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PHOSPHOPROTEIN B-50 IN NERVE GROWTH CONES FROM FETAL RAT BRAIN

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The presynaptic, nervous tissue-specific phosphoprotein B-50 is present in infant and adult rat brain. In the present study we demonstrate that B-50 is a major phosphoprotein in nerve growth cones obtained from fetal rat brain. As this protein is an endogenous substrate for protein kinase C, an enzyme linked to cell growth and proliferation, a role for B-50 in nerve growth cone function is suggested.

A number of phosphoproteins in neural membranes have been implicated in the mechanism of receptor activation, receptor-mediated transmembrane signal transduction and ion conductance [14]. One of these proteins is the nervous tissue-specific B-50 protein (M_r 48,000, IEP 4.5 [11, 23]), which is exclusively localized in presynaptic terminals and plasma membranes [5, 21]. Presumably this protein is identical to the protein γ-5 [8], F_1 [1] and P54p(Ca) [13].

In synaptic plasma membranes (SPM) this B-50 protein is a prominent substrate for the adrenocorticotropin hormone (ACTH)- and Ca^{2+}-sensitive lipid-dependent protein kinase C [1–3], an enzyme implicated in transmembrane signal transduction [4, 16]. Indeed, a variety of evidence suggests that the degree of phosphorylation of B-50 is important to transmembrane signal transduction, perhaps by exerting a negative feedback control in receptor-activated phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis [6, 10]. In view of the restricted presynaptic localization and the fact that the B-50 is already present at early stages of brain development [18], it was of interest to study whether in fact the protein is present in outgrowing neurites and specifically in growth cones of maturing neurons [9]. In the present paper we report evidence that the phosphoprotein B-50 is associated with the brain subcellular fraction enriched in nerve growth cones obtained from fetal rat brain.

Nerve growth cones were isolated essentially according to Pfenninger et al. [20]. Briefly, brains of fetal Wistar rats (TNO, Zeist, The Netherlands), removed after 17–

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19 days gestation, were gently homogenized by 5 strokes in a Teflon–glass homogenizer (clearance 0.250 mm) in 8 vols. (w/v) of 0.32 M sucrose containing 1 mM $MgCl_2$ and 1 mM Tris-HCl, pH 7.3. The homogenate was centrifuged for 15 min at 1600 g_{max}, and the resulting supernatant was loaded onto a discontinuous sucrose-density gradient (0.75 M, 1.0 M and 2.66 M sucrose) and centrifuged for 40 min at 245,000 g_{max} in a Beckman VTi-50 rotor. The material floating on top of the 0.75 M sucrose layer, and containing the nerve growth cones, was collected and diluted with 20 vols. of a buffer consisting of 10 mM Na-acetate, 10 mM Mg-acetate and 0.1 mM Ca-acetate (pH 6.5). After centrifugation for 20 min at 100,000 *g*, a pellet containing nerve growth cone particulate material (GC_P) was obtained. The isolation of light SPM from adult rat brain, the endogenous protein phosphorylation assay and the one- and two-dimensional separation of membrane proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF)–PAGE were performed as described before [11]. The B-50 protein was purified from rat brain and phosphorylated in vitro by protein kinase C as described by Zwiers et al. [25]. Protein determinations were carried out according to Lowry et al. [12] using bovine serum albumin as standard. The immuno-identification of the B-50 protein by means of affinity-purified anti-B-50 immunoglobulins was performed on Western blots from SDS–PAGE gels according to Oestreicher et al. [19] and Nielsen et al. [15].

An electron micrograph of an ultrathin section of the material floating on top of

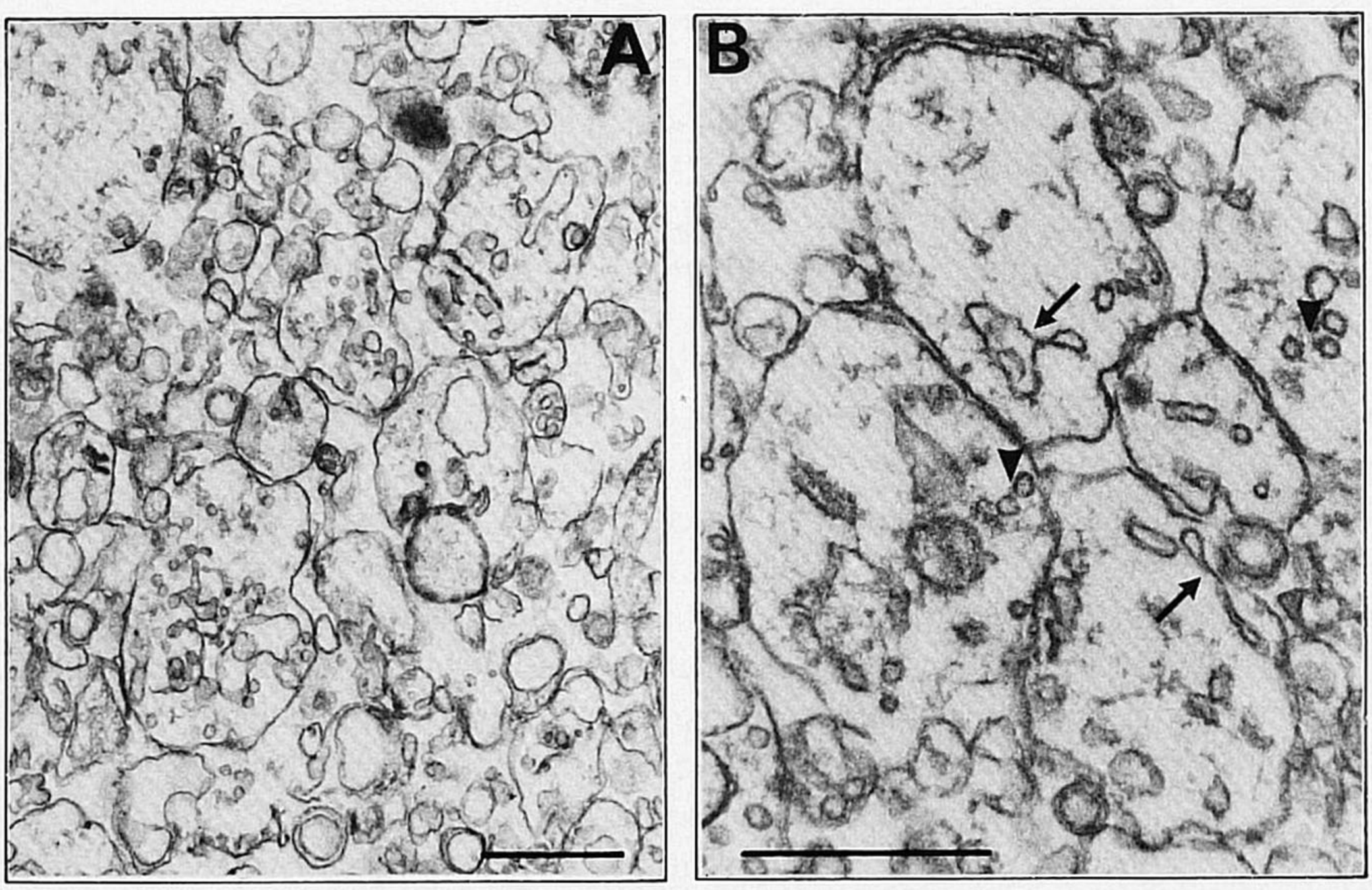

Fig. 1. Electron micrograph of nerve growth cones obtained from fetal rat brain. The picture shows a homogeneous population of vesicular structures (A). At higher magnification (B) cisternae of smooth endoplasmic reticulum (arrow) and clear vesicles (arrowhead) can be seen within these structures. Bar = 0.5 μm.

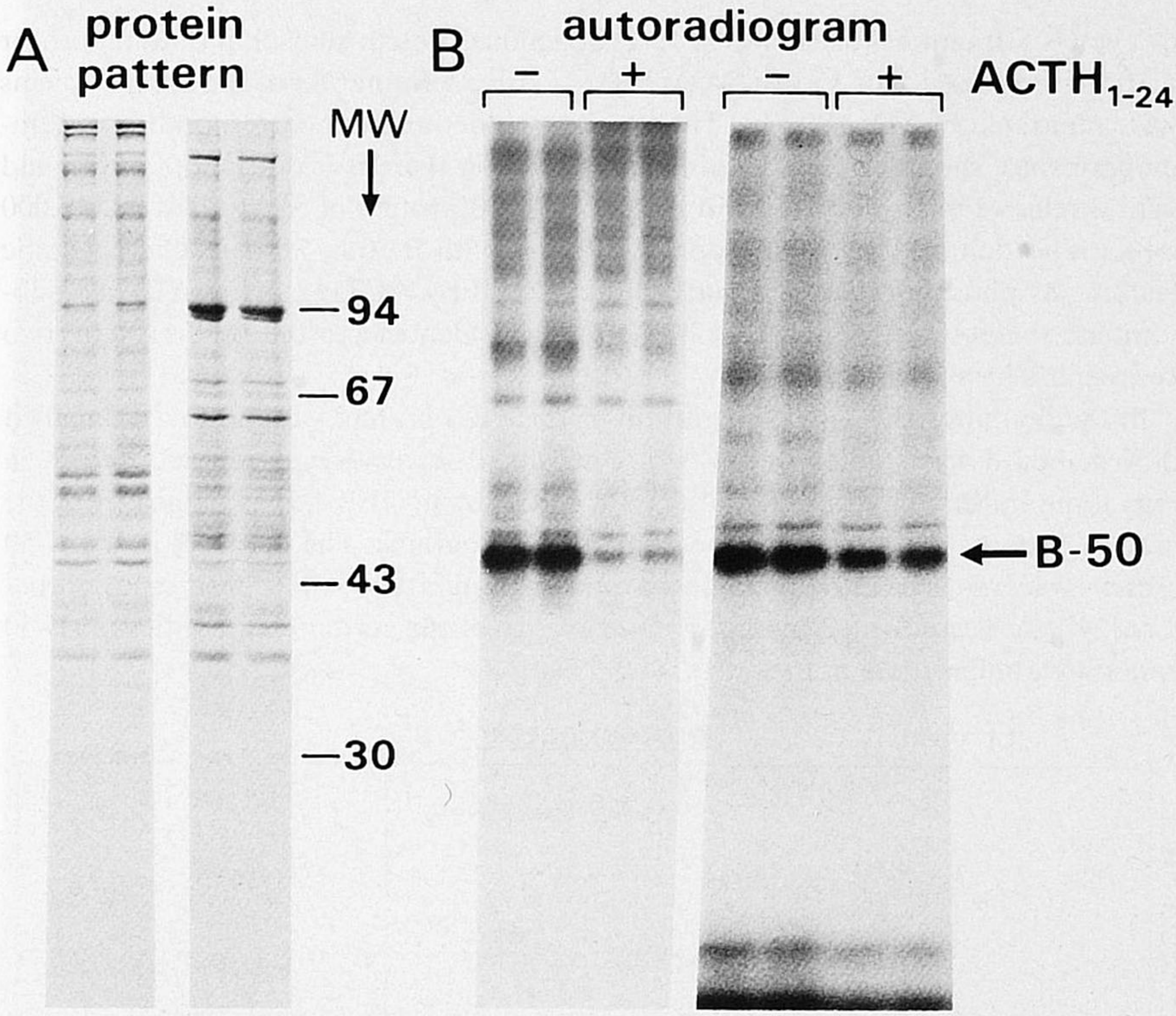

Fig. 2. Protein-staining patterns (A) and autoradiograms (B) of nerve growth cone particulate material (CG_P) and SPM subjected to SDS–PAGE (10 μg protein per lane) after endogenous phosphorylation in the absence and presence of $ACTH_{1-24}$ (3×10^{-6} M). Molecular weight markers (MW) in kdalton.

the 0.75 M sucrose layer is shown in Fig. 1. Prior to fixation (2% glutaraldehyde) and embedding in Araldite, the fraction was diluted to isotonic conditions by careful stepwise suspension in 50 mM phosphate buffer, pH 7.4, containing 0.4 mM $CaCl_2$, ensuring preservation of vesicular structures. The fraction contains a homogeneous population of vesicular structures enclosing various cellular organelles, primarily smooth endoplasmic reticulum and clusters of large clear polymorph vesicles. Thus, the structural composition of this fraction is highly similar to that described by Pfenninger et al. [20]. Presumably due to the careful osmotic manipulation of the subcellular fraction, the ultrastructural appearance resembles that of the subcellular fraction characterized as nerve growth cones by Gordon-Weeks and Lockerbie [7]. Furthermore, the amount of particulate material recovered from this fraction as determined by protein content is similar to that reported by Pfenninger et al. [20] (14 ± 4 μg/mg protein of the original homogenate; mean $\pm$ S.E.M. from 6 preparations). In keeping with Pfenninger et al. [20] and Gordon-Weeks and Lockerbie [7], we refer to this material as nerve growth cones.

The GC_P fraction was subjected to endogenous protein phosphorylation with or without the presence of 3×10^{-6}M $ACTH_{1-24}$. After termination of the assay proteins were separated by SDS–PAGE. The protein profiles and the corresponding autoradiograms are shown in Fig. 2. In the GC_P fraction there is a phosphoprotein band with a relative migration identical to that of B-50 protein in SPM. This M_r 48,000 (48K; K = kdalton) GC_P phosphoprotein shares with B-50 in SPM its characteristic feature: its phosphorylation is markedly inhibited by $ACTH_{1-24}$ (Fig. 2B). To substantiate the notion that the GC_P 48K protein is identical to the B-50 protein, two approaches have been used.

In the first approach, the comigration of this GC_P protein with added ^{32}P-labelled B-50 standard was studied in the two-dimensional separation system. As shown in Fig. 3, the tracer comigrates with the GC_P 48K protein (IEP 4.5) as judged from the protein patterns and their corresponding autoradiograms. The impurity in the B-50 protein tracer is caused by breakdown during preparation as has been reported before [24] and is confirmed by the cross-reactivity of the contaminant with anti-B-50 immunoglobulins (data not shown).

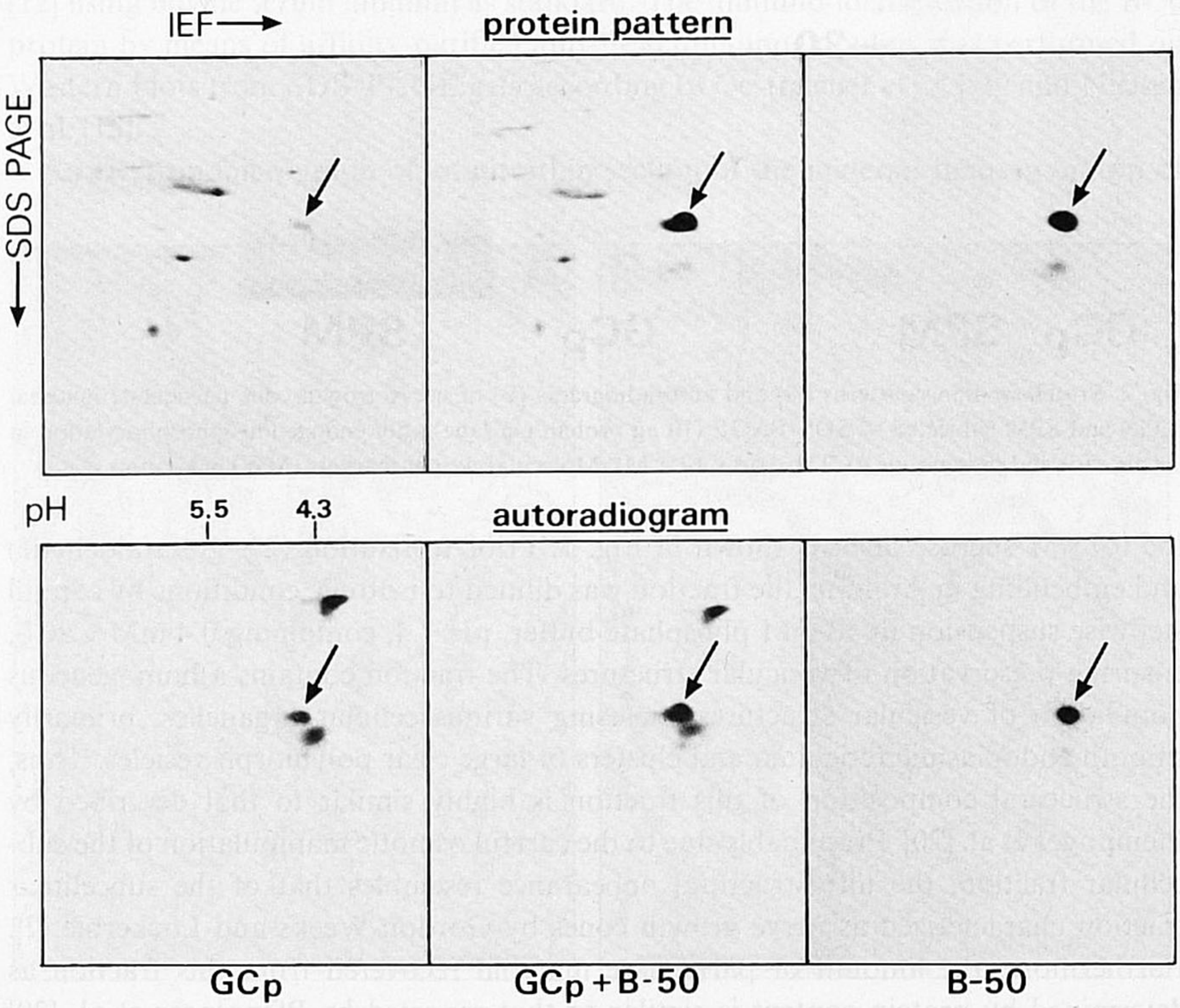

Fig. 3. Comigration of the 48K protein in GC_P and purified B-50 after two-dimensional electrophoresis. Protein-staining pattern and autoradiogram of endogenous phosphorylated GC_P is shown in the absence (GC_P) and presence (GC_P + B-50) of purified ^{32}P-labelled B-50 (B-50). Arrow denotes position of B-50. Only the 120–25 kdalton molecular weight region of the gels is shown.

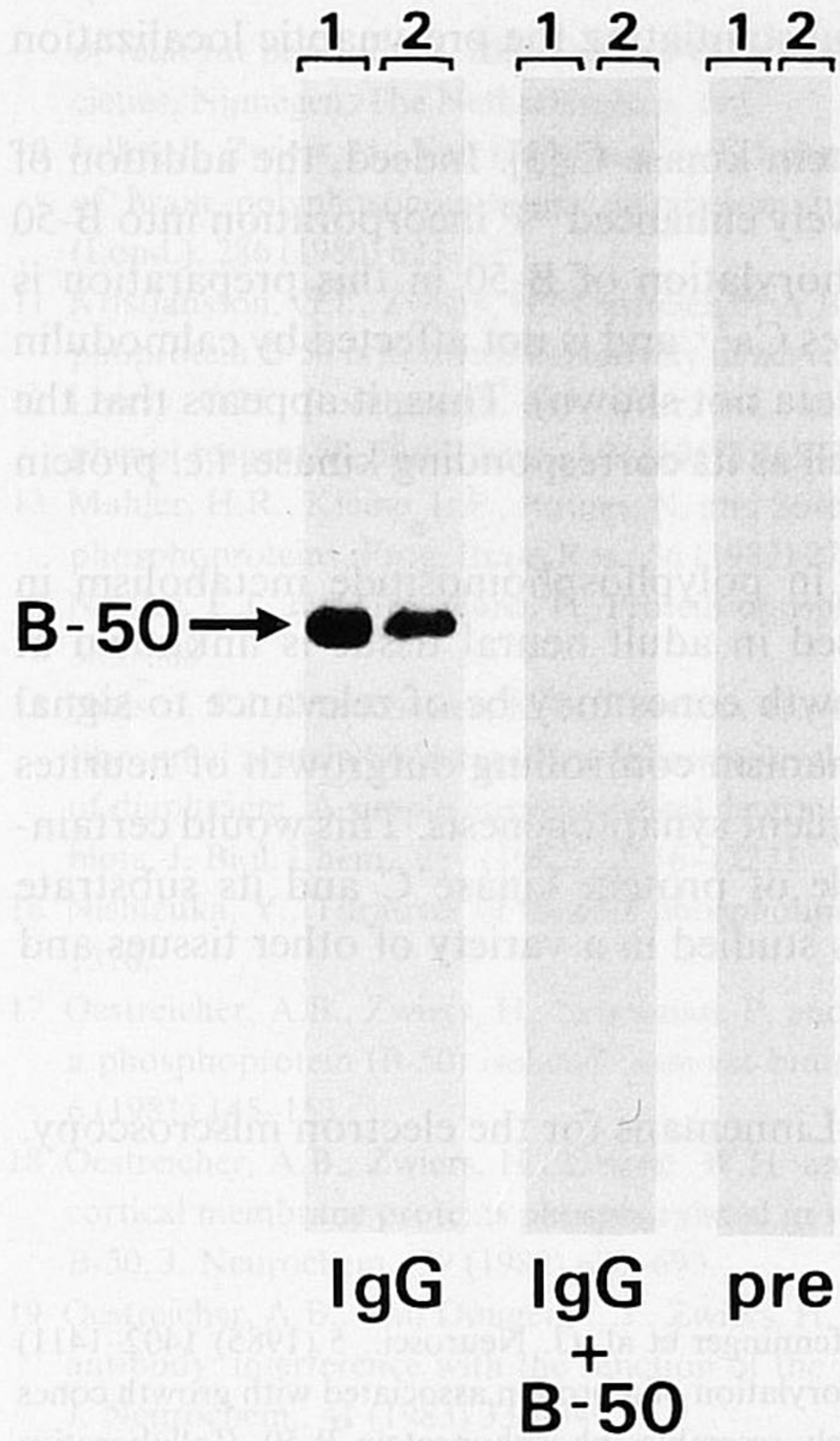

Fig. 4. Cross-reactivity of affinity-purified anti-B-50 immunoglobulins (from antiserum number 8103) with the 48K protein in GC_P (1) and SPM (2) as shown by immunoblotting. IgG, anti-B-50 immunoglobulin (dilution 1:2000); IgG + B-50, anti-B-50 immunoglobulins, preabsorbed with purified B-50; Pre, preimmune immunoglobulins.

In the second approach, the immunoreactivity of the GC_P 48K protein with affinity-purified anti-B-50 immunoglobulins was studied. As can be seen in Fig. 4, the GC_P 48K protein is the only protein cross-reacting with the anti-B-50 immunoglobulins. There is no staining when preimmune immunoglobulins are used instead of anti-B-50 immunoglobulins or when the anti-B-50 immunoglobulins are preabsorbed with purified B-50. These data confirm the suggestion that the GC_P 48K protein is indeed the B-50 protein.

Immunocytochemical localization of B-50 in adult rat brain has shown that B-50 immunoreactivity is most pronounced in brain regions enriched in synaptic contacts and is virtually absent in the white matter [17]. Electron microscopical detection of affinity-purified anti-B-50 immunoglobulins by protein A–gold techniques revealed that B-50 is predominantly localized at presynaptic sites of the nerve terminals [5]. Already in the early stages of postnatal development, the presence of B-50 in rat brain SPM could be demonstrated [18]. The data presented here unequivocally show the presence of B-50 in the brain of fetal rats at 17–19 days gestation. Moreover, the data provide strong evidence for the presence of B-50 in nerve growth cones, pinched-

off fragments of outgrowing nerves, thus substantiating the presynaptic localization of B-50.

B-50 is an endogenous substrate for protein kinase C [3]. Indeed, the addition of protein kinase C to the GC_P fraction selectively enhanced ^{32}P incorporation into B-50 (data not shown). The endogenous phosphorylation of B-50 in this preparation is strongly inhibited by ACTH (Fig. 2), requires Ca^{2+} and is not affected by calmodulin or 3′,5′-cyclic adenosine monophosphate (data not shown). Thus, it appears that the growth cones contain the B-50 protein as well as its corresponding kinase, i.e. protein kinase C [3].

Whether B-50 plays a modulatory role in polyphosphoinositide metabolism in nerve growth cones similar to that proposed in adult neural tissue is unknown at present. However, its presence in nerve growth cones may be of relevance to signal transduction involved in the guidance mechanism controlling outgrowth of neurites towards appropriate target areas and subsequent synaptogenesis. This would certainly be in line with current views on the role of protein kinase C and its substrate proteins in growth and tumor promotion as studied in a variety of other tissues and cells (see for instance ref. 22).

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NOTE ADDED IN PROOF

After this manuscript was submitted, a paper by Pfenninger et al. (J. Neurosci., 5 (1985) 1402–1411) came to our attention. This group reported the phosphorylation of a protein associated with growth cones designated pp46, which in several characteristics closely resembles phosphoprotein B-50. Collaborative experiments are planned to evaluate the relationship of these two proteins.

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