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Modification of Store-operated Channel Coupling and Inositol Trisphosphate Receptor Function by 2-Aminoethoxydiphenyl Borate in DT40 Lymphocytes*

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Store-operated channels (SOCs) provide an important means for mediating longer-term Ca^{2+} signals and replenishment of Ca^{2+} stores in a multitude of cell types. However, the coupling mechanism between endoplasmic reticulum stores to activate plasma membrane SOCs remains unknown. In DT40 chicken B lymphocytes, the permeant inositol trisphosphate receptor ($InsP_3R$) modifier, 2-aminoethoxydiphenyl borate (2-APB), was a powerful activator of store-operated Ca^{2+} entry between 1–10 μM. 2-APB activated authentic SOCs because the entry was totally selective for Ca^{2+} (no detectable entry of Ba^{2+} or Sr^{2+} ions), and highly sensitive to La^{3+} ions (IC_{50} 30–100 nM). To assess the role of $InsP_3Rs$ in this response, we used the DT40 triple $InsP_3R$-knockout (ko) cell line, DT40$InsP_3R$-ko, in which the absence of full-length $InsP_3Rs$ or $InsP_3R$ fragments was verified by Western analysis using antibodies cross-reacting with N-terminal epitopes of all three chicken $InsP_3R$ subtypes. The 2-APB-induced activation of SOCs was identical in the DT40$InsP_3R$-ko, cells indicating $InsP_3Rs$ were not involved. With both wild type (wt) and ko DT40 cells, 2-APB had no effect on Ca^{2+} entry in store-replete cells, indicating that its action was restricted to SOCs in a store-coupled state. 2-APB induced a robust activation of Ca^{2+} release from stores in intact DT40wt cells but not in DT40$InsP_3R$-ko cells, indicating an $InsP_3R$-mediated effect. In contrast, 2-APB blocked $InsP_3Rs$ in permeabilized DT40wt cells, suggesting that the stimulatory action of 2-APB was restricted to functionally coupled $InsP_3Rs$ in intact cells. Uncoupling of ER/PM interactions in intact cells by calyculin A-induced cytoskeletal rearrangement prevented SOC activation by store-emptying and 2-APB; this treatment completely prevented 2-APB-induced $InsP_3R$ activation but did not alter $InsP_3R$ activation mediated by phospholipase C-coupled receptor stimulation. The results indicate that the robust bifunctional actions of 2-APB on both SOCs and $InsP_3Rs$ are dependent on the coupled state of these channels and suggest that 2-APB may target the coupling machinery involved in mediating store-operated Ca^{2+} entry.

Ca^{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, 2). The cytosolic Ca^{2+} signals generated in response to receptors are complex involving two closely coupled components: rapid, transient release of Ca^{2+} stored in the endoplasmic reticulum (ER)[1] followed by slowly developing extracellular Ca^{2+} entry (1, 3–7). G protein-coupled receptors and tyrosine kinase receptors, through activation of phospholipase C, generate the second messenger, $InsP_3$, which diffuses rapidly within the cytosol to interact with $InsP_3Rs$ on the ER; the $InsP_3Rs$ serve as Ca^{2+} channels to release luminally stored Ca^{2+} and generate the initial Ca^{2+} signal phase (1, 4). The resulting depletion of Ca^{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 channels" (SOCs), which mediate capacitative Ca^{2+} entry (3, 5–8). This second Ca^{2+} entry phase of Ca^{2+} signals serves to mediate longer-term cytosolic Ca^{2+} elevations and provides a means of replenishing intracellular stores (3, 5). The mechanism for coupling ER Ca^{2+} store depletion with Ca^{2+} entry remains a crucial but unresolved question (5–8).

Coupling to activate SOCs has been hypothesized to involve direct coupling between the ER and plasma membrane (9, 10), and evidence indicates that physical docking of the ER with the plasma membrane may be involved in SOC activation (11–14). Recent evidence has indicated that the $InsP_3R$ may be a component in mediating the coupling process between stores and Ca^{2+} entry channels. In particular, a role for the $InsP_3R$ has been implicated in the activation of the TRP family of receptor-activated channels, which share a number of functional parameters with SOCs including reports that they are store-operated (15–21). Evidence from reconstitution studies has indicated a direct functional communication between TRP channels and $InsP_3Rs$ (17, 22–24), and a number of reports have revealed a physical interaction between TRP channels and the $InsP_3R$ (24–29).

Recently, we have probed the role played by $InsP_3Rs$ in Ca^{2+} entry mechanisms utilizing cells from the DT40 chicken B lymphocyte line (DT40$InsP_3R$-ko) in which the $InsP_3R$ genes have been disrupted (30). It was observed that these cells have store-operated Ca^{2+} entry that is functionally the same as that occurring in the wild type DT40 cells (30–32). Moreover, we observed that TRPC3 channels transiently expressed in

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[1] The abbreviations used are: ER, endoplasmic reticulum; TRP, transient receptor potential; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; fura-2/AM, fura-2 acetoxymethylester; 2-APB, 2-aminoethoxydiphenyl borate; SOC, store-operated channel; ko, knockout; wt, wild type; ICM, intracellular-like medium.

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DT40$InsP_3$R-ko cells or DT40wt cells were in both cases functionally activated in response to phospholipase C-coupled receptors (33). These results suggested that the $InsP_3$R was not necessary for SOC or TRP channel activation. Recent work has also focused on the permeant $InsP_3$ receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB) (34), to probe the role of the $InsP_3$R in coupling to activate SOC and TRP channels. At concentrations around 50 μM, the 2-APB molecule inhibits store-operated Ca^{2+} entry, blocking the activation of SOCs in response to store depletion with Ca^{2+} pump blockers or ionomycin (35, 36). 2-APB also blocks activation of mammalian TRPC3 channels (35) and TRP channels mediating the *Drosophila* light response (37). However, in the case of both of these TRP channels, the action of 2-APB does not appear to be directly upon the TRP channels themselves but rather upon an upstream target that is involved perhaps in controlling the activation of the channels (35, 37). We recently determined that the blocking action of 2-APB on SOCs was still observed in the DT40$InsP_3$R-ko cells (31), suggesting that this action of 2-APB was not mediated by the $InsP_3$R.

Considering these recent results and the fact that they appeared to contradict a large body of evidence implicating a role for $InsP_3$Rs in SOC activation, it was crucial to obtain more information on the function of SOCs and the actions of 2-APB in the DT40 cell lines. It was also essential to determine more definitively whether the DT40$InsP_3$R-ko cells were truly devoid of $InsP_3$R translation products. Thus, it was suggested (17) that truncated $InsP_3$R genes replacing the original genes in these cells could give rise to expression of C-terminally truncated $InsP_3$Rs (17), which might be sufficient for the activation of Ca^{2+} entry channels (22–25, 28, 29). Here we provide the results of exhaustive Western analyses establishing that the DT40$InsP_3$R-ko cells are devoid of detectable $InsP_3$Rs or fragments thereof. We also reveal that robust bifunctional actions of 2-APB on both SOCs and $InsP_3$Rs in DT40wt cells are dependent on the coupled state of these channels and suggest that 2-APB may target the coupling machinery involved in mediating store-operated Ca^{2+} entry.

EXPERIMENTAL PROCEDURES

Culture of Cells—Cells of both the wild type DT40 chicken B cell line (DT40wt) and triple $InsP_3$ receptor knockout cell line (DT40$InsP_3$R-ko) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine, as described previously (30).

Measurement of Intracellular Calcium—Cells grown on coverslips for 1 h 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 25 min at 20 °C. Cells were washed, and dye was allowed to de-esterify 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 (38, 39). 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 groups of 10–12 cells. Resting Ca^{2+} levels in the two DT40 cell lines were similar at ~100–130 nM. All measurements shown are representative of a minimum of three, and in most cases, a larger number of independent experiments.

$InsP_3$R Immunodetection—Cells of both of the DT40 lines as well as the A7r5 smooth muscle and Chinese hamster ovary cell lines (CHO; used as $InsP_3$R positive controls) were homogenized and treated exactly as described previously (40), with the exception that for both DT40wt and DT40$InsP_3$R-ko cells the analyses used cell homogenates and not isolated membranes. All samples were rapidly frozen in liquid N_2 and stored at −80 °C, protein determinations were performed by the Lowry procedure. For $InsP_3$R detection, samples were boiled for 2 min in sample buffer, separated on 3–12% Laemmli-type linear gradient gels, transferred to Immobilon-P, and probed with the following antibody preparations directed against $InsP_3$R N-terminal domains: (*a*) the polyclonal anti-cytI3b-1 and anti-cytI3b-2 antisera directed against Ca^{2+}-binding site 3b (amino acids 378–450) of the type-1 $InsP_3$R (41); (*b*) the polyclonal Rbt226 and Rbt227 antisera directed against amino acids 330–344 of the type-2 $InsP_3$R (42); (*c*) the monoclonal antibody MMA-type3 against amino acids 22–230 of the type-3 $InsP_3$R (I31220, Transduction Laboratories) (43). Immunodepletion of the polyclonal anti-cytI3b-1 antiserum was performed by a 2-h preincubation of the antiserum with an excess of antigenic peptide. A new antibody was developed recognizing a conserved epitope localized in the N-terminal portion of the three $InsP_3$R isoforms. The synthetic peptide KSNKY-LTVNKRLPAL corresponding to amino acids 127–141 of the human type-1 $InsP_3$R, which is conserved between isoforms and between diverse vertebrate species, was coupled to keyhole limpet hemocyanin through an additional C-terminal cysteine. Two rabbits (Rbt475 and Rbt476) were repeatedly immunized with the coupled peptide. Both antisera reacted with high affinity against each of the $InsP_3$R isoforms from various sources. These antisera were further affinity-purified against the peptide. Finally, for some control experiments the N-terminal $InsP_3$R antiserum AbC (44) was also used. Analysis of the immunoreactive bands was performed after incubation with secondary antibodies coupled to alkaline phosphatase detected by using Vistra™ ECF (Amersham Life Sciences, Inc.) and fluorimaged as described before (45).

Cell Permeabilization—The procedures for cell permeabilization cells were as described earlier (46, 47). Briefly, suspensions of DT40 cells (1×10^6 cells/ml) were stirred gently and incubated with 0.0025% saponin in an intracellular-like medium (ICM) (comprising 140 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, and 10 mM Hepes-KOH, pH 7.0) at 37 °C until 95% permeabilization occurred (normally after 6–7 min). After permeabilization, cells were washed twice in saponin-free ICM at 4 °C and kept cold before use in experiments. To avoid problems of lipid dilution of added hydrophobic compounds, the final cell concentration in all experiments was kept at exactly 5×10^5 cells/ml.

Calcium Flux Experiments—Ca^{2+} flux measurements were conducted as described previously (47–49). The accumulation of $^{45}Ca^{2+}$ into intracellular organelles was measured using permeabilized DT40 cells (5×10^5 cells/ml) maintained by gentle stirring at 37 °C in ICM containing 50 μM $CaCl_2$ (with 150 Ci/mol $^{45}Ca^{2+}$), EGTA to buffer free Ca^{2+} to exactly 0.1 μM, 3% polyethylene glycol, and 5 μM ruthenium red (to prevent mitochondrial Ca^{2+} accumulation) in a total volume of 2 ml. Effectors mentioned in the figures (2-APB) were added at the times shown. Oxalate with GTP when present was added immediately prior to the start of uptake. At the required times, 200-μl aliquots were removed from the stirred uptake medium, diluted immediately into 4 ml of ice-cold ICM containing 1 mM $LaCl_3$, and then rapidly vacuum-filtered on glass fiber filters (Whatman GF/B), washed, and counted. The figures show ATP-dependent Ca^{2+} accumulation with that component of Ca^{2+} retained by the cells and filters in the absence of ATP, subtracted (~0.1% of total Ca^{2+}). The experiments shown are typical of at least three separate experiments.

Materials and Miscellaneous Procedures—ATP, GTP, EGTA, polyethylene glycol, saponin, ruthenium red, Hepes, and oxalate were purchased from Sigma. 2-APB was from Tocris (Ballwin, MO). Thapsigargin was from LC Services (Woburn, MA). Fura-2/AM was from Molecular Probes (Eugene, OR). The DT40 cell lines were generously provided by Dr. Tomohiro Kurosaki (Kyoto, Japan). For Ca^{2+} flux experiments, free Ca^{2+} concentrations were controlled using EGTA computing all complexes between EGTA, ATP, Ca^{2+}, Mg^{2+}, monovalent cations, and protons, as described previously (50). Anti-chicken IgM (supernatant, M-4 clone) was from Southern Biotechnology Associates (Birmingham, AL). The AbC antiserum was a generous gift from Dr. C. W. Taylor (University of Cambridge, UK).

RESULTS AND DISCUSSION

2-APB Is a Powerful Activator of SOC Activity in DT40wt Cells—Considering the potential significance of 2-APB in targeting the activation of SOCs and TRP channels, we examined the details of the kinetics of action of 2-APB. Surprisingly, 2-APB was observed to induce a robust biphasic modification of SOC function in DT40 chicken B-lymphocytes. Using DT40wt cells in which Ca^{2+} stores had been previously depleted with thapsigargin, the addition of Ca^{2+} resulted in the onset of SOC-mediated Ca^{2+} entry, as shown in Fig. 1. After SOC activation, the addition of low levels of 2-APB (1–10 μM) induced a rapid and profound stimulation of Ca^{2+} entry (Fig. 1, *A–C*) with a maximal effect at 10 μM. In contrast, at higher levels (25–75 μM), the action of 2-APB was strongly inhibitory

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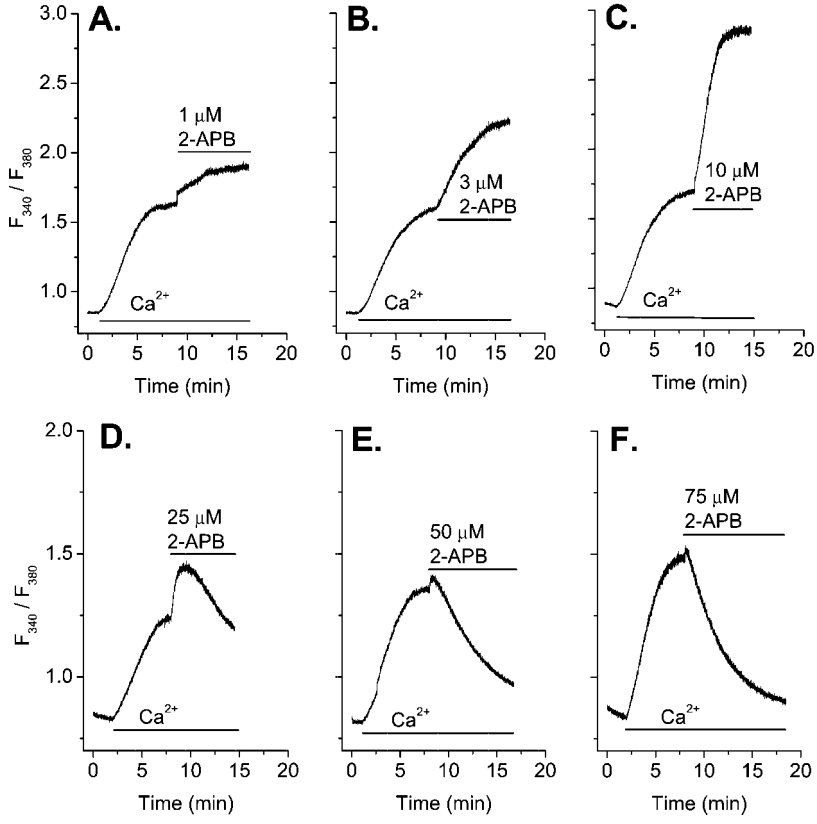

FIG. 1. **Biphasic modification of store-operated Ca^{2+} entry by 2-APB in DT40wt cells.** Cytosolic Ca^{2+} was measured in fura-2-loaded wild type DT40 cells as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate times of replacement with external medium containing 1 mM Ca^{2+} and/or indicated concentrations of 2-APB. *A*, store-operated Ca^{2+} entry was observed in the wild type DT40 cells pretreated with 1 μM thapsigargin upon the addition of Ca^{2+} (*bar*) and the further addition of 1 μM 2-APB (*bar*). *B*, same as in *A* but with 3 μM 2-APB (*bar*). *C*, same as in *A* but with 10 μM 2-APB (*bar*). *D*, same as in *A* but with 25 μM 2-APB (*bar*). *E*, same as in *A*, but inhibition of SOC was observed upon the addition of 50 μM 2-APB (*bar*). *F*, same as in *E* but with 75 μM 2-APB (*bar*).

on SOCs (Fig. 1, *D–F*). Thus, although there was an initial transient increase in Ca^{2+} entry, this subsided to reveal a powerful blocking action. At the higher levels of 2-APB, the stimulatory effect was extremely brief such that at 75 μM there was an almost imperceptible activation followed rapidly by almost complete inhibition of Ca^{2+} entry. Indeed, we had previously observed the inhibitory action of 2-APB on SOCs (31, 35) but had not examined the effects of acute addition of submaximally effective 2-APB levels on preactivated SOC activity. The biphasic effects of 2-APB on SOCs were not unique to the DT40 cells because we observed similar effects of 2-APB in HEK293 cells, DC3-F fibroblasts, and DDT$_1$MF-2 smooth muscle cells (data not shown). In these cells the stimulatory effect with low levels of 2-APB was smaller, but the overall biphasic effect was similar.

One particular reason why the nature and coupling of SOCs has remained elusive is that modifiers of the activation process have not been identified. Whereas there have been reports on a variety of agents that can inhibit SOC activity (51), almost invariably such agents have been shown to be highly nonselective, modifying a range of other channels for Ca^{2+} and/or other ions. In contrast, the action of 2-APB on SOCs reflects a much more selective function, 2-APB having little or no effect on a range of Ca^{2+} and related channels including ryanodine receptors (34), voltage-sensitive Ca^{2+} entry channels (34), arachidonic acid-activated Ca^{2+} entry channels (52), Ca^{2+} entry channels activated by *S*-nitrosylation (36), Ca^{2+}-activated Cl^- entry channels (37, 53), or purinergic P2X receptor Ca^{2+} entry channels.[2] The stimulation of SOCs by a defined modifier, 2-APB, provides a potentially much more significant probe for assessing the nature and coupling of SOCs.

2-APB Activates Authentic SOCs—It was important to determine whether the stimulatory action of 2-APB on Ca^{2+} entry represents activation of authentic SOCs as opposed to the activation of some other channel activity. One of the distinguishing characteristics of SOCs is their high cation specificity, allowing the passage of Ca^{2+} ions but excluding passage of closely related ions such as Sr^{2+} and Ba^{2+} (5, 33, 35). We therefore determined whether the activation of entry induced by 2-APB had similar selectivity. The entry of Ba^{2+} or Sr^{2+} can easily be monitored by a change in fura-2 ratio-fluorescence, which is similar to the change induced by Ca^{2+} entering the cytosol (35). As shown in Fig. 2*A*, after emptying stores with thapsigargin, the addition of Ba^{2+} resulted in no change in fluorescence, and there was no further change upon the subsequent addition of 10 μM 2-APB, *i.e.* the condition inducing maximal entry of Ca^{2+} (see Fig. 1*C*). Identical results were obtained with Sr^{2+} addition (data not shown). These results show that activated SOCs and the additional entry activated by 2-APB are highly selective for Ca^{2+} over Sr^{2+} or Ba^{2+}. We also considered that the observed lack of Ba^{2+} or Sr^{2+} entry induced by 2-APB in these experiments could reflect the absence of an elevated cytosolic Ca^{2+} level, *i.e.* it was possible that the 2-APB activation of Ca^{2+} entry observed in Fig. 1*C* was dependent on the increased Ca^{2+} level preceding 2-APB addition. However, when either Ba^{2+} or Sr^{2+} was added together with 10 μM 2-APB at 3 min after thapsigargin addition (simultaneously with the elevated cytosolic Ca^{2+} following thapsigargin-induced Ca^{2+} release), there was again no ion entry (data not shown). In contrast, the addition of Ca^{2+} and 10 μM 2-APB under the same conditions resulted in the expected large and rapid activation of Ca^{2+} entry.

A second parameter defining SOC activity is an exceptional sensitivity to blockade by the La^{3+} ion (5, 35). The data in Fig. 2*B* reveal that the extremely high La^3 sensitivity of thapsigargin-induced SOC-mediated Ca^{2+} entry is indistinguishable from the La^{3+} sensitivity of Ca^{2+} entry stimulated by the subsequent addition of 10 μM 2-APB. Thus both components of Ca^{2+} entry were reduced to ~50% by the addition of 100 nM

[2] H.-T. Ma, K. Venkatachalam, and D. L. Gill, unpublished observations.

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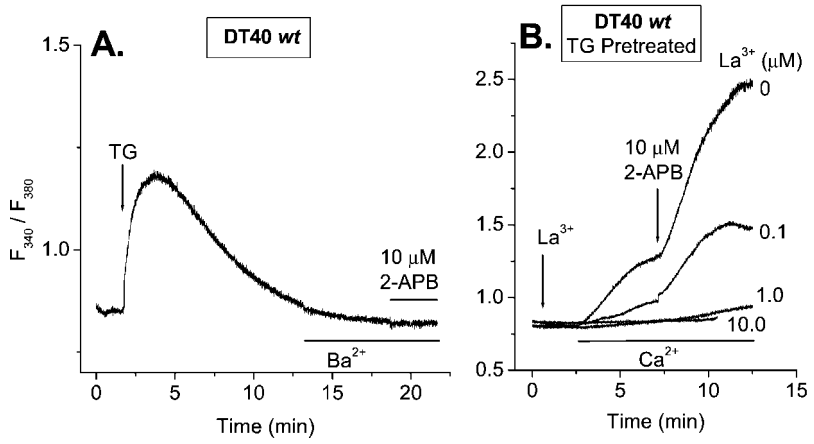

FIG. 2. **Activation of store-operated Ca^{2+} entry by 10 μM 2-APB in DT40wt cells has high selectivity for divalent cations and high sensitivity to La^{3+}.** Ca^{2+} measurements were as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate the times with medium containing 1 mM Ba^{2+} or 1 mM Ca^{2+} and/or 10 μM 2-APB. *A*, Ca^{2+} pools were released by the addition of 1 μM thapsigargin (*TG*; *arrow*), and no entry was observed upon the addition of 1 mM Ba^{2+} (*bar*) and 10 μM 2-APB (*bar*). *B*, stores were depleted with 1 μM thapsigargin prior to the addition of 0, 100 nM, 1 μM, or 10 μM La^{3+} added (*arrow*) prior to the addition of 1 mM Ca^{2+} (*bar*) followed by 10 μM 2-APB (*arrow*).

La^{3+} and were completely abolished by La^{3+} at 1 μM or higher (Fig. 2*B*). These data are consistent with the known cation selectivity of SOCs (5) and provide evidence that the 2-APB-induced activation of Ca^{2+} entry is mediated by authentic SOCs. We may conclude that 2-APB acts either directly or indirectly to stimulate SOCs.

Analysis of $InsP_3R$ Expression in DT40wt and DT40$InsP_3R$-ko Cells—Because 2-APB is an effective $InsP_3R$ modifier (34, 35) and, as described above, there is substantial evidence indicating a role for $InsP_3Rs$ in the activation of SOCs and TRP channels, it was logical to determine whether the stimulatory action of 2-APB on SOCs was mediated by or in any way related to $InsP_3Rs$. This question could be answered directly by utilizing the DT40$InsP_3R$-ko cells. However, a frequently raised concern about the use of these knockout cells is that the absence of $InsP_3R$ proteins or fragments therefrom has not been definitively established. Whereas the parent DT40 cells express all three $InsP_3R$ isoforms (30), studies have shown that the DT40InsP3R-ko cells have no detectable $InsP_3R$ message and appear devoid of functional $InsP_3Rs$ based on Ca^{2+} release and $InsP_3$ binding (30–32). However, an important point raised by Kiselyov *et al.* (17) was that the "deleted" genes in these knockout cells were actually replaced by truncated constructs lacking coding sequences for ~100 amino acids at the C-terminal region of the $InsP_3Rs$ (30). Thus, the possibility remains that these cells might still express N-terminal domains of the $InsP_3Rs$. It was argued, therefore, that truncated $InsP_3R$ translation products might fulfill a "coupling" role for activating SOCs even though they might be functionally devoid of channel properties (17). Indeed, studies reveal that the N-terminal domain of the $InsP_3R$ interacts with and may mediate coupling to activate TRP channels and SOCs (22–25, 28, 29).

Thus, before proceeding further with the use of DT40$InsP_3R$-ko cells, it was important to assess the expression of $InsP_3R$ translation products (both full-length and fragments) in these cells. Such analysis had not previously been undertaken; the only former analysis at the protein level involved assessment of full-length $InsP_3Rs$ in the DT40$InsP_3R$-ko cells with antibodies directed against the C terminus of type-1 and type-2 $InsP_3Rs$ (54). To investigate the possible translation of N-terminal domains, we analyzed the presence of full-length $InsP_3Rs$ as well as $InsP_3R$ fragments using antibodies raised against epitopes located in the N-terminal portion of each of the three $InsP_3R$ subtypes. Because, if present, such N-terminal fragments might lack C-terminal transmembrane domains and hence remain soluble, we analyzed DT40 cell homogenates. The polyclonal antibodies, anti-cytI3b-1 and anti-cytI3b-2, directed against Ca^{2+}-binding site 3b (amino acids 378–450) of the type-1 $InsP_3R$ (41) detected full-length type-1 $InsP_3R$ in DT40wt cells (Fig. 3*A*). The monoclonal MMAtype3 antibody

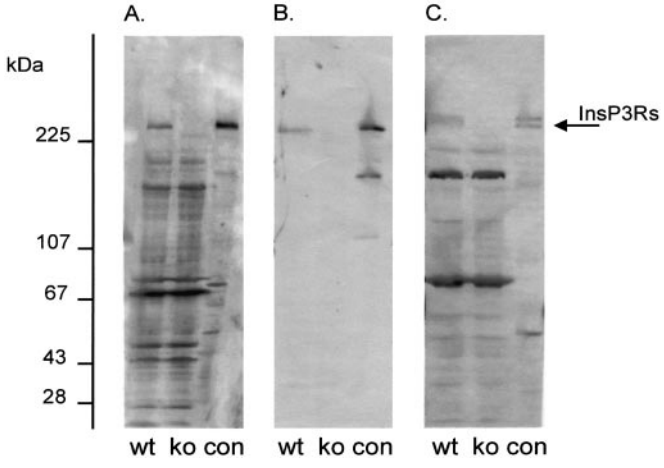

FIG. 3. **Western analysis of $InsP_3R$ proteins in DT40wt and DT40Ins P_3R-ko cells.** $InsP_3R$ were detected using anti-cytI3b-1 (*A*), MMAtype3 (*B*), or affinity-purified Rbt475 (*C*) in gels loaded with 200 μg of cell homogenate from DT40wt (*wt*) or DT40$InsP_3R$-ko cells (*ko*) or 50 μg of microsomes from A7r5 microsomes (*A* and *C*) or Chinese hamster ovary cells (*B*) used as positive controls (*con*). The antibodies were used at dilutions of 1/750, 1/300, and 1/2000, respectively. The *arrow* at the *right* indicates the position of full-length $InsP_3Rs$. In *panel C*, with A7r5 microsomes the two $InsP_3R$ isoforms, type-1 and type-3, can be clearly identified as distinct. The positions of the molecular mass standards are indicated at the *left*. The results are typical of three experiments.

directed against amino acids 22–230 of the type-3 $InsP_3R$ (43) recognized full-length type-3 $InsP_3R$ in DT40wt cells (Fig. 3*B*). However, the available Rbt226 and Rbt227 polyclonal antibodies against the 330–344-amino acid region of the N terminus of type-2 $InsP_3Rs$ (42) did not react in DT40 cells, presumably because of significant species differences between the chicken and mammalian sequences in this region. To circumvent this problem we used the new antibodies Rbt475 and Rbt476 for which the epitope (amino acids 127–141) is conserved between the three $InsP_3R$ isoforms and between diverse species including *Xenopus* oocytes and carp brain. Both antibodies reacted with full-length $InsP_3Rs$ in DT40wt cells giving a broad band, which likely reflects the simultaneous detection of multiple $InsP_3R$ isoforms (Fig. 3*C*).

Significantly, none of the antibodies used detected any full-length $InsP_3R$ in the DT40$InsP_3R$-ko cells nor was there any identifiable N-terminally derived fragment that was specifically recognized by the antibodies. Thus, for each particular antibody, the lower molecular mass band pattern was consistently identical between the DT40wt and DT40$InsP_3R$-ko cells. For the polyclonal antibodies used, nonspecific staining was more apparent in the DT40 cell homogenates than in the A7r5

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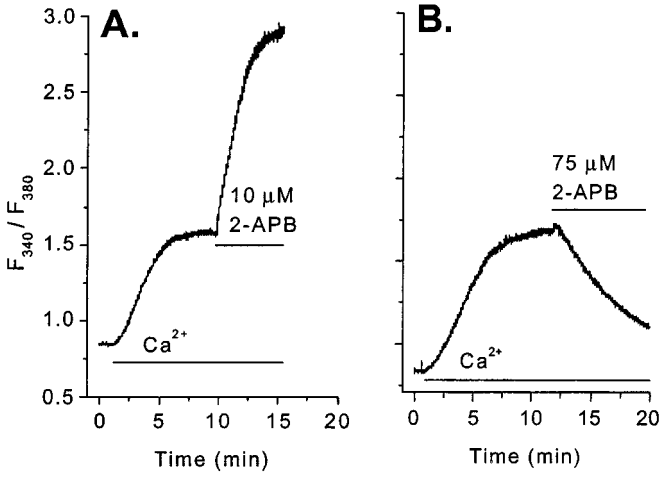

FIG. 4. **Action of 2-APB on SOC-mediated Ca^{2+} entry DT40InsP$_3$R-ko cells.** Cytosolic Ca^{2+} was measured in fura-2-loaded DT40InsP$_3$R-ko cells as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate times of replacement with external medium containing 1 mM Ca^{2+} and/or indicated concentrations of 2-APB. Cells were store-depleted by prior treatment (15 min) with 1 μM thapsigargin followed by the addition of 1 mM Ca^{2+} (*bar*) and either (*A*) 10 μM 2-APB or (*B*) 75 μM 2-APB (*bar*).

microsomes used as positive controls, which likely reflects a broader array of proteins in the homogenates and the 4-fold higher quantity of DT40 cell protein loaded onto gels to make detection possible. Because at the high concentrations of anti-cytI3b-1 and anti-cytI3b-2 antibodies used there was substantial recognition of nonspecific bands (Fig. 3*A*), the antiserum was immunodepleted with antigen. Using this immunodepleted antiserum, only the full-length type-1 InsP$_3$R band disappeared in both DT40wt cell homogenates and A7r5 microsomes (data not shown). For the same reason, the Rbt475 and the Rbt476 antisera were affinity-purified against the peptide used for immunization. After affinity purification only two nonspecific reaction targets were observed, and the recognition of full-length InsP$_3$Rs exclusively in DT40wt and not in DT40InsP$_3$R-ko cells remained identical (Fig. 3*C*). Finally, using the unrelated AbC antibody, which also recognizes a common N-terminal epitope of InsP$_3$Rs and cross-reacts with DT40wt InsP$_3$R, a different pattern of nonspecific bands was observed with no evidence of any commonly recognized N-terminal fragment (data not shown). In conclusion, these results indicate that in DT40InsP$_3$R-ko cells there is no detectable full-length or N-terminal InsP$_3$R fragment present.

SOC Activation by 2-APB in DT40 Cells Is InsP$_3$R-independent—Because it is clear that SOCs exist and can be activated normally in the DT40InsP$_3$R-ko cells (30–32), these results provide significant weight to our conclusion that the InsP$_3$R protein, or a fragment therefrom, is not required for SOC activation. In addition, we can extend our previous conclusions against the requirement for InsP$_3$Rs in the activation of TRPC3 channels functionally expressed in DT40InsP$_3$R-ko (33). Turning our attention back to 2-APB, we determined whether the biphasic modification of SOCs by 2-APB was altered in any way by the absence of InsP$_3$Rs in DT40InsP$_3$R-ko cells. As shown in Fig. 4, the effects of 2-APB on Ca^{2+} entry in the DT40InsP$_3$R-ko cells were identical to the effects in DT40wt cells. Thus, at 10 μM 2-APB, there was still a powerful and sustained activation of SOCs (Fig. 4*A*), whereas at 75 μM there was clear inhibition of SOC activity following a very small and transient increase in entry (Fig. 4*B*). We may therefore conclude that both actions of 2-APB are independent of the InsP$_3$R.

2-APB Selectively Activates Store-coupled SOCs—Having determined that 2-APB can act as a powerful stimulator of SOCs, we undertook experiments to examine whether the state of the channels is important in their modification by 2-APB. Thus we wished to know whether SOCs need to be in an activated state (store-coupled) or whether 2-APB could activate SOCs in their closed state. We therefore assessed the action of 10 μM 2-APB on Ca^{2+} entry in cells in which stores were replete. As shown in Fig. 5*A*, using DT40wt cells in the presence of external Ca^{2+}, the addition of 2-APB induced only a very slight increase in cytosolic Ca^{2+}. In the DT40InsP$_3$R-ko cells the effect of 2-APB was even smaller (Fig. 5*B*). When 10 μM 2-APB was added to DT40wt cells in the absence of external Ca^{2+}, a small but significant fluctuation in response was observed (Fig. 5*C*) suggesting a low level intermittent release of Ca^{2+} from stores. The DT40InsP$_3$R-ko cells showed no such fluctuations in response to 10 μM 2-APB in the absence of Ca^{2+} nor any entry of Ca^{2+} when Ca^{2+} was added back to the medium (Fig. 5*D*).

The lack of any sizable Ca^{2+} entry in response to 2-APB added to store-replete cells indicates that the activation of SOCs by 2-APB requires that stores are emptied. Thus, 2-APB does not appear to activate SOCs in their inactive state, suggesting that the action of 2-APB to stimulate SOCs is limited to those channels that are in an actively coupled state. 2-APB may function either to increase the conductance state of opened, SOCs or it may induce the recruitment of more SOCs to become opened, perhaps by increasing the efficiency of store coupling. Overall, the robust action of 2-APB to stimulate store-coupled SOCs (as opposed to nonactivated channels) suggests that its target is a component involved in the coupling process itself.

2-APB Activates InsP$_3$R-mediated Ca^{2+} Release in Intact DT40 Cells—2-APB was determined to be an inhibitor of InsP$_3$Rs in a number of cell types (34, 35). Interestingly, it appears that its actions on InsP$_3$Rs display some significant parallels with those on SOCs. As noted above, low 2-APB levels appeared to induce some release of Ca^{2+} from stores (Fig. 5*C*). As shown in Fig. 6*A*, using 25 μM 2-APB, the fluctuations in Ca^{2+} in DT40wt cells were larger and more apparent, whereas in DT40InsP$_3$R-ko cells no change in Ca^{2+} occurred in response to 2-APB (Fig. 6*B*). The addition of 75 μM 2-APB caused a very clear release of Ca^{2+} from stores in DT40wt cells. Indeed, as shown in Fig. 6*C*, the release of Ca^{2+} was sufficient enough that the addition of thapsigargin induced no further Ca^{2+} release. In other words, the effect of 2-APB was to completely empty stores. In stark contrast, the addition of 75 μM 2-APB to the DT40InsP$_3$R-ko cells produced only a very slight increase in Ca^{2+}, and the subsequent addition of thapsigargin caused a normal large release response (Fig. 5*D*) indicating that stores were not significantly altered. These results show that 2-APB can function to activate the InsP$_3$R in the DT40wt cells and can cause a very effective release of stored Ca^{2+}. Indeed, the action of 2-APB was sufficient enough that upon removal of 2-APB from cells in the presence of external Ca^{2+}, entry of Ca^{2+} was observed, presumably as a result of SOC activation (Fig. 6*C*). Obviously, because SOCs were inhibited by 75 μM 2-APB (see Fig. 1*F*), there was no entry until after 2-APB was removed. The observed activation of SOCs after 2-APB-induced store depletion was not dependent on thapsigargin because entry was similar whether or not thapsigargin was added (data not shown). Indeed, the entry of Ca^{2+} after 2-APB removal in DT40wt cells (Fig. 6*C*) was similar to that observed after removal of 2-APB in the experiment shown in Fig. 6*D* in which SOCs were activated by thapsigargin-induced store emptying.

2-APB Blocks InsP$_3$Rs in Permeabilized DT40 Cells—This action of 2-APB to apparently activate InsP$_3$Rs was unusual and was not observed in a number of other cell lines. Indeed, we have substantial data indicating that 2-APB functions to pre-

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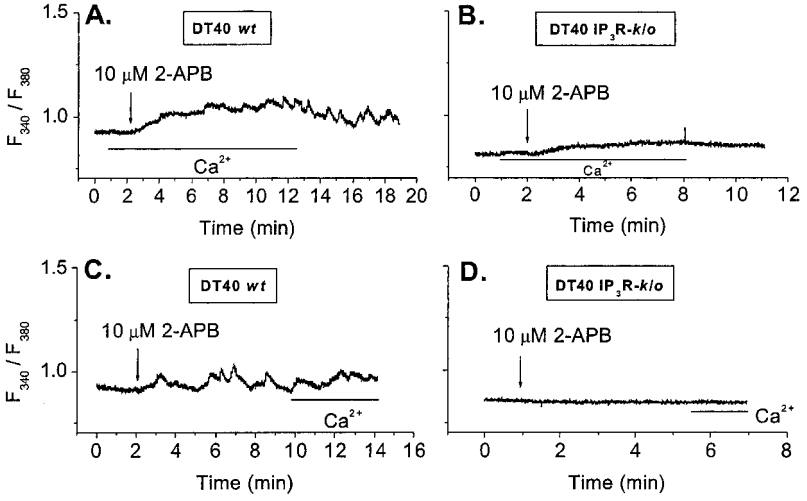

FIG. 5. **Enhanced Ca^{2+} entry with 10 μM 2-APB is observed only after pool depletion in both DT40wt and DT40InsP_3R-ko cells.** Cytosolic Ca^{2+} was measured in fura-2-loaded DT40 cells as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate times of replacement with external medium containing 1 mM Ca^{2+}. *A*, 1 mM Ca^{2+} was added (*bar*) prior to the addition of 10 μM 2-APB (*arrow*) in the DT40wt cells. *B*, same as in *A* but in DT40InsP_3R-ko cells. *C*, same as in *A*, but 10 μM 2-APB (*arrow*) was added prior to the addition of 1 mM Ca^{2+} (*bar*) in DT40wt cells. *D*, same as in *C* but in DT40IP_3R-ko cells.

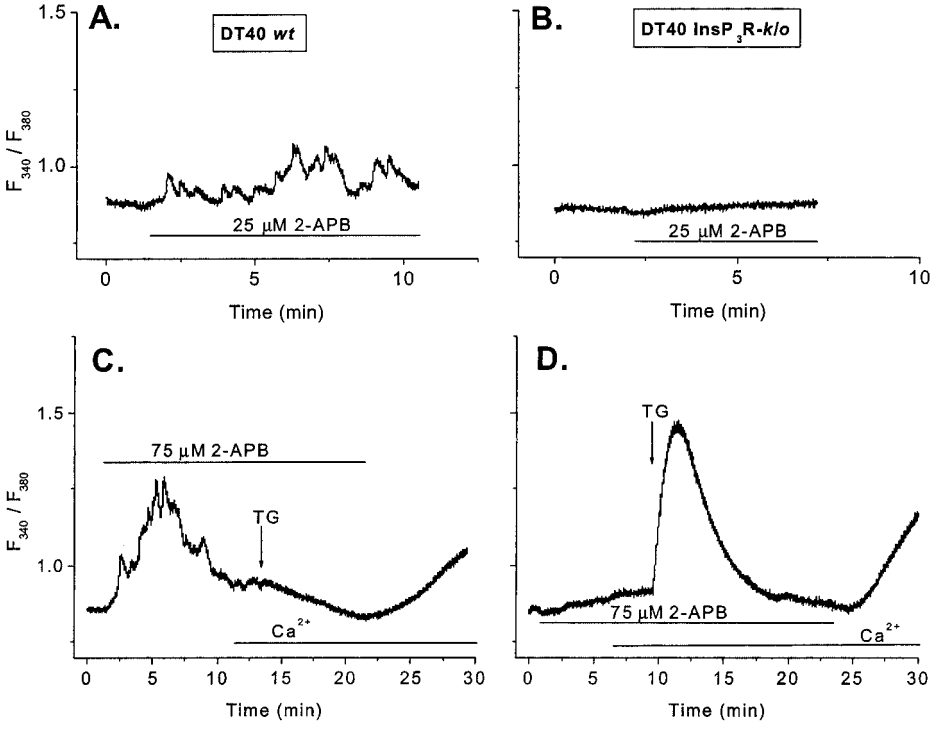

FIG. 6. **2-APB releases Ca^{2+} from stores in DT40wt cells but not in DT40InsP_3R-ko cells.** Cytosolic Ca^{2+} was measured in fura-2-loaded DT40 cells as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate times of replacement with external medium containing indicated concentration of 2-APB and/or 1 mM Ca^{2+}. *A*, addition of 25 μM 2-APB (*bar*) in the absence of external Ca^{2+} in DT40wt cells. *B*, same as in *A* but DT40InsP_3R-ko cells. *C*, addition of 75 μM 2-APB (*bar*) causes complete depletion of Ca^{2+} stores, and no further release is observed upon addition of 1 μM thapsigargin (*TG*; *arrow*). The removal of 2-APB and the addition of 1 mM Ca^{2+} (*bars*) led to recovery of store-operated Ca^{2+} entry. *D*, same as in *C* but using DT40InsP_3R-ko cells where 75 μM 2-APB (*bar*) caused almost no store depletion. The subsequent addition of 1 μM thapsigargin (*TG*; *arrow*) released stores, and in the presence of 1 mM Ca^{2+} (*bar*) the recovery of store-operated Ca^{2+} entry followed removal of 2-APB.

vent Ca^{2+} release responses activated by phospholipase C-coupled receptor agonists, presumably as a result of inhibiting $InsP_3$Rs (35). Moreover, we have observed that in permeabilized DT40wt cells, 2-APB prevented $InsP_3$ from activating Ca^{2+} release through $InsP_3$Rs (31). Thus, the direct addition of $InsP_3$ to release $^{45}Ca^{2+}$ from stores in permeabilized DT40wt cells was blocked by 2-APB in a dose-dependent manner (31). In those experiments, however, we did not examine the action of 2-APB in the absence of $InsP_3$. Thus we considered that $InsP_3$Rs might exhibit complex biphasic modification by 2-APB, perhaps similar to its action on SOCs. We therefore examined the action of 2-APB on $InsP_3$ receptor-mediated Ca^{2+} release from stores in permeabilized DT40 cells, the results indicating that 2-APB has no direct activating effect on $InsP_3$Rs. Opening of $InsP_3$ receptor Ca^{2+} release channels was measured by their ability to prevent $^{45}Ca^{2+}$ accumulation into stores mediated by Ca^{2+} pumping activity. Thus, as shown in Fig. 7, whereas concentrations of 2-APB in the range of 15–100 μM did reduce Ca^{2+} accumulation, there was no significant difference in the action of 2-APB on uptake into permeabilized DT40wt cells (Fig. 7*A*) as opposed to DT40InsP_3R-ko cells (Fig. 7*B*). Concentrations of 2-APB up to 50 μM induced only modest changes in Ca^{2+} uptake; at 100 μM 2-APB there was a more significant reduction in uptake. Such effects are consistent with an inhibitory action of high levels of 2-APB on sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump activity, which has been reported previously (34, 55). Indeed, this modest reduction in Ca^{2+} pump function is likely the cause of the small increase in cytosolic Ca^{2+} observed with high 2-APB in the DT40InsP_3R-ko cells as shown in Fig. 6*D*. However, this release should not be confused with that activated by 2-APB in intact cells, which differs profoundly between the DT40wt and DT40InsP_3R-ko cells. Nor is this release the cause of SOC activation because that occurs at a low 2-APB level and requires the stores to have been previously emptied.

The Actions of 2-APB on SOCs and $InsP_3$Rs Depend on ER-PM Interactions—From these results, the actions of 2-APB on InsP_3R-mediated Ca^{2+} release present an apparent paradox. Thus, the clear stimulatory action of 2-APB on $InsP_3$Rs in intact DT40wt cells is in stark contrast to the inhibitory action of 2-APB

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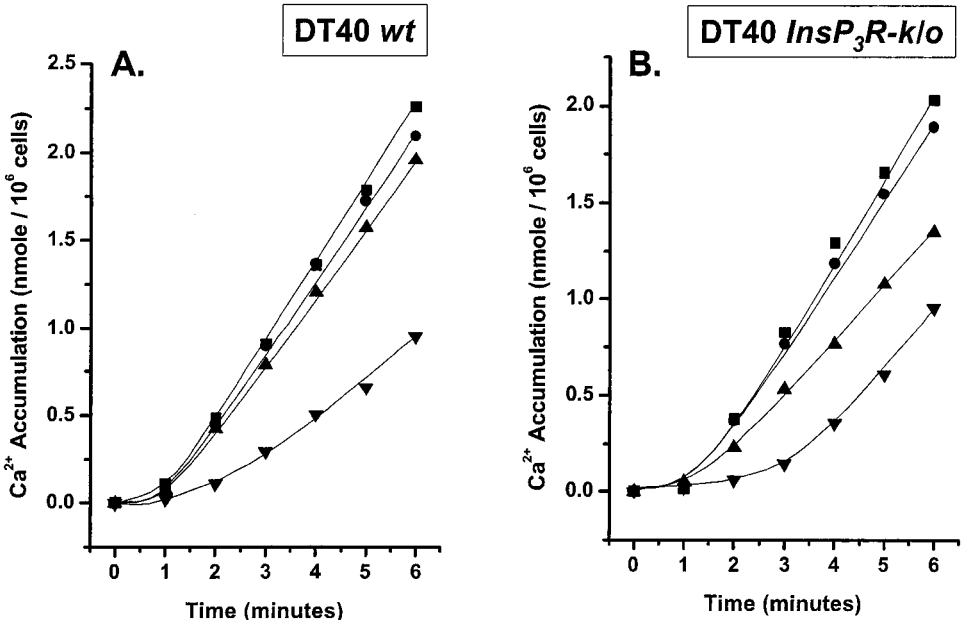

FIG. 7. **2-APB blocks accumulation of $^{45}Ca^{2+}$ in permeabilized wild type as well as triple $InsP_3$-receptor knockout cells.** Cell permeabilization and ATP-dependent Ca^{2+} accumulation in the presence of 20 μM GTP and 10 mM oxalate were as described under "Experimental Procedures." All additions were made before the initiation of Ca^{2+} accumulation by ATP addition at time zero. *A*, in the DT40wt cells, uptake was under control conditions (■) with 15 μM 2-APB (●), 50 μM 2-APB (▲), or 100 μM 2-APB (▼). *B*, same as in *A* but in DT40$InsP_3$R-ko cells.

on $InsP_3$Rs in permeabilized DT40wt cells (31). In view of the substantial data that $InsP_3$Rs in the ER can "conformationally couple" to components in the plasma membrane (17, 22–24), we considered the possibility that the physical and functional elimination of the plasma membrane in permeabilized cells resulted in removal of a controlling element for the $InsP_3$R, which is either the target for 2-APB or modifies the response to 2-APB. We therefore sought an independent means of modifying the interaction between the plasma membrane and the ER. In recent work (11), we defined conditions to induce physical rearrangement of the actin cytoskeleton to prevent plasma membrane-ER interactions and uncouple the activation process for SOCs. Most effective was the treatment of cells with low concentrations of the phosphatase inhibitor, calyculin A, which in all cells types examined resulted in translocation of existing actin filaments to form a tight band of cortical actin immediately subjacent to the plasma membrane (11, 35). As shown in Fig. 8*A*, this calyculin A-induced uncoupling of SOC activation was clearly observed in DT40wt cells. After treatment with 100 nM calyculin A, the activation of SOCs in response to thapsigargin was completely blocked. Moreover, the stimulatory action of 2-APB on SOCs was also eliminated. As in other cell types, calyculin A altered neither the basal cytosolic Ca^{2+} levels nor the size of the stores released by thapsigargin (not shown). These data support the view that the action of 2-APB to stimulate SOCs requires close coupling between the plasma membrane and ER.

Interestingly, the action of 2-APB to stimulate Ca^{2+} release via $InsP_3$Rs in DT40wt was also completely blocked by calyculin A treatment. In contrast, receptor-induced $InsP_3$-mediated Ca^{2+} release was not blocked by calyculin A. The Ca^{2+} release response to addition of anti-IgM antibody to stimulate B cell receptors through phospholipase C-γ activation in normal DT40wt cells in the absence of external Ca^{2+} is shown in Fig. 8*B*. After pretreatment of cells with calyculin A, the addition of 75 μM 2-APB induced no detectable Ca^{2+} release (Fig. 8*C*). Yet, the response to subsequent addition of anti-IgM (Fig. 8*C*) was almost the same as observed in untreated cells (Fig. 8*B*). The latter result is consistent with much previous evidence that redistribution of the cytoskeleton to form a dense cortical actin layer does not present a barrier to the chemical message mediated by $InsP_3$

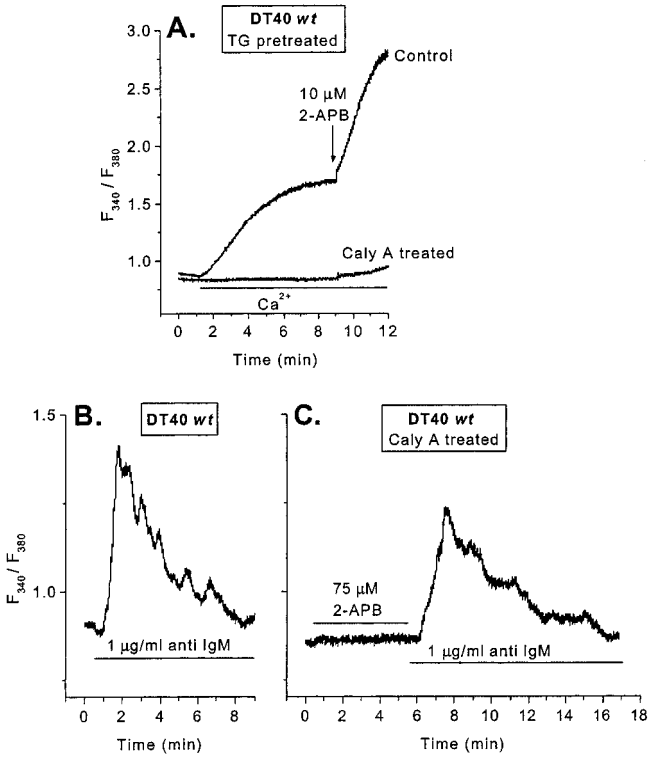

FIG. 8. **Uncoupling of ER-PM interactions with calyculin A prevents SOC activation by 2-APB and $InsP_3$R-mediated Ca^{2+} release in response to 2-APB but not B cell receptor activation.** Cytosolic Ca^{2+} was measured in fura-2-loaded wild type DT40 cells as described under "Experimental Procedures." Standard conditions included nominally divalent cation-free external medium. The *bars* indicate times of replacement with external medium containing 1 mM Ca^{2+} and/or either 10 μM or 75 μM 2-APB and 1 μg/ml anti-IgM. *A*, store-operated Ca^{2+} entry and its further activation by 10 μM 2-APB (*arrow*) in the DT40wt cells pretreated (2 h) with 100 nM calyculin A (*Caly A*). 1 μM thapsigargin was added 15 min before time zero, and 1 mM Ca^{2+} was added as shown (*bar*). *B*, Ca^{2+} release response to anti-IgM in untreated DT40wt cells. *C*, DT40wt cells were pretreated with 100 nM calyculin A (1 h), and 75 μM 2-APB was added (*bar*) followed by the addition of 1 μg/ml anti-IgM (*bar*).

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diffusing from the plasma membrane to the ER to activate release via $InsP_3Rs$ (11, 35). Therefore, the action of 2-APB to stimulate $InsP_3Rs$ differs from the receptor-induced process and appears to require the coupling between stores and plasma membrane. If, in an experiment similar to that shown in Fig. 8*B*, anti-IgM was added with 2-APB still present, the Ca^{2+} release response to anti-IgM was largely reduced (data not shown). This finding indicates that 2-APB was not only without any stimulatory action in the calyculin A-treated cells but also that it reverted to become an inhibitor of $InsP_3R$-mediated release, an effect that appears consistent with its direct action in the permeabilized cells.

Concluding Remarks—Much recent data have accrued from the use of the DT40$InsP_3R$-ko cells to indicate that the function of SOCs and TRP channels is independent of the $InsP_3R$ (30–33, 54). Thus, the absence of detectable $InsP_3R$ translation products, either of full or partial length, adds considerable weight to the validity of these conclusions. The results presented here also reveal 2-APB to be a powerful modifier of both SOCs and $InsP_3Rs$. Moreover, the actions of 2-APB on these two channels provide some intriguing parallels and point to 2-APB modifying potentially important regulatory targets. In both cases, 2-APB can act as both a stimulator and an inhibitor of function, but in neither case is the target of 2-APB necessarily the channel itself. In the case of SOCs, 2-APB can act as a powerful stimulator of the activation process mediated by store emptying. However, 2-APB appears to have no direct action on uncoupled channels in store-replete cells and therefore may not be an intrinsic direct activator of SOCs. Even after stores are emptied, 2-APB does not activate channels when coupling is prevented by calyculin A. Thus, the target for 2-APB may be a component of the coupling process itself. In the case of $InsP_3Rs$, the action of 2-APB to stimulate the release channel also does not appear to be a direct action and may instead be upon a regulatory component coupling to the $InsP_3R$. When plasma membrane interactions with the ER are disrupted either by permeabilization or by calyculin A-induced cellular rearrangement, 2-APB does not have any stimulatory action and instead functions as an inhibitor of the channel. Considering the actions of 2-APB, a significant question is whether it directly modifies channels, perhaps in their conformationally coupled state, or whether it targets the coupling machinery itself. In the case of mammalian and *Drosophila* TRP channels, the modification by 2-APB does not appear to be direct, based on the lack of action of 2-APB when TRP channels are activated directly, *i.e.* independently of receptor-coupled phospholipase C (33, 35, 37). This suggests that 2-APB targets the coupling process to activate the channels and not necessarily the channels themselves (33, 35, 37). Indeed, there is little to suggest that TRP channels and $InsP_3R$ proteins are structurally related and present a common target for 2-APB. Finally, although we have not yet pinpointed the molecular targets of 2-APB, these are likely to play important roles in the control of the physiological functioning of and the possible coupling between SOCs, TRP channels, and $InsP_3Rs$.

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