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MODULATION OF THE ACTIVITY OF PURIFIED PHOSPHATIDYLINOSITOL 4-PHOSPHATE KINASE BY PHOSPHORYLATED AND DEPHOSPHORYLATED B-50 PROTEIN

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Summary: To investigate the modulation of phosphatidylinositol 4-phosphate kinase activity by the degree of phosphorylation of the B-50 protein, the enzyme was purified from rat brain cytosol by ammonium sulphate precipitation and DEAE-cellulose column chromatography. Purified rat brain B-50 was phosphorylated with protein kinase C and dephosphorylated with alkaline phosphatase. Incubation of the semi-purified phosphatidylinositol 4-phosphate kinase with 1 µg of the B-50 preparation enriched in the dephospho-form, resulted in a small reduction of phosphatidylinositol 4-phosphate kinase activity (-16%), whereas incubation with the phospho B-50 preparation inhibited the enzyme activity by 40%. The effect of exogenous B-50 was studied in the presence of 10 µg albumin to minimize aspecific protein-protein interactions. The present data on the effect of exogenous B-50 protein on phosphatidylinositol 4-phosphate kinase activity, further support our hypothesis that the phosphorylation state of B-50 may be a regulatory factor in phosphoinositide metabolism in rat brain.

Phosphorylation of membrane components is thought to be of importance to the function of nerve membranes (1). Both the phosphorylation of proteins and of a special class of phospholipids, the (poly)phosphoinositides, are implicated in the regulation of selective neuronal membrane permeability and chemical neurotransmission (1,2). In fact there is increasing evidence that the phosphorylation of certain proteins is closely linked to the turnover of phosphoinositides (3-5).

We have previously shown in rat brain synaptic plasma membranes (SPM) that ACTH inhibits the phosphorylation of a brain-specific protein (B-50; M_r 48 kDa, IEP 4.5) (6,7), and simultaneously stimulates the formation of phosphatidylinositol 4,5-bisphosphate (PIP_2) from phosphatidylinositol 4-

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phosphate (PIP) (4,8). Jolles et al. (4) demonstrated that in a semi-purified B-50/B-50 kinase fraction from rat brain, the extent of phosphorylation of B-50 protein was inversely correlated with the amount of labeled phosphate incorporated into PIP_2. Similarly, incubation of SPM with affinity-purified antibodies directed against B-50 resulted in an inhibition of B-50 phosphorylation concomitantly with a stimulation of the labeling of PIP_2 (8). These findings led to the hypothesis that changes in the phosphorylation state of the B-50 protein may be a regulatory factor in the metabolism of polyphosphoinositides (9,10).

To test this hypothesis in a more direct manner the PIP kinase was purified from the cytosolic fraction of rat brain homogenate. Purified B-50 was phosphorylated or dephosphorylated and subsequently the effect of these phospho- and dephospho-B-50 preparations was tested on the activity of PIP kinase.

MATERIALS AND METHODS

Purification of PIP kinase

The purification of PIP kinase was performed as described by Van Dongen et al. (11). In short, a cytosolic fraction from the brains of 30 rats (male albino rats, 150-180 g, of an inbred Wistar strain, TNO, Zeist, NL) was prepared and subjected to ammonium sulphate precipitation (ASP). The protein fraction precipitating between 20 and 40% saturation was further purified by DEAE-cellulose column chromatography (see also 7). The proteins were eluted by stepwise enhancing the NaCl concentration (22.5 ml 50 mM, 22.5 ml 100 mM and 36 ml 150 mM NaCl), followed by a linear gradient (300 ml total volume) of 150 to 250 mM NaCl in buffer A (10 mM Tris-HCl, pH 7.4 and 0.1 mM dithiothreitol). Fractions of 4.5 ml were collected, dialyzed and assayed for PIP kinase activity. The fractions with maximal PIP kinase activity were combined and stored at $-20^{o}C$.

Isolation of B-50

B-50 protein from rat brain SPM was isolated by a procedure, involving alkaline extraction and ammonium sulphate precipitation (12). The ASP protein fraction was further purified by isoelectric focusing on a Sephadex IEF flat bed (for details see 8). The B-50-containing fractions were pooled and applied to a pre-packed Sephadex G-25 column (Pharmacia, PD-10) and eluted with 10 mM NH_4-formiate, pH 7.0. The void volume was collected, lyophilized and dissolved in 10 mM sodium acetate buffer, pH 6.5 (approx. 1 µg/µl) and dialyzed overnight against 1 l of the same buffer to remove trace amounts of ampholines. The purity of the isolated B-50 was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D gel electrophoresis (see below).

Phosphorylation of B-50

Phosphorylation of B-50 was performed according to Zwiers et al. (12). Five hundred µg of the isolated B-50 was incubated at $30^{o}C$ with 120 µg of a protein kinase C preparation isolated as described by Aloyo et al. (13) and

100 µM ATP in a phosphorylation buffer consisting of 10 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM calcium acetate, pH 6.5 (buffer B). Phosphatidylserine (PS) (a gift from Dr. B. Poorthuis, Dept. of Biochemistry, State University of Utrecht, NL) was present in a final concentration of 20 µg/ml (the final volume was 1 ml).

After 60 min 50 µl 40 mM EGTA was added to terminate the incubation and to prevent proteolysis of B-50. The incubate was applied to the flat bed of Sephadex IEF and separated as described above. The two most acidic B-50-containing fractions were pooled, separated from the ampholines as described under "Isolation of B-50". The effect of phosphorylation was assessed by 2D gel electrophoresis using a narrow pH gradient (12; see also Fig. 2).

Dephosphorylation of B-50

Dephosphorylation of B-50 was carried out according to Zwiers et al. (12). Five hundred µg of the isolated B-50 was incubated at 30°C with 500 µg alkaline phosphatase (E. coli, Worthington, USA) in 50 mM Tris-HCl, pH 8.0 (the final volume was 1 ml) (12). After 20 min 50 µl 40 mM EGTA was added to terminate the reaction. The total incubate was applied to the flat bed of Sephadex IEF and separated as described above. The two most basic B-50-containing fractions were pooled and separated from ampholines as described under "Isolation of B-50". The effect of dephosphorylation was assessed by 2D gel electrophoresis using a narrow pH gradient (12; see also Fig. 2).

Determination of PIP kinase activity

PIP kinase activity was assayed as described previously (11). In brief, DEAE-cellulose fractions (approx. 1 µg protein) were incubated with 48 µM PIP (Sigma Chemical Co., USA), added protein, 7.5 µM ATP and 1 µCi $|\gamma\text{-}^{32}P|$-ATP in buffer B (the final volume was 25 µl). These conditions allowed linear incorporation of labeled phosphate into PIP_2 and the reaction rate was proportional to the amount of enzyme. After a preincubation of 5 min at 30°C the incubation was started by the addition of radioactive ATP in buffer solution. The reaction was terminated 5 min later by the addition of 2 ml ice-cold chloroform-methanol-12 M HCl (200:100:0.75, by vol.).

Lipid extraction and thin-layer chromatography

After termination of the phosphorylation reaction, carrier polyphosphoinositide (10 µg) (14) was added. The extraction procedure and thin-layer chromatography were carried out as described in detail by Jolles et al. (15). The amount of radioactivity incorporated into PIP_2 was measured by liquid-scintillation counting (15).

Other analyses

1D SDS-PAGE was performed on 11% slab gels (6,12). 2D gel electrophoresis was performed as described by Kristjansson et al. (16) with the modifications described by Zwiers et al. (12).

Total protein was determined according to Lowry et al. (17) using bovine serum albumin (BSA) as a standard. The amount of pure B-50 was estimated by densitometric scanning of the Fast Green stained gel pattern using known amounts of BSA on the same gel as a standard. Scanning was performed at 650 nm with a Zeiss PM-QII spectrophotometer with KM_3 chromatography attachment.

Immunochemical detection of B-50 in DEAE-cellulose column fractions was performed as described earlier (8).

RESULTS AND DISCUSSION

A 40% enrichment in PIP kinase activity was obtained after ammonium sulphate precipitation (between 20-40% saturation) of the cytosolic fraction of rat brain. This ASP(20-40%) fraction was further purified by DEAE-

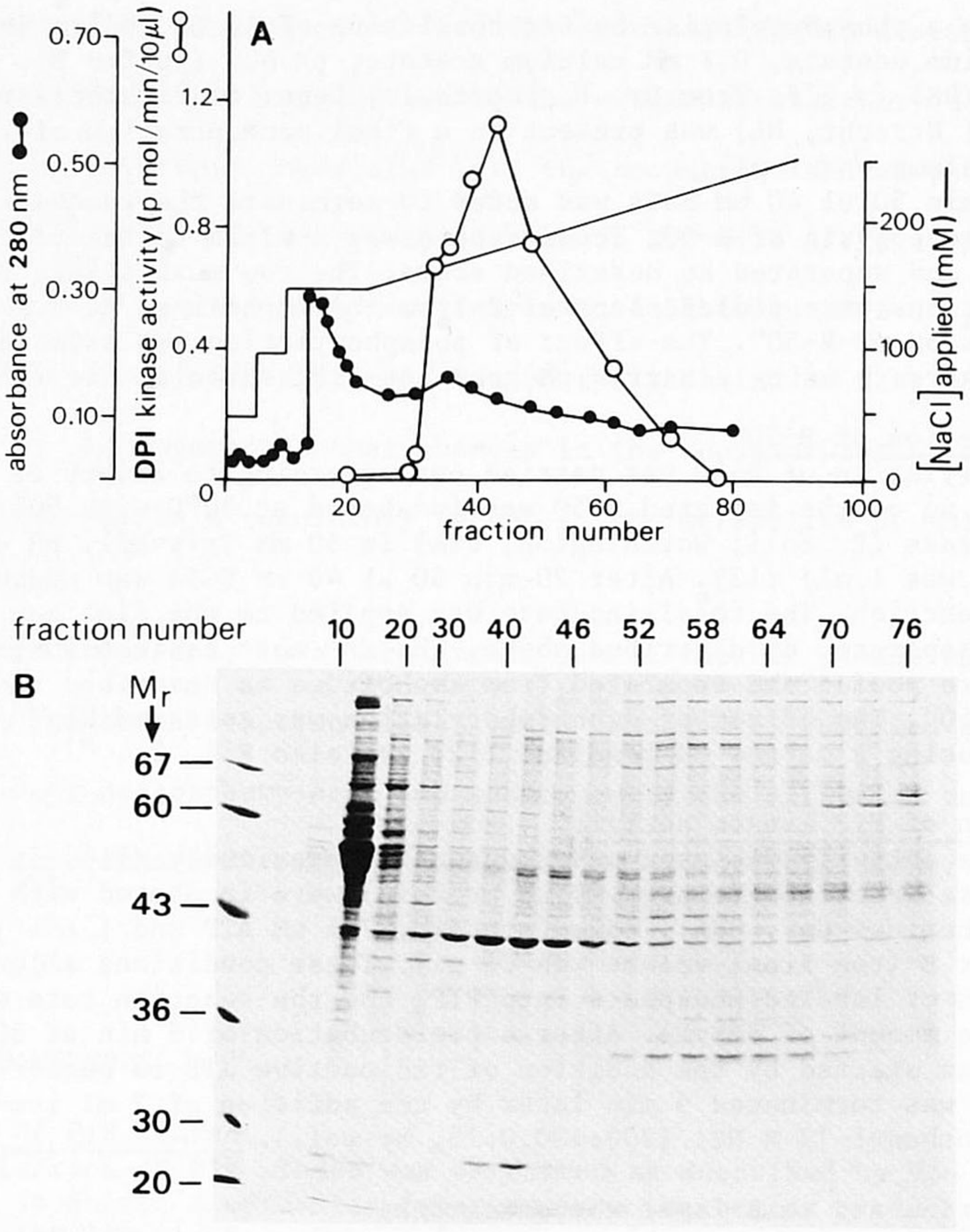

Fig. 1. Purification of PIP kinase by DEAE-cellulose column chromatography. The ASP(20-40%) protein fraction (210 mg) obtained from rat brain cytosol was applied to a DEAE-cellulose column. A: the proteins were eluted by stepwise enhancement of NaCl concentration followed by a linear gradient from 150-250 mM NaCl (-). Fractions of 4.5 ml were collected. The extinction was measured at 280 nm (o-o). PIP kinase activity was assessed using 10 µl per fraction (I) (o-o). B: protein-staining pattern of fractions. Thirty µl of each fraction was applied to a SDS-PAGE slab gel. After electrophoresis the gel was fixed and stained with Fast Green.

cellulose column chromatography. The protein was eluted by a stepwise enhancement of the ionic strength with NaCl, followed by a NaCl gradient (see Fig. 1A). After applying 150 mM NaCl to the column, the bulk of the proteins was eluted. In contrast, the PIP kinase activity was only eluted after application of the shallow gradient (Fig. 1A). DEAE-cellulose fractions 30 to 60 were pooled.

The specific activity of PIP kinase after ammonium sulphate precipitation and DEAE-cellulose column chromatography was enhanced 5.6-fold and the reco-

very of the activity was 22%. The protein pattern of the DEAE-cellulose fractions was visualized by SDS-PAGE (Fig. 1B). Fractions with PIP kinase activity showed an intensively stained band with a molecular weight of 45 kDa (fractions 30-60). Recently, we have shown that the PIP kinase activity is contained in this band (11). In the DEAE-cellulose column fractions B-50 protein was not detectable using affinity-purified anti-B-50 immunoglobulins (data not shown). This result is in agreement with the observations of Kristjansson et al. (16), who concluded that B-50 is present only in particulate fractions of rat brain.

As shown previously (18,19) addition of proteins to semi-purified enzyme preparations may influence the activity of these enzymes in an aspecific manner. The semi-purified system may be even more complex due to the use of exogenous PIP as substrate. The physico-chemical properties of the added lipid (artificial micelle formation) may codetermine the rate of enzyme activity in the in vitro assay (20-22). In order to minimize the influence of such factors we decided to study the effects of B-50 on PIP kinase activity in the presence of an excess of albumin. Therefore, before studying the effect of the degree of phosphorylation of added B-50 on the PIP kinase activity, we investigated the effect of various concentrations of BSA in the PIP kinase assay. Low concentrations of albumin (0.5-5 µg) stimulated the enzyme by 24-55% (Table I). Addition of 10 µg albumin to the incubation medium had no further effect on PIP kinase activity, whilst addition of 100 µg albumin caused an inhibition (-32%) (Table I). In contrast, addition of albumin to a cytosolic or SPM fraction did not affect the PIP kinase activity in these subcellular brain fractions (Table I). Therefore, we assume that the albumin effect is related to the artificial incubation conditions used in the reconstituted assay system and is of an aspecific nature.

The influence of B-50 on the activity of semi-purified PIP kinase was studied in the presence of 10 µg albumin. The advantage of such an approach is that aspecific protein-protein interactions possibly affecting PIP kinase activity (see Table I) are minimized. On the other hand, the experiments

Table I. Effect of bovine serum albumin on PIP kinase activity of DEAE-cellulose pool (fractions 30-60, see Fig. 1), cytosol and SPM. DEAE-cellulose pool (1 µg protein) and cytosol (1 µg protein) were incubated for 5 min as described under Materials and Methods. SPM (10 µg) were incubated with 7.5 µM ATP for 20 s as described by Oestreicher et al. (8). The amount of added albumin was varied.

Fraction used	Amount of albumin (µg)	PIP kinase activity
DEAE-cellulose pool	0	0.62 ± 0.05[+]
	0.5	0.81 ± 0.06[*]
	5.0	0.97 ± 0.05[*]
	10.0	0.63 ± 0.05
	100.0	0.42 ± 0.04[*]
Cytosol[1]	0	0.11 ± 0.01[+]
	5.0	0.11 ± 0.01
	10.0	0.12 ± 0.01
SPM	0	5.06 ± 0.36[++]
	5.0	5.20 ± 0.34
	10.0	5.10 ± 0.30

[+] The results are expressed as pmol/µg protein/min.
[++] The results are expressed as fmol/µg protein/20 s.
[1] The cytosol fraction was obtained after centrifuging the brain homogenate at 150,000 g for 60 min (11).
[*] Significantly different from control value, n = 3; 2 $p < 0.001$ (Student's t-test).

using this artificial, reconstituted PIP kinase/B-50 system are carried out under stimulated PIP kinase conditions and may not easily be compared to the actual situation in the brain cell membrane.

Batches of B-50 enriched in the phospho- and dephospho-forms were prepared as described under Materials and Methods, using the findings of Zwiers et al. (12) that B-50 shows microheterogeneity upon isoelectric focusing in a narrow pH gradient. Differences in the state of phosphorylation underly this observed heterogeneity (see also Fig. 2). B-50 batches enriched in the phospho- and dephospho-forms were assessed by 2D gel electrophoresis. As can be seen in Fig. 2A, more B-50 protein was visible after phosphorylation in the more acidic spots 3 and 4, confirming that more B-50 was in the phospho-form (12). After dephosphorylation more B-50 protein was detectable in spots 1 and 2, confirming that most of the B-50 was in the dephospho-form (Fig. 2B).

The analytical 2D separation system yields insufficient amounts of maximally phosphorylated (spot 4) and dephosphorylated (spot 1) B-50 to study the modulation of PIP kinase activity. Therefore it was decided to use the

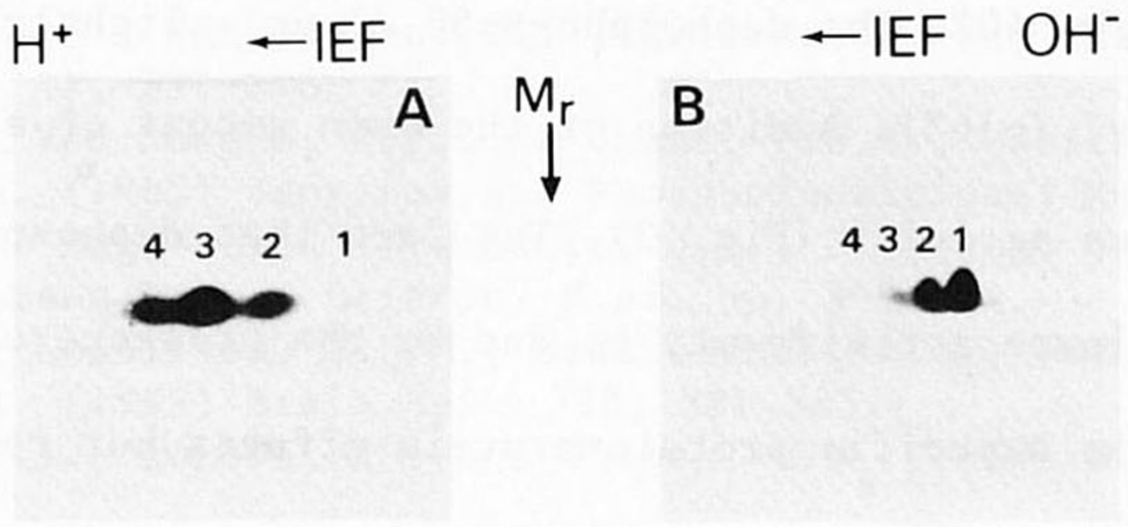

Fig. 2. Protein-staining pattern of phospho- and dephospho-B-50 (3 µg) after 2D gel electrophoresis. B-50 was purified by flat bed isoelectric focusing. Part of this B-50 was phosphorylated with protein kinase C and ATP. Another part of the purified B-50 was dephosphorylated using alkaline phosphatase (E. coli). The phosphorylated (A) and dephosphorylated (B) B-50 batches were assessed by 2D gel electrophoresis.

two most acidic fractions of the phosphorylated batch and the two most basic of the dephosphorylated batch in the modulation studies. Thus, although the two preparations differed considerably in degree of phosphorylation of B-50, by no means do they represent pure phospho- or dephospho-B-50.

Nonetheless, as shown in Fig. 3, there are incubation conditions which clearly show a differential effect of the preparations enriched in the phospho- and dephospho-B-50 on the activity of PIP kinase. Addition of 0.5 µg B-50 of the phospho- or the dephospho preparation did not affect PIP kinase activity. Addition of 1 µg B-50 enriched in the phospho-form inhibited the

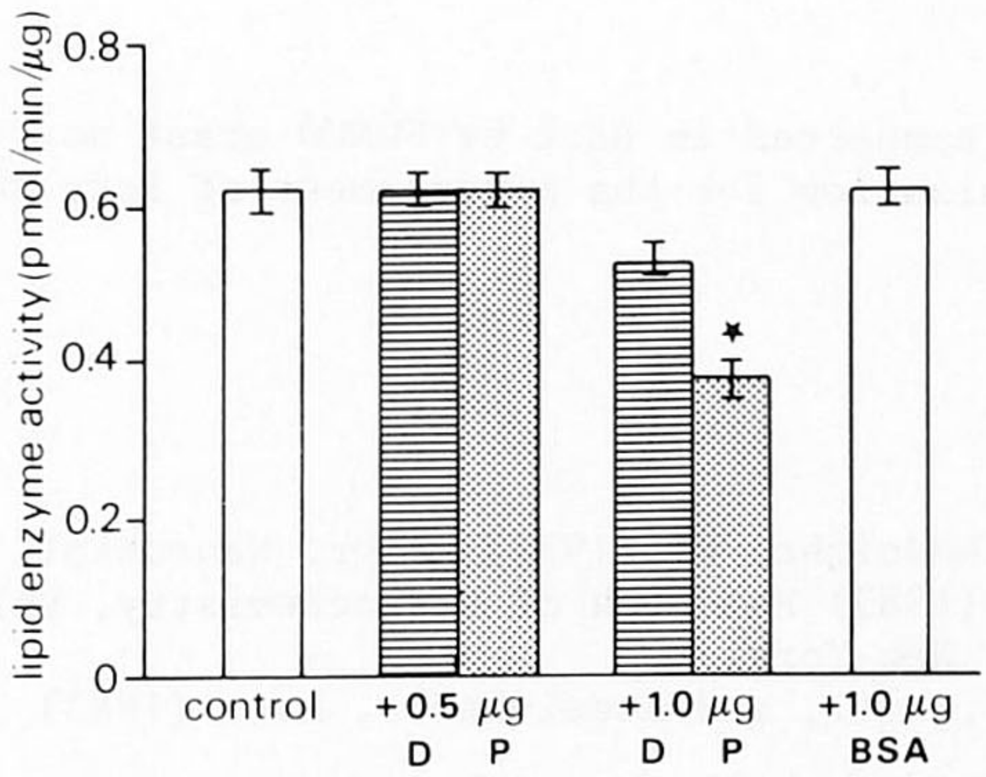

Fig. 3. Effect of B-50 on PIP kinase activity. One µg of the DEAE-cellulose pool (fractions 30-60) was incubated under standard assay conditions in the presence of 10 µg albumin. The amount of B-50 enriched in the phospho (P) or dephospho (D) form was varied. SEM (n = 3) is indicated by the bars. The experiment was repeated once with similar results. In addition, the effect of extra albumin on PIP kinase activity was studied. ★ Significantly different from control value. 2 $p < 0.05$ (Student's t-test).

PIP kinase activity by 40%. The dephospho-B-50 (1 µg) slightly reduced the lipid kinase activity (-16%). Addition of the same amount of albumin did not affect the PIP kinase activity (Fig. 3). The fact that dephospho-B-50 does not stimulate PIP kinase activity may be due to the presence of albumin. The albumin minimizes aspecific protein-protein effects but renders the PIP kinase in the activated state and may not allow further stimulation by addition of the dephospho-B-50. The slight inhibition seen with the dephosphorylated B-50 preparation may have been caused by the presence of sufficient amounts of phospho-B-50 in the preparation used.

The fact that B-50 enriched in the phospho-form inhibited PIP kinase activity more than did B-50 enriched in the dephospho-form, is in line with previous studies from this laboratory (4,8,9,23). These studies have indicated an inverse relationship between the degree of phosphorylation of B-50 and the activity of PIP kinase in membranes, broken cell preparations or tissue slices of rat brain. In this paper we present further evidence that the phosphorylation state of B-50 protein may be a regulatory factor in the metabolism of phosphoinositides in rat brain. Further investigation is needed to determine whether PIP kinase in other tissues is also modulated by a phosphoprotein as suggested by Akhtar et al. (3).

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