Novel Function of Phosphoinositide 3-Kinase in T Cell Ca\(^{2+}\) Signaling

A PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE-MEDIATED Ca\(^{2+}\) ENTRY MECHANISM*

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This study presents evidence that phosphoinositide (PI) 3-kinase is involved in T cell Ca\(^{2+}\) signaling via a phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P \(_3\)) sensitive Ca\(^{2+}\) entry pathway. First, exogenous PI(3,4,5)P \(_3\) at concentrations close to its physiological levels induces Ca\(^{2+}\) influx in T cells, whereas PI(3,4)P \(_2\), PI(4,5)P \(_2\), and PI(5)P have no effect on [Ca\(^{2+}\)]\(_i\). This Ca\(^{2+}\) entry mechanism is cell type-specific as B cells and a number of cell lines examined do not respond to PI(3,4,5)P \(_3\) stimulation. Second, inhibition of PI-3-kinase by wortmannin and by overexpression of the dominant negative inhibitor Ap85 suppresses anti-CD3-induced Ca\(^{2+}\) response, which could be reversed by subsequent exposure to PI(3,4,5)P \(_3\). Third, PI(3,4,5)P \(_3\) is capable of stimulating Ca\(^{2+}\) efflux from Ca\(^{2+}\)-loaded plasma membrane vesicles prepared from Jurkat T cells, suggesting that PI(3,4,5)P \(_3\) interacts with a Ca\(^{2+}\) entry system directly or via a membrane-bound protein. Fourth, although D-myoinositol 1,3,4,5-tetrasphosphate (Ins(1,3,4,5)P \(_4\)) mimics PI(3,4,5)P \(_3\) in many aspects of biochemical functions such as membrane binding and Ca\(^{2+}\) transport, we raise evidence that Ins(1,3,4,5)P \(_4\) does not play a role in anti-CD3- or PI(3,4,5)P \(_3\)-mediated Ca\(^{2+}\) entry. This PI(3,4,5)P \(_3\)-stimulated Ca\(^{2+}\) influx connotes physiological significance, considering the pivotal role of PI-3-kinase in the regulation of T cell function. Given that PI-3-kinase and phospholipase C\(_γ\)-coupled, either directly through conformational coupling or indirectly via diffusible factors, to the Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry (TROCE) mechanism is activated in response to TCR-CD3 stimulation (2). However, this putative pathway is less well characterized. It is known to be independent of the depletion of intracellular Ca\(^{2+}\) and inhibited by SKF96365, a Ca\(^{2+}\)-channel blocker, and phorbol esters (9).

In this paper, we present data suggesting a new function of phosphoinositide (PI) 3-kinase in T cell Ca\(^{2+}\) regulation via a PI(3,4,5)P \(_3\)-sensitive Ca\(^{2+}\) entry mechanism. In response to TCR activation, PI-3-kinase and other signaling molecules such as PLC-\(γ\)1 are recruited to the plasma membrane to form multifunctional complexes (10–12). Activation of PI-3-kinase results in a transient accumulation of \(μ\)M levels of PI(3,4,5)P \(_3\) and phosphatidylinositol 3,4-bisphosphate (PI(3,4)P \(_2\)) both absent in quiescent T cells (13). To date, a clear consensus on the mode of action of these lipid messengers in regulating TCR signaling has yet to emerge. Putative downstream effectors for PI(3,4,5)P \(_3\) and PI(3,4)P \(_2\) in receptor-stimulated signaling include Ca\(^{2+}\)-independent PKC isozymes (δ, ε, η, ζ), PLC-\(γ\), Akt, and so forth (14). The results of this study suggest that PI(3,4,5)P \(_3\) mediates a novel Ca\(^{2+}\) entry mechanism on plasma membranes. Given the intimate relationship between PI 3-kinase (PI(3,4,5)P \(_3\)), PLC-\(γ\)1, D-myoinositol 1,4,5-trisphosphate; Ins(1,3,4,5)P \(_4\), D-myoinositol 1,3,5-tetrasphosphate; ER, endoplasmic reticulum; di-C\(_8\)-PI(3,4,5)P \(_3\), 1-4 and 2-4-octanoyl-sn-glycero-3-phosphoryl-di-myoinositol 3,4,5-trisphosphate; AM, acetoxymethyl ester; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HA, hemagglutinin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

...of signaling cascades that culminate in the activation and proliferation of T lymphocytes. One of the early signaling events is a biphasic increase in intracellular Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_i\)), which is characterized by a high transient spike of [Ca\(^{2+}\)]\(_i\), followed by a long-lasting plateau phase (1, 2). It is believed that the initial phase of Ca\(^{2+}\) response is attributable to the action of inositol 1,4,5-trisphosphate (Ins(1,4,5)P \(_3\)) that releases Ca\(^{2+}\) from the endoplasmic reticulum (ER). Next, depletion of the ER Ca\(^{2+}\) store signals an influx of Ca\(^{2+}\) across the plasma membrane to sustain the wave of Ca\(^{2+}\) signaling. Three discrete mechanisms have been proposed for the sustained influx of Ca\(^{2+}\) (2). First, Ins(1,4,5)P \(_3\) receptors are present on the plasma membranes of T lymphocytes (3–5). Thus, Ins(1,4,5)P \(_3\) may play a dual role of releasing Ca\(^{2+}\) from ER stores and stimulating Ca\(^{2+}\) influx across plasma membranes concurrently. Second, the capacitative Ca\(^{2+}\) entry model (6) dictates that the emptying of the intracellular Ca\(^{2+}\) store is coupled, either directly through conformational coupling or indirectly via diffusible factors, to the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel (7, 8). Third, a TCR-operated Ca\(^{2+}\) entry (TROCE) mechanism is activated in response to TCR-CD3 stimulation (2). However, this putative pathway is less well characterized. It is known to be independent of the depletion of intracellular Ca\(^{2+}\) and inhibited by SKF96365, a Ca\(^{2+}\)-channel blocker, and phorbol esters (9).

Engagement of the TCR-CD3 complex stimulates an array...
nase and PLC-γ in T cell activation, these data prompt a new hypothesis that PI(3,4,5)P3-sensitive Ca2+ entry plays a concerted role with Ins(1,4,5)P3-induced Ca2+ release and capacitative Ca2+ entry in TCR-mediated Ca2+ signaling.

MATERIALS AND METHODS

d-myosinotol 1,4,5-trisphosphate (Ins(1,4,5)P3), d-ino-sylositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4), 1-O-(2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-d-myosinotol 3,4,5-trisphosphate (PI(3,4,5)P3), 1-O-(1,2-di-O-acetyl-sn-glycero-3-phosphoryl)-d-myosinotol 3,4,5-trisphosphate (PI(3,4,5)P3), 1-O-(1,2-di-O-acetyl-sn-glycero-3-phosphoryl)-d-myosinotol 3,4,5-trisphosphate (d-Ins(1,3,4,5)P4), and 1-O(1,2-di-O-acetyl-sn-glycero-3-phosphoryl)-d-myosinotol 3-monophosphate (PI(3)P) were synthesized as previously reported (15, 16). The synthesis of the biotinylated PI(3,4,5)P3 (+)-1-O-1-(4-[(N-biotinyloxyiminio)-octanoyl]-2-O-acetyl-sn-glycero-3-phosphoryl)-d-myosinotol 3,4,5-trisphosphate (Biotin-PIP3) is described elsewhere (17). The identity and purity of all insitol phosphates and inositol lipids were examined by 1H and 31P NMR and high resolution mass spectrometry. Phorbol 12-myristate 13-acetate, wortmannin, SKF96365, indol-3-ace-toxymethyl ester (AM), fura-2 AM were purchased from Calbiochem.

Mouse spleen cells (107) were treated with 5 × 10−8M leupeptin and 1-(2-aminoethyl)benzoxycarbonylmethyl ester (AM), fura-2 AM were purchased from Calbiochem. Nifedipine was obtained from ICN. Leupeptin and 4-(2-aminoethyl)benzoxycarbonyl (AM), fura-2 AM were purchased from Calbiochem.

Flow Cytometric Cell Sorting and Analysis of Intracellular Ca2+—Mouse spleen cells (107) were treated with 5 μg of anti-Thy-1.2 MAb or anti-B-220 MAb-fluorescein (FITC) conjugates in 200 μl of culture medium consisting of 4.3 mM Na2HPO4, 24.3 mM NaH2PO4, 4.3 mM K2HPO4, 113 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2.2 mM MgCl2, 2.2 mM CaCl2, and 5.6 mM glucose. Aliquots containing 1 × 106 cells were each resuspended in 0.3 ml of the aforementioned assay buffer containing 10 μM fluo-3 AM and the membrane-bound activity was analyzed by liquid scintillation spectrometry. Nonspecific binding was measured in the presence of 10 μM Ins(1,3,4,5)P4.

Fluorescence Spectrophotometric Measurement of Intracellular Ca2+—Ca2+ was monitored by the change in the fluorescence intensity of fura-2-loaded cells. Jurkat T cells (1 × 106 cells/ml) were incubated with 30–60 μM fura-2 AM in the dark for 1 h at 37 °C. The cells were then pelleted by centrifugation at 1,500 g for 10 min, washed with assay buffer twice, and resuspended at approximately 8 × 107 cells/ml in the same buffer containing 1 mM Ca2+. The effect of anti-CD3 MAb or various inositol lipids on [Ca2+]i was examined by fura-2 fluorescence in a Hitachi F-2000 spectrofluorimeter at 37 °C with excitation and emission wavelengths at 340 and 510 nm, respectively. The maximum fura-2 fluorescence (Fmax) in Jurkat cells was determined by adding A23187 (1 μM), and the minimum fluorescence (Fmin) was determined following depletion of external Ca2+ by 5 mM EGTA. The [Ca2+]i was calculated according to the equation [Ca2+]i = Kd × (F − Fm)/(Fm − F), where Kd denotes the apparent dissociation constant (∼22 μM) of the fluorescence dye-Ca2+ complex (21).

[3H]inositol Phosphate Turnover Analysis—The examination of phos-photoinositide turnover was carried out according to a modification of the procedure reported by Sei et al. (9). In brief, Jurkat T cells were incubated with myo-[2-3H]inositol (10 μCi/106 cells/ml) in isoinositol-free RPMI medium supplemented with 10% fetal bovine serum. The cells were then washed with 20 mM Hepes, pH 7.4, containing 285 mM NaCl, 11 mM KC1, 1.3 mM Na2HPO4, 1 mM KH2PO4, 8.5 mM NaHCO3, 1.6 mM MgSO4, 2.2 mM MgCl2, 2.2 mM CaCl2, and 5.6 mM glucose. Aliquots containing 1 × 106 cells were each resuspended in 0.3 ml of the aforementioned assay buffer plus 1 mM CaCl2 and 100 μM EGTA and transferred to 1.5-microcentrifuge tubes. Each sample was incubated with 1.5 μg of anti-CD3 MAb or 20 μM PI(3,4,5)P3, for the indicated times and quenched by adding 0.25 ml of 6% trichloroacetic acid. The tubes were centrifuged for 2 min at 12,000 × g. The supernatant (200 μl) was analyzed by high performance liquid chromatography on a 5-μM MDorfosorbex SAX column (4.6 × 200 mm) equilibrated with H2O. The [3H]inositol phosphates were eluted with a linear gradient of 0–0.9 M NH4H2PO4 in 60 min at a flow rate of 1 ml/min. Fractions were collected every 1 ml, and their radioactivity was measured by liquid scintillation. Synthetic [3H]Ins(1,4,5)P3, [3H]Ins(1,4,5,8)P4, [3H]Ins(4,5,8)P3, Ins(4)P were used as standards. The respective retention times were 60, 48, 43, and 31 min. Detection of PI(3,4,5)P3-binding Proteins in T Cell Plasma Mem-branes—T cell plasma membranes were treated with 5% [-[3-cholami-dopropyl(dimethylamino)-1-propylphosphonic acid for 1 h on ice and centrifuged at 40,000 × g for 1 h. The supernatant (100 μg of protein) was incubated with 100 μCi Biotin-PIF, for 1 h, and 200 μl of strepta
dialyzed against distilled water for 12 h, proteins eluted at 2 M urea. The pattern remained unaltered even 5 min after PI(3,4,5)P3 treatment (data not shown). The experimental procedures are described under “Materials and Methods.”

vihadine beads (Roche Molecular Biochemicals) were added. The mixture was incubated for an additional hour and centrifuged at 12,000 × g for 5 min. The beads were washed with 1 ml of each of the following solutions in tandem: 10 mM Tris, pH 7.5, containing 5 mM EDTA and 150 mM NaCl, phosphate-buffered saline, and 2 M urea. After being dialyzed against distilled water for 12 h, proteins eluted at 2 M urea were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by silver staining.

RESULTS

**PI(3,4,5)P3 Induced Intracellular Ca2+ Increase in Mouse Splenic T Cells but Not in B Cells**—Previously, we reported that treatment of washed platelets with exogenous PI(3,4,5)P3 induced Ca2+ influx across plasma membranes, resulting in immediate cell aggregation (22). This finding prompted us to study PI(3,4,5)P3-induced Ca2+ response in other cell types. Using fluorescence-activated cell sorting, we examined the effect of PI(3,4,5)P3 on Indo-1-loaded mouse spleen cells stained with FITC-conjugates of anti-Thy1.2 or anti-B-220 mAb. As shown, 10 mM PI(3,4,5)P3 induced a significant increase in [Ca2+]i in Thy1.2-positive mouse spleen cells as soon as 15 s after stimulation. This PI(3,4,5)P3-stimulated Ca2+ increase was also confirmed in human peripheral T cells and Jurkat T cells by fluorescence spectrophotometry. In contrast, [Ca2+]i in the B-220-positive cell population remained unaffected even in the presence of 20 mM PI(3,4,5)P3 after a prolonged exposure up to several minutes (Fig. 1).

In addition, a number of cell lines, including NIH3T3 fibroblast cells, PC-12 pheochromocytoma cells, Hep G2 hepatocarcinoma cells, LNCaP prostate adenocarcinoma cells, were examined. None of these cells showed appreciable Ca2+ response following PI(3,4,5)P3 stimulation (data not shown). This cell-type specificity underscores a fundamental difference in the role of PI 3-kinase in Ca2+ regulation in different cells.

The direct introduction of micellar PI(3,4,5)P3 to intact cells is also worth comment. Although how PI(3,4,5)P3 permeates cell membranes remains unclear, published data from this and other laboratories show that exogenous PI(3,4,5)P3 can readily fuse with cell membranes and exert cellular and biochemical responses in different cell types including platelets, NIH3T3 cells, and adipocytes (22–24).

**PI(3,4,5)P3 Induces Ca2+ Entry in T Cells**

- **A** depicts that the effect of PI(3,4,5)P3 on [Ca2+]i in splenic T cells was dose-dependent, with a threshold of about 2 mM, and was saturable. As shown, treatment with the lipid messenger in excess of 10 mM did not further enhance the amplitude of Ca2+ response.

- **B** shows that PI(3,4,5)P3-induced Ca2+ response was independent of the stage of cell development. PI(3,4,5)P3 does not release Ca2+ from internal stores.

**PI(3,4,5)P3 Does Not Release Ca2+ from Internal Stores**—Several lines of evidence suggest that the PI(3,4,5)P3-induced Ca2+ response was attributable to Ca2+ influx from the medium. First, this Ca2+ increase was completely abrogated by pretreatment with EGTA (Fig. 3A). Second, although PI(3,4,5)P3 has been reported to activate PLC-γ via distinct mechanisms (25), the PLC inhibitor U73122 did not exhibit appreciable inhibition on PI(3,4,5)P3-elicted Ca2+ response (Fig. 3A). Furthermore, the PI(3,4,5)P3 concentration needed to elicit Ca2+ response (<10 mM) was an order of magnitude lower than the threshold required for PLC-γ activation. Thus, PI(3,4,5)P3-induced Ca2+ response was independent of Ins(1,4,5)P3 formation. Third, PI(3,4,5)P3 had no effect on thapsigargin-sensitive Ca2+ pools (Fig. 3B). Jurkat T cells were treated with 10 mM PI(3,4,5)P3 in a Ca2+-depleted milieu, fol-
mobilization across the plasma membrane because deprivation of external Ca\(^{2+}\) by EGTA inhibited 70% of the Ca\(^{2+}\) signal (Fig. 5A, inset).}

Pretreatment with wortmannin (1 \(\mu\)M) attenuated the amplitude of anti-CD3-induced Ca\(^{2+}\) influx by EGTA in Jurkat T cells. Cells were loaded with fura-2 and incubated with Me\(_2\)SO vehicle (trace b) or Me\(_2\)SO vehicle (trace a) for 5 min before stimulation with 10 \(\mu\)g/ml anti-CD3, followed by 10 \(\mu\)M PI(3,4,5)P\(_3\) and 10 \(\mu\)M A23187, alone or in combination, on anti-CD3-induced Ca\(^{2+}\) response in Jurkat T cells. Cells were loaded with fura-2 and incubated with Me\(_2\)SO vehicles (Control), wortmannin (1 \(\mu\)M), U73122 (10 \(\mu\)M), or the combination of wortmannin (1 \(\mu\)M) and U73122 (10 \(\mu\)M) for 10 min before adding anti-CD3 mAb (10 \(\mu\)g/ml). The relative anti-CD3-induced Ca\(^{2+}\) response was calculated based on [Ca\(^{2+}\)]\(_{i}\) at the plateau following individual treatments vis-à-vis that of control.

PI(3,4,5)P\(_3\) Does Not Perturb Membrane Permeability to Ca\(^{2+}\)—Due to the extremely high charge density, it has been speculated that PI(3,4,5)P\(_3\) might directly affect the properties of cellular membranes. Therefore, one might raise a concern that PI(3,4,5)P\(_3\) facilitated Ca\(^{2+}\) translocation across plasma membranes by acting like a detergent. To refute this possibility, we examined the effect of PI(3,4,5)P\(_3\) on the permeability of liposomal vesicles to Ca\(^{2+}\). Fura-2-loaded multilamellar vesicles with a lipid composition similar to that of the plasma membrane were exposed to PI(3,4,5)P\(_3\) (1 \(\mu\)M) in the absence of 5 mM EGTA, fura-2-loaded Jurkat T cells were treated with 10 \(\mu\)M fura-2, warmed up to 40 °C for 5 min, and vortexed for 30 s. The suspension was centrifuged at 12,000 \(\times\) g for 10 min, and the liposomal pellet was suspended in assay buffer (described under “Materials and Methods”) containing 1 mM CaCl\(_2\). The fura-2-loaded liposomes were treated with 10 \(\mu\)M PI(3,4,5)P\(_3\), 7 \(\mu\)M 25-hydroxycholecalciferol (25(OH)D\(_3\)), or 1 \(\mu\)M A23187, and the influx of Ca\(^{2+}\) was monitored by fura-2 fluorescence.

PI(3,4,5)P\(_3\) Did Not Perturb Membrane Permeability to Ca\(^{2+}\) in T Cells—Due to the extremely high charge density, it has been speculated that PI(3,4,5)P\(_3\) might directly affect the properties of cellular membranes. Therefore, one might raise a concern that PI(3,4,5)P\(_3\) facilitated Ca\(^{2+}\) translocation across plasma membranes by acting like a detergent. To refute this possibility, we examined the effect of PI(3,4,5)P\(_3\) on the permeability of liposomal vesicles to Ca\(^{2+}\). Fura-2-loaded multilamellar vesicles with a lipid composition similar to that of the plasma membrane were exposed to PI(3,4,5)P\(_3\) (1 \(\mu\)M) in the absence of 5 mM EGTA, fura-2-loaded Jurkat T cells were treated with 10 \(\mu\)M fura-2, warmed up to 40 °C for 5 min, and vortexed for 30 s. The suspension was centrifuged at 12,000 \(\times\) g for 10 min, and the liposomal pellet was suspended in assay buffer (described under “Materials and Methods”) containing 1 mM CaCl\(_2\). The fura-2-loaded liposomes were treated with 10 \(\mu\)M PI(3,4,5)P\(_3\), 7 \(\mu\)M 25-hydroxycholecalciferol (25(OH)D\(_3\)), or 1 \(\mu\)M A23187, and the influx of Ca\(^{2+}\) was monitored by fura-2 fluorescence.

Role of PI 3-Kinase in TCR-mediated Ca\(^{2+}\) Signaling—The activation of Ca\(^{2+}\) influx by PI(3,4,5)P\(_3\) suggested a potential link between PI 3-kinase and T cell Ca\(^{2+}\) signaling. To test this premise, a combination of pharmacological and molecular approaches was employed to characterize the role of PI 3-kinase in anti-CD3-mediated Ca\(^{2+}\) response.

We first examined the effect of wortmannin, a potent PI 3-kinase inhibitor, on anti-CD3 mAb-induced Ca\(^{2+}\) response in fura-2-loaded Jurkat T cells. Fig. 5 shows that ligation of the TCR-CD3 complex by anti-CD3 mAb provoked a 4-fold increase in cytosolic Ca\(^{2+}\) (trace a). Subsequent exposure to exogenous PI(3,4,5)P\(_3\) (10 \(\mu\)M) only augmented the Ca\(^{2+}\) response to a small extent. The anti-CD3-induced Ca\(^{2+}\) response largely stemmed from Ca\(^{2+}\) mobilization across the plasma membrane because deprivation of external Ca\(^{2+}\) by EGTA inhibited 70% of the Ca\(^{2+}\) signal (Fig. 5A, inset).
that elicited by PI(3,4,5)P_3 in anti-CD3-stimulated cells (not shown). Samples were calibrated in reference to actin as internal standard (data not shown). Δp85 inhibits anti-CD3-induced Ca^{2+} response in a dose-dependent manner. Cells expressing different levels of Δp85 (a–d, as indicated above) were collected at the 6th day post-transfection and tested for anti-CD3-stimulated Ca^{2+} response.

We hypothesized that PI 3-kinase acted in concert with PLC-γ in initiating Ca^{2+} signaling following TCR activation. This premise is supported by the observation that when used alone, wortmannin and the PLC inhibitor U73122 (10 μM) exerted 57% and 50% inhibition, respectively, on anti-CD3-induced Ca^{2+} increase, whereas a combination of these two inhibitors could virtually abolish the Ca^{2+} response (Fig. 5B).

We also took an independent nonpharmacological approach to confirm the above results, in which Jurkat T cells were transiently transfected with a vector expressing HA epitope-tagged Δp85. It is well documented that the deletion of the binding motif for the catalytic p110 subunit in Δp85 confers PI 3-kinase dominant negative activity (19). Overexpression of this dominant negative inhibitor in T cells has been shown to down-regulate TCR-mediated interleukin-2 gene expression (29).

To confirm the above results, in which Jurkat T cells were transiently transfected with a vector expressing HA epitope-tagged Δp85. It is well documented that the deletion of the binding motif for the catalytic p110 subunit in Δp85 confers PI 3-kinase dominant negative activity (19). Overexpression of this dominant negative inhibitor in T cells has been shown to down-regulate TCR-mediated interleukin-2 gene expression (29).

Western analysis using anti-HA antibodies verified the expression of HA-Δp85 in transfected Jurkat T cells (Fig. 6A). It is noteworthy that the level of Δp85 expression displayed a direct correlation with the amount of cDNA used in transfection. Accordingly, transfected Jurkat T cells expressing varying levels of Δp85 were tested for Ca^{2+} response in response to anti-CD3 stimulation. In line with the wortmannin data, Δp85 suppressed anti-CD3-induced Ca^{2+} response in a dose-dependent manner, ranging from 5% to 40% in accordance with the level of Δp85 expression (Fig. 6B, traces a–d). Since both wortmannin and Δp85 gave consistent results in inhibiting anti-CD3-stimulated Ca^{2+} response, these data strongly support the involvement of PI 3-kinase in TCR-mediated Ca^{2+} signaling.

Characterization of PI(3,4,5)P_3-induced Ca^{2+} Entry—To characterize the underlying mechanism, we examined the effect of various pharmacological inhibitors on PI(3,4,5)P_3-induced Ca^{2+} entry. These included nifedipine (a voltage-gated Ca^{2+} channel blocker; 20 μM), phorbol 12-myristate 13-acetate (an inhibitor of TCR-mediated Ca^{2+} entry (9); 10 μg/ml), SKF96365 (a blocker of receptor-gated Ca^{2+} channels and store-operated Ca^{2+} entry (30); 10 μM), and forskolin (an inhibitor of store-operated Ca^{2+} entry (9); 50 μM). Among these inhibitors, only SKF96365 could effectively inhibit PI(3,4,5)P_3-induced Ca^{2+} influx (% of [Ca^{2+}]) control, 25 ± 3%, n = 3). The extent of inhibition was similar to that observed in the effect of SKF96365 on anti-CD3-induced Ca^{2+} response (Fig. 7). Other inhibitors examined failed to exert significant inhibition on PI(3,4,5)P_3-exerted Ca^{2+} influx (% of [Ca^{2+}] control, 88–97%).

Structurally, PI(3,4,5)P_3 contained Ins(1,3,4,5)P_4 as the head group, which raised a possibility that PI(3,4,5)P_3 might facilitate Ca^{2+} entry by activating the putative Ins(1,3,4,5)P_4 or Ins(1,3,4,5)P_4 receptor in plasma membranes. To test this theory, we first examined the displacement of [H]Ins(1,3,4,5)P_4 binding to the plasma membrane of Jurkat T cells by di-C_8-PI(3,4,5)P_3 (Fig. 8). Among these inhibitors, only SKF96365 could effectively inhibit PI(3,4,5)P_3-induced Ca^{2+} influx (% of [Ca^{2+}] control, 25 ± 3%, n = 3). The extent of inhibition was similar to that observed in the effect of SKF96365 on anti-CD3-induced Ca^{2+} response (Fig. 7). Other inhibitors examined failed to exert significant inhibition on PI(3,4,5)P_3-exerted Ca^{2+} influx (% of [Ca^{2+}] control, 88–97%).
between Ins(1,3,4,5)P4 and PI(3,4,5)P3 raised a crucial issue, whether anti-CD3 and PI(3,4,5)P3-induced Ca2+ release was inhibited by SKF96365, which is consistent with that observed with the whole cell (Fig. 7). Also noteworthy is that Ca2+ release induced by PIP3 or Ins(1,3,4,5)P4 was not augmented by subsequent challenge with either agonist or Ins(1,4,5)P3 (indicated by the arrows). The lack of Ca2+ response was likely due to desensitization or saturation of the binding site instead of the depletion of Ca2+ since the addition of 1 μM A23187 following PIP3 treatment triggered the release of large amounts of Ca2+ (Fig. 9, inset).

In contrast, for Ins(1,4,5)P3-stimulated Ca2+ release, subsequent stimulation with PIP3 or Ins(1,3,4,5)P4 caused additional release of Ca2+. Taken together with the binding data, this observation suggests that the putative PIP3 or Ins(1,3,4,5)P4 receptor might be discrete from the Ins(1,4,5)P3 receptor. Furthermore, the presence of Ins(1,4,5)P3 receptors in T cell plasma membranes was confirmed by using two specific antibodies against the type I and type III receptors. Western blot analysis showed significantly more labeling of the plasma membrane with the type III receptor antibodies than with type I receptor antibodies (Fig. 10). It is noteworthy that this Ins(1,4,5)P3 receptor subtype distribution is similar to that reported for platelet plasma membranes (31).

**Fig. 8.** Inhibition of specific [3H]Ins(1,3,4,5)P4 binding to plasma membranes of Jurkat T cells by increasing concentrations of PIP3, Ins(1,3,4,5)P4, and Ins(1,4,5)P3. The displacement assay was carried out as described under “Materials and Methods.” Nonspecific binding was measured in the presence of 30 μM Ins(1,3,4,5)P4. Each data point represents the mean of three determinations.

In cells, the 3-phosphorylation of Ins(1,4,5)P3 by Ins(1,4,5)P3-specific 3-kinase accounts for a major pathway for the formation of Ins(1,3,4,5)P4 (32). Thus, stimulated Ins(1,4,5)P3 accumulation leads to Ins(1,3,4,5)P4 increase in T cells (2). Fig. 11A (left panel) demonstrates that treatment of myo-[3H]inositol-labeled Jurkat T cells with anti-CD3 mAb stimulated a transient increase in [3H]Ins(1,4,5)P3, accompanied by a concurrent rise in [3H]Ins(1,3,4,5)P4 over a 10-min period. Considering the time course of anti-CD3-induced Ca2+ response (Fig. 11B, control), the production of [3H]Ins(1,3,4,5)P4 slightly lagged behind the rise in [Ca2+]i in response to anti-CD3. The Ins(1,3,4,5)P4 level peaked about 5 min post-treatment and returned to a near basal level, as indicated by the arrow. The external Ca2+ concentration returned to a near baseline level, the membrane vesicles were washed with 10 mM Hepes, pH 7.0, four times and suspended in the same buffer. The specific [3H]Ins(1,3,4,5)P4 binding to plasma membrane vesicles was linear up to 25 μM Ins(1,3,4,5)P4 (indicated by the arrows). The lack of Ca2+ response was likely due to desensitization or saturation of the binding site instead of the depletion of Ca2+ since the addition of 1 μM A23187 following PIP3 treatment triggered the release of large amounts of Ca2+ (Fig. 9, inset).

In contrast, for Ins(1,4,5)P3-stimulated Ca2+ release, subsequent stimulation with PIP3 or Ins(1,3,4,5)P4 caused additional release of Ca2+. Taken together with the binding data, this observation suggests that the putative PIP3 or Ins(1,3,4,5)P4 receptor might be discrete from the Ins(1,4,5)P3 receptor. Furthermore, the presence of Ins(1,4,5)P3 receptors in T cell plasma membranes was confirmed by using two specific antibodies against the type I and type III receptors. Western blot analysis showed significantly more labeling of the plasma membrane with the type III receptor antibodies than with type I receptor antibodies (Fig. 10). It is noteworthy that this Ins(1,4,5)P3 receptor subtype distribution is similar to that reported for platelet plasma membranes (31).

**Fig. 9.** Ca2+ release from plasma membrane vesicles. Jurkat T cell plasma membranes were prepared as described under “Materials and Methods” and were treated with 30 μM Mg2+-ATP, 1 mM CaCl2, 1 μM thapsigargin, and 2.5 μg/ml oligomycin on ice for 10 min. The membrane vesicles were washed with 10 mM Hepes, pH 7.0, four times and suspended in the same buffer. The assay medium consisted of 0.2–0.25 mg of membrane proteins and 1 μM fura-2 in 2 ml of 10 mM Hepes, pH 7.0, and treated with 25 μM di-C8-PI(3,4,5)P3 (A), 10 μM Ins(1,4,5)P3 (B), or 10 μM Ins(1,4,5)P3 (C), as indicated by the arrows. Until the external Ca2+ concentration returned to a near baseline level, the membrane vesicles were stimulated with 10 μM Ins(1,3,4,5)P4 or 25 μM di-C8-PI(3,4,5)P3 as indicated. The inset indicates the sequential additions of 25 μM PIP3, 25 μM Ins(1,3,4,5)P4, and 1 μM A23187.

**Fig. 10.** Western blot analysis of Ins(1,4,5)P3 receptor (IP3R) isoforms in T cell plasma membranes.
The role of PI(3,4,5)P3 in anti-CD3-mediated Ca2+ signaling has been investigated. Given the observation that PI(3,4,5)P3 stimulates PLC-g in vitro (25), the time course of [3H]Ins(1,4,5)P3 and [3H]Ins(1,3,4,5)P4 production in response to PI(3,4,5)P3 stimulation was examined. As PI(3,4,5)P3 is not susceptible to hydrolysis by any known PLC (34), it does not contribute to Ins(1,3,4,5)P4 formation in vivo. As shown in Fig. 12A, exogenous PI(3,4,5)P3 did not display any stimulatory effect on the production of either inositol phosphates. In fact, PI(3,4,5)P3 suppressed the formation of [3H]Ins(1,3,4,5)P4 without affecting Ins(1,4,5)P3 production. B, effect of adriamycin (10 μM) on anti-CD3-induced Ca2+ increase in Jurkat T cells. No appreciable difference was noted in the Ca2+ response between adriamycin-treated and adriamycin-untreated cells, even though the inhibitor completely blocked Ins(1,3,4,5)P4 synthesis.

Affinity Probing of PI(3,4,5)P3-binding Proteins in T Cell Plasma Membranes—We further prepared a biotinylated analog of PI(3,4,5)P3, Biotin-PIP3 (Fig. 13A), to confirm the existence of PI(3,4,5)P3-binding proteins in T cell plasma membranes. This affinity ligand has been successfully applied to the purification of PI(3,4,5)P3-binding proteins even with a Kd as high as 100 μM (17). The plasma membrane fraction was treated with 5% CHAPS, and the solubilized proteins were incubated with Biotin-PIP3 followed by streptavidin beads. The adsorbed beads were spun down by centrifugation, washed with 150 mM NaCl, and eluted with 2 M urea. SDS-polyacrylamide gel electrophoresis analysis of solubilized proteins from T cell plasma membranes and affinity-purified proteins, visualized by silver staining, indicated two major protein bands with apparent molecular masses of 67 kDa and 59 kDa (Fig. 13B). Molecular mass markers: myosin H-chain (228 kDa), phosphorylase b (102 kDa), bovine serum albumin (71 kDa), and ovalbumin (46 kDa).

**DISCUSSION**

This study presents both pharmacological and molecular genetic evidence that PI 3-kinase plays an obligatory role in TCR-mediated Ca2+ signaling via a PI(3,4,5)P3-sensitive Ca2+ influx system on plasma membranes. This unique Ca2+ entry mechanism connotes physiological significance considering the pivotal role of PI 3-kinase in the regulation of T cell function and may serve as a potential target for the modulation of T cell immunity.

Substantial evidence indicates that triggering of T cells through the TCR-CD3 complex leads to membrane recruitment...
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of signaling proteins such as PI 3-kinase, PLC-γ₁, and Grb2 to form multi-molecular signaling complexes (12). These proteins initiate distinct signaling cascades that culminate in cell proliferation and induction of effector functions like interleukin-2 secretion. However, in contrast to PLC-γ₁ and Grb2, the precise role of PI 3-kinase in TCR signaling remains elusive. Recent evidence suggests that PI 3-kinase is required for Erk2 activation (28), NFAT activation (29), and interleukin-2 production (27) in stimulated T cells. The present data demonstrate that PI(3,4,5)P₃, the primary output signal of PI 3-kinase, can generate Ca²⁺ stimuli that synergize with Ins(1,4,5)P₃-induced Ca²⁺ release and capacitative Ca²⁺ entry for sustaining elevated [Ca²⁺], a driving force underlying many cellular responses.

This Ca²⁺ entry mechanism is directly activated by PI(3,4,5)P₃. In cells, PI(3,4,5)P₃ is subjected to rapid turnover by three discrete pathways: dephosphorylation by multiple 5-phosphatases to form PI(3,4,5)P₂ (35–37), dephosphorylation by PTEN to form PI(4,5)P₂ (38), and hydrolysis by phosphoinositide-specific phospholipases to form Ins(3,4,5)P₃ (39). None of these metabolites was capable of eliciting Ca²⁺ response in the whole cells or plasma membrane vesicles. This PI(3,4,5)P₃-induced Ca²⁺ influx displays several unique features. First, among various cell types examined to date, only T cells and platelets display Ca²⁺ influx in response to PI(3,4,5)P₃ treatment, whereas mouse splenic B cells, NIH3T3 cells, PC-12 cells, Hep G2 cells, and LNCaP cells were insensitive to PI(3,4,5)P₃. In the literature, based on studies with PI 3-kinase inhibitors, PI 3-kinase has also been implicated in antigen-stimulated Ca²⁺ influx in mast cells (40, 41). Taken together, these data suggest that PI(3,4,5)P₃-sensitive Ca²⁺ entry pathway exists in specific hematopoietic cells. This cell-type specificity warrants further investigation because it underlines a distinct function of PI 3-kinase in Ca²⁺ regulation.

Second, this Ca²⁺ entry does not require depletion of internal Ca²⁺ pools, indicating that the PI(3,4,5)P₃-activated Ca²⁺ influx is independent of signals from empty stores (capacitative Ca²⁺ entry). In addition, PI(3,4,5)P₃ does not disturb Ins(1,4,5)P₃-sensitive or thapsigargin-sensitive Ca²⁺ release from plasma membrane vesicles. This in vitro cross-reactivity, due to the largely shared structural motifs, raises an interesting question with regard to which species representing the physiologically relevant ligand responsible for the Ca²⁺ entry. To date, published data on the role of Ins(1,3,4,5)P₄ in Ca²⁺ mobilization across plasma membranes remain inconclusive. Although several reports implicated Ins(1,3,4,5)P₄ in mediating Ca²⁺ entry in certain types of electrically nonexcitable cells such as sea urchin eggs (42), Xenopus oocytes (43), and platelets (44), other studies indicated that Ins(1,3,4,5)P₄ did not have a significant effect, if any, on potentiating Ca²⁺ influx in other cells like mouse lacrimal acinar cells (45) and Jurkat T cells (33). The data obtained in this study support the latter view that Ins(1,3,4,5)P₄ does not play a role in anti-CD3- or PI(3,4,5)P₃-elicited Ca²⁺ influx.

Meanwhile, several research groups have isolated an Ins(1,3,4,5)P₄-binding protein, GAP1P₄BP, from platelet plasma membranes (44, 46–48). GAP1P₄BP was found to be a GTPase-activating protein with a molecular mass of 10 kDa. It remains enigmatic how this GAP protein is involved in Ca²⁺ entry. However, our affinity ligand study indicates that although many PI(3,4,5)P₃-binding proteins existed in the T cell plasma membrane, none of these proteins displayed a molecular mass in line with that of GAP1P₄BP. This finding dampened the possibility that GAP1P₄BP was involved in the PI(3,4,5)P₃-induced Ca²⁺ influx in Jurkat T cells.

In summary, although the mechanism by which PI(3,4,5)P₃ mediates Ca²⁺ entry remains unclear, this PI(3,4,5)P₃-sensitive pathway not only provides molecular insights into T cell Ca²⁺ regulation but also represents a potential target for the modulation of cell function in T lymphocytes. Unlike inositol phosphates, PI(3,4,5)P₃ is membrane-permanent. Thus, it is plausible to design PI(3,4,5)P₃ analogues as antagonists of the putative receptors for therapeutic uses. However, outstanding questions that remain are as follows. What is its relationship with the Ins(1,4,5)P₃ receptor on plasma membranes? Is there cross-communication with other Ca²⁺ channels (such as Ca²⁺ release-activated Ca²⁺ channels) on plasma membranes to regulate Ca²⁺ entry? To address these questions, sequence analysis of the putative PI(3,4,5)P₃-binding proteins is currently under way in this laboratory.

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