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

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**The mechanism for coupling between $Ca^{2+}$ 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 ($InsP_3R$) was examined using the permeant $InsP_3R$ 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 $InsP_3R$-mediated store release and carbachol-induced $Sr^{2+}$ entry through TRPC3 channels were both reversed at high agonist levels, suggesting $InsP_3Rs$ 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 $InsP_3R$-independent since $InsP_3Rs$ are dispensable for the light response. We used triple $InsP_3R$ knockout DT40 chicken B-cells to further assess the role of $InsP_3Rs$ in SOC activation. $^{45}Ca^{2+}$ flux analysis revealed that although DT40 wild-type cells retained normal $InsP_3Rs$ mediating 2-APB-sensitive $Ca^{2+}$ release, the DT40$InsP_3R$-k/o cells were devoid of functional $InsP_3Rs$. Using intact cells, all parameters of $Ca^{2+}$ store function and SOC activation were identical in DT40wt and DT40$InsP_3R$-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 $IC_{50}$) were identical. The results indicate that (*a*) the action of 2-APB on $Ca^{2+}$ entry is not mediated by the $InsP_3R$ 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.**

Intracellular $Ca^{2+}$ 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 $Ca^{2+}$ 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, inositol 1,4,5-trisphosphate ($InsP_3$), that diffuses rapidly within the cytosol to interact with $InsP_3$ receptors on the ER, which serve as $Ca^{2+}$ channels to release luminal-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 the process known as 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 to replenish intracellular stores (3, 5). Whereas receptor-induced generation of $InsP_3$ and the function of $Ca^{2+}$ release channels to mediate the initial $Ca^{2+}$ signaling phase is well understood, 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 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 $InsP_3$ 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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[1] The abbreviations used are: ER, endoplasmic reticulum; TRP, transient receptor potential, $InsP_3$, 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.

ryanodine 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 (DT40w/t) and triple $InsP_3$ receptor knock-out cell line (DT40$InsP_3$R-k/o) were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine, as described previously (38). Cells of the T3–65 clone of HEK293 cells stably expressing the human TRPC3 channel were cultured as 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 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 $\mu$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. Approximately 95% of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization (39, 40). 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. Details of these $Ca^{2+}$ measurements were described previously for the T3–65 clone of HEK293 cells (17, 21). Resting $Ca^{2+}$ levels in the two DT40 cell lines were similar, ~100–130 nM, and in T3–65 HEK293 cells, resting $Ca^{2+}$ was 50–100 nM. All measurements shown are representative of a minimum of three and, in most cases, a larger number of independent experiments.

*Cell Permeabilization*—The procedures for cell permeabilization were as described earlier (41, 42). Briefly, suspensions of DT40 cells ($1 \times 10^6$ cells/ml) were gently stirred and incubated with 0.0025% saponin in an intracellular-like medium (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 intracellular-like medium 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 $5 \times 10^5$ cells/ml.

*Calcium Flux Experiments*—$Ca^{2+}$ flux measurements were conducted as previously described (42–44). The accumulation of $^{45}Ca^{2+}$ into intracellular organelles was measured using permeabilized DT40 cells ($5 \times 10^5$ cells/ml) maintained with gentle stirring at 37 °C in intracellular-like medium containing 50 $\mu$M $CaCl_2$ (with 150 Ci/mol $^{45}Ca^{2+}$), buffered to 0.1 $\mu$M free $Ca^{2+}$ with EGTA, 3% polyethylene glycol, and 5 $\mu$M ruthenium red (to prevent mitochondrial $Ca^{2+}$ accumulation) in a total volume of 2 ml. Effectors mentioned in the figures ($InsP_3$, adenophostin-A, and 2-APB) were added at the times shown. Oxalate with GTP when present were added immediately before the start of uptake. At the required times, 200-$\mu$l aliquots were removed from the stirred uptake medium, diluted immediately into 4 ml of ice-cold intracellular-like medium containing 1 mM $LaCl_3$, 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 cells and filters in the absence of ATP subtracted (~0.1% of total $Ca^{2+}$). All experiments shown are typical of at least three and, in most cases, a larger number of separate experiments.

*Electroretinogram Recordings in Drosophila Retina*—Wild-type (Canton S), *trp*, and *trpl* mutant flies were immobilized on coverslips in bee's wax, and electroretinogram (ERG) recordings were performed as previously described using orange (580 nm) light (45). To introduce 2-APB or the control solution into the retina of a live fly, a small hole was generated near the edge of the eye using a glass needle. The hole was covered immediately with Vaseline to prevent dehydration of the exposed tissue. 2-APB (100 mM in $Me_2SO$) or $Me_2SO$ (negative control) was diluted 1:200 into Ringer's solution, and glass needles (tip diameter ~8 $\mu$m) containing these solutions were inserted into the holes in the retina. The chemicals were driven into the eye by application of brief pressure using a 1-ml syringe connected to the needles. The injections caused an immediate change in the ERG amplitudes due to an alteration in electrical resistance. This initial change was also observed with vehicle only and therefore did not represent an effect of the 2-APB.

*Materials and Miscellaneous Procedures*—$InsP_3$ and adenophostin-A were from Calbiochem. ATP, GTP, EGTA, carbachol, polyethylene glycol, saponin, ruthenium red, Hepes, and oxalate were purchased from Sigma. 2-APB used in intact cell studies was from Sigma. For the $^{45}Ca^{2+}$ studies using permeabilized DT40 cells, we utilized 2-APB from Tocris (Ballwin, MO) since Sigma 2-APB induced significantly greater inhibition of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase pump-mediated $Ca^{2+}$ uptake. Thapsigargin was from LC Services, Woburn, MA. Fura-2/acetoxymethylester was from Molecular Probes, Eugene, OR. The DT40 cell line was from 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 previously described (46).

## 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 particularly 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

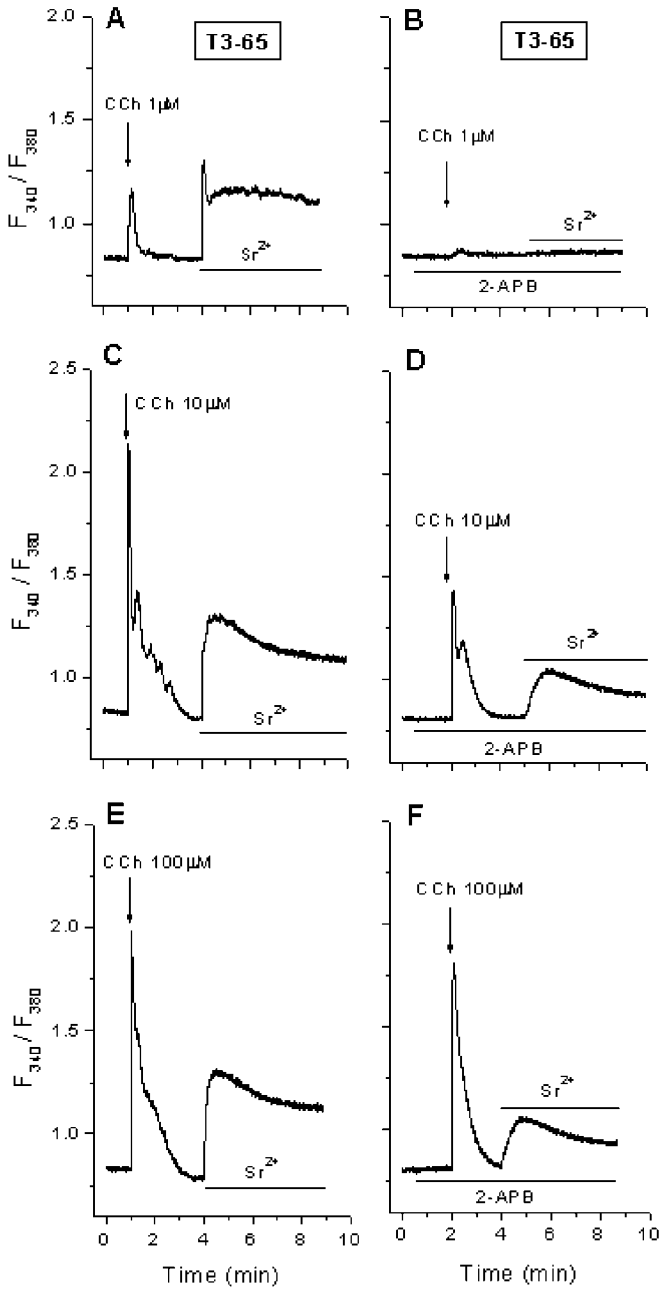


FIG. 1. **Inhibition by 2-APB of muscarinic receptor-induced $Ca^{2+}$ release and $Sr^{2+}$ entry through TRPC3 channels stably transfected HEK293 cells is partially reversed by increased agonist stimulation.** Cytosolic $Ca^{2+}$ was measured in fura-2-loaded T3–65 clone of the HEK293 cells as described under "Experimental Procedures." Standard conditions included 1 mM $Ca^{2+}$ in the external medium; *bars* indicate times of replacement of nominally divalent cation-free medium with medium containing 1 mM $Sr^{2+}$ and/or 75 $\mu$M 2-APB. *A*, TRPC3 channels were activated by the addition of 1 $\mu$M carbachol (*CCh*) (*arrow*) followed by the addition of medium containing 1 mM $Sr^{2+}$. *B*, as in *A*, but in the presence of 75 $\mu$M 2-APB (*bar*). *C*, as in *A*, but TRPC3 channels were activated by the addition of 10 $\mu$M *CCh* (*arrow*) followed by the addition of medium containing 1 mM $Sr^{2+}$. *D*, as in *C*, but in the presence of 75 $\mu$M 2-APB (*bar*). *E*, as in *A*, but hTRP3 channels were activated by the addition of 100 $\mu$M carbachol (*arrow*) followed by the addition of medium containing 1 mM $Sr^{2+}$. *F*, as in *E*, but in the presence of 75 $\mu$M 2-APB (*bar*). *F*, fluorescence.

$Ca^{2+}$, 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. 1*B*). 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. 1*D*), and at 100 $\mu$M carbachol, release and entry were reduced by only ~50% by 2-APB (Fig. 1*F*). 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* 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. 2*A*, *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. 2*A*, *second trace*). The *trp* and *trpl* mutants were more sensitive to the inhibitory effects of 2-APB than wild-type flies (Fig. 2*A*, *third* and *fourth traces*). A plot of the time course of inhibition of light response amplitude by 2-APB is shown in Fig. 2*B*. 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

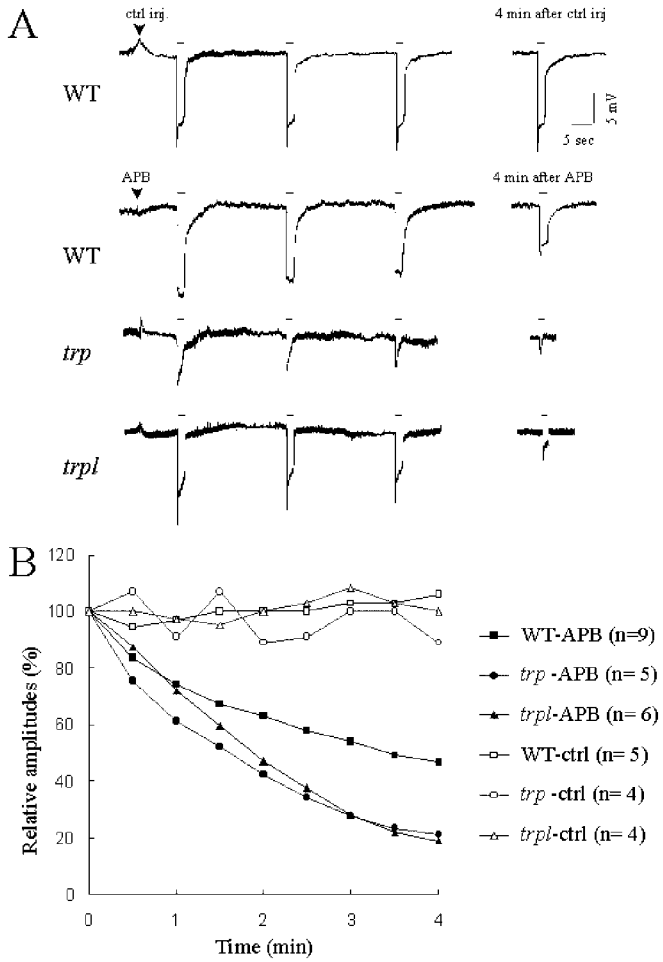


FIG. 2. **The TRP channel-mediated light responses in *Drosophila* eyes are inhibited by 2-APB.** *A*, ERG recording in wild-type (*WT*) flies (Canton S strain) after introduction of the vehicle alone ($Me_2SO$) diluted in Ringer's solution (*ctrl inj*). The flies were exposed to a series of 2-s orange light stimuli with a frequency of 2 pulses/min. An ERG recorded 4 min after the control injection is included to the right. Similar control injections were performed with *trp* and *trpl* flies (data not shown). *trp* flies were exposed to light stimuli of slightly shorter (1.7 s) to avoid the inactivation resulting from repeated exposure to light of 2-s duration. As was the case with wild-type flies, the amplitudes of the *trp* and *trpl* ERGs were largely unchanged over the course of the experiment (data not shown). Event markers showing the initiation and cessation of the light stimuli are indicated above, and time and millivolt scales are presented to the right of the ERGs. *B*, ERG recordings after the introduction of 2-APB (500 $\mu$M final concentration) to the retinas of wild-type (*WT*), *trp*, and *trpl* flies. WT and *trpl* flies were exposed to light pulses of 2-s duration, and *trp* flies were stimulated with 1.7-s light pulses. *C*, relative amplitudes of ERGs in wild-type, *trp*, and *trpl* flies after exposure to 2-APB or the control injections. Shown are the mean ERG amplitudes at a frequency of 2 light pulses/min over the course of 4 min. The numbers of flies assayed (*n*) are indicated. The zero time point represents the first light response 7 s after the injections.

of the TRP channels mediating the *Drosophila* light-induced current appears normal in the absence of $InsP_3$ receptors, the results indicate that the action of 2-APB may be independent of the $InsP_3$ 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/TRPL heteromultimers, and TRPL/TRP$\gamma$ heteromultimers (54, 57). Because TRP/TRPL 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/TRPL heteromultimers to 2-ABP. From these studies it is probable that the action of 2-APB on blocking TRP channels, at least in fly retinal cells, is through a target other than the $InsP_3$ receptor.

Considering this information, it was important to assess whether the action of 2-APB on SOC activation in vertebrate cells was also independent of the $InsP_3$ receptor. An important tool for such analysis was the $InsP_3$ 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 $InsP_3$ receptor (38). The loci of the three $InsP_3$ receptor subtypes were disrupted sequentially by the targeted introduction of mutations of the alleles of each $InsP_3$ receptor subtype into the wild-type loci by homologous recombination (38). The resulting triple-$InsP_3$ receptor gene knock-out cells (DT40$InsP_3$R-k/o) have been reported to have no phospholipase C-coupled receptor-induced $Ca^{2+}$ release responses, yet apparently retain $Ca^{2+}$ entry in response to store-depletion (38). It has been suggested that the triple $InsP_3$R-k/o cells could be expressing truncated variants of the $InsP_3$ receptor and that certain functions of the $InsP_3$ 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 $InsP_3$R-k/o cells, we sought first to assess and compare $InsP_3$ receptor function directly. Radioactive $Ca^{2+}$ flux measurements using permeabilized cells provide a sensitive means for assessing the $Ca^{2+}$ accumulation and release properties of $Ca^{2+}$ stores (43, 46) and have not previously been conducted in the $InsP_3$R-k/o cells. Saponin-permeabilized DT40w/t cells displayed sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase-mediated $Ca^{2+}$ 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 $Ca^{2+}/10^6$ cells) was somewhat less, likely as a result of the comparatively smaller size of DT40 cells. In DT40w/t cells, application of 10 $\mu$M $InsP_3$ induced rapid release of more than 50% of accumulated $Ca^{2+}$ (Fig. 3*A*). $Ca^{2+}$ accumulation was almost identical in the DT40$InsP_3$R-k/o cells; however, in contrast to DT40w/t cells, there was no measurable release of $Ca^{2+}$ in response to $InsP_3$ (Fig. 3*B*). Moreover, adenophostin A, a powerful $InsP_3$ receptor agonist with 100-fold greater potency than $InsP_3$ (58), also had no significant effect on $Ca^{2+}$ release in the DT40$InsP_3$R-k/o cells (Fig. 3*C*).

The somewhat small accumulation of $Ca^{2+}$ within DT40 cells made it difficult to conclude with certainty that there was no $InsP_3$ receptor function. Uptake of $Ca^{2+}$ 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 subcompartments within permeabilized cells, resulting in luminal $Ca^{2+}$ accumulation reflecting the combined activity of a much large number of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase pumps (42, 46, 59). The $InsP_3$-sensitive $Ca^{2+}$ pool is selectively permeable to carboxylate anions, including oxalate, which precipitate $Ca^{2+}$ and greatly increase the $Ca^{2+}$ capacity of the ER lumen (41, 42, 46). Whereas 10 mM oxalate alone has a marginal effect, in combination with 20 $\mu$M GTP there is an ~100-fold increase in the accumulation of $Ca^{2+}$ specifically within the $InsP_3$-sensitive $Ca^{2+}$ pool (59, 60). In this respect, DT40 cells were similar, and $Ca^{2+}$ accumulation in the pres-

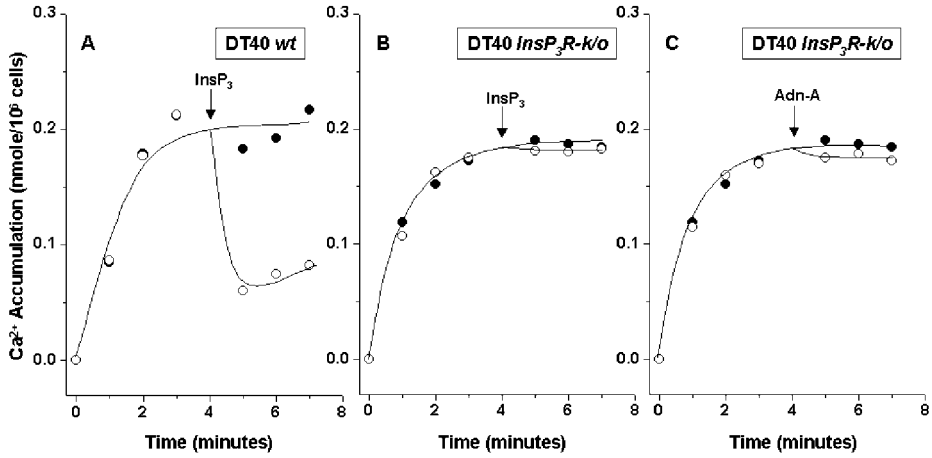


FIG. 3. **$InsP_3$- and adenophostin A-mediated effects on $Ca^{2+}$ release in permeabilized wild-type and triple $InsP_3$ receptor knockout variants of the DT40 cell line.** Cell permeabilization and ATP-dependent $Ca^{2+}$ accumulation were as described under "Experimental Procedures." *A*, uptake was under control conditions (●) or with 10 $\mu$M $InsP_3$ (○) added at 4 min (*arrow*) in wild-type DT40 cells (DT40wt). *B*, as in *A*, but cells were the triple $InsP_3$ receptor knockout variant of the DT40 line (DT40$InsP_3$R-k/o). *C*, as with *B*, but with 500 nM adenophostin A (*Adn-A)* (○) added at 4 min (*arrow*).

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. 4*A*). From a number of experiments the effect of $InsP_3$ was to prevent 80–90% of the $Ca^{2+}$ accumulation in DT40w/t cells (Fig. 4*A*). In complete contrast, there was no effect of $InsP_3$ on $Ca^{2+}$ accumulation in DT40$InsP_3$R-k/o cells even though there was an almost identical enhancement of $Ca^{2+}$ accumulation in the presence of GTP and oxalate (Fig. 4*B*). 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 DT40$InsP_3$R-k/o cells. The slight enhancement of $Ca^{2+}$ accumulation observed with adenophostin A in Fig. 4*D* 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 DT40$InsP_3$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 resumption of store-operated $Ca^{2+}$ entry, confirming the earlier work of Sugawara *et al.* (38). In many cell types, removal and

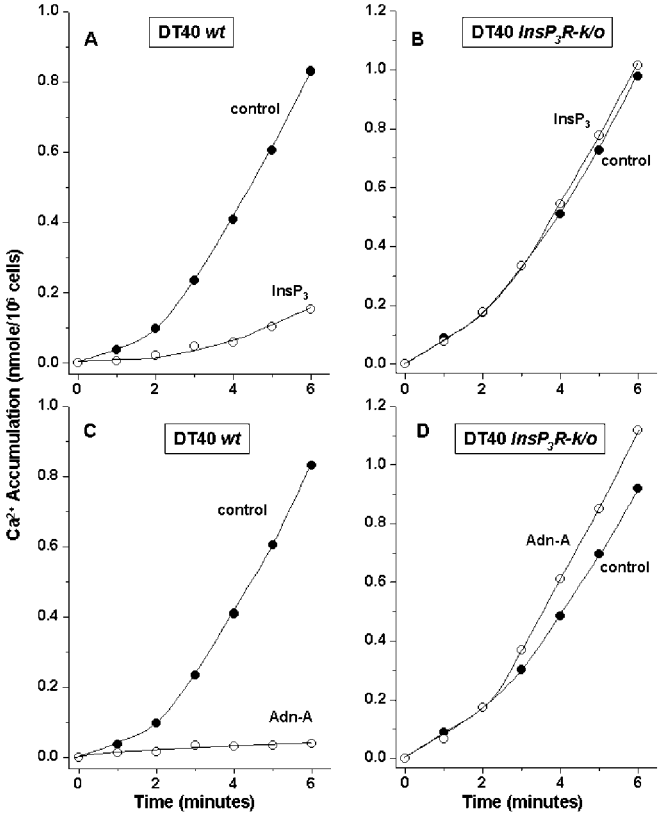


FIG. 4. **Effects of $InsP_3$ and adenophostin-A on $Ca^{2+}$ sequestration enhanced by GTP and oxalate in permeabilized wild-type DT40 cells and permeabilized cells of the $InsP_3$-receptor knock-out variant of the DT40 cell line.** ATP-dependent $Ca^{2+}$ accumulation was measured in the presence of 20 $\mu$M GTP and 10 mM oxalate. All additions were made before initiation of $Ca^{2+}$ accumulation by ATP addition. The conditions were as follows: *A*, control conditions with 20 $\mu$M GTP and 10 mM oxalate (●) or with 10 $\mu$M $InsP_3$ (○) in the wild-type DT40 cells (DT-40wt); *B*, as in *A*, but using the $InsP_3$ receptor knockout variant of the DT40 cells (DT40$InsP_3$R-k/o); *C*, as in *A*, but with 500 nM adenophostin A (*Adn-A)* (○); *D*, as in *B*, but with 500 nM *Adn-A* (●).

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

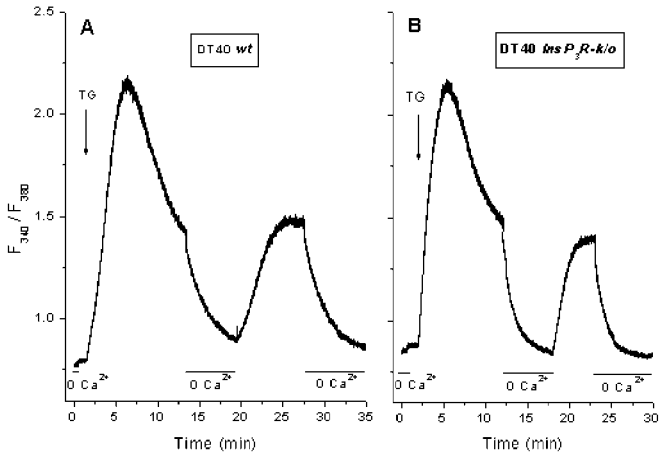


FIG. 5. **$Ca^{2+}$ store mobilization induced by thapsigargin and subsequent store-operated $Ca^{2+}$ entry in intact DT40 wild-type cells and the DT40 triple $InsP_3$ receptor knockout variant cell line.** $Ca^{2+}$ measurements were as described under "Experimental Procedures"; *bars* indicate times of replacement of medium with nominally $Ca^{2+}$-free media (*0 $Ca^{2+}$*). *A*, $Ca^{2+}$ levels were monitored after store depletion with 1 $\mu$M thapsigargin (*arrow*) and transient $Ca^{2+}$ removal in the wild-type DT40 cells (DT40wt). *B*, as in *A*, but in the $InsP_3$ receptor knockout DT40 variant cells (DT40$InsP_3$R-k/o). *F*, fluorescence.

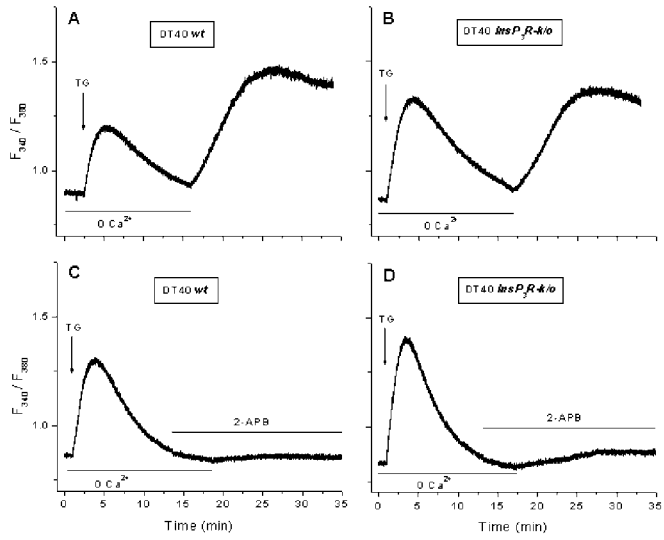


FIG. 6. **Store-operated $Ca^{2+}$ entry is blocked by 2-APB in both wild-type DT40 cells and the $InsP_3$ receptor knockout variant DT40 cell line.** $Ca^{2+}$ measurements using the wild-type and $InsP_3$ receptor knockout variant of the DT-40 cell line were as described in the legend to Fig. 5; *bars* indicate times of replacement of medium with nominally $Ca^{2+}$-free media (*0 $Ca^{2+}$*) and/or 75 $\mu$M 2-APB. *A*, $Ca^{2+}$ release induced by the addition of 1 $\mu$M thapsigargin (*arrow*) in $Ca^{2+}$-free media followed by $Ca^{2+}$ entry after the addition of medium containing 1 mM $Ca^{2+}$ in the wild-type DT-40 cells (DT40wt). *B*, as in *A*, but in the $InsP_3$ receptor knockout variant of the DT40 cells (DT40$InsP_3$R-k/o). *C*, as in *A*, but in the presence of 75 $\mu$M 2-APB (*bar*) added before the addition of medium containing 1 mM $Ca^{2+}$. *D*, as in *B*, but in the presence of 75 $\mu$M 2-APB (*bar*) added before the addition of medium containing 1 mM $Ca^{2+}$. *F*, fluorescence.

was sensitive to the $InsP_3$ receptor antagonist, 2-APB. The answer to this question is clearly affirmative, as shown from the data in Fig. 6. In this experiment, $Ca^{2+}$ entry was activated by $Ca^{2+}$ addition after thapsigargin-induced store depletion in the absence of extracellular $Ca^{2+}$. A comparison of release and entry for DT40w/t and DT40$InsP_3$R-k/o cells under this condition is shown in Fig. 6, *A* and *B*, respectively. If 75 $\mu$M 2-APB was added 5 min before $Ca^{2+}$ addition, the store-operated $Ca^{2+}$ entry was almost completely blocked in both cell types (Fig. 6, *C* and *D*). As important as determining whether 2-APB could alter SOC activity was to determine if the presence or absence of functional $InsP_3$ receptors might influence the kinetics of action of 2-APB on SOC function. The data shown in Fig. 7 address this point. After emptying stores in DT40-w/t cells with thapsigargin, the addition of $Ca^{2+}$ resulted in sustained $Ca^{2+}$ entry lasting for 10s of minutes. The addition of 2-APB caused an immediate inhibition of SOC-mediated $Ca^{2+}$ entry, and $Ca^{2+}$ levels rapidly fell (Fig. 7*A*). This action of 2-APB was identical in the DT40$InsP_3$R-k/o cells (Fig. 7*B*). Moreover, the presence or absence of $InsP_3$ receptors did not influence the return of functional SOC activity after removal of 2-APB. Thus, after the blockade of SOC-mediated $Ca^{2+}$ entry, removal of 2-APB caused a clear return of $Ca^{2+}$ entry in DT40w/t cells (Fig. 7*C*), and this reversal of action of 2-APB was almost identical in DT40$InsP_3$R-k/o cells (Fig. 7*D*). Experiments also assessed the concentration dependence of action of 2-APB to block store-operated $Ca^{2+}$ entry in the two cell types. There was no significant difference in the 2-APB sensitivity of $Ca^{2+}$ entry between DT40w/t and DT40$InsP_3$R-k/o cells, the $IC_{50}$ in both cases being 15–20 $\mu$M (data not shown). Therefore, the actions of 2-APB on store-operated $Ca^{2+}$ entry does appear to be independent of whether or not cells have functional $InsP_3$ receptors.

Considering these results, one further important question about 2-APB was whether it truly did have a direct action upon the $InsP_3$ receptor within DT40 cells. Until now we have used intact cells and shown that 2-APB clearly blocks phospholipase C-coupled receptor agonist responses in a variety of cell types, and this has been presumed to reflect blockade of $InsP_3$-induced activation of $InsP_3$ receptors (17, 21). However, since the actions of 2-APB on $Ca^{2+}$ entry in DT40 cells appear independent of $InsP_3$ receptors, we considered the possibility that the $InsP_3$ 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 DT40w/t cells. As shown in Fig. 8, 2-APB was a highly effective functional blocker of $InsP_3$ receptors in DT40w/t cells. Thus, under standard conditions in which $InsP_3$ induced rapid $Ca^{2+}$ release (Fig. 8*A*), the prior addition of 50 $\mu$M 2-APB completely prevented the action of $InsP_3$ (Fig. 8*B*). 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. 8*C*) was completely blocked by 2-APB (Fig. 8*D*). In these experiments 2-APB had a modest inhibitory effect on $Ca^{2+}$ pumping activity in DT40w/t cells; this was the same for pumping activity in DT40$InsP_3$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 DT40w/t cells (Fig. 9). The $IC_{50}$ for 2-APB on $InsP_3$-mediated $Ca^{2+}$ release was ~12 $\mu$M (Fig. 9, *inset*).[2] 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 $\mu$M. 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 $\mu$M and 10–15 $\mu$M, respectively, in HEK293 cells (17).

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

[2] 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 $\mu$M. The difference may be cell type-related or may reflect differences between the sources of 2-APB as described under "Materials and Miscellaneous Procedures."

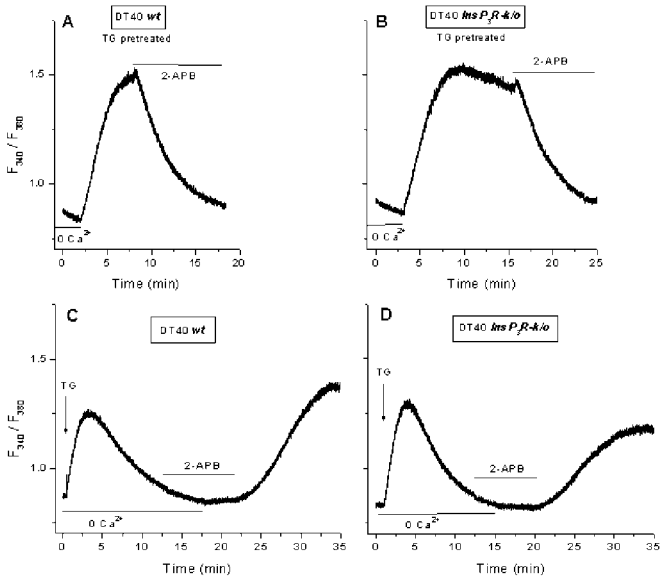


FIG. 7. **Acute addition of 2-APB rapidly blocks store-operated $Ca^{2+}$ entry after store depletion, and this inhibition by 2-APB is reversible in both the wild-type and the $InsP_3$-receptor knockout variant of the DT-40 cell line.** $Ca^{2+}$ measurements using the wild-type and $InsP_3$ receptor knockout variant of the DT-40 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 $\mu$M 2-APB. *A*, $Ca^{2+}$ entry upon the addition of medium containing 1 mM $Ca^{2+}$ in wild-type DT40 cells (DT40wt) after prior pool depletion with 1 $\mu$M thapsigargin (not shown) can be blocked by the acute addition of 75 $\mu$M 2-APB (*bar*). *B*, as in *A*, but in the $InsP_3$ receptor knockout variant of the DT40 cell line (DT40$InsP_3$R-k/o). *C*, recovery of store-operated $Ca^{2+}$ entry after the removal of 75 $\mu$M 2-APB in wild-type DT40 cells (DT-40wt) after pool depletion with 1 $\mu$M thapsigargin (*arrow*). *D*, as in *C*, but in the $InsP_3$ receptor knockout variant of the DT40 cells (DT40$InsP_3$R-k/o). *F*, fluorescence.

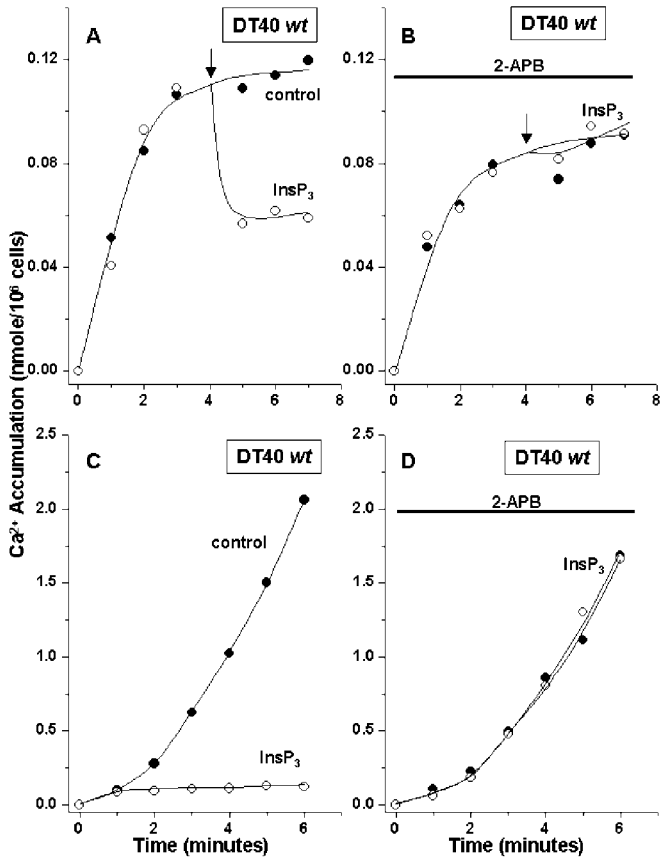


FIG. 8. **$InsP_3$-induced $Ca^{2+}$ store release is completely blocked by 2-APB in permeabilized wild-type DT-40 cells.** ATP-dependent $Ca^{2+}$ accumulation and oxalate-induced GTP-dependent $Ca^{2+}$ sequestration in permeabilized wild-type DT40 cells were measured as described in the legends to Figs. 3 and 4. *A*, uptake was under control conditions (●) or with 10 $\mu$M $InsP_3$ (○) added at 4 min (*arrow*). *B*, as in *A*, but with 50 $\mu$M 2-APB present throughout (*bar*). *C*, ATP-dependent $Ca^{2+}$ accumulation was measured under control conditions in the presence of 20 $\mu$M GTP and 10 mM oxalate (●) or in the presence of 10 $\mu$M $InsP_3$ (○). All additions were made before $Ca^{2+}$ accumulation. *D*, as in *C*, but with 50 $\mu$M 2-APB present throughout (*bar*).

channels. The actions of 2-APB in the $InsP_3$ receptor knock-out cells 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.[3]

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, $G_q$, or phospholipase C, suggesting it is at a late coupling stage downstream of the light-induced pathway and perhaps upon the channel directly. However, 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.[4] This situation is intriguingly analogous to

[3] T. Kurosaki, unpublished observations.

[4] I. Chorna-Ornan, T. Joel-Almagor, H. C. Ben-Ami, S. Frechter, B.

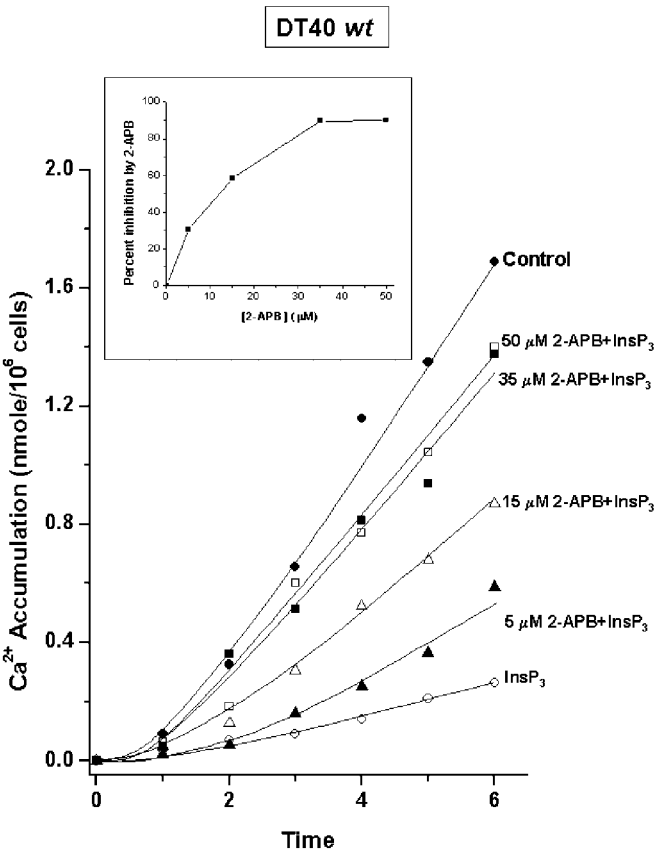


FIG. 9. **Concentration dependence of 2-APB-induced inhibition of $Ca^{2+}$ release through $InsP_3$ receptors in permeabilized wild-type DT40 cells.** ATP-dependent $Ca^{2+}$ accumulation and oxalate-induced GTP-dependent $Ca^{2+}$ sequestration in permeabilized wild-type DT40 cells were measured as described in the legends to Fig. 3. Uptake was under control conditions (●) or with 10 μM $InsP_3$ and either without 2-APB (○) or with 5 μM 2-APB (▲), 15 μM 2-APB (△), 35 μM 2-APB (□), or 50 μM 2-APB (■) present throughout. All additions were made prior to $Ca^{2+}$ accumulation. *Inset*, plot of the concentration dependence of the action of 2-APB on $InsP_3$ receptor-mediated $Ca^{2+}$ release in permeabilized DT40 cells, revealing an $IC_{50}$ of ~12 μM.

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),[4] or purinergic P2X receptor $Ca^{2+}$ entry channels.[5] 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 $InsP_3$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.

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