Assessment of the Role of the Inositol 1,4,5-Trisphosphate Receptor in the Activation of Transient Receptor Potential Channels and Store-operated Ca\textsuperscript{2+} Entry Channels*

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The mechanism for coupling between Ca\textsuperscript{2+} stores and store-operated channels (SOCs) is an important but unresolved question. Although SOCs have not been molecularly identified, transient receptor potential (TRP) channels share a number of operational parameters with SOCs. The question of whether activation of SOCs and TRP channels is mediated by the inositol 1,4,5-trisphosphate receptor (InsP\textsubscript{3}R) was examined using the permeant InsP\textsubscript{3}R antagonist, 2-aminoethoxydiphenyl borate (2-APB) in both mammalian and invertebrate systems. In HEK293 cells stably transfected with human TRPC3 channels, the actions of 2-APB to block carbachol-induced InsP\textsubscript{3}R-mediated store release and carbachol-induced Sr\textsuperscript{2+} entry through TRPC3 channels were both reversed at high agonist levels, suggesting InsP\textsubscript{3}Rs mediate TRPC3 activation. However, electroretinogram recordings of the light-induced current in Drosophila revealed that the TRP channel-mediated responses in wild-type as well as trp and trpl mutant flies were all inhibited by 2-APB. This action of 2-APB is likely InsP\textsubscript{3}R-independent since InsP\textsubscript{3}Rs are dispensable for the light response. We used triple InsP\textsubscript{3}R knockout DT40 chicken B-cells to further assess the role of InsP\textsubscript{3}Rs in SOC activation. \textsuperscript{45}Ca\textsuperscript{2+} flux analysis revealed that although DT40 wild-type cells retained normal InsP\textsubscript{3}Rs mediating 2-APB-sensitive Ca\textsuperscript{2+} release, the DT40InsP\textsubscript{3}R-k/o cells were devoid of functional InsP\textsubscript{3}Rs. Using intact cells, all parameters of Ca\textsuperscript{2+} store function and SOC activation were identical in DT40wt and DT40InsP\textsubscript{3}R-k/o cells. Moreover, in both cell lines SOC activation was completely blocked by 2-APB, and the kinetics of action of 2-APB on SOCs (time dependence and IC\textsubscript{50}) were identical. The results indicate that (a) the action of 2-APB on Ca\textsuperscript{2+} entry is not mediated by the InsP\textsubscript{3}R and (b) the effects of 2-APB provide evidence for an important similarity in the function of invertebrate TRP channels, mammalian TRP channels, and mammalian store-operated channels.

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Intracellular Ca\textsuperscript{2+} signals control diverse 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\textsuperscript{2+} signals generated in response to receptors are complex, involving two closely coupled components: rapid, transient release of Ca\textsuperscript{2+} stored in the endoplasmic reticulum (ER) followed by slowly developing extracellular Ca\textsuperscript{2+} entry (1, 3–7). G protein-coupled receptors and tyrosine kinase receptors, through activation of phospholipase C, generate the second messenger, inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) that diffuses rapidly within the cytosol to interact with InsP\textsubscript{3}Rs on the ER, which serve as Ca\textsuperscript{2+} channels to release luminal-stored Ca\textsuperscript{2+} and generate the initial Ca\textsuperscript{2+} signal phase (1, 4). The resulting depletion of Ca\textsuperscript{2+} stored within the ER lumen serves as the primary trigger for a message that is returned to the plasma membrane, resulting in the slow activation of store-operated channels (SOCs), which mediate the process known as capacitative Ca\textsuperscript{2+} entry (3, 5). Whereas receptor-induced generation of InsP\textsubscript{3} and the function of Ca\textsuperscript{2+} release channels to mediate the initial Ca\textsuperscript{2+} signaling phase is well understood, the mechanism for coupling ER Ca\textsuperscript{2+} store depletion with Ca\textsuperscript{2+} entry remains a crucial but unresolved question (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 ER with the plasma membrane may be involved in SOC activation (11–14). The mammalian TRP family of receptor-operated ion channels appears to share some operational parameters with SOCs (15, 16). Experiments reveal that the activation of TRPC3 channels like SOCs may require close coupling between the ER and plasma membrane (11, 17). Kiselyov et al. (18, 19) provide evidence that activation of human TRPC3 channels stably expressed in HEK293 cells occurs through a process involving endogenous InsP\textsubscript{3} receptors. This activation appears to reflect a specific molecular interaction between the two channel proteins (20) analogous in some ways to the coupling between

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The abbreviations used are: ER, endoplasmic reticulum; TRP, transient receptor potential; InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; fura-2/AM, fura-2 acetoxymethylester; 2-APB, 2-aminoethoxydiphenyl borate; SOC, store-operated channel; ERG, electroretinogram; HEK cells, human embryonic kidney cells.
rynoide receptors and Ca\(^{2+}\) entry channels in fast muscle triad junctions (1). Experiments reveal that the activation of both TRP channels and SOCs is prevented by the action of two different membrane-permeant InsP\(_3\) receptor antagonists, xestospongin C and 2-aminoethoxydiphenyl borate (2-APB) (17, 21), providing further evidence for the involvement of the InsP\(_3\) receptor in coupling to activate both entry channel types. The analogy between the operation of SOCs and TRP channels is further strengthened by reports indicating that specific members of the TRP family of channels can operate in a store-dependent manner (18, 19, 22–27). However, there are also reports indicating that the ionic selectivity of TRP channels does not correspond with known SOC activities (28–31) and that TRP channels can operate independently of store depletion (31–37).

Given the uncertainty with respect to the function and coupling of store-operated Ca\(^{2+}\) entry, the current report focuses on two central questions: first, whether InsP\(_3\) receptors are involved in the operation of SOCs and TRP channels; and second, whether members of the TRP family of channels are operationally related to SOCs. The studies provide further assessment of the involvement of the InsP\(_3\) receptor in coupling to entry channels by comparing vertebrate and invertebrate TRP channel function with store-operated channels functioning in mammalian cells in which all three of the InsP\(_3\) receptor subtypes have been genetically deleted. The results indicate that although InsP\(_3\)Rs do not mediate the action of 2-APB on Ca\(^{2+}\) entry, the effects of 2-APB provide evidence for an important similarity in the function of invertebrate TRP channels, mammalian TRP channels, and mammalian store-operated channels.

**EXPERIMENTAL PROCEDURES**

**Culture of Cells**—Cells of both the wild-type DT40 chicken B cell line (DT40/wt) and triple InsP\(_3\) receptor knockout cell line (DT40/InsP\(_3\)-Rk/o) were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glucose, as described previously (38). Cells of the T3–65 clone of HEK293 cells stably expressing the human TRPC3 channel were described previously (17, 21).

**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\(_4\), 1.2 mM CaCl\(_2\), 11.5 mM glucose, 0.1% bovine serum albumin, 20 mM Hepes-KOH, pH 7.4) and loaded with fura-2/AM (2 \(\mu\)M) 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. Fluorescence emission at 505 nm was monitored with excitation at 340 nm. Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (39, 40). The accumulation of 45Ca\(^{2+}\) into intracellular organelles was measured using permeabilized DT40 cells (5 \(\times\) 10\(^6\) cells/ml) maintained with gentle stirring at 37 °C in intracellular-like medium containing 50 \(\mu\)M CaCl\(_2\) (with 150 Ci/mol 45Ca\(^{2+}\)), buffered to 0.1 \(\mu\)M free Ca\(^{2+}\) with EGTA, 3% polyethylene glycol, and 5 \(\mu\)M ruthenium red (to prevent mitochondrial Ca\(^{2+}\) accumula-

**RESULTS AND DISCUSSION**

The mechanism of coupling between intracellular Ca\(^{2+}\) stores and the activation of store-operated Ca\(^{2+}\) entry channels has remained an elusive process. Support for the conformational-coupling model (9, 10) has arisen from recent evidence that InsP\(_3\) receptors are involved in the activation of TRP channels and SOCs (17–20). Availability of the membrane-permeant antagonist of the InsP\(_3\) receptor, 2-APB (47), provided some new information on the possible role of the InsP\(_3\) receptor in capacitative Ca\(^{2+}\) entry (17). 2-APB blocks the activation of capacitative Ca\(^{2+}\) entry in response to store depletion with Ca\(^{2+}\) pump blockers or ionomycin (17, 21). In addition, 2-APB blocks receptor-induced activation of mammalian TRPC3 channels (17, 21). TRPC3 channels can also be activated directly by application of diacylglycerol (17, 36); however, this direct stimulation of TRPC3 channels is not blocked by 2-APB, suggesting its action is not directly on the Ca\(^{2+}\) entry channels but, instead, on the coupling process, leading to channel activation in response to receptor stimulation (17).

Using the T3–65 clone of HEK293 cells stably expressing TRPC3 channels (48), the experiment shown in Fig. 1 appeared to reinforce this conclusion. In these cells, TRPC3 channels can be distinguished from SOCs by their ability to mediate a substantial entry of Sr\(^{2+}\) in response to agonists of phospholipase C-coupled receptors (17). Carbachol acting through endogenous muscarinic receptors was observed to be a more effective activator of TRPC3 channels. Compared with other phospholipase C-coupled receptors in the same cells, for example purinergic receptors (17), muscarinic receptor-induced InsP\(_3\) levels are sustained longer as a result of less efficient receptor desensitization (49). At a low dose (1 \(\mu\)M), carbachol induced a modest transient release of Ca\(^{2+}\) from internal stores in the absence of external entry channels (18,889

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Ca\(^{2+}\), and a substantial entry of Sr\(^{2+}\) upon the addition of the divalent cation, typical of the activation of TRPC3 channels in these cells. In the presence of 75 \(\mu\)M 2-APB, both the release and entry responses to 1 \(\mu\)M carbachol were abolished (Fig. 1B). At higher concentrations of carbachol (Fig. 1, C and E), both the release phase and the entry phase were more pronounced. However, the inhibitory action of 2-APB was substantially decreased. With 10 \(\mu\)M carbachol, significant release and entry were observed in the presence of 2-APB (Fig. 1D), and at 100 \(\mu\)M carbachol, release and entry were reduced by only ~50% by 2-APB (Fig. 1F). This could suggest that the higher levels of InsP\(_3\) induced by muscarinic activation prevent the action of 2-APB on both InsP\(_3\) receptor-mediated Ca\(^{2+}\) release and TRPC3 channel activation. Such an effect would be consistent with the action of 2-APB on the InsP\(_3\) receptor, which has been shown to be reduced at higher InsP\(_3\) concentrations (47). The implication from this is that the InsP\(_3\) receptor could be the mediator of both release and entry, a conclusion consistent with the substantial evidence indicating a direct interaction between TRP channels and InsP\(_3\) receptors (18–20). It is also possible that the higher activation of phospholipase C results in more diacylglycerol (as well as InsP\(_3\)) to stimulate TRPC3 channels (36), resulting in the observed decrease in 2-APB sensitivity since the action of diacylglycerol on TRPC3 channels is insensitive to 2-APB (17). Whether or not this is the case, it still remains that 2-APB blocks receptor-induced TRPC3 activation.

The central question of whether the action of 2-APB on TRPC3 channels reflects its modification of InsP\(_3\) receptors was addressed by analyzing the TRP channels that mediate the visual response in Drosophila. Invertebrate phototransduction occurs through rhodopsin-induced phospholipase C activation, resulting in activation of the family of retinal-specific TRP channels that mediate the light-induced current (16). The activation of these channels had been considered a paradigm for store-operated channels; however, the role of Ca\(^{2+}\) stores in the light response is controversial, and evidence indicates they may not be involved (16, 37). Moreover, genetic studies indicate that the single InsP\(_3\) receptor gene product in Drosophila (DOP-3R) is dispensable with respect to the light response (50, 51). Based on the action of 2-APB on the mammalian TRPC3 channels, it was important to assess the agent’s action on Drosophila TRP channels. Drosophila photoreceptor cells express three members of the TRP family, TRP, TRPL, and TRP\(_{\gamma}\) (52–54). Loss-of-function mutations in two of these channels, TRP and TRPL, have been described, demonstrating that they are essential for a normal light response (55, 56). To determine whether 2-APB had any effect on the light-stimulated channel activities, the drug was injected into fly eyes, and ERG recordings were performed. ERGs are extracellular recordings that measure the summed response of all cells in the retina. Since TRP, TRPL, and TRP\(_{\gamma}\) form the light-stimulated channels in photoreceptor cells, the ERG amplitudes reflect the activities of these TRP family members. Stimulation of wild-type eyes with light results in a corneal negative response that rapidly returns to base line after cessation of the light stimulus. Control injections in wild-type retinas had no effect on the response amplitudes of the ERG over the 4-min course of the experiment (Fig. 2A, top trace). The ERGs in trp and trpl flies were also unchanged after control injections (data not shown). Injection of 2-APB caused a gradual decrease in amplitude of the wild-type ERG responses (Fig. 2A, second trace). The trp and trpl mutants were more sensitive to the inhibitory effects of 2-APB than wild-type flies (Fig. 2A, third and fourth traces). A plot of the time course of inhibition of light response amplitude by 2-APB is shown in Fig. 2B. Four minutes after injection of 2-APB, the ERG amplitudes of wild-type flies were decreased to 46.5 ± 4.1%, whereas those in trp or trpl flies were inhibited to 21.1 ± 3.7% and 18.8 ± 2.1% respectively.

Given the information from the laboratories of Zuker and co-workers (50) and Hardie and co-workers (51) that activation...
of the TRP channels mediating the Drosophila light-induced current appears normal in the absence of InsP₃ receptors, the results indicate that the action of 2-APB may be independent of the InsP₃ receptor. Although the action of 2-APB on the Drosophila ERGs may appear slower than its action on SOCs and TRP channels in mammalian cells (17, 21), it should be noted that the ERG is a measurement of the sum of responses from all retinal cells, only some of which are in close apposition to the site of 2-APB application. Diffusion of the drug to more distant cells likely accounts for the slower onset of its action. Indeed, it appears that almost complete inhibition of TRP channel activation can be obtained with 2-APB within the 4-min time period. The principle channels in wild-type photoreceptor cells appear to be TRP homomultimers, TRP/TRP₂ heteromultimers, and TRP₁/TRPγ heteromultimers (54, 57). Because TRP/TRP₂ heteromultimers are absent in both trp and trpl, the greater sensitivity of the mutant flies to the effects of 2-ABP may reflect a relative lower sensitivity of TRP/TRP₂ heteromultimers to 2-ABP. From these studies it is probable that the action of 2-ABP on blocking TRP channels, at least in fly retinal cells, is through a target other than the InsP₃ receptor.

Considering this information, it was important to assess whether the action of 2-ABP on SOC activation in vertebrate cells was also independent of the InsP₃ receptor. An important tool for such analysis was the InsP₃ receptor knock-out cell line developed by Sugawara et al. (38). The wild-type DT40 chicken B cell line is a useful tool for generating gene knock-outs and expresses all three mammalian subtypes of the InsP₃ receptor (38). The loci of the three InsP₃ receptor subtypes were disrupted sequentially by the targeted introduction of mutations of the alleles of each InsP₃ receptor subtype into the wild-type loci by homologous recombination (38). The resulting triple-InsP₃ receptor gene knock-out cells (DT40InsP₃R-K/o) have been reported to have no phospholipase C-coupled receptor-induced Ca²⁺ release responses, yet apparently retain Ca²⁺ entry in response to store-depletion (38). It has been suggested that the triple InsP₃R-K/o cells could be expressing truncated variants of the InsP₃ receptor and that certain functions of the InsP₃ receptor (for example, physical coupling to plasma membrane entry channels) could be retained in the cells (18); however, there is at present no evidence that a corresponding transcript or protein is expressed in these cells.

Using both wild-type DT40 cells and the DT40 triple InsP₃R-K/o cells, we sought first to assess and compare InsP₃ receptor function directly. Radioactive Ca²⁺ flux measurements using permeabilized cells provide a sensitive means for assessing the Ca²⁺ accumulation and release properties of Ca²⁺ stores (43, 46) and have not previously been conducted in the InsP₃R-K/o cells. Saponin-permeabilized DT40wt cells displayed sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase-mediated Ca²⁺ pumping activity qualitatively similar to many other cell types (42–44), although the absolute uptake capacity of stores (between 0.1 and 0.2 nmol of Ca²⁺/10⁶ cells) was somewhat less, likely as a result of the comparatively smaller size of DT40 cells. In DT40wt cells, application of 10 μM InsP₃ induced rapid release of more than 50% of accumulated Ca²⁺ (Fig. 3A). Ca²⁺ accumulation was almost identical in the DT40InsP₃R-K/o cells; however, in contrast to DT40wt cells, there was no measurable release of Ca²⁺ in response to InsP₃ (Fig. 3B). Moreover, adenophostin A, a powerful InsP₃ receptor agonist with 100-fold greater potency than InsP₃ (58), also had no significant effect on Ca²⁺ release in the DT40InsP₃R-K/o cells (Fig. 3C).

The somewhat small accumulation of Ca²⁺ within DT40 cells made it difficult to conclude with certainty that there was no InsP₃ receptor function. Uptake of Ca²⁺ within the ER lumen can be dramatically enhanced by application of a combination of GTP and oxalate to the uptake medium (41, 42, 59). GTP induces self-association and communication between ER sub-compartments within permeabilized cells, resulting in luminal Ca²⁺ accumulation reflecting the combined activity of a much larger number of sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase pumps (42, 46, 59). The InsP₃-sensitive Ca²⁺ pool is selectively permeable to carboxylate anions, including oxalate, which precipitate Ca²⁺ and greatly increase the Ca²⁺ capacity of the ER lumen (42, 46, 46). Whereas 10 mM oxalate alone has a marginal effect, in combination with 20 μM GTP there is an ∼100-fold increase in the accumulation of Ca²⁺ specifically within the InsP₃-sensitive Ca²⁺ pool (59, 60). In this respect, DT40 cells were similar, and Ca²⁺ accumulation in the pres-
ence of GTP and oxalate continued at a steady rate (Fig. 4). This rate of uptake was sustained for 10s of minutes, resulting in accumulation of Ca\(^{2+}\) 20–50-fold higher than the steady state reached in the absence of GTP and oxalate. Almost all of this Ca\(^{2+}\) uptake was within the InsP\(_3\)-sensitive Ca\(^{2+}\) pool. Thus, inclusion of InsP\(_3\) in the uptake medium resulted in a substantial reduction of Ca\(^{2+}\) accumulation (Fig. 4A).

From a number of experiments the effect of InsP\(_3\) was to prevent 80–90\% of the Ca\(^{2+}\) accumulation in DT40 cells (Fig. 4A). In complete contrast, there was no effect of InsP\(_3\) on Ca\(^{2+}\) accumulation in DT40InsP\(_3\)-R-k/o cells even though there was an almost identical enhancement of Ca\(^{2+}\) accumulation in the presence of GTP and oxalate (Fig. 4B). Even more compelling were the differences in effectiveness of adenophostin A under the same conditions (Fig. 4, C and D); almost 100\% of the accumulation was prevented in DT40w/t cells, whereas there was no releasing action in the DT40InsP\(_3\)-R-k/o cells. The slight enhancement of Ca\(^{2+}\) accumulation observed with adenophostin A in Fig. 4D was not consistent; in four separate experiments with knock-out cells, there was no measurable decrease in uptake with adenophostin A. Thus, under conditions enormously enhancing the amount of Ca\(^{2+}\) sequestered within the InsP\(_3\)-sensitive Ca\(^{2+}\) store and, hence, the Ca\(^{2+}\) release signal mediated by InsP\(_3\) receptors, the results clearly reveal the absence of any functional InsP\(_3\) receptors in the knock-out cells, whereas the responses of wild-type cells are essentially normal. An interesting corollary to this is that, in answer to an earlier question (11), the action of GTP to induce communication between ER subcompartments does not appear related to InsP\(_3\) receptor function.

Given this remarkable divergence in store function measured in the permeabilized cells, it was crucial to assess whether there were any corresponding changes in the operation of stores and the activation of store-operated Ca\(^{2+}\) entry in the intact cells. The data in Fig. 5, A and B, reveal that store function and store-operated Ca\(^{2+}\) entry were each virtually identical in the intact DT40w/t and DT40InsP\(_3\)-R-k/o cells. The release of Ca\(^{2+}\) in response to thapsigargin was the same in both amplitude and rate, indicating that the size of stores and the leak rate from stores after pump blockade were unaffected by the complete absence of functional InsP\(_3\) receptors. This is an interesting result in itself since it indicates that leak of Ca\(^{2+}\) is not attributable to the function of InsP\(_3\) receptors. After removal of extracellular Ca\(^{2+}\), the decrease in cytosolic Ca\(^{2+}\) indicated that the component of the response resulting from store-operated Ca\(^{2+}\) entry was clearly the same in the two cell lines. Readdition of Ca\(^{2+}\) resulted in the almost identical reaccumulation of store-operated Ca\(^{2+}\) entry, confirming the earlier work of Sugawara et al. (38). In many cell types, removal and readdition of Ca\(^{2+}\) after store-emptying results in a large overshoot of store-operated Ca\(^{2+}\) entry, reflecting transient reversal of a Ca\(^{2+}\)-sensitive negative feedback mechanism on SOC activation (11, 61). In contrast, the entry response upon the readdition of Ca\(^{2+}\) in both DT40 cell lines did not include an overshoot, entry merely returning to an approximate steady state level.

Since SOC activation occurred essentially normally in the two cell types, the important question was whether the entry
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**FIG. 5.** Ca\(^{2+}\) store mobilization induced by thapsigargin and subsequent store-operated Ca\(^{2+}\) entry in intact DT40 wild-type cells and the DT40 triple InsP\(_3\) receptor knockout variant cell line. Ca\(^{2+}\) measurements were as described under “Experimental Procedures”; bars indicate times of replacement of medium with nominally Ca\(^{2+}\)-free media (0 Ca\(^{2+}\)). A, Ca\(^{2+}\) levels were monitored after store depletion with 1 μM thapsigargin (arrow) and transient Ca\(^{2+}\) removal in the wild-type DT40 cells (DT40w/t), B, as in A, but in the InsP\(_3\) receptor knockout DT40 variant cells (DT40InsP\(_3\)R-k/o). F, fluorescence.

**FIG. 6.** Store-operated Ca\(^{2+}\) entry is blocked by 2-APB in both wild-type DT40 cells and the InsP\(_3\) receptor knockout variant DT40 cell line. Ca\(^{2+}\) measurements using the wild-type and InsP\(_3\) receptor knockout variant of the DT40 cell line were as described in the legend to Fig. 5; bars indicate times of replacement of medium with nominally Ca\(^{2+}\)-free media (0 Ca\(^{2+}\)) and/or 75 μM 2-APB. A, Ca\(^{2+}\) release induced by the addition of 1 μM thapsigargin (arrow) in Ca\(^{2+}\)-free media followed by Ca\(^{2+}\) entry after the addition of medium containing 1 μM Ca\(^{2+}\) in the wild-type DT-40 cells (DT40wt). B, as in A, but in the InsP\(_3\) receptor knockout variant of the DT40 cells (DT40InsP\(_3\)R-k/o). C, as in A, but in the presence of 75 μM 2-APB (bar) added before the addition of medium containing 1 μM Ca\(^{2+}\). D, as in B, but in the presence of 75 μM 2-APB (bar) added before the addition of medium containing 1 μM Ca\(^{2+}\). F, fluorescence.

InsP\(_3\) receptor may not be a direct target of 2-APB in these or even other cells. It was important to investigate the direct action of 2-APB on the InsP\(_3\) receptors in permeabilized DT40/wt cells. As shown in Fig. 8, 2-APB was a highly effective functional blocker of InsP\(_3\) receptors in DT40/wt cells. Thus, under standard conditions in which InsP\(_3\) induced rapid Ca\(^{2+}\) release (Fig. 8A), the prior addition of 50 μM 2-APB completely prevented the action of InsP\(_3\) (Fig. 8B). Moreover, under conditions in which maximal sustained Ca\(^{2+}\) accumulation within the InsP\(_3\)-sensitive Ca\(^{2+}\) pool was achieved in the presence of GTP and oxalate, the action of InsP\(_3\) to effect release and prevent Ca\(^{2+}\) accumulation (Fig. 8C) was completely blocked by 2-APB (Fig. 8D). In these experiments 2-APB had a modest inhibitory effect on Ca\(^{2+}\) pumping activity in DT40/wt cells; this was the same for pumping activity in DT40InsP\(_3\)R-k/o cells (data not shown), indicating that this was not a reflection of an action of 2-APB on the InsP\(_3\) receptor. Last, we undertook experiments to determine the sensitivity of action of 2-APB on the InsP\(_3\) receptor in permeabilized DT40/wt cells (Fig. 9). The IC\(_{50}\) for 2-APB on InsP\(_3\)-mediated Ca\(^{2+}\) release was ~12 μM (Fig. 9, inset). Although not identical, this value is close to the IC\(_{50}\) values for 2-APB on inhibition of store-operated Ca\(^{2+}\) entry in intact DT40 cells, both wild-type and knock-outs, which as described above were both 15–20 μM. Moreover, these values are very similar to the IC\(_{50}\) values for 2-APB on store-operated channels and TRPC3 channels, which were measured as 10 μM and 10–15 μM, respectively, in HEK293 cells (17).

Overall, these studies provide significant new information on the activation of TRP channels and store-operated Ca\(^{2+}\) entry

In work published by Missiaen et al. (66) subsequent to the submission of this paper, the IC\(_{50}\) for 2-APB on InsP\(_3\) receptor-induced Ca\(^{2+}\) release in permeabilized A7r5 cells was measured as 36 μM. The difference may be cell type-related or may reflect differences between the sources of 2-APB as described under “Materials and Miscellaneous Procedures.”
channels. The actions of 2-APB in the InsP$_3$ receptor knock-out variant of the DT-40 cell line provide compelling evidence that the target through which 2-APB modifies store-operated Ca$^{2+}$ entry is unlikely to be any of the known InsP$_3$ receptor gene products. Certainly, our data provide strong evidence that the DT40-InsP$_3$R-k/o cells are devoid of any functional InsP$_3$ receptors. The only uncertainty about this conclusion is the possibility that the inserted mutated InsP$_3$ receptor gene sequences could give rise to expression of truncated InsP$_3$ receptors deficient in part of the C-terminal pore-forming domain, as recently suggested (18). In experiments of Kiselyov et al. (18), a 154-amino acid C-terminal deletion construct of the type I InsP$_3$ receptor, when expressed in HEK293 cells, was shown to couple and allow InsP$_3$-mediated activation of co-expressed TRPC3 channels. Thus, whereas the C-terminal transmembrane domains of the InsP$_3$ receptor form the release channel, the large cytoplasmic domain appears to couple directly with plasma membrane entry channels (19, 20). However, although the expression of C-terminal deletions could provide an InsP$_3$ receptor target for 2-APB and account for inhibition of SOC activation in the DT40-InsP$_3$R-k/o cells, at present there is no evidence for the existence of any transcript or translation product corresponding to a fragment of the InsP$_3$ receptor in these cells.  

The conclusion that a target other than the InsP$_3$ receptor mediates the action of 2-APB on entry channels gains further support from the ability of 2-APB to inhibit TRP channel activation in Drosophila phototransduction. In Drosophila, there is only a single InsP$_3$ receptor subtype, and homozygous elimination of the InsP$_3$ receptor in fly eyes in the experiments of Acharya et al. (50) and Raghu et al. (51) establish that this InsP$_3$ receptor is not required for rhodopsin-mediated activation of the wild-type light-induced current response. Therefore, the action of 2-APB to inhibit the visual TRP channels may be either by direct channel inhibition or it may reflect interaction of 2-APB with another regulatory protein component that is not the InsP$_3$ receptor. Our results indicate that the action of 2-APB is not restricted to a particular member of the family of TRP channels mediating phototransduction. Thus, it would seem that activation of at least two and possibly all three Drosophila TRP channels is blocked by 2-APB.

However, recent work suggests that 2-APB may not necessarily have a direct inhibitory action on the TRP channels themselves. Thus the Drosophila retinal TRP channels can be reversibly activated by induction of metabolic stress, for example anoxia, ATP depletion, or mitochondrial uncoupling (62). This activation occurs independently of light stimulation and does not require rhodopsin, G$_\text{q}$, or phospholipase C, suggesting it is at a late coupling stage downstream of the light-induced pathway and perhaps upon the channel directly. Whereas light induction of TRP channels is blocked by 2-APB, their direct activation in response to metabolic stress is not blocked by 2-APB. This situation is intriguingly analogous to

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3 T. Kurosaki, unpublished observations.

4 I. Chorna-Ornan, T. Joel-Almagor, H. C. Ben-Ami, S. Frechter, B.
the actions of 2-APB on mammalian TRPC3 channels. Thus, activation of TRPC3 channels by physiological phospholipase C-coupled receptors is blocked by 2-APB, but direct channel activation by application of diacylglycerol is resistant to 2-APB (17). These results would lead us to conclude that the target for 2-APB mediates a step that is upstream from the channel in the pathway leading to physiological activation of TRP channels. Whereas we cannot rule out the possibility that 2-APB is a direct modifier of Ca$^{2+}$ entry channels, we should consider that its actions are rather specific. Thus, 2-APB has little effect on a range of Ca$^{2+}$ and related channels including Ryanodine receptors (47), voltage-sensitive Ca$^{2+}$ entry channels (47), arachidonic acid-activated Ca$^{2+}$ entry channels (63), Ca$^{2+}$ entry channels activated by S-nitrosylation (21), Ca$^{2+}$-activated Cl$^{-}$ entry channels (64), or purinergic P2X receptor Ca$^{2+}$ entry channels. Instead, its action appears restricted to preventing activation of TRP channels, SOCs, and, of course, InsP$_3$ receptors.

Recent data from Braun et al. (65) indicates that 2-APB can block a Ca$^{2+}$ channel activity measured in excised patches from rat basophilic leukemia cells. However, it is not clear whether this current reflects authentic store-operated channels mediating the Ca$^{2+}$ release-activated current (I$_{CRAC}$). The suggestion was that the action of 2-APB was directly at the plasma membrane. One could argue that since 2-APB may be a direct modifier of InsP$_3$ receptors (especially considering the data presented in Fig. 8), then it could as easily be a direct modifier of TRPs and SOCs. However, this would predict that TRPs, SOCs, and InsP$_3$ receptors would share some underlying functional/structural motifs. Considering all these arguments, perhaps a reasonable hypothesis for the action of 2-APB is that it interacts with a target, possibly a regulatory protein, which directly mediates control over SOCs, TRP channels, and InsP$_3$ receptors. Certainly, the important conclusions to be drawn from this work are that, although InsP$_3$R$_5$ do not mediate the action of 2-APB on Ca$^{2+}$ entry, the effects of 2-APB provide evidence for an important similarity in the function of invertebrate TRP channels, mammalian TRP channels, and mammalian store-operated channels.

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