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The Kinase C Substrate Protein B-50 and Axonal Regeneration

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VERHAAGEN, J., C. O. M. VAN HOOFF, P. M. EDWARDS, P. N. E. DE GRAAN, A. B. OESTREICHER, P. SCHOTMAN, F. G. I. JENNEKENS AND W. H. GISPEN. *The kinase C substrate protein B-50 and axonal regeneration.* BRAIN RES BULL **17**(6) 737–741, 1986.—As reported previously the prominent protein kinase C substrate protein B-50 is present in growth cones isolated from fetal rat brain and in outgrowing hippocampal neurites. These findings suggest that B-50 plays a role in axonal growth during development of the nervous system. In the present paper the fate of B-50 is investigated in regenerating rat sciatic nerve. Using affinity-purified anti-B-50 antibodies B-50 levels have been compared in crushed and contralateral intact nerves by means of immunoblotting and radioimmunoassay. B-50 levels in the crushed nerve increased 5.3-fold as compared to non-crushed controls. Furthermore, the cellular localization of B-50 has been assessed by immunohistochemistry. Virtually no B-50 immunoreactivity was seen in control nerves, but bright immunofluorescence appeared in regenerating sprouts. Our data are in line with current evidence from several laboratories that B-50 is a member of a small family of growth-associated proteins and support the hypothesis that B-50 is involved in axonal growth.

Axonal regeneration Phosphoprotein B-50 Immunochemistry Growth-associated proteins

THE neuron-specific phosphoprotein B-50 (MW 48 kDa, IEP 4.5) [38] is a major endogenous substrate of protein kinase C [1] and may be involved in a feedback mechanism in the receptor-mediated hydrolysis of polyphosphoinositides [8, 13, 27, 32]. In adult rat brain the B-50 protein is predominantly localized in cell membranes of presynaptic terminals [9,29]. The relatively high levels of endogenous B-50 phosphorylation in fetal and neonatal rat brain membranes [11,20] and the presence of B-50 in outgrowing hippocampal neurites [22] and in nerve growth cones isolated from fetal rat brain [6] suggest a role of this phosphoprotein in neurite outgrowth. In the present paper B-50 is studied during neurite outgrowth in the regenerating rat sciatic nerve following crush damage. We report here that during regenerative axonal outgrowth the amount of B-50 in the sciatic nerve increases 5.3-fold. B-50 immunoreactivity is localized in regenerating axons and newly formed sprouts that cross the lesion. Therefore this study demonstrates the involvement of an immunochemically characterized and quantitated growth-associated phosphoprotein in outgrowing neural sprouts.

METHOD

Surgical Procedures and Dissection

Female rats of an inbred Wistar strain (TNO, Zeist, NL; body weight 120–140 g) were subjected to a unilateral crush lesion of the sciatic nerve 27 mm distal from the sciatic notch as described previously [3]. Crush lesioning was performed under Hypnorm (Philips Duphar, Amsterdam, NL; 0.1 ml Hypnorm/100 g body weight) anaesthesia. The proximal border of the 2.0 mm wide crush lesion was marked with an epineural suture (Ethicon 6.0) in order to enable reproducible dissection.

Gel Electrophoresis and Immunoblotting

Six days following the surgery, rats were sacrificed by decapitation and the sciatic nerves were isolated. Five mm nerve pieces taken at the position of the crush were dissected out from the crushed and contralateral control nerve. The nerve pieces were homogenized by 10 up and down strokes of a Potter-type homogenizer (clearance 0.125 mm). Im-

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TABLE 1
THE AMOUNT OF B-50 IN RAT SCIATIC NERVE

Tissue	Days Following Crush	ng B-50/ mg Protein	n
Control Sciatic Nerve	—	5.1 ± 0.2	8
Crush Region	6	26.8 ± 1.8*	8
Crush Region	36	10.9 ± 0.9*	4
Proximal to Crush	6	9.1 ± 0.7*	4
Distal to Crush	6	15.2 ± 0.6*	4
Distal to Transection	6	6.6 ± 0.3	3

B-50 was quantitated in 5 mm nerve pieces with a radioimmunoassay as described in the Method section. Five mm nerve pieces were dissected from crushed and contralateral rat sciatic nerve, 6 or 36 days following the surgery. The pieces were taken (1) 2–1.5 cm proximal to the epineural suture marking the proximal border of the crush site ("proximal to crush"), (2) the region containing the crush site starting 2.5 mm proximal to the suture ("crush region"), (3) the region immediately distal to the piece taken as crush region ("distal to crush"). In one experimental group instead of a crush, the sciatic nerve was transected and ligated and 6 days later the pieces distal to the transection were taken ("distal to transection"). Data are presented as means ± SEM and statistical analysis was performed by one-way ANOVA followed by a supplemental *t*-test. *Significantly different from control sciatic nerve, $F(5,30)=54.7$, $p<0.05$.

mediately following homogenization, a sample mix was added to the homogenate to give (final concentrations): Tris-HCl 62.5 mM, pH 6.8, SDS 1%, glycerol 10%, bromophenol blue 0.001%, 2-mercaptoethanol 5%. The homogenates were heated for 10 min at 60°C in sample mix prior to electrophoresis in 11% SDS-polyacrylamide slab gels as described by Zwiers *et al.* [38]. Transfer of protein from the SDS-polyacrylamide gels to nitrocellulose paper was undertaken according to the method of Towbin *et al.* [30]. Nitrocellulose papers with transferred proteins (immunoblots) were rinsed for 30 min in Tris-buffered saline (TBS), pH 7.4. Immunoblots were incubated for 1 hr at room temperature with affinity-purified anti-B-50 antibodies (antiserum 8420, 1/500) [21] in TBS containing 0.1% Tween-20 and 0.1% gelatin. Subsequently, the immunoblots were rinsed in TBS containing 0.1% Tween-20 and incubated for 2 hr with horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulins. Antigen-antibody binding was detected by the staining method of Buckel and Zehelein [4].

The B-50 Radioimmunoassay

The amount of B-50 was quantitated in 5 mm nerve pieces obtained from various regions of crushed and control sciatic nerves as specified in Table 1. The radioimmunoassay was performed as described by Oestreicher *et al.* [23]. A B-50 tracer was prepared with high specific radioactivity (10–30 μCi/μg protein) by phosphorylation of 3 μg of purified B-50 [21] with carrier-free [γ-^{32}P]-ATP (500 μCi; 7.5 μM) catalyzed by 3 μg purifed protein kinase C [1]. The radioimmunoassay was performed using affinity-purified anti-B-50 immunoglobulins (antiserum 8420) in detergent containing medium with purified B-50 as a standard. The detection range was 0.1–10 ng.

Immunohistochemistry

Nerves were fixed for 2.5 hr in 1% paraformaldehyde/50 mM phosphate buffer, pH 7.4, containing 0.1 M lysine and 0.2% sodium periodate [17]. The nerves were cryoprotected by immersion in graded sucrose (7.5, 15, 25 and 35%). The double labelling experiments were performed at room temperature, using mouse monoclonal anti-70 kD neurofilament (NF70) antibodies (Monosan, Leiden, NL) and affinity-purified rabbit anti-B-50 antibodies (antiserum 8420). Four μm thick cyrostat sections were reacted for 24 hr with a mixture of NF70 (dilution 1:20) and B-50 (dilution 1:2400) antibodies in phosphate-buffered saline (PBS), containing 0.2% Triton X-100 (Triton, v/v) and 0.2% bovine serum albumin (BSA, w/v). After extensive rinsing with PBS-Triton, sections were incubated for 1 hr with rhodamine-conjugated horse anti-rabbit immunoglobulins (dilution 1:200; Central Laboratory for Blood Transfusion, Amsterdam, NL) in PBS-Triton-BSA. Subsequently, the sections were incubated for 1 hr with 1% normal rabbit immunoglobulins (Miles Lab. Ltd., Merseyside, UK) in PBS. Finally, fluorescein-conjugated rabbit anti-mouse antibodies (Dako, Copenhagen, Denmark) dissolved in PBS-Triton-BSA, supplemented with 1% normal rabbit immunoglobulins were applied to the sections also for 1 hr. The sections were mounted in 90% glycerol in veronal-buffered saline, containing 0.1%

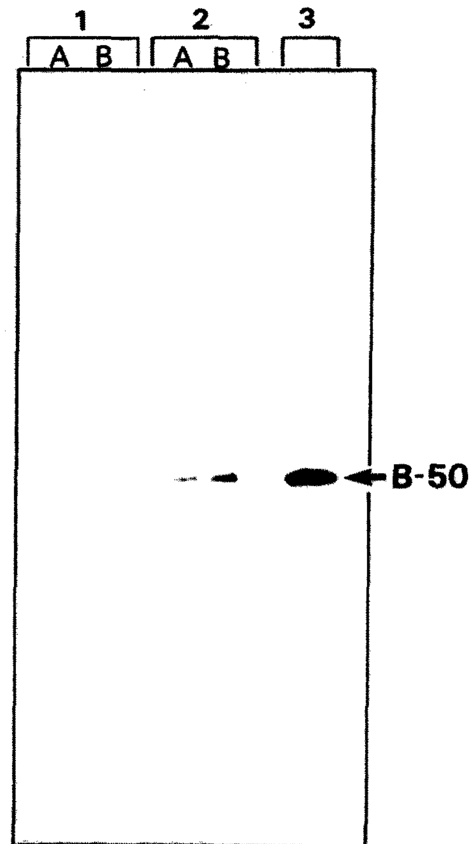

FIG. 1. B-50 immunoreactivity on Western blots loaded with samples, obtained from crushed (lanes 2) and non-crushed (lanes 1) rat sciatic nerve pieces (region 2, see legend Table 1). A: 20 μg protein; B:40 μg protein. Ten μg protein of light synaptosomal plasma membranes (lane 3) isolated according to [16] was used as a reference for B-50 localization on the blot. Anti-B-50 antiserum 8420 was used in a 1:500 dilution. Preimmune serum and secondary antibody controls were negative (results not shown).

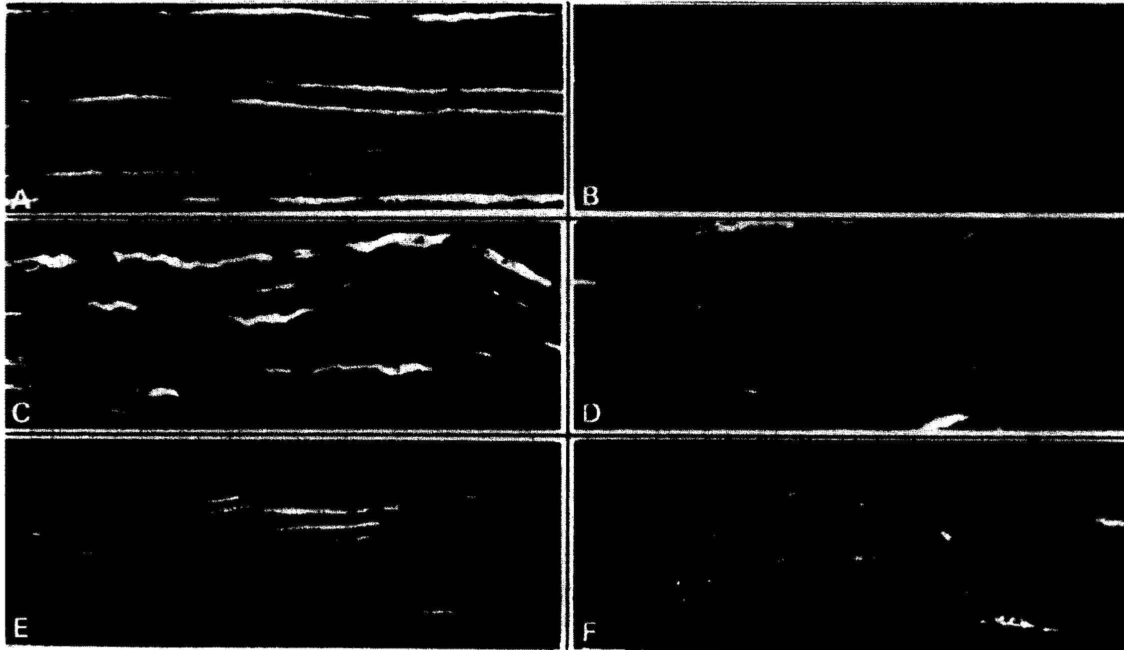

FIG. 2. Longitudinal sections through an intact (A,B) and crushed (C, D, E, F) rat sciatic nerve. In these sections neurites were selectively labelled with monoclonal mouse anti-70 kDa neurofilament (NF70) antibodies. Double label immunofluorescence microscopy with anti-NF70 antibodies (1:20) and rabbit affinity-purified anti-B-50 antibodies (1:2400), shows corresponding fluorescein (A, C, E) and rhodamine (B, D, F) staining in the same section. Six days following surgery sections were taken at the position of the epineural suture that marked the proximal border of the crush (C,D). Eleven days following surgery the sections were taken at 5 mm distal from the epineural suture (E,F). Control sections (A,B) were taken from the non-crushed contralateral nerve at approximately the same distance from the sciatic notch as the epineural suture. Note the absence of B-50 immunoreactivity in the axons (B) as identified by the NF70 immunostaining (A). At 6 and 11 days following surgery, most axon profiles around the proximal border of the crush (C,D) and in the distal portion (E,F) of the regenerating nerve are labelled with both NF70 (C,E) and B-50 (D,F) antibodies. Near the crush the immunostained profiles represent a mixture of parent axons and newly formed sprouts, whereas in the nerve part distal to the crush only newly formed sprouts are visualized. Control experiments indicated no significant leakage between fluorescein and rhodamine channels. Furthermore, it was shown that the normal rabbit immunoglobulins could completely prevent crossreaction between the horse anti-rabbit and rabbit anti-mouse immunoglobulins. Original magnification 125×.

paraphenylene diamine. The sections were examined with a Leitz Orthoplan microscope, equipped with a Ploem epi-illuminator.

Protein Determination

Protein concentrations were measured using the method of Lowry *et al.* [16].

RESULTS

Immunolabelling of Western blots of homogenates from nerve pieces containing the crush region with affinity-purified rabbit anti-B-50 immunoglobulins revealed a single immunoreactive protein band in the piece taken from the crushed nerve but not in that taken from the control nerve. This immuno-band comigrates with B-50 in synaptosomal plasma membranes (Fig. 1).

The amount of B-50 in the nerve homogenates was quantitated by means of a radioimmunoassay, again employing the affinity-purified anti-B-50 antibodies (Table 1). In the control nerve the B-50 content per mg protein is approximately 1.5% of that in the cortex cerebrum (342.0±14.0 ng B-50/mg protein). Six days following crush lesioning the amount of B-50 in the crushed region of the nerve is increased 5.3-fold (Table 1). Furthermore, the B-50 content in pieces taken proximal and distal from the crush is higher than in control nerves with a significantly higher amount in the distal as compared to the proximal nerve piece ($p<0.05$). No increase in B-50 was observed in that distal portion when the nerve was transected and ligated. Thirty-six days following the crush lesion the amount of B-50 has decreased but it still is significantly higher than that found in control nerves (Table 1).

In order to study the cellular localization of the increased amount of B-50, longitudinal sections taken from the intact and regenerating nerve were subjected to immunohistochemical analysis using anti-B-50 immunoglobulins and anti-NF70 antibodies. The latter antibodies were employed to identify regenerating axons and sprouts.

As is shown in Fig. 2A, numerous axons could be identified in longitudinal sections of intact nerves by means of immunocytochemical staining with mouse monoclonal anti-NF70 antibodies. Virtually no labelling was obtained with the anti-B-50 antibodies in these sections (Fig. 2B). However, in tissue sections from crushed nerves 6 days following the lesion, a nearly perfect co-localization of NF70 and B-50 immunoreactivity was observed (Fig. 2C,D). In response to a crush lesion, newly formed sprouts cross the crushed zone and grow into the distal portion of the crushed

nerve. These newly formed sprouts could be visualized in nerve sections taken at 5 mm distal from the crush site 11 days post-crush, using anti-neurofilament antibodies [31,34]. In these sprouts also good co-localization of both B-50 and neurofilament immunoreactivity was found (Fig. 2E,F).

DISCUSSION

Recently it has been reported that the neuron-specific presynaptically localized kinase C substrate B-50 is present in abundant amounts in outgrowing hippocampal neurites [22] and in nerve growth cones isolated from fetal rat brain [6]. These findings prompted us to make a detailed study of the fate of B-50 during regenerative axonal outgrowth in the crushed sciatic nerve of the rat.

The amount of B-50 increased substantially in the sciatic nerve following a crush lesion. On immunoblots of homogenates of nerve pieces taken from the crushed region of the damaged nerve one immuno-band comigrating with B-50 in synaptosomal plasma membranes appeared. This band was not present on immunoblots prepared from control nerve homogenates. In tissue sections incubated with a mixture of anti-B-50 and anti-NF70 antibodies, B-50 immunoreactivity was found to be confined to the damaged axon profiles (Fig. 2C,D) and was virtually absent in intact axons (Fig. 2A,B).

Quantification of the B-50 content of nerve pieces obtained from crushed and control nerves learned that following nerve crush the amount of B-50 increased 5.3-fold in the crushed region of the nerve (Table 1). Although the enhanced level of B-50 in the crushed nerve may merely reflect accumulation of B-50 as a result of hampered axonal transport, the data presented argue against this formal possibility. The level of B-50 in the distal region of the nerve containing the very thin newly formed sprouts is higher than that proximal to the crush site (Table 1). This implies that these thin sprouts, that have passed the crush zone, contain very high levels of B-50. Indeed, we demonstrated that bright immunofluorescence distal to the crush site is specifically localized in the newly formed sprouts (Fig. 2E,F). Taken together, the virtual absence of B-50 protein in the contralateral nerve piece and the abundance of B-50 in the regenerating axons and newly formed sprouts, support the notion that the B-50 protein is part of the regeneration response of the crushed nerve.

The drop in B-50 content in the crush region 36 days following the lesion is of interest. Several explanations may be plausible. This observation may serve as first indication that the B-50 content returns to normal levels after reinnervation of the target muscle. Alternatively, it may be that disappearance of newly formed outgrowing growth sprouts in that region is reflected by a decrease in the B-50 level. Both possibilities are subject of further study.

Several authors have shown the existence of a family of growth-associated proteins (GAPs) both in embryonic, maturing and in regenerating neurones, using [^{35}S]-methionine metabolic labelling [37]. The present paper suggests that B-50 is a member of this protein family. Based on several characteristics (IEP, MW, etc.), B-50 may be similar to GAP-43 [12, 18, 36], GAP-48 [2], pp46 [14] and F1 [10,25]. It has been shown that GAPs are expressed during axonal maturation and regeneration [28]. The fact that B-50 is present in growth cones isolated from fetal rat brain [6, 19, 25] and in regenerating fibers (this paper) is in line with such a notion. Furthermore, Holmes and Rodnight [11] reported that in brain the endogenous phosphorylation of B-50 reaches a maximum at 15 days after birth and then declines as a function of age. We have obtained evidence using the B-50 radioimmunoassay that the amount of B-50 in rat brain cortex is highest in neonatal rats and then declines with age resulting in a 40% reduction in 28 month old as compared to 2 month old male rats [24].

It remains to be shown what role B-50 plays in growth-associated cellular mechanisms. In the adult rat brain, B-50 is particularly localized in presynaptic terminals and may be part of a feedback mechanism in transmembrane signal transduction, involving activation of protein kinase C by the receptor-mediated generation of diacylglycerol [8, 13, 32]. The resulting stimulation of B-50 phosphorylation diminished phosphatidylinositol 4-phosphate kinase activity, thereby decreasing the amount of phosphatidylinositol 4,5-bisphosphate available for further receptor activation [8]. The characteristics of the endogenous phosphorylation of B-50 are similar in synaptic plasma membranes isolated from adult rat brain and in growth cone particulate material isolated from fetal rat brain [6]. Moreover, phorbol 12,13-dibutyrate stimulates B-50 phosphorylation in adult rat brain synaptic plasma membranes [7,26] and in primary cultured embryonic neurones [5]. This suggests that signal transduction processes involving protein kinase C are operative in the activation and/or guidance of outgrowing neurites. This would certainly be in line with current views on the role of protein kinase C in growth and tumor promotion [33,35].

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