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Phosphorylation of B-50 Protein by Calcium-Activated, Phospholipid-Dependent Protein Kinase and B-50 Protein Kinase

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Abstract: B-50 is a brain-specific phosphoprotein, the phosphorylation state of which may play a role in the regulation of (poly)phosphoinositide metabolism. Several kinases were tested for their ability to phosphorylate purified B-50 protein. Only calcium-activated, phospholipid-dependent protein kinase (kinase C) and B-50 protein kinase were able to use B-50 protein as a substrate. Furthermore, kinase C specifically phosphorylates B-50 when added to synaptic plasma membranes. We further characterized the sensitivity of kinase C and B-50 kinase to ACTH (and various fragments), phospholipids, chlorpromazine, and proteolytic activation. Since the sensitivities of both kinases were similar, we conclude that B-50 protein kinase is a calcium-dependent, phospholipid-stimulated protein kinase of the same type as kinase C.
Key Words: Kinase C—Phosphoprotein B-50—ACTH—Chlorpromazine—Phospholipids. **Aloyo V. J. et al.** Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. *J. Neurochem.* **41,** 649–653 (1983).

B-50 protein is a membrane-bound, brain-specific phosphoprotein which is apparently presynaptically located (Sörensen et al., 1981; Kristjansson et al., 1982). Treatment both *in vivo* and *in vitro* with the behaviorally active peptide $ACTH_{1-24}$ leads to changes in the degree of phosphorylation of the B-50 protein in rat brain (Zwiers et al., 1976; 1977). Previously, data were obtained that suggest that the phosphorylation state of the B-50 protein plays a regulatory role in phosphatidyl *myo*-inositol 4-phosphate (DPI) phosphorylation (Jolles et al., 1980).

B-50 protein is phosphorylated by a calcium-sensitive, cyclic nucleotide-independent protein kinase, which has been isolated and purified together with the B-50 protein from rat brain membranes (Gispen et al., 1979; Zwiers et al., 1980).

In recent years there have been several reports on the partial purification and characterization of cyclic nucleotide-independent protein kinases from rat brain (Inoue et al., 1977; Greengard, 1979; Zwiers et al., 1980; Miyamoto et al., 1981). One of these, a calcium-dependent, phospholipid-sensitive protein kinase (also called kinase C) has been partially purified from the cytosolic fraction of rat brain (Inoue et al., 1977). The activity of this kinase is enhanced by either a calcium-dependent protease (Inoue et al., 1977) or by any of several phospholipids (Takai et al., 1979). Although this kinase was originally isolated from the soluble fraction of rat brain, it has recently been found in the particulate fraction as well (Kuo et al., 1980). It appears that in the presence of calcium the soluble kinase binds to membranes, resulting in its activation (Takai et al., 1979).

During our studies on the regulation of endogenous B-50 phosphorylation in synaptosomal membranes, we noted several apparent similarities with the reported properties of kinase C. This paper details the experiments that led us to conclude that B-50 protein kinase shares many properties with kinase C and is also a phospholipid-dependent protein kinase.

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Abbreviations used: ASP_{57-82}, Proteins precipitated by ammonium sulfate between 57 and 82% of saturation; PS, Phosphatidyl serine; SPM, Synaptic plasma membranes.

MATERIALS AND METHODS

Kinase C was prepared as described by Inoue et al. (1977) from the soluble fraction of whole brains (minus cerebellum) of female Wistar rats of approximately 150 g body weight, by means of DEAE-cellulose and gel filtration chromatography. The peak of the kinase C activity eluted from the Sephadex G-100 column was used for all further experiments. The kinase was activated by reaction with either trypsin or calcium-dependent protease (Inoue et al., 1977), or by the addition of phosphatidyl serine (PS) (Takai et al., 1979). Rat brain calcium-dependent protease was partially purified by the method of Inoue et al. (1977), by DEAE-cellulose and Sephadex G-100 chromatography. Protease activity was assayed by the ability of the preparation to activate kinase C in the presence of calcium (Inoue et al., 1977).

B-50 protein and its corresponding kinase were purified from the membrane fraction of rat brain by the procedure of Zwiers et al. (1980). The Triton X-100-solubilized fraction was separated by DEAE/cellulose chromatography, on a linear NaCl gradient. The fractions containing endogenous B-50 protein-phosphorylating activity were pooled and further fractionated by ammonium sulfate precipitation (Zwiers et al., 1980). The proteins precipating between 57 and 82% saturation (ASP_{57-82}) were used. Purified B-50 protein and B-50 protein kinase were prepared from ASP_{57-82} by isoelectric focusing (Zwiers et al., 1980).

Light synaptic plasma membranes (SPM) were prepared from rat brain cortex by the method of Terenius (1973) as detailed in Bär et al. (1982). Cyclic AMP-dependent protein kinases (Type I, rabbit muscle, Sigma P4890; Type II, beef heart, Sigma P5511; catalytic subunit, beef heart, Sigma P2645) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Protein was determined by the method of Lowry et al. (1951).

RESULTS

Kinase C and three different preparations of cyclic AMP-dependent protein kinase (Type I, rabbit muscle; Type II, beef heart; catalytic subunit from beef heart) were compared for their ability to use purified B-50 protein as a substrate. Table 1 shows that the B-50 protein is poorly phosphorylated by the cyclic AMP-dependent kinases, whereas kinase C does phosphorylate B-50. Addition of kinase C (but not the catalytic subunit from beef heart cyclic AMP-dependent protein kinase–data not shown) to SPM results in a threefold increase of B-50 protein phosphorylation (Fig. 1A). Analysis by two-dimensional gel electrophoresis of the SPM proteins phosphorylated with or without added kinase C confirms that the protein phosphorylated is indeed B-50 protein (molecular weight 48,000; IEP 4.5; Fig. 1B). However, the phosphorylation of other membrane proteins (for example, the protein of molecular weight 78,000, IEP 4.0 in Fig. 1B) is not significantly increased by the presence of kinase C.

Because of these results we further compared the properties of B-50 protein kinase with those of kinase C. As previously reported (Inoue et al., 1977; Zwiers et al., 1980), we have observed that both kinases are cyclic nucleotide-independent (data not shown). In addition, we have confirmed the observation of Mori et al. (1980) that chlorpromazine inhibits kinase C. Chlorpromazine is also an inhibitor of B-50 phosphorylation in ASP_{57-82} (Table 2).

B-50 protein phosphorylation in both SPM and in ASP_{57-82} is inhibited by several ACTH fragments (Zwiers et al., 1978; 1980). Addition of $ACTH_{1-24}$ to kinase C also results in dose-dependent inhibition of B-50 phosphorylation (Fig. 2). From the dose-response curves of the inhibiton of B-50 phosphorylation by $ACTH_{1-24}$ shown in Fig. 2, a concentration resulting in 50% inhibition (IC_{50}) of 0.82 μM $ACTH_{1-24}$ for kinase C and 0.99 μM $ACTH_{1-24}$ for ASP_{57-82} can be calculated. B-50 phosphorylation by kinase C is also inhibited by (Lys^{17},Lys^{18})-$ACTH_{5-18}$, but not by $ACTH_{1-10}$. A similar pattern of B-50 inhibition in ASP_{55-80} has previously been reported by Zwiers et al. (1980).

The sensitivity of the B-50 protein kinase to phospholipid activation was determined by measuring the endogenous B-50 protein phosphorylation in both the DEAE-cellulose eluate fractions and the ASP_{57-82} fraction with and without added PS. As discussed in detail previously (Zwiers et al., 1979), the endogenous B-50 protein-phosphorylating activity is eluted from the DEAE-cellulose column at approximately 0.2 *M* NaCl. Figure 3 shows that this activity is markedly stimulated by the addition of PS (20 μg/ml final concentration). This same concentration of PS results in an approximately fourfold stimulation of B-50 protein phosphorylation in the ASP_{57-82} fraction (Table 2). Furthermore, addition of PS to highly purified B-50 protein kinase prepared from ASP_{57-82} by isoelectric focusing–poly-

TABLE 1. *Ability of several kinases to phosphorylate B-50 protein*

Kinase	Activity (fmol/min)
cAMP-dependent (beef heart) (0.60 μg)	0.36 ± 0.13
cAMP-dependent (rabbit muscle) (2.33 μg)	0.10 ± 0.20
Catalytic subunit of cAMP-dependent kinase (beef heart) (0.056 μg)	0.06 ± 0.21
Kinase C (1.56 μg total protein)	37.90 ± 0.81

The kinases were assayed with 0.3 μg B-50 as the substrate under the following conditions: buffer A (10 m*M* sodium acetate, 10 m*M* magnesium acetate, 0.1 m*M* calcium acetate, 6 m*M* Tris-HCl, pH 7.4), 10 μ*M* ATP, 2 μCi [γ-^{32}P]ATP, final volume 25 μl. The mixture was prewarmed at 30°C for 5 min, and then the reaction was initiated by the addition of ATP. After 5 min, the reaction was terminated by the addition of a denaturing solution, giving a final concentration of 2% sodium dodecyl sulfate. The proteins were separated by sodium dodecyl sulfate PAGE, and the incorporation of [^{32}P]phosphate into B-50 protein was determined by liquid scintillation counting of the excised gel band. In this experiment, 1 fmol phosphate corresponds to 17 dpm of [^{32}P]phosphate. Cyclic AMP (5 μ*M* final concentration) was added to the cyclic AMP-dependent protein kinases and PS (20 μg/ml final concentration) was added to kinase C. Under identical conditions the amount of each kinase indicated in the table effected the incorporation of 5–6 pmol phosphate/min into 30 μg histone.

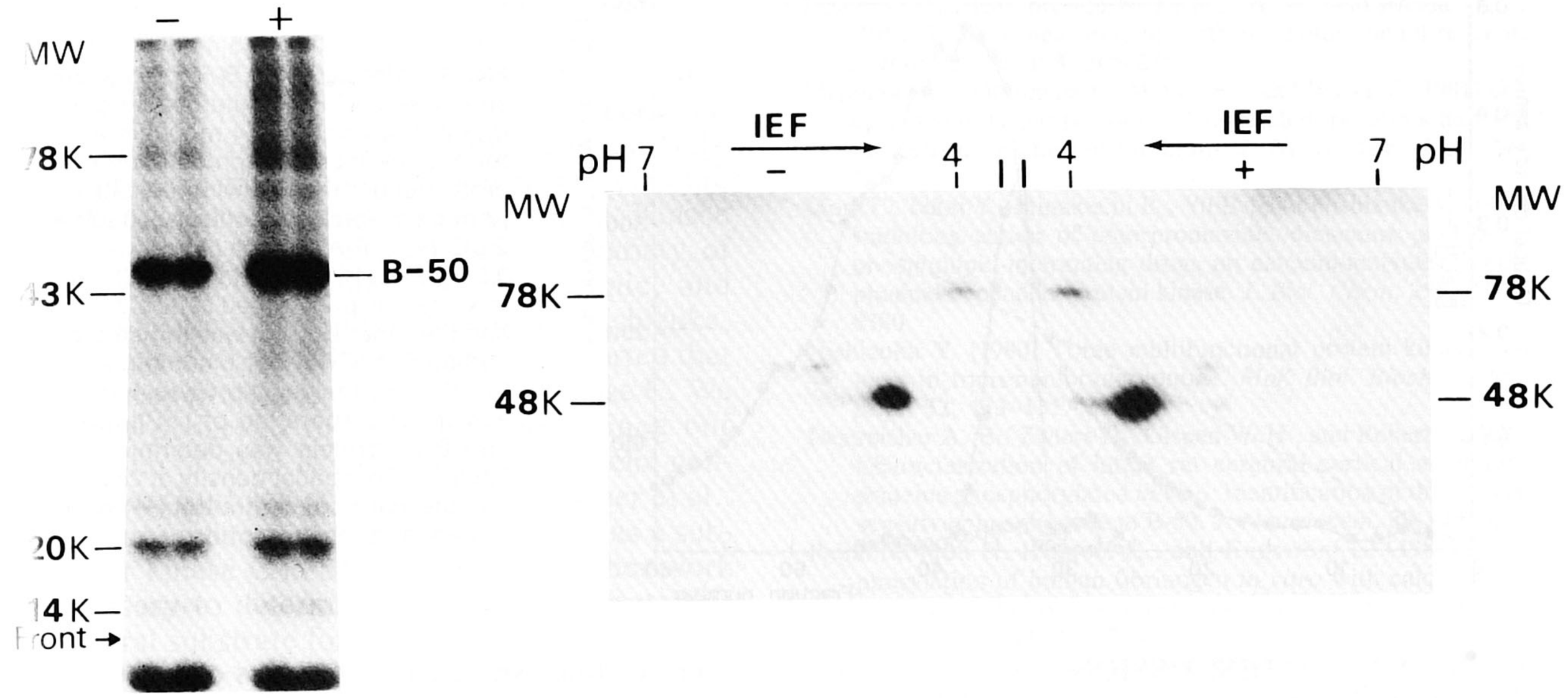

FIG. 1. Phosphorylation of SPM proteins by kinase C. The endogenous phosphorylation of SPM proteins was determined using 10 μg of rat brain SPM protein, 10 μ*M* ATP, 22 μCi [γ^{-32}P]ATP in buffer containing 10 m*M* Tris-HCl, 10 m*M* sodium acetate, 1 m*M* magnesium acetate, and 1 m*M* calcium acetate, pH 7.4, final volume 275 μl without (−) or with (+) 11 μg (total protein) kinase C preparation. After a 15-s incubation, the phosphorylation reaction was stopped by either adding a denaturing solution containing sodium dodecyl sulfate or by immersion in liquid nitrogen. (**A,** left) The proteins from duplicate incubations stopped by sodium dodecyl sulfate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE). Phosphorylated proteins were detected by autoradiography. B-50 protein and the molecular weights of several standard proteins are indicated. (**B,** right) The proteins from the reaction stopped by liquid nitrogen were separated by two-dimensional gel electrophoresis (first dimension: isoelectric focusing PAGE; second dimension: sodium dodecyl sulfate PAGE). Phosphorylated proteins were detected by autoradiography. The pH of the isoelectric focusing gel lanes is indicated at the top of the figure, and the molecular weights of the two major phosphoproteins are indicated on the side. The incorporation of phosphate into B-50 protein (48K) was 0.57 ± 0.07 pmol without kinase C and 1.55 ± 0.21 pmol with kinase C; for the 78K protein, the incorporation was 0.12 ± 0.03 pmol and 0.15 ± 0.03 pmol, respectively, mean ± SEM (n = 3).

acrylamide gel electrophoresis also results in a stimulation of kinase activity (Fig. 4).

We have confirmed the result of Inoue et al. (1977) that partially purified rat brain calcium-dependent protease activates kinase C in the presence of calcium (data not shown). Similarly, preincubation of ASP_{57-82} with the same protease preparation results in a nearly fourfold stimulation of B-50 protein phosphorylation (Table 2).

TABLE 2. *Effects of various treatments on the endogenous B-50 phosphorylation in ASP_{57-82}*

Treatment	Relative amount of incorporation into B-50 protein
None	1.0
Addition of chlorpromazine	0.5
Addition of PS	4.1
Addition of PS and chlorpromazine	1.6
Preincubation with protease	3.8

The ASP_{57-82} was assayed for endogenous phosphorylation of B-50 protein under the following conditions: ASP_{57-82} proteins (2.5 μg total protein) in buffer A, 10 μ*M* ATP, 2 μCi [γ-^{32}P]ATP, pH 7.4, final volume 25 μl. The mixture was prewarmed for 5 min at 30°C, and then the reaction was initiated by the addition of ATP. After incubation for 15 s, the reaction was terminated and the incorporation of [^{32}P]phosphate into B-50 protein was determined as described in Table 1. PS and chlorpromazine were added at a final concentration of 20 μg/ml and 100 μ*M*, respectively. The ASP_{57-82} was preincubated at 30°C with the partially purified calcium-dependent protease (8 μg total protein) in the presence of 2 m*M* Ca^{2+} for 10 min before assaying.

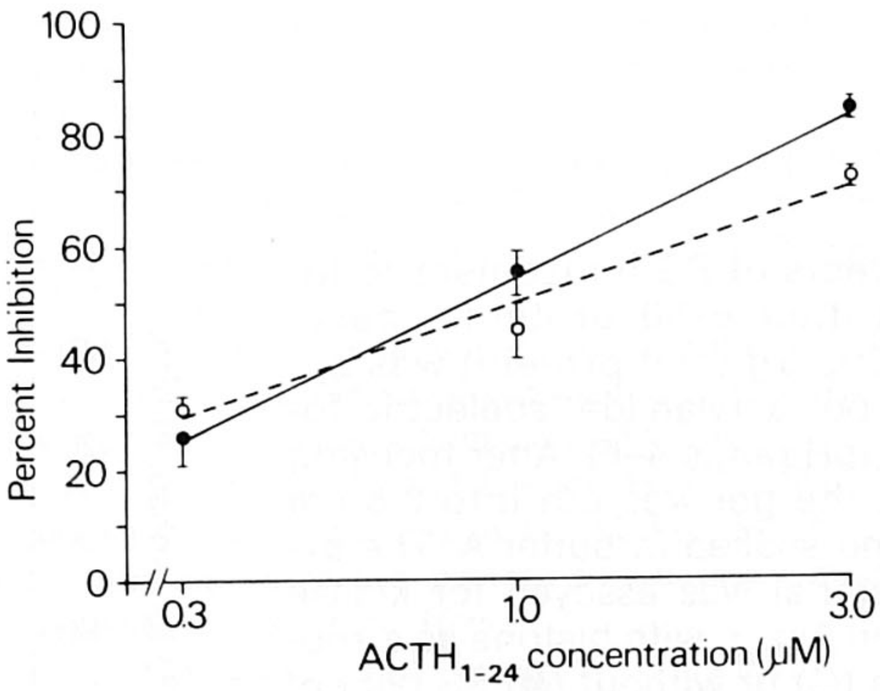

FIG. 2. Inhibition of kinase C (●) and B-50 protein kinase (○) by $ACTH_{1-24}$. Kinase C (0.9 μg total protein), with 0.3 μg of B-50 protein as the substrate, and ASP_{57-82} (2.5 μg total protein) were assayed under the conditions used in Table 1, except that the Ca^{2+} concentration was 30 μ*M*. $ACTH_{1-24}$ was added 10 s prior to the addition of the ATP. The incorporation of [^{32}P]phosphate in B-50 was determined as in Table 1 (mean ± SEM; n = 3). Regression analysis showed that the two lines are not significantly different ($F = 6.68$; $p < 0.05$)

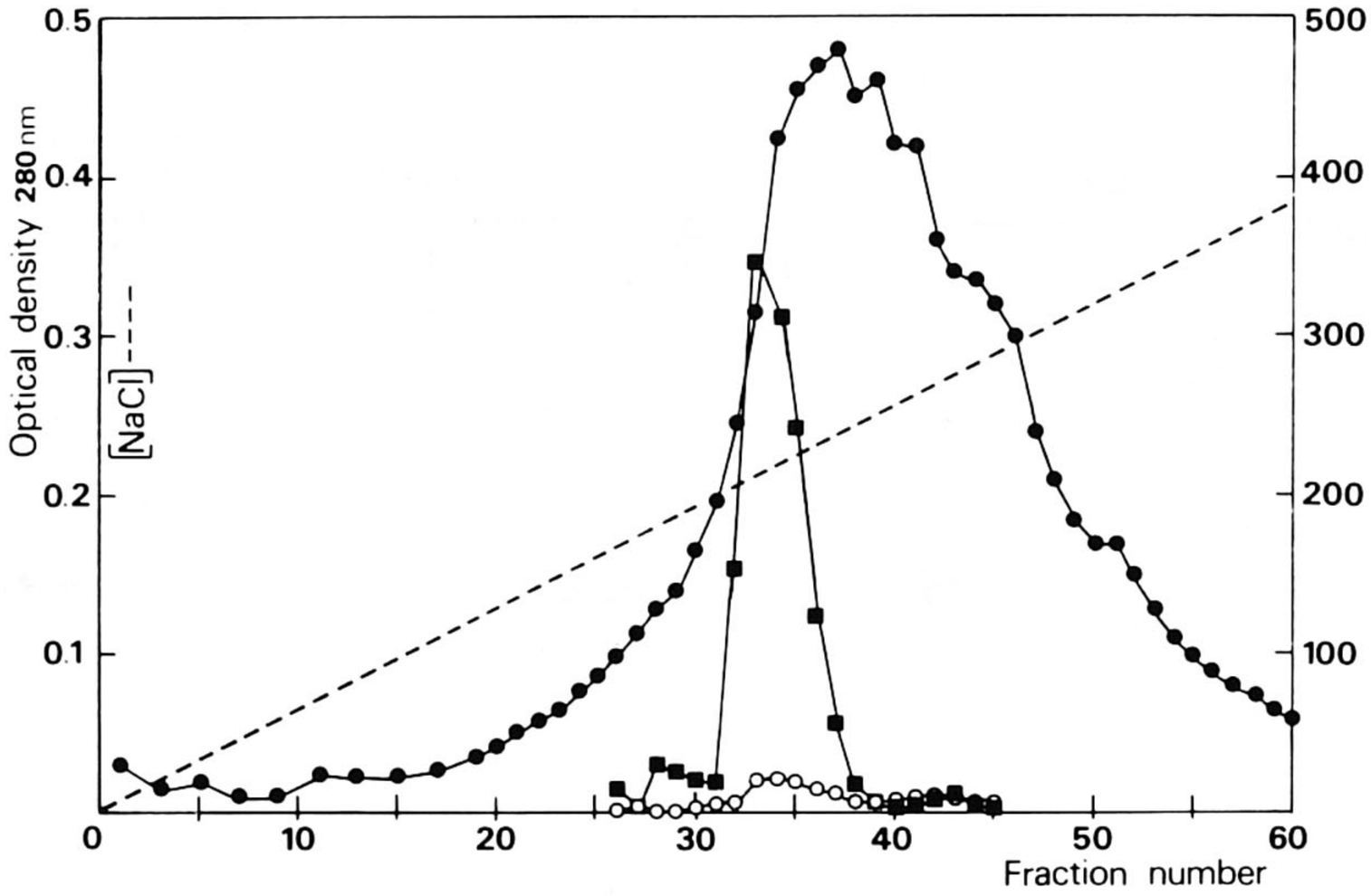

FIG. 3. Purification of B-50 protein and B-50 kinase by DEAE-cellulose chromatography. The fractions (4 ml) were assayed for endogenous phosphorylation of B-50 protein under the following conditions: 10 μl of each fraction, buffer A, 10 μ*M* ATP, 2 μCi [γ−^{32}P]ATP, with (■) or without (○) 0.5 μg PS, pH 7.4, final volume 25 μl. The mixture was prewarmed at 30°C for 5 min, and then the reaction was initiated by the addition of ATP. After incubating at 30°C for 10 min, the reaction was terminated and the incorporation of [^{32}P]phosphate into B-50 protein was determined as in Table 1. The optical density at 280 nm (●) and the NaCl concentration (---) were determined for each fraction.

DISCUSSION

B-50 protein kinase as first described by Zwiers et al. (1980) is characterized by its ability to phosphorylate B-50 protein. Therefore, we have compared several kinases for their ability to phosphorylate B-50. Of the kinases tested, only kinase C was able to use purified B-50 protein as a substrate. Furthermore, when added to SPM, kinase C is able to phosphorylate B-50, but not several other proteins. A further characteristic of B-50 protein kinase is the inhibition of its activity by ACTH and other behaviorally active neuropeptides (Zwiers et al., 1980; 1981). Similar to B-50 protein kinase, kinase C (using B-50 protein as substrate) was inhibited by $ACTH_{1-24}$ and (Lys^{17},Lys^{18})-$ACTH_{5-18}$, but not by $ACTH_{1-10}$. This same structure-activity relationship was found when endogenous B-50 phosphorylation was assayed in SPM (Zwiers et al., 1978).

The distinguishing characteristics of kinase C are its activation by PS and the Ca^{2+}-dependent protease and its inhibition by chlorpromazine (Inoue et al., 1977; Takai et al., 1979; Mori et al., 1980). We report here that B-50 protein kinase is also activated by both PS and the Ca^{2+}-dependent protease. In addition, chlorpromazine is an inhibitor of B-50 protein kinase activity. Furthermore, both B-50 protein kinase and kinase C are cyclic nucleotide-independent protein kinases (Inoue et al., 1977; Zwiers et al., 1980).

We conclude that B-50 protein kinase is a phospholipid-sensitive protein kinase that is very similar to kinase C. In this regard it must be noted that Papanikolaou et al. (1982) have also reported a phospholipid-sensitive protein kinase which uses fibrinogen as a substrate. This enzyme was also shown to be very similar to kinase C (Papanikolaou et al., 1982). It may be that there exist many phospholipid-

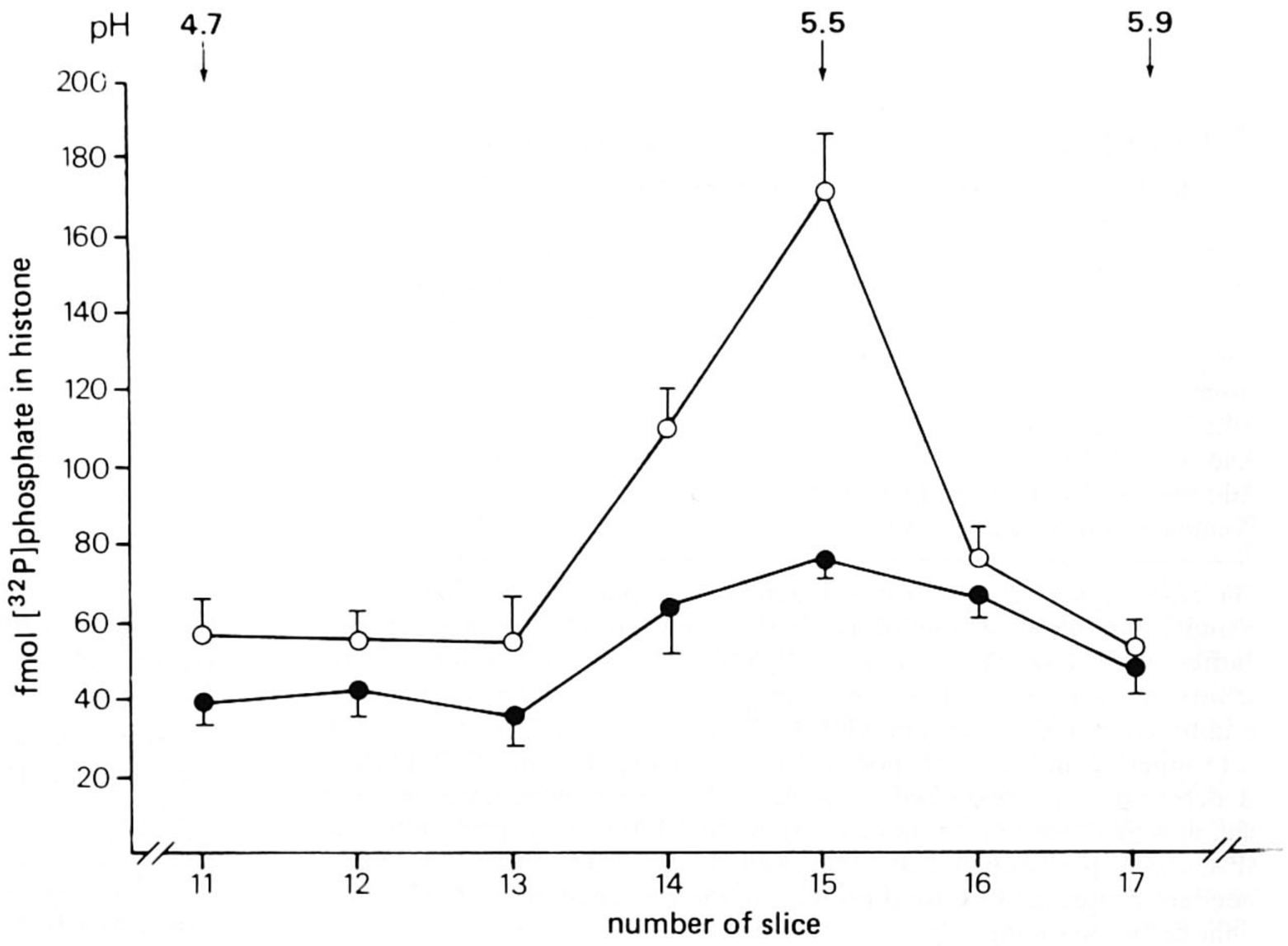

FIG. 4. Effects of PS on isoelectric focusing-purified B-50 protein kinase. ASP_{57-82} (200 μg total protein) was applied to a polyacrylamide isoelectric focusing gel (pH range 4–6). After focusing overnight, the gel was cut into 0.5-cm sections and soaked in buffer A. The extracted material was assayed for kinase activity as in Fig. 3, with histone as a substrate, with (○) or without (●) PS (20 μg/ml final concentration). The pH of corresponding gel slices is indicated at the top.

nsitive protein kinases, each having its own specific substrate, e.g., B-50 protein kinase or fibrinogen kinase. However, the same kinase C may exist in many tissues, as reported by Kuo et al. (1980) and the restricted localization of the substrate proteins may determine which proteins are phosphorylated when kinase C is activated. Kinase C has been shown to phosphorylate a variety of substrate proteins of nuclear, cytoplasmic, and membrane origin (Wrenn et al., 1980; Nishizuka, 1980). Wise et al. (1982) have recently reported that myelin basic protein is a substrate for kinase C. We have not observed this phenomenon, since our preparation of SPM is virtually free of myelin contamination (Burbach et al., 1981; Oestreicher et al., 1982). Our evidence points to B-50 protein as a substrate for kinase C in SPM; however, further work is necessary to determine whether B-50 protein is the natural substrate for kinase C in rat brain membranes.

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