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# Affinity-Purified Anti-B-50 Protein Antibody: Interference with the Function of the Phosphoprotein B-50 in Synaptic Plasma Membranes

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**Abstract:** Affinity-purified anti-B-50 protein antibodies were used to study the previously proposed relationship of the phosphorylation state of B-50 protein and polyphosphoinositide metabolism in synaptic plasma membranes. Antibodies were raised against a membrane extract enriched in the B-50 protein and its adrenocorticotropin-sensitive protein kinase, obtained from rat brain. Anti-B-50 protein immunoglobulins were purified by affinity chromatography on a solid immunosorbent prepared from B-50 protein isolated by an improved procedure. The purified antibodies reacted only with the B-50 and B-60 protein, a proteolysis derivative (of B-50), as assessed by the sodium dodecyl sulfate-gel immunoperoxidase method. These antibodies inhibited specifically the endogenous phosphorylation of B-50 protein in synaptic plasma membranes, without affecting notably the phosphorylation of other membrane proteins. This inhibition was accompanied by changes of the formation of phosphatidylinositol 4,5-diphosphate and phosphatidic acid in synaptic plasma membranes, whereas formation of phosphatidylinositol 4-phosphate was not altered. Inhibition by $ACTH_{1-24}$ of the endogenous phosphorylation of B-50 protein in membranes was associated only with an enhancement of the phosphorylation of phosphatidylinositol 4-phosphate to phosphatidylinositol 4,5-diphosphate. These data support our hypothesis on the functional interaction of B-50 protein and phosphatidylinositol 4-phosphate kinase in rat brain membranes. The evidence shows that purified anti-B-50 protein antibodies can be used to probe specifically the function of B-50 protein in membranes. **Key Words:** Antibodies—Phosphoprotein—Polyphosphoinositides—Affinity purification—Synaptic plasma membranes—ACTH. **Oestreicher A. B. et al.** Affinity-purified anti-B-50 protein antibody: Interference with the function of the phosphoprotein B-50 in synaptic plasma membranes. *J. Neurochem.* **41**, 331–340 (1983).

Adrenocorticotropin (ACTH) and its N-terminal peptides have been shown to influence specific behaviors in animals and humans (De Wied and Gispen, 1977). During studies to characterize those neurochemical processes that determine or are associated with the central action of ACTH peptides (Dunn and Gispen, 1977), $ACTH_{1-24}$ was found to affect both protein phosphorylation (Zwiers et al., 1976, 1980*a*) and polyphosphoinositide (polyPI) metabolism (Jolles et al., 1980, 1981*a*) in rat brain membranes. These processes are considered to be of great significance for the function of the cell membrane (Greengard, 1979; Michell, 1979), and in neurons seem to be involved in the regulation of membrane permeability and synaptic transmission (for reviews, see Greengard, 1978; Oestreicher et al., 1982; Rodnight, 1982).

Zwiers et al. (1976, 1978) demonstrated that be-

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*Abbreviations used:* ACTH, Adrenocorticotropin; ASP, Ammonium sulfate precipitated proteins; ASP (57–82%), Proteins precipitated between 57 and 82% saturation of ammonium sulfate; BSA, Bovine serum albumin; IEP, Isoelectric point; IgG, Immunoglobulin G; PA, Phosphatidic acid; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate-buffered saline; PI, DPI, TPI, Phosphatidylinositol, -4-phosphate, -4,5-diphosphate; PIP, Phosphorylation inhibiting peptide; Po, Old procedure; PolyPI, Polyphosphoinositide(s); Pn, New procedure; SDS, Sodium dodecyl sulfate; SGIP, SDS-gel-immunoperoxidase; SPM, Synaptic plasma membranes.

haviorally active ACTH peptides cause a dose-dependent inhibition of phosphorylation *in vitro* of at least five protein bands in synaptic plasma membranes (SPM). Subsequently, Zwiers et al. (1978) showed that the neuropeptide inhibited the protein kinase(s) activity of SPM, but influenced the activity of neither protein phosphatases nor that of ATPases of SPM. The ACTH-sensitive protein kinase and one of its substrate proteins (the B-50 protein) have been extracted from rat brain membranes (Zwiers et al., 1979), purified, and characterized (Zwiers et al., 1980*a*). B-50 is a phosphoprotein with a molecular weight (Mr) of 48,000 and an isoelectric point (IEP) of 4.5. Zwiers et al. (1980*b*) have reported that on dialysis of an ammonium sulfate-precipitated protein fraction [ASP(57–82%)] enriched in B-50 protein kinase and B-50 protein, a basic peptide was produced concurrently with proteolytic degradation of the B-50 protein. The peptide (Mr 1600) inhibited the phosphorylation of B-50 in ASP and SPM and was called phosphorylation inhibiting peptide (PIP). Recently Zwiers et al. (1982*a*) demonstrated that specific proteolysis of the B-50 protein in ASP occurs only in the absence of calcium ions, producing a large fragment, the B-60 protein (Mr 46,000), and the small peptide PIP.

The B-50 protein appears to be localized exclusively in particulate fractions of nervous tissue (Kristjansson et al., 1982) and is concentrated, throughout the rat brain, in regions rich in synaptic contacts (Oestreicher et al., 1981), presumably in the presynaptic membranes (Sörensen et al., 1981).

Jolles et al. (1980) reported that when a membrane protein fraction enriched in B-50 protein substrate/B-50 protein kinase [ASP(55–80%)] was incubated with added phosphatidylinositol 4-phosphate (DPI) and [$\gamma$-$^{32}$P]ATP, $ACTH_{1-24}$ increased in a dose-dependent manner the formation of phosphatidylinositol 4,5-diphosphate (TPI), and simultaneously inhibited the phosphorylation of the B-50 protein. The extent of prephosphorylation of B-50 protein was inversely correlated with the amount of radioactive phosphate incorporated in TPI (Jolles et al., 1980). In rat membrane/cytosol fraction, $ACTH_{1-24}$ inhibited $^{32}$P incorporation into the B-50 protein and concomitantly stimulated the production of $^{32}$P-labeled TPI and inhibited that of $^{32}$P-labeled phosphatidic acid (PA). The neuropeptide effect was abolished by increasing concentrations of calcium (Jolles et al., 1981*a*,*b*). Moreover, the same amino acid sequences of the ACTH molecule were found to be active in the peptide effect on B-50 protein phosphorylation, TPI/PA formation, and behavioral activity *in vivo* (Zwiers et al., 1978; Gispen et al., 1979; Jolles et al., 1981*b*).

These findings led to the hypothesis that changes in the extent of phosphorylation of the B-50 protein may be a regulatory factor in polyPI metabolism in SPM (Jolles et al., 1980, 1981*a*). In the present investigation we studied this proposed functional relationship. We report the affinity purification of anti-B-50 protein antibodies and their application to inhibit specifically endogenous phosphorylation of the B-50 protein and to stimulate TPI formation in SPM.

## MATERIALS AND METHODS

### Chemicals

All chemicals were analytical grade. [$\gamma$-$^{32}$P]ATP was bought from the Radiochemical Centre (Amersham, England). Protein A-Sepharose, CNBr-activated Sepharose 4B, Sephadex IEF, and molecular weight marker proteins were purchased from Pharmacia Chemicals (Sweden) and 3.5–10 LKB Ampholines from LKB-produkter AB (Sweden). Goat anti-rabbit immunoglobulin conjugated to peroxidase were obtained from Nordic Immunological Laboratory (Tilburg, The Netherlands); synthetic $ACTH_{1-24}$ from Organon International BV (Oss, The Netherlands).

### Isolation of B-50 protein

Rats (150–200 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). The B-50 protein was purified by two procedures. All fractions of the purification procedures were analyzed for enrichment of the B-50 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Zwiers et al. (1976). The first, old procedure (Po) has been reported in detail by Zwiers et al. (1980*a*) and was used except for some minor modifications (see Zwiers et al., 1982*a*; see below). To limit proteolytic degradation of the B-50 protein during purification, calcium was omitted from the buffers unless specified otherwise (Zwiers et al., 1982*a*). The final purification step consisted of separating the B-50 protein from the ASP (57–82%) fraction by preparative isoelectric focusing on a flat bed of Sephadex-IEF containing 5% (wt/vol) Ampholines yielding a pH gradient of 4–6 (Radola, 1973). The yield was approximately 300 μg B-50 protein from 90 rat brains.

The second, new procedure (Pn) was developed to increase the yield of the B-50 protein per rat brain. Here, we checked also if the B-50 protein-enriched fractions conserved the specific immunoreactivity. Pn consisted of the following steps.

(a) A standard crude mitochondrial pellet was prepared from 90 rat brains (Zwiers et al., 1976). This fraction was subjected to hypo-osmotic shock, 5 ml double-distilled water/g wet weight for 15 min, followed by centrifugation for 20 min at 10,000 *g* (Sorvall SS-34).

(b) The pellet was suspended in 1 m*M* magnesium acetate; the pH of the suspension was adjusted to 11.5 with 1 *M* NaOH to a final volume of 10 ml/g wet weight;

(c) After centrifugation for 20 min at 48,000 *g* the supernatant containing the alkaline-extracted proteins was acidified to pH 5.5 with 1 *M* sodium acetate-acetic acid buffer at pH 5.0. Centrifugation for 20 min at 48,000 *g* yielded a supernatant containing the major fraction of the B-50 protein.

(d) The supernatant was treated with ammonium sulfate, at first to yield 57% saturation. Precipitated proteins were removed by centrifugation for 20 min at 40,000 *g* in a Sorvall OTD-2 centrifuge (Beckman SW 27.1 rotor) and the supernatant was saturated to 82%. The precipitate,

ASP (57–82%), was collected and dialyzed for at least 3 h against double-distilled water.

(e) The final step of Pn was identical to that of Po. The yield from 90 rat brains was 1–3 mg B-50 protein.

### Immunization procedure

White New Zealand rabbits were immunized as described previously by Oestreicher et al. (1981). The immunization was started by injecting 300 μg of a B-50 protein-enriched preparation [ASP (57–82%)], followed by seven boosters: at first, at intervals of 3 weeks and then of a month over a period of 6 months; finally, after a rest period of 9 months a booster of 100 μg pure B-50 protein was given. This booster enhanced greatly the titer of the sampled antiserum of rabbit 8002 as measured by enzyme-linked immunosorbent assay (see below). This antiserum 8002 was used for affinity chromatography. Another antiserum (8106) was obtained by immunizing the rabbit repeatedly with only purified B-50 protein (100 μg/injection). This antiserum was characterized in a previous study (Kristjansson et al., 1982). Serum samples were aliquoted and stored at −20°C.

### Detection of antibodies

Development of the formation and purification of antibodies to the B-50 protein was monitored by an enzyme-linked immunosorbent assay (Engvall, 1980). The specificity of the serum samples was checked by the SDS-gel-immunoperoxidase (SGIP) method (Van Raamsdonk et al., 1977). This method was applied as reported previously (Oestreicher et al., 1981), except for some adaptations described below. Antigens were separated by electrophoresis in SDS 11% (wt/vol) polyacrylamide slab gels of 3 mm thickness. After the electrophoresis, gel pieces of 42 × 42 × 3 mm, each containing six or seven sample lanes, were cut. The gel pieces were placed on a sample holder and quick-frozen in a Bright cryostat microtome at −30°C. Longitudinal sections of 50 μm were sliced from the frozen gel piece at −30°C. It was discovered that during the prolonged period of incubation and washes, purified B-50 protein, in contrast with membrane-bound B-50, was gradually extracted from the thin gel sections if these had been fixed only in 15% (vol/vol) glacial acetic acid and 85% (vol/vol) ethanol. The loss of B-50 was prevented by applying an extra protein fixation step; thus, when the gel sections, after the slicing in the cryostat microtome, were fixed for 15 min in 2.5% (wt/vol) glutardialdehyde about 80% of the purified B-50 was retained, as determined by liquid scintillation counting of $^{32}$P-labeled B-50. Immunostaining of B-50, B-60, and of other proteins was not notably affected by the extra fixation step. Sections were stored at −20°C in a protein fixation solution, containing 15% (vol/vol) glacial acetic acid and 85% (vol/vol) ethanol.

Another check of the specificity of the antibodies was carried out by absorption of the antibody samples for 2 h at 4°C with an excess of purified B-50 protein or of membranes. An antibody sample of 1 μl original antiserum was diluted five times in phosphate-buffered saline (PBS) containing 5 μg bovine serum albumin (BSA) and 1 μg B-50 protein or 60 μg of protein of PBS washed SPM or 0.25 mg PBS-washed heart particulate homogenate. Finally, particulate material was removed from the absorbed samples by centrifugation.

### Purification of antibodies

The B-50 protein immunosorbent column was prepared as follows: Two milligrams B-50 protein purified by Po and exhaustively dialyzed was mixed with 1 g swollen CNBr-activated Sepharose 4B in 0.1 *M* $NaHCO_3$ and 0.5 *M* NaCl (pH 9.0). The coupling reaction was allowed to proceed for 16 h at 21°C by shaking this mixture in a stoppered small column (diameter, 1.2 cm). The reaction was monitored by analyzing samples taken after 2 min and 16 h on SDS-PAGE and scanning of the protein-stained gel. Sixty percent of the added B-50 protein (1.2 mg) had been coupled. The reaction was terminated by collection of the void volume effluent and treatment with 2 ml of 1 *M* glycine (pH 8.0) for 90 min. Then, the column was alternatively washed with portions of 4 ml of 1 *M* NaCl in 0.1 *M* sodium acetate (pH 4.0) and in 0.1 *M* sodium borate (pH 8.5). Finally the column was rinsed with 0.1 *M* ammonium formate (pH 2.7) and subsequently neutralized with PBS. The column was stored at −20°C. Affinity chromatography was carried out in a batchwise procedure. One milliliter of anti-B-50 protein antiserum (fraction 1 of Fig. 3) was added to the B-50 protein column and allowed to react for 1 h at room temperature with gentle agitation. The unbound proteins were collected as the first effluent (Fig. 3, fraction 2). The B-50 column was washed subsequently with five times 4 ml of PBS (fractions 3 of Fig. 3) and specifically bound antibodies were eluted with six times 4 ml of 100 m*M* ammonium formate (pH 2.7) (see Fig. 3, fractions 4 and 5). The eluates were immediately neutralized to pH 7 with 1.0 *M* ammonia and concentrated by lyophylization. Purified antibodies were stored at −20°C. Control immunoglobulins were isolated from immune serum or from preimmune or normal rabbit serum by affinity chromatography on a Protein A-conjugated Sepharose column (2 ml of swollen gel) by a procedure similar to that described above. When immune serum was used, the total immunoglobulin fraction was first isolated by affinity chromatography on a protein A-column, followed by separation in two fractions, control and anti B-50 IgGs, on the B-50 immunosorbent.

### Phosphorylation assay

Endogenous phosphorylation activity was assayed as described previously (Zwiers et al., 1976; 1978; 1979). In brief, the incubations were carried out under the following conditions: 7.5 μ*M* ATP, 2–3 μCi of [γ-$^{32}$P]ATP (approx. 3000 Ci/mmol), 5 μl of membrane fraction (approx. 15 μg of protein), 10 m*M* sodium acetate, 10 m*M* magnesium acetate, 0.1 m*M* $CaCl_2$ at pH 6.5, in a final volume of 25 μl at 30°C. The membrane samples were preincubated for 5 min. Then buffer solution plus or minus $ACTH_{1-24}$ was added 20 s before zero time, at which the phosphorylation reaction was started by addition of the radioactive ATP. The reaction was terminated 20 s later.

The effect of immunoglobulins was studied as follows. The membrane samples were incubated with the IgGs for 10 min in ice, followed by the preincubation and phosphorylation at 30°C.

Lipid and protein phosphorylation was studied in parallel samples of the same experiment as reported by Jolles et al. (1981*a*). The lipid phosphorylation reaction was terminated by addition of 2 ml ice-cold chloroform-methanol-12 *M* HCl (200:100:0.75, by vol) (Shaikh and Palmer, 1977). The protein phosphorylation reaction was stopped by addition of a denaturing solution resulting in

final concentrations of 62.5 m*M* Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.001% (wt/vol) bromphenol blue, and 5% (wt/vol) 2-mercaptoethanol. The denatured membrane proteins were separated by SDS-PAGE. After staining with Fast Green FCF and autoradiography of the gel the labeled B-50 protein band was excised from the gel and measured for radioactivity by liquid scintillation counting (Zwiers et al., 1978, 1980*a*; Wiegant et al., 1978).

### Lipid extraction and TLC

After termination of the phosphorylation reaction carrier polyPI was added and the extraction procedure and TLC were carried out as described in detail by Jolles et al. (1981*a*).

### Preparation of subcellular fractions

SPM was prepared from total rat brain as described previously by Kristjansson et al. (1982).

### Other analyses

SDS-PAGE and determination of incorporated radioactivity were performed as described by Jolles et al. (1981*a*). Proteins were determined by the method of Lowry et al. (1951). The protein content of the purified B-50 preparation was estimated by densitometric scanning of Fast Green-stained bands in SDS-polyacrylamide gels with BSA as a standard.

## RESULTS

### Characterization of purified B-50 antigen

Purification of the B-50 protein was monitored by analysis with SDS-PAGE and checked for preservation of specific immunoreactivity to anti-B-50 protein antiserum by the SGIP method (see Fig. 1). In Fig. 1 one of these immunochemical analyses is presented. The original antiserum (8002) immunostained the 48K band of the alkaline extract (track 7), ASP (track 6), and the purified B-50 protein (track 3), three samples isolated by Pn, similarly to the B-50 protein band in the reference antigen preparations, SPM (track 1), ASP of Po (track 5), and brain particulate (track 4). Some other protein bands were also stained (see tracks 2, 5, and also Fig. 4). Figure 2A presents the protein profiles of the ASP (57–82%) fractions of Po and Pn. The Pn resulted in an ASP fraction containing more total protein, more B-50 protein, and a greater variety of other proteins than Po. It was found that the composition and relative quantities of proteins isolated in the ASP fractions showed some variation from preparation to preparation (Fig. 2A versus Fig. 1A). The variation was larger for Pn than for Po. However, when the proteins of the ASP (57–82%) fraction of Pn were further fractionated by flat bed isoelectric focusing, B-50 protein was separated out. A protein band of 48K and IEP of 4.5 was observed in three fractions (Fig. 2B). The anti-B-50 protein antiserum reacted with the 48K and 46K bands of these fractions, thus detecting the isolated B-50 and its proteolytic breakdown product B-60 (see Figs. 1 and 4B). One fraction (indicated by an arrow) contained the major portion of the B-50 protein. It was virtually free of endogenous proteolysis products. The total yield of Pn was 0.1–0.2 μg B-50 protein/mg protein of rat brain. This fraction was used to prepare the solid immunosorbent.

### Purification of anti-B-50 protein IgG from the original antiserum

Since rabbit 8002 was initially immunized with ASP, formation of various antibody populations was detected (Fig. 1, tracks 2, 5, and 6). A sample (1 ml) of the antiserum was applied to the column of B-50 protein covalently bound to Sepharose beads. After equilibration unbound serum proteins were

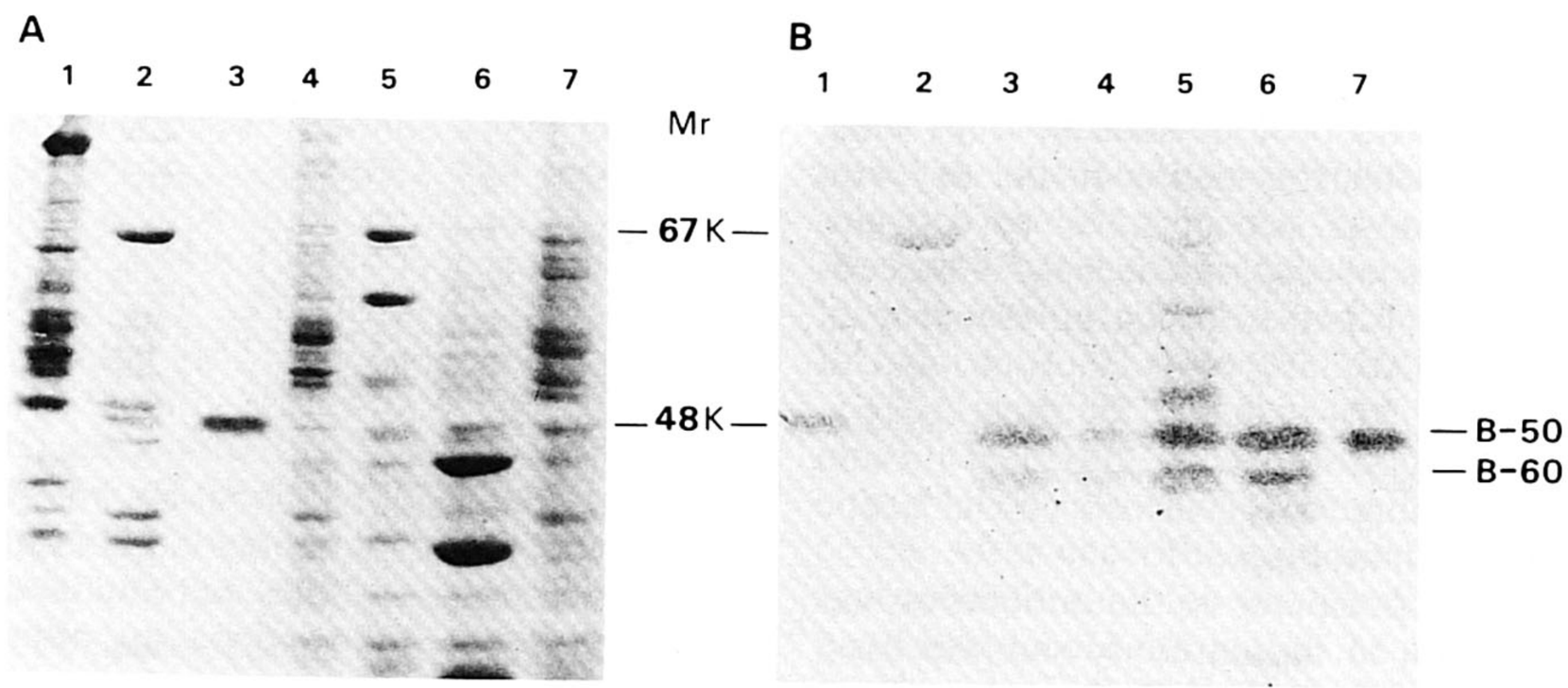


**FIG. 1.** Immunochemical analysis of B-50 protein-enriched fractions of Pn and Po. Samples of various antigens [track 1, 50 μg SPM proteins; track 2, 100 μg protein of cytosol fraction; track 3, 3 μg B-50 protein; track 4, 100 μg protein of particulate fraction from rat brain; track 5, 50 μg ASP (57–82%) of Po; track 6, 150 μg ASP (57–82%) of Pn; track 7, 100 μg protein of alkaline extract of Pn] were separated by electrophoresis on an 11% (wt/vol) SDS-polyacrylamide gel of 3 mm thickness as described by Oestreicher et al. (1981). After the electrophoresis square pieces of 4.5 × 4.5 cm were excised, frozen, and sectioned to 50-μm thick sections in a cryostat microtome. The remaining gel piece was stained with Fast Green to reveal the pattern of separated protein bands in the gel **(A). (B)** Immunostaining after incubation of the fixed gel sections with 1:40 diluted original anti-B-50 protein antiserum.

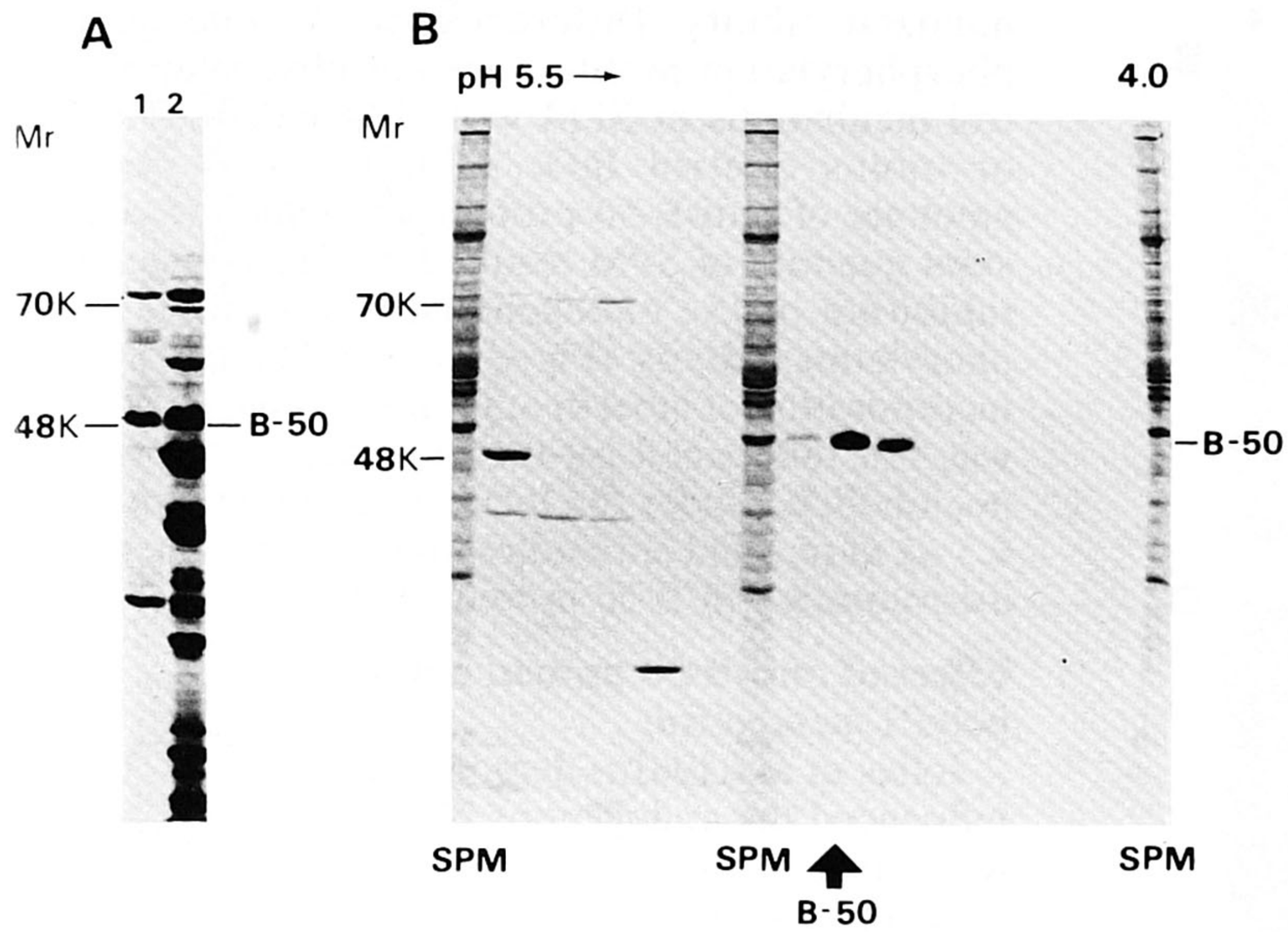


**FIG. 2.** Protein profiles of SDS-PAGE gels showing ASP (57–82%) fractions of Po and Pn **(A)** and B-50 protein purified from the ASP fraction of Pn **(B).** (A) ASP (57–82%) fractions obtained by Po (track 1, 5 μg of total protein) and by Pn (track 2, 50–100 μg of total protein) were analyzed by SDS-PAGE. (B) Purified B-50 protein was obtained from alkaline-extracted membranes (Pn) after preparation of ASP (57–82%) (see procedure Pn) and separation of these proteins by isoelectric focusing. The purest B-50 protein was detected in the fraction of IEP 4.5 (arrow).

removed thoroughly. Subsequently, the anti-B-50 protein antibodies were dissociated and eluted as described under Materials and Methods. Figure 3 shows the analyses by SDS-PAGE. The first eluted fractions had a protein profile (Fig. 3, fraction 2) very similar to that of the original antiserum sample (fraction 1). The subsequent wash fraction (Fig. 3, fraction 3) contained only a small amount of protein, mainly albumin. After the change from the wash buffer to the acidic formate (pH 2.7), the first two eluted fractions contained very little of the heavy (H) chains of immunoglobulins (fraction 4), but these were followed by fractions (Fig. 3, fraction 5) with increasing amounts. Staining of the proteins in the gel with Fast Green revealed clearly the 50K bands of the H chains of immunoglobulins. The 25K bands of the light (L) chains could be clearly visualized by silver staining (Merril et al., 1981) of the same gel (data not shown). The analysis by the SGIP method of the immunoreactivity of the fractions from the B-50 protein column is presented in Fig. 4. When the immunostaining profile (Fig. 4B) of the original antiserum is compared with the protein staining profile (Fig. 4A) of the same gel, one notices that the anti-B-50 protein serum reacts only with a few protein bands, e.g., the 48K B-50 and 46K B-60 protein band, 67K and some other bands of ASP, whereas all the other bands are nonreactive. Incubation of thin sections of the same gel with isolated IgG of fraction 5 (Fig. 4D) yielded solely specific immunostaining of the B-50 protein in SPM (track 2), and of B-50 and its cleavage product B-60 (Zwiers et al., 1982*a*) in the isolated B-50 protein preparation (track 3) and of the ASP (track 4) preparation. The first effluent, fraction 2 (Fig. 4C) containing unbound serum proteins and contaminating antibodies showed immunostaining of other protein bands (track 4) and the 67K albumin (track 1). The average recovery of immunoreactivity to B-50 was 40%. The content of anti-B-50 IgGs in the antiserum 8002 was about 3% of the total immunoglobulins. In other experiments antibody specificity was dem-

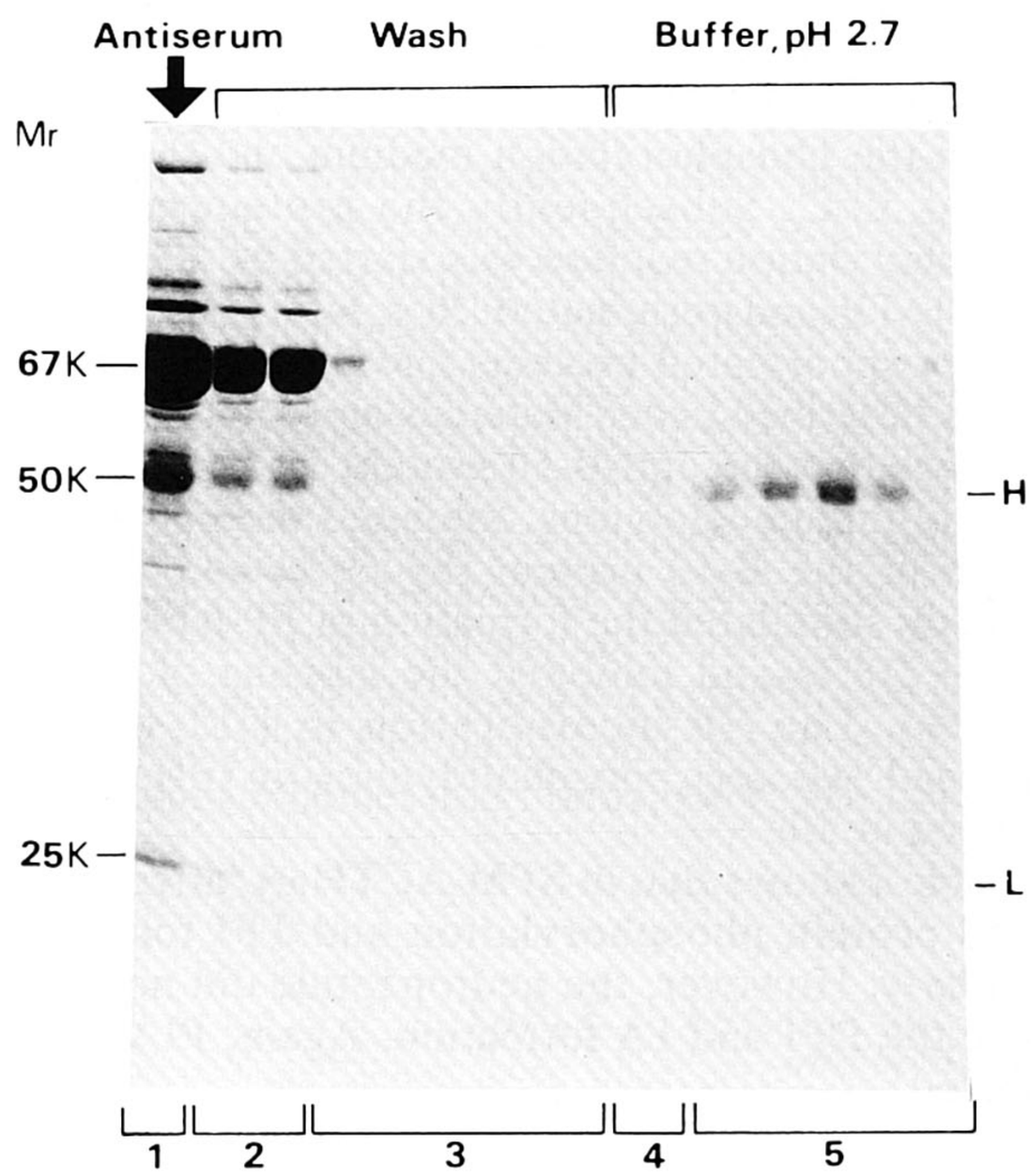


**FIG. 3.** SDS-PAGE of the anti-B-50 protein antiserum and of fractions containing anti-B-50 protein immunoglobulins, obtained after affinity chromatography on a B-50 protein conjugated column. The protein-staining pattern of the fractions subsequently eluted from the affinity column is shown: fraction 1, the antiserum [0.2% (vol/vol) of the amount applied to the immunosorbent]; fraction 2, fractions eluted containing unbound serum proteins; fraction 3, wash fractions; fractions 4 and 5, eluted after treatment with acidic buffer to dissociate the bound antibodies. The heavy (H) and light (L) chains of the immunoglobulins eluted in fraction 5 (2.5% of total recovered IgG).

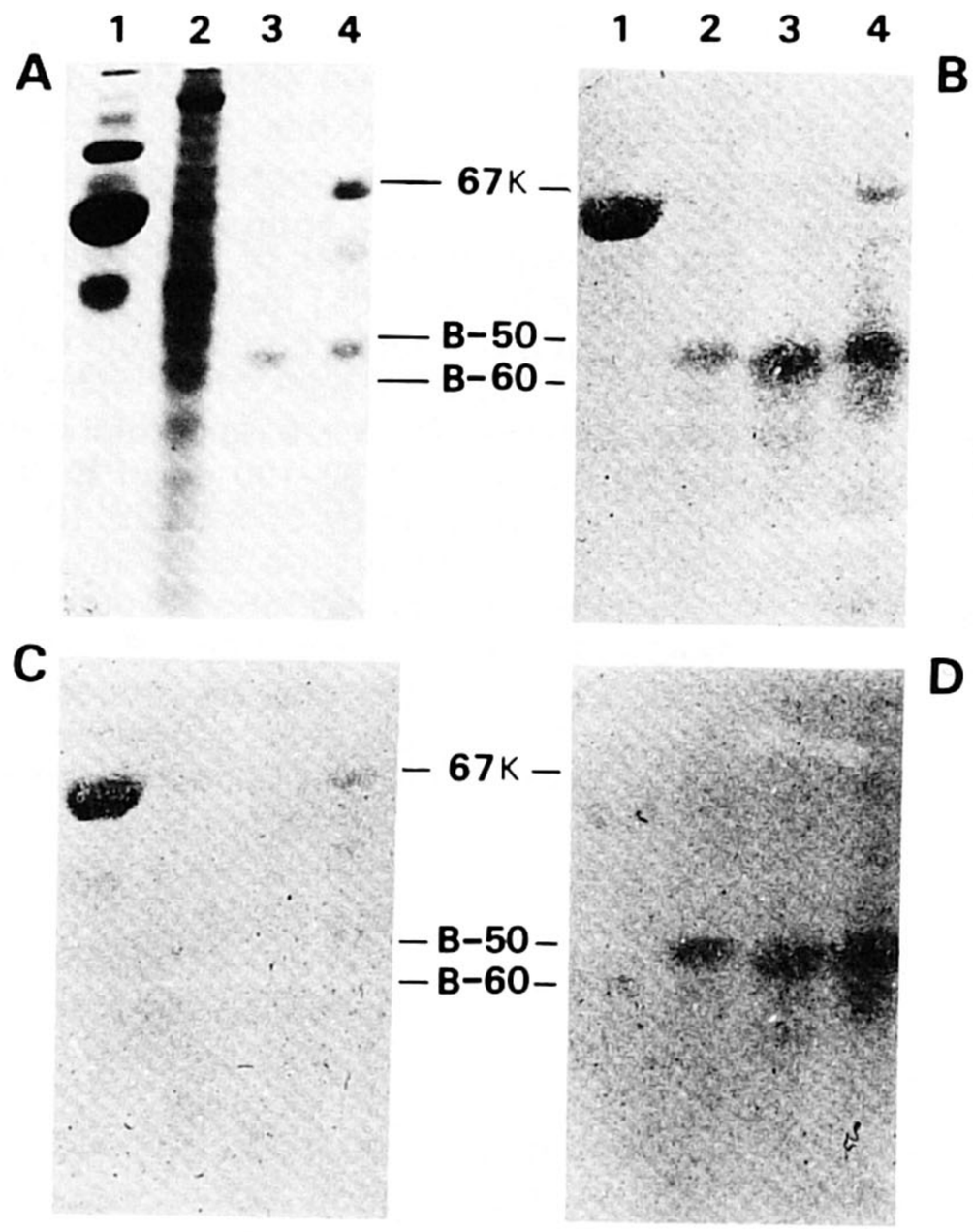


**FIG. 4.** Specificity analysis of anti-B-50 protein antiserum and the purified anti-B-50 protein immunoglobulins by the SGIP method. Samples of various antigens [track 1, 50 μg of rat serum proteins; track 2, 50 μg protein of SPM; track 3, 3 μg B-50 protein and track 4, 20 μg ASP (57–82%) ] were electrophoresized on an 11% (wt/vol) SDS-polyacrylamide gel of 3 mm thickness as described by Oestreicher et al. (1981) and under Materials and Methods. **(A)** Protein pattern of separated bands in the gel. **(B, C, and D)** Immunostaining: B, crude anti-B-50 protein antiserum (1:40 diluted); C, effluent fraction 2 with the unbound serum proteins (1:5 diluted); D, purified anti-B-50 protein immunoglobulins of fraction 5 (0.08 μg protein/μl, 1:15 diluted).

onstrated by absorption. When thin gel sections similar to those of Fig. 4 were incubated with the anti-B-50 antiserum or anti-B-50 IgG previously absorbed with either purified B-50 protein or with PBS washed SPM, no immunostaining of the B-50 and B-60 protein bands could be demonstrated (data not shown). However, the immunostaining of contaminating antibodies (e.g., the 67K band) in the original antiserum was not removed by this absorption (conf. Fig. 4C).

### Effect of anti-B-50 protein antibodies on the endogenous phosphorylation of B-50 protein in rat brain membranes

Figure 5A shows that the purified anti-B-50 protein antibodies (+) inhibited the endogenous phosphorylation of the B-50 protein in SPM (control track 1) by about 55% without affecting the phosphorylation of other major phosphoprotein bands (e.g., 52K). The inhibition of the B-50 phosphorylation was found with both anti-B-50 protein IgG preparations purified from the antisera of the two immunized rabbits. Differences in the endogenous phosphorylation profile were not observed in control incubations of SPM with $^{32}$P-labeled ATP plus or minus control IgG. Addition of increasing amounts of anti-B-50 protein antibodies to a constant quantity of SPM resulted in a dose-dependent inhibition of the endogenous B-50 protein phosphorylating activity (Fig. 5B). Simultaneously, the incorporation of label in other protein bands of SPM was not influenced by the presence of the immunoglobulins. Under the incubation conditions used, 0.3 μg of purified antibodies inhibited the phosphate incorporation in B-50 protein by 50%.

### Effect of anti-B-50 protein antibodies on polyPI metabolism

Jolles et al. (1981*a*) demonstrated that $ACTH_{1-24}$ influenced the endogenous phosphorylation of proteins, one of them being the B-50 protein, and of lipids in a membrane/cytosol fraction prepared from a lysed crude mitochondrial synaptosomal fraction of rat brain. Since the anti-B-50 protein antibodies had an inhibitory effect on the endogenous phosphorylation of B-50 protein in SPM similar to $ACTH_{1-24}$ (Zwiers et al., 1976), the effect of IgG on the phosphorylation of metabolites of polyPI was studied and compared with that on B-50 phosphorylation in these synaptic membranes. SPM was preincubated for 10 min at 0°C with immunoglobulins in the phosphorylation medium and then for 5 min at 30°C. Subsequently the protein and lipid phosphorylation reaction was started by addition of [γ-$^{32}$P]ATP and terminated 20 s later. Analysis of the phosphorylated proteins and lipids was carried out as described under Materials and Methods. Table 1 shows that the anti-B-50 protein IgG, but not the control immunoglobulins, inhibited the phosphorylation of the B-50 protein (by 69%) and simultaneously stimulated the formation of $^{32}$P-labeled TPI (by +218%) and inhibited the labeling of PA (by −63%). The $^{32}$P incorporation in DPI was not changed, whereas phosphatidylinositol (PI) was not labeled at all.

Table 2 shows that in SPM $ACTH_{1-24}$ affected the B-50 protein phosphorylation and TPI formation similarly. However, the neuropeptide did not influence the DPI and PA formation. Again, PI was not labeled.

## DISCUSSION

The present paper describes the purification of anti-B-50 protein antibodies by affinity chromatography on a B-50 protein column and the application of the specific antibodies to study their effect on the phosphorylation of B-50 protein and on polyPI metabolism in SPM.

When the original anti-B-50 protein antiserum was examined only by immunostaining at the position

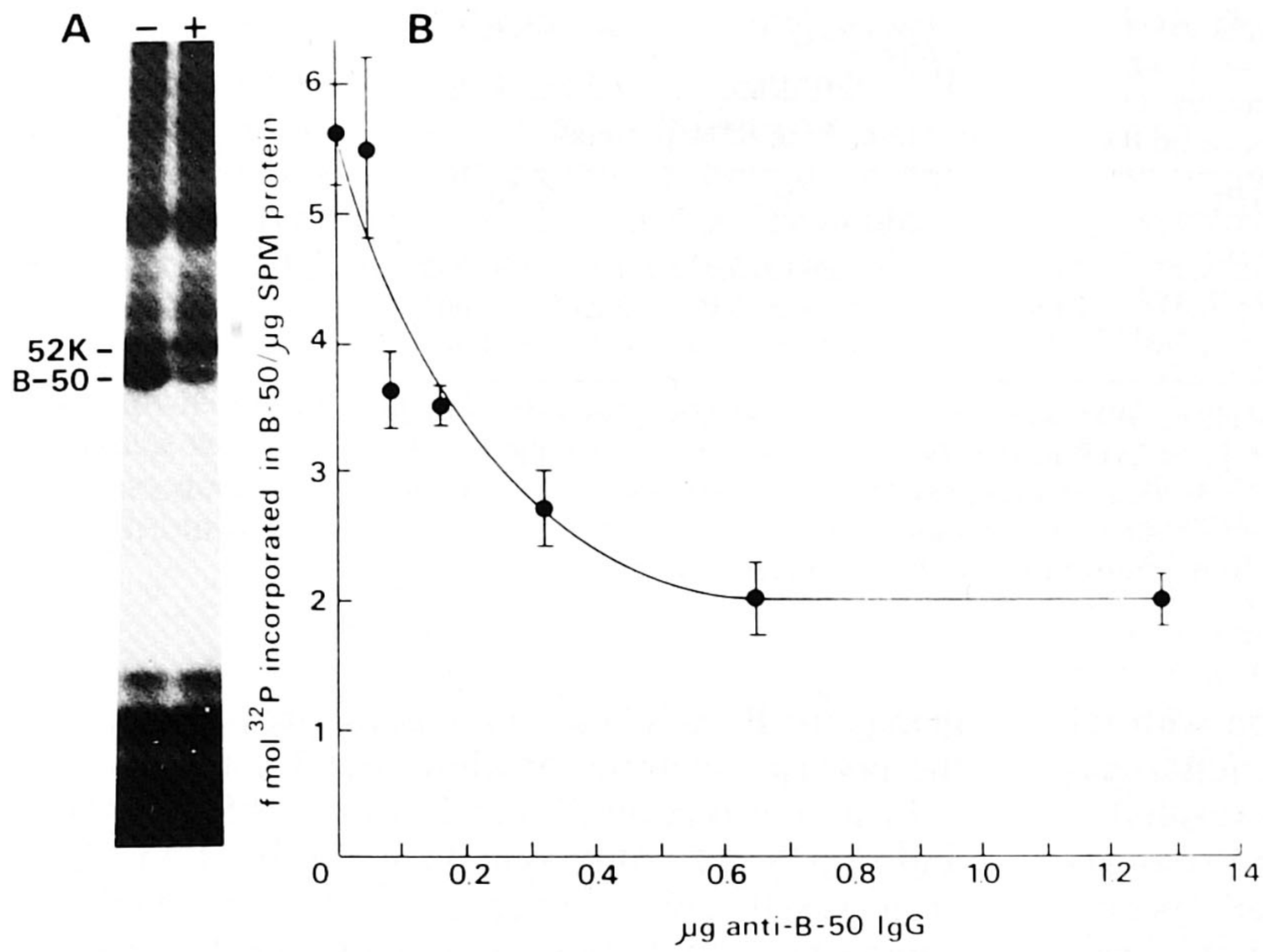


**FIG. 5.** Effect of anti-B-50 protein immunoglobulins on the endogenous phosphorylation of the B-50 protein in SPM. Aliquots (20 μg of protein) of SPM were phosphorylated in the presence of: **(A)** 1 μg of protein of control IgG (–) or of anti-B-50 protein IgG (+); **(B)** increasing amounts (0.03–1.3 μg) of anti-B-50 protein IgG. The phosphorylation assay, analysis by SDS-PAGE, autoradiography, and measurement of incorporation of radioactive phosphate in B-50 were carried out as described under Materials and Methods. SEM (n = 3) is indicated by the bars.

of the B-50 protein of SPM, membranes (Figs. 1 and 4) and homogenate of rat brain were found. With use of other protein fractions like the ASP, enriched in B-50 protein and its protein kinase, immunostaining of other protein bands was observed as well: its specific proteolytic product B-60 protein and some protein bands, e.g., in the region 67-70K (Zwiers et al., 1980, 1982*a*). To study solely the specific interaction of anti-B-50 protein antibodies with phosphorylation processes in membranes, isolation of pure anti-B-50 protein IgG became a prerequisite. This required the availability of amounts of purified B-50 protein larger than used formerly and prompted us to develop an improved purification procedure. The pellet obtained after osmotic shock of the mitochondrial-synaptosomal $P_2$ fraction (Zwiers et al., 1980*a*) was used as additional source of B-50 protein. In this fraction heavy SPMs tend to accumulate (Jones and Matus, 1974; Van Leeuwen et al., 1976). Next, we solubilized the acidic B-50 protein from the membranes by alkaline extraction at pH 11.5, a purification step adapted from the isolation procedure of the basic synaptic protein I (Ueda and Greengard, 1977; Strömbom et al., 1979). The alkaline treatment did not notably affect immunoreactivity of the B-50 enriched fraction, as was shown by the SGIP method (Fig. 1B, tracks 5 and 6). Further isolation of B-50 protein from the neutralized alkaline extract was carried out by similar steps as described formerly by Zwiers et al. (1980*a*). Indeed, the new procedure, Pn, resulted in an increased yield, 10–15 μg B-50 protein/g wet weight of rat brain, whereas that obtained by Po yielded 3 μg of native B-50 protein/g wet weight. The affinity-purified immunoglobulins reacted only with B-50 protein and its proteolytic breakdown product B-60 (Fig. 4D). These antibodies could be completely absorbed by either purified B-50 protein or B-50 pro-

**TABLE 1.** *Effects of anti-B-50 immunoglobulins on $^{32}$P-incorporation into lipids and B-50 protein of SPM*

| Addition | $^{32}$P incorporation (fmol P/μg SPM protein) | | | |
|---|---|---|---|---|
| | TPI | DPI | PA | B-50 |
| None | 5.06 ± 0.36 | 9.80 ± 0.53 | 1.88 ± 0.10 | 2.89 ± 0.07 |
| Anti-B-50 IgG | 16.1 ± 1.1[a] | 8.80 ± 0.57 | 0.70 ± 0.08[a] | 0.90 ± 0.04[a] |
| Control IgG | 5.96 ± 0.63 | 10.2 ± 0.57 | 1.75 ± 0.27 | 2.46 ± 0.07 |

Aliquots (8 μg of protein) of SPM were phosphorylated in the absence or the presence of 1 μg of anti-B-50 IgG or of control IgG. Phosphorylation and assay conditions were as described under Materials and Methods. The results (means ± SEM, n = 3–4) are expressed as fmol P/μg protein/20 s. The experiment was repeated three times with similar results.

[a] Significantly different from control (no addition); Student's *t*-test.

**TABLE 2.** *Inhibition by $ACTH_{1-24}$ of phosphorylation of lipids and B-50 protein in SPM*

| Addition | $^{32}P$ incorporation (fmol P/μg SPM protein) | | | |
|---|---|---|---|---|
| | TPI | DPI | PA | B-50 |
| None | 3.39 ± 0.19 | 7.48 ± 0.38 | 1.26 ± 0.16 | 0.451 ± 0.033 |
| 0.1 m*M* ACTH | 5.58 ± 0.57[a] | 7.06 ± 0.75 | 1.25 ± 0.09 | 0.239 ± 0.005[a] |
| 0.01 m*M* ACTH | 3.82 ± 0.26[a] | 6.31 ± 0.12 | 1.36 ± 0.07 | 0.319 ± 0.008[a] |

Aliquots (13 μg of protein) of SPM were incubated as described under Materials and Methods for 20 s with [γ-$^{32}$P]ATP in the presence or absence of $ACTH_{1-24}$. The results (means ± SEM; n = 3–4) are expressed as fmol P incorporated/μg of protein in 20 s. Similar results were obtained in two other experiments.

[a] Significantly different from control (no addition); Student's *t*-test.

tein containing preparations, but absorption with rat heart homogenate did not remove the immunoreactivity of the anti-B-50 IgGs (unpublished results).

Naito and Ueda (1981) have reported that affinity-purified anti-protein I IgG inhibited specifically the endogenous phosphorylation of this protein in various subcellular fractions and also in rat cerebrum homogenate. This suggested to us that anti-B-50 protein IgGs might also be used as a specific tool to manipulate the phosphorylation state of B-50 protein. Indeed, addition of anti-B-50 protein IgG to SPM markedly inhibited the endogenous phosphorylation of B-50 protein (Fig. 5A). By two-dimensional analysis rat brain SPM has been shown to contain only one phosphoprotein at the 48K position: the B-50 protein (Zwiers et al., 1980*a*, Bär et al., 1982). The anti-B-50 protein IgG was virtually without inhibition on other strongly phosphorylated protein bands of SPM; see, for example, the 52K phosphoband (Fig. 5A). Moreover, we observed inhibition by anti-B-50 protein IgG of the endogenous phosphorylation of solely the antigen protein band in a fraction enriched in synaptic vesicles and myelin containing SPM as contaminant (Zwiers, unpublished). The inhibition was not observed with control IgG. The extent of inhibition was dependent on the dose of anti-B-50 protein IgG. With the highest dose used 64% inhibition was obtained (Fig. 5B). Fifty percent inhibition was already reached with a threefold lower dose (0.3 μg). Steric blocking and other factors (e.g., denaturation of IgG, multiple sites of phosphorylation) may be responsible for the incomplete inhibition. However, one may also assume that at any time a certain fraction of the B-50 protein is buried in the membrane and therefore not accessible to the IgG.

The function of B-50 protein may be intimately related to the subcellular localization. By immunohistochemical staining methods with an anti-B-50 protein antiserum we have determined that B-50 protein is localized throughout rat brain in areas characterized by their abundance of synaptic contacts (Oestreicher et al., 1981). Studies at the ultrastructural level by immuno electron microscopy using anti-B-50 IgG are now in progress to examine the precise synaptic location of B-50.

In studies on polyPI metabolism in ASP (55–80%) and in a lysed crude synaptosomal fraction, Jolles et al. (1980, 1981*a,b*) reported evidence leading to a hypothesis that the state of phosphorylation of the B-50 protein influences the DPI kinase activity and polyPI metabolism in rat brain membranes. Since, similar to $ACTH_{1-24}$, the anti-B-50 IgG were shown to alter the extent of phosphorylation of B-50 in SPM (Fig. 5), we applied the IgG to examine the proposed hypothesis. Table 1 shows that binding of anti-B-50 protein IgG to SPM resulted in inhibition of B-50 protein phosphorylation and concomitantly stimulation of TPI formation. The same correlation was observed when the effect of the anti-B-50 protein IgG was studied at a higher ATP concentration (0.5 m*M*) (data not shown). The effect of the anti-B-50 protein IgG was only partially analogous to that of $ACTH_{1-24}$ on polyPI metabolism in SPM (Table 2). $ACTH_{1-24}$ did not influence the formation of PA in SPM, in contrast with the observations with a lysed synaptosomal fraction (Jolles et al., 1981*a,b*). It may be that the differential effect of $ACTH_{1-24}$ and anti-B-50 protein IgG on PA formation originates from the difference in target of the two ligands, i.e., B-50 protein kinase versus B-50 protein (Zwiers et al., 1982*b*). There appears to be another regulatory link between polyPI turnover and the phosphorylation state of the B-50 protein. Aloyo et al. (1981, 1982) found that purified B-50 protein kinase (Zwiers et al., 1980*a*) and lipid-activated protein kinase C purified from the particulate fraction of rat brain (Kuo et al., 1980) shared several important properties (e.g., similar molecular weight, isoelectric point, and peptide map).

Several models have been proposed to explain the significance of the interaction of protein kinase/B-50 protein and polyPI metabolism for the events occurring in the membrane during neurotransmission (Jolles et al., 1982; Takai et al., 1982). Our hypothesis states that interaction of neuropeptide(s) with the synaptic membrane will influence membrane characteristics by effects on the following

chain of reactions: B-50 protein kinase → B-50 protein → DPI kinase, possibly with consequences for the calcium influx (Zwiers et al., 1982*b*). In conclusion, at present we report experiments that further support our hypothesis of the possible regulatory role of the B-50 protein in polyPI metabolism of rat brain SPM.

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