lethality of *ess1* mutants in budding yeast, but the C109A mutant is inactive in the complementation assay (*31*). Together, these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Pin1 participates in the replication checkpoint in a manner requiring its catalytic activity. Target-specific inhibition of mitosis-promoting kinase activity provides a biochemical mechanism for Pin1's role in enabling the replication checkpoint. Pin1 could mediate inhibition of Cdc25 hyperphosphorylation and act in concert with 14-3-3–mediated cytoplasmic sequestration of the phosphatase to prolong the G_2 phase by preventing functional interaction of Cdc25 with Cdc2 under checkpoint conditions. This function is consistent with previous observations that the G_2 phase is prolonged when Pin1 is overexpressed. In the absence of DNA perturbation, consequences of Pin1 removal may not be manifested; this would explain the lack of apparent phenotype in Pin1-null metazoans. Our demonstration of the essential role of Pin1 in the replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.

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39. The Cdc25 antibody was a gift from E. Shibuya. The MPM-2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

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Requirement of the Inositol Trisphosphate Receptor for Activation of Store-Operated Ca^{2+} Channels

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The coupling mechanism between endoplasmic reticulum (ER) calcium ion (Ca^{2+}) stores and plasma membrane (PM) store-operated channels (SOCs) is crucial to Ca^{2+} signaling but has eluded detection. SOCs may be functionally related to the TRP family of receptor-operated channels. Direct comparison of endogenous SOCs with stably expressed TRP3 channels in human embryonic kidney (HEK293) cells revealed that TRP3 channels differ in being store independent. However, condensed cortical F-actin prevented activation of both SOC and TRP3 channels, which suggests that ER-PM interactions underlie coupling of both channels. A cell-permeant inhibitor of inositol trisphosphate receptor ($InsP_3R$) function, 2-aminoethoxydiphenyl borate, prevented both receptor-induced TRP3 activation and store-induced SOC activation. It is concluded that $InsP_3Rs$ mediate both SOC and TRP channel opening and that the $InsP_3R$ is essential for maintaining coupling between store emptying and physiological activation of SOCs.

Receptor-induced Ca^{2+} signals comprise two interdependent components—rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating PM SOCs. The trigger for SOC activation is decreased Ca^{2+} in the ER lumen (*1, 2*). However, despite intense study, the ER-derived signal coupling store depletion with SOC activation remains unknown (*3*). Direct coupling between ER and PM has been hypothesized (*4, 5*), and evidence indicates that physical docking of ER with the PM is involved in SOC activation (*6–8*). The mammalian TRP family of receptor-operated ion channels has been suggested to share some operational

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parameters with SOCs (*9*). Kiselyov *et al.* (*10, 11*) provided evidence that human TRP3 channel activation results from interaction with $InsP_3Rs$. However, other evidence indicates that diacylglycerol (DAG), not $InsP_3$, is the phospholipase C (PLC) product that mediates activation of TRP3 channels (*12*) and that TRP3 channels operate independently of stores (*12–15*). We show here that physical interaction between ER and PM is necessary for activation of both TRP and SOC, and we provide new evidence that the $InsP_3R$ is an essential component for mediating and maintaining coupling between store emptying and physiological activation of SOCs.

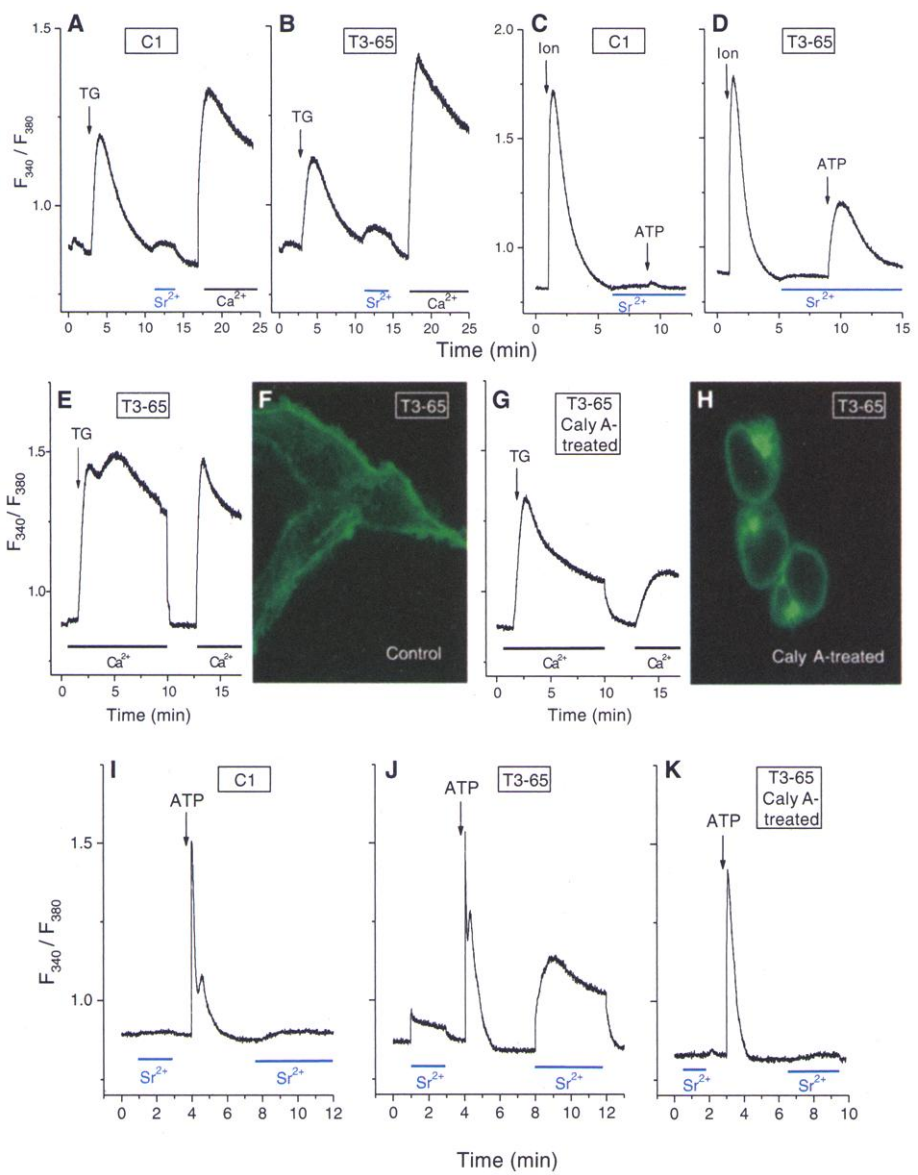

Fig. 1. Correlation of ER-PM interactions with activation of SOC and TRP3 channels. (**A** to **D**) Comparison of Sr^{2+} and Ca^{2+} entry in fura-2-loaded clonal T3-65 HEK293 cells stably transfected with the hTRP3 gene and control-transfected clonal C1 cells (*14*). Cytosolic Sr^{2+} and Ca^{2+} was measured by ratiometric (F_{340}/F_{380}) fluorescence of groups of 5 to 10 fura-2-loaded cells as described (*23*). (A and B) TG (1 μM) (arrows) was added to C1 or T3-65 cells in the absence of Ca^{2+} followed by addition of 1 mM Sr^{2+}. Medium was replaced with divalent cation-free medium, to which 1 mM Ca^{2+} was then added (bars). (C and D) Cells were treated with 5 μM ionomycin (Ion) (arrows) in the absence of Ca^{2+}; 1 mM external Sr^{2+} (bars) and 100 μM ATP (arrows) were then added to the external medium. (**E** to **H**) Effects of 50 nM calyA treatment (45 min, 22°C) on TG-induced Ca^{2+} responses and F-actin distribution in T3-65 cells. Cytosolic Ca^{2+} was measured in response to 1 μM TG (arrows) in control (E) or calyA-treated (G) T3-65 cells with or without external Ca^{2+} (bars). Confocal images of F-actin labeled with fluorescein isothiocyanate–conjugated phalloidin in control (F) or calyA-treated (H) T3-65 cells. Imaging and F-actin analysis were as described (*6*). (**I** to **K**) Cytosolic fura-2 ratiometric measurements in Ca^{2+}-free medium in response to 100 μM ATP (arrows) in the presence (bars) or absence of 1 mM external Sr^{2+} in C1 cells (I), control T3-65 cells (J), or T3-65 cells treated with calyA (K) as above. TG, ionomycin, and ATP were maintained after addition.

To directly compare the function of endogenous SOCs and TRP3 channels, we used T3-65 clonal human embryonic kidney (HEK293) cells stably transfected to express the human TRP3 (hTRP3) channel (*14*). Both channels function in T3-65 cells, whereas only SOCs function in the control-transfected clonal C1 line. We used Sr^{2+} entry to identify and distinguish SOC and TRP3 channel function. After release of Ca^{2+} from stores with the Ca^{2+} pump blocker thapsigargin (TG) in the absence of external Ca^{2+}, Sr^{2+} addition resulted in little Sr^{2+} entry in either C1 or T3-65 cells (Fig. 1, A and B). Ca^{2+} addition resulted in Ca^{2+} entry in both cell lines, which is typical of SOC activation (*16*). The ionophore ionomycin induced more rapid and complete release of stored Ca^{2+} and again little Sr^{2+} entry in either cell type (Fig. 1, C and D). Addition of adenosine triphosphate (ATP) to stimulate PLC-coupled purinergic receptors caused entry of Sr^{2+} in T3-65 cells but not in C1 cells, revealing TRP3 channel function in T3-65 cells. Thus, the TRP3 channel is clearly not activated by store emptying alone, with either TG or ionomycin; instead, it appears to be activated by a product of receptor-induced PLC. Redistribution of actin (caused, for example, by phosphatase inhibitors) to form a dense cortical layer beneath the PM displaced cortical ER and prevented SOC activation by store emptying, providing evidence that ER-PM interactions are required for SOC activation (*6*). In T3-65 cells, the slowly developing SOC-mediated Ca^{2+} entry in response to store emptying (Fig. 1E) was substantially reduced after treatment with the phosphatase inhibitor calyculin A (calyA) (Fig. 1G); this action correlated with the formation of a dense cortical actin layer (Fig. 1, F and H). If TRP3 were directly activated by receptors in the PM, we reasoned that TRP3 activation might be insensitive to calyA. However, whereas calyA had no effect on receptor-induced ($InsP_3$-mediated) Ca^{2+} release, it completely blocked receptor-mediated TRP3 activation in T3-65 cells (Fig. 1, I to K), suggesting that TRP activation, as for SOCs, involves coupling between the PM and another organelle.

TRP3-mediated Sr^{2+} entry was increased by the membrane permeable DAG analog 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Fig. 2A) and increased less effectively by another permeable analog, 1,2-dioctanoyl-*sn*-glycerol (DOG) (Fig. 2B). The DAG lipase inhibitor RHC80267 (RHC) also induced Sr^{2+} entry (Fig. 2C). These results are consistent with activation of TRP channels by DAG (*12*). They appear to be a specific reflection of TRP3 channels because there was no action of any agent with C1 cells (Fig. 2, A to C).

We obtained similar results for TRP-mediated Ca^{2+} entry (Fig. 2, D to F), indicating that DAG is not activating SOC. Even after agonist, OAG induced no Sr^{2+} entry (Fig. 2G), again suggesting no endogenous TRP activity in C1 cells. After ATP-induced Sr^{2+} entry through TRP3 channels in T3-65 cells, the addition of OAG induced a small further increase in Sr^{2+} entry (Fig. 2H). We compared the action of calyA on both agonist- and DAG-induced TRP3 activation. Whereas calyA treatment eliminated agonist-induced Sr^{2+} entry, the action of OAG was unaffected (Fig. 2I). This indicates that ER-PM interactions mediate only agonist-induced TRP3 activation, whereas DAG may activate TRP3 directly. The inability of agonist to stimulate TRP3 in calyA-treated cells suggests that DAG does not contribute to PLC-coupled receptor-induced TRP3 activation, which implies that receptors activate TRP3 only through $InsP_3$.

Much evidence indicates that TRP3 channels operate independently of Ca^{2+} stores (*12–15*) (Fig. 1). We therefore questioned how related SOC and TRP really are and whether $InsP_3Rs$ play any role in their activation. The membrane-permeant $InsP_3R$ antagonist 2-aminoethoxydiphenyl borate (2-APB) (*17*) proved to be a remarkably effective probe for assessing $InsP_3R$ involvement in situ. 2-APB at 75 μM blocked receptor-induced store emptying in intact HEK293 cells and all other cells tested, regardless of PLC-coupled agonist (Fig. 3, A to E). 2-APB had no effect on basal cytosolic Ca^{2+} or on the size of ionophore-releasable stores in intact cells, which indicates no change in Ca^{2+} homeostasis. In broken cells, 2-APB directly blocks $InsP_3R$-mediated Ca^{2+} release from ER (*18*). 2-APB has no effect on $InsP_3$ binding, does not alter $InsP_3$ production through agonist-sensitive PLC, and does not modify the function of ryanodine receptors or voltage-operated Ca^{2+} channels (*17*).

In T3-65 cells, ATP-induced Ca^{2+} release and subsequent TRP3-mediated Sr^{2+} entry were almost completely inhibited by 75 μM 2-APB (Fig. 3, F and G). However, the action of OAG was unaffected. To test whether the block of ATP-induced store emptying might have prevented TRP3 activation, we conducted the same experiment after complete store emptying with ionomycin and obtained similar results (Fig. 3, H and I). Thus, the $InsP_3R$ antagonist blocked receptor-induced TRP3 activation. The lack of effect of 2-APB on DAG-induced TRP3 activation confirms the distinction between TRP3 activation by DAG and agonist and indicates that 2-APB does not block the TRP3 channel per se.

Another permeant $InsP_3R$ antagonist, xestospongin C (xestC) (*19*), induced similar effects. However, the action of xestC was very slow, requiring 20 min at 20 μM to prevent TRP activation, and only partly prevented agonist-induced Ca^{2+} release (Fig. 3J). This likely reflects a latency of blockade of $InsP_3Rs$ deeper within cells (*20*). Upon removal of xestC, there was no reversal of inhibition (Fig. 3J). In contrast, 2-APB rapidly prevented Ca^{2+} release and TRP-mediated Sr^{2+} entry and was fully reversible. Thus, removal of 2-APB resulted in immediate return of entry (Fig. 3K).

We also conducted this experiment after store depletion with ionomycin (to eliminate Ca^{2+} release through reactivated $InsP_3Rs$), revealing the return of TRP-mediated Sr^{2+} entry alone after 2-APB removal (Fig. 3L). Prior application and removal of 2-APB resulted in the return of full agonist-induced store release and Sr^{2+} entry (Fig. 3M). The 2-APB dose-response curve for TRP-mediated Sr^{2+} entry was close to that for receptor-induced Ca^{2+} store release (Fig. 3N), with median inhibitory concentration (IC_{50}) values of 10 and 25 μM, respectively. The small difference in IC_{50} again may reflect a latency of action of 2-APB on more remote $InsP_3Rs$ mediating Ca^{2+} release as opposed to those coupled to PM TRP3 channels.

The results with calyA and 2-APB pro-

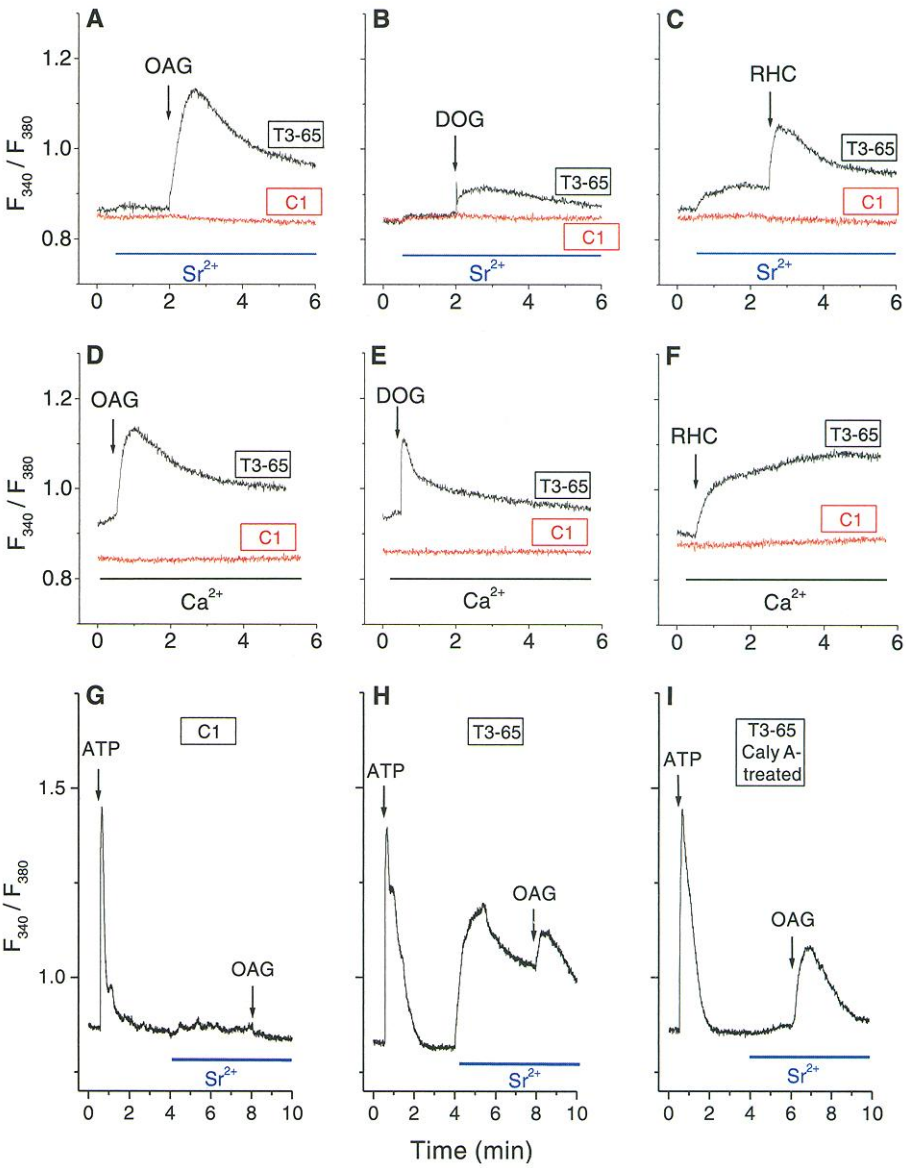

Fig. 2. Activation of TRP3 channels by two distinct mechanisms. (**A** to **C**) Cytosolic fura-2 ratiometric measurements in T3-65 cells or C1 cells in response to 100 μM OAG (A), 100 mM DOG (B), or 50 μM RHC (C) in the presence of 1 mM external Sr^{2+} (bar). (**D** to **F**) Cytosolic fura-2 measurements in T3-65 cells or C1 cells in response to 100 μM OAG (D), 100 μM DOG (E), or 50 μM RHC (F) with 1 mM external Ca^{2+} (bar). (**G** to **I**) Cytosolic fura-2 measurements in Ca^{2+}-free medium in response to 100 μM ATP (arrows) and 100 μM OAG (arrows) in the presence (bars) or absence of 1 mM external Sr^{2+} in C1 cells (G), control T3-65 cells (H), or T3-65 cells treated with calyA (I) as above. ATP and DAG analogs were maintained after addition.

vide good evidence that ER $InsP_3Rs$ are required for coupling PLC activation to TRP3 channel opening. Considering the important differences between TRP and SOC function described here and by others (*12–15*), a crucial question was whether SOC activation has any similar $InsP_3R$ requirement. SOC was measured after TG-induced store emptying in C1 cells (Fig. 4A); removal and readdition of external Ca^{2+} revealed the familiar overshoot response due to reactivation of SOCs (*6, 16*). The store-release component was observed in the absence of external Ca^{2+}, and later addition of Ca^{2+} again resulted in full SOC-mediated Ca^{2+} entry (Fig. 4B). Addition of 2-APB just before addition of TG resulted in complete inhibition of SOC activation; even the response to Ca^{2+} removal and readdition was eliminated (Fig. 4C). 2-APB did not prevent Ca^{2+} release with TG (*21*). Prolonged incubation with xestC reduced but did not eliminate TG-induced SOC activation (Fig. 4D). The action of both 2-APB and xestC on SOC indicates that $InsP_3Rs$ are required for SOC activation. The dose-response relationship for 2-APB on SOC blockade was close to that for TRP blockade (IC_{50} 10 to 15 μM). After complete ionomycin-induced store emptying, 2-APB eliminated Ca^{2+} entry (Fig. 4E).

Addition of 2-APB while SOC-mediated entry was maximally active resulted in rapid termination of SOC (Fig. 4F). This indicates that the $InsP_3R$ is required for maintenance as well as activation of SOCs. Analysis of the 2-APB–induced reversal of SOC revealed a 12-s delay (Fig. 4G), whereas 2-APB–induced reversal of TRP3-mediated Sr^{2+} entry had no detectable lag (Fig. 4H). The rapid action of 2-APB caused us again to question whether the action of 2-APB on Ca^{2+} entry was $InsP_3R$ related. 2-APB blocked intracellular $InsP_3Rs$ extremely rapidly (Fig. 4I); added with agonist, $InsP_3R$-mediated Ca^{2+} release from stores was reduced 50%; added 20 s before agonist release, it was reduced 80%. Thus, 2-APB can reach and block function of intracellular $InsP_3Rs$ with sufficient rapidity to account for its blockade of SOC activation. After 2-APB–induced SOC blockade, removal of 2-APB resulted in a slow return of SOC activity over several minutes (Fig. 4J). PLC activation with carbachol (CCh) to produce a high level of cytosolic $InsP_3$ resulted in larger and faster reappearance of Ca^{2+} entry (Fig. 4K), whereas CCh induced no return of Ca^{2+} entry if 2-APB remained present (Fig. 4E). This supports the conclusion that the activation and return of SOC function is mediated through $InsP_3R$ activation.

Our results support a central role of the $InsP_3R$ in mediating Ca^{2+} entry. The results also highlight differences in the operation of the two entry channels. TRP3 is activated rapidly in response to PLC-coupled receptors or DAG but is not directly activated by store emptying. SOC activity develops relatively slowly in response to store emptying (*6, 22*) but is not activated by DAG. Despite these differences, both channels can be uncoupled by calyA-induced cortical actin rearrangement, which suggests that physiological activation in each case requires ER-PM interactions. Receptor-induced activation of the

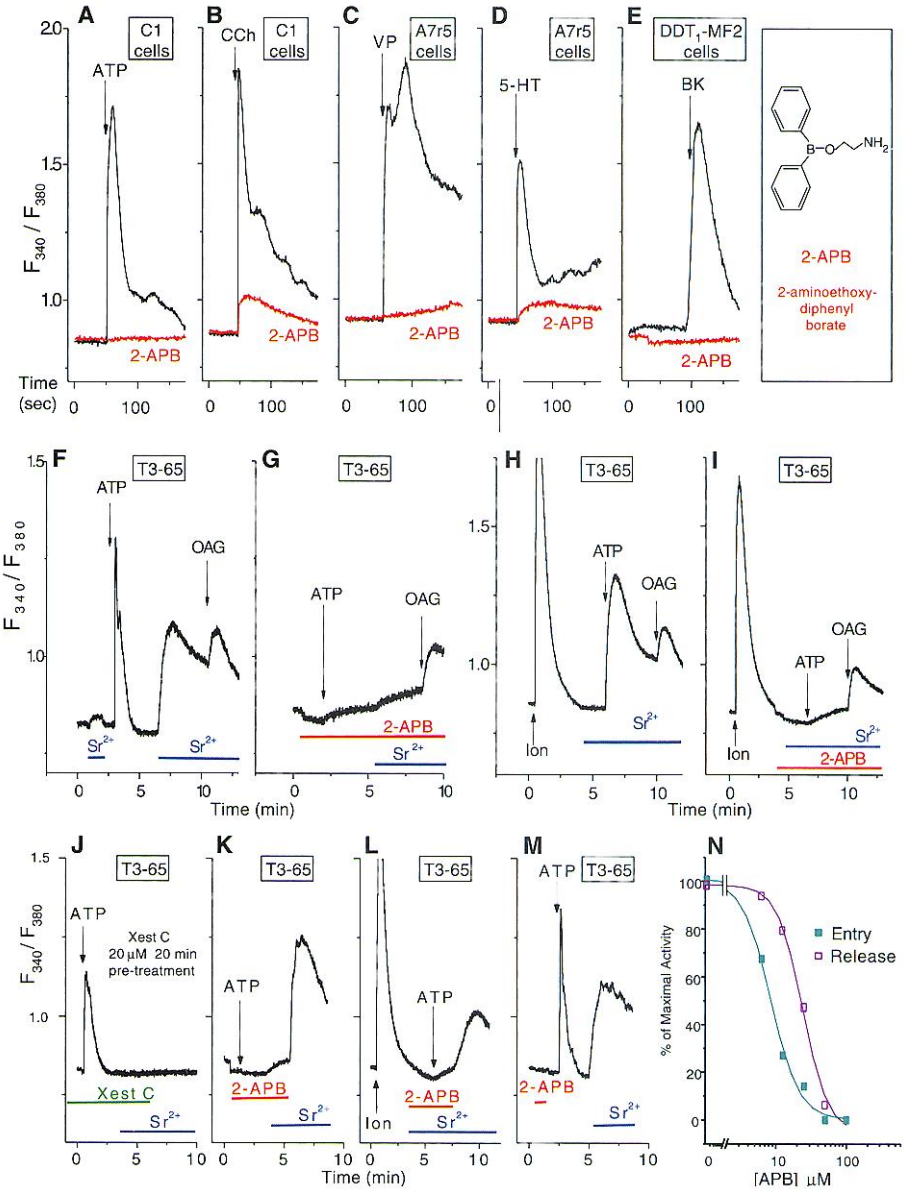

Fig. 3. Inhibition by 2-APB of TRP3 channel activation through PLC-coupled receptors. (**A** to **E**) 2-APB (75 μM) prevented PLC-coupled receptor-induced Ca^{2+} release in response to 100 μM ATP and 10 μM CCh in C1 cells (A and B), 1 nM vasopressin (VP) or 1 μM serotonin (5-HT) in A7r5 cells (C and D) or 10 μM bradykinin (BK) in DDT_1MF2 cells (E). (**F** to **I**) Ca^{2+} release and TRP3-mediated Sr^{2+} entry activated by ATP were blocked by 2-APB in T3-65 cells; DAG-induced TRP3 activation was unaffected. (F and G) Fura-2 responses to 100 μM ATP (arrows) and 100 μM OAG (arrows) in the presence or absence of 1 mM external Sr^{2+} with (F) or without (G) 75 μM 2-APB. (H and I) Effects of 100 μM ATP (arrows) and 100 μM OAG (arrows) on Sr^{2+} entry after store depletion with 5 μM ionomycin (Ion) (arrows). (**J**) Fura-2 measurements in response to 100 μM ATP (arrow) in the presence or absence of 1 mM external Sr^{2+} in T3-65 cells pretreated with 20 μM xestC for 20 min and maintained until removal. (**K** to **M**) Effect of transient 2-APB (75 μM) addition on ATP-induced Sr^{2+} entry in T3-65 cells either without ionomycin (K and M) or after Ca^{2+} store release with 5 μM ionomycin (L). (**N**) Concentration dependence of 2-APB–induced inhibition of ATP-mediated Ca^{2+} release and TRP3-mediated Sr^{2+} entry in T3-65 cells. Both activities were measured consecutively on one coverslip after addition of 2-APB 90 s before addition of 100 μM ATP (in Ca^{2+}-free medium) followed by addition of Sr^{2+} 3.5 min later. Results are single measurements made on a series of coverslips. A7r5 and DDT_1MF2 smooth muscle cells were used as described (*6*). 2-APB synthesis was described (*17*) and xestC was from I. Pessah (University of California, Davis).

TRP3 channel appears to be mediated by the $InsP_3R$. Furthermore, the elusive coupling of the physiologically important endogenous SOC itself appears to be $InsP_3R$-mediated. Thus, SOC and TRP can both be considered $InsP_3R$-mediated channels, a conclusion highly compatible with the evidence here and previously (*6*) for the role of ER-PM interactions in SOC activation. We suggested that store emptying promotes reversible docking of ER with PM to activate SOC (*6, 7*). Yao *et al.* (*8*) considered that a similar docking process might activate Ca^{2+} entry through insertion of SOCs into the PM. Our results reveal that $InsP_3R$ blockade rapidly deactivates already activated SOCs, suggesting that channel activation is maintained by continued close contact with $InsP_3Rs$ and militating against an insertion model. Instead, it provides strong evidence in favor of a conformational coupling model for SOC (*4, 5*). The latency in SOC activation, together with our recent structural and functional evidence (*6*), suggests that SOC activation requires that $InsP_3Rs$ on the ER membrane are moved to the vicinity of SOCs and that this trafficking is the basis of activation by store emptying.

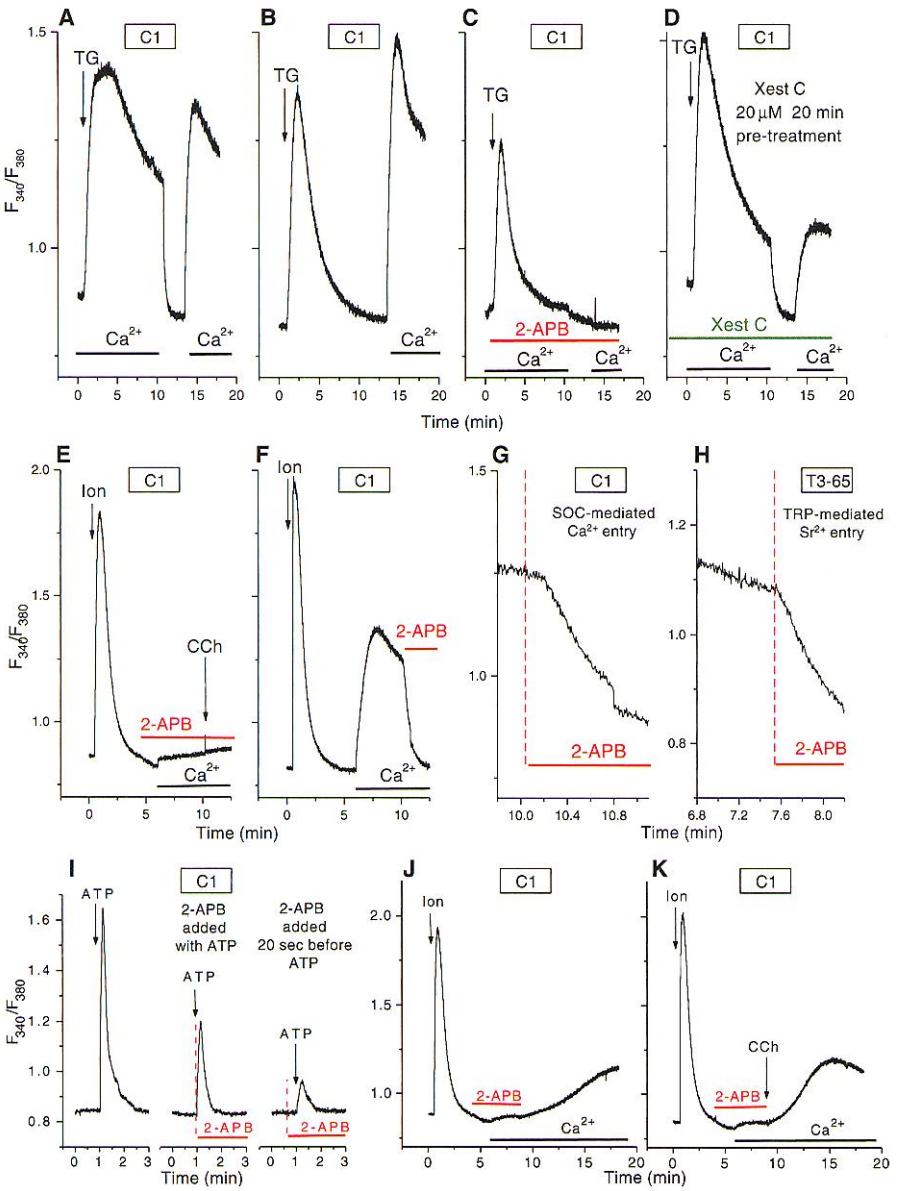

Fig. 4. Inhibition and reversal of SOC opening by $InsP_3R$ inhibitors. (**A** and **B**) Ca^{2+} release and entry in control-transfected C1 cells activated by 1 μM TG in the presence or absence of external Ca^{2+}. (**C**) Ca^{2+} entry after 1 μM TG in the presence of 1 mM Ca^{2+} and after subsequent removal and addition of Ca^{2+} was blocked by 75 μM 2-APB. (**D**) TG-induced Ca^{2+} entry as for (C) was partly blocked after xestC treatment (20 min, 20 μM). (**E**) SOC-mediated Ca^{2+} entry after Ca^{2+} release with 5 μM ionomycin was blocked by 75 μM 2-APB; addition of 100 μM CCh with continued 2-APB was without effect. (**F**) Sustained SOC-mediated Ca^{2+} entry after 5 μM ionomycin-induced store release was rapidly terminated by addition of 75 μM 2-APB. (**G** and **H**) Comparison of 2-APB–induced termination of sustained SOC-mediated Ca^{2+} entry in C1 cells (G) and sustained TRP3-mediated Sr^{2+} entry in T3-65 cells (H). Results in (G) were from an experiment identical to that shown in (F) and mean lag time was 12.4 ± 0.6 s (n = 4). Results in (H) were for Sr^{2+} entry in T3-65 cells activated by 100 μM ATP with addition of 75 μM 2-APB; lag-time was <3 s. (**I**) Rapidity of action of 2-APB to inhibit ATP-induced Ca^{2+} store release in C1 cells: 100 μM ATP was added either alone (left), simultaneously with 75 μM 2-APB (middle), or 20 s after addition of 75 μM 2-APB (right). (**J** and **K**) Receptor-induced $InsP_3$ production enhanced reversal of 2-APB–induced blockade of SOC activated by ionomycin in C1 cells. (J) 2-APB (75 μM) was added (bar) after store release with 5 μM ionomycin, and Ca^{2+} was added and 2-APB was removed. (K) Identical to (J) except 100 μM CCh was added and 2-APB was removed.

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20. The IC_{50} of xestC on $InsP_3$ receptor-mediated Ca^{2+} release in microsomes is 358 nM (*19*); the slow and insensitive action of xestC on intact cells likely reflects poor membrane permeability. $InsP_3$ receptors close to the PM may be selectively sensitive.
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23. The hTRPC3-transfected T3-65 and control-transfected C1 clonal line HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing G418 at 400 μg/ml as described (*14*). Cells were passaged twice weekly and plated on coverslips overnight before fura-2 loading and ratiometric analysis of cytosolic Ca^{2+} as described (*6*). Results are typical of at least three and usually more separate experiments. TG, $InsP_3$, ionomycin, calyA, OAG, DOG, and RHC80267 were from Calbiochem.

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