Expression, Purification, and Regulation of Two Isoforms of the Inositol 1,4,5-Trisphosphate 3-Kinase*

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The level of inositol 1,4,5-trisphosphate in the cytoplasm is tightly regulated by two enzymes, the inositol 1,4,5,5-phosphatase and the inositol 1,4,5-trisphosphate 3-kinase. Two isoforms of the inositol 1,4,5-trisphosphate 3-kinase have been identified, the A form and the B form. The regulatory properties of the two isoforms were compared following overexpression and purification of the proteins from a v-src transformed mammalian cell line. The highly purified, recombinant inositol 1,4,5-trisphosphate 3-kinases were differentially regulated by calcium/calmodulin and via phosphorylation by protein kinase C or the cyclic AMP-dependent protein kinase. Both enzymes had similar affinities for inositol 1,4,5-trisphosphate ($K_m$ 2–5 μM). Calcium/calmodulin stimulated the activity of isoform A about 2.5-fold, whereas the activity of isoform B was increased 20-fold. The cyclic AMP-dependent protein kinase phosphorylated the inositol 1,4,5-trisphosphate 3-kinase A to the extent of 9.9 mol/mol and isoform B to 1 mol/mol. Protein kinase C phosphorylated isoform A to the extent of 2 mol/mol and isoform B to 2.7 mol/mol. Phosphorylation of isoform A by the cyclic AMP-dependent protein kinase caused a 2.5-fold increase in its activity when assayed in the absence of calcium/calmodulin, whereas phosphorylation by protein kinase C decreased activity by 72%. The activity of isoform B in the absence of calcium/calmodulin was not affected by phosphorylation using either kinase. When assayed in the presence of calcium/calmodulin, phosphorylation of isoform A by the cyclic AMP-dependent protein kinase increased activity 1.5-fold, whereas phosphorylation of isoform B decreased activity by 45%. Phosphorylation of either isoform A or B by protein kinase C resulted in a 70% reduction of calcium/calmodulin-stimulated activity. Differential expression and regulation of the two inositol 1,4,5-trisphosphate 3-kinase isoforms provides multiple mechanisms for regulating the cytosolic level of inositol 1,4,5-trisphosphate in cells.

The second messenger inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) mediates the biological response of a large number of hormones and neurotransmitters in target cells by regulating calcium release from intracellular stores (1, 2). In keeping with its biological role, the levels of Ins(1,4,5)P$_3$ are tightly regulated by two mechanisms; dephosphorylation via an Ins(1,4,5)P$_3$ 5-phosphatase to Ins(1,4)P$_2$ or by phosphorylation with the inositol 1,4,5-trisphosphate 3-kinase (IP3K) to Ins(1,3,4,5)P$_4$. The former enzyme initiates the pathway which recycles the inositol moiety to the plasma membrane as phosphatidylinositols. The latter enzyme produces Ins(1,3,4,5,6)P$_5$, which has been suggested to have roles in controlling calcium homeostasis, transferring calcium between intracellular stores, and/or regulating calcium entry across the plasma membrane (5). In addition, Ins(1,3,4,5)P$_4$ may also play a role in regulating cross-talk between the calcium and other signaling pathways as an Ins(1,3,4,5)P$_4$ binding protein has been identified that can stimulate the GTPase activity of the ras and rap small GTP binding proteins (6, 7). Further metabolism of Ins(1,3,4,5)P$_4$ leads to the formation of Ins(1,3,4,5,6)P$_6$ and eventually Ins(1,2,3,4,5,6)P$_6$. Thus, the activity of the inositol 1,4,5-trisphosphate 3-kinase is responsible for regulating the levels of a large number of inositol polyphosphates that are important in cellular signaling (1, 2, 5).

Perhaps because of its key position in inositol polyphosphate metabolism, the IP3K is tightly regulated. Both calcium/calmodulin and protein phosphorylation mechanisms are documented to control its activity (8). Preparations of IP3K purified from rat brain (9–11) or rat liver (12) can be activated 2–3-fold by addition of calcium/calmodulin, while the IP3K purified from other sources can be activated in the range of 4–17-fold by calcium/calmodulin (13–16). The IP3K is also a substrate for the cyclic AMP-dependent protein kinase, the calcium/calmodulin-dependent protein kinase II, and protein kinase C in vitro. When the IP3K purified from rat brain or bovine smooth muscle is phosphorylated by the cyclic AMP-dependent protein kinase in vitro, its activity is increased about 2-fold (17, 18) while phosphorylation of the protein by protein kinase C reduced activity to about 25% of the basal activity (17–19). Phosphorylation of IP3K by brain with the calcium/calmodulin-dependent protein kinase II increases its $V_{max}$ about 9-fold and decreases the $K_m$ for calmodulin from 52 to 2 nm (20). Finally, a number of reports document that treatment of cells with hormones, kinase activators, or phosphatase inhibitors alters the activity of IP3K in cell extracts (20–26).

Efforts to clone cDNAs encoding the inositol 1,4,5-trisphosphate 3-kinase have identified two isoforms, designated as the A and B isoforms (27–31). The A isoform of the protein has a predicted molecular mass of 51 kDa (27) and is abundant in...
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brain and testis (32). The B isoform has a predicted molecular weight of 74 kDa (31) and is more widely expressed (32). The C-terminal regions of these proteins contain the catalytic and calmodulin binding domains and are roughly 80% similar at the amino acid level. However, there is little identity between the N-terminal regions of the two isoforms (31), suggesting that they may be regulated differently. Most of the experiments exploring the regulatory properties of IP3K have been performed with proteins that are (11, 17, 20, 28) or closely resemble (18) the A isoform, and thus little is known about the effects of calcium/calmodulin and phosphorylation on the activity of the B isoform. To compare the regulation of the two isoforms of IP3K, we have established stable cell lines over-expressing each of the two rat IP3K isoforms. A double affinity tag (hexahistidine and the FLAG epitope) was engineered into the cDNAs encoding the IP3K, and the proteins were overexpressed in B31 rat fibroblasts. This strategy provides a means to purify significant amounts of each protein quickly and efficiently. Examination of the regulatory properties of these purified, recombinant IP3Ks shows that the two isoforms are regulated very differently by calcium/calmodulin and via phosphorylation with protein kinase A or C. These observations provide a basis for understanding the complex regulation of IP3K in different types of cells.

EXPERIMENTAL PROCEDURES

Maintenance of Tissue Culture Cells—Cells were grown in Dulbecco's minimum essential medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator (37 °C with 5% CO$_2$ and 95% air). B31 cells, a Rat-1 fibroblast cell line expressing pp60v-src of rat brain cDNA using the polymerase chain reaction. The brain cDNA for IP3K A was amplified from 0.1 mg of brain cDNA with proteins that are (11, 17, 20, 28) or closely resemble (18) the A isoform, and thus little is known about the effects of calcium/calmodulin and phosphorylation on the activity of the B isoform. To compare the regulation of the two isoforms of IP3K, we have established stable cell lines over-expressing each of the two rat IP3K isoforms. A double affinity tag (hexahistidine and the FLAG epitope) was engineered into the cDNAs encoding the IP3K, and the proteins were overexpressed in B31 rat fibroblasts. This strategy provides a means to purify significant amounts of each protein quickly and efficiently. Examination of the regulatory properties of these purified, recombinant IP3Ks shows that the two isoforms are regulated very differently by calcium/calmodulin and via phosphorylation with protein kinase A or C. These observations provide a basis for understanding the complex regulation of IP3K in different types of cells.

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The reaction was initiated by the addition of the PuF polymerase and allowed to proceed for 25 cycles with cycling segments of 1 min, 94 °C; 1 min, 55 °C; 1 min, 72 °C. The reaction was terminated by the addition of 95% ethanol to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. The PCR product was then diluted in water to give a final concentration of 95% ethanol. 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reaction was stopped by blotting onto Whatman P81 cation exchange paper, and the filters were washed for 1 h in 50 mM NaCl. The molar ratio of phosphorylation was estimated from the amount of radioactivity bound to the filters. Controls were performed without the IP3K protein, and the minimal level of autophosphorylation of PKA or PKC was subtracted when calculating the molar ratio of phosphorylation. Minor amounts of PKA or PKC autophosphorylation were observed (5–10% of total). Due to the low concentration of protein used in this assay, the amount of IP3K was estimated by resolving the proteins on SDS gels, staining the gels with silver (37), and comparing the bands intensities with ovalbumin standards. The protein concentrations were quantified using the BioImage WholeBand software.

Hydroxylamine Digestion—Phosphorylated proteins were resolved on 7.5% polyacrylamide gels, and the IP3K protein was excised from the gel after locating the radioactive protein by autoradiography. The gel slice was placed in a solution containing 2 mM guanidine hydrochloride, 2 mM hydroxylamine, and 0.2 M K2CO3, pH 9, to cleave the protein between asparagine and glycine residues (39). After incubation at 45 °C for 1.5 h, the gel slice was washed for 30 min with deionized water and for 15 min with SDS gel sample buffer, homogenized, and loaded onto a 16.5% Tricine SDS-polyacrylamide gel (40). After electrophoresis, the proteins were transferred to nitrocellulose prior to Western analysis. The N-terminal region of the IP3K containing the FLAG epitope was identified with FLAG monoclonal antibodies and alkaline phosphatase conjugated antibodies to IP3K or the FLAG antibody, the reactive proteins were transferred to nitrocellulose and probed with anti-FLAG antibodies (anti-FLAG antibody 34) and vector-transfected cells (lane 2) as well as the stably transfected cells all contained endogenous proteins that reacted with the antibody (bands at 35, 48, and 87 kDa). The molecular masses of standards in kDa are shown at the left.

RESULTS AND DISCUSSION

Expression and Purification of Ins(1,4,5)P3 3-Kinase

Overexpression of IP3K in Mammalian Cells—Although the A isoform of IP3K from rat brain has been overexpressed in NIH-3T3 and CCL39 fibroblasts (41), we were unable to develop cell lines overexpressing either isoform of IP3K in normal Rat-1 fibroblasts (data not shown). However, we were able to establish stable B-31 Rat-1 cell lines overexpressing each of the IP3K isoforms as described under “Experimental Procedures.” Clonal populations of cells were obtained by purifying neomycin-resistant colonies produced from a single cell. The expanded colonies were monitored for protein expression by Western blotting cell extracts with the FLAG antibody. Colonies that reacted positively with the FLAG antibody were assayed for IP3K activity. Two cell lines were selected for further study and termed B31-IP3K-A and B31-IP3K-B. Western analysis of extracts from these cell lines resolved on SDS gels and blotted to nitrocellulose is shown in Fig. 1. Note that the FLAG antibody reacts strongly with bands of 59 kDa (lane 3) and 92 kDa (lane 4). These molecular weights correspond to the predicted molecular masses of the native proteins (50 kDa for isoform A and 74 kDa for isoform B (27, 31)). When duplicate gels were transferred to nitrocellulose and probed with antibodies to IP3K or the FLAG antibody, the reactive proteins eluting from the FLAG and Ni2+-NTA column bound the recombinant proteins permitted stringent washing conditions. Both column wash buffers contained 0.1% CHAPS, and the buffers used with the Ni2+-NTA column contained 100 mM β-mercaptoethanol. In addition, 25–50 mM imidazole was used to remove weakly adsorbing proteins from the Ni2+-NTA resin. The final product was eluted from the Ni2+-NTA column with 200 mM imidazole. This two-column purification procedure was simple, efficient (20–50% recovery of activity), and could be completed in 4 h. The resulting proteins were highly purified (Fig. 2).

The purified, recombinant IP3K is essentially homogeneous as judged by silver-stained gels (arrows in Fig. 2, A and B, lanes 7). The apparent molecular mass of the purified A isoform is about 59 kDa and that of the B isoform is about 92 kDa. As expected, the affinity-tagged proteins migrated slower than the predicted molecular masses of the native proteins (51 kDa for isoform A and 74 kDa for isoform B (27, 31)). When duplicate gels were transferred to nitrocellulose and probed with antibodies to IP3K or the FLAG antibody, the reactive proteins eluting from the FLAG and Ni2+-NTA columns migrated identically with the purified proteins indicated by the arrows in Fig. 2 (data not shown). The slower mobility of the recombinant proteins made them easily distinguishable from the low levels of endogenous IP3K when using the IP3K A antibody to perform Western analysis on cell extracts and the FLAG column wash fractions (not shown). The activity of IP3K was monitored throughout the purification, and results from two representative purifications are shown in Tables I and II. A typical protein yield from 20 150-mm tissue culture plates of cells as described under “Experimental Procedures.” The extract was first applied to a 1-ml FLAG antibody column, eluted in buffer containing 200 μg/ml FLAG peptide, and the eluate was applied directly to a Ni2+-NTA column. The high affinity with which these two columns bound the recombinant proteins permitted stringent washing conditions. Both column wash buffers contained 0.1% CHAPS, and the buffers used with the Ni2+-NTA column contained 100 mM β-mercaptoethanol. In addition, 25–50 mM imidazole was used to remove weakly adsorbing proteins from the Ni2+-NTA resin. The final product was eluted from the Ni2+-NTA column with 200 mM imidazole. This two-column purification procedure was simple, efficient (20–50% recovery of activity), and could be completed in 4 h. The resulting proteins were highly purified (Fig. 2).

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IP3K has been purified from numerous sources including rat brain, bovine brain, rat liver, rat thymus, human platelets, and porcine muscle. The rat brain IP3K has been purified by three separate groups, and it appears that the protein isolated in these studies is most likely isoform A based on its size, anti-
Expression and Purification of Ins(1,4,5)P₃ 3-Kinase

**TABLE I**

Purification of the A isoform of Ins(1,4,5)P₃ 3-kinase

| Fraction | Protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Yield (%) | Purification (fold) |
|----------|--------------|----------------------------|---------------------------------|-----------|---------------------|
| Cell extract | 220ᵃ | 726 | 3.30 | 100 | 1.0 |
| Cytosol | 101ᵇ | 505 | 5.00 | 69 | 1.5 |
| Anti-FLAG | 0.14₀ᵇ | 243 | 1733 | 34 | 530 |
| Nickel | 0.004ᵇ | 145 | 36317 | 21 | 11005 |

ᵃ Protein concentration in these fractions was determined by the method of Lowry (35).
ᵇ Protein concentration in these fractions was determined by silver staining (36) as described under "Experimental Procedures.

**TABLE II**

Purification of the B isoform of Ins(1,4,5)P₃ 3-kinase

Forty 150-mm plates of B31 IP3K B Rat-1 fibroblasts were homogenized, a cytosolic extract was prepared, and the IP3K was purified as described under "Experimental Procedures.

| Fraction | Protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Yield (%) | Purification (fold) |
|----------|--------------|----------------------------|---------------------------------|-----------|---------------------|
| Cell extract | 422ᵃ | 152 | 0.36 | 100 | 1.0 |
| Cytosol | 246ᵃ | 143 | 0.58 | 94 | 1.6 |
| Anti-FLAG | 1.30ᵇ | 89 | 70.5 | 58 | 196 |
| Nickel | 0.005ᵇ | 71 | 15770 | 47 | 43806 |

ᵃ Protein concentration in these fractions was determined by the method of Lowry (35).
ᵇ Protein concentration in these fractions was determined by silver staining (36) as described under "Experimental Procedures.

The A isoform was more susceptible than the B isoform to proteolysis during the purification. The protease inhibitors leupeptin, aprotinin, bacitracin, benzamidine, peptatin, Pefabloc, and calpain inhibitors I and II were added to homogenization and elution buffers to prevent degradation of the protein. When degradation did occur, it typically occurred early in the purification procedure and could be observed as loss of the N-terminal end of the molecule. The degraded protein was apparent in the flow through of the first column as a band of ~50 kDa. This protein did react with IP3K antibodies but did not respond to FLAG antibodies following Western analysis (data not shown). As other investigators have reported (11), β-mercaptoethanol and the detergents Triton X-100 or CHAPS stabilized enzyme activity during purification and were used routinely. Poor stability of the protein was also observed at dilute protein concentrations; addition of bovine serum albumin stabilized activity in keeping with previous results (9). When the purified protein was stored at ~70 °C in a buffer containing detergent and reducing agent at pH 7–8, activity was maintained for at least 3 months.
Comparison of the Regulatory Properties of the Inositol 1,4,5-Trisphosphate 3-Kinases

Regulation of the Two IP3K Isoforms by Calcium/Calmodulin—The concentrations of calcium needed to activate each form of IP3K was determined in the presence of 10 μM calmodulin. The activity of both isoforms was significantly stimulated by calcium as shown in Fig. 3, A and B. The purified IP3K A isoform was maximally stimulated at 2 μM calcium and the IP3K B isoform at 5 μM concentrations of calcium above 10 μM inhibited the maximal activity of both isoforms, possibly because of competition between Ca2+ and Mg2+ in the ATP binding domain of the IP3K. The A isoform was almost completely inactive when calcium was raised to 100–500 μM, but the activity of the B isoform remained above basal. Using the optimal free calcium concentrations determined in Fig. 3, the effect of calcium/calmodulin on the activity of the two proteins was examined as a function of Ins(1,4,5)P3 concentration. As shown in Fig. 4, the B isoform was activated to a much greater extent than the A isoform. Note that the activity of the B isoform is increased about 20-fold over basal (range of 17–23 in five experiments). In agreement with the findings of other investigators (10, 16, 45), the recombinant IP3K isoforms became more sensitive to stimulation by calcium/calmodulin, phosphorylation is an alternative activation mechanism by calcium/calmodulin as they were purified from other cytosolic proteins. For example, the maximal calcium/calmodulin stimulation of the enzyme observed in crude cytosolic extracts was 1.5–2-fold for isoform A and 5–8-fold for isoform B (data not shown). It was also found that the extent of stimulation was highly dependent on the composition of the assay buffer. Maximal stimulation was observed only when BSA, detergent, and magnesium concentrations were omitted or their concentrations optimized (see “Experimental Procedures” for details). The ability of calcium/calmodulin to stimulate activity of the IP3K is well established; however, different degrees of activation have been reported. For example, the A isoform of IP3K isolated from rat brain is activated 2–3-fold and an uncharacterized isoform from human platelets is stimulated about 17-fold (9–11, 16). However, because the activation of IP3K by calcium/calmodulin depends greatly on the purity of the protein and the assay conditions, the magnitude of the activation observed in different studies does not clearly establish the presence of the different isoforms in particular tissues. More direct examination of the isoforms present in each cell type using antibodies, Northern analysis, or protein sequencing will be needed to predict the regulatory properties of IP3K in different cells.

Regulation of IP3K Activity by Phosphorylation—In addition to stimulation by calcium/calmodulin, phosphorylation is another well documented mode of regulating IP3K activity (17–26). To compare the effect of phosphorylation on the activity of the two isoforms of IP3K, the purified, recombinant proteins were phosphorylated in vitro with pure PKA and PKC and examined for the stoichiometry of phosphorylation and changes in activity. The time course of phosphorylation of the A isoform of IP3K by PKA and PKC is shown in Fig. 5, A and B, respectively. Fig. 5, C and D, illustrates analogous experiments using isoform B. Stoichiometric phosphorylation of each isoform occurred within 15 min at 30 °C. The stoichiometry of phosphorylation of IP3K A was similar to that previously reported (17): PKA caused incorporation of about 0.9 mol of phosphate/mol of enzyme (Fig. 5A), and PKC caused incorporation of 1.7–2 mol/mol (Fig. 5B). IP3K B was phosphorylated to a stoichiometry of about 1 and 2.7 mol of phosphate/mol of enzyme by PKA and PKC, respectively (Fig. 5, C and D). Phosphorylation reactions run in the presence of [γ-32P]ATP were resolved on SDS gels and autoradiographs made from the dried gels. The insets in Fig. 5, A–D, show sections of the autoradiographs surrounding the migration positions of the two IP3K isoforms. Analysis of the films indicated that no radioactivity was incorporated into IP3K in the absence of added protein kinase and that greater than 90% of the radioactive phosphate was incorporated into the purified IP3K A isoforms. The remainder of the radioactivity was incorporated into the protein kinase because of autophosphorylation (see “Experimental Procedures” for details). Western analysis using the FLAG antibody was performed on duplicate gels transferred to nitrocellulose. The reactive proteins migrated identically with the radioactive IP3K isoforms identified in the four insets (data not shown).

Since engineering the affinity tags into the N-terminal region of the IP3K isoforms also introduced a new serine into the A isoform and a serine and threonine into the B isoform (see “Experimental Procedures”), it is conceivable that new phosphorylation sites were created in the proteins. Thus, the observed changes in phosphorylation and activity might be due to the added residue. This possibility seems unlikely because the stoichiometry and changes in activity determined using our
FIG. 5. Phosphorylation of purified IP3K A by PKA and PKC. The time course of phosphorylation of each purified, recombinant IP3K isoform was determined at 30 °C using PKA and PKC. A, the A isoform of IP3K phosphorylated with PKA; B, the A isoform of IP3K phosphorylated with PKC; C, the B isoform of IP3K phosphorylated with PKA; D, the B isoform of IP3K phosphorylated with PKC. Kinase reactions were performed as described under “Experimental Procedures” in a total volume of 500 μl from which 25–50-μl aliquots were withdrawn and blotted onto P81 cation exchange paper at the indicated times. The molar ratio of phosphorylation was determined as described under “Experimental Procedures” after background due to autophosphorylation of PKA or PKC was subtracted. Insets, phosphorylation reactions performed in the presence of [γ-32P]ATP were resolved on SDS gels, and autoradiographs were prepared. The insets show sections of the autoradiographs surrounding the migration positions of the IP3K isoforms following incubation with (+) or without (−) the indicated protein kinases. The major phosphorylated proteins in the reactions were the IP3K isoforms. Minor amounts of PKA or PKC autophosphorylation occurred (−5–10% of total), and no other phosphoproteins were observed. Data represent typical results obtained from three time course experiments performed on three separate preparations of IP3K A or IP3K B.

To determine if phosphorylation of the IP3K isoforms altered the kinetic properties of the enzymes, the activity of each isoform was measured after phosphorylation with PKA or PKC. Activity was measured at Ins(1,4,5)P3 concentrations ranging from 0–50 μM in the absence or presence of a maximal amount of calcium/calmodulin. The substrate-velocity curves and corresponding Lineweaver-Burk plots were analyzed for each IP3K isoform following phosphorylation with either kinase. Phosphorylation of IP3K by PKA increases its activity by increasing the \( V_{\text{max}} \) without a significant change in its \( K_m \) for Ins(1,4,5)P3 (−2–5 μM). Analogous experiments using both protein kinases and the A or B isoforms of IP3K as the substrates showed that when phosphorylation caused a change in IP3K activity, the \( V_{\text{max}} \) of the enzyme was changed without a significant alteration in the \( K_m \) (data not shown).

The effects of phosphorylation with PKA and PKC on the observed \( V_{\text{max}} \) of IP3K assayed in the presence and absence of calcium and calmodulin are summarized in Fig. 6. Fig. 6A demonstrates that PKA increases the activity of the A isoform of IP3K about 2–3-fold when assayed in the presence of calcium/calmodulin and about 1.5–2-fold if assayed in its presence. In contrast, phosphorylation of the A isoform with PKC caused a significant decrease in activity whether assayed in the presence or absence of calcium/calmodulin (to −25% of the unphosphorylated enzyme activity). The effects of phosphorylation on the activity of the B isoform of IP3K are markedly different. Fig. 6B demonstrates that phosphorylation using either PKA or PKC had no effect when IP3K B activity was assayed in the absence of calcium/calmodulin. However, when assayed in the presence of calcium/calmodulin, the activity of the B isoform was decreased following phosphorylation by either protein kinase. Note that activity was reduced to 50% of the unphosphorylated enzyme activity following treatment with PKA and to a greater degree (26%) following treatment with PKC. This result suggests that the interaction of the B isoform with calcium/calmodulin is affected by its phosphorylation state.

The data in Fig. 6 shows that phosphorylation of either IP3K isoform with purified protein kinases markedly affects their activity. Possible mechanisms to explain these observations include: 1) phosphorylation may alter the catalytically active

recombinant isoform A match those determined by Sim et al. (17) using protein purified from rat brain. However, to specifically address this issue, we digested the A isoform of IP3K with hydroxylamine, which selectively cleaves amide bonds between asparagine and glycine residues. Based on the known sequence of the A isoform of IP3K, hydroxylamine would be predicted to cleave the protein once between amino acids 59 and 60, resulting in two peptides with molecular masses of 8.5 and 47.5 kDa. The 47.5 kDa C-terminal portion will contain both the PKA and PKC phosphorylation sites determined by Sim, et al. (17). When the phosphorylated IP3K A was digested with hydroxylamine, greater than 90% of the radioactivity was associated with the 47.5-kDa C terminal peptide, and the 8.5-kDa N-terminal peptide reacted with the FLAG antibody (data not shown). Identical results were obtained when IP3K was phosphorylated with either PKA or PKC. Therefore, it does not appear that the amino-terminal serine or threonine residues introduced with the affinity tags are phosphorylated by either protein kinase.

2 The smaller calcium/calmodulin stimulation of isoform B activity observed in these experiments as compared with those in Fig. 4 is due to the BSA added to stabilize IP3K activity when it was diluted into the IP3K assay.
conformation of the protein and/or 2) phosphorylation may interfere with the ability of calcium/calmodulin to stimulate the enzyme. The data in Fig. 6 suggests that phosphorylation may affect the activity of isoform A differently than isoform B. The structural details concerning the regulation of the A isoform are much more complete. Previous studies using this isoform purified from rat brain determined that the major phosphorylation sites in the protein are Ser119 for PKA and Ser119 and Ser185 for PKC (17, 28). Our current results expand on this earlier work and demonstrate that both basal and calcium/calmodulin-stimulated IP3K activity are inhibited to the same extent following phosphorylation by PKC (about 75%, Fig. 6A). However, the increment in activity caused by calcium/calmodulin (−2–3-fold) remains after phosphorylation by PKC, suggesting that introduction of negatively charged phosphate residues at Ser119 and/or Ser185 does not interfere with the binding of calmodulin. When the A isoform is phosphorylated by PKA, calcium/calmodulin is still able to stimulate IP3K activity although the -fold increment is reduced slightly as compared with the unphosphorylated enzyme (2.5-versus 1.8-fold in Fig. 6A). Overall, the data are most consistent with the interpretation that phosphorylation of the A isoform by either protein kinase alters the catalytic conformation of the enzyme without greatly affecting the ability of calcium/calmodulin to interact with the protein. Future studies will need to address these issues directly.

In marked contrast, phosphorylation of the B isoform of IP3K only affects its activity in the presence of calcium/calmodulin (Fig. 6B), thus phosphorylation may interfere with the ability of calcium/calmodulin to interact with the enzyme. While definitive information is lacking about the phosphorylation sites and the calmodulin binding domain in isoform B, comparison of the amino acid sequences of the two isoforms provides some potential insights into its mode of regulation. First, Ser119 in the A isoform is conserved in the B isoform as Ser224, whereas there is no serine located in the B isoform in a position analogous to Ser185 in isoform A. Second, while the calmodulin binding domain of the B isoform has not been mapped by mutagenesis studies, the sequences of the two isoforms can be aligned using the invariant tryptophan (Trp165) in the −33 amino acid calmodulin binding domain of the A isoform (42, 46). Alignment of these two calmodulin binding domains shows that the corresponding tryptophan in the B isoform is Trp380. The alignment indicates a potential amphipathic helix in the B isoform between Thr368 and Leu405. This region contains 2–3 potential phosphorylation sites that do not appear in the sequence of the A isoform (Arg-Val-Ser277, Ser279, Trp-Lys, and Ser306, V2-Phe-Lys). This information, combined with the data in Fig. 5D showing that there is an additional phosphorylation site for PKC in the B isoform, suggests that phosphorylation of the calmodulin binding domain could be a regulatory mechanism in the B isoform. Future studies will need to identify these phosphorylation sites to completely understand the regulation of this protein.

It has already been reported that the A isoform of IP3K is also a substrate for the calcium/calmodulin-dependent protein kinase II. This kinase phosphorylates IP3K A at Thr311 both in intact cells and in vitro, increasing activity of the enzyme and markedly decreasing the Km for calmodulin (20). Thus, in addition to the opposing effects of PKA and PKC on the Vmax of IP3K A, phosphorylation by a calmodulin-sensitive kinase modifies IP3K activity by another mechanism, an increased affinity for its allosteric activator, calcium/calmodulin. This finding implies that, in cells such as neurons with high concentrations of IP3K A and calcium/calmodulin-dependent kinase II, signals that raise Ca2+ cause a rapid activation of the enzyme and potentially large increases in Ins(1,3,4,5)P4 levels. Interestingly, the site phosphorylated by the calcium/calmodulin-dependent kinase (Thr311) is well removed from the calmodulin binding domain of IP3K A, and calmodulin stimulates both the phosphorylated and dephosphorylated forms of the protein to the same extent (about 2.5-fold). It will be interesting to determine if phosphorylation at Thr311 directly modifies the affinity of calmodulin for its binding domain in IP3K or changes the active conformation of the enzyme by other mechanisms. Since both the phosphorylation site (as Thr522) and the surrounding amino acids are conserved in the sequence of IP3K B, it is possible that the calcium/calmodulin-dependent protein kinase will cause similar changes in the activity of the B isoform. However, as phosphorylation of this enzyme by either PKA or PKC inhibits calcium/calmodulin-stimulated activity (Fig. 6B), IP3K B exhibits potentially opposing regulatory mechanisms. Therefore, it will be important to determine if phosphorylation by PKA or PKC can still inhibit its activity following phosphorylation by the calmodulin-dependent kinase. Because of the complex and differential regulation of the two isoforms of IP3K, there is a need to define the predominant isoforms in each cell type and to account for the effects of the multiple signaling molecules activated after agonist stimulation to understand the regulation of InsP4 levels.

In summary, this work demonstrates that recombinant, affinity-tagged IP3K A or IP3K B can be expressed to high levels in mammalian cells and efficiently purified. The purified preparations of both isoforms have a high specific activity and can be acutely regulated by calcium/calmodulin and phosphorylation in vitro. Both of these regulatory mechanisms are likely to be used in intact cells to regulate inositol phosphate metabolism. Indeed, exposing cells to protein kinase activators or protein phosphatase inhibitors alters IP3K activity in cell extracts (18, 20–26). However, differing and conflicting changes in activity were observed in these studies, perhaps due to differing assay conditions or the existence of the different IP3K isoforms in the cells studied. The new data presented in this study on the markedly different regulation of the IP3K isoforms may help explain the conflicting results seen in the studies noted above. It will be important to explore the role of these different regulatory mechanisms in the ability of different types of cells to synthesize or utilize Ins(1,3,4,5)P4 and other inositol polyphosphates.

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3 The cDNA for the A isoform of IP3K indicates two ATG’s that could be used to initiate transcription (17, 28). The amino acid numbering used here corresponds to the longer form of the protein (28).
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