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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(3)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 Δp85 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-*myo*-inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) 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 form multifunctional complexes downstream of many receptor signaling pathways, we hypothesize that $PI(3,4,5)P_3$-induced Ca^{2+} entry acts concertedly with $Ins(1,4,5)P_3$-induced Ca^{2+} release in initiating T cell Ca^{2+} signaling. By using a biotinylated analog of $PI(3,4,5)P_3$ as the affinity probe, we have detected several putative $PI(3,4,5)P_3$-binding proteins in T cell plasma membranes.

Engagement of the TCR[1]-CD3 complex stimulates an array 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 inflow 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 diffusable 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).

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-γ1 are recruited to the plasma membrane to form multifunctional complexes (10–12). Activation of PI 3-kinase results in a transient accumulation of μM 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-ki-

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[1] The abbreviations used are: TCR, T cell receptor; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PI(3)P, phosphatidylinositol 3-monophosphate; Biotin-PIP_3, biotinylated $PI(3,4,5)P_3$; $Ins(1,4,5)P_3$, D-*myo*-inositol 1,4,5-trisphosphate; $Ins(1,3,4,5)P_4$, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; ER, endoplasmic reticulum; di-C_8-$PI(3,4,5)P_3$, 1-*O*-(1, 2-di-*O*-octanoyl-*sn*-glycero-3-*O*-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate; AM, acetoxymethyl ester; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

nase and PLC-γ in T cell activation, these data prompt a new hypothesis that PI(3,4,5)P_3-sensitive Ca^{2+} entry plays a concerted role with Ins(1,4,5)P_3-induced Ca^{2+} release and capacitative Ca^{2+} entry in TCR-mediated Ca^{2+} signaling.

MATERIALS AND METHODS

D-*myo*-Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), D-*myo*-inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-*O*-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate (PI(3,4,5)P_3), 1-*O*-(1,2-di-*O*-octanoyl-*sn*-glycero-3-*O*-phosphoryl)-D-*myo*-inositol 3,4,5-trisphosphate (di-C_8-PI(3,4,5)P_3), 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-*O*-phosphoryl)-D-*myo*-inositol 3,4-bisphosphate (PI(3,4)P_2), and 1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-*O*-phosphoryl)-D-*myo*-inositol 3-monophosphate PI(3)P were synthesized as previously reported (15, 16). The synthesis of the biotinylated PI(3,4,5)P_3, (+)-1-*O*-(1-*O*-[8-(*N*-biotinoylamino)-octanoyl]-2-*O*-octanoyl-*sn*-glycero-3-phosphoryl)-*myo*-inositol 3,4,5-trisphosphate (Biotin-PIP$_3$), is described elsewhere (17). The identity and purity of all inositol phosphates and inositol lipids were examined by ^{1}H and ^{31}P NMR and high resolution mass spectrometry. Phorbol 12-myristate 13-acetate, wortmannin, SKF96365, indo-1 acetoxymethyl ester (AM), fura-2 AM were purchased from Calbiochem. Nifedipine was obtained from ICN. Leupeptin and 4-(2-aminoethyl)benzenesulfonyl fluoride were products from Sigma. [^{3}H]Ins(1,3,4,5)P_4 was purchased from NEN Life Science Products. Anti-human CD3 mAb (21-L5) was obtained from Santa Cruz. Anti-Thy1.2 mAb-FITC and anti-B-220 mAb-FITC were products from Pharmingen.

Flow Cytometric Cell Sorting and Analysis of Intracellular Ca^{2+}—Mouse spleen cells (10^7) were treated with 5 μg of anti-Thy1.2 mAb- or anti-B-220 mAb-fluorescein (FITC) conjugates in 200 μl of culture medium consisting of a 1:1 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 nutrient mixtures, 10% fetal bovine serum, 2 mM glutamine, 50 nM 2-mercaptoethanol, 20 units/ml bovine insulin, 20 nM progesterone, 5 μg/ml transferrin, and 1 μg/ml gentamicin for 30 min on ice. Anti-Thy1.2 and anti-B-220 are antibodies against the cell surface markers of T and B cells, respectively. The suspension was washed three times and resuspended in 500 μl of the same medium. For intracellular Ca^{2+} analysis, these cells were loaded with 1 μM indo-1 AM for 30 min at 37 °C, washed twice, and suspended in 1 ml of assay buffer consisting of 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, 113 mM NaCl, 5 mM glucose, pH 7.4. A FACStar plus cell sorter (Becton Dickinson) was used for cell sorting and to monitor indo-1 fluorescence. FITC-stained cells were analyzed by monitoring the emission at 530 nm with excitation at 488 nm. Intracellular Ca^{2+} was measured by comparing the ratio of indo-1 emission at 405 nm and 520 nm with excitation at 350 nm as described previously (18).

Transient Transfection—The construct expressing hemagglutinin (HA)-tagged Δp85 was a kind gift from Professor Alex Toker (Harvard Medical School). Δp85 is a deletion mutant that lacks a region required for tight association with p110 but is still able to bind to appropriate phosphotyrosine targets. Thus, Δp85 can compete with native p85 for binding to essential signaling proteins and behaves as a dominant negative mutant (19). Jurkat T cells were grown to a density of 5×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum. Cells were harvested, washed with serum-free Opti-MEM (Life Technologies, Inc.), and suspended in the same medium (5×10^5 cells/ml). Transfection was carried out according to a modification of the protocol supplied by the manufacturer. Aliquots containing 0.5 μg, 1.5 μg, and 3 μg of the HA·Δp85 expression vector or 3 μg of a control pCMV/blue plasmid in 500 μl of Opti-MEM were incubated with 30–60 μl of the Plus reagent from the LipofectAMINE Plus reagent kit at 25 °C for 15 min, and the mixture was added to 40 μl of the LipofectAMINE reagent in 500 μl of Opti-MEM. The mixture was incubated at 25 °C for 15 min and added to 5 ml of the cell suspension. After 5 h at 37 °C, the transfection media were replaced with 5 ml of the RPMI 1640–10% fetal bovine serum medium. The transfected cells were allowed to grow for 6 days with the medium changed every other day to express foreign DNA. The collected cells were analyzed for anti-CD3-induced Ca^{2+} response by fluorescence spectrometry and for HA·Δp85 expression by Western blot using anti-HA antibodies.

Preparation of Jurkat T Cell Plasma Membranes—Two different methods were employed for the plasma membrane preparations. For the Ins(1,3,4,5)P_4 receptor binding assay, the membrane fraction was prepared according to a method described by Khan *et al.* (5). For the Ca^{2+} release assay, the plasma membrane was purified by a modification of the method described by Neville (20). In brief, Jurkat T cells (4×10^8 cells) were washed with phosphate-buffered saline and suspended in 5 ml of PM buffer consisting of 20 mM Hepes, pH 7.2, 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM dithiothreitol, 1 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 20 μg/ml leupeptin. The cell suspension was homogenized in a Dounce homogenizer using the loose pestle with 5 strokes up and down. The homogenate was centrifuged at $1,500 \times g$ for 10 min. The pellet was suspended in 3.125 ml of PM buffer and mixed with 5.5 ml of 69% (w/w) sucrose to make a final 44% (w/w) sucrose-membrane mixture. The mixture was overlaid with 42.3% (w/w) sucrose, and the two-phase suspension was subjected to centrifugation at $90,000 \times g$ for 2 h in a swinging bucket rotor. The membrane material at the interface of the phases contained the greatest enrichment in plasma membranes based on the activity of (Na^+-K^+)-ATPase. This fraction was collected, suspended in 5 ml of 10 mM Hepes, pH 7.5, containing 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl-)benzenesulfonyl fluoride, and 20 μg/ml leupeptin and centrifuged at $25,000 \times g$ for 10 min. The pellet was suspended in 1 ml of the same buffer.

Displacement of [^{3}H]Ins(1,3,4,5)P_4 Binding to Jurkat T Cell Plasma Membranes by di-C_8-PI(3,4,5)P_3 and Inositol Polyphosphates—Since PI(3,4,5)P_3 contains Ins(1,3,4,5)P_4 as its head group, it is plausible that this inositol phospholipid shares the binding site on the plasma membrane with Ins(1,3,4,5)P_4. To assess this possibility, we examined the displacement of [^{3}H]Ins(1,3,4,5)P_4 binding by di-C_8-PI(3,4,5)P_3, a water-soluble derivative, Ins(1,3,4,5)P_4, and Ins(1,4,5)P_3. The freshly prepared membrane preparation (200 μg of protein) was incubated with 2 nM [^{3}H]Ins(1,3,4,5)P_4 (30 Ci/mmol) in 10 mM Hepes, pH 7.5, containing 100 mM KCl, 20 mM NaCl, and 1 mM EDTA in the presence of various concentrations of the competitive ligand, with a final volume of 0.3 ml. The mixture was incubated at 4 °C for 15 min, and the reaction was terminated by centrifugation at $16,000 \times g$ for 5 min. The membrane-bound radioactivity was analyzed by liquid scintillation spectrometry. Nonspecific binding was measured in the presence of 30 μM Ins(1,3,4,5)P_4.

Fluorescence Spectrophotometric Measurement of Intracellular Ca^{2+}—$[Ca^{2+}]_i$ was monitored by the change in the fluorescence intensity of fura-2-loaded cells. Jurkat T cells (1×10^7 cells/ml), suspended in the aforementioned assay buffer containing 0.5% bovine serum albumin and 2 mM probenacid, were incubated with 10 μM fura-2 AM in the dark for 1 h at 37 °C. The cells were then pelleted by centrifugation at $1,000 \times g$ for 10 min, washed with assay buffer twice, and resuspended at approximately 8×10^5 cells/ml in the same buffer containing 1 mM Ca^{2+}. The effect of anti-CD3 mAb or various inositol lipids on $[Ca^{2+}]_i$ 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 intensity (F_{max}) in Jurkat cells was determined by adding A23187 (1 μM), and the minimum fluorescence (F_{min}) was determined following depletion of external Ca^{2+} by 5 mM EGTA. The $[Ca^{2+}]_i$ was calculated according to the equation $[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F)$, where K_d denotes the apparent dissociation constant (=224 nM) of the fluorescence dye-Ca^{2+} complex (21).

[^{3}H]Inositol Phosphate Turnover Analysis—The examination of phosphoinositol 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-^{3}H]inositol (10 μCi/10^6 cells/ml) in inositol-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 KCl, 1.3 mM Na_2HPO_4, 1 mM KH_2PO_4, 8.3 mM $NaHCO_3$, 1.6 mM $MgSO_4$, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, and 5.6 mM glucose. Aliquots containing 1×10^6 cells were each resuspended in 0.3 ml of the aforementioned assay buffer plus 1 mM $CaCl_2$ and 100 μM EGTA and transferred to 1.5-ml microcentrifuge tubes. Each sample was incubated with 1.5 μg of anti-CD3 mAb or 20 μM PI(3,4,5)P_3 for the indicated times and quenched by adding 0.25 ml of 6% trichloroacetic acid. The tubes were centrifuged for 2 min at $12,000 \times g$. The supernatant (200 μl) was analyzed by high performance liquid chromatography on a 5-μm Adsorbosphere Sax column (4.6 × 200 mm) equilibrated with H_2O. The [^{3}H]inositol phosphates were eluted with a linear gradient of 0–0.9 M $NH_4H_2PO_4$ 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 [^{3}H]Ins(1,3,4,5)P_4, [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(4,5)P_2, Ins(4)P were used as standards. The respective retention times were 60, 48, 43, and 31 min.

Detection of PI(3,4,5)P_3-binding Proteins in T Cell Plasma Membranes—T cell plasma membranes were treated with 5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid for 1 h on ice and centrifuged at $40,000 \times g$ for 1 h. The supernatant (100 μg of protein) was incubated with 100 μM Biotin-PIP$_3$ for 1 h, and 200 μl of strepta-

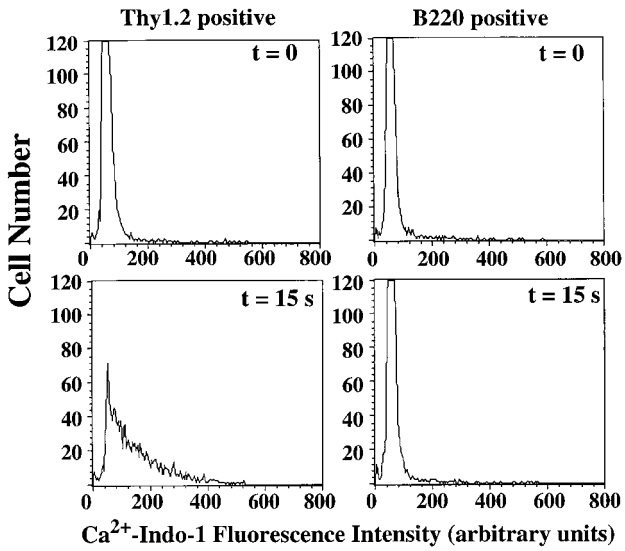

FIG. 1. **Representative fluorescence intensity distributions from flow cytometry.** *Left*, indo-1 fluorescence ratio of Thy1.2-positive mouse splenic cells at 0 and 15 s following stimulation with 10 μM PI(3,4,5)P_3. *Right*, indo-1 fluorescence ratio of B-220-positive mouse splenic cells at 0 and 15 s following stimulation with 10 μM PI(3,4,5)P_3. The pattern remained unaltered even 5 min after PI(3,4,5)P_3 treatment (data not shown). The experimental procedures are described under "Materials and Methods."

vidin 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)P_3 Induced Intracellular Ca^{2+} 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)P_3 induced Ca^{2+} influx across plasma membranes, resulting in immediate cell aggregation (22). This finding prompted us to study PI(3,4,5)P_3-induced Ca^{2+} response in other cell types. Using fluorescence-activated cell sorting, we examined the effect of PI(3,4,5)P_3 on indo-1-loaded mouse spleen cells stained with FITC-conjugates of anti-Thy1.2 or anti-B-220 mAb. As shown, 10 μM PI(3,4,5)P_3 induced a significant increase in $[Ca^{2+}]_i$ in Thy1.2-positive mouse spleen cells as soon as 15 s after stimulation. This PI(3,4,5)P_3-stimulated Ca^{2+} increase was also confirmed in human peripheral T cells and Jurkat T cells by fluorescence spectrophotometry. In contrast, $[Ca^{2+}]_i$ in the B-220-positive cell population remained unaffected even in the presence of 20 μM PI(3,4,5)P_3 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 Ca^{2+} response following PI(3,4,5)P_3 stimulation (data not shown). This cell-type specificity underscores a fundamental difference in the role of PI 3-kinase in Ca^{2+} regulation in different cells.

The direct introduction of micellar PI(3,4,5)P_3 to intact cells is also worth comment. Although how PI(3,4,5)P_3 permeates cell membranes remains unclear, published data from this and other laboratories show that exogenous PI(3,4,5)P_3 can readily fuse with cell membranes and exert cellular and biochemical responses in different cell types including platelets, NIH3T3 cells, and adipocytes (22–24).

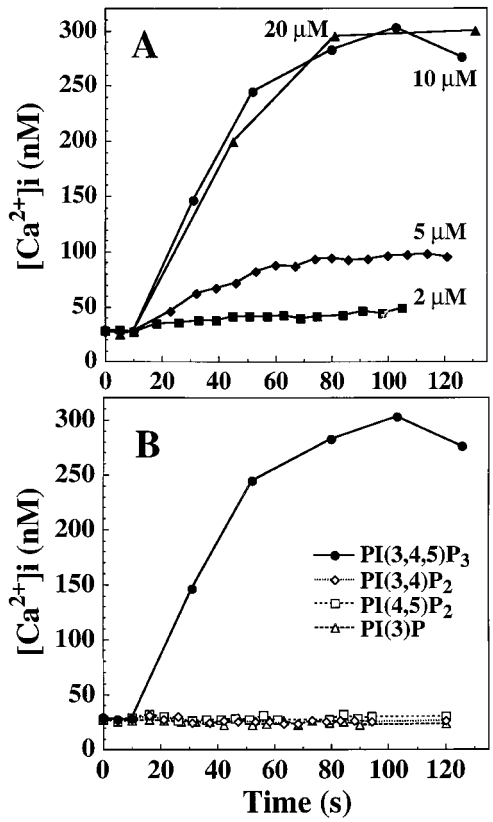

FIG. 2. *A*, time course and dose dependence of the effect of PI(3,4,5)P_3 on $[Ca^{2+}]_i$ in Thy1.2-positive mouse splenic cells. *B*, phosphoinositide specificity in eliciting a $[Ca^{2+}]_i$ increase in T cells. Concentrations used: PI(3,4,5)P_3, 10 μM; PI(3,4)P_2, 20 μM; PI(4,5)P_2, 20 μM; PI(3)P, 20 μM. $[Ca^{2+}]_i$ was analyzed by flow cytometry as described in Fig. 1.

Fig. 2*A* depicts that the effect of PI(3,4,5)P_3 on $[Ca^{2+}]_i$ in splenic T cells was dose-dependent, with a threshold of about 2 μM, and was saturable. As shown, treatment with the lipid messenger in excess of 10 μM did not further enhance the amplitude of Ca^{2+} response.

In line with our previous finding in platelets (22), the induction of Ca^{2+} response displayed stringent specificity for PI(3,4,5)P_3, underlying its second messenger role. Other phosphoinositides examined, including PI(3,4)P_2, PI(4,5)P_2, PI(3)P, failed to exert an appreciable change in $[Ca^{2+}]_i$ at 20 μM (Fig. 2*B*). Moreover, fura-2 fluorimetry showed that the effect of PI(3,4,5)P_3 on $[Ca^{2+}]_i$ was also demonstrated in different subtypes of T cells, including mouse thymocytes, Jurkat T cells, and human peripheral T cells (data not shown). Taken together, these data indicate that exogenous PI(3,4,5)P_3 stimulated Ca^{2+} influx in T cells regardless the stage of cell development.

PI(3,4,5)P_3 Does Not Release Ca^{2+} from Internal Stores—Several lines of evidence suggest that the PI(3,4,5)P_3-induced Ca^{2+} response was attributable to Ca^{2+} influx from the medium. First, this Ca^{2+} increase was completely abrogated by pretreatment with EGTA (Fig. 3*A*). Second, although PI(3,4,5)P_3 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)P_3-elicited Ca^{2+} response (Fig. 3*A*). Furthermore, the PI(3,4,5)P_3 concentration needed to elicit Ca^{2+} response (<10 μM) was an order of magnitude lower than the threshold required for PLC-γ activation. Thus, PI(3,4,5)P_3-induced Ca^{2+} response was independent of Ins(1,4,5)P_3 formation. Third, PI(3,4,5)P_3 had no effect on thapsigargin-sensitive Ca^{2+} pools (Fig. 3*B*). Jurkat T cells were treated with 10 μM PI(3,4,5)P_3 in a Ca^{2+}-depleted milieu, fol-

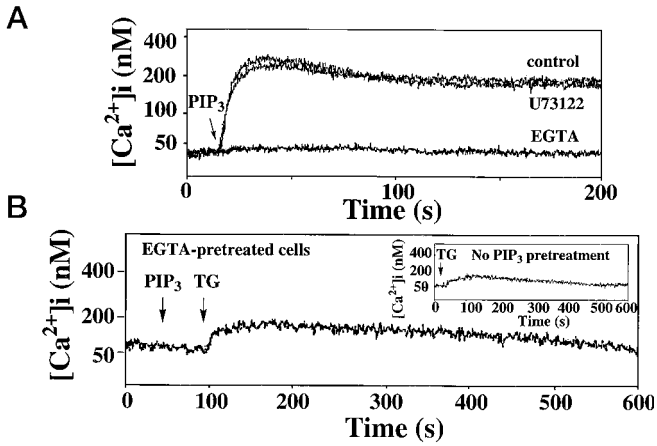

FIG. 3. *A*, PI(3,4,5)P_3-induced Ca^{2+} response is due to external Ca^{2+} influx and is independent of PLC activation. Fura-2-loaded Jurkat T cells were treated with 10 μM PI(3,4,5)P_3 alone (*control*) or they were exposed to 10 μM U73122 or 5 mM EGTA for 10 min before 10 μM PI(3,4,5)P_3 stimulation. $[Ca^{2+}]_i$ was analyzed by fluorescence spectrophotometry as described under "Materials and Methods." *B*, PI(3,4,5)P_3 does not affect thapsigargin (*TG*)-sensitive internal Ca^{2+} pools. In the presence of 5 mM EGTA, fura-2-loaded Jurkat T cells were treated with, in tandem, 10 μM PI(3,4,5)P_3 and 1 μM thapsigargin. *Inset*, in the presence of 5 mM EGTA, fura-2-loaded Jurkat T cells were treated with 1 μM thapsigargin alone (*inset*). The *arrow* indicates the time of addition for individual compounds. Representative *traces* from three observations are shown.

lowed by exposure to thapsigargin (1 μM). As shown, PI(3,4,5)P_3 exposure did not affect the extent of thapsigargin-induced Ca^{2+} response as compared with that without PI(3,4,5)P_3 pretreatment (Fig. 3*B*, *inset*). These results indicate that PI(3,4,5)P_3-induced Ca^{2+} increase was attributable to Ca^{2+} inflow from the medium.

PI(3,4,5)P_3 Did 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 *vis à vis* A23187 and 25-hydroxycholecalciferol, a sterol known to increase membrane permeability to Ca^{2+} (26). As shown in Fig. 4, A23187 (1 μM) caused a rapid and robust increase in fura-2 fluorescence, whereas 25(OH)D_3 (7 μM) induced an immediate but more modest rise.

In contrast, 10 μM PI(3,4,5)P_3 did not elicit any appreciable effect on fura-2 fluorescence. Taken together with the aforementioned cell-type and ligand specificity data, one could conclude that PI(3,4,5)P_3 does not perturb membrane permeability to Ca^{2+}.

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 μM) only augmented the Ca^{2+} response to a small extent. The anti-CD3-induced Ca^{2+} response largely

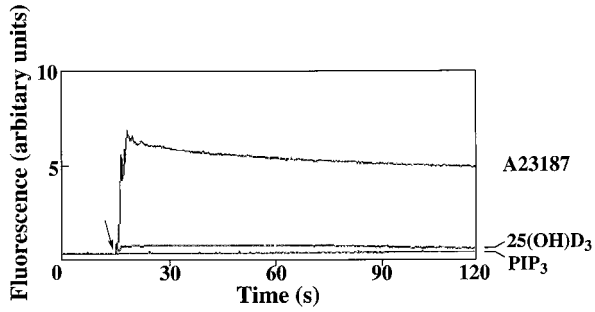

FIG. 4. **PI(3,4,5)P_3 does not perturb the permeability of lipid vesicles to Ca^{2+}.** Fura-2-loaded multilamellar lipid vesicles were prepared as follows. A mixture of 50 mol % phosphatidylcholine, 30 mol % cholesterol, 10 mol % phosphatidylserine, and 10 mol % phosphatidylethanolamine was dissolved in $CHCl_3$ and dried under a stream of N_2 followed by vacuum overnight. The lipids were dispersed in 0.1 M Hepes, pH 7.0, containing 10 μM fura-2, warmed up to 40 °C for 5 min, and vortexed for 30 s. The suspension was centrifuged at 12,000 × *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 μM PI(3,4,5)P_3, 7 μM 25-hydroxycholecalciferol (*25(OH)D_3*), or 1 μM A23187, and the influx of Ca^{2+} was monitored by fura-2 fluorescence.

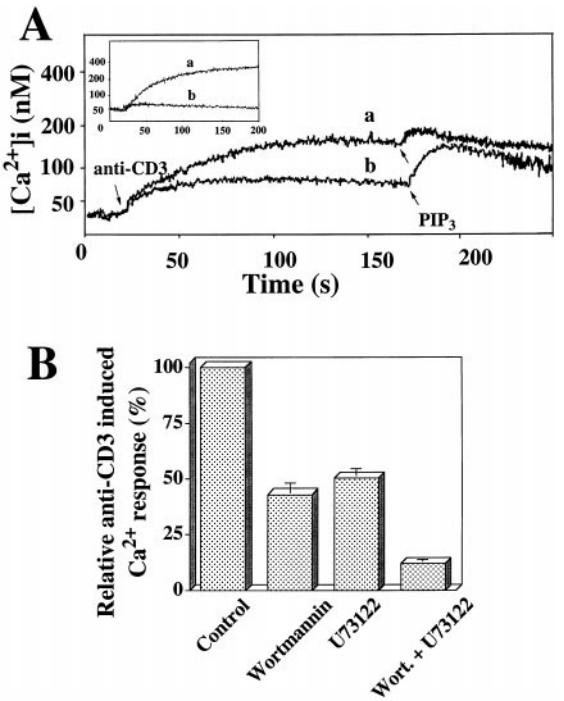

FIG. 5. *A*, PI(3,4,5)P_3 rescues the inhibitory effect of wortmannin on anti-CD3 mAb-induced Ca^{2+} response in Jurkat T cells. Cells were loaded with fura-2 and incubated with wortmannin (1 μM) (*trace b*) or Me_2SO vehicle (*trace a*) for 5 min before stimulation with 10 μg/ml anti-CD3, followed by 10 μM PI(3,4,5)P_3. The *arrow* indicates the time of ani-CD3 or PI(3,4,5)P_3 addition. The *inset* depicts the effect of EGTA on anti-CD3 mAb-induced $[Ca^{2+}]_i$ response in Jurkat T cells (*trace a*, control; *trace b*, 1 mM EGTA). Representative *traces* from three observations are shown. *B*, effect of wortmannin and U73122, 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_2SO vehicles (*Control*), wortmannin (1 μM), U73122 (10 μM), or the combination of wortmannin (1 μM) and U73122 (10 μM) for 10 min before adding anti-CD3 mAb (10 μg/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.

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. 5*A*, *inset*).

Pretreatment with wortmannin (1 μM) attenuated the am-

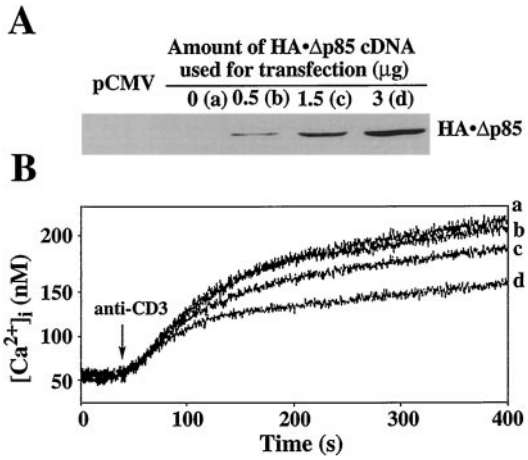

FIG. 6. **Overexpression of Δp85 inhibits anti-CD3-induced Ca^{2+} response.** *A*, expression levels of HA·Δp85 in Jurkat T cells that had been infected with a control pCMV/blue plasmid or with increasing amounts of the HA·Δp85-expressing plasmid (*a*, 0 μg; *b*, 0.5 μg; *c*, 1.5 μg; *d*, 3 μg) for 6 days. The Western analysis was carried out using antibodies against the HA tag. The loading amounts of individual samples were calibrated in reference to actin as internal standard (data not shown). *B*, 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.

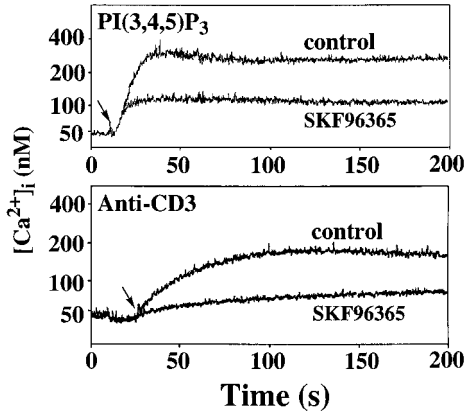

FIG. 7. **Effect of SKF96365 on PI(3,4,5)P_3-induced Ca^{2+} response (*A*) and on anti-CD3-induced Ca^{2+} response (*B*).** Fura-2-loaded Jurkat T cells were treated with 10 μM SKF96365, followed by 10 μM PI(3,4,5)P_3 or 10 μg/ml anti-CD3. The *arrow* indicates the time of agonist stimulation.

plitude of the anti-CD3-stimulated Ca^{2+} influx by nearly 60%, which, however, could be rescued by the subsequent exposure to 10 μM PI(3,4,5)P_3 (*trace b*). As shown, PI(3,4,5)P_3 could restore the $[Ca^{2+}]_i$ of wortmannin-treated cells to that of the control. Moreover, the extent of Ca^{2+} increase greatly exceeded that elicited by PI(3,4,5)P_3 in anti-CD3-stimulated cells (*trace a*), suggesting that the reversal of wortmannin inhibition by PI(3,4,5)P_3 was not simply due to an additive effect.

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. 5*B*).

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 (27), Erk2 (extracellular signal-regulated protein kinase) activation (28), and NFAT (nuclear factor of activated T cells) activation (29).

Western analysis using anti-HA antibodies verified the expression of HA·Δp85 in transfected Jurkat T cells (Fig. 6*A*). 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+} entry 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. 6*B*, *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+} inflow (% 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(1,4,5)P_3 or Ins(1,3,4,5)P_4 receptor in plasma membranes. To test this theory, we first examined the displacement of [^{3}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 *vis à vis* Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 (Fig. 8).

The di-C_8 analog of PI(3,4,5)P_3, a water-soluble derivative, was used in lieu of micellar di-C_{16}-PI(3,4,5)P_3 because the latter would be readily incorporated into membranes. The displacement curves for di-C_8-PI(3,4,5)P_3 and Ins(1,3,4,5)P_4 were in agreement with those reported for platelet membranes (22). The potencies in displacing [^{3}H]Ins(1,3,4,5)P_4 were in the order of di-C_8-PI(3,4,5)P_3 > Ins(1,3,4,5)P_4 ≫ Ins(1,4,5)P_3, with IC_{50} values of 15, 24, and 650 nM, respectively.

Ca^{2+}-releasing Activity of PI(3,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,4,5)P_3—The inside-out plasma membrane of Jurkat T cells displayed ATP-dependent Ca^{2+} sequestering activity. Thus, the plasma membrane vesicles were loaded with Ca^{2+} by exposing to ATP in the presence of thapsigargin (1 μM) and oligomycin (5 μg/ml). The Ca^{2+}-loaded membrane vesicle was washed and analyzed for agonist-induced Ca^{2+} release that was reflective of Ca^{2+} influx in intact cells. Fura-2 fluorescence indicates that addition of di-C_8-PI(3,4,5)P_3 (25 μM), Ins(1,3,4,5)P_4 (10 μM), or Ins(1,4,5)P_3 (10 μM) caused immediate Ca^{2+} release followed by slow re-uptake, which was, presumably, due to small quantities of residual ATP in the milieu (Fig. 9). The potencies of these three agonists in eliciting Ca^{2+} release were in line with the respective binding affinity shown in Fig. 8. Other inositol metabolites examined, including PI(3,4)P_2, PI(4,5)P_2, Ins(1,3,4)P_3, and Ins(3,4,5)P_3 could not induce such Ca^{2+} efflux. This finding argued against the pos-

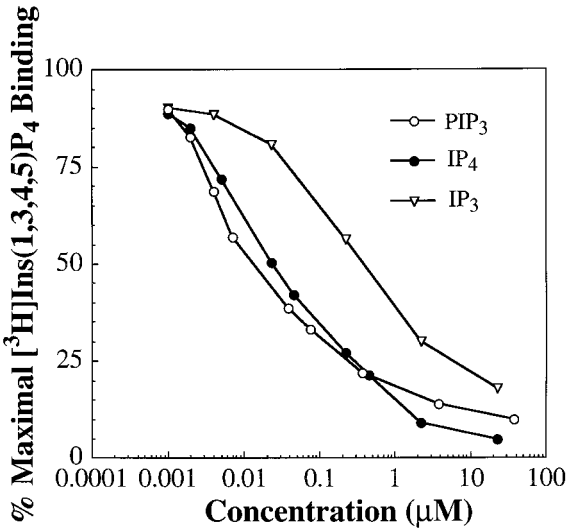

FIG. 8. **Inhibition of specific [^{3}H]Ins(1,3,4,5)P_4 binding to plasma membranes of Jurkat T cells by increasing concentrations of PI(3,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,4,5)P_3.** The displacement assay was carried out as described under "Materials and Methods." Nonspecific binding was measured in the presence of 30 μM Ins(1,3,4,5)P_4. Each data point represents the mean of three determinations.

sibility that the Ca^{2+} response arose from the metabolite(s) of PI(3,4,5)P_3 or Ins(1,3,4,5)P_4.

Moreover, the di-C_8-PI(3,4,5)P_3- or Ins(1,3,4,5)P_4-stimulated Ca^{2+} release was inhibited by SKF96365, which is consistent with that observed with the whole cell (Fig. 7). Also noteworthy is that Ca^{2+} release induced by PI(3,4,5)P_3 or Ins(1,3,4,5)P_4 was not augmented by subsequent challenge with either agonist or Ins(1,4,5)P_3 (indicated by the *arrows*). The lack of Ca^{2+} response was likely due to desensitization or saturation of the binding site instead of the depletion of Ca^{2+} since the addition of 1 μM A23187 following PI(3,4,5)P_3 treatment triggered the release of large amounts of Ca^{2+} (Fig. 9, *inset*).

In contrast, for Ins(1,4,5)P_3-stimulated Ca^{2+} release, subsequent stimulation with PI(3,4,5)P_3 or Ins(1,3,4,5)P_4 caused additional release of Ca^{2+}. Taken together with the binding data, this observation suggests that the putative PI(3,4,5)P_3 or Ins(1,3,4,5)P_4 receptor might be discrete from the Ins(1,4,5)P_3 receptor. Furthermore, the presence of Ins(1,4,5)P_3 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)P_3-receptor subtype distribution is similar to that reported for platelet plasma membranes (31).

Ins(1,3,4,5)P_4 Is Not a Physiological Relevant Ligand in anti-CD3- or PI(3,4,5)P_3-induced Ca^{2+} Entry—The cross-reactivity between Ins(1,3,4,5)P_4 and PI(3,4,5)P_3 raised a crucial issue, *i.e.* which one was the "physiologically relevant" ligand responsible for anti-CD3- and PI(3,4,5)P_3-induced Ca^{2+} influx? To address this issue, we examined the kinetics of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production in response to the stimulation by anti-CD3 and PI(3,4,5)P_3 and the effect of the Ins(1,4,5)P_3 3-kinase inhibitor adriamycin on these Ca^{2+} responses.

In cells, the 3-phosphorylation of Ins(1,4,5)P_3 by Ins(1,4,5)P_3-specific 3-kinase accounts for a major pathway for the formation of Ins(1,3,4,5)P_4 (32). Thus, stimulated Ins(1,4,5)P_3 accumulation leads to Ins(1,3,4,5)P_4 increase in T cells (2). Fig. 11*A* (*left panel*) demonstrates that treatment of *myo*-[2-^{3}H]inositol-labeled Jurkat T cells with anti-CD3 mAb stimulated a transient increase in [^{3}H]Ins(1,4,5)P_3, accompanied by a concurrent rise in [^{3}H]Ins(1,3,4,5)P_4 over a 10-min period. Considering the time course of anti-CD3-induced Ca^{2+} response (Fig. 11*B*, *control*), the production of [^{3}H]Ins(1,3,4,5)P_4 slightly lagged behind the rise in $[Ca^{2+}]_i$ in response to anti-CD3. The Ins(1,3,4,5)P_4 level peaked about 5 min post-treatment *vis à vis* 90 s for $[Ca^{2+}]_i$ to reach maximum. Moreover, pretreatment of the cells with the Ins(1,4,5)P_3 3-kinase inhibitor adriamycin (10 μM) (33) completely blocked Ins(1,3,4,5)P_4 formation (Fig. 11*B*) without interfering with Ins(1,4,5)P_3 production. It is noteworthy that the inhibition of Ins(1,3,4,5)P_4 formation by adriamycin treatment had no appreciable effect on anti-CD3-induced Ca^{2+} response (Fig. 11*B*). These data reaffirmed the earlier conclusion by Guse and coworkers (33) that Ins(1,3,4,5)P_4 does not play a major stimula-

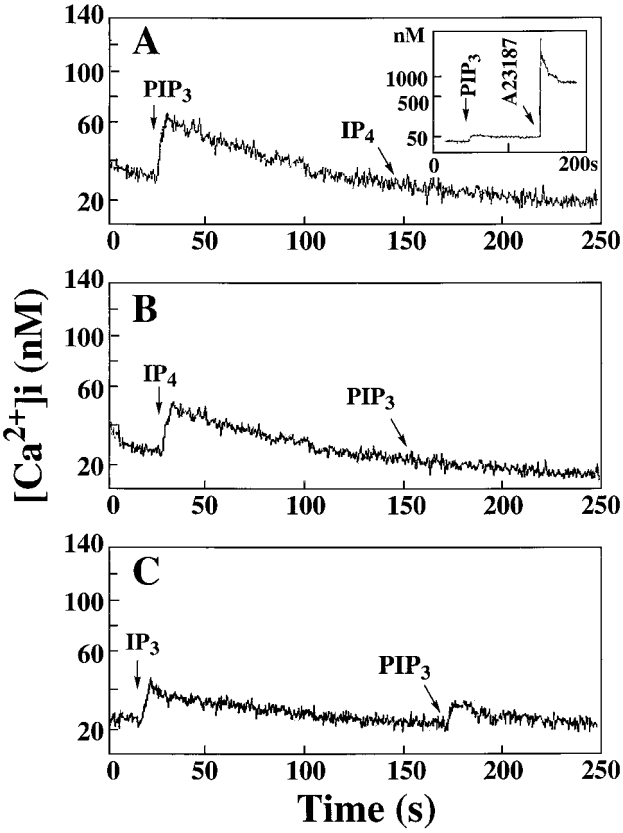

FIG. 9. **Ca^{2+} release from plasma membrane vesicles.** Jurkat T cell plasma membranes were prepared as described under "Material and Methods" and were treated with 30 μM Mg^{2+}-ATP, 1 mM $CaCl_2$, 1 μM thapsigargin, and 2.5 μg/ml oligomycin on ice for 10 min. The membrane vesicles were washed with 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-C_8-PI(3,4,5)P_3 (*A*), 10 μM Ins(1,3,4,5)P_4 (*B*), or 10 μM Ins(1,4,5)P_3 (*C*), as indicated by the *arrow*. Until the external Ca^{2+} concentration returned to a near base level, the membrane vesicles were stimulated with 10 μM Ins(1,3,4,5)P_4 or 25 μM di-C_8-PI(3,4,5)P_3 as indicated. The *inset* indicates the sequential additions of 25 μM PI(3,4,5)P_3 and 1 μM A23187.

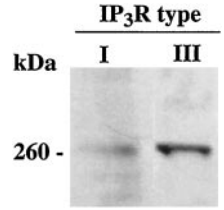

FIG. 10. **Western blot analysis of Ins(1,4,5)P_3 receptor (IP_3R)isotypes in T cell plasma membranes.**

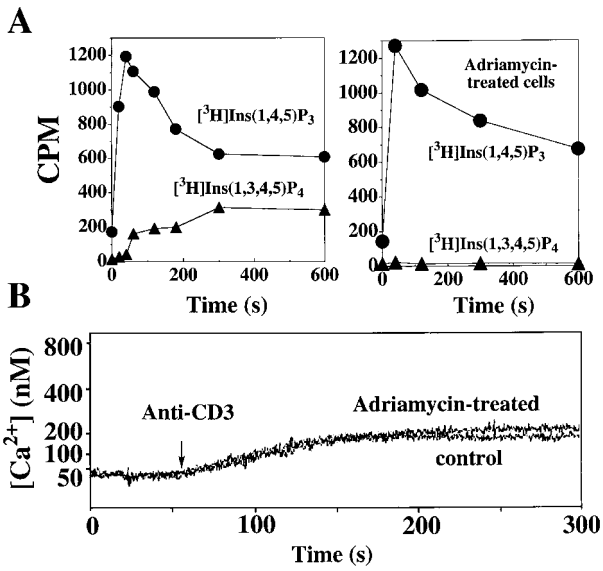

FIG. 11. **Evidence that Ins(1,3,4,5)P_4 is not a physiologically relevant ligand during the course of anti-CD3- induced Ca^{2+} response.** *A*, kinetics of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 production in [2-^{3}H]inositol-labeled Jurkat T cells in response to 10 μg/ml anti-CD3 mAb (*left panel*). In the *right panel*, [2-^{3}H]inositol-labeled Jurkat T cells were exposed to 10 μM adriamycin for 2 h before anti-CD3 stimulation. As shown, the Ins(1,4,5)P_3 3-kinase inhibitor completely suppressed the formation of [^{3}H]Ins(1,3,4,5)P_4 without affecting Ins(1,4,5)P_3 production. *B*, effect of adriamycin (10 μM) on anti-CD3-induced Ca^{2+} increase in Jurkat T cells. No appreciable difference was noted in the Ca^{2+} response between adriamycin-treated and adriamycin-untreated cells, even though the inhibitor completely blocked Ins(1,3,4,5)P_4 synthesis.

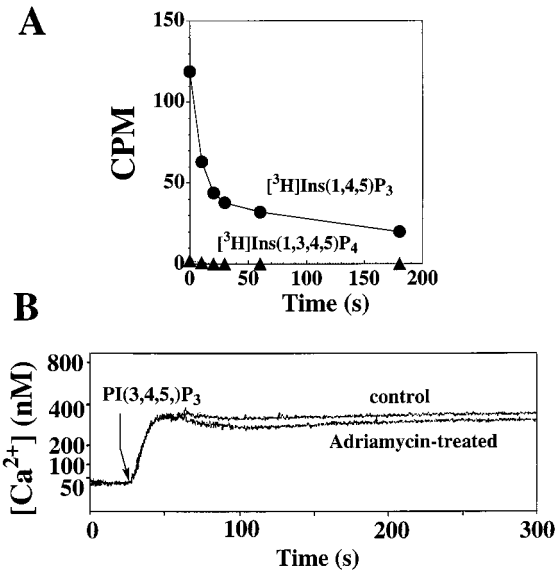

FIG. 12. **Evidence that Ins(1,3,4,5)P_4 is not involved in PI(3,4,5)P_3-induced Ca^{2+} response.** *A*, kinetics of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 production in [2-^{3}H]inositol-labeled Jurkat T cells in response to 10 μM PI(3,4,5)P_3. As shown, [^{3}H]Ins(1,3,4,5)P_4 was undetectable throughout the course of the examination. *B*, effect of adriamycin (10 μM) on PI(3,4,5)P_3-induced Ca^{2+} response in Jurkat T cells.

tory role in anti-CD3-mediated Ca^{2+} signaling.

Given the observation that PI(3,4,5)P_3 stimulates PLC-γ *in vitro* (25), we also investigated the time course of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 production in response to PI(3,4,5)P_3 stimulation. As PI(3,4,5)P_3 is not susceptible to hydrolysis by any known PLC (34), it does not contribute to Ins(1,3,4,5)P_4 formation *in vivo*. As shown in Fig. 12*A*, exogenous PI(3,4,5)P_3 did not display any stimulatory effect on the production of either inositol phosphates. In fact, PI(3,4,5)P_3 suppressed the formation of [^{3}H]Ins(1,4,5)P_3, and [^{3}H]Ins(1,3,4,5)P_4 was virtually undetectable throughout the course of examination. In addition, adriamycin had no appreciable effect on PI(3,4,5)P_3-elicited Ca^{2+} response (Fig. 12*B*). These results indicate that Ins(1,3,4,5)P_4 is not physiologically relevant in either anti-CD3- or PI(3,4,5)P_3-induced Ca^{2+} response.

Affinity Probing of PI(3,4,5)P_3-binding Proteins in T Cell Plasma Membranes—We further prepared a biotinylated analog of PI(3,4,5)P_3, Biotin-PIP$_3$ (Fig. 13*A*), to confirm the existence of PI(3,4,5)P_3-binding proteins in T cell plasma membranes. This affinity ligand has been successfully applied to the purification of PI(3,4,5)P_3-binding proteins even with a K_d as high as 100 μM (17). The plasma membrane fraction was treated with 5% CHAPS, and the solubilized proteins were incubated with Biotin-PIP$_3$, 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 the eluted proteins, visualized by silver staining, indicates two major protein bands with apparent molecular masses of 67 kDa and 59 kDa and several minor bands at and below 42 kDa (Fig. 13*B*). No protein band with a molecular mass greater than 70 kDa was detected.

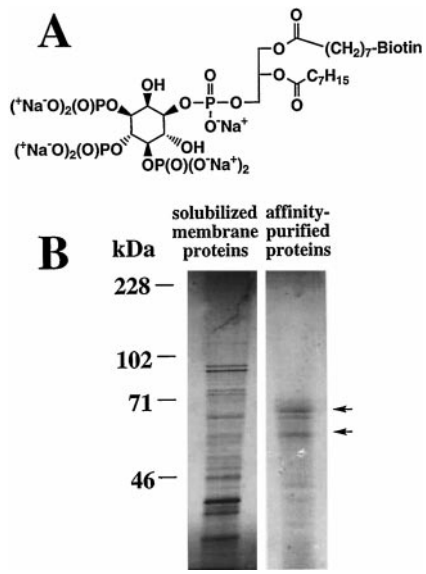

FIG. 13. **Affinity identification of PI(3,4,5)P_3-binding proteins in T cell plasma membranes.** *A*, structure of the affinity ligand Biotin-PIP$_3$. *B*, SDS-polyacrylamide gel electrophoresis analysis of solubilized proteins from T cell plasma membranes and affinity-purified proteins, visualized by silver staining. The *arrows* indicated the two major protein bands with apparent masses of 67 kDa and 59 kDa. 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 Ca^{2+} signaling via a PI(3,4,5)P_3-sensitive Ca^{2+} influx system on plasma membranes. This unique Ca^{2+} 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

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)P_3, the primary output signal of PI 3-kinase, can generate Ca^{2+} stimuli that synergize with Ins(1,4,5)P_3-induced Ca^{2+} release and capacitative Ca^{2+} entry for sustaining elevated $[Ca^{2+}]_i$, a driving force underlying many cellular responses.

This Ca^{2+} entry mechanism is directly activated by PI(3,4,5)P_3. In cells, PI(3,4,5)P_3 is subjected to rapid turnover by three discrete pathways: dephosphorylation by multiple 5-phosphatases to form PI(3,4)P_2 (35–37), dephosphorylation by PTEN to form PI(4,5)P_2 (38), and hydrolysis by phosphoinositide-specific phospholipases to form Ins(3,4,5)P_3 (39). None of these metabolites was capable of eliciting Ca^{2+} response in the whole cells or plasma membrane vesicles.

This PI(3,4,5)P_3-induced Ca^{2+} influx displays several unique features. First, among various cell types examined to date, only T cells and platelets display Ca^{2+} influx in response to PI(3,4,5)P_3 treatment, whereas mouse splenic B cells, NIH3T3 cells, PC-12 cells, Hep G2 cells, and LNCaP cells were insensitive to PI(3,4,5)P_3. In the literature, based on studies with PI 3-kinase inhibitors, PI 3-kinase has also been implicated in antigen-stimulated Ca^{2+} influx in mast cells (40, 41). Taken together, these data suggest that PI(3,4,5)P_3-sensitive Ca^{2+} 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 Ca^{2+} regulation.

Second, this Ca^{2+} entry does not require depletion of internal Ca^{2+} pools, indicating that the PI(3,4,5)P_3-activated Ca^{2+} inflow is independent of signals from empty stores (capacitative Ca^{2+} entry). In addition, PI(3,4,5)P_3 does not disturb Ins(1,4,5)P_3-sensitive or thapsigargin-sensitive Ca^{2+} pools.

Third, 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+} 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 Ca^{2+} entry. To date, published data on the role of Ins(1,3,4,5)P_4 in Ca^{2+} mobilization across plasma membranes remain inconclusive. Although several reports implicated Ins(1,3,4,5)P_4 in mediating Ca^{2+} 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)P_4 did not have a significant effect, if any, on potentiating Ca^{2+} 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)P_4 does not play a role in anti-CD3- or PI(3,4,5)P_3-elicited Ca^{2+} influx.

Meanwhile, several research groups have isolated an Ins(1,3,4,5)P_4-binding protein, $GAP1^{IP4BP}$, from platelet plasma membranes (44, 46–48). $GAP1^{IP4BP}$ was found to be a GTPase-activating protein with a molecular mass of 104 kDa. It remains enigmatic how this GAP protein is involved in Ca^{2+} entry. However, our affinity ligand study indicates that although many PI(3,4,5)P_3-binding proteins existed in the T cell plasma membrane, none of these proteins displayed a molecular mass in line with that of $GAP1^{IP4BP}$. This finding dampened the possibility that $GAP1^{IP4BP}$ was involved in the PI(3,4,5)P_3-induced Ca^{2+} influx in Jurkat T cells.

In summary, although the mechanism by which PI(3,4,5)P_3 mediates Ca^{2+} entry remains unclear, this PI(3,4,5)P_3-sensitive pathway not only provides molecular insights into T cell Ca^{2+} regulation but also represents a potential target for the modulation of cell function in T lymphocytes. Unlike inositol phosphates, PI(3,4,5)P_3 is membrane-permanent. Thus, it is plausible to design PI(3,4,5)P_3 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)P_3 receptor on plasma membranes? Is there cross-communication with other Ca^{2+} channels (such as Ca^{2+} release-activated Ca^{2+} channels) on plasma membranes to regulate Ca^{2+} entry? To address these questions, sequence analysis of the putative PI(3,4,5)P_3-binding proteins is currently under way in this laboratory.

REFERENCES

1. Cardenas, M. E., and Heitman, J. (1995) *Adv. Second Messenger Phosphoprotein Res.* **30,** 281–298
2. Guse, A. H. (1998) *Crit. Rev. Immunol.* **18,** 419–448
3. Kuno, M., and Gardner, P. (1987) *Nature* **326,** 301–304
4. Khan, A. A., Steiner, J. P., Klein, M. G., Schneider, M. F., and Snyder, S. H. (1992) *Science* **257,** 815–818
5. Khan, A. A., Steiner, J. P., and Snyder, S. H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2849–2853
6. Putney, J. W., Jr. (1986) *Cell Calcium* **7,** 1–12
7. Zweifach, A., and Lewis, R. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6295–6299
8. Premack, B. A., McDonald, T. V., and Gardner, P. (1994) *J. Immunol.***152,** 5226–5240
9. Sei, Y., Takemura, M., Gusovsky, F., Skolnick, P., and Basile, A. (1995) *Exp. Cell Res.* **216,** 222–231
10. de Aos, I., Metzger, M. H., Exley, M., Dahl, C. E., Misra, S., Zheng, D., Varticovski, L., Terhorst, C., and Sancho, J. (1997) *J. Biol. Chem.* **272,** 25310–25318
11. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) *Cell* **92,** 83–92
12. Bruyns, E., Marie-Cardine, A., Kirchgessner, H., Sagolla, K., Shevchenko, A., Mann, M., Autschbach, F., Bensussan, A., Meuer, S., and Schraven, B. (1998) *J. Exp. Med.* **188,** 561–575
13. Ward, S. G., Ley, S. C., MacPhee, C., and Cantrell, D. A. (1992) *Eur. J. Immunol.* **22,** 45–49
14. Rameh, L. E., and Cantley, L. C. (1999) *J. Biol. Chem.* **274,** 8347–8350
15. Lu, P. J., Gou, D. M., Shieh, W. R., and Chen, C. S. (1994) *Biochemistry* **33,** 11586–11597
16. Wang, D.-S., and Chen, C.-S. (1996) *J. Org. Chem.* **61,** 5905–5910
17. Wang, D.-S., Ching, T.-T., St. Pyrek, J., and Chen, C.-S. (2000) *Anal. Biochem.*, in press
18. Muthusamy, N., Baluyut, A. R., and Subbarao, B. (1991) *J. Immunol.* **147,** 2483–2492
19. Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994) *EMBO J.* **13,** 511–521
20. Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta* **153,** 540–552
21. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
22. Lu, P. J., Hsu, A. L., Wang, D. S., and Chen, C. S. (1998) *Biochemistry* **37,** 9776–9783
23. Derman, M. P., Toker, A., Hartwig, J. H., Spokes, K., Falck, J. R., Chen, C.-S., Cantley, L. C., and Cantley, L. G. (1997) *J. Biol. Chem.* **272,** 6465–6470
24. Gagnon, A., Chen, C. S., and Sorisky, A. (1999) *Diabetes* **48,** 691–698
25. Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S.-R., Kwon, K.-S., and Rhee, S. G. (1998) *J. Biol. Chem.* **273,** 4465–4469
26. Holmes, R. P., and Yoss, N. L. (1983) *Nature* **305,** 637–638
27. Eder, A. M., Dominguez, L., Franke, T. F., and Ashwell, J. D. (1998) *J. Biol. Chem.* **273,** 28025–28031
28. Von Willebrand, M., Jascur, T., Bonnefoy-Berard, N., Yano, H., Altman, A., Matsuda, Y., and Mustelin, T. (1996) *Eur. J. Biochem.* **235,** 828–835
29. Jascur, T., Gilman, J., and Mustelin, T. (1997) *J. Biol. Chem.* **272,** 14483–14488
30. Chung, S. C., McDonald, T. V., and Gardner, P. (1994) *Br. J. Pharmacol.* **113,** 861–868
31. El-Daher, S. S., Patel, Y., Siddiqua, A., Hassock, S., Edmunds, S., Maddison, B., Patel, G., Goulding, D., Lupu, F., Wojcikiewicz, R. J. H., and Authi, K. S. (2000) *Blood*, in press
32. Johnson, R. M., Wasilenko, W. J., Mattingly, R. R., Weber, M. J., and Garrison, J. C. (1989) *Science* **246,** 121–124
33. da Silva, C. P., Emmrich, F., and Guse, A. H. (1994) *J. Biol. Chem.* **269,** 12521–12526
34. Serunian, L. A., Haber, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J. M., and Cantley, L. C. (1989) *J. Biol. Chem.* **264,** 17809–17815
35. Jackson, S. P., Schoenwaelder, S. M., Matzaris, M., Brown, S., and Mitchell, C. A. (1995) *EMBO J.* **14,** 4490–4500
36. Woscholski, R., Waterfield, M. D., and Parker, P. J. (1995) *J. Biol. Chem.* **270,**

31001–31007
37. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1689–1693
38. Maehama, T., and Dixon, J. E. (1998) *J. Biol. Chem.* **273,** 13375–13378
39. Ching, T. T., Wang, D. S., Hsu, A. L., Lu, P. J., and Chen, C. S. (1999) *J. Biol. Chem.* **274,** 8611–8617
40. Barker, S. A., Lujan, D., and Wilson, B. S. (1999) *J. Leukocyte Biol.* **65,** 321–329
41. Setoguchi, R., Kinashi, T., Sagara, H., Hirosawa, K., and Takatsu, K. (1998) *Immunol. Lett.* **64,** 109–118
42. Irvine, R. F., and Moor, R. M. (1986) *Biochem. J.* **240,** 917–920
43. Guse, A. H., Gercken, G., Boysen, H., Schwarz, J. R., and Meyerhof, W. (1991) *Biochem. Biophys. Res. Commun.* **179,** 641–647
44. O'Rourke, F., Matthews, E., and Feinstein, M. B. (1996) *Biochem. J.* **315,** 1027–1034
45. Bird, G. St. J., and Putney, J. W., Jr. (1996) *J. Biol. Chem.* **271,** 6766–6770
46. Cullen, P. J., Hsuan, J. J., Truong, O., Letcher, A. J., Jackson, T. R., Dawson, A. P., and Irvine, R. F. (1995) *Nature* **376,** 527–530
47. Cullen, P. J., Loomis-Husselbee, J., Dawson, A. P., and Irvine, R. F. (1997) *Biochem. Soc. Trans.* **25,** 991–996
48. Lockyer, P. J., Bottomley, J. R., Reynolds, J. S., McNulty, T. J., Venkateswarlu, K., Potter, B. V., Dempsey, C. E., and Cullen, P. J. (1997) *Curr. Biol.* **7,** 1007–1010

Novel Function of Phosphoinositide 3-Kinase in T Cell Ca^{2+} Signaling: A PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE-MEDIATED Ca2+ ENTRY MECHANISM

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