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Identification of a B-50-like protein in frog brain synaptosomes

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Vestibular compensation in the frog following unilateral labyrinthectomy is accompanied by distinct changes in the endogenous phosphoprotein patterns in total frog brain homogenate and isolated synaptosomes. The purpose of this study was to characterize one of these proteins, an acidic 45-kDa synaptosomal protein, resembling in some of its features the growth-associated protein GAP-43/B-50. Our results demonstrate by comparative analysis with purified rat B-50/GAP-43 that the 45-kDa protein (IP 4.8) in synaptosomal membranes of frog brain is phosphorylated by added purified PKC, cross-reacts with affinity-purified rabbit antibodies to rat B-50 and exhibits a *Staphylococcus aureus* V8 protease peptide digestion pattern corresponding to rat B-50. Therefore, we conclude that the acidic 45-kDa synaptosomal protein is a growth-associated B-50-like protein, probably involved in processes responsible for compensatory reorganization of the vestibular structures after hemilabyrinthectomy in the frog.

INTRODUCTION

Unilateral destruction of the vestibular nerve in the frog *Rana temporaria* leads to severe malfunctions in posture and movement. These functional deficits, however, are restored during the process of vestibular compensation, which involves the adaptive reorganization of the remaining structures involved in the control of posture and locomotion and possibly requires (a) the modulation of synaptic efficacy in existing synapses and (b) the formation of new synapses by axonal sprouting[12,14,37,47].

In a previous study we have shown that the process of vestibular compensation is accompanied by distinct changes in the endogenous phosphorylation pattern of frog brain proteins[13,21,23], and that some of these proteins represent consituents of synaptosomes[22]. In particular, an acidic 45-kDa synaptosomal phosphoprotein (IP 4.8) was observed, with biochemical properties similar to the synaptic plasma membrane protein B-50 as described by Zwiers et al.[49]. The phosphoprotein B-50, which is identical to F1, GAP-43, GAP-48, pp 46 or neuromodulin (P57)[5,15,42], is a major constituent of neuronal growth cone membranes[9,25,31,41]. B-50 (GAP-43) is present in outgrowing neurites in fetal and neonatal brains and spinal cord[16,20,33,44]. It has been suggested that B-50 (GAP-43) plays a role in transmembrane signal transduction[10], the modulation of neurotransmitter release[11,18] and in alterations occurring during long-term potentiation[17,30]. In addition, it has been proposed that growth-associated proteins, in particular GAP-43/B-50, participate in mechanisms of axonal outgrowth and repair following lesions of the nervous system[4,19,24,29,39,40].

The purpose of the present study was to characterize the 45-kDa frog brain synaptosomal phosphoprotein, which is significantly affected by the process of vestibular compensation in the frog. The present study shows that this protein, which is correlated with plastic events occurring in the frog brain during vestibular compensation, is similar to the rat B-50 protein, because of its immunochemical cross-reactivity and PKC phosphorylation and therefore may be a member of the family of growth-associated proteins.

MATERIALS AND METHODS

Tissue preparation

Synaptosomes were isolated from frog brains (*Rana temporaria*) according to Whittaker and Greengard[46] by means of sucrose density gradient centrifugation. The pelleted synaptosomal fraction was resuspended in 500 μl 10 mM Tris/HCL pH 7.4 (buffer A) containing 10 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and stored at –80°C for further analysis.

Protein was determined according to the method of Bradford[7], using bovine serum albumin as the standard.

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Protein phosphorylation

Endogenous phosphorylation of frog brain synaptosomes was carried out in the absence or presence of 1 mM $CaCl_2$ or 20 μg/ml phosphatidyl serine in a final reaction volume of 50 μl containing 60 μg protein in buffer A. After 5 min of preincubation, the reaction was started by addition of 5 μCi [γ-^{32}P]ATP (S.A. 3000 Ci/mmol, Amersham, F.R.G.; 2 μM final ATP-concentration). The phosphorylation reaction was carried out at 20°C (room temperature) for 3 min and stopped by adding 50 μl of a urea-containing solution (9.5 M urea, 2% β-mercaptoethanol, 4% Triton X-100, 2% SDS, 3.2% ampholines pH 5–7, 0.8% ampholines pH 2–11) followed by immediate freezing in liquid nitrogen. Samples were separated by two-dimensional gel electrophoresis.

Phosphorylation by added protein kinase C (PKC) of heat-inactivated synaptosomal fractions and purified B-50, was assayed as described by Oestreicher et al.[34]. PKC (100 ng/μg protein), purified according to Kikkawa et al.[26], was added to the incubation mixture containing 60 μg of synaptosomal proteins or 1 μg B-50, 5 μCi [γ-^{32}P]ATP (final concentration 2 μM) and 20 μg/ml phosphatidyl serine in buffer A. The reaction was carried out for 20 min at 37°C, in a final incubation volume of 50 μl and was terminated either by the addition of a concentrated SDS-denaturing buffer (30% glycerol, 3% β-mercaptoethanol, 9% SDS, 187.5 mM Tris/HCl, pH 6.8) and immediate boiling for SDS polyacrylamide gel electrophoresis (SDS–PAGE) or by the addition of a urea-containing solution (see above) followed by immediate freezing in liquid nitrogen.

Gel electrophoresis and autoradiography

SDS–PAGE or two-dimensional gel electrophoresis was performed according to the methods of Laemmli[28] or O'Farrell[35]. The gels for isoelectrofocusing (IEF) contained 4% ampholines pH 5–7 and 1% ampholines pH 2–11. For SDS–PAGE gradient slab gels of 5–15% acrylamide were used. Gels were stained with 0.2% Coomassie brilliant blue in 50% methanol/10% acetic acid and destained in 10% methanol/10% acetic acid, dried on 3MM Whatman paper and exposed to Kodak X-R-5 X-ray films for time periods of 1–10 days.

Immunoblotting

Proteins of frog brain synaptosomal fractions and purified B-50 protein were separated by IEF and SDS–PAGE and subsequently transferred to nitrocellulose, according to Towbin et al.[43]. The nitrocellulose blots were incubated in the absence (control) or presence of 1:2000 diluted anti-B-50 immunoglobulins (IgGs) affinity-purified from the rabbit antiserum 8613[32]. Immunobinding was detected by goat anti-rabbit IgG coupled to alkaline phosphatase (Promega). The specificity of the detection method was checked by omitting the primary antibody, with the result that immunostaining of specific protein bands did not occur.

Proteolysis

Proteolytic digestion of previously radiolabeled B-50 protein and B-50-like immunoreactive protein of frog brain synaptosomes with *Staphylococcus aureus* V8 protease (SAP) was carried out during electrophoresis as described by Cleveland et al.[8]. Briefly, following two-dimensional gel electrophoresis the B-50 and B-50-like protein spots were excised from the wet 5–15% gradient gels. The pieces were soaked in buffer B (0.125 M Tris/HCl, pH 6.8, 0.1% SDS) and 1 mM EDTA for 30 min, placed in a slot of a second 15% SDS–PAGE gel, covered with 10 μl buffer B containing 20% glycerol and 10 μl buffer B containing 10% glycerol and SAP (20 ng/μl). Electrophoresis was performed as described above except that the stacking gel was longer (5 cm) and that the current was turned off for 45 min when the Bromphenol blue dye was close to the bottom of the stacking gel. The gel was fixed in 50% methanol and silver stained as described by Wray et al.[48].

RESULTS

Electrophoretic and biochemical properties of the 45-kDa frog brain synaptosomal protein

When lysed frog brain synaptosomes were phosphorylated with [γ-^{32}P]ATP in the presence of either 1 mM $CaCl_2$ or 20 μg/ml phosphatidyl serine (PS), the two-dimensional phosphoprotein patterns revealed that the phosphorylation of a 45-kDa (IP 4.8) protein and of a set of 3 24-kDa proteins (IPs 5.0–6.0) were strongly affected by both reagents (Fig. 1). These spots were excised and the incorporated radioactivity was determined in a scintillation counter. As compared to the untreated control, the incorporation of ^{32}P into the 45-kDa component (IP 4.8) was significantly increased in the presence of 1 mM $CaCl_2$ by 139 ± 21% S.E.M., and in the presence of 20 μg/ml PS by 133 ± 10% S.E.M. ($P < 0.001$, $n = 3$). ^{32}P incorporation into the 24-kDa components (IPs 5.0–6.0) was also significantly stimulated by 50 ± 8% S.E.M. in the presence of calcium, and by 120 ± 3% S.E.M. in the

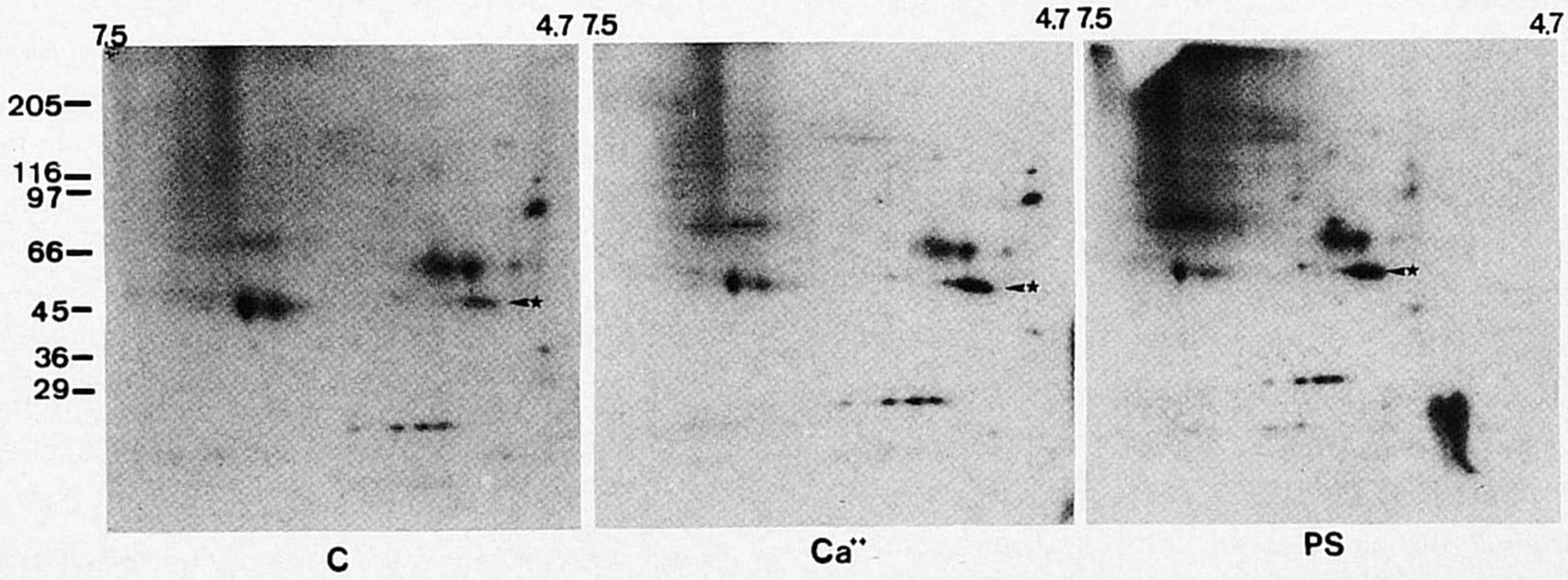

Fig. 1. Ca^{2+}- and phospholipid-stimulated phosphorylation of frog brain synaptosomal proteins, analyzed by two-dimensional separation. Aliquots (60 μg) of the synaptosomal fraction were phosphorylated with [γ-^{32}P]ATP in the absence (C) or presence of 1 mM $CaCl_2$ (Ca^{2+}) or in the presence of 20 μg/ml phosphatidyl serine (PS). Arrow with asterisk (*) denotes position of the acidic 45-kDa protein. Its endogenous phosphorylation was substantially enhanced in the presence of Ca^{2+} and PS.

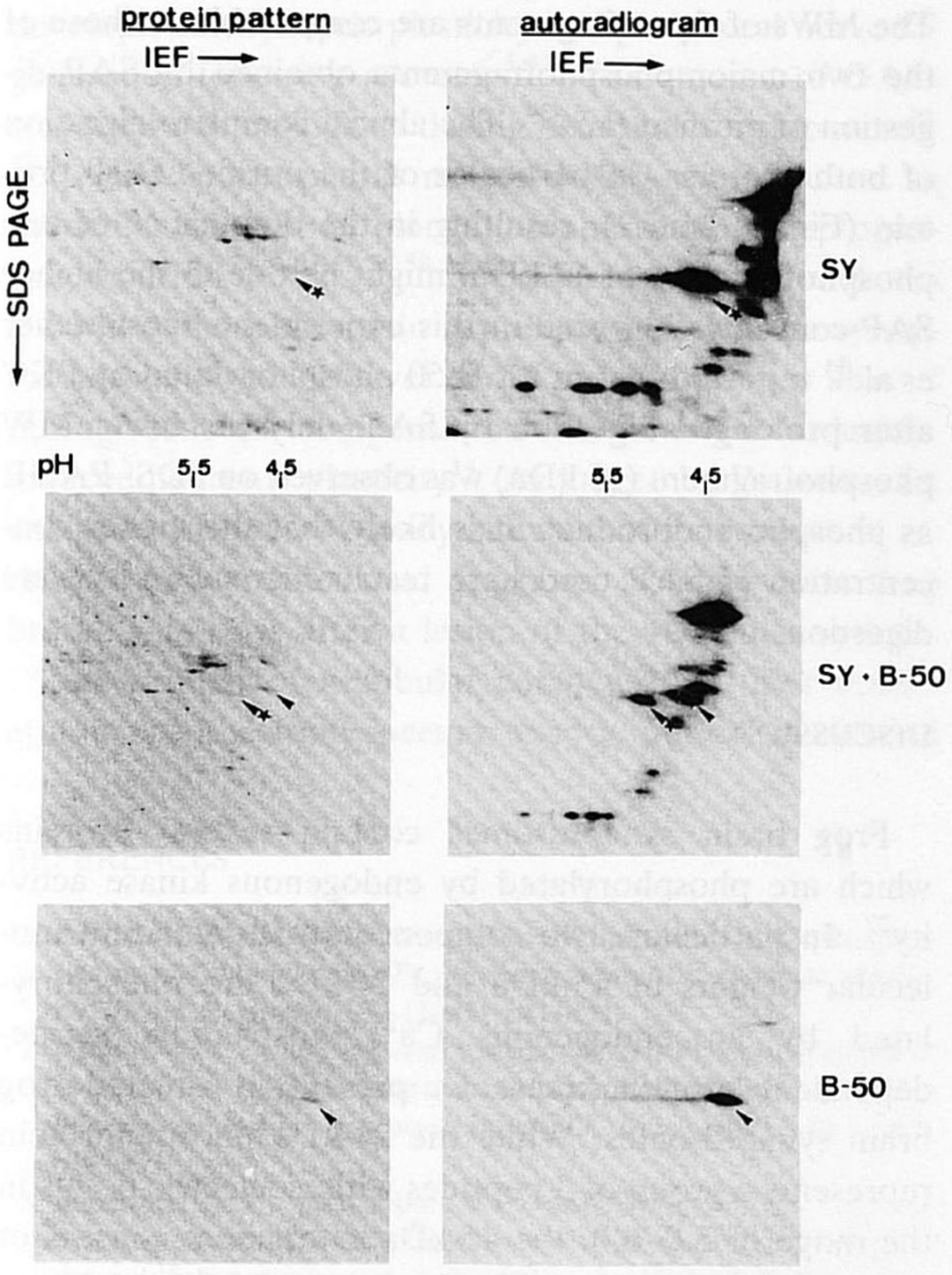

Fig. 2. Comparison of the electrophoretic mobilities of the 45-kDa frog brain synaptosomal protein with B-50. Protein staining patterns and autoradiograms of a two-dimensional gel system separating B-50 protein (1 μg) and frog brain synaptosomes (60 μg protein) phosphorylated by added PKC in the absence (SY) or presence of B-50 (SY + B-50). The B-50-like protein of the synaptosomal fraction is marked by an arrow with an asterisk, while the position of B-50 is denoted by an arrow.

presence of phosphatidyl serine ($P < 0.001$, $n = 3$). Thus, the endogenous protein phoshorylation in frog brain synaptosomes is in part accomplished and regulated by protein kinase C (PKC). While the phosphorylation of the 45-kDa protein was equally well stimulated in both conditions, the phosphorylation of the 24-kDa components was most effectively stimulated in the presence of phosphatidyl serine.

To invesitgate whether the acidic 45-kDa phosphoprotein in frog brain synaptosomes is related to the PKC-substrate protein B-50, we used a two-dimensional separation system to prove its comigration with added purified B-50. Aliquots (60 μg protein) of the frog brain synaptosomal fraction were therefore heat-inactivated and then, like purified B-50 (1 μg), phosphorylated in the presence of freshly added PKC and PS.

As shown in Fig. 2, frog brain synaptosomes contain a prominent 45-kDa PKC-substrate (IP 4.8 arrow with an *) which has a slightly lower molecular weight and is less

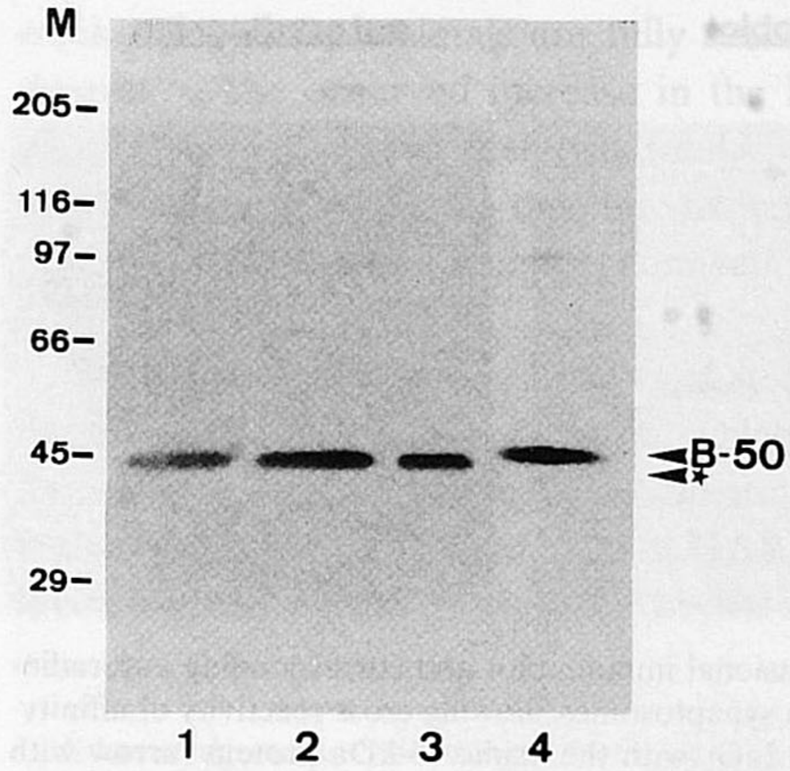

Fig. 3. Cross-reactivity of affinity-purified anti-B-50 IgGs with the 45-kDa protein in frog brain synaptosomes and purified B-50 (1 μg, lane 4). Lane 1 indicates reactivity with the 45-kDa synaptosomal protein of unlesioned animals. Lanes 2 and 3 indicate the time course of changes in immunoreactivity at 7 and 14 days, respectively, following the lesion of the vestibular system. Lanes 1–3 each contain 60 μg of synaptosomal proteins. The positions of B-50 and the B-50-like-immunoreactive 45-kDa protein (*) are denoted.

acidic than the purified B-50 protein (48 kDa, IP 4.5), as judged by the protein patterns and their corresponding autoradiograms.

Cross-reactivity of rabbit polyclonal rat B-50 antibodies with the acidic 45-kDa frog brain protein

In a second approach the immunological relationship of the 45-kDa frog brain synaptosomal protein with B-50 was investigated using affinity-purified polyclonal rabbit antibodies raised against rat B-50. Western blot analysis revealed that the antibody reacted specifically with both the 45-kDa frog brain synaptosomal protein (Fig. 3, lanes 1–3 and Fig. 4) and purified B-50 (Fig. 3, lane 4). Additionally, the blot shown in Fig. 3 supports the previously mentioned finding, that the B-50-immunoreactive protein in frog brain synaptosomes has a slightly lower apparent molecular weight (MW) compared to purified B-50 from rat brain.

Fig. 3 also indicates that the amount of the B-50-immunoreactive protein in frog brain synaptosomes is increased in animals which were subjected to hemilabyrinthectomy and allowed to compensate for 7 (Fig. 3, lane 2) and 14 days (Fig. 3, lane 3). Densitometric quantification revealed that the amount of the B-50-like protein in frog brain synaptosomes is increased 30% in lesioned animals which were allowed to compensate for 7 days and 22% in animals which compensated for 14 days.

Proteolysis of purified B-50 and the 45-kDa B-50-immunoreactive protein

The similarity of the [^{32}P]-labeled 45-kDa protein of

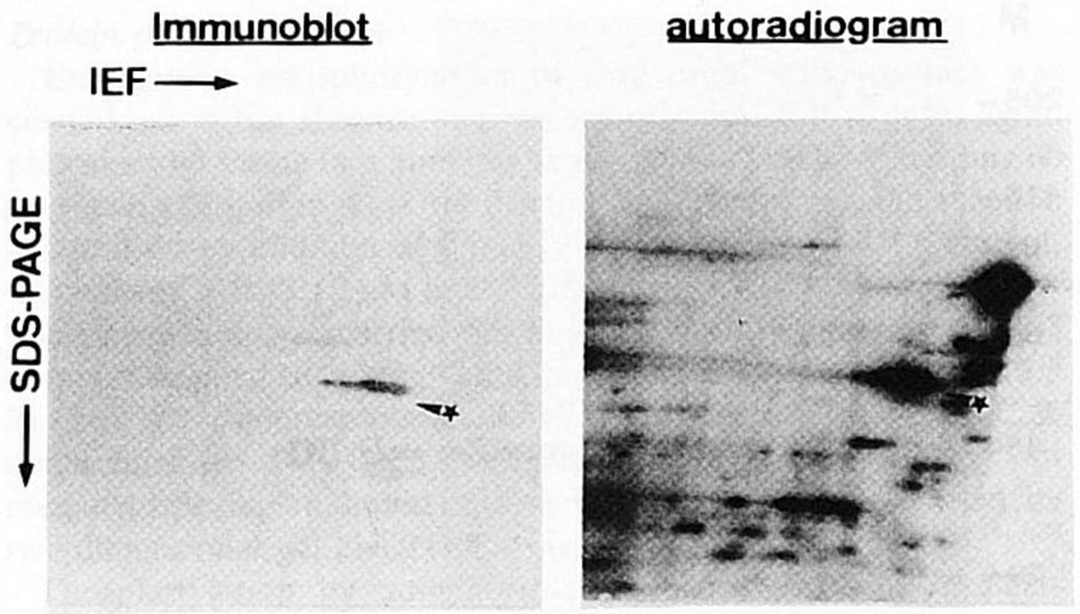

Fig. 4. Two-dimensional immunoblot and corresponding autoradiogram of frog brain synaptosomes showing cross-reactivity of affinity-purified anti-B-50 IgGs with the acidic 45-kDa protein (arrow with asterisk). Synaptosomal proteins (60 μg) were phosphorylated by added PKC, separated in the two-dimensional gel system and transferred to nitrocellulose. Blots were incubated with 1:2000 diluted anti-B-50 IgGs. The corresponding autoradiogram shows that the anti-B-50 IgGs recognized the strongly [^{32}P]-labelled acidic 45-kDa protein in frog brain synaptosomes (arrow with asterisk).

frog brain synaptosomes and B-50 protein was further substantiated by limited proteolysis with SAP. Fig. 5 shows that proteolytic digestion of the 45-kDa protein (Fig. 5, lane 1) and B-50 (Fig. 5, lane 2) generates similar phosphopeptide maps with fragments of 29 and 17 kDa.

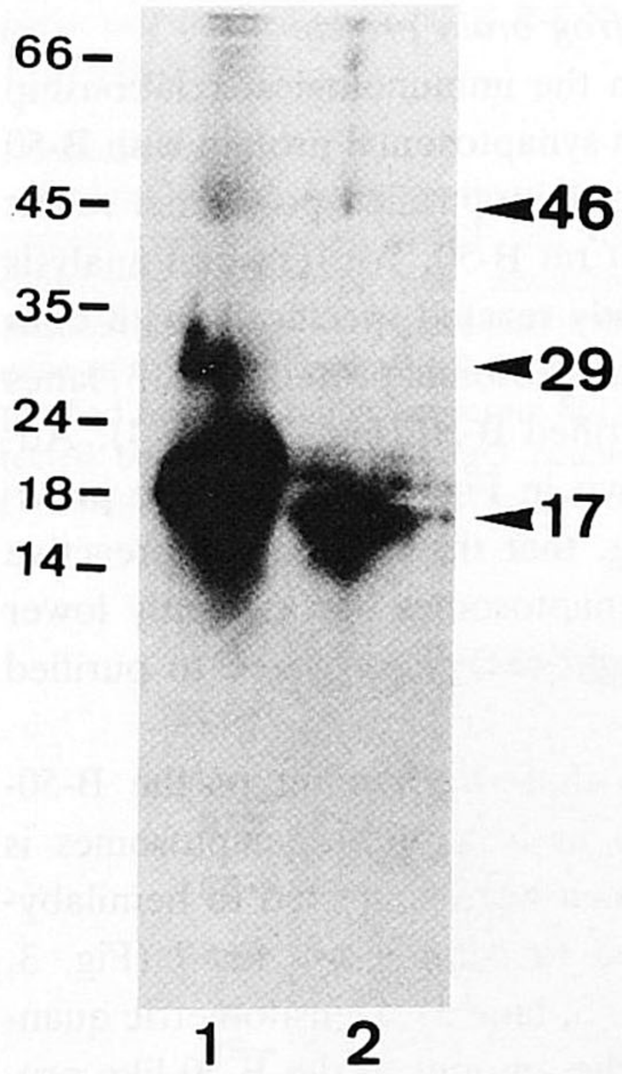

Fig. 5. Autoradiogram of digestion with SAP of [^{32}P]-labelled B-50 and the 45-kDa B-50-immunoreactive protein. Proteins (60 μg of frog brain synaptosomal fraction and 1 μg B-50) were phosphorylated by added PKC and separated by two-dimensional gel electrophoresis. The radiolabelled B-50 and acidic 45-kDa proteins were excised from the gels, digested with SAP (20 ng/μl) during SDS–PAGE on a second 15% polyacrylamide gel and this gel was subjected to autoradiography. Results are shown for the acidic 45-kDa protein (lane 1) and purified B-50 (lane 2).

The MWs of these fragments are comparable to those of the two major phosphofragments obtained by SAP digestion of purified B-50[34]. The almost complete digestion of both proteins, in particular of the purified B-50 protein (Fig. 5, lane 2), resulting in the dominance of one phosphofragment of 17 kDa, might be due to the higher SAP-concentration used in this experiment. Oestreicher et al.[34] reported that in rat B-50 phosphorylated by PKC after prolonged digestion by SAP, only the lower MW phosphofragment (15 kDa) was observed on SDS–PAGE as phospho endproduct. It is likely that the higher concentration of SAP used here resulted in such complete digestion.

DISCUSSION

Frog brain synaptosomes contain several proteins which are phosphorylated by endogenous kinase activity[22]. In particular, two components with apparent molecular weights of 45-kDa and 24-kDa are phosphorylated by an endogenous Ca^{2+}/phosphatidyl serine-dependent protein kinase C, present in isolated frog brain synaptosomes. While the 24-kDa phosphoprotein represents a group of 3 peptides with isoelectric points in the range of 5.0–6.0, the 45-kDa component consists of a single peptide with an IP of 4.8, and thus appears as a rather acidic protein resembling in its properties the B-50 rat brain growth-associated protein. By means of phosphorylation by added PKC of heat-inactivated proteins of frog brain synaptosomes and rat B-50 followed by two-dimensional analysis, it is shown that the acidic 45-kDa frog brain protein is a substrate for PKC, as is well-established for B-50.

Immunochemical characterization reveals that the 45-kDa acidic frog brain protein is recognized by the polyclonal B-50 antibodies and, therefore, appears to have some epitope(s) immunologically related to rat B-50. Peptide digestion of rat brain B-50 and frog brain 45-kDa phosphoprotein with protease SAP, results in the appearance of two major peptides with similar molecular weights. This further substantiates the biochemical similarity of both components.

Our data reveal that frog brain synaptosomes contain a B-50-like phosphoprotein migrating slightly faster in SDS–PAGE than B-50 of the rat. This difference might reflect a difference in SDS-binding, which has been previously observed among GAPs, or could be due to a lack of a sequence of acidic amino acids. Such a difference has been observed in bovine and human, but not rodent, GAPs, the former being about one kDa larger than the rodent equivalent[27,45].

Growth-associated proteins which are substrates for PKC-mediated phosphorylation[1,2] play a role in neuronal

development, synaptic plasticity and peripheral nerve regeneration following axotomy[5,42]. The amount of GAPs was shown to be highest during neuronal development[20,33,41], and in peripheral nerves during regeneration, in particular when axon elongation and active synaptogenesis occurred[3,4,6,24,29,36,38,39,40]. Our data indicate that a B-50-like phosphoprotein is present in frog brain synaptosomes and that its level of ^{32}P incorporation is altered during vestibular compensation[22], a paradigm for lesion-induced neuronal plasticity[14]. As judged by Western blot analysis, the present study shows that its content is increased in synaptosomal fractions isolated from frog brains 7–14 days after a lesion of the vestibular system.

The process of vestibular compensation after unilateral labyrinthectomy occurs over a period of several weeks. Generally, animals are fully recovered after 2–3 months[12]. The observed increase in the B-50-like phosphoprotein during the first two weeks after the lesion might indicate that during this time axonal sprouting and the reorganization of synaptic connections occur most actively.

In conclusion, this study shows that frog brain contains a B-50-like phosphoprotein which is modulated during the process of vestibular compensation in the frog brain. This is another example of a GAP participating in lesion-induced plastic events in the central nervous system.

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