A and B are rapid unless FPP–Mg^{2+} binds the associate complex of A–B. It is reasonable to assume that A–B–FPP–Mg^{2+} is an intermediary state upon which the condensation with IPP occurs and proceeds until hexaprenyl diphosphate ($C_{30}PP$) is produced. The results of gel filtration of the preincubated mixture containing component A, component B, Mg^{2+}, FPP, and [1-^{14}C]IPP (Figure 5, panel a) show that $C_{30}PP$ is not bound to any form of the components. This implies that the complex A–B–$C_{30}PP$–Mg^{2+} must be dissociated into A–B and $C_{30}PP$–Mg^{2+} every time upon turnover of catalysis. Probably, A–B is rapidly or simultaneously dissociated into A and B which are ready to associate again with FPP to give A–B–FPP–Mg^{2+}. The dissociation of A–B might be essential to release $C_{30}PP$–Mg^{2+} from A–B–$C_{30}PP$–Mg^{2+}. The $C_{30}PP$–Mg^{2+} dissociated from the enzyme will form large micellar aggregates, as indicated by the elution volume by Superose 12 (Figure 5). This must be a crucial point of the reaction mechanism by which the catalytic reaction yielding insoluble material can turn over without aid of detergent.

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Bovine Brain Contains Two Types of Phosphatidylinositol Kinase†

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ABSTRACT: Two phosphatidylinositol (PI) kinases from bovine brain were separated by rate zonal sucrose gradient centrifugation of detergent-solubilized membranes. Of the total PI kinase activity, 43% migrates on sucrose gradients with a size of approximately 55 kilodaltons (kDa); this kinase has properties similar to one of two PI kinase activities characterized in fibroblasts [Whitman, M., Kaplan, D. R., Roberts, T., & Cantley, L. (1987) *Biochem. J.* (in press)] and has been termed type 2. The remainder of the activity migrates in a second peak with a size of approximately 230 kDa. This enzyme possesses properties which are unlike both fibroblast PI kinase activities and has been termed type 3. The type 2 and type 3 enzymes have very different affinities for adenine nucleotides and are readily distinguishable by their sensitivities to inhibition by adenosine. The K_Ms of types 2 and 3 kinases for ATP are 54 and 742 μM, and the K_is for adenosine are 18 and 1520 μM, respectively. The two enzymes also differ in their affinities for PI, phosphatidylinositol 4-phosphate, and Mg^{2+} as well as in stimulation and inhibition by other phospholipids. When PI kinase from erythrocyte ghosts is fractionated by sucrose gradient centrifugation, only one peak of activity is observed which is indistinguishable from brain type 2 PI kinase.

The stimulated metabolism of membrane inositol phospholipids is among the first responses of cells to a variety of neurotransmitters, growth factors, and hormones (Berridge, 1984). It is thought that the initial response to ligand/receptor interaction is the activation of a specific phospholipase C which hydrolyzes phosphatidylinositol 4-phosphate (PIP)[1] and PIP_2

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[1] Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; kDa, kilodalton(s); Con A, concanavalin A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TLC, thin-layer chromatography.

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present in the membrane and that increased turnover of PI occurs subsequently as PI is sequentially phosphorylated by PI and PIP kinases in order to maintain levels of PIP and PIP_2 (Hokin, 1985). The products of phosphoinositide hydrolysis by phospholipase C include the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. DAG has been shown to mediate many cellular responses including promotion of growth by activating protein kinase C (Nishizuka, 1984), and inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+} (Berridge & Irvine, 1984).

In addition to regulation of PI turnover at the level of phospholipase C, it has been proposed that regulation of PI and PIP phosphorylation is also important in determining the magnitude of the cellular response (Sugimoto et al., 1984; Whitman et al., 1985; Macara et al., 1984; Taylor et al., 1984; Martin et al., 1986; Chahwala et al., 1987). Upon stimulation of cells by a variety of ligands, PIP and PIP_2 levels decrease initially. However, when the concentrations of these phospholipids have been followed with time, resynthesis of PIP and PIP_2 to levels equal to or above the starting concentrations has been observed within a few minutes of the addition of stimulus in hepatocytes (Creba et al., 1983), fibroblasts (Chu et al., 1985), thymocytes (Taylor et al., 1984), and neutrophils (Della Bianca et al., 1986). Furthermore, following stimulation of neutrophils with fMet-Leu-Phe (Della Bianca et al., 1986), or thymocytes with Con A (Taylor et al., 1984), the ratio of PIP to PI has been shown to steadily increase within 1 min. It has also been demonstrated that in rat pituitary cells (Martin et al., 1986; Straub & Gershengorn, 1986) and in fibroblasts (Chawala et al., 1987), the presence of ATP is necessary for maximal release of inositol phosphates, presumably to allow continued synthesis of PIP and PIP_2. It is therefore likely that the activities of PI and PIP kinases are modulated during the stimulation of phosphoinositide turnover, in order to maintain and in some cases increase the pools of PIP and PIP_2, but it is not clear whether this modulation is due to positive regulation of the enzymes or to release from feedback inhibition. Although PI kinases have been described in many tissues and species (Colodzin & Kennedy, 1965; Kai et al., 1966; Harwood & Hawthorne, 1969; Bostwick & Eichberg, 1981; Gould et al., 1983; Kurosawa & Parker, 1986; O'Shea et al., 1986), none has been purified, and very little is known about their regulation. We now report the separation of two PI kinases from bovine brain which are distinct in both physical and kinetic properties. These properties are discussed in the context of the regulation of PI turnover in response to stimulation of cells.

Experimental Procedures

Materials. PI (bovine liver), phosphatidylcholine (bovine brain and 95% egg lecithin), phosphatidylethanolamine (bovine liver), and phosphatidylserine (bovine brain) were obtained from Avanti Polar Lipids. PIP, PIP_2, DAG, Triton X-100, aprotinin, leupeptin, phenylmethanesulfonyl fluoride, dithiothreitol, ATP, ADP, AMP, DEAE-Sephacel, and cholic acid were from Sigma. Cholic acid was recrystallized 3 times from 70% ethanol and prepared as a 10% solution of the sodium salt, pH 8. Molecular weight standards for chromatography were also obtained from Sigma: bovine serum albumin (66K), β-amylase (200K), and apoferritin (443K). Ammonium vanadate (Fisher) was prepared by heating a 20 mM solution at 90 °C for 30 min and immediately adjusting the pH to 8 with NaOH. Adenosine was obtained from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP was from New England Nuclear (3000 Ci/mmol). Ultrogel AcA34 was from LKB.

Preparation and Solubilization of Brain Membranes. All connective tissue and blood vessels were dissected out of bovine brain cortex, which was then homogenized in 5 volumes of buffer A [50 mM Tris, pH 8, 5% sucrose, 10 mM $MgCl_2$, 1 mM dithiothreitol, and a mixture of protease/phosphatase inhibitors (1 mM EDTA, 1 mg/L soybean and lima bean trypsin inhibitors, 10 mg/L aprotinin, 0.5 mg/L leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, and 0.2 mM vanadate)]. Following centrifugation of the homogenate (50000*g*, 30 min), the pellet was resuspended and recentrifuged sequentially in low-salt buffer (10 mM Tris, pH 8, plus protease/phosphatase inhibitors) followed by high-salt buffer (buffer A + 1 M NaCl). The pellet was resuspended in buffer A containing 0.5% cholate, incubated at 4 °C for 1 h, and centrifuged at 100000*g* for 1 h. The solubilized extract (approximately 5 mg of protein/mL) was either used immediately or frozen in a dry ice/ethanol mixture for storage at −70 °C. Some extracts were purified 3-fold by precipitation with ammonium sulfate at a final concentration of 35%. Pellets were resuspended in buffer A containing 10% glycerol and no sucrose by sonication in a bath sonicator for 1 min.

Preparation and Solubilization of Erythrocyte Ghosts. Erythrocyte ghosts were prepared from fresh human blood as described previously (Fairbanks et al., 1971). The lysis buffer contained 5 mM sodium phosphate, pH 8, 1 mM dithiothreitol, and protease/phosphatase inhibitors. Ghosts were washed with lysis buffer containing 1 M NaCl, resuspended in 5 volumes of buffer A containing 0.5% cholate and 0.1 M NaCl, incubated at 4 °C for 30 min, and centrifuged at 25000*g* for 30 min. The supernatant was concentrated 5-fold by centrifugation in 30 000 molecular weight cutoff microconcentrators (Amicon) to a final protein concentration of 1 mg/mL. Protein was measured by the method of Lowry (Lowry et al., 1951) after removal of interfering substances (Bensadoun & Weinstein, 1976).

Sucrose Gradient Centrifugation. A discontinuous sucrose gradient was formed from 4 mL of 10% sucrose, 3 mL of 20% sucrose, and 4 mL of 30% sucrose in buffer B (50 mM Tris, pH 8, 0.1 M NaCl, 1 mM dithiothreitol, protease/phosphatase inhibitors, 0.5% cholate, and 0.5 mg/mL phosphatidylcholine); 0.5-mL samples of detergent extracts of brain membranes (or a 35% ammonium sulfate cut) or erythrocyte ghosts were layered on top of the 10% sucrose, and the gradients were centrifuged at 41 000 rpm for 24 h at 4 °C in an SW41 Ti rotor (Beckman); 1-mL fractions were removed and numbered starting from the top of the tube.

Assay of PI Kinase Activity. PI kinase was assayed in 20 μL of a reaction mix containing (unless otherwise indicated) 250 μg/mL PI, 50 mM Hepes, pH 7.1, 1 mM EGTA, 1 mM sodium phosphate, 0.1% Triton X-100 [phospholipids were dried with N_2 and sonicated for 10 min in a bath sonicator (Heat Systems-Ultrasonics) together with buffer and detergent], [γ-^{32}P]ATP (0.5 mM and 400 dpm/pmol), and 10 mM $MgCl_2$. The enzyme was diluted to $\leq$200 μg of protein/mL with buffer D (50 mM Tris, pH 8, 20% glycerol, 1 mM dithiothreitol, protease/phosphatase inhibitors, and 0.1% Triton X-100), and 5 μL of this dilution was included in the 20-μL assay mixture. Reactions were carried out at room temperature for 15 min and stopped by addition of 75 μL of 1 N HCl. (The production of PIP was linear for at least 30 min under these conditions.) One hundred fifty microliters of chloroform/methanol (1:1) was added, tubes were mixed 30 s, and 50 μL of the lower layer was washed with 50 μL of methanol/1 N HCl (1:1). Thirty microliters of the washed lower layer was analyzed by thin-layer chromatography as described (Sugimoto et al., 1984). Foil-backed silica gel 60 plates

(Merck) were coated with a solution of 1% potassium oxalate and were activated at 100 °C for 1 h before use. Plates were developed in chloroform (60)/methanol (47)/ammonia (2)/H_2O (11). The radioactive reaction product was located by autoradiography, cut from the plate, and quantitated by liquid scintillation counting. Internal standards of PIP which could be visualized with iodine were used to verify the identity of the radioactive reaction product.

The affinities of types 2 and 3 PI kinases for adenine nucleotides were determined by measuring the reaction rate under linear conditions at several concentrations of ATP and several concentrations of the inhibitors. For these determinations, 500 μg/mL phosphatidylcholine was also included in the reaction mixture.[2] K_Ms and V_Ms were calculated by fitting the data to the equation $V = V_M[ATP]/([ATP] + K_M)$ by using a nonlinear least-squares fitting procedure. K_is were determined by fitting pooled K_Ms from two or three experiments to the equation $K_M(\text{apparent}) = (K_M + [\text{inhibitor}])(K_M/K_i)$ by linear regression. The correlation coefficients for these lines were between 0.85 and 0.95.

Phosphorylation of Types 2 and 3 PI Kinase Reaction Products by PIP Kinase. The standard PI kinase assay was used to prepare [^{32}P]PIP using both types 2 and 3 PI kinases. Samples were prepared in parallel using unlabeled ATP; 1 μg of PIP was added as a carrier after the reactions were stopped. PIP was scraped from the TLC plates and eluted from the silica gel in chloroform/methanol/0.1% phosphoric acid (6:4.7:1.3). The unlabeled extract from the type 2 kinase reaction mixture was added to the ^{32}P-labeled extract from the type 3 kinase reaction mixture, and conversely, so that the composition of the two extracts was identical except for the [^{32}P]PIP. The dried lipids were resuspended in H_2O and neutralized with NaOH, buffer was added (final concentrations 100 mM Hepes, pH 7.1, 2 mM EGTA, 2 mM sodium phosphate, and 0.01% Triton X-100), and the mixture was sonicated. ATP and $MgCl_2$ were added to final concentrations of 0.5 and 20 mM, respectively. PIP kinase (3 μg, 9 nmol min^{-1} mg^{-1} at 80 μg/mL PIP) was added, and the mixture was incubated for 30 min (final volume 50 μL). PIP kinase was purified 4260-fold from red blood cells (L. Ling, J. Schultz, and L. C. Cantley, unpublished results). PI was phosphorylated by this enzyme at a rate less than 5% that of PIP phosphorylation. PIP_2 was resolved from PIP by TLC as described above.

Results

Separation of Two PI Kinases from Bovine Brain. PI kinase activity from bovine brain was associated almost exclusively with particulate matter. The enzyme remained in the 50000*g* pellet through low- and high- (1 M NaCl) salt washes; 80% of the activity was solubilized from the membranes by 0.5% cholate or 0.1% Triton X-100. The specific activity of PI kinase in the extract was approximately 1 nmol min^{-1} (mg of protein)$^{-1}$; the activity was enriched 3-fold by ammonium sulfate fractionation. Rate zonal sucrose gradient centrifugation of the ammonium sulfate cut resolved two peaks of PI kinase activity which migrated with estimated sizes of 55 and 230 kDa (Figure 1A). (These sizes are approximations which are intended only for comparative purposes.) The activity from

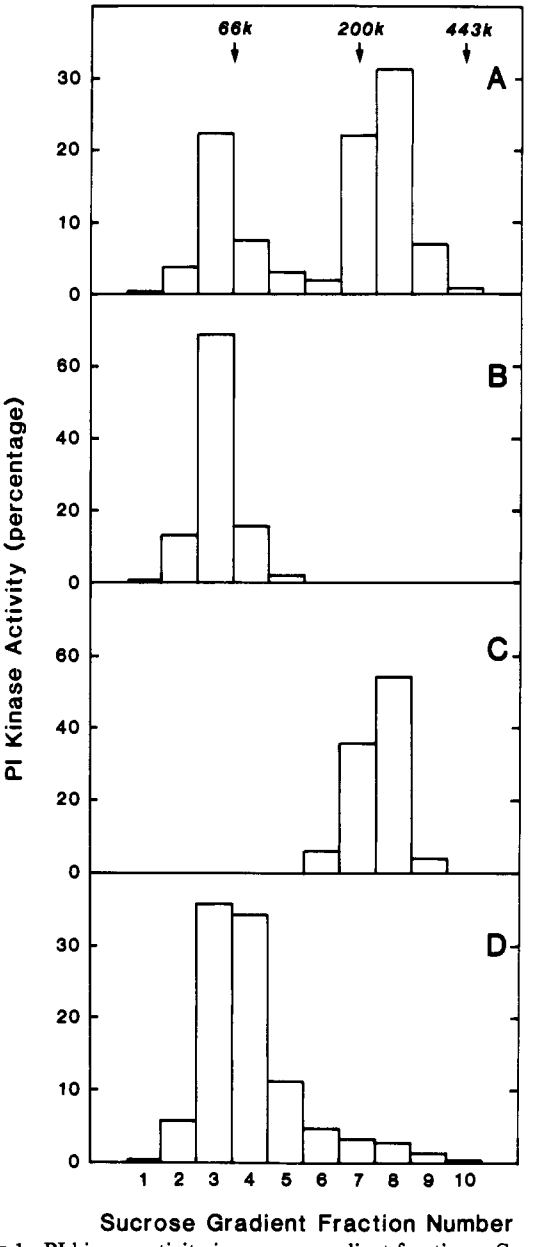

FIGURE 1: PI kinase activity in sucrose gradient fractions. Samples were fractionated by rate zonal centrifugation on 10–30% sucrose gradients containing cholate + phosphatidylcholine. Fraction 1 corresponds to the top of the gradient. (A) Cholate extract of brain membranes. The arrows indicate the migration positions of molecular weight markers: bovine serum albumin (66K), β-amylase (200K), apoferritin (443K). (B) Fraction 3 from the gradient pictured in (A) was rerun on a second identical gradient. (C) Fraction 8 from the gradient pictured in (A) was rerun on a second identical gradient. (D) Cholate extract of erythrocyte ghosts fractionated on an identical sucrose gradient.

either peak migrated with the same mobility when recentrifuged under identical conditions (Figure 1B,C). Approximately 20% of the activity loaded on the gradients was recovered after centrifugation, of which the lower mobility form and the higher mobility form constituted 43% ± 2% and 57% ± 2% (mean ± SEM, $n = 6$) of the activity, respectively, in six sucrose gradient preparations using several different membrane extracts. The distribution of the two kinases was independent of the type of detergent (cholate or Triton X-100) used for the initial extract or the presence of either detergent in the gradients.

Kinetic Characterization of the Two PI Kinases. We have designated the lower mobility PI kinase type 2, on the basis of it possessing similar properties to one of two activities re-

[2] Under standard assay conditions, the affinity of type 2 kinase for ATP was dependent on the concentration of PI if no other lipid was present. When phosphatidylcholine, but not phosphatidylserine or phosphatidylethanolamine, was included in the assay, PI no longer had this effect. Therefore, all kinetic analyses were carried out in the presence of phosphatidylcholine.

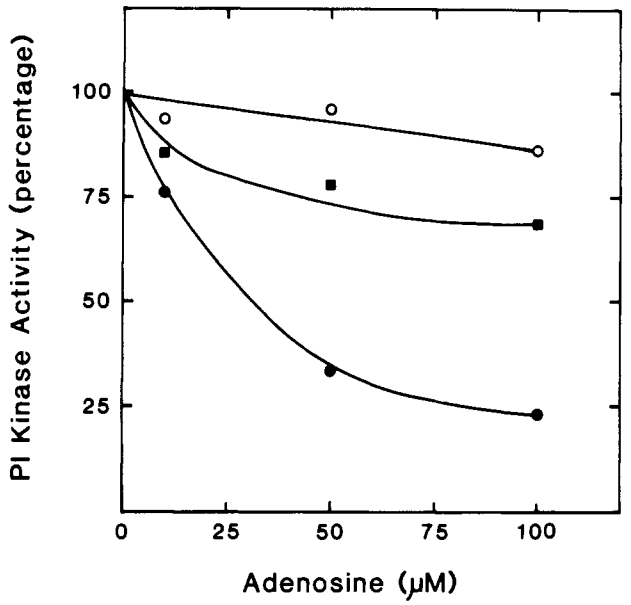

FIGURE 2: Inhibition of PI kinases by adenosine. PI kinase activity in a cholate extract of brain membranes (■), sucrose gradient fractions 3 + 4 (type 2) (●), or sucrose gradient fractions 8 + 9 (type 3) (○) were assayed in the presence of increasing concentrations of adenosine, with 25 μM ATP. These data are representative of several determinations at the same and other ATP concentrations.

Table I: Affinities of Brain and Erythrocyte Ghost PI Kinases for Substrates and Inhibitors

	erythrocyte ghost	brain type 2	brain type 3
K_M(ATP)[a] (μM)	44 (2)	54 ± 3 (8)	742 ± 50 (6)
K_i(adenosine)[b] (μM)	10	18	1520
K_i(ADP)[b] (μM)		27	570
K_i(AMP)[b] (μM)		240	2820
$K_M(Mg^{2+})$[c] (mM)		0.6	2.6
K_M(PI)[c] (μM)		17 (15 μg/mL)	127 (112 μg/mL)
K_i(apparent)-(PIP)[c,d] (μM)		243 (235 μg/mL)	774 (747 μg/mL)

[a] Mean ± SEM (n); the K_M(ATP) for types 2 and 3 PI kinases differ significantly, $p < 0.001$. [b] Values are obtained by pooling data from two to three experiments (see inserts to Figure 3 and Experimental Procedures). [c] Values are the average from two to three experiments. [d] The apparent K_i for PIP was determined at a PI concentration of 200 μg/mL.

cently identified in NIH 3T3 fibroblasts (Whitman et al., 1987). The higher mobility form has been designated type 3, on the basis of its dissimilarity from any previously described activity.

The two PI kinases were affected very differently by adenosine, a previously identified inhibitor of PI kinase (Buckley, 1977; Doctrow & Lowenstein, 1985). Type 2 PI kinase was inhibited by adenosine with high affinity and type 3 with low affinity (Figure 2). Inhibition by adenosine of PI kinase activity in an unfractionated extract of brain membranes consisted of both high- and low-affinity components (Figure 2), consistent with it containing approximately 43% type 2 and 57% type 3 kinase as indicated in Figure 1A.

The affinities of types 2 and 3 PI kinases for ATP were determined by mesuring the reaction rate under linear conditions at several concentrations of ATP[2] (see Experimental Procedures). The K_Ms of types 2 and 3 PI kinases for ATP are 54 and 742 μM, respectively (Figure 3 and Table I). Since the reaction rates for both kinases were linear at the lowest concentration of ATP, the difference in K_Ms is not due to the

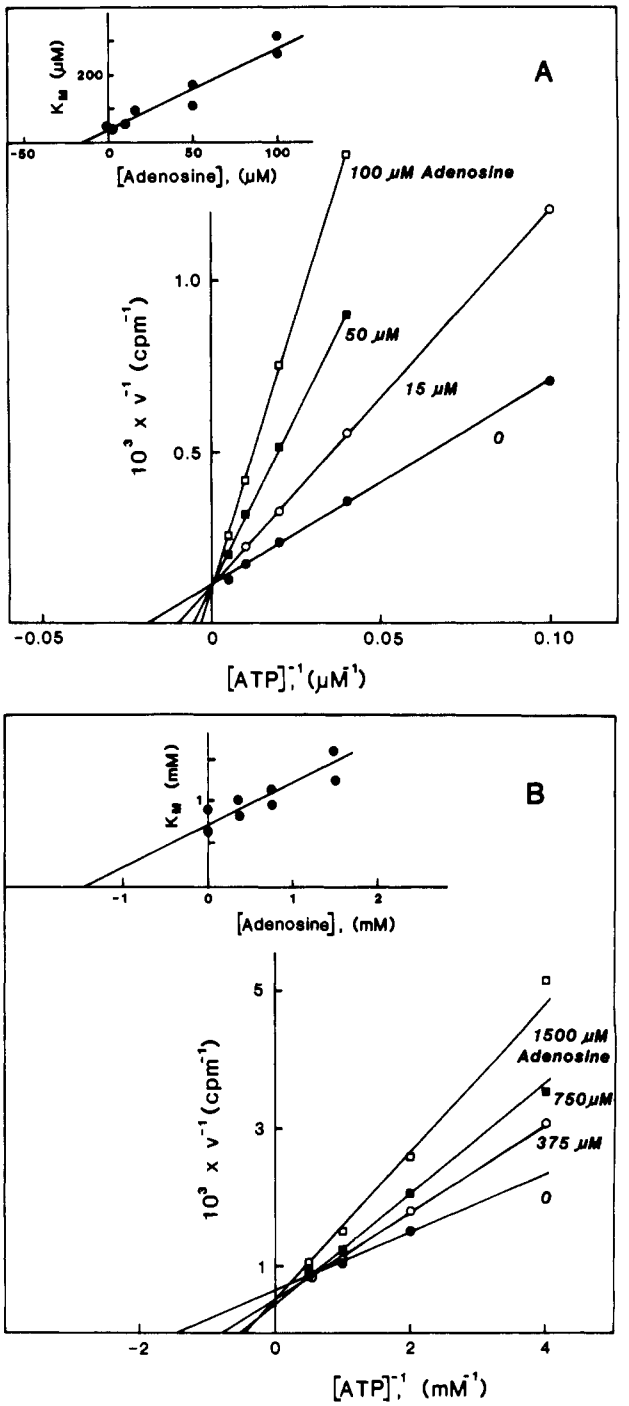

FIGURE 3: Inhibition of types 2 and 3 PI kinase by adenosine. Type 2 (A) and type 3 (B) PI kinases were measured under linear conditions at several concentrations of ATP for each of several concentrations of adenosine. A Lineweaver–Burk plot of a representative experiment is shown for each case. Data were used to calculate K_Ms as described under Experimental Procedures. (A) [adenosine] = (●) 0, (○) 15, (■) 50, or (□) 100 μM. (B) [adenosine] = (●) 0, (○) 375, (■) 750, or (□) 1500 μM. (Inserts A and B) Data from two or three experiments were pooled and used to calculate K_is as described under Experimental Procedures.

differential depletion of ATP by ATPases. Further, the radioactive reaction product [^{32}P]PIP was found to be stable to a chase with excess unlabeled ATP under standard assay conditions (data not shown). This indicates that the activities of PIP kinase, PIP phosphomonoesterase, and PIP phosphodiesterase are insignificant under these conditions and are not artifactually lowering the apparent PI kinase activity.

Inhibition by adenosine, ADP, and AMP was assessed by measuring enzymatic activity at several concentrations of ATP

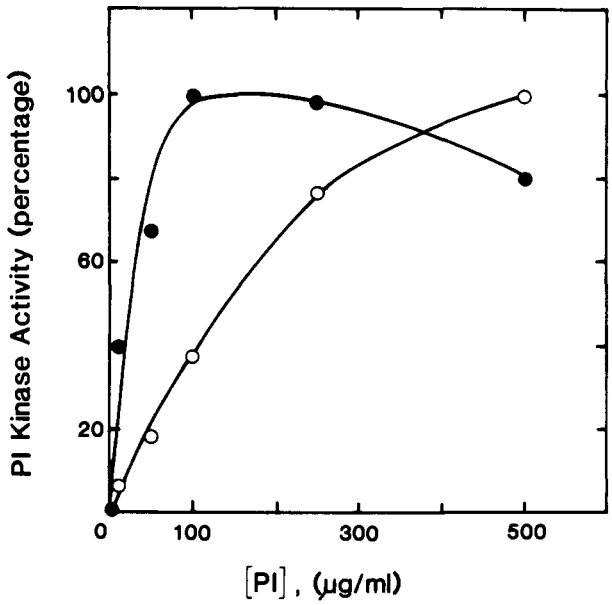

FIGURE 4: Affinities of types 2 and 3 PI kinases for PI. Type 2 PI kinase (●) and type 3 PI kinase (○) were assayed with various concentrations of PI as described under Experimental Procedures.

and inhibitors. Double-reciprocal plots of the data demonstrate that inhibition of type 2 PI kinase by adenosine is competitive with ATP (K_i = 18 μM; Figure 3A). Adenosine inhibits type 3 PI kinase weakly (K_i = 1520 μM). This inhibition is primarily competitive with ATP, although the failure of the double-reciprocal plots to intersect precisely at the y axis suggests some mixed inhibition (Figure 3B). ADP and AMP (data not shown) also inhibited both kinases competitively with ATP, although as the intersections of the double-reciprocal plots deviate slightly from the y axis, some mixed inhibition is possible. The inhibition constants for these compounds derived from the effects on the apparent K_M for ATP (see Experimental Procedures) are summarized in Table I.

Types 2 and 3 PI kinases also differ in their affinities for Mg^{2+}, PI, and PIP, and in stimulation or inhibition by various phospholipids and detergents. K_Ms for Mg^{2+} are 0.6 and 2.6 mM for types 2 and 3 kinases, respectively (Table I). Type 2 PI kinase has a significantly higher affinity for PI (Figure 4) and is more sensitive to inhibition by PIP (Figure 5A) and phosphatidylserine (Figure 5B) compared to type 3 kinase. In contrast, phosphatidylcholine inhibits type 3 kinase more strongly than type 2 kinase (Figure 5D). Both kinases are maximally active in 0.1–0.5% Triton X-100; however, only type 3 kinase is active in 0.1% cholate (data not shown).

Physical Characterization of the Two PI Kinases. Two PI kinases were resolved with Ultrogel AcA34, a gel filtration medium which is also a strong cation exchanger. Type 3 PI kinase eluted with an apparent molecular size above the linear range, between 350 and 750 kDa. The elution position of type 2 kinase was dependent on pH, salt, and detergent conditions. Its apparent molecular size was 25 kDa in 1% Triton X-100/50 mM Tris, pH 8, but was 150 kDa in the same buffer plus 0.5 M NaCl. The identity of the two kinases was assessed by their sensitivity to inhibition by adenosine; the inhibition curves were indistinguishable from those shown in Figure 2 (data not shown).

Type 2 PI kinase could be resolved from type 3 by anion-exchange chromatography on DEAE-Sephacel in 1% Triton X-100 (50 mM Tris, pH 7.8). Under these conditions, type 2 activity (assessed by sensitivity to inhibition by adenosine) did not bind to the gel and could be recovered in the flow-through and wash fractions, while type 3 activity eluted at 0.2 M NaCl (data not shown).

Specificity of Types 2 and 3 PI Kinases. We assessed the ability of PIP made by types 2 and 3 PI kinase to serve as substrate for a highly purified PIP kinase from red blood cell ghosts (L. Ling, J. Schultz, and L. Cantley, unpublished results). After isolation from reaction mixtures, [^{32}P]PIP made by types 2 and 3 PI kinase was converted to [^{32}P]PIP_2 with efficiencies of 13.1% and 19.7%, respectively (values are averages from two separate experiments). This conversion was dependent on the addition of PIP kinase. In view of the five possible phosphorylation sites on PI, the fact that the reaction products of both PI kinases are good substrates for PIP kinase argues against a nonspecific lipid kinase being responsible for either activity.

We further investigated the possibility that one type of PI kinase was due to a lipid kinase of broad specificity. The other lipid kinases are PIP kinase and DAG kinase; in sucrose gradient fractions containing type 2 PI kinase, the activities of the three lipid kinases are approximately equal (at equal substrate concentrations under the assay conditions described for PI kinase). However, in a more purified preparation of type 2 PI kinase (40 nmol min^{-1} mg^{-1}), no phosphorylation of PIP or DAG is observed. In sucrose gradient fractions containing type 3 PI kinase, DAG and PIP kinase activities are only 8% and 14% of PI kinase activity, respectively, at equal substrate concentrations. Thus, it appears unlikely that either kinase activity results from a lipid kinase whose physiological substrate is not PI.

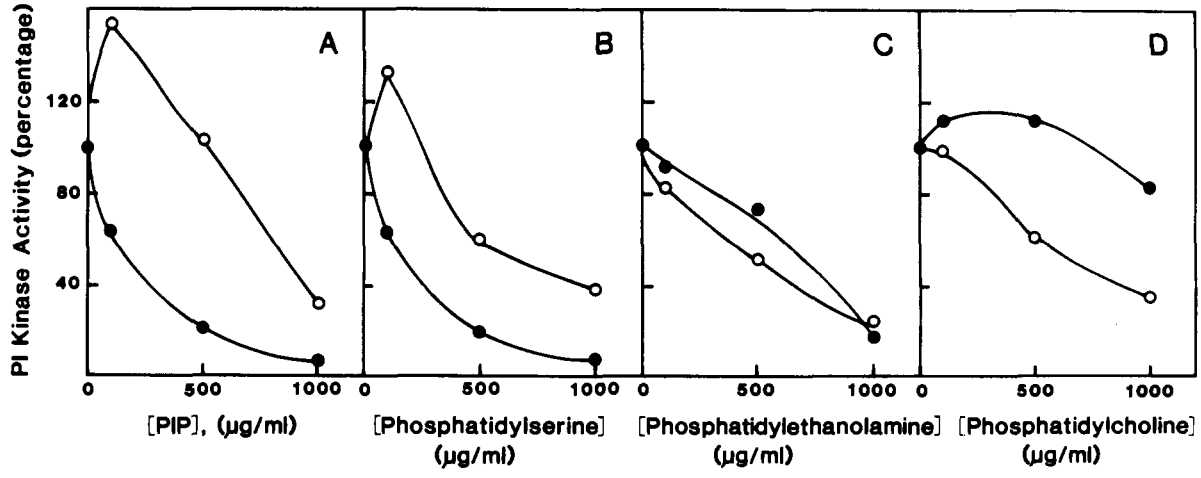

FIGURE 5: Effects of phospholipids on types 2 and 3 PI kinases. Type 2 PI kinase (●) and type 3 PI kinase (○) were assayed with 200 μg/mL PI in the presence of various phospholipids as indicated.

Similarity of Erythrocyte Ghost PI Kinase to Brain Type 2 PI Kinase. PI kinase in erythrocyte ghosts was not removed by low- or high-salt extractions but was effectively solubilized by 0.5% cholate. When a cholate extract of ghosts was fractionated by sucrose density gradient centrifugation, all of the recovered PI kinase activity migrated with a mobility comparable to the lower mobility (type 2) activity from brain. This enzyme has a K_M for ATP of 44 μM and is inhibited by adenosine with a K_i of 10 μM (Table I), also similar to brain type 2 PI kinase.

Discussion

In this paper, we describe the separation and characterization of two PI kinases from bovine brain. These enzymes can be physically separated by centrifugation on sucrose gradients, by anion-exchange chromatography in Triton X-100, or with Ultrogel AcA34 and can be distinguished biochemically by their affinities for a variety of substrates and inhibitors. Rat brain also possesses activities which are similar to bovine brain types 2 and 3 PI kinases (data not shown), while human erythrocyte ghosts contain only the type 2 activity. Two PI kinases with distinct biochemical properties have also been identified in extracts of murine (NIH 3T3, BALB 3T3) and rat (F 111) fibroblasts by others in this laboratory (Whitman et al., 1987). One of these enzymes, type 2, appears to be the same as the lower mobility species we have identified when brain and human erythrocyte ghost extracts are fractionated by density gradient centrifugation. These enzymes have very similar K_Ms for ATP, are inhibited with high affinity by adenosine and ADP, and are dependent on nonionic detergent for activity. We present evidence that brain type 3 PI kinase is distinct from the types 1 and 2 PI kinases described in fibroblast lines. Although both brain type 3 and fibroblast type 1 PI kinases are inhibited by adenosine wih low affinity, they differ greatly in their K_Ms for ATP (742 vs 10 μM). In addition, both type 2 and type 3 PI kinases are active in nonionic detergent, unlike type 1 kinase from fibroblasts. This preliminary characterization of PI kinases from various tissues and species suggests that the type 2 enzyme is rather ubiquitous while the types 1 and 3 kinases are expressed in a tissue-specific manner.

Types 2 and 3 PI kinases differ in regulation by substrate availability, product inhibition, and phospholipid environment. The two enzymes have different affinities for the cofactor Mg^{2+}, but both are suboptimally active at the cellular free Mg^{2+} concentration of 0.37 mM (Corkey et al., 1986). Type 3 kinase requires a higher concentration of PI for maximal activity than does type 2 kinase, although the physiological relevance of this parameter in an assay containing mixed micelles of phospholipid and detergent is unclear. PI constitutes about 6% of cellular phospholipids (Schroeder et al., 1976). Type 2 kinase is inhibited 50% when the PI:PIP ratio is 1:1, while type 3 kinase is poorly inhibited by PIP. Since the ratio of PI to PIP is approximately 40:1 in fibroblasts (Fleischman et al., 1986), it is unlikely that the increase in phosphorylation of PI following stimulation of PIP_2 breakdown is due to release from feedback inhibition by PIP. Other phospholipids modulate the activity of PI kinases as well. Phosphatidylserine inhibits type 2 kinase with high affinity. Phosphatidylcholine slightly stimulates type 2 kinase at low concentrations and inhibits type 3 kinase at higher concentrations. Thus, the activity of these enzymes could be affected by the phospholipid composition of the membrane, which varies among membranes of different organelles and can be modulated by the availability of head groups and fatty acids for phospholipid synthesis (Schroeder et al., 1976).

Since the K_is for ADP are lower than the K_Ms for ATP, both types 2 and 3 PI kinases are potentially subject to regulation by the energy charge of the cell. Neither kinase would be inhibited at the normal intracellular ATP:ADP ratio (60:1) (Veech et al., 1979). The overall energy charge would have to decrease dramatically to inhibit either kinase significantly; however, it has been suggested that the energy charge is not uniform across the cell (Aw & Jones, 1985). If a gradient exists between sites of ATP synthesis and utilization (such as the plasma membrane), the subcellular location of the kinase may determine whether it is subject to regulation by the energy charge of the cell.

The two PI kinases from bovine brain can be distinguished by their sensitivity to inhibition by adenosine. High-affinity inhibition by adenosine has been reported for PI kinases from erythrocyte ghosts (Buckley, 1977) and aortic smooth muscle (Doctrow & Lowenstein, 1985). These enzymes appear to have K_is for adenosine which are similar to those for brain type 2 PI kinase, while type 3 kinase is much less sensitive to inhibition by adenosine. Adenosine is thought to have a physiological role as a vasodilator (Berne et al., 1979). Since vascular smooth muscle contraction is associated with increased turnover of inositol phospholipids, it has been suggested that inhibition of PI kinase may contribute to the vasodilator activity of adenosine (Doctrow & Lowenstein, 1985). The intracellular level of adenosine is normally $\sim$10 μM; it can reach 100 μM during anoxic conditions (Doctrow & Lowenstein, 1985). Inhibition by adenosine is competitive with ATP, and at physiological levels of ATP, 100 μM adenosine would have little effect on either kinase. However, if the ATP:ADP ratio also declined due to anoxia, inhibition of type 2 kinase by adenosine and ADP together could become important.

Both type 2 and 3 PI kinases remain associated with membranes through high- and low-salt washes and can be solubilized only with detergent. Thus, they must either be integral membrane proteins, be tightly associated with an integral membrane protein, or be fatty acylated. Type 2 kinase migrates on sucrose gradients with a size of approximately 55 kDa while type 3 kinase migrates with a size of approximately 230 kDa. The type of detergent used in the gradient (cholate or Triton X-100, which form micelles of 1.7 and 90 kDa, respectively; Hjelmeland & Chrambach, 1984) did not affect the migration of either kinase. This indicates that neither enzyme incorporates into a detergent micelle and suggests that neither is an integral membrane protein. Although the two kinases can be resolved with Ultrogel AcA34, the apparent molecular sizes obtained with this gel filtration medium vary with the salt, detergent, and pH, most likely because of ionic and hydrophobic interactions.

The large apparent size of the type 3 kinase raises the possibility that it is present in a complex of proteins. While it is possible that the type 3 kinase could be derived from the type 2 kinase, by formation of a homotetramer or a complex with another protein, it seems unlikely that formation of such a complex could result in the large differences between the two enzymes in affinities for the soluble substrate ATP and the inhibitors adenosine, ADP, and AMP. If such a complex is responsible for the large molecular weight of the type 3 activity, it must be quite stable since the size was not affected by repeated centrifugation on sucrose gradients in the presence of detergent (Figure 1C). It is also possible that the type 2 enzyme arises from proteolytic degradation of the type 3 enzyme. This seems unlikely as the type 2 PI kinase is present in red cells and fibroblasts, which have no detectable type 3 activity.

It has been reported that PI kinase activity from sarcoplasmic reticulum copurifies with phosphorylase kinase, a multisubunit enzyme of 415 kDa (Georgoussi & Heilmeyer, 1986). However, the specific activity of the PI kinase in the purified phosphorylase kinase is no higher than the activity in crude membranes (1 nmol min^{-1} mg^{-1}); it is likely that the two membrane proteins are interacting nonspecifically due to the absence of detergent in the purification procedures. It has also recently been reported that about 2% of A431 cell membrane PI kinase activity copurifies with the epidermal growth factor receptor through affinity chromatography on epidermal growth factor–resin or anti-epidermal growth factor receptor IgG–Sepharose (Thompson et al., 1985). To determine whether PI kinase in brain associates with a membrane glycoprotein, we measured binding to lectin–agarose gels (G. Endemann and L. Cantley, unpublished experiments). Of the PI kinase activity in a brain extract, 5% binds specifically to wheat germ lectin–agarose, suggesting that this activity may be associated with a glycoprotein. This activity possesses exactly the same K_M for ATP and resistance to inhibition by adenosine as type 3 PI kinase. However, the majority of the type 3 PI kinase failed to bind to the lectin, indicating that the high apparent molecular weight cannot be entirely accounted for by association with a wheat germ lectin binding glycoprotein.

The identification of two PI kinases in brain which are regulated differently raises several possibilities. One is that types 2 and 3 PI kinases could be derived from different cell types present in brain cortex. A second possibility is that the two kinases are involved in response to activation by different ligands. This could be due to their physical proximity to or association with distinct receptors, or because they are regulated by different second messengers. A third possibility is that one type of PI kinase contributes to stimulated turnover of PIP and PIP_2, and the other fulfills some "housekeeping" function. There appear to be biochemically distinct pools of PIP and PIP_2 which do not turn in response to stimulation of cells (Berridge, 1984; Chahwala et al., 1987). One type of PI kinase could be involved in maintaining those pools while the other maintained the pools associated with ligand-stimulated turnover. If this were the case, it would be expected that one type of PI kinase would be enriched in the plasma membrane, where ligand-stimulated turnover of phosphoinositides occurs. These possibilities are under investigation.

Previous studies on the regulation of brain PI kinase have considered the enzyme as a single species (Colodzin & Kennedy, 1965; Kai et al., 1966). The partial purification of PI kinase from brain myelin has recently been reported (Saltiel et al., 1987). This activity shows a biphasic dependence on PI as a substrate which is most likely due to the presence of both types 2 and 3 PI kinase. Determination of the roles for the two brain PI kinases in membrane signal transduction will require further purification of these activities and study of the regulation of these enzymes in response to various stimuli.

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Registry No. ATP, 56-65-5; Mg, 7439-95-4; ADP, 58-64-0; AMP, 61-19-8; PI kinase, 37205-54-2; adenosine, 58-61-7.

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Steady-State and Time-Resolved Spectroscopic Studies on the Hematoporphyrin–Lipoprotein Complex†

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ABSTRACT: The interaction of hematoporphyrin (Hp) with the isolated rabbit lipoprotein fractions very low density lipoproteins, low-density lipoproteins, and high-density lipoproteins has been studied by steady-state and time-resolved spectroscopy. The porphyrin appears to be bound to both the apoprotein and the lipid phase. The two populations of lipoprotein-bound Hp molecules can be distinguished on the basis of the fluorescence excitation spectrum, decay constants of the lowest excited singlet and triplet states, and accessibility to oxygen. Upon Hp binding, the intrinsic fluorescence emission of apolipoproteins is quenched at least in part via singlet–singlet energy transfer from tryptophyl residues to the porphyrin moiety. The binding of Hp with the protein matrix can be adequately described on the basis of Scatchard analysis, whereas the interaction of Hp with the lipid core can be described as the partitioning of the dye between a hydrophobic and an aqueous phase. The Hp binding capacity of lipoproteins is maximal for very low density lipoproteins.

Plasma lipoproteins serve as an important means of transport of lipids to cells. Lipid delivery occurs after internalization of the lipoprotein particles by cells; the process takes place by two parallel compartmented routes: (i) receptor-mediated endocytosis via coated pits/coated vesicles which are degraded at the lysosome level and (ii) trapping in plasmalemmal vesicles which are taken up through the invagination of noncoated regions of cell membrane (Goldstein et al., 1979; Chapman, 1980). The receptor-mediated pathway is especially active for cells characterized by a high mitotic index, where the need for an extra supply of cholesterol and phospholipids for membrane synthesis induces the formation of a large number of low-density lipoprotein (LDL)[1] receptors on the cell surface (Devon et al., 1981; Slater et al., 1984).

These observations suggested the use of LDL as carriers of cytostatic drugs to tumor cells in vivo (Norata et al., 1984). Recently, we showed that lipoproteins play a major role in the transport of hematoporphyrin (Hp) in the bloodstream (Jori et al., 1984a) and the delivery of porphyrins to tumors in experimental animals (Jori, 1985). The specific localization of some porphyrins in neoplastic tissues is the basis of a novel modality of tumor treatment, termed photodynamic therapy (Dougherty, 1984). Significant amounts of porphyrins are also accumulated by other tissues showing cell hyperproliferation, including atheromatous plaques (Spears et al., 1983), psoriatic areas (Berns et al., 1984), and metastatic abscesses (Venezio et al., 1985).

Therefore, it appears of interest to characterize the complexes of Hp with the most important classes of serum lipoproteins, namely, VLDL, LDL, and HDL. We have studied the Hp–lipoprotein system by means of steady-state and time-resolved emission spectroscopy, which is very sensitive to the nature of the Hp microenvironment (Jori & Spikes, 1984). Our studies have been centered on those lipoprotein fractions which can be isolated by standard sequential density flotation; hence, they are not intended to define the detailed binding mode of Hp with specific sites; rather, they describe the properties of the complexes between Hp and systems, though heterogeneous, which are important for its transport and tissue delivery in vivo.

Materials and Methods

Hematoporphyrin. Hp was purchased from Porphyrin Products (Logan, UT). Analysis of the sample by high-pressure liquid chromatography showed the presence of about 12% impurities including protoporphyrin IX (3–4%), [(hydroxyethyl)vinyl]deuteroporphyrin IX (5%), and some highly aggregated material.

The concentration of Hp aqueous solutions was determined spectrophotometrically using the molar extinction coefficient $\epsilon = 4.23 \times 10^5\ M^{-1}\ cm^{-1}$ at 401 nm in 1 M HCl (Marks, 1969).

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[1] Abbreviations: LDL, low-density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL, high-density lipoprotein(s); Hp, hematoporphyrin IX; SDS, sodium dodecyl sulfate; HSA, human serum albumin.

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