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Evidence That the Synaptic Phosphoprotein B-50 Is Localized Exclusively in Nerve Tissue

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Abstract: The localization of the phosphoprotein B-50 (molecular weight 48,000, isoelectric point 4.5) in the rat has been studied. Inspection of endogenous phosphorylation patterns of the particulate as well as the cytosolic subcellular fractions from a variety of peripheral organs failed to demonstrate phosphorylation of a molecular weight 48,000 protein. Only in the particulate fractions from brain tissue was there endogenous phosphorylation of the B-50 protein. Two-dimensional analysis (isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis) and an immunochemical detection method employing an anti-B-50 antiserum revealed the presence of B-50 in particulate material from brain, but not in that of other tissues. Therefore the data were interpreted as pointing to the localization of B-50 in nervous tissue. In addition, the regional distribution of endogenous B-50 phosphorylation was studied using synaptosomal plasma membranes (SPM) obtained from individual rat brain regions. The highest value was found in SPM of septal origin, the lowest in SPM from the medulla spinalis. The relationship of the high value for B-50 phosphorylation in the septum to the sensitivity of that brain area to $ACTH_{1-24}$ is discussed. **Key Words:** Phosphoprotein—Immunochemistry—$ACTH_{1-24}$—Brain-specific protein. **Kristjansson G. I. et al.** Evidence that the synaptic phosphoprotein B-50 is localized exclusively in nerve tissue. *J. Neurochem.* **39**, 371–378 (1982).

Exposure of rat synaptosomal plasma membranes to [γ-^{32}P]ATP results in the labeling of a number of constituent phosphoproteins (Rodnight et al., 1975). Several different endogenous membrane protein kinases have been characterized by their sensitivity to cyclic nucleotides, calcium/calmodulin, lipids, and neuropeptides (Greengard, 1978; Rodnight, 1979; DeBlas et al., 1979; Nishizuka, 1980; Zwiers et al., 1980*a*). One such kinase, characterized by its inhibition by ACTH and other behaviorally active neuropeptides (Zwiers et al., 1978; 1980*b*; 1981), has been isolated and purified from rat brain membranes (Zwiers et al., 1979; 1980*a*). One of its endogenous substrate proteins, B-50 (molecular weight 48,000, isoelectric point 4.5; Zwiers et al., 1980*a*) was shown by immunohistochemical techniques to have a synaptic localization throughout the brain (Oestreicher et al., 1981). Recently, Sorensen et al. (1981), who used a variety of subcellular fractionation techniques, reported on the intrasynaptic localization of this B-50 protein. They concluded that it is a constituent of presynaptic membranes of a low buoyant density. In the present paper we report, on the basis of one- and two-dimensional separation procedures in combination with immunochemical detection methods, that the B-50 phosphorylation could be found only in particulate fractions prepared from nervous tissue, and not in liver, heart, lung, adrenal cortex, skeletal muscle, and spleen. In addition, we describe the regional distribution of the endogenous B-50 phosphorylating activity in rat brain.

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Abbreviations used: IEF, Isoelectric focusing; IEP, Isoelectric point; SDS, Sodium dodecyl sulfate; SGIP, SDS-gel immunoperoxidase; SPM, Synaptosomal plasma membranes.

MATERIALS AND METHODS

Tissue dissection

Male Wistar rats of an inbred strain (TNO, Zeist) were used for all experiments. The rats, weighing 150–200 g, were killed by decapitation, and the brain, liver, spleen, heart, adrenal cortex, lung, and skeletal muscle were carefully dissected. In some experiments, the brains were further dissected into medulla spinalis, medulla oblongata, mesencephalon, thalamus, hypothalamus, septum, hippocampus, caudal and rostral cortex cerebrum, neostriatum, and cerebellum according to the method of Gispen et al. (1972).

Subcellular fractionation

Crude membrane and cytosol fraction. Heart and muscle were minced with a McIlwain tissue chopper. The other tissues (liver, spleen, adrenal cortex, lung, and brain) were cut into small pieces with scissors. All tissue fractions were weighed and homogenized in 9 volumes of 0.25 *M* sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle (700 r.p.m.; clearance 0.125 mm; five up-and-down strokes). The homogenates were centrifuged for 10 min at 1000 *g* to remove cell debris, and the resulting supernatant was centrifuged in the Beckman 50-Ti rotor at 150,000 *g* for 30 min. The supernatant of this separation was taken as the cytosol fraction and the pellet as the particulate fraction (see also Uno et al., 1976). The particulate fraction was subjected to an osmotic shock in the hypotonic buffer A (10 m*M* sodium acetate, 10 m*M* magnesium acetate, 1 m*M* calcium acetate, pH 6.5), centrifuged for 30 min at 150,000 *g*, and subsequently resuspended in buffer A (5 mg protein/ml).

Synaptosomal plasma membranes. Total rat brain tissue or the individual brain regions from 10 rats were separately homogenized in 0.32 *M* sucrose (1:10, wt/vol) with a glass Potter-Elvehjem homogenizer (see above). Aliquots of 3.5 ml of the homogenates were used to isolate synaptosomal plasma membranes according to a modification of the procedure described by Zwiers et al. (1976). In short, the P_2 fraction was lysed and centrifuged at 10,000 *g* for 20 min. The resulting supernatant (3.5 ml) was layered on a discontinuous sucrose gradient (8 ml 1.0 *M*, 4 ml 0.4 *M*) and spun for 80 min at 100,000 g_{max} in the Beckman SW-27.1 rotor. The material floating on the 1.0-*M* layer was collected, washed, and resuspended in buffer A (5 mg protein/ml).

Phosphorylation assay

Endogenous phosphorylation of proteins was studied as described by Zwiers et al. (1976), with minor modifications. In short, 20 μg of protein from the fraction to be assayed was preincubated for 5 min at 30°C (volume of 20 μl). The phosphorylation reaction was started by the addition of 2 μCi of [γ-^{32}P]ATP (5 μl, containing 187 pmol ATP). The reaction was terminated by the addition of 12.5 μl of a stop solution containing sodium dodecyl sulfate (SDS).

When the effect of $ACTH_{1-24}$ (Organon Int. BV, Oss, The Netherlands) was studied, this peptide was added in a volume of 5 μl of buffer A 10 s prior to the addition of the ATP.

Separation of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins were separated on SDS polyacrylamide slab gels (acrylamide 11%; bisacrylamide 0.2%) as described previously (Zwiers et al., 1976). After termination of the run, to visualize the separated proteins the gels were stained with Fast Green, destained, and dried. Incorporation of [^{32}P]phosphate into individual proteins was detected by autoradiography using Kodak Royal X-Omat x-ray film (see Zwiers et al., 1976). Quantitative determination of the radioactivity incorporated into the phosphoprotein B-50 was achieved by liquid scintillation counting as described by Wiegant et al. (1978).

Two-dimensional analysis of proteins

Proteins from the cytosol and particulate fractions of all the tissues and from synaptosomal plasma membranes (SPM) from brain tissue were separated in the first dimension by isoelectric focusing (IEF) in polyacrylamide slab gels. The procedure for casting and running the IEF gel was similar to that described by Zwiers et al. (1980*a*), except that the gel used here contained 8.5 *M* urea and 0.5% Triton X-100. The protein samples (5–100 μg of protein, volume 20 μl) were pretreated with urea and Triton X-100, resulting in the final concentrations of 8.5 *M* urea and 0.5% Triton X-100. The mixtures were vigorously shaken for 1 min on a Vortex mixer, and subsequently ampholines (2.5%, wt/vol; pH range 3.5–10.0) and sucrose (5%, wt/vol) (final concentration) were added. The IEF gel was run overnight at 200 V at room temperature. Individual tracks were excised and incubated for 30 min in 8 ml of the following wash solution: 62.5 m*M* Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.001% bromophenol blue; and 5% 2-mercaptoethanol. These washed tracks were run in the second dimension on SDS slab gels as described by Zwiers et al. (1980*a*). Gels were stained for protein with Fast Green and (in some cases) dried and subjected to autoradiography.

Determination of protein

Quantitative protein determinations were carried out by the method of Lowry et al. (1951) and, in the case of the cytosolic fractions, by the method of Spector (1978).

Immunochemical detection of B-50

Antiserum. The antigen, the B-50 protein, was prepared according to the procedure described by Zwiers et al. (1980*a*). B-50 protein, 100 μg dissolved in 1 ml saline, was mixed with the same volume of Freund's complete adjuvant. This mixture was used for the immunization of one white New Zealand rabbit. The immunization was effected by intradermal injections on multiple sites on the back of the animal. The injection was repeated after 5 weeks. After two immunizations an antiserum was produced which reacted with the B-50 protein. The specificity of the antiserum was checked as described by Oestreicher et al. (1981).

Immunochemical detection. The particulate fractions (100 μg protein/sample) obtained from various rat tissues were analyzed by means of the SDS-gel–immunoperoxidase (SGIP) method of van Raamsdonk et al. (1977). This method was carried out as described previously (Oestreicher et al., 1981). In order to decrease background staining and to increase the sensitivity of the method, the detection by goat anti-rabbit IgG peroxidase was replaced by the unlabeled antibody enzyme method of Sternberger (1974). Gel sections were incubated with 300 μl of an appropriate dilution (usually 1:40) of antiserum. Control sections were incubated with normal and preimmune serum to check for nonspecific adsorp-

ion of immunoglobulins. Control sections showed no immunostaining of the B-50 protein.

RESULTS

Protein profiles and autoradiograms of the particulate cell fractions

The particulate cell fraction obtained from a variety of tissues was used in an endogenous phosphorylation assay. Subsequently the proteins of these fractions were separated by SDS polyacrylamide gel electrophoresis (PAGE). The protein profile as obtained after staining with Fast Green is shown in Fig. 1A. This picture clearly shows a marked heterogeneity among the tissues studied. It is especially evident in the region of interest containing proteins within the M_r range 40,000–60,000. In particular, heart and adrenal tissues seem to have a relatively large amount of protein in this region (lanes 1 and 7). In contrast, the particulate fractions of lung and spleen contain relatively few proteins in this region (lanes 2 and 4). Comparison of crude brain particulate material (lane 6) with the purified synaptic plasma membrane fraction (lane 8) demonstrates that in SPM fewer proteins are present. The arrow at the 48,000 mark denotes the presumed position of the B-50 protein. Certainly this band may consist of more than one protein; therefore, two-dimensional separation and autoradiography of phosphorylated B-50 were essential.

By far the most active endogenous phosphorylation takes place in the particulate material from brain (Fig. 1B, lane 6). The SPM fraction (lane 8) shows a marked phosphorylation of a protein of M_r 48,000. The phosphorylation is exclusively in protein B-50 (Zwiers et al., 1980*a*). Judging from the autoradiogram after one-dimensional separation by SDS-PAGE, it seems that only particulate fractions of brain tissue contain a labeled phosphoprotein of M_r 48,000 (lanes 6 and 8). In nearly all crude particulate fractions a phosphoprotein band at 43,000–45,000 is visible, but this band was not detected in SPM.

Identification of B-50 by molecular weight and isoelectric point

Previously it was demonstrated by two-dimensional separation techniques that the B-50 protein has an M_r of 48,000 and an isoelectric point (IEP) of 4.5 (Zwiers et al., 1980*a*). Using these properties as a guide in identifying B-50, we performed two-dimensional separation of the proteins of the 1000 *g* supernatant and the crude particulate and cytosol fractions of the various tissue homogenates. The protein patterns were compared with that obtained from SPM. The lowest amount of SPM protein that gives detectable B-50 staining is approximately 5 μg. Thus, absence of B-50 staining indicates that there is less B-50 than that present in 5 μg of SPM protein. The detection of the exact position of B-50 was ensured by adding a minimal quantity of phosphorylated B-50 (1000 cpm [^{32}P]-B-50, corresponding to approximately 1 ng of B-50, prepared according to Zwiers et al., 1980*a*) to the samples of the other tissues prior to the two-dimensional separation. Autoradiography was used

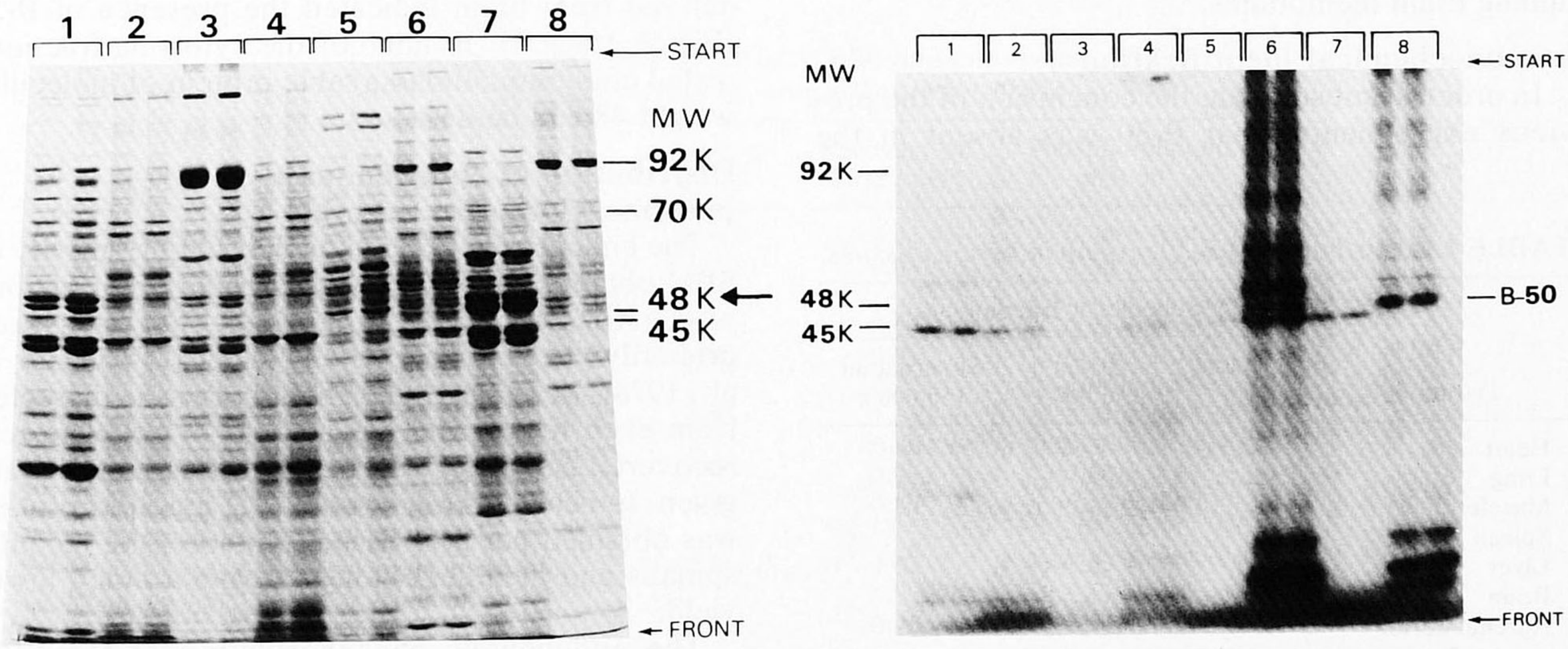

FIG. 1. Protein pattern and corresponding autoradiograph of membrane fractions from various tissues. Duplicate samples of 20 μg of protein from the crude membrane fraction obtained from various tissues and from SPM were phosphorylated under standard assay conditions. The proteins were separated on a SDS slab gel, stained, dried, and subjected to autoradiography. The numbers at the top refer to the tissues used: 1. heart; 2. lung; 3. muscle; 4. spleen; 5. liver; 6. brain; 7. adrenal cortex; 8. SPM. (**A,** *left*) Protein staining pattern. At the right the positions of standard proteins, such as phosphorylase b (92,000), the B-50 protein kinase (70,000), B-50 (48,000, arrow), and ovalbumin (45,000), are indicated. (**B,** *right*) The corresponding autoradiogram. At the right the phosphoprotein B-50 is indicated. The presented experiment was repeated twice with similar results.

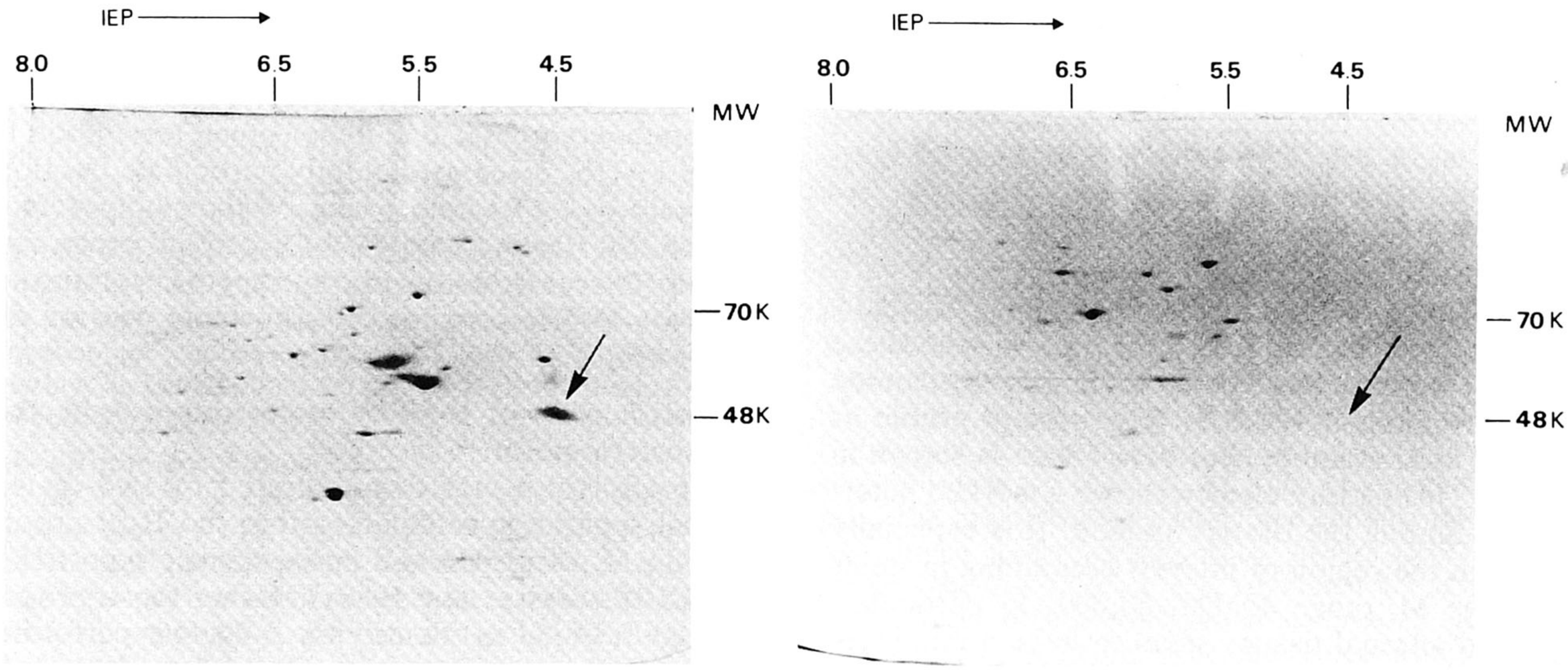

FIG. 2. Typical two-dimensional protein patterns of SPM (**A,** *left*) and crude membrane fraction of spleen (**B,** *right*). Both patterns are from 100 μg total protein. The arrow indicates the position of B-50; at the top the pH gradient is given, at the right side the position of relevant M_r markers. The analysis was repeated twice with similar results.

to detect this labeled protein after separation. In Fig. 2A the two-dimensional separation pattern of SPM from rat brain cortex is shown, and the position of the B-50 protein is indicated by a black arrow. In Fig. 2B the comparable pattern of spleen particulate material is shown, and here the black arrow signifies the absence of stained material at the spot where B-50 is found in SPM. In Table 1 a summary of the results of the two-dimensional analysis of the presence of B-50 is given. These results show that B-50 could only be detected in fractions containing brain membranes.

Immunochemical identification

In order to substantiate the conclusion of the previous experiments—that B-50 was absent in the particulate fractions of tissues other than the brain—the SGIP method using antiserum to B-50 was carried out with thin longitudinal gel sections containing the separated proteins from the particulate and cytosolic fractions of heart, lung, skeletal muscle, spleen, brain adrenal cortex, and liver. SPM was used as reference. In Fig. 3 the results obtained with the particulate fractions and SPM are shown. In SPM the only protein band that stained coincided with the 48,000 band (Fig. 3, lane 8), whereas of the seven particulate fractions only that derived from brain indicated the presence of B-50 (Fig. 3, track 5). In none of the cytosolic fractions tested could immunoassayable protein of molecular weight 48,000 be detected.

TABLE 1. *Distribution of B-50 protein in various tissues*

Tissue	Subcellular fraction		
	Supernatant 1000 *g*	Pellet 150,000 *g*	Supernatant 150,000 *g*
Heart	–[a]	–	–
Lung	–	–	–
Muscle	–	–	–
Spleen	–	–	–
Liver	–	–	–
Brain	+	+	–
Adrenal cortex	–	–	–

The presence of B-50 was determined by visual inspection of the two-dimensional protein staining patterns.

[a] Minus (–) indicates that there is less B-50 in 100 μg of total protein applied on the gel than in the lowest amount of SPM protein (5 μg) that gives detectable B-50 staining. + indicates the presence of B-50 protein. Results are based on two independent experiments.

Distribution of endogenous B-50 phosphorylation in rat brain

The endogenous B-50 phosphorylating activity of SPM obtained from various dissected brain regions was estimated under conditions that measured primarily the initial reaction velocity (Wiegant et al., 1978). In Table 2, the amount of tissue dissected from each region and the amount of SPM protein recovered per g wet weight of starting material are given. In nearly all regions 0.8–1.1 mg SPM protein was obtained per g of tissue. Notably, the medulla spinalis and medulla oblongata gave a much lower yield.

The endogenous phosphorylation of the SPM from the various regions was studied with 20 μg of SPM protein per assay. After the completion of the assay, the SDS-gel protein staining patterns of all 11 SPMs were quite similar (data not shown). However, the endogenous phosphorylation patterns with respect to B-50 were different. Low B-50 phosphor-

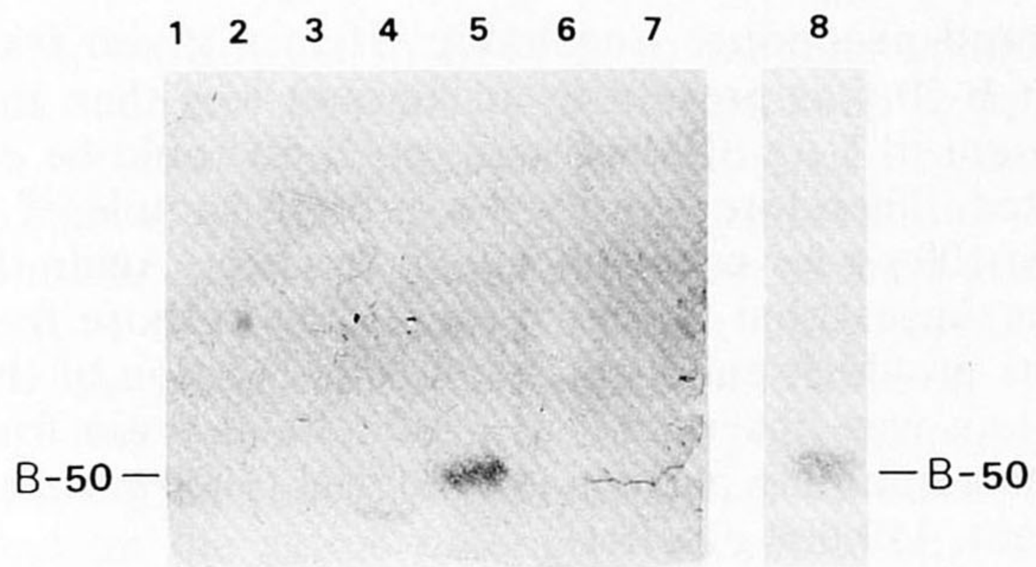

FIG. 3. Immunochemical detection of B-50 on sections of slab gels by the SGIP method. The samples tested are the particulate fractions obtained from 1. liver; 2. heart; 3. lung; 4. muscle; 5. brain; 6. spleen; 7. adrenal cortex. Track 8 shows the immunostaining of a gel section containing SPM. The position of B-50 is indicated. The immunochemical analysis was performed three times, with the use of two separately isolated batches of particulate fractions.

ylation was observed in medulla spinalis, medulla oblongata and cerebellum. Regions with high activity were septum, cortex cerebrum, neostriatum and hippocampus. In Fig. 4, the SPM protein pattern and autoradiogram of the regions with lowest B-50 phosphorylation (medulla spinalis) and the highest (septum) are shown. As can be seen in panel A of Fig. 4, there are only minute differences between the two regions with respect to SPM protein composition. However, there is a slight overall enhancement of phosphorylating activity and a marked increase in the phosphorylation of B-50 in the septum as compared to the material obtained from medulla spinalis (Fig. 4, panel B).

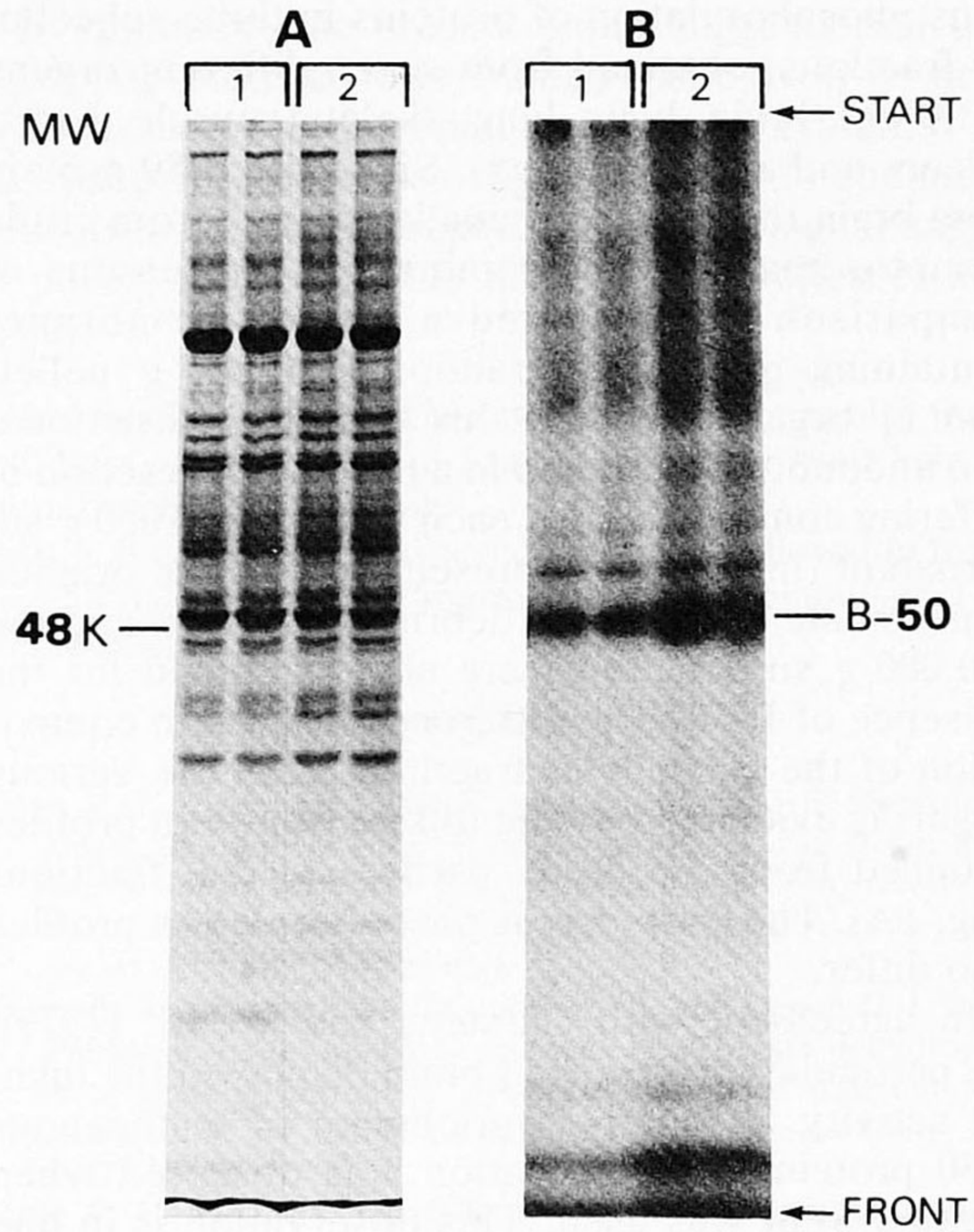

FIG. 4. Comparison of endogenous phosphorylation of duplicate samples (20 μg total protein) of SPM obtained from medulla spinalis (1) and septum (2). **(A)** Protein staining pattern; **(B)** the corresponding autoradiogram.

DISCUSSION

In the present paper we have presented evidence for a specific localization of the phosphoprotein B-50 in nervous tissue. We monitored the endoge-

TABLE 2. *Amounts of tissue, SPM, and endogenous B-50 phosphorylation in various rat brain areas*

Brain area	Amount of tissue[a]	Recovery of SPM (mg)[b]	Phosphorylation of B-50[c]
Medulla spinalis	0.9	0.44	0.38 ± 0.01
Medulla oblongata (including pons)	1.5	0.44	1.50 ± 0.02
Mesencephalon	1.5	0.64	5.61 ± 1.30
Hypothalamus	0.5	0.91	4.16 ± 0.28
Thalamus	0.4	0.77	5.68 ± 0.01
Neostriatum	0.6	1.14	8.41 ± 0.03
Septum	0.1	0.86	10.83 ± 0.20
Hippocampus	0.9	0.93	7.17 ± 0.86
Cortex cerebri (caudal)	4.0	0.84	9.00 ± 0.40
Cortex cerebri (rostral)	2.9	1.03	8.63 ± 0.06
Cerebellum	2.0	0.76	2.73 ± 0.07

[a] Total amount of tissue per area obtained from 10 rats.
[b] Recovery of mg SPM protein per g wet weight tissue.
[c] B-50 phosphorylation in the different SPMs was determined under standard phosphorylation conditions (see Materials and Methods). Incorporation is expressed as fmol phosphate in B-50 per μg total SPM protein. Each value represents the mean ± SEM (n = 3). This experiment was repeated twice with similar results.

nous phosphorylation of proteins in three subcellular fractions, obtained from seven different organs in the rat (brain, liver, lung, skeletal muscle, heart, spleen, and adrenal cortex). Since the B-50 protein from brain tissue was originally isolated from crude synaptosomal plasma membranes, for reasons of comparison we prepared a crude, membrane-containing particulate fraction (150,000 *g* pellet) from all organs. Because this subcellular fractionation undoubtedly resulted in a particulate fraction of differing composition for each organ, the 1000 *g* supernatant (in essence representative of the original homogenate without cell debris and nuclei) and the 150,000 *g* supernatant were also examined for the presence of B-50. The difference in protein composition of the subcellular fractions from the various organs is evidenced by the different protein profiles obtained from the crude particulate cell fractions (Fig. 1A). The endogenous phosphorylation profiles also differ.

In agreement with Carstens and Weller (1979), the particulate fractions of brain displayed the highest activity. A relative enrichment of endogenous B-50 protein phosphorylation was observed when purified SPM was used. This observation is in line with previous studies indicating that in brain tissue the B-50 protein is mainly found in regions rich in synaptic projections (Oestreicher et al., 1981) and, in the synaptic region, predominantly in the presynaptic membranes (Sorensen et al., 1981). Endogenous phosphorylation of a protein of M_r 48,000, which could be inhibited by $ACTH_{1-24}$, was only observed in the 1000 *g* supernatant and particulate fraction from brain. The absence of a phosphorylated protein band of M_r 48,000 in the other organs studied is not in itself convincing evidence that the phosphate acceptor protein was not present. For in the absence of the corresponding protein kinase, no phosphorylation of B-50 would be measured, despite the fact that B-50 itself was present in the fractions studied. In preliminary experiments with brain SPM, it was found that addition of purified B-50 protein kinase only slightly enhanced the phosphorylation of endogenous B-50 as compared with the phosphorylation of the B-50 by the endogenous B-50 kinase. Even combination of purified B-50 and purified B-50 kinase leads to trace amounts of radiolabeled B-50 (Zwiers et al., 1980*a*). Thus, the use of the appropriate kinase to monitor the presence of the acceptor protein did not appear to be a fruitful approach. Therefore, we focused on two-dimensional separation and immunochemical identification of the B-50 protein itself.

The two-dimensional separation technique, consisting of IEF followed by SDS-PAGE, could only be used in a qualitative manner, since the scanning of the protein patterns obtained was highly dependent on the shape of the various stained protein spots, rendering the peak-heights-above-background procedure unreliable. If in a given fraction B-50 was present in an amount less than that present in 5 μg SPM protein, no B-50 could be detected. Therefore, we used a protein sample of at least 100 μg for each subcellular fraction. Again the data suggest that B-50 is present only in those fractions in which endogenous phosphorylation of this protein was observed, e.g., subcellular brain fractions containing particulate material (1000 *g* supernatant, 150,000 *g* pellet).

To substantiate further this tentative conclusion that B-50 is exclusively localized in brain particulate material, gel sections containing SDS-PAGE-separated proteins obtained from the various subcellular fractions were incubated with the antiserum to B-50. Under the conditions of the SGIP method, the B-50 present in 1 μg of SPM per section could be demonstrated clearly. Thus, in agreement with our two-dimensional PAGE results, this immunochemical procedure did not demonstrate the presence of B-50 in cell fractions of organs other than the particulate fraction of brain tissue.

The antiserum to B-50 showed some staining with that of M_r 44,000 protein in muscle (Fig. 3, track 4). However, control sections incubated with preimmune serum also revealed immunostaining of the same protein band in muscle membranes, implying that the antibodies to a muscle protein were present in the rabbit serum at the start of the immunization.

Finally, we studied the regional distribution of endogenous B-50 phosphorylating activity in brain tissue. From our previous immunohistochemical studies (Oestreicher et al., 1981) it appeared that the B-50 protein can be found in regions rich in synaptic projections throughout the brain. Two-dimensional analysis of SPM from various regions reveals no phosphoprotein with a molecular weight near 48,000, other than B-50 (data not shown). Others have reported on the involvement of multiple protein kinases in the endogenous phosphorylation of brain proteins (Huttner et al., 1981; Kennedy and Greengard, 1981), and thus some caution is warranted in making the assumption that the same protein kinase/phosphoprotein system is studied in all brain regions (Table 2). However, recent evidence of Aloyo et al. (1982) suggests that the B-50 protein is exclusively phosphorylated by its corresponding protein kinase (Zwiers et al., 1980*a*) and not by various other protein kinases.

These data and the fact that all B-50 phosphorylating systems were found to be inhibited by $ACTH_{1-24}$ suggest that the same B-50 phosphorylating system is present in all the regions studied. Table 2 shows a difference in the phosphorylation of endogenous B-50 between the brain regions studied. Under the conditions used for these experiments, the phosphorylation observed is entirely due to the initial rate (Wiegant et al., 1978). The highest value was found in SPM from septal

origin, the lowest in SPM from medulla spinalis. With such an approach, one should keep in mind that the data thus obtained are influenced by both the kinase activity present in the preparations and the initial degree of phosphorylation of B-50 at the onset of the endogenous phosphorylation assay *in vitro*. As previously documented, it is unlikely that persisting eventual differences in phosphatase activity during the phosphorylation assay have any effect on the actual extent of incorporation (Wiegant et al., 1978; Zwiers et al., 1978). It is also unlikely that the observed differences in endogenous B-50 phosphorylation are due to differences in the degree of contamination with other subcellular constituents of the SPM preparations used. This is shown by the absence in the various SPM preparations (Fig. 4) of specific phosphoproteins, namely: (1) the α subunit of pyruvate dehydrogenase (45,000) localized in mitochondria (Morgan and Routtenberg, 1981; Browning et al., 1981); (2) tubulin (52,000) localized in synaptic vesicles (Burke and DeLorenzo, 1981); and (3) myelin basic proteins (15,000–20,000) localized in myelin (Sulakhe et al., 1980). Phosphorylation of these marker phosphoproteins is high in the crude particulate fraction of brain (Fig. 1B, lane 6), but absent from the SPM preparations. The fact that the protein staining patterns of the SPM obtained from the different regions of the brain all contain B-50 and are so markedly similar makes it unlikely that the observed phosphorylation differences are due to regional differences in protein composition. Yet, unknown variables may have added to the observed regional differences. In view of the above, one is forced to interpret such differences with caution. However, it may be that these data actually reflect meaningful differences in an endogenous membrane protein complex across brain regions.

It is of interest that in SPM of septal origin $ACTH_{1-24}$ is at least 10 times more effective in inhibiting B-50 protein kinase compared with SPM from whole brain (Zwiers et al., 1982). The septal area has previously been reported to be essential for the expression of some of the behavioral effects of ACTH-like peptides (Van Wimersma Greidanus et al., 1975). Also, other studies point to a special relationship between ACTH and the septum: stereospecific uptake mechanism (Verhoef et al., 1977*a,b*); specific transport of pituitary ACTH into the septal complex (Mezey et al., 1978); ACTH-containing nerve fiber projections (Krieger et al., 1980) and ACTH-induced increase in septal cyclic AMP *in vivo* (Wiegant et al., 1979).

That a neuropeptide modulates the degree of phosphorylation of a membrane phosphoprotein may underlie some of its behavioral activities, the specificity of the ACTH–B-50 protein kinase interaction being provided by local delivery at peptidergic synapses in the brain.

In summary, the present paper suggests that the phosphoprotein B-50 is located exclusively in nervous tissue. The fact that it is primarily found in presynaptic membranes suggests a role in chemical neurotransmission, presumably involving changes in polyphosphoinositide metabolism (Jolles et al., 1980).

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REFERENCES

Aloyo V. J., Zwiers H., and Gispen W. H. (1982) B-50 protein kinase and kinase C in rat brain, in *Progress in Brain Research, Vol. 56: Brain Phosphoproteins—Characteristics and Function* (Gispen W. H. and Routtenberg A., eds.), (in press). Elsevier, Amsterdam.

Browning M., Baudry M., Bennett W. F., and Lynch G. (1981) Phosphorylation-mediated changes in pyruvate dehydrogenase activity influence pyruvate-supported calcium accumulation by brain mitochondria. *J. Neurochem.* **36**, 1932–1940.

Burke B. E. and DeLorenzo R. J. (1981) Ca^{2+} and calmodulin dependent phosphorylation of neurotubulin. *Proc. Natl. Acad. Sci. USA* **78**, 991–995.

Carstens M. and Weller M. (1979) A comparison of the intrinsic protein kinase activities of membrane preparations from various tissues. *Biochim. Biophys. Acta* **551**, 420–431.

DeBlas A. L., Wang Y. J., Sorensen R., and Mahler H. R. (1979) Protein phosphorylation in synaptic membranes regulated by adenosine 3′:5′-monophosphate: Regional and subcellular distribution of the endogenous substrates. *J. Neurochem.* **33**, 647–659.

Gispen W. H., Schotman P., and De Kloet E. R. (1972) Brain RNA and hypophysectomy: A topographical study. *Neuroendocrinology* **9**, 285–296.

Greengard P. (1978) Phosphorylated proteins as physiological effectors. Protein phosphorylation may be a final common pathway for many biological regulatory agents. *Science* **199**, 146–152.

Huttner W. B., DeGennaro L. J., and Greengard P. (1981) Differential phosphorylation of multiple sites in purified protein I by cyclic AMP-dependent and calcium-dependent protein kinases. *J. Biol. Chem.* **256**, 1482–1488.

Jolles J., Zwiers H., Van Dongen C., Schotman P., Wirtz K. W. A., and Gispen W. H. (1980) Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation. *Nature* **286**, 623–625.

Kennedy M. B. and Greengard P. (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. *Proc. Natl. Acad. Sci. USA* **78**, 1293–1297.

Krieger D. T., Liotta A. S., Brownstein M. J., and Zimmerman E. A. (1980) ACTH, β-lipotropin and related peptides in brain, pituitary and blood. *Recent Prog. Horm. Res.* **36**, 272–344.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Mezey E., Palkovits M., De Kloet E. R., Verhoef J., and De Wied D. (1978) Evidence for pituitary-brain transport of a behaviorally potent ACTH analog. *Life Sci.* **22**, 831–838.

Morgan D. G. and Routtenberg A. (1981) Evidence that a 41,000 dalton brain phosphoprotein is pyruvate dehydrogenase. *Biochem. Biophys. Res. Commun.* **95**, 569–576.

Nishizuka Y. (1980) Three multifunctional protein kinase systems in trans-membrane control. *Mol. Biol. Biochem. Biophys.* **32**, 113–135.

Oestreicher A. B., Zwiers H., Schotman P., and Gispen W. H. (1981) Immunohistochemical localization of a phosphoprotein (B-50) isolated from rat brain synaptosomal plasma membranes. *Brain Res. Bull.* **6**, 145–153.

Rodnight R., Reddington M., and Gordon M. (1975) Methods for studying protein phosphorylation in cerebral tissues, in *Research Methods in Neurochemistry, Vol. 3* (Marks N. and Rodnight R., eds), pp. 324–367. Plenum Press, New York.

Rodnight R. (1979) Cyclic nucleotides as second messenger in synaptic transmission. *Int. Rev. Biochem.* **26**, 1–80.

Sorensen R. G., Kleine L. P., and Mahler H. R. (1981) Presynaptic localisation of phosphoprotein B-50. *Brain Res. Bull.* **7**, 57–61.

Spector T. (1978) Refinement of the Coomassie Blue method of protein quantitation. *An. Biochem.* **86**, 142–146.

Sternberger L. A. (1974) *Foundation of Immunology Series: Immunochemistry* Prentice Hall, Englewood Cliffs, New Jersey.

Sulakhe P. V., Petrali E. H., Davis E. R., and Thiessen B. J. (1980) Calcium ion stimulated endogenous protein kinase catalyzed phosphorylation of basic proteins in myelin subfractions and myelin-like membrane fraction from rat brain. *Biochemistry* **19**, 5363–5371.

Uno I., Ueda T., and Greengard P. (1976) Differences in properties of cytosol and membrane-derived protein kinases. *J. Biol. Chem.* **251**, 2192–2195.

Van Raamsdonk W., Pool C. W., and Heyting C. (1977) Detection of antigen and antibodies by an immuno-peroxidase method applied on thin longitudinal sections of SDS-polyacrylamide gels. *J. Immunol. Methods* **17**, 337.

Van Wimersma Greidanus Tj.B., Bohus B., and De Wied D. (1975) CNS sites of action of ACTH, MSH and vasopressin in relation to avoidance behavior, in *Anatomical Endocrinology* (Stumph W. E. and Grant L. D., eds), pp. 284–289. Basel, Karger.

Verhoef J., Palkovits M., and Witter A. (1977a) Distribution of a behaviorally highly potent $ACTH_{4-9}$ analog in rat brain after intraventricular administration. *Brain Res.* **126**, 89–104.

Verhoef J., Witter A., and De Wied D. (1977b) Specific uptake of a behaviorally potent ^3H-$ACTH_{4-9}$ analog in the septal area after intraventricular injection in rats. *Brain Res.* **131**, 117–128.

Wiegant V. M., Zwiers H., Schotman P., and Gispen W. H. (1978) Endogenous phosphorylation of rat brain synaptosomal plasma membranes *in vitro*: Methodological aspects. *Neurochem. Res.* **3**, 443–453.

Wiegant V. M., Dunn A. J., Schotman P., and Gispen W. H. (1979) ACTH-like neurotropic peptides: Possible regulators of rat brain cyclic AMP. *Brain Res.* **168**, 565–584.

Zwiers H., Veldhuis H. D., Schotman P., and Gispen W. H. (1976) ACTH, cyclic nucleotides and brain protein phosphorylation *in vitro*. *Neurochem. Res.* **1**, 669–677.

Zwiers H., Wiegant V. M., Schotman P., and Gispen W. H. (1978) ACTH-induced inhibition of endogenous rat brain protein phosphorylation *in vitro*: Structure-activity study. *Neurochem. Res.* **3**, 455–463.

Zwiers H., Tonnaer J., Wiegant V. M., Schotman P., and Gispen W. H. (1979) ACTH-sensitive protein kinase from rat brain membranes. *J. Neurochem.* **33**, 247–256.

Zwiers H., Schotman P., and Gispen W. H. (1980*a*) Purification and some characteristics of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. *J. Neurochem.* **34**, 1689–1699.

Zwiers H., Verhoef J., Schotman P., and Gispen W. H. (1980*b*) A new phosphorylation-inhibiting peptide (PIP) with behavioral activity from rat brain membranes. *FEBS Lett.* **112**, 168–172.

Zwiers H., Aloyo V., and Gispen W. H. (1981) Behavioral and neurochemical effects of the new opioid peptide dynorphin(1-13): Comparison with other neuropeptides. *Life Sci.* **28**, 2545–2553.

Zwiers H., Jolles J., Oestreicher A. B., and Gispen W. H. (1982) Peptides and membrane phosphorylation, in *Progress in Brain Research: Vol. 56: Brain Phosphoproteins—Characteristics and Function* (Gispen W. H. and Routtenberg A., eds), (in press). Elsevier, Amsterdam.