Proc. West. Pharmacol. Soc. 29:183-185 (1986)

PROTEOLYSIS OF NERVOUS TISSUE SPECIFIC PHOSPHOPROTEIN B-50 YIELDING A NEW PEPTIDE WITH BEHAVIOURAL ACTIVITY

H. ZWIERS & W.H. GISPEN[1]

Department of Pharmacology & Therapeutics, The University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1 and [1]Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology & Institute of Molecular Biology, State University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands

In recent years we have isolated and characterized an ACTH sensitive membrane protein kinase and substrate protein from rat brain tissue. The evidence available suggests that this protein kinase is very similar, if not identical, to protein kinase C. The substrate protein B-50 (mol. wt. 48 kDa) is one of the most acidic proteins present in synaptic membranes. It is localized exclusively in neuronal tissue and electron microscopic studies point to a presynaptic localization. A variety of data suggest that this phosphoprotein may play a regulatory role in receptor mediated polyphosphoinositide metabolism. In the purification procedure of B-50 and B-50 protein kinase, dialysis of the protein fraction was one of the tools used. We noted that after dialysis the endogenous phosphorylation of B-50 was markedly enhanced. This suggested the presence of a small molecular weight entity that interfered with the B-50 protein kinase activity. We will review our efforts to characterize this active principle and will discuss its possible role in vivo in view of recent findings concerning the further characterization of B-50.

METHODS: The purification of rat B-50/B-50-kinase complex (ASP 57-82) was performed as described before (1). Phosphorylation assays, SDS-PAGE, dialysis, HPLC chromatography and characterization of phosphorylation inhibiting peptide (PIP) are described in (2).

RESULTS & DISCUSSION: The dialysate of ASP_{57-82} proteins obtained from 150 g rat brain tissue was lyophilized, resuspended in water and subjected to HPLC in a system developed to separate small molecular weight peptides (MW<4000). When tested in the endogenous phosphorylation assay only one peak displayed the inhibiting activity. Based on its sensitivity to pronase or acid hydrolysis and the nature of the separation system used, the factor was thought to be a small peptide. Indeed, amino acid analysis of the inhibiting fraction revealed the presence of a basic peptide of approximately 15 amino acids with a molecular weight of the order of 1600 (phosphorylation inhibiting peptide, PIP). Preliminary evidence showed that during dialysis PIP occurred in the dialysate as the result of a specific proteolytic cleavage of the B-50 protein (2). The ammonium sulfate precipitate containing the highest endogenous B-50 phosphorylating activity, also contains protease activity. When incubated in the absence of calcium a time-dependent decrease of the protein B-50 is observed with a concomitant appearance of phosphoprotein B-60 and PIP (Fig. 1). Addition of calcium and/or calmodulin enhanced the protease activity whereas the substrate specificity is lost. Results of both isoelectric focusing and peptide mapping indicate that B-50 and B-60 are related proteins (3).

Previously, a marked correlation for ACTH fragments was found between the structural requirements of peptides to inhibit B-50 protein phosphorylation

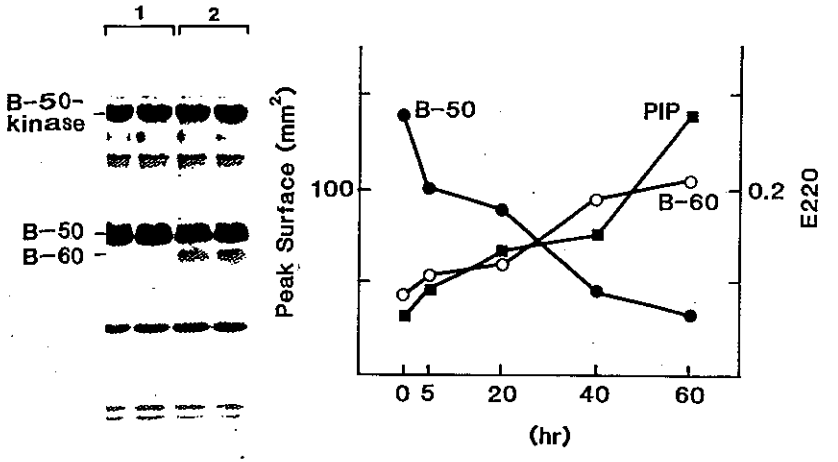

FIGURE 1: Left part: Protein staining pattern of ASP_{57-82} proteins. Duplicate samples (3 µg of total protein, 30 µl, containing 1 mM EGTA), were incubated for 20 h (sample 2) or 0 h (sample 1) at 4°C. Incubation was terminated by the addition of 15 µl of a stop solution containing SDS. Proteins were separated by SDS-polyacrylamide gel electrophoresis. Right part: Amounts of B-50, B-60 and PIP as a function of dialysis time at 4°C. Densitometric scans of Fast-Green stained protein profiles (at 650 mm) of ASP_{57-82} revealed relative amounts of B-50 and B-60 by measuring peak surface. Amounts of PIP were determined by measurement of the extinction at 220 nm of the HPLC fraction containing PIP.

and to induce excessive grooming in the rat (4). Therefore in a preliminary experiment we tested whether PIP was also capable of inducing excessive grooming after intracerebroventricular application in rats. Indeed, like ACTH, PIP induced the display of excessive grooming (Table 1).

TABLE 1: Induction of excessive grooming in the rat

Treatment	Dose	% of maximal grooming score (n,4; means ± S.E.M.)
Vehicle		16 ± 4
$ACTH_{1-24}$	0.3 µg/3 µl	76 ± 3
PIP	0.1 µg/3 µl	45 ± 5

Although the data obtained thus far indicate that PIP is an important peptide in having ACTH like effects both in in vitro systems (inhibition of B-50) and in in vivo systems (induction of excessive grooming in the rat), until recently there has been little experimental evidence that the conversion of B-50 to B-60 also occurs in synaptosomal plasma membranes and in intact nervous tissue (unpublished). However, recent findings concerning the localization and further characterization of B-50 may indicate conditions under which PIP is produced in vivo. Electron microscopic 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. Recently, evidence was reported that B-50 is associated with the brain subcellular fraction enriched in nerve growth cones obtained from fetal rat brain (6). On the autoradiograms obtained after phosphorylation and two-dimensional separation of nerve growth cone proteins, an intense phosphorylated protein is detected having identical chromatographic properties as B-60 (6,7). This indicates that in fetal nerve growth cones there is a high level of proteolytic activity converting B-50 into B-60 with the concomitant formation of PIP. The second line of evidence relates to the possibility that B-50 is a growth associated protein (GAP). Based on obvious similarities in 2D separation characteristics we started, in 1981, collaborative studies to find out if rabbit antibodies against rat B-50 would crossreact with a prominent, rapidly transported, growth associated protein of MW 49 kDa synthesized in regenerating goldfish optic nerve (8). Unfortunately these rabbit antibodies probably did not crossreact with goldfish proteins (9) and proof that B-50 is a GAP protein is lacking. Examination of 2D patterns of these goldfish GAP's shows that the 49 kDa protein is accompanied by a slightly lower molecular weight entity (GAP_{44}). Analogues to the rat B-50/B-60 conversion, also goldfish GAP_{49}, might have been partially converted to GAP_{44} with the release of a small fragment. Recently it was found that ACTH facilitates recovery of nerve function after crush lesion (10,11).

Since we observed some similarities in biological activity of ACTH and PIP, it might well be that PIP by local production has a function in synaptogenesis, and is involved in processes leading to recovery of damaged nerve tissue.

ACKNOWLEDGEMENTS: The authors would like to thank M. Devlin, for typing, and to Dr. K. Lederis.

REFERENCES

1. Zwiers, H., Schotman, P. & Gispen, W.H.: J. Neurochem. 34:1689 (1980).
2. Zwiers, H., Verhoef, J., Schotman, P. & Gispen, W.H.: Febs Lett. 112:168 (1980).
3. Zwiers, H., Gispen, W.H., Kleine, L. & Mahler, H.R.: Neurochem. Res. 7:127 (1982).
4. Zwiers, H., Aloyo, V.J. & Gispen, W.H.: Life Sci. 28:1545, 1981.
5. Gispen, W.H., Leunissen, J.J.M., Oestreicher, A.H., Verkley, A.J. & Zwiers, H.: Brain Res. 328:381 (1985).
6. DeGraan, P.N.E., VanHooff, C.O.M., Tilly, B.C., Oestreicher, A.B., Schotman, P. & Gispen, W.H.: Neurosci. Lett. 61:235 (1985).
7. Katz, F., Ellis, L. & Pfenninger, K.H.: J. Neurosci. 5:1402 (1985).
8. Benowitz, L.I. & Lewis, E.R.: J. Neurosci. 3:2153 (1983).
9. Oestreicher, A.B., Van Duin, M., Zwiers, H. & Gispen, W.H.: J. Neurochem. 43:935 (1984).
10. Bylsma, W.A., Schotman, P., Jennekens, F.G.I., Gispen, W.H. & DeWied, D.: Eur. J. Pharmacol. 92:231 (1983).
11. Edwards, P.M., Van der Zee, C.E.E.M., Verhaagen, J., Schotman, P., Jennekens, F.G.I. & Gispen, W.H.: J. Neurological Sci. 64:333 (1984).