca^{2+} was essentially unaffected, whereas the entry of Sr^{2+} was completely eliminated (Fig. 7B). Added during the sustained entry of Sr^{2+} mediated by the TRP3 channel, MDL terminated the channel activity (Fig. 7C). If MDL was added simultaneously with Sr^{2+} addition, there was a brief increase in Sr^{2+} entry which disappeared shortly after (Fig. 7D). Therefore, it is clear that MDL blocks receptor-induced activation of the TRP3 channel and that sensitivity to MDL is a property shared by TRP3 and store-operated Ca^{2+} entry channels.

In a recent report, Hofmann *et al.* (40) revealed that certain TRP channels, including the TRP3 channel, could be directly activated by diacylglycerol, and this effect was quite independent of protein kinase C activation. The conclusion from this study was that DAG, and not $InsP_3$, was the PLC-derived product responsible for agonist-induced activation TRP3 channels (40). However, our results indicated that TRP3 channels could be activated by both PLC-derived products (17). As shown in Fig. 8A, the membrane permeant DAG analogue, OAG, induced a clear activation of the TRP3 channel. This property of the TRP3 channel is quite distinct from SOC, which is

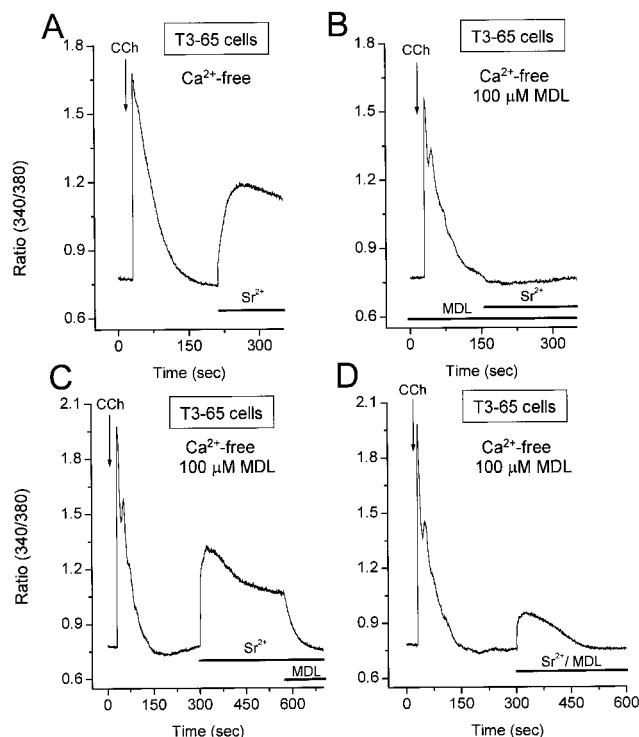


FIG. 7. Inhibition of Sr^{2+} entry through hTRP3 channels stably transfected in HEK293 cells. Ca^{2+} measurements using the T3-65 clone of HEK293 cells were as in Fig. 1; bars indicate times of replacement of nominally divalent cation-free medium with medium containing 1 mM Sr^{2+} and/or addition of 100 μ M MDL. A, TRP3 channels were activated by addition of 100 μ M carbachol (CCh) followed by addition of 1 mM Sr^{2+} . B, as in A, in the presence of 100 μ M MDL. C, as in A, but MDL added during sustained Sr^{2+} entry. D, as in A, but MDL added concurrently with Sr^{2+} .

insensitive to DAG analogues (17). The $InsP_3$ receptor blocker 2-APB did not alter OAG-induced activation of TRP3 channels (Fig. 8B), suggesting that DAG may activate the channel directly and independently from the $InsP_3$ receptor (17). In contrast, inclusion of MDL in the medium prior to OAG addition almost completely eliminated the OAG response (Fig. 8C). This provides useful further evidence that OAG is indeed acting on the TRP3 channel. When added simultaneously with OAG, the action of MDL was significantly diminished (Fig. 8D), that is, it was less effective in blocking. If MDL was added after OAG-induced TRP3 activation the inhibitory action of MDL was yet further reduced (not shown). The results suggest that both MDL and OAG may directly modify the TRP3 channel. Considering the diminished ability of MDL to reverse prior activation by OAG (in contrast to its very rapid termination of TRP3 activated by carbachol, as shown in Fig. 7C), it is possible that the diacylglycerol and MDL compete for a common site of action.

Overall, the results presented here provide some interesting similarities and differences in the operation of three distinct but related Ca^{2+} entry mechanisms. The coupling mechanism by which store-operated Ca^{2+} entry is activated has proven elusive. Most likely, the process involves "conformational coupling" between components of the ER and plasma membrane (7-9), and it appears that the $InsP_3$ receptor is a required ER component mediating the coupling process (15, 17). A significant problem in assessing operation of store-operated entry is its apparent heterogeneity between cells perhaps indicating that a number of related channels can be store-coupled. Here we have compared endogenous store-operated Ca^{2+} entry with two other distinct but related entry mechanisms. The TRP3-

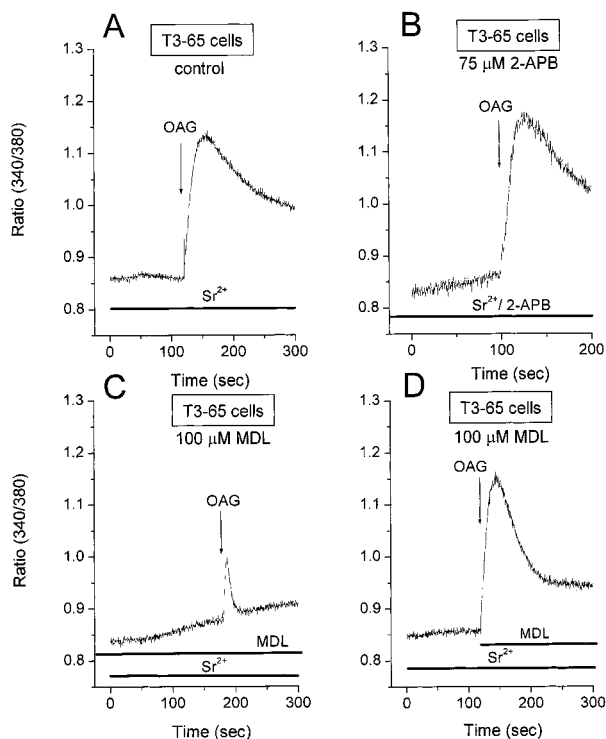


FIG. 8. MDL and 2-APB have differing effects on the activation of hTRP3 by the diacylglycerol analogue, OAG. Experiments using the T3-65 clone of HEK293 cells were performed in the presence of 1 mM external Sr^{2+} and in the presence of 100 μM MDL or 75 μM 2-APB, as shown. *A*, TPR3 channels were activated by addition of 100 μM OAG (arrow). *B*, as in *A*, but in the presence of 2-APB added for 2.5 min prior to OAG. *C*, as in *A*, but in the presence of 100 μM MDL added 4 min prior to OAG. *D*, as in *C*, but MDL added simultaneously with OAG.

mediated Ca^{2+} entry operates independently of stores, yet its activation appears to require the $InsP_3$ receptor. The *S*-nitrosylation-dependent entry mechanism is potentiated by store emptying but appears to reflect a direct activation process not mediated or maintained by the $InsP_3$ receptor; this may indicate that coupling between stores and entry channels is not always mediated by the $InsP_3$ receptor. Whereas these distinctions in coupling exist, a common feature of all three entry mechanisms is their sensitivity to the cycloalkyl lactamamide blocker, MDL-12,330A. Whereas sensitivity in the 10 μM range may not indicate a high affinity interaction, the similarity of sensitivity of the entry mechanisms to MDL is a significant finding. It should also be considered that, thus far, few reliable direct modifiers of store-operated entry have been identified. The econozoles, including SKF93635, have been utilized as blockers (42); however, our analysis of econozole action indicates great variation in their effectiveness between different cell types. In some cells SKF93635 may block in the high micromolar range (28), whereas in others there is little effect on Ca^{2+} entry and/or spurious changes in fluorescence.³ The reason for this variation is unclear but may reflect differences in channel subtypes and/or coupling state. In contrast, the action of MDL is very reliable between cells. Interestingly, there have been reports of direct actions of MDL in the low micromolar range on voltage-operated Ca^{2+} channels (41, 43, 44), suggesting that there may be some wider structural relationship between Ca^{2+} entry channels. Any relationship between these

entry mechanisms might be further elucidated from examination of the actions of molecules structurally related to MDL.

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³ R. L. Patterson and D. L. Gill, unpublished observations.