Role of the Phospholipase C-Inositol 1,4,5-Trisphosphate Pathway in Calcium Release-activated Calcium Current and Capacitative Calcium Entry*

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We investigated the putative roles of phospholipase C, polyphosphoinositides, and inositol 1,4,5-trisphosphate (IP3) in capacitative calcium entry and calcium release-activated calcium current (Ica) in laerical acinar cells, rat basophilic leukemia cells, and DT40 B-lymphocytes. Inhibition of phospholipase C with U73122 blocked calcium entry and Ica activation whether in response to a phospholipase C-dependent agonist or to calcium store depletion with thapsigargin. Run-down of cellular polyphosphoinositides by concentrations of wortmannin that block phosphatidylinositol 3-kinase completely blocked calcium entry and Ica. The membrane-permeant IP3 receptor inhibitor, 2-aminoethoxydiphenyl borane, blocked both capacitative calcium entry and Ica. However, it is likely that 2-aminoethoxydiphenyl borane does not inhibit through an action on the IP3 receptor because the drug was equally effective in wild-type DT40 B-cells and in DT40 B-cells whose genes for all three IP3 receptors had been disrupted. Intracellular application of another potent IP3 receptor antagonist, heparin, failed to inhibit activation of Ica. Finally, the inhibition of Ica activation by U73122 or wortmannin was not reversed or prevented by direct intracellular application of IP3. These findings indicate a requirement for phospholipase C and for polyphosphoinositides for activation of capacitative calcium entry. However, the results call into question the previously suggested roles of IP3 and IP3 receptor in this mechanism, at least in these particular cell types.

Activation of cell surface receptors coupled to phospholipase C (PLC) leads to generation of the second messenger inositol 1,4,5-trisphosphate (IP3). IP3 is known to bind to and activate receptors present on intracellular calcium stores, the endoplasmic reticulum, allowing calcium to be released into the cytosol (1). In most cells, the emptying of these calcium stores subsequently activates calcium influx across the plasma membrane through the “capacitative calcium entry” pathway (2, 3). It is unclear how empty intracellular stores signal activation of plasma membrane capacitative calcium entry. However, a rise in cytosolic calcium is not required, nor is the activation of plasma membrane receptors, because agents such as the calcium-ATPase inhibitor thapsigargin and the calcium ionophore ionomycin, which deplete calcium stores independently of receptor-coupled events, can fully activate capacitative calcium entry (3). Two general models are proposed as underlying mechanisms for capacitative calcium entry activation. One is based on the requirement for a diffusible messenger generated upon store depletion (4, 5). The other hypothesizes a conformational coupling between proteins on the intracellular stores (e.g. the IP3 receptor) and capacitative calcium entry channels or associated proteins in the plasma membrane (6, 7).

The diffusible messenger hypothesis proposes that a decrease in the concentration of stored calcium leads to the release of a factor that diffuses to the plasma membrane and activates capacitative calcium entry channels. Evidence in support of this model comes from reports of an unidentified calcium influx factor (4, 5, 8, 9) and patch clamping experiments in Xenopus oocytes (10). Evidence from Xenopus oocytes indicates that if the signal is diffusible then its diffusion is somewhat limited, because the signal remains confined to the area of calcium release (11–13).

The conformational coupling model proposes the direct relay of a signal through protein-protein interactions. In the model’s simplest form, IP3 receptors in the endoplasmic reticulum interact with capacitative calcium entry channels in the plasma membrane (6, 7). A change in the conformation of the IP3 receptor, which occurs after a drop in endoplasmic reticulum luminal calcium (14), may then be transmitted directly to the capacitative calcium entry channels causing them to open (6, 7). This theory was originally proposed by analogy with ryanodine receptors on the sarcoplasmic reticulum stores, which bind directly to dihydropyridine calcium channels in the plasma membrane of skeletal muscle (15). The theory has subsequently gained some experimental support. A number of studies suggest that the regulation of capacitative calcium entry is dependent upon an intimate interaction between the endoplasmic reticulum and plasma membrane. Pharmacological or physical dislocation of the plasma membrane away from the endoplasmic reticulum prevents activation of capacitative calcium entry by store depletion (16–19).

However, there is more direct evidence that an IP3 receptor-couplage capacitative calcium entry channel complex bridges the gap...
between endoplasmic reticulum and plasma membrane. Calcium flux through endogenous capacitative calcium entry channels (20) or overexpressed TRP3 channels (a candidate capacitative calcium entry channel (21)) can be recorded in the cell-attached configuration but ceases when the patch is excised. The addition of IP$_3$ and the IP$_3$ receptor to the patch (but not the IP$_3$ receptor alone) reconstitutes capacitative calcium entry activity. A similar requirement for the IP$_3$ receptor is revealed by the use of the IP$_3$ receptor inhibitors 2-aminoethoxydiphenyl borane (2-APB) and xestospongin C, which uncouple store depletion from the activation of capacitative calcium entry (22). Thus, there is substantial evidence that IP$_3$ receptors are required for activation of capacitative calcium entry channels and also evidence that these IP$_3$ receptors need to be liganded with IP$_3$.

The requirement for IP$_3$ for capacitative calcium entry presents something of a paradox, because, as discussed above, many laboratories have confirmed that store depletion alone, in the absence of phospholipase C activation, is capable of full activation of capacitative calcium entry. Thus, it has been suggested that the requirement for IP$_3$ must normally be fulfilled by its basal production, presumably by a phospholipase C located in close proximity to the channel-IP$_3$ receptor complex. The cells were then removed from the trypsin by centrifugation, followed by the use of the IP$_3$ receptor inhibitors 2-aminoethoxydiphenyl borane (2-APB) and xestospongin C, which uncouple store depletion from the activation of capacitative calcium entry (22). Thus, there is substantial evidence that IP$_3$ receptors are required for activation of capacitative calcium entry channels and also evidence that these IP$_3$ receptors need to be liganded with IP$_3$.

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To this end, we monitored capacitative calcium entry channel activity directly by measuring the calcium release-activated calcium current ($I_{\text{calc}}$) in RBL cells and indirectly by measuring the cytosolic calcium concentration in RBL, mouse lacrimal, and DT40 cells. In the last case, we used both wild-type DT40 cells and cells whose IP$_3$ receptor genes were disrupted by targeted homologous recombination (24). IP$_3$ receptor function was inhibited pharmacologically with the membrane-permeant IP$_3$ receptor inhibitor 2-APB and the membrane-impermeant inhibitor, low molecular weight heparin. Basal IP$_3$ formation was prevented with a phospholipase C inhibitor (U73122), and the levels of the precursor polyphosphoinositides were decreased by use of a phosphatidylinositol 4-kinase inhibitor (wortmannin). Intracellular calcium stores were subsequently depleted, independent of PLC and the IP$_3$ receptor, using thapsigargin and ionomycin, to test whether capacitative calcium entry could still be activated by store depletion. Our results show that maneuvers that are expected to disrupt basal PLC activity prevent activation of capacitative calcium entry upon store depletion. However, the specific function of PLC is uncertain, since we are unable to demonstrate a role for IP$_3$ or IP$_3$ receptors in this pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Measurement of Intracellular Calcium—**Rat basophil leukemia cells stably expressing the muscarinic m1 receptor (RBL-2H3 m1) were a gift from Dr. M. Beaven (National Institutes of Health, Bethesda, MD) (25). Cells were cultured in Earle’s minimal essential medium with Earle’s salts, 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin, (37 °C, 5% CO$_2$). For experiments, cells were passaged onto glass coverslips (number 1 ½) and used 12–26 h after plating.

Mouse lacrimal cells were isolated as described previously (26). Briefly, the excised glands from three male (male CD-1; 30–40 g) were finely minced and treated for 1 min with 0.2 mg/10 ml trypsin (Sigma). The cells were then removed from the trypsin by centrifugation, followed by a 5-min incubation with 2.5 mg/10 ml soybean trypsin inhibitor (Sigma) in the presence of 2.5 mM EGTA. Finally, the acinar cells were isolated after treating the tissue with 5 mg/10 ml collagenase (Roche Molecular Biochemicals) for 10 min. Throughout, all enzyme solutions were prepared in Dulbecco’s modified Eagle’s medium. Following isolation, the cells were washed and suspended in sterile Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 units/ml streptomycin. The cells were allowed to attach to glass coverslips (number 1) coated with Matrigel. Lacrimal cells were incubated on glass coverslips at least 3 h before use.

Lacrimal cells attached to glass coverslips were mounted in a Teflon chamber and incubated with 0.5 μM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature. The cells were then washed and bathed in a HEPES-buffered physiological saline solution (mM): NaCl, 154.5; KCl, 5.3; MgSO$_4$, 0.8; CaCl$_2$, 1.8; and MgCl$_2$, 1.1. The cells were subsequently bathed in a HEPES-buffered physiological saline solution (mM): NaCl, 120; KCl, 5.3; MgSO$_4$, 0.8; CaCl$_2$, 1.8; and MgCl$_2$, 2.0. The cells were maintained in culture at 40 °C in a humified 95% air, 5% CO$_2$ incubator, and at a cell density that ranged between 25 × 10$^3$ and 1 × 10$^4$ cells/ml. Both cell types were allowed to attach to glass coverslips (number 1) coated with Matrigel, and maintained in the RPMI 1640 medium described above.

**Fluo-4 measurements** were performed with the same imaging system (Universal Imaging) mounted on a Zeiss Axiosvert 35 inverted microscope equipped with a Zeiss × 40 (1.3 NA) Neofluor objective. The fluorescence light source was a Deltascan D101 (Photon Technology International Ltd.), equipped with a light path chopper and dual excitation monochromators. The light path chopper enabled rapid interchange between two excitation wavelengths (340 and 380 nm) and a photomultiplier tube monitored the emission fluorescence at 510 nm, selected by a barrier filter (Omega). All experiments were performed at room temperature. The data were expressed as a ratio of Fura-2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm $(F_{340}/F_{380})$.

The immortalized chicken B-lymphocyte cell line, DT-40 (RIKEN Cell Bank number RCB1464), and a mutant version with genes for all three IP$_3$ receptor types disrupted (RIKEN Cell Bank number RCB1467) were maintained in suspension with RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum, 4 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 50 μg/ml 2-mercaptoethanol. The cells were incubated in a standard whole cell recording configuration, using RBL-2H3 m1 cells.

**Electrophysiology—**Patch clamp experiments were conducted in the standard whole cell recording configuration, using RBL-2H3 m1 cells.
FIG. 1. The IP₃ receptor inhibitor 2-APB, but not heparin, blocks \( I_{\text{crac}} \) and capacitative calcium entry. A and B, extracellular application of 500 nM ionomycin (IONO) was used to deplete intracellular calcium stores and activate \( I_{\text{crac}} \) in RBL-2H3 m1 cells. The IP₃ receptor inhibitor 2-APB (100 \( \mu \)M) was applied extracellularly either after (A, \( n = 4 \)) or before (B, \( n = 3 \)) application of ionomycin for the time indicated by the bars. C, 12 mg/ml heparin was included in the patch pipette (open circles, \( n = 6 \); controls are filled circles, \( n = 6 \)). 200 or 400 s (not shown) after forming the whole cell mode (time 0), ionomycin (500 nM) was added to deplete intracellular calcium stores and activate \( I_{\text{crac}} \). D, 30 \( \mu \)M caged IP₃ was included in the patch pipette in the absence (filled circles, \( n = 7 \)) or presence (open circles, \( n = 10 \)) of 12 mg/ml heparin. 200 s after forming the whole cell mode (time 0), UV light was applied to release the caged IP₃, allowing intracellular calcium store depletion and \( I_{\text{crac}} \) activation. E, Fura-2-loaded mouse lacrimal cells were treated with 1 mg/ml thapsigargin to deplete intracellular calcium stores and activate capacitative calcium entry, either in the presence (i, \( n = 5 \)) or absence (ii, \( n = 5 \)) of 30 \( \mu \)M 2-APB. For patch clamp experiments, the whole cell configuration was established at time 0, at which time the voltage protocol described under “Experimental Procedures” was initiated. \( I_{\text{crac}} \) was measured once every 5 s at \( -100 \) mV. Current is normalized against cell capacitance, and the current density (pA/pF) is plotted. Values are mean \( \pm \) S.E.

Materials—IP₃, caged IP₃, ionomycin, U73122, and U73343 were from Calbiochem. Thapsigargin was from LC laboratories. Heparin and Cs₄-1,2-bis(2-aminophenoxy)ethane-N,N',N''-N'''-tetracetic acid—Cs₄ (with free calcium set to 100 nM, calculated using MaxChelator software, version 6.60), pH 7.2. Bath solution (HBSS) was as described above, except CaCl₂ was increased to 10 mM for calcium-HBSS, or 10 mM MgCl₂ was included in nominally calcium-free HBSS (10 mM MgCl₂ was included in nominally calcium-free HBSS).

In all experiments, upon forming the whole cell configuration, the cell membrane potential was held at 0 or \(+20\) mV. Once every 5 s, the membrane potential was stepped to \(+100\) mV (for 20 ms to assess \( I_{\text{crac}} \), and then a voltage ramp to \(+60\) mV over a period of 160 ms was applied. Currents are normalized to cell capacitance. All voltages are corrected for a 10-mV liquid junction potential. Membrane currents were amplified with an Axopatch-1C amplifier (Axon Instruments, Burlingham, CA). Voltage clamp protocols were implemented, and data acquisition was performed with PCLAMP 6.1 software (Axon Instruments). Currents were filtered at 1 kHz and digitized at 200-\( \mu \)s intervals.

Measurement of \( [\text{3H}] \)inositol-labeled Phosphatidylinositol 4-Phosphate (PIP) and Phosphatidylinositol 4,5-Bisphosphate (PIP²)—RBL-2H3 cells were labeled with \( [\text{3H}] \)inositol and incubated in the presence or absence of methacholine, wortmannin, or both according to the protocol for examining effects on \( I_{\text{crac}} \) (see “Results”). \( [\text{3H}] \) Labeled lipids were extracted, deacylated, and separated by HPLC as previously described (28, 29). The \( [\text{3H}] \)inositol-labeled polyphosphoinositide levels were determined by liquid scintillation spectroscopy of the HPLC fractions corresponding to the retention times of authentic PIP and PIP² standards.

Materials—IP₃, caged IP₃, ionomycin, U73122, and U73343 were from Calbiochem. Thapsigargin was from LC laboratories. Heparin and wortmannin were from Sigma. Cs₄-1,2-bis(2-aminophenoxy)ethane-N,N',N''-N'''-tetracetic acid and Fura-2 were from Molecular Probes.

RESULTS

Inhibition of IP₃ Receptor Function with 2-APB, but Not Heparin, Blocks \( I_{\text{crac}} \) and Capacitative Calcium Entry—RBL-2H3 cells have a well defined calcium release-activated calcium current (\( I_{\text{crac}} \)), activated upon intracellular calcium store depletion (30). We used the whole cell patch clamp technique to measure \( I_{\text{crac}} \) in RBL-2H3 cells, stably transfected with the muscarinic m1 receptor (25). Extracellular application of ionomycin (500 nM) led to rapid and full activation of \( I_{\text{crac}} \) (Fig. 1A). Subsequently, extracellular application of the IP₃ receptor inhibitor 2-APB (100 \( \mu \)M) rapidly blocked \( I_{\text{crac}} \). Cells incubated with 2-APB for 3 min before exposure to ionomycin failed to show any detectable activation of \( I_{\text{crac}} \) (Fig. 1B). The inhibition was not readily reversible upon removal of 2-APB (Fig. 1, A and B).

In contrast to the results with 2-APB, blockade of IP₃ receptor function with the competitive IP₃ receptor antagonist heparin (10 mg/ml) failed to block ionomycin-activated \( I_{\text{crac}} \) (Fig. 1C). Heparin is cell-impermeant and was delivered to the cell interior by inclusion in the patch pipette. An effective concentration clearly entered the cell, because 200 s after forming the whole cell mode, flash photolysis of caged IP₃ (30 \( \mu \)M) failed to release stored calcium or activate \( I_{\text{crac}} \) in cells exposed to heparin (Fig. 1D, open circles). In the absence of heparin, \( I_{\text{crac}} \) was readily activated by UV flash photolysis of caged IP₃ (Fig. 1D, closed circles).

Heparin has previously been reported to have no effect on the rise in cytosolic calcium induced by thapsigargin-activated capacitative calcium entry in mouse lacrimal cells (31). However, the IP₃ receptor antagonist 2-APB (30 \( \mu \)M) does prevent capacitative calcium entry in response to store depletion with thapsigargin in mouse lacrimal cells (Fig. 1E).

Capacitative Calcium Entry Is Sensitive to Inhibition of PLC by U73122—Methacholine, a muscarinic receptor agonist, evokes a sustained rise in intracellular calcium in mouse lacrimal cells...
rimal cells, when added at both a low (1 mM) and high (100 mM) concentration (Fig. 2A). The calcium signal is composed of the IP$_3$-mediated release of stored calcium and plasma membrane capacitative calcium entry (32). We aimed to prevent this agonist response by blocking activation of PLC and IP$_3$ generation with a membrane-permeable PLC inhibitor, U73122 (33). A 3–5-min pretreatment of cells with 10 mM U73122 has previously been documented to fully and irreversibly prevent PLC activation upon agonist stimulation (33, 34). Likewise, a 5-min preincubation of mouse lacrimal cells with 10 mM U73122 was sufficient to prevent the intracellular calcium response to low or high doses of methacholine, consistent with a blockade of agonist-activated PLC (Fig. 2A). A 5-min pretreatment of cells with 1–5 μM U73122 resulted in variable degrees of inhibition of the calcium signal (data not shown). A 5-min preincubation of cells with 10 μM U73343, a less potent analogue of U73122, had no effect on the agonist-evoked responses (not shown).

The addition of the Ca$^{2+}$-ATPase inhibitor thapsigargin to lacrimal cells in the absence of extracellular calcium caused a transient increase in intracellular calcium, due to release of calcium from stores. Upon the addition of extracellular calcium, a second more sustained rise in intracellular calcium concentration occurs, due to calcium influx via capacitative calcium entry (Fig. 2B). A 5-min pretreatment of cells with 10 μM U73122 had no effect on the ability of thapsigargin to release intracellular calcium stores but fully prevented the rise in intracellular calcium concentration upon the readdition of extracellular calcium (Fig. 2B). U73343 had no effect on thapsigargin-induced capacitative calcium entry (Fig. 2B). Thus, inhibition of PLC activity with U73122 appears to result in a specific block of capacitative calcium entry after store depletion.

$I_{crac}$ Is Sensitive to Inhibition of PLC Activity by U73122—To ensure the effects of U73122 were not indirect (for example, due to changes in cell membrane potential or stimulation of the calcium removal processes), we examined the effects of this reagent on $I_{crac}$ in RBL-2H3 m1 cells, measured under voltage-clamped conditions. The extracellular addition of either thapsigargin (1 μM) or ionomycin (500 nM) was sufficient to deplete intracellular calcium stores and fully activate $I_{crac}$ in control cells (Fig. 3A, open circles). A 5-min pretreatment of cells with 10 μM U73122 (10 μM), prevented activation of $I_{crac}$ upon store depletion with either thapsigargin (Fig. 3A, open circles) or ionomycin (Fig. 3B, open circles). A 5-min pretreatment of cells with 5 μM U73122 (a dose that failed to consistently block either agonist or thapsigargin-induced calcium signals in mouse lacrimal cells) failed to block activation of $I_{crac}$ in RBL-2H3 m1 cells, although $I_{crac}$ was inhibited to 38 ± 4% (n = 9) of controls (see also Fig. 4B). A 5-min pretreatment of cells with the inactive analogue U73343 (10–15 μM) did not affect activation of $I_{crac}$ upon store depletion with either thapsigargin or ionomycin (Fig. 3B, gray circles).

The addition of 10 μM U73122 to RBL-2H3 cells once $I_{crac}$ had been activated resulted in a much smaller inhibition of $I_{crac}$ and never caused a complete block. $I_{crac}$ in U73122-treated cells averaged 73 ± 12% of controls. This suggests that $I_{crac}$ is much less sensitive to inhibition of PLC once it is initiated (Fig. 3, C and D).

The Addition of Exogenous IP$_3$ and Diacylglycerol Fails to Restore U73122-inhibited Capacitative Calcium Entry—In the context of the conformational coupling model, the apparent dependence of capacitative calcium entry on PLC might reflect a requirement for IP$_3$ on the channel-associated IP$_3$ receptor (6, 7, 23). Thus, we next addressed whether the addition of exogenous IP$_3$ to the patch pipette would overcome the block of $I_{crac}$ activation in cells treated with U73122. Intracellular delivery of F-IP$_3$ (50 or 500 μM), a slowly metabolizable analogue of IP$_3$, rapidly activated $I_{crac}$ in control cells after forming the whole cell mode (Fig. 4A). However, a 5-min pretreatment of cells with 10 μM U73122 fully prevented activation of $I_{crac}$ by delivery of 50 μM F-IP$_3$ (Fig. 4A). A combination of 100 μM IP$_3$ with 100 μM 1-oleyl-2-acetyl-sn-glycerol, a membrane permeant diacylglycerol, also failed to restore $I_{crac}$ in response to a combination of thapsigargin and ionomycin (not shown, n = 4).

Because the addition of exogenous IP$_3$ was not sufficient to restore $I_{crac}$ after inhibition of PLC, we tried to increase endogenous IP$_3$ levels. Hence, we aimed to inhibit PLC activity partially using submaximal U73122 (5 μM, 5 min pretreatment); subsequently, we added a high dose of methacholine (100 μM) in an attempt to drive residual PLC activity. Pretreatment of RBL-2H3 m1 cells with 5 μM U73122 inhibited ionomycin-activated $I_{crac}$ to 38 ± 4% of control levels. The subsequent addition of 100 μM methacholine failed to increase $I_{crac}$ significantly (Fig. 4B). In RBL-2H3 m1 cells not pretreated with U73122, a time-matched addition of 100 μM methacholine caused $I_{crac}$ activation, showing that the failure of response in U73122-treated cells is not due to a general lack of responsiveness after 600 s in the whole cell mode (Fig. 4B, dotted trace).
phosphorylates phosphatidylinositol to PIP, results in inhibition of capacitative calcium entry in platelets (37). In SH-SY-5Y cells at least, although PIP is depleted by a 10-min preincubation with 10 μM wortmannin, depletion of PIP2 is facilitated by agonist stimulation (38). Hence, we attempted to reduce cellular PIP and PIP2 levels by incubating cells with wortmannin for an extended period (10–20 μM; 40 min) and to further deplete PIP2 to low levels with agonist stimulation (methacholine, 10–15 min). A final 15–20 min in the presence of 20 μM wortmannin but in the absence of methacholine ensured that I_{crac} would not be preactivated by the agonist. We

FIG. 3. The phospholipase C inhibitor U73122 blocks I_{crac}. A–D, RBL-2H3 m1 cells were first exposed to 10 μM U73122 or 10 μM U73343 for 5 min or were untreated. Subsequently, thapsigargin (TG, 1 μM) or ionomycin (IONO, 500 nM) was applied extracellularly to deplete intracellular calcium stores and activate I_{crac}; HBSS containing 10 mM calcium was replaced with nominally calcium-free HBSS at the times indicated. Filled circles represent control experiments, without U73122. In B, the gray circles represent cells treated with 10 μM U73343. Values are mean ± S.E. (n ≥ 8).

FIG. 4. The addition of exogenous IP_3 fails to relieve the inhibition of I_{crac} produced by inhibition of PLC. A, RBL-2H3 m1 cells were pretreated for 5 min with either 10 μM U73122 (n = 18, open symbols) or 10 μM U73343 (n = 5, gray symbols) or were untreated (n = 21, black symbols). After pretreatment, the whole cell configuration was established (time 0), and 50 or 500 μM (not shown) F-IP_3 was delivered to the cell interior through the patch pipette. B, RBL-2H3 m1 cells were exposed to 5 μM U73122 for 5 min (black symbols, n = 10), after which time 500 nM ionomycin (IONO) was applied extracellularly (as indicated). After a further 200 s, 100 μM methacholine (MeCh) was added. In control cells, not treated with U73122 (broken trace, no symbols), the addition of 100 μM methacholine 600 s after forming the whole cell mode rapidly activated I_{crac}; HBSS containing 10 mM calcium was replaced with nominally calcium-free HBSS at the times indicated.

FIG. 5. Wortmannin-induced inhibition of I_{crac}. A, RBL-2H3 m1 cells were pretreated with 20 μM wortmannin for 20–30 min, and 100 μM methacholine (MeCh) was added for a further 10–15 min, followed by a 10–15-min recovery period in the absence of agonist. The whole cell mode was then established, and 1 μM thapsigargin/ionomycin was applied extracellularly as indicated to activate I_{crac}. Extragranular calcium was removed as indicated. Traces are mean ± S.E. (n ≥ 4). B, RBL-2H3 m1 cells were pretreated with 20 μM wortmannin and 100 μM methacholine as described above. After this pretreatment, the whole cell mode was established, and 500 μM F-IP_3, included in the patch pipette, was delivered to the cell interior. pA/pF, pA/picofarads
then assessed the effect of this protocol on \( I_{\text{crac}} \) (Fig. 5). This pretreatment of RBL 2H3 m1 cells almost completely inhibited \( I_{\text{crac}} \) activated by thapsigargin and ionomycin (13 ± 3% of control cells, Fig. 5A, open circles). Control cells pretreated with 100 \( \mu \text{M} \) methacholine, for 10 min, in the absence of wortmannin, displayed normal \( I_{\text{crac}} \) activation upon treatment with thapsigargin and ionomycin (107 ± 15% of control cells, Fig. 5A, closed circles). This indicates that methacholine pretreatment per se is not responsible for the inhibition.

In cells pretreated with 20 \( \mu \text{M} \) wortmannin for only 5 min, in the absence of agonist (a treatment shown not to deplete PIP2 levels in a previous study (38)), \( I_{\text{crac}} \) was not inhibited (95.8 ± 3% of controls, \( n = 4 \), not shown). Moreover, a 40–60-min pretreatment of cells with 10 \( \mu \text{M} \) wortmannin to inhibit phosphatidylinositol 3-kinase, but not phosphatidylinositol 4-kinase, also failed to inhibit \( I_{\text{crac}} \) (106 ± 11% of controls, \( n = 5 \), not shown).

The Addition of Exogenous IP\(_3\) Fails to Restore Wortmannin-inhibited \( I_{\text{crac}} \)—In cells pretreated with 20 \( \mu \text{M} \) wortmannin (40 min) and 100 \( \mu \text{M} \) methacholine (10 min) to inhibit phosphatidylinositol 4-kinase and deplete polyphosphoinositides, we attempted to overcome the inhibition of \( I_{\text{crac}} \) activation by the addition of exogenous IP\(_3\). Intracellular delivery of 500 \( \mu \text{M} \) F-IP\(_3\) failed to restore the \( I_{\text{crac}} \) activation in wortmannin-pretreated cells (Fig. 5B).

Effect of Wortmannin on Levels of PIP and PIP\(_2\) in RBL-2H3 Cells—The above results suggest that the inhibitory action of wortmannin is not likely to be due to a diminished ability to produce IP\(_3\) from PIP2. Thus, we utilized a \(^{3}H\)inositol labeling protocol to examine the effects of wortmannin on levels of PIP and PIP\(_2\) in RBL-2H3 cells. The results are summarized in Table I. Using a protocol similar to that for the \( I_{\text{crac}} \) measurements, wortmannin efficiently depleted cellular PIP, whether or not combined with agonist treatment. Despite this striking effect on PIP levels, PIP\(_2\) levels were little affected. PIP\(_2\) was reduced somewhat only with the combination of wortmannin and agonist treatment. Despite this striking effect on PIP levels, PIP\(_2\) levels were little affected. PIP\(_2\) was reduced somewhat only with the combination of wortmannin and agonist, but it seems unlikely that this would be sufficient to completely prevent PLC-mediated formation of IP\(_3\).

2-APB and Wortmannin Block Capacitative Calcium Entry in DT-40 IP\(_3\) Receptor Knockout Cells—Sugawara et al. (24) generated a line of DT-40 cells (an immortalized B-cell line) with the genes for all three IP\(_3\) receptor types disrupted by targeted homologous recombination. These cells were negative for all three types of IP\(_3\) receptors by Northern analysis. The knockout cells were shown to produce normal phospholipase C responses following activation of appropriate surface membrane receptors, but produced no detectable [Ca\(^{2+}\)]\(_i\) signal (24). However, normal capacitative calcium entry responses to thapsigargin were observed. We have utilized the cell line with all three IP\(_3\) receptors knocked out to examine the apparent roles of IP\(_3\) receptors and inositol lipids in capacitative calcium entry in this cell type. We have used fluorimetric analysis of [Ca\(^{2+}\)]\(_i\), changes; for technical reasons, we have not been able to successfully measure store-operated ion currents in this cell type.

It has been argued that because of the site of gene disruption, these cells could, in theory, produce a truncated form of IP\(_3\) receptor that might still serve to couple to plasma membrane channels (21). Thus, we measured specific binding of \(^{3}H\)IP\(_3\) to membranes prepared from wild-type DT-40 cells and the IP\(_3\) receptor knockout cells. The wild-type cells contained 16.8 ± 2.1 fmol/mg of protein of specific IP\(_3\) binding sites. The knockout cells were found to contain 1.3 ± 1.7 fmol/mg of protein of specific IP\(_3\) binding sites, a value not significantly different from zero.

The data shown in Fig. 6 confirm the earlier observations of Sugawara et al. (24). The wild-type cells exhibited irregular [Ca\(^{2+}\)]\(_i\) oscillations in response to activation of the B-cell receptor in ~70% of the cells. In the IP\(_3\) receptor knockout cells, no response to B-cell receptor activation was seen in any of the cells (Fig. 6A). Fig. 6B confirms that DT-40 cells exhibit a capacitative calcium entry response to thapsigargin and that

### Table I

|                | Control | Methacholine | Wortmannin | Wortmannin + methacholine |
|----------------|---------|--------------|------------|----------------------------|
| **PIP**        | 12,619 ± 421 | 13,598 ± 686 | 16,073 ± 330 | 9,153 ± 421             |
| **PIP\(_2\)**  | 16,897 ± 505 | 18,417 ± 603 | 2,890 ± 108\(^a\) | 2,425 ± 116\(^b\)        |

\(^a\)Significantly different from all other PIP\(_2\) values.

\(^b\)Significantly different from control and methacholine PIP values.
In the protocol depicted in Fig. 7, DT-40 cells were sequentially treated with an activator of the B-cell receptor and with thapsigargin. In this experiment, examining multiple cells simultaneously, the asynchrony of the oscillatory responses to B-cell receptor activation resulted in a somewhat blunted average response. In Fig. 7A, the absence of the agonist response in the knockout cells, together with a normal response to thapsigargin, is again evident. A similar experiment, carried out in the presence of 75 μM 2-APB, is shown in Fig. 7B. 2-APB completely blocked the B-cell receptor response in the wild-type cells and reduced the thapsigargin response to a small, transient response in both the wild-type and knockout cells. This response is similar to that seen in the absence of external Ca2+ (not shown) and presumably reflects intracellular release of Ca2+ from internal stores.

Unfortunately, we were not able to examine the effects of U73122 on Ca2+ signaling in DT-40 cells, because concentrations of the drug required for phospholipase C inhibition were toxic to the cells and on their own caused large irregular rises in [Ca2+]. However, we were able to utilize wortmannin to examine the possible role of phosphorylated inositol lipids. As shown in Fig. 8, wortmannin treatment caused a marked attenuation of the Ca2+ entry response to thapsigargin, and this effect was similar in the wild type and mutant cell lines.

**DISCUSSION**

The current study employed a variety of pharmacological reagents that would be expected to disrupt the phospholipase C-IP3 pathway at distinct points. The rationale for this approach was to gain insight into the role played by this pathway in coupling intracellular Ca2+ store depletion to the activation of capacitative calcium entry channels in the plasma membrane. The model under consideration is one in which basal activity of phospholipase C and basal levels of IP3 in the vicinity of coupling complexes, are required for signaling to take place. Surprisingly, our findings call into question a role for IP3 in signaling capacitative calcium entry, yet the data support a critical role of a phospholipase C and phosphorylated inositol lipids.

In a previous study, Berven and Barritt (39) observed inhibition by U73122 of thapsigargin-induced calcium entry in hepatocytes, but since they had no reason to suspect a role for phospholipase C in this process, they concluded that this represented a nonspecific effect of the drug. However, our results suggest that the inhibitory effect of this drug is indeed likely due to its action against PLC. First, U73122, but not the less potent isomer U73343, completely inhibited both capacitative calcium entry and Icrac. Second, the minimum concentration of the drug that inhibited calcium entry and Icrac was also the minimum concentration reported to inhibit PLC-dependent signaling. Third, U73122 was much less effective when applied after activation of Icrac had already taken place, indicating that it is not acting as a direct channel blocker.

At the concentrations employed in the current study, wortmannin inhibits PI 4-kinase, and under these conditions the drug blocked capacitative calcium entry and Icrac. As for U73122, inhibition did not occur if the drug was added after activation had taken place. However, while this drug effec-
tively depleted cellular PIP levels, PIP2 levels were minimally affected.

The original rationale for examining the roles of PLC and PIP2 was based on a model according to which PLC-mediated breakdown of PIP2 would provide IP3, a requirement for coupling IP3 receptors to plasma membrane capacitative calcium entry or CRAC channels (23). In support of this idea, we found that the membrane-permeant IP3 receptor antagonist, 2-APB, completely inhibited capacitative calcium entry and I\textsubscript{crac}. This result confirms the previous findings of Ma et al. (22). These investigators concluded that 2-APB was unlikely to be acting as a channel blocker, because it was able to block the activation of TRP3 channels when the channels were activated by a PLC-linked stimulus but not when the channels were activated by diacylglycerol. We have confirmed this observation in a stable, TRP3-expressing cell line in our own laboratory. However, in the case of capacitative calcium entry channels it seems unlikely that the inhibitory action of 2-APB involves IP3 receptors. In the current study, in DT-40 cells lacking all three types of IP3 receptors (24), 2-APB was as effective in inhibiting capacitative calcium entry. For the case of PLC, there may be an analogy with the coupling mechanism in some cell types but not in others. There is an obvious analogy with the modes of coupling between ryanodine receptors and I-type calcium channels. This direct mode of interaction apparently occurs only in skeletal muscle, while coupling in other systems (heart for example) involves a diffusible messenger, in this case calcium itself.

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Role of the Phospholipase C-Inositol 1,4,5-Trisphosphate Pathway in Calcium Release-activated Calcium Current and Capacitative Calcium Entry
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