Characterization of a Soluble Adrenal Phosphatidylinositol 4-Kinase Reveals Wortmannin Sensitivity of Type III Phosphatidylinositol Kinases†

Gregory J. Downing, Stanley Kim, Satoshi Nakanishi,‡ Kevin J. Catt, and Tamas Balla*

Endocrinology and Reproduction Research Branch, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: Phosphorylation of phosphatidylinositol (PtdIns) by PtdIns 4-kinases is the first step in the synthesis of polyphosphoinositides, the lipid precursors of intracellular signaling molecules. We have recently identified a cytosolic PtdIns 4-kinase (cPI4K) in the bovine adrenal cortex that is distinguished from previously known PtdIns 4-kinases by its sensitivity to the PtdIns 3-kinase inhibitor wortmannin (WT). The present study has further characterized this soluble enzyme and compared its properties to those of the membrane-bound, type II PtdIns 4-kinase activity of the adrenal cortex and the type III enzyme of bovine brain. The enzymatic activity of adrenal cPI4K was inhibited not only by WT (IC_{50} ∼ 50 nM) but also by LY-294002 (IC_{50} ∼ 100 μM), another inhibitor of PtdIns 3-kinase, and neither compound affected type II PtdIns 4-kinase at concentrations that inhibited cPI4K. In contrast to the type II enzyme, cPI4K had a significantly higher K_m for ATP, was relatively insensitive to inhibition by adenosine (K_i ∼ 800 μM vs ∼40 μM), had lower affinity for PtdIns, and was not inhibited by Ca^{2+} ions. These properties identify the WT-sensitive adrenal cPI4K as a type III PtdIns 4-kinase that is distinct from the tightly membrane-bound, Ca^{2+}- and adenosine-sensitive, type II PtdIns 4-kinase. The type III PtdIns 4-kinase prepared from bovine brain exhibited similar kinetic parameters as the adrenal cPI4K, and was also inhibited by WT with an IC_{50} of 30−50 nM. Since WT inhibits the synthesis of agonist-regulated phosphoinositide pools in intact cells at micromolar concentrations, these findings indicate that type III rather than type II PtdIns 4-kinases are responsible for the maintenance of the precursor phospholipids required for intracellular signaling through the inositol phosphate/Ca^{2+} pathway.

Inositol phospholipids participate in the regulation of multiple aspects of cellular function. Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2][1] is the plasma membrane precursor of two pivotal messengers, Ins(1,4,5)P_3 and DG, that are produced by phospholipase C-catalyzed hydrolysis when numerous cell surface receptors are activated by their specific ligands (Berridge, 1993; Nishizuka, 1984). While this early signaling role of PtdIns(4,5)P_2 is well recognized and has been extensively studied, it has become evident that polyphosphoinositides are also involved in the regulation of more diverse cellular functions. Actin binding proteins such as profilin have been shown to bind PtdIns(4,5)P_2, and this interaction has a significant impact on the ability of phospholipase C isozymes to hydrolyze the lipid (Goldschmidt-Clermont et al., 1991). Adaptor proteins that participate in clathrin-coated pit assembly (and hence receptor-mediated endocytosis) also bind PtdIns(4,5)P_2 (as well as some highly phosphorylated inositol derivatives), and such interactions affect their function (Beck & Keen, 1991). A separate nuclear phosphoinositide system is regulated by as yet unknown mechanisms that are distinct from those involved in the classical plasma membrane phosphoinositide system (Divecha et al., 1993). More recently, the critical role of PtdIns(4,5)P_2 in the G nucleotide-dependent activation and association of phospholipase D with various small G proteins (ARFs and Rho) has been the focus of numerous studies (e.g., Liscovitch et al., 1994).

In contrast to these multiple functions of PtdIns(4,5)P_2, the manner in which 3-phosphorylated inositol phospholipids influence growth responses and other important cell functions has yet to be defined. The 3-phosphorylated inositides represent only a small fraction of the total phosphoinositide population and are produced by PtdIns 3-kinases. These enzymes are activated by receptor tyrosine kinases as well as by oncogenic tyrosine kinases, and are important for several aspects of cell regulation (Fry, 1994). Since most (if not all) of the known actions of phosphoinositides require the phosphorylation of PtdIns, the phospholipid kinases are essential participants in the regulation of multiple cell functions. Although the importance of 4-phosphorylated inositides was recognized much earlier than that of the 3-phosphorylated forms, considerably more is known about PtdIns 3-kinases than about PtdIns 4-kinases. The first PtdIns 3-kinase to be isolated and cloned (Hiles et al., 1992; Otsu et al., 1991) has 110 kDa catalytic and 85 kDa regulatory subunits, and phosphorylates PtdIns and PtdIns(4)P as well as PtdIns(4,5)P_2. Additional 3-kinases have been described recently, including a PtdIns-specific 3-kinase that

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* To whom correspondence should be addressed at the Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Building 49, Room 6A35, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301)-496-2136. FAX: (301)-480-8010.

‡ Present address: Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan.

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[1] Abbreviations: PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; GroPtdIns(4)P, glycerophosphatidylinositol 4-phosphate; DG, diacylglycerol; cPI4K, cytosolic PtdIns 4-kinase; WT, wortmannin; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; BSA, bovine serum albumin.

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phosphorylates only PtdIns but not its phosphorylated derivatives (Stephens et al., 1994a), and others that are regulated by G protein $\beta\gamma$ subunits (Stephens et al., 1994b; Stoyanov et al., 1995). These enzymes are soluble and can be inhibited by the fungal metabolite wortmannin with potencies that vary among the individual forms of the enzyme (Stephens et al., 1994a; Stack & Emr, 1994).

Although the receptor-regulated formation of the calcium-mobilizing second messenger, Ins(1,4,5)P_3, is highly dependent on the formation of PtdIns(4,5)P_2 by PtdIns 4-kinase and PtdIns(4)P 5-kinase, the PtdIns 4-kinase involved in this pathway has not yet been identified. PtdIns 4-kinase activity is most abundant in the membrane fraction of homogenized tissues and can be solubilized by detergents, so most attempts to purify the enzyme have utilized the membrane-bound forms. In the brain, two forms of detergent-soluble PtdIns 4-kinase have been distinguished: type II, a smaller ($\sim$56 kDa) adenosine-sensitive enzyme; and type III, a larger ($>$200 kDa) form that is less sensitive to inhibition by adenosine (Carpenter & Cantley, 1990). In addition, we have identified a cytosolic PtdIns 4-kinase (cPI4K) in the bovine adrenal cortex (Nakanishi et al., 1995). This novel enzyme is sensitive to the PtdIns 3-kinase inhibitor, wortmannin, and is essential for the maintenance of agonist-sensitive PtdIns(4,5)P_2 pools in several cell types. In the present study, the characteristics of this soluble kinase have been determined and compared to those of the membrane-bound type II adrenal PtdIns 4-kinase and the brain type III enzyme.

EXPERIMENTAL PROCEDURES

Preparation of PtdIns 4-Kinase. The previously described procedure for preparation of adrenal PtdIns 4-kinase (Nakanishi et al., 1995) was slightly modified to provide larger quantities of the enzyme. Briefly, cortical tissue from 9−10 bovine adrenal glands was dispersed in 200 mL of ice-cold buffer A (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin) containing 1 M NaCl with a glass Dounce homogenizer and stirred on ice for 1 h. After centrifugation at 100000*g* for 60 min, the supernate was removed and the pellet was rehomogenized in the same buffer containing 1% Triton X-100. The suspension was stirred on ice for 1 h and was then centrifuged at 100000*g* for 60 min. The soluble PtdIns 4-kinase activity, which was present in the first supernate, was recovered by 40% $(NH_4)_2SO_4$ precipitation followed by dialysis against buffer A containing 30 mM NaCl. The solution was then loaded onto a DEAE-Sepharose column (2.5 × 30 cm) preequilibrated with the same buffer. The column was washed with 200 mL of the same buffer, and proteins were subsequently eluted with a linear gradient of 0.03−1 M NaCl in buffer A (300 mL total volume). Fractions were assayed for PtdIns 4-kinase activity in the absence or presence of WT. Active fractions were pooled, dialyzed, concentrated, and stored at −80 °C for at least 2 weeks without loss of activity. The second supernatant, containing the Triton-solubilized, originally membrane-bound PtdIns 4-kinase activity, was used without further purification.

The brain type III enzyme was prepared by the method of Endemann et al. (1987), and about 50% of the cholate-solubilized membrane fraction obtained during this procedure was found to be WT-sensitive. Interestingly, application of this method to the adrenal cortex did not yield detergent-extractable WT-sensitive activity and, as in our original protocol (Nakanishi et al., 1995), the WT-sensitive PtdIns 4-kinase was largely recovered in the salt-wash fraction.

Assay of PtdIns 4-Kinase. The activity of PtdIns 4-kinase was measured as the incorporation of radioactivity from [γ-^{32}P]ATP into PtdIns (Nakanishi et al., 1995). The standard reaction mixture for PtdIns 4-kinase (50 μL final volume) contained 50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM PtdIns, 0.4% Triton X-100, 0.5 mg/mL BSA, 100 μM (1μCi) [γ-^{32}P]ATP, and the enzyme. All assay components, except [γ-^{32}P]ATP, were preincubated with inhibitors for 10 min at 30 °C. Reactions were started by addition of [γ-^{32}P]ATP and after 10 min were terminated by the addition of 3 mL of $CHCl_3$:CH_3OH:*cc* HCl [200:100:0.75 (v/v)]. The organic solvent phase was separated from [γ-^{32}P]ATP by adding 0.6 mL of 0.6 N HCl, mixing vigorously, and standing for phase separation. The upper (aqueous) phase was discarded, and 1.5 mL of $CHCl_3$:CH_3OH:0.6 N HCl [3:48:47, (v/v)] was added to the lower phase, followed by mixing and phase separation. The lower phase was then transferred to scintillation vials, and after evaporation, its radioactivity was measured by liquid scintillation spectrometry.

For studies on Ca^{2+}-sensitivity, the reaction buffer was slightly modified (100 mM Tris/HCl and 2 mM EGTA) and $CaCl_2$ was added to achieve the indicated free Ca^{2+} concentrations, which were determined by fluorescence assay with Fura-2 (free acid). For determinations of K_m and adenosine sensitivity, adenosine was added together with [γ-^{32}P]ATP and increasing concentrations of unlabeled ATP.

PtdIns 3-Kinase Measurements. PtdIns 3-kinase activity was immunoprecipitated with anti-p85 antibody from primary cultures of adrenal glomerulosa cells, and its activity was assayed as described previously (Nakanishi et al., 1995).

Deacylation of Phospholipid Products. The products of the enzyme reactions were resolved by TLC (Balla et al., 1988), and the PtdIns(4)P was cut out and dissolved in 2 mL of methylamine reagent (monomethylamine/methanol/butanol, 100:80:20). After incubation at 50 °C for 1 h in tightly capped tubes and subsequent cooling, 1 mL of propanol was added and the samples were dried. After redissolving in 0.4 mL of H_2O and extraction with 0.48 mL of butanol/petrol ether/ethyl formate (20:4:1), the aqueous phase was analyzed on a strong anion exchange HPLC column (Partisphere SAX, Alltech, Deerfield, IL) using $(NH_4)_2HPO_4$ as the eluant (Auger et al., 1990). Radioactivity was determined by an on-line scintillation flow-detector. In parallel reactions, [^{3}H]PtdIns(4)P standard was also deacylated to serve as a control for the elution of GroPtdIns(4)P.

Size-Exclusion Chromatography. One hundred microliters of partially purified enzyme was loaded onto a Superose-12 FPLC column (Pharmacia Biotech, Piscataway, NJ) that had been preequilibrated with buffer A containing 0.5 M NaCl. The column was eluted with the same buffer at a flow rate of 1 mL/min, and 0.5 min fractions were collected on ice. The column was calibrated with dextran-blue (void), γ-globulin (158 kDa), BSA (66 kDa), ovalbumin (42 kDa), and carbonic anhydrase (29 kDa), and corrections were made for the delay between the UV monitor and fraction collector (0.5 min). The PtdIns 4-kinase activity of the individual fractions was assayed as described above.

Sucrose Gradient Separation of PtdIns 4-Kinases. Three hundred microliters of partially purified PtdIns 4-kinase was layered on top of 10 mL continuous gradients of 10−35% sucrose in buffer A containing 1% Triton X-100. For the brain preparations, sucrose gradients were prepared in the buffer described by Edelmann et al. (1987). All gradients were centrifuged at 100000*g* for 24 h at 4 °C. After fractionation into 1 mL aliquots, the density and PtdIns 4-kinase activity of the individual fractions was measured.

Materials. [^{3}H]PtdIns(4)P and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from DuPont-NEN (Wilmington, DE). Fura-2 free acid was from Molecular Probes, Inc. (Eugene, OR). Adenosine, 5′-chloro-5′-deoxyadenosine and all phospholipids were from Sigma. DEAE-Sepharose fast flow media, Superose-12 and Mono-Q HR 5/5 columns, and protein A−Sepharose were from Pharmacia Biotech Inc. (Piscataway, NJ). The anti-p85 antibody for PtdIns 3-kinase was purchased from UBI (Lake Placid, NY). WT was a gift of Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, and LY 294002 was kindly provided by Dr. Chris Vlahos, Eli Lilly and Co. (Indianapolis, IN). All other reagents were of analytical or HPLC grade.

RESULTS

cPI4K Is Inhibited by PtdIns 3-Kinase Inhibitors. As reported earlier (Nakanishi et al., 1995), only about 30% of the total PtdIns 4-kinase activity of the adrenal homogenate was found in the soluble fraction, and the majority of the activity remained tightly associated with the membranes. About 40−50% of the soluble activity was inhibited by WT, and this was separated from the WT-resistant component by DEAE-Sepharose chromatography, which retained the former but not the latter activity. The WT-sensitive activity could be further enriched by chromatography on SP-Sepharose, heparin, Mono-Q, and Mono-S columns (not shown). Most of the experiments described in the present report were performed with the more stable DEAE-purified enzyme, but similar results were obtained when using highly purified but less stable preparations. The properties of the WT-sensitive enzyme activity were compared with those of the tightly membrane-bound PtdIns 4-kinase after solubilization of the latter with 1% Triton X-100. The characteristics of the latter enzyme were consistent with those of a type II PtdIns 4-kinase (see below), and it is hereafter referred to as type II PtdIns 4-kinase.

As shown in Figure 1 (panel B), cPI4K activity was completely inhibited by both WT and another known PtdIns 3-kinase inhibitor, LY-294002, with IC_{50} values of ~50 nM and ~100 μM, respectively. In contrast, neither of these agents inhibited the type II PtdIns 4-kinase at concentrations up to 100 μM (panel A). The inhibitory potencies of both WT and LY-294002 were significantly lower for cPI4K than those reported for PtdIns 3-kinase. This was confirmed by assaying their effects on adrenal PtdIns 3-kinase immunoprecipitated from adrenal glomerulosa cells with anti-p85 antibody (IC_{50} ~1 nM and ~2 μM for WT and LY-294002, respectively) (Figure 1C).

Identification of the Product of cPI4K. The phosphorylation product of cPI4K action was characterized by deacylation and HPLC analysis, as shown in Figure 2. The deacylated ^{32}P-labeled PtdInsP product comigrated with the [^{3}H]GroPtdIns(4)P standard prepared from [^{3}H]PtdIns(4)P and was clearly separated from the deacylated ^{32}P-labeled product of PtdIns 3-kinase phosphorylation. These results demonstrate that the WT- and LY-294002-sensitive activity is indeed a PtdIns 4-kinase and not a PtdIns 3-kinase.

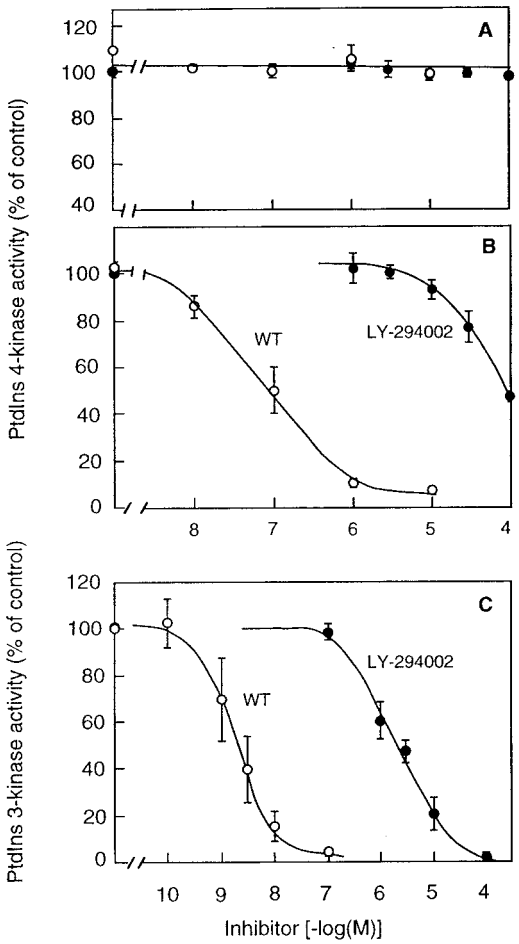

FIGURE 1: Effects of wortmannin (WT) and LY-294002 on adrenal PtdIns kinase activities. PtdIns 4-kinase (panels A and B) and PtdIns 3-kinase (panel C) activities were prepared from bovine adrenocortical homogenates and adrenal glomerulosa cells, respectively, as described under Experimental Procedures. The membrane-bound PtdIns 4-kinase (A) was solubilized with Triton X-100, and the cytosolic PtdIns 4-kinase (B) was purified by DEAE-Sepharose chromatography. Kinase activities were measured as the incorporation of ^{32}P into organic solvent-extractable material from [γ-^{32}P]-ATP using PtdIns as a substrate after 10 min preincubations with the indicated concentrations of WT (○) or LY-294002 (●). Results are expressed as the percent of the control activity measured without inhibitors and are means ± SEM of data from three separate experiments.

Comparison of the Kinetic Parameters of cPI4K and Type II PtdIns 4-Kinase. PtdIns kinases have been classified previously based on their K_m values for ATP and their sensitivities to inhibition by adenosine (Pike, 1992). As shown in Figure 3, the K_m value for ATP was significantly higher for cPI4K than for the type II enzyme [410 ± 24 μM (n = 9) and 120 ± 14 μM (n = 5), respectively], and the K_i value for adenosine was about 20 times higher for the cPI4K enzyme [852 ± 185 μM (n = 4) and 38.7 ± 7 μM (n = 2), respectively (means ± SEM)]. To achieve the half-maximal initial velocity of the conversion of PtdIns to PtdIns(4)P, higher PtdIns concentrations were required for the cPI4K than for the type II enzyme, regardless of whether the Triton X-100 concentration or the PtdIns/Triton X-100 ratio was kept constant (not shown). In neither case was it possible

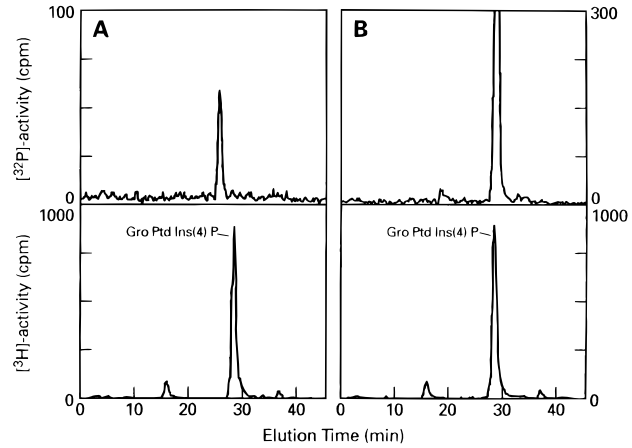

FIGURE 2: HPLC elution profiles of the deacylated reaction products of PtdIns phosphorylation by adrenal PtdIns 3-kinase (A) and the cytosolic PtdIns 4-kinase (B). PtdIns kinase reactions were performed with [γ-^{32}P]ATP, and the reaction products were extracted, separated by TLC, and deacylated as described under Experimental Procedures. The deacylation product of [^{3}H]PtdIns(4)P was used as GroPtdIns(4)P standard (lower panels). The HPLC column (Partisphere SAX, Alltech) was eluted with water for 5 min, followed by a gradient of $(NH_4)_2HPO_4$ (0−300 mM from 5 to 60 min).

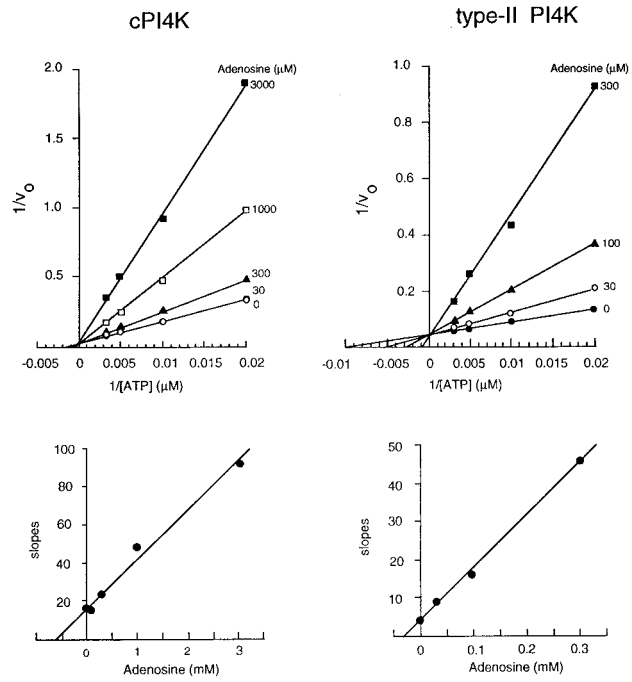

FIGURE 3: Kinetic parameters of cPI4K (left) and type II PtdIns 4-kinase (right). The initial velocities of PtdIns phosphorylation by the respective enzymes were determined with [γ-^{32}P]ATP at various concentrations of unlabeled ATP and adenosine. The double-reciprocal plots (upper panels) show the competitive nature of the inhibition by adenosine and the higher sensitivity of the type II enzyme. The K_i values for the inhibitory effects of adenosine were calculated by plotting the slopes of the double-reciprocal plots as a function of adenosine concentration (Orsi & Tipton, 1979) (lower panels).

to obtain a linear relationship in double-reciprocal plots with the cPI4K enzyme.

Size Estimation of the Adrenal PtdIns 4-Kinases. When the cPI4K preparation was subjected to size-exclusion chromatography, the DEAE-purified activity migrated as a single but relatively broad peak of around 200 kDa (Figure 4A). Further purification of the kinase on a Mono-Q column

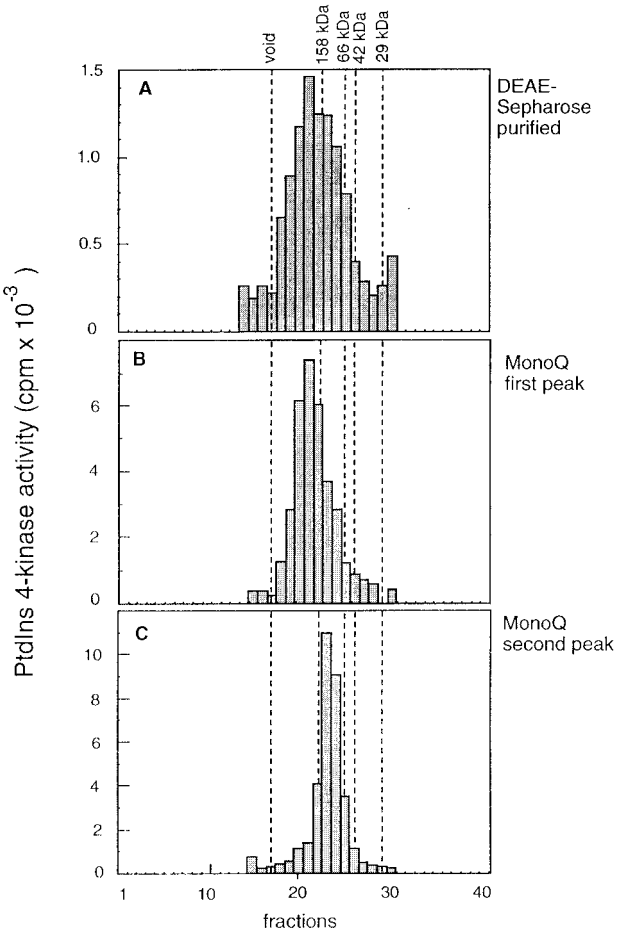

FIGURE 4: Distribution of PtdIns 4-kinase activity during Superose-12 size-exclusion chromatography. Soluble PtdIns 4-kinase activities partially purified by DEAE-Sepharose chromatography (panel A) or by FPLC on Mono-Q HR columns (panels B and C) were applied in 100 μL volume to a Superose-12 size-exclusion column equilibrated and eluted with 0.5 M NaCl in buffer A as described under Experimental Procedures. The PtdIns 4-kinase activities of 0.5 min fractions were assayed as described in the legend to Figure 1. The column was calibrated with dextran blue (void), γ-globulin (158 kDa), BSA (66 kDa), ovalbumin (42 kDa), and carbonic anhydrase (29 kDa). The relatively broad peak of activity observed in the DEAE-Sepharose-purified enzyme (panel A) was resolved by FPLC on a Mono-Q column into two peaks, the first eluting at ~200 kDa (panel B) and the second at ~110 kDa (panel C) on the size-exclusion column.

resulted in the separation of two peaks of enzyme activity (not shown). These activities showed apparent sizes of 200 and 110 kDa (Figure 4B,C), the larger species eluting first from the Mono-Q column. Both activities showed the same sensitivity to inhibition by WT and were indistinguishable by their K_m for ATP or K_i for adenosine (not shown). Based on these observations, it is possible that the larger form is a homodimer or a heterodimer that contains a regulatory component analogous to the p85 of PtdIns 3-kinase. However, the possibility that the smaller activity represents a proteolytic fragment of the larger enzyme cannot be excluded.

To compare the native size of the cPI4K with that of the type II enzyme, these activities were separated by sucrose density gradient centrifugation. Cholate-solubilized bovine brain membranes have been shown to contain two distinct PtdIns 4-kinases that are separated by density gradient centrifugation into a smaller type II and a larger type III

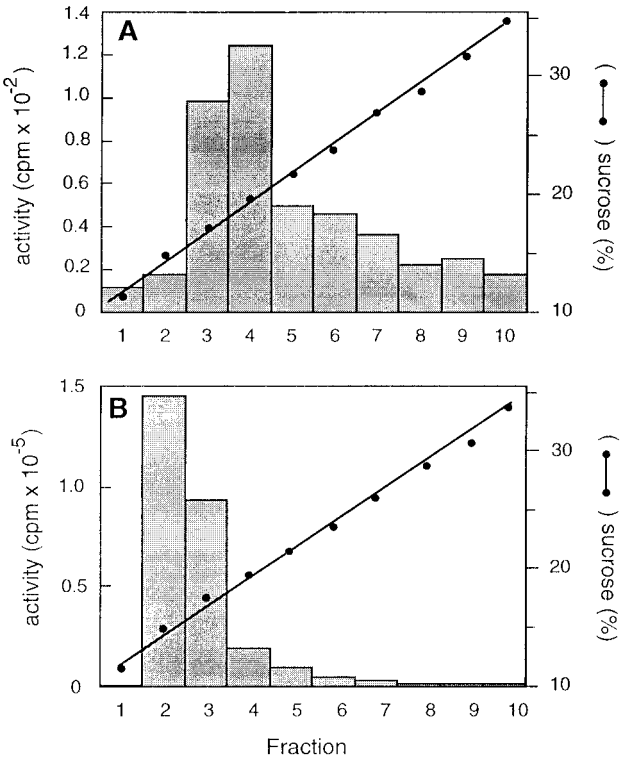

FIGURE 5: Distribution of cPI4K and type II PtdIns 4-kinase activities on sucrose density gradients. The soluble PtdIns 4-kinase partially purified by DEAE-Sepharose chromatography (panel A) and the membrane-bound type II enzyme solubilized by 1% Triton X-100 (panel B) were layered on 10 mL continuous sucrose gradients (10−35%) and centrifuged at 100000*g* for 24 h. 1 mL fractions were assayed for their PtdIns 4-kinase activity (bars) and their actual densities determined (●). A representative result from one of two experiments is shown.

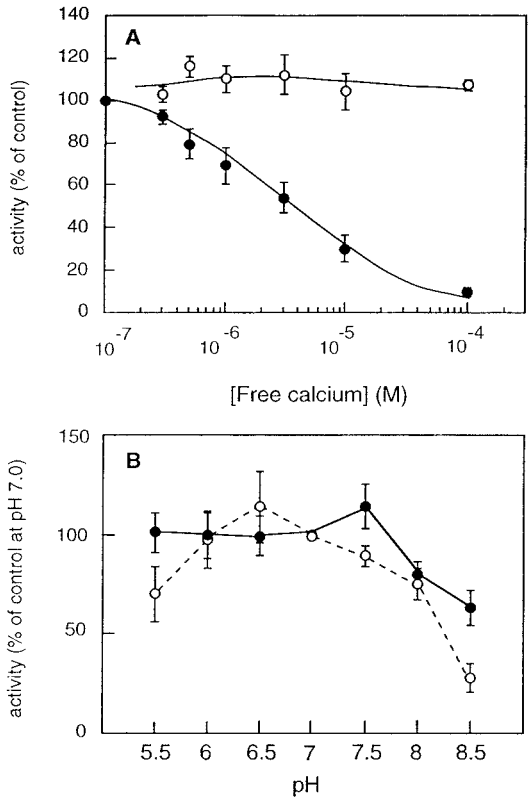

FIGURE 6: Calcium and pH sensitivities of cPI4K and type II PtdIns 4-kinase enzymes. The soluble PtdIns 4-kinase partially purified by DEAE-Sepharose chromatography (○) and the membrane-bound type II enzyme solubilized by 1% Triton X-100 (●) were assayed at selected free Ca^{2+} concentrations (upper panel) or pH values (lower panel) as described under Experimental Procedures. The results are expressed as percent of control activities measured under Ca^{2+}-free (10^{-7} M) conditions (upper panel) or at pH 7.0 (lower panel). Means ± SEM of three observations are shown.

enzyme (Endemann et al., 1987). As shown in Figure 5, the solubilized adrenal membranes contained a single peak of activity that sedimented at a density similar to that reported for the type II PtdIns 4-kinase of brain and red blood cell membranes. In contrast, the cPI4K activity sedimented at a higher density, and its peak was broader than that of the type II enzyme. These results indicate that detergent-solubilized adrenal membranes do not contain significant amounts of type III activity and that the soluble cP14K of this tissue has a higher apparent size than the type II enzyme. Since it has not been possible to renature and detect PtdIns 4-kinase activity after SDS−PAGE, the molecular size of the denatured protein remains to be determined.

Cation Sensitivity of cPI4K and Type II PtdIns 4-Kinase Activities. Previous studies have shown that type II PtdIns 4-kinase is inhibited by millimolar concentrations of Ca^{2+}. Comparison of the Ca^{2+} sensitivities of the cPI4K and the solubilized type II PtdIns 4-kinase activities (Figure 6A) showed that the type II enzyme was potently inhibited by increasing concentrations of Ca^{2+}. In contrast, the WT-sensitive cPI4K was neither stimulated nor inhibited by the divalent cation in the concentration range used. Ca^{2+} was also without effect on cPI4K when bovine brain calmodulin was included in the assays (not shown). Both cPI4K and type II PtdIns 4-kinase showed a broad pH optimum, the cPI4K being less active at higher pH (Figure 6B).

As shown in Figure 7, both enzymes required Mg^{2+} for optimal activity and exhibited similar sensitivities to the bivalent cation. However, the type II enzyme was activated poorly and only at low concentrations of Mn^{2+}, while cPI4K could be stimulated by increasing concentrations of Mn^{2+} to almost 50% of its Mg^{2+}-activated activity (Figure 7A,B).

WT Sensitivity of the Brain Type III Enzyme. Since the properties of the adrenal cPI4K resembled those of the brain type III PtdIns 4-kinase enzyme, we characterized the latter activity prepared by the protocol of Endemann et al. (1987). As previously reported, the bovine brain contained a significant amount of membrane-associated PtdIns 4-kinase activity that was resistant to salt extraction but was solubilized by 0.5% cholate. However, unlike the adrenal cortex (regardless of which method was used for homogenization and fractionation), about 40% of the detergent-solubilized PtdIns 4-kinase from brain membranes was inhibited by 10 μM WT. Sucrose density gradient analysis revealed that the WT-sensitive and WT-insensitive components corresponded to the type III and type II enzymes, respectively (Figure 8). The soluble brain fractions also contained WT-sensitive and WT-insensitive PtdIns 4-kinase activities with the same size-distribution on sucrose gradients as the detergent-extracted material (Figure 8). The WT-sensitive activities from the soluble and detergent-extracted fractions were purified and separated from the WT-insensitive forms on Mono-Q columns (not shown), and their WT sensitivity and kinetic parameters were determined. Both soluble and detergent-extracted enzymes were inhibited by WT with similar IC_{50} values (Figure 8, insets), and their respective K_m values for ATP (565 and 705 μM) and K_i values for adenosine (1357

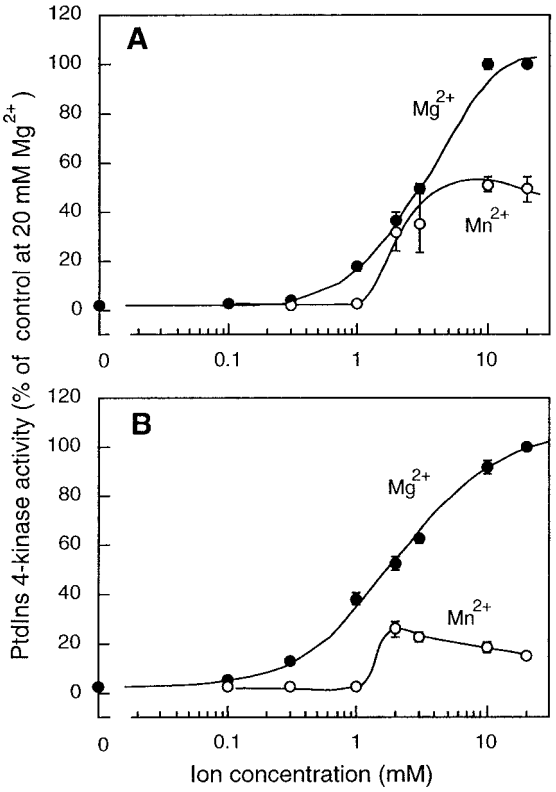

FIGURE 7: Dependence of PtdIns 4-kinase activities on Mg^{2+} and Mn^{2+}. The soluble PtdIns 4-kinase partially purified by DEAE-Sepharose chromatography (panel A) or the membrane-bound type II enzyme solubilized by 1% Triton X-100 (panel B) were assayed at various concentrations of Mg^{2+} (●) or Mn^{2+} (○). The results are expressed as percent of control activities measured at 20 mM Mg^{2+}. Means ± SEM of four separate observations are shown.

μM and 1057 μM) were consistent with both of these activities being type III enzymes. There was no apparent size difference between the two activities and the larger form of cPI4K (Downing and Balla, unpublished observation). Whether these activities represent cytosolic and membrane-associated forms of the same enzyme, or are distinct enzymes in the same family of PtdIns 4-kinases, is currently under investigation. Nevertheless, these results demonstrate that both cytosolic and membrane-bound PtdIns 4-kinases of the type III variety are WT-sensitive enzymes.

DISCUSSION

This study has characterized a soluble PtdIns 4-kinase activity isolated from the adrenal cortex and compared its properties to those of the previously described membrane-associated type II and type III PtdIns 4-kinase activities. As reported earlier (Nakanishi et al., 1995), this enzyme is unique among the PtdIns 4-kinases in being sensitive to the PtdIns 3-kinase inhibitor, WT, albeit at higher concentrations than required to inhibit PtdIns 3-kinase(s). The adrenal cPI4K was also inhibited by another known PtdIns 3-kinase inhibitor, LY-294002, which is structurally unrelated to WT (Vlahos et al., 1994). Again, LY-294002 inhibited cPI4K at higher concentrations than the adrenal PtdIns 3-kinase and was without effect on the membrane-associated type II PtdIns 4-kinase activity. Since both of these compounds are less potent inhibitors of cPI4K than of the PtdIns 3-kinases of adrenal glomerulosa cells, they are still relatively specific inhibitors of PtdIns 3-kinase when used at low concentrations.

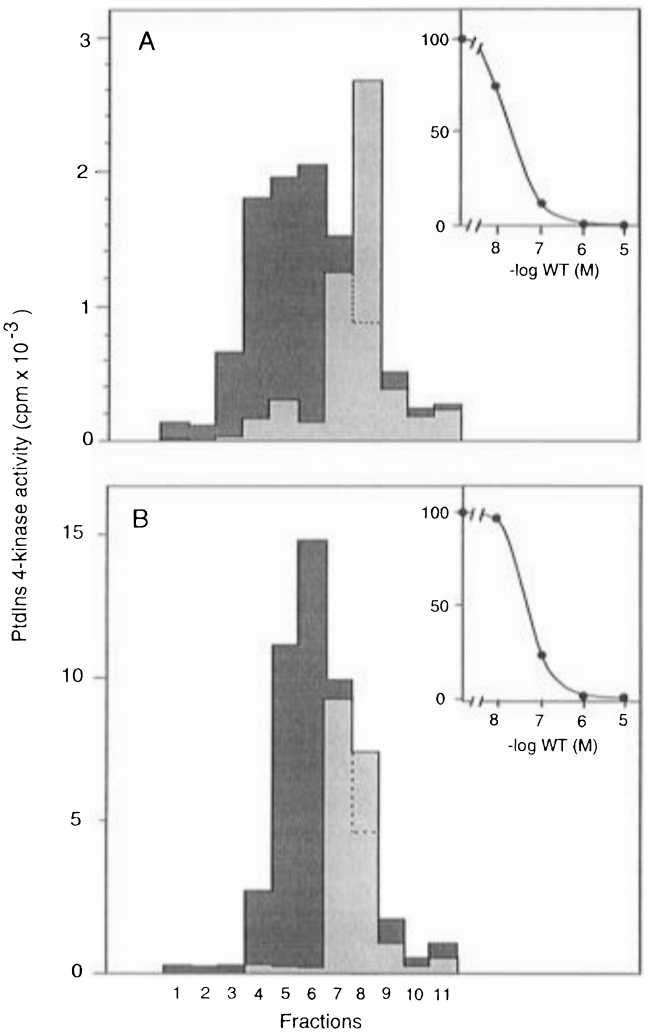

FIGURE 8: Sucrose gradient separation of soluble (A) and detergent-solubilized (B) phosphatidylinositol 4-kinases of bovine brain. Samples of enzymes prepared from bovine brain as described under Experimental Procedures were loaded on 10 mL linear sucrose gradients (10−35%) and centrifuged at 100000*g* for 24 h. Gradients were separated into 1 mL fractions, and their PtdIns 4-kinase activity was measured in the presence and absence of 10 μM WT. The WT-sensitive and WT-insensitive PtdIns 4-kinase activities are shown as light and dark columns, respectively. The inset shows the dose-dependent inhibition by WT of the MonoQ-purified type III PtdIns 4-kinase activities.

Mammalian PtdIns 4-kinases have been largely isolated from membrane fractions and are usually considered to be one of two types (Carpenter & Cantley, 1990). The type II enzyme, a prototype of which is present in the red blood cell membrane (Wetzker et al., 1991; Graziani et al., 1992), has a low K_m for ATP and high sensitivity to inhibition by adenosine [$K_i \sim 10-70$ μM (Pike, 1992)]. This enzyme has been purified from several sources (Wetzker et al., 1991; Porter et al., 1988; Walker et al., 1988) and has a molecular mass of about 56 kDa on SDS−PAGE. It has also been reported to interact with EGF receptors and to be activated by stimulation with EGF (Kauffmann-Zeh et al., 1994; Cochet et al., 1990). The type III enzyme, on the other hand, has a larger molecular mass based on its migration on sucrose gradients and is estimated to be over 200 kDa in its native form. It also differs from the type II enzyme in its higher K_m value for ATP, lower affinity for PtdIns, and relative resistance to inhibition by adenosine ($K_i \sim 1.5$ mM) (Endemann et al., 1987). The type III kinase has not been

purified, and an attempt to clone a 90 kDa membrane-bound PtdIns 4-kinase of rat brain that was believed to be a type III enzyme resulted in the isolation of a long-chain fatty acid-CoA-ligase (Yamakawa & Takenawa, 1988).

Very few data are available about soluble mammalian PtdIns 4-kinases, but a soluble yeast PtdIns 4-kinase, PIK1, has been purified and subsequently cloned from *Saccharomyces cerevisiae* (Flanagan & Thorner, 1992; Flanagan et al., 1993). Interestingly, the same protein has been cloned as part of the nuclear pore complex (Garcia-Bustos et al., 1994), indicating that it can also function as a membrane-associated enzyme. Among the various yeast PtdIns kinases, this enzyme was found to be essential for cell growth since its genetic deletion resulted in a lethal phenotype (Flanagan et al., 1993). An additional PtdIns 4-kinase (STT4) cloned from *S. cerevisiae* was found to be dispensable for cell growth in the presence of osmotic stabilizers, and appears to be involved in the protein kinase C pathway (Yoshida et al., 1994). The mammalian homolog of this enzyme (termed PI4Kα) was recently cloned from human placenta and is enzymatically and immunologically related to the type II family of PtdIns 4-kinase(s) (Wong & Cantley, 1994). Based on sequence similarity, further PtdIns kinase homologs (TOR1 and TOR2) have been isolated from yeast (Kunz et al., 1993). These proteins, as well as their recently isolated mammalian homologs (Brown et al., 1994; Sabatini et al., 1994), are targets of the immunophilin drug rapamycin. Additional PtdIns kinase homologs, such as the mutated gene (ATM) responsible for the genetic disease ataxia telangiectasia (Savitsky et al., 1995), that are involved in cell-cycle control and DNA repair have recently been identified (Keith & Schreiber, 1995). It is not yet clear whether these enzymes possess significant PtdIns kinase activity, or function primarily as protein kinases (Hunter, 1995).

The adrenal cPI4K characterized in the present study is clearly distinct from the type II PtdIns 4-kinase of the adrenal and other tissues, and in many regards resembles the type III PtdIns 4-kinase of bovine brain. The enzyme can be activated by detergents, which clearly distinguishes it from the PtdIns 3-kinase(s). It has a relatively high K_m for ATP and a lower affinity to PtdIns, and its K_i value for adenosine is significantly higher than that of the type II PtdIns 4-kinase. Unlike the type II enzyme, cPI4K is not inhibited by Ca^{2+} ions. Size-exclusion chromatography and sucrose-density centrifugation revealed that cPI4K is a larger enzyme in its native form than the type II PtdIns 4-kinase. These characteristics of the adrenal PtdIns 4-kinase enzyme raised the question of whether the type III PtdIns 4-kinase of the brain is a WT-sensitive enzyme. Unlike the adrenal cortex, the bovine brain was found to contain a membrane-associated, detergent-extractable PtdIns 4-kinase activity that fulfilled the criteria of a type III enzyme and was inhibited by WT in a similar manner as the cPI4K of the adrenal. Interestingly, the soluble brain fraction also contains a WT-sensitive, type III PtdIns 4-kinase activity. Further studies are in progress to clarify the relationship between these enzymes, but preliminary estimates of the size of these molecules argue against the soluble activity being a proteolytic fragment of the membrane-bound enzyme.

Given the similarities between the WT sensitivities and kinetic properties of cPI4K and the type III PtdIns 4-kinase, it is possible that the two are identical and can be recovered in either the membranes or the soluble fractions depending on the tissue and the homogenization conditions. This would be comparable to the case of the yeast PIK1 enzyme, which was cloned as a cytosolic enzyme as well as part of the nuclear-pore protein complex. It is not known at this point whether the PIK1 enzyme can be inhibited by WT or LY-294002. It is interesting to note that the properties of the cPI4K resemble those of a soluble PtdIns 4-kinase that was partially purified from bovine uterus, the only mammalian soluble PtdIns 4-kinase that has been previously characterized (Li et al., 1989).

The complexity of the multiple PtdIns kinases raises the question of the roles of these enzymes in cell regulation. The sustained generation of Ins(1,4,5)P_3 requires the conversion of PtdIns to PtdIns(4)P and PtdIns(4,5)P_2, and this has been believed to be a function of the type II and type III PtdIns 4-kinases. However, we recently demonstrated that WT, at concentrations higher than those required to inhibit PtdIns 3-kinase(s), abolishes the agonist-stimulated production of labeled PtdIns(4)P in several cell types and prevents the sustained generation of Ins(1,4,5)P_3 and calcium signaling (Nakanishi et al., 1995). Since WT at this concentration does not inhibit type II PtdIns 4-kinase(s), these data indicate that the PtdIns 4-kinase involved in hormone-stimulated PtdIns turnover belongs to the type III rather than the type II family.

Our findings also raise a question about the function of the membrane-bound, WT-insensitive PtdIns 4-kinase(s), such as the type II enzyme. Recently, this enzyme has been shown to be important in supporting GTPγS-stimulated phospholipase D activity (Pertile et al., 1995). Also, the yeast homolog of the mammalian PtdIns-specific form of PtdIns 3-kinase, Vps34p, is involved in vacuolar sorting (Stack & Emr, 1994). These data emphasize that cellular functions not directly related to hormone-regulated PtdInsP_2 hydrolysis can also be affected by PtdIns kinases. Since both PtdIns 3-kinase and Vps34p have been shown to possess protein kinase activity (Carpenter et al., 1993; Stack & Emr, 1994; Dhand et al., 1994), it has yet to be determined whether PtdIns phosphorylation is central to their biological function(s).

In summary, we have characterized a soluble, WT-sensitive PtdIns 4-kinase of the adrenal cortex. This enzyme belongs to the type III group of PtdIns 4-kinases and is clearly distinct from the tightly membrane-bound type II enzyme. In addition, the type III PtdIns 4-kinase of bovine brain has proven to be a WT-sensitive enzyme. Our observations suggest that WT-sensitive type III enzymes are of critical importance in maintaining the phosphoinositide pools necessary for agonist-stimulated inositol phosphate production and Ca^{2+} signaling. The multiple enzymes that phosphorylate PtdIns at various positions of its inositol ring emerge as activities of critical importance, with potential functions far beyond their role in hormone-induced PtdIns turnover. The molecular cloning and characterization of these enzymes will be crucial in understanding the multiple roles of inositol phospholipids in controlling a wide range of diverse cell activities.

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