

## Calmodulin Activates Phosphatidylinositol 3-Kinase\*

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**Calmodulin and phosphatidylinositol 3-kinase are vital components of a number of common intracellular events. Calmodulin, a ubiquitous  $\text{Ca}^{2+}$ -dependent effector protein, regulates multiple processes in eukaryotic cells, including cytoskeletal organization, vesicular trafficking, and mitogenesis. Phosphatidylinositol 3-kinase participates in events downstream of the receptors for insulin and other growth factors. Here we demonstrate by coimmunoprecipitation and affinity chromatography that  $\text{Ca}^{2+}$ /calmodulin associates with Src homology 2 domains in the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase, thereby significantly enhancing phosphatidylinositol 3-kinase activity *in vitro* and in intact cells. Furthermore, CGS9343B, a calmodulin antagonist, inhibited basal and  $\text{Ca}^{2+}$ -stimulated phosphorylation of phosphatidylinositol in intact cells. These data demonstrate a novel mechanism for modulating phosphatidylinositol 3-kinase and provide a direct link between components of two fundamental signaling pathways.**

A critical aspect of protein and/or lipid kinase regulation is the tight coupling of catalytic activity to various extracellular or intracellular signals. Alterations in levels of intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ )<sup>1</sup> mediate the action of several hormones through the regulation of key enzymes; these signaling events are often orchestrated by the  $\text{Ca}^{2+}$  effector, calmodulin (CaM) (1). Activation of phosphatidylinositol 3-kinase (PI3-kinase) in many tissues and cell types is required for mitogenesis, neuronal differentiation, and enhanced glucose transport (2). PI3-

kinase and CaM are common components of several fundamental intracellular processes. For example, wortmannin, an inhibitor of PI3-kinase, and CGS9343B impede insulin-induced glucose uptake (3–5). Both CaM (6, 7) and PI3-kinase (8) participate in early endosome fusion. In the cytoskeleton, PI3-kinase has been linked to actin rearrangement (9), binds  $\alpha$ -,  $\beta$ - and  $\gamma$ -tubulin (10), and plays a role in platelet-derived growth factor- and insulin-induced membrane ruffling (11, 12). Overexpression of CaM alters cell morphology and the arrangement of microfilaments within the cell (13). In addition, CaM binds to a variety of cytoskeletal proteins (1) including the family of unconventional myosins (14) and has been implicated in osteoclast membrane ruffling (15) and the formation of microspikes in neuronal cells (16). Finally, both PI3-kinase (17) and CaM, via modulation of the association of IQGAP1 with Cdc42 (18), may participate in the regulation of Rho family GTPases. Since CaM and PI3-kinase modulate similar cellular events, we evaluated a possible interaction between these two signaling components.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Lysis**—Sf9 cells were maintained in Grace's medium supplemented with 10% fetal bovine serum and infected with baculovirus as described previously (19). CHO cells were grown to 80% confluence in Ham's F-12 medium with 10% fetal bovine serum. 32D cells and 32D cells expressing rat IRS-1 (32D/IRS-1) were cultured as described previously (20). The medium was removed, cells were washed 3 times with phosphate-buffered saline, and 1 ml of lysis buffer (50 mM Tris base, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.1  $\mu\text{g}/\text{ml}$  leupeptin, aprotinin, and pepstatin, and 1% Triton X-100) was added. Where indicated  $\text{CaCl}_2$  or EGTA was included. The cells were collected and quick-frozen in methanol/dry  $\text{CO}_2$ .

**Antibodies**—The specific anti-calmodulin monoclonal antibody has been previously described (21). The anti-myoglobin monoclonal antibody (IgG<sub>1c</sub>) was highly purified by Dr. J. Ladenson (Washington University Medical Center, St. Louis). The anti-p85 antibody was prepared by immunizing rabbits with a glutathione S-transferase (GST) fusion protein containing the inter-SH2 region of p85. The anti-p85 monoclonal and the anti-GST and anti-p110 polyclonal antibodies were purchased from Upstate Biotechnology Inc.

**CaM-Sepharose**—Cell lysates were incubated for 2 h at 4 °C with 20  $\mu\text{l}$  of CaM-Sepharose or Sepharose alone in the presence of 0.1 mM  $\text{CaCl}_2$  or 1 mM EGTA. GST fusion proteins were incubated with CaM-Sepharose for 1 h at 25 °C in the presence of 0.1 mM  $\text{CaCl}_2$  or 1 mM EGTA. Samples were washed 5 times in lysis buffer containing 0.1 mM  $\text{CaCl}_2$  or 1 mM EGTA, resolved by SDS-PAGE, and immunoblotted as described below.

**Immunoprecipitation and Immunoblotting**—Equal amounts of protein lysate were immunoprecipitated with either anti-CaM monoclonal antibody, anti-myoglobin monoclonal antibody, anti-p85 antibody, or preimmune serum. Samples were washed five times in lysis buffer, resolved by SDS-PAGE, and transferred to PVDF, and immunoblots were probed with anti-p85, anti-p110, or anti-CaM antibody. Complexes were visualized with the appropriate horseradish peroxidase-conjugated secondary antibody and developed by ECL.

**PI3-kinase Activity**—Anti-CaM immunoprecipitates, anti-p85 immunoprecipitates, or CaM-Sepharose beads were resuspended in 15 mM Hepes, pH 7.4, 5 mM  $\text{MgCl}_2$ , 0.3 mM EGTA, 0.24 mg/ml phosphatidylinositol, and 20  $\mu\text{M}$  [ $\gamma$ -<sup>32</sup>P]ATP in a final volume of 100  $\mu\text{l}$ . In selected experiments, 0.1 mM  $\text{CaCl}_2$  (EGTA was omitted), 0.5, 2, or 5  $\mu\text{M}$  CaM, 100  $\mu\text{M}$  LY294002, or 100  $\mu\text{M}$  pY608 were added singly or in combination as indicated in the figure legends. The reaction was stopped with 100  $\mu\text{l}$  of 1 M HCl, and 200  $\mu\text{l}$  of 1:1  $\text{CHCl}_3$ :methanol was added. After centrifugation, the lower phase was spotted onto Silica Gel 60 plates, and thin-layer chromatography was performed using  $\text{CHCl}_3$ /methanol/ $\text{NH}_4\text{OH}/\text{dH}_2\text{O}$  (45:35:1.5:8.5) as the solvent. Where indicated, phosphatidylinositol phosphate (PtdIns-P) was located by autoradiography and excised, and <sup>32</sup>P was determined by liquid scintillation counting. For HPLC analysis, PtdIns-P was extracted from the silica gel, deacyl-

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<sup>1</sup> The abbreviations used are:  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$ ; CaM, calmodulin; PI3-kinase, phosphatidylinositol 3-kinase; CHO, Chinese hamster ovary; IRS, insulin receptor substrate; 32D/IRS-1, 32D cells expressing rat IRS-1; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HPLC, high pressure liquid chromatography; PtdIns-P, phosphatidylinositol phosphate; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-3,4-P<sub>2</sub>, phosphatidylinositol 3,4-diphosphate; PtdIns-3,4,5-P<sub>3</sub>, phosphatidylinositol 3,4,5-triphosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-4,5-P<sub>2</sub>, phosphatidylinositol 4,5-diphosphate.

ated with methylamine, and subjected to anion exchange chromatography using an on-line radiochemical detector as described previously (22). Deacylated [ $^3\text{H}$ ]PtdIns-4-P was used as an internal standard.

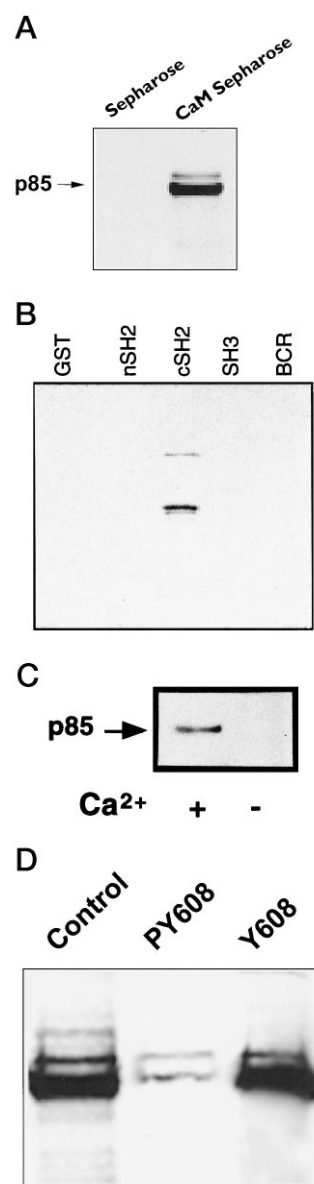
**$^{32}\text{P}$  Labeling of Phospholipids**—CHO cells were incubated for 2 h in serum-free, phosphate-free RPMI 1640 medium. 0.5 mCi of [ $^{32}\text{P}$ ]orthophosphate was added to each culture dish for an additional 2 h. To some cells, 40  $\mu\text{M}$  CGS9343B was added during the final 30 min of the incubation. Cells were then treated with or without 5  $\mu\text{M}$  A23187 for 15 min, washed three times in cold phosphate-buffered saline, and lysed in 0.75 ml of 1:1 methanol, 1 M HCl. Lipids were extracted three times with 0.3 ml of chloroform, dried under nitrogen, and spotted onto Silica Gel 60 plates that had been prechromatographed with 1.2% potassium oxalate in  $\text{dH}_2\text{O}$ :methanol (60:40) and dried. Thin-layer chromatography of the lipids was performed using  $\text{CHCl}_3$ /acetone/methanol/acetic acid/ $\text{dH}_2\text{O}$  (80:30:26:24:14) as the solvent. Radioactive spots corresponding to phosphatidylinositol phosphates were identified by autoradiography, scraped from the plates, and analyzed by HPLC as described above.

#### RESULTS AND DISCUSSION

The 85-kDa regulatory subunit of PI3-kinase (p85) was precipitated from lysates of baculovirus-infected Sf9 cells expressing p85 with CaM-Sepharose but not with Sepharose alone, demonstrating an interaction between these two proteins (Fig. 1A). To identify the CaM-binding region of p85, CaM-Sepharose was incubated with GST fusion proteins containing various regions of p85. Probing with anti-GST antibody disclosed that the carboxyl-terminal SH2 domain bound to the CaM-Sepharose (Fig. 1B). Longer exposure of the blot revealed association of the amino-terminal SH2 domain with CaM-Sepharose, suggesting that this region also interacts with CaM albeit with a lower affinity (data not shown). Binding to either SH2 domain was significantly reduced when  $\text{Ca}^{2+}$  was chelated with EGTA (data not shown). Furthermore, endogenous p85 from CHO cell lysates bound to CaM-Sepharose only in the presence of  $\text{Ca}^{2+}$  (Fig. 1C). No binding of CaM to the SH3 or breakpoint cluster homology regions was detected.

Insulin activates PI3-kinase by inducing the association of the SH2 domains of p85 with specific phosphotyrosine-containing motifs of IRS proteins (19). It has been demonstrated that a phosphorylated YMXM peptide (pY608), derived from amino acids 605–615 of IRS-1, activates PI3-kinase by binding to its SH2 domains (23). Incubation with pY608, but not with the corresponding nonphosphorylated peptide (Y608), resulted in displacement of baculovirus-expressed p85 from CaM-Sepharose (Fig. 1D). These data suggest that CaM binds in or near the pocket occupied by phosphorylated YMXM motifs. Among the functional possibilities that may be inferred from this observation are that CaM may directly activate PI3-kinase or may modulate PI3-kinase activity by competing with tyrosine-phosphorylated proteins for binding to the SH2 domains of p85.

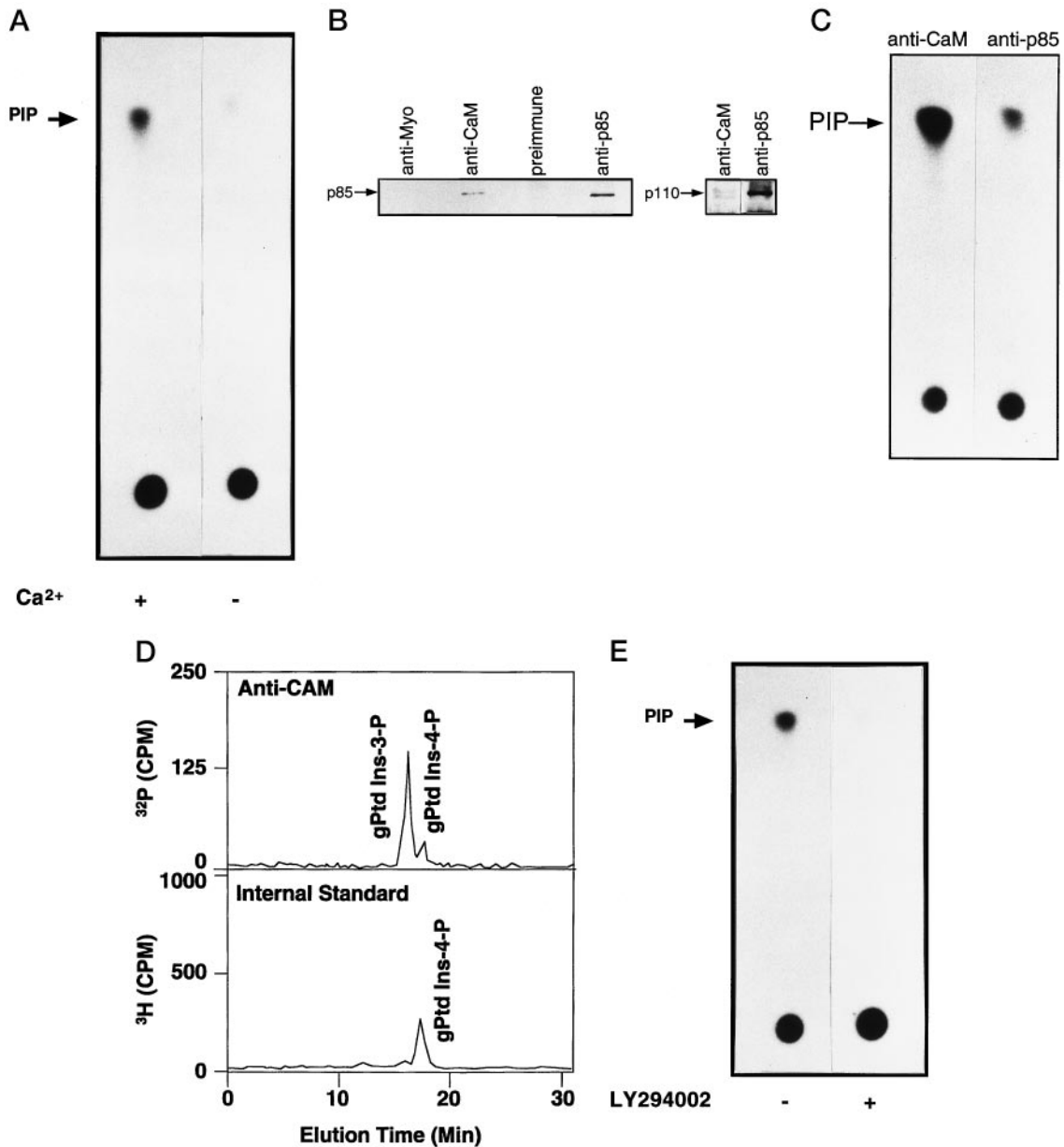
To resolve this question, the PI3-kinase activity associated with CaM was examined. The binding of PI3-kinase from CHO cell lysates to CaM-Sepharose was  $\text{Ca}^{2+}$ -dependent (Fig. 2A). Anti-CaM and anti-p85 antibodies immunoprecipitated p85 and p110, the catalytic subunit of PI3-kinase, from CHO cell lysates (Fig. 2B); however, p85 was not coimmunoprecipitated with an irrelevant isotype-identical monoclonal antibody (anti-myoglobin) or preimmune serum (Fig. 2B). Even though more p85 and p110 were present in anti-p85 immunoprecipitates than in anti-CaM immunoprecipitates, the PI3-kinase activity in the anti-CaM immunoprecipitates was 6-fold greater than that in anti-p85 immunoprecipitates (Fig. 2C). These data, coupled with the relatively small amount of catalytic p110 subunit bound to CaM, strongly support the contention that CaM significantly stimulates PI3-kinase activity. HPLC analysis of the PtdIns-P produced by PI3-kinase in the anti-CaM immunoprecipitate revealed that 80% was phosphatidylinositol 3-phosphate (PtdIns-3-P) (Fig. 2D). Greater than 95% of the phosphatidylinositol kinase activity in CHO cell lysates was



**FIG. 1. Binding of CaM to p85.** A, Sf9 cells infected with baculovirus-expressing p85 were lysed and incubated with Sepharose or CaM-Sepharose in the presence of 0.1 mM  $\text{CaCl}_2$ . B, purified GST or GST fusion proteins containing various regions of p85 including the amino-terminal SH2 domain (*nSH2*), carboxyl-terminal SH2 domain (*cSH2*), SH3 domain, or breakpoint cluster homology region (*BCR*) were incubated with CaM-Sepharose in the presence of 0.1 mM  $\text{CaCl}_2$ . The upper band corresponds to the predicted migration of GST-*cSH2*, whereas the lower band is most likely a fragment of the GST-*cSH2* fusion protein. C, CHO cells were lysed and incubated with CaM-Sepharose in the presence or absence of 0.1 mM  $\text{Ca}^{2+}$ . D, lysates from Sf9 cells infected with baculovirus-expressing p85 were incubated with CaM-Sepharose in the presence of 100  $\mu\text{M}$  Y608 or pY608. After washing the beads, proteins were resolved by SDS-PAGE and transferred to PVDF. Blots depicted in A, C, and D were probed for p85, and the blot in B was probed for GST. Data are representative of two independent experimental determinations.

phosphatidylinositol 4-kinase (data not shown), supporting a specific interaction of CaM and PI3-kinase. Furthermore, the PI3-kinase inhibitor LY294002 (24) decreased the PI3-kinase activity in the anti-CaM immunoprecipitates by greater than 90% (Fig. 2E), further substantiating the identity of this enzyme as PI3-kinase.

Since CaM binds to IRS-1 in a  $\text{Ca}^{2+}$ -sensitive manner (25), the possibility of a ternary complex between CaM, p85, and IRS-1 was evaluated in 32D cells, which lack endogenous IRS-1 and IRS-2 (26). Essentially identical amounts of p85 from ly-



**FIG. 2. Isolation of p85 and PI3-kinase activity from CHO cells.** *A*, CHO cell lysates were incubated with CaM-Sepharose. After washing, PI3-kinase activity was determined by incubating the beads with [ $\gamma$ - $^{32}$ P]ATP and phosphatidylinositol, and resolving the samples by TLC. An autoradiograph is shown with the position of migration of PtdIns-3-P (*PIP*) indicated. *B*, CHO cells were lysed, and equal amounts of protein were immunoprecipitated with anti-myoglobin (*anti-Myo*), anti-CaM, preimmune serum (*preimmune*), or anti-p85 antibody and treated as in *A*. The immunoblots were probed with antibody to p85 or p110 and visualized with a horseradish peroxidase-conjugated secondary antibody. The positions of migration of the p85 and p110 subunits of PI3-kinase are indicated. *C*, PI3-kinase activity in the anti-CaM and anti-p85 immunoprecipitates was measured as described in *A*. An autoradiograph is shown with the position of migration of PtdIns-3-P indicated. *D*, following TLC, PtdIns-P from the anti-CaM sample was isolated from the TLC plate and analyzed by HPLC. The migration of PtdIns-3-P (16 min) and PtdIns-4-P (17.5 min) is indicated. *E*, anti-CaM immunoprecipitates of CHO cell lysates were preincubated with or without 100  $\mu$ M LY294002, and PI3-kinase activity was measured. In all cases, representative data from two independent experiments are shown.

sates of 32D and 32D/IRS-1 cells were precipitated with CaM-Sepharose (Fig. 3), indicating that the binding is independent of IRS proteins.

To further confirm our results, activation of PI3-kinase by pY608 or CaM was compared using anti-p85 immunoprecipitates of CHO cell lysates. In anti-p85 immunoprecipitates, 5  $\mu$ M CaM stimulated PI3-kinase activity by 50%, while 100  $\mu$ M pY608 enhanced PI3-kinase activity by 38% (Fig. 4). EGTA did not significantly alter basal or pY608-stimulated PI3-kinase activity in the anti-p85 immunoprecipitates (data not shown) but abolished the activation of PI3-kinase by CaM (Fig. 4). These data verify that CaM stimulated PI3-kinase in a  $\text{Ca}^{2+}$ -dependent manner. The activity of baculovirus-expressed PI3-

kinase was similarly augmented by CaM (data not shown). Thus, the activation of PI3-kinase by CaM was comparable to that obtained by occupancy of the SH2 domains with a phosphopeptide, implying that the binding of CaM to the SH2 domains of p85 is a novel mechanism for the regulation of PI3-kinase activity.

To examine whether  $\text{Ca}^{2+}$ /CaM activates PI3-kinase *in situ*, [ $\text{Ca}^{2+}$ ] $_i$  was increased with the ionophore A23187 (25) in CHO cells preloaded with  $^{32}$ P.  $^{32}$ P-Labeled phospholipids were then extracted and resolved by TLC, and phosphatidylinositol phosphates were excised and examined by HPLC. Treatment with A23187 increased PtdIns-3-P by 30% (Table I). The CaM antagonist, CGS9343B, decreased basal levels of PtdIns-3-P and

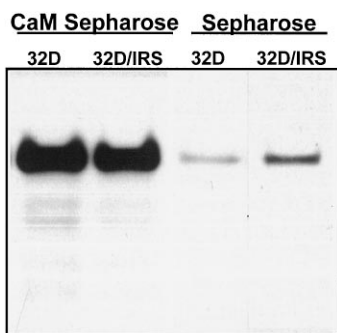


FIG. 3. Isolation of p85 from 32D and 32D/IRS-1 cells. 32D and 32D/IRS-1 cells were lysed and incubated with Sepharose or CaM-Sepharose in the presence of 0.1 mM  $\text{CaCl}_2$ . Proteins were separated by SDS-PAGE and transferred to PVDF, and blots were probed for p85. The data are representative of two separate experimental determinations.

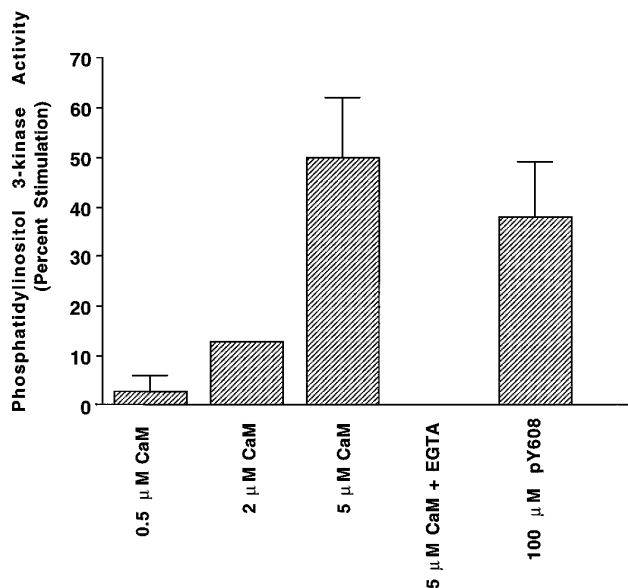


FIG. 4. CaM stimulation of PI3-kinase. PI3-kinase activity was measured in anti-p85 immunoprecipitates preincubated with various concentrations of CaM or 100  $\mu\text{M}$  pY608. 100  $\mu\text{M}$   $\text{Ca}^{2+}$  was present in all samples, except where chelated with EGTA. The data, expressed as the mean  $\pm$  range ( $n = 2$ ), are the percent stimulation of PI3-kinase activity relative to control samples (no additions).

TABLE I  
Phosphatidylinositol phosphate levels in CHO cells

	Control <sup>a</sup>	A23187 <sup>a</sup>	CGS9343B	A23187 + CGS9343B
PtdIns-3-P	1900 $\pm$ 437	2461 $\pm$ 167 (+30%) <sup>b</sup>	1138 (-67%)	957 (-99%)
PtdIns-4-P	105,940 $\pm$ 6213	185,300 $\pm$ 7590 (+75%)	62,458 (-65%)	56,482 (-88%)
PtdIns-3,4-P <sub>2</sub>	ND <sup>c</sup>	993 $\pm$ 134	ND	ND
PtdIns-4,5-P <sub>2</sub>	152,820 $\pm$ 10,934	147,520 $\pm$ 26,463 (-4%)	137,391 (-11%)	112,566 (-35%)
PtdIns-3,4,5-P <sub>3</sub>	ND	ND	ND	ND

<sup>a</sup> Data are expressed as mean  $\pm$  S.E.,  $n = 3$ .

<sup>b</sup> Values denote percent change versus control.

<sup>c</sup> ND, not detected.

prevented the stimulation by increased  $[\text{Ca}^{2+}]_i$ . A23187 induced the formation of phosphatidylinositol 3,4-diphosphate (PtdIns-3,4-P<sub>2</sub>) (which was not detected in lipids extracted from control cells), and this effect was abrogated by concomitant incubation with CGS9343B. Phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P<sub>3</sub>), the predominant phospholipid produced in response to insulin and other growth factors (2, 27, 28), was not detected under any conditions examined. The

production of phosphatidylinositol 4-phosphate (PtdIns-4-P), but not phosphatidylinositol 4,5-diphosphate (PtdIns-4,5-P<sub>2</sub>), was also enhanced by increased  $[\text{Ca}^{2+}]_i$  and was sensitive to CGS9343B (Table I). The CaM-sensitive PI4-kinase activity may be mediated by a mammalian CaM-stimulated inositol trisphosphate 4-kinase similar to that identified in plants (29).

This study establishes a novel direct interaction between  $\text{Ca}^{2+}$ /CaM and the SH2 domains of p85 resulting in the activation of PI3-kinase. Increased  $[\text{Ca}^{2+}]_i$ , acting through CaM, modulates PI3-kinase activity in intact cells. Interestingly, the specific phosphatidylinositol phosphates generated by  $\text{Ca}^{2+}$ /CaM differ from those induced by incubating cells with insulin or growth factors (2, 27, 28). We demonstrate that changes in  $[\text{Ca}^{2+}]_i$  regulate PI3-kinase in a manner distinct from the canonical phosphotyrosine-dependent pathway, providing an additional level of control of this fundamental enzyme. Our findings expand the repertoire of enzymes that are regulated by  $\text{Ca}^{2+}$ /CaM and accentuate the myriad interconnections between intracellular signaling pathways.

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