A NEW PHOSPHORYLATION-INHIBITING PEPTIDE (PIP) WITH BEHAVIORAL ACTIVITY FROM RAT BRAIN MEMBRANES

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Received 25 January 1980

1. Introduction

We have described the isolation and purification of a protein kinase from rat brain membranes, which was inhibited by ACTH and not stimulated by cAMP [1–3]. Dialysis of the protein fraction was one of the tools used in the purification of this membrane-bound enzyme. We noted that after dialysis, the endogenous phosphorylation of one substrate protein (B-50, MW 48K, IEP 4.5) was markedly enhanced. A general enhancement of endogenous membrane protein was found phosphorylation after dialysis [4]. Here, the isolation, characterization and possible origin of a brain peptide which inhibits the B-50 protein phosphorylation after dialysis was found [4]. of the effects brought about by ACTH, are reported.

2. Materials and methods

2.1. *Fractionation of the ACTH-sensitive, membrane-bound protein kinase*

The purification of this enzyme and one of its substrate proteins (B-50) has been detailed in [3]. In short, crude synaptosomal plasma membrane fractions were prepared from rat cortex (27 g wet wt). The proteins were partly solubilized with 0.5% Triton X-100 in 75 mM KCl [2] and then subjected to column chromatography over DEAE-cellulose using a NaCl gradient to elute the endogenous B-50 phosphorylating activity [2]. The active eluate fractions were pooled (DEAE pool) and treated with ammonium sulphate (55% saturation). Precipitated proteins (ASP 0–55%) were removed by centrifugation and to the supernatant more ammonium sulphate was added till 80% saturation. This precipitate (ASP 55–80%) was again collected by centrifugation and served as source for the isolation of the inhibiting factor. Among other proteins, the ASP 55–80% contains 1 protein kinase and 1 major phosphoprotein (B-50) [3].

2.2. *Endogenous phosphorylation assay and SDS polyacrylamide gel electrophoresis*

Endogenous B-50 protein kinase activity was assayed under the following conditions: 7.5 μM ATP, 1 μCi [γ-^{32}P] ATP (± 3000 Ci/mmol; Amersham). 15 μl protein sample (Triton/KCl extract, DEAE column fractions, ammonium sulphate fractions), 50 mM Na-acetate, 10 mM Mg-acetate, 1 mM Ca-acetate, pH 6.5 in 25 μl final vol. Synthetic $ACTH_{1-24}$ (Organon Int. BV, The Netherlands) or the inhibiting factor was added 10 s prior to the addition of ATP. To test the effect of the inhibiting factor, the phosphorylation was carried out for 1 min [1], using a freshly prepared dialyzed ASP 55–80% fraction [3]. After termination of the reaction, phosphoproteins were separated on SDS–polyacrylamide slab gels (11%). The gels were then dried and stained for proteins. Autoradiography and liquid scintillation counting of labelled phosphoproteins were done as in [5].

2.3. *Isolation of the phosphorylation inhibiting peptide (PIP)*

The ASP 55–80% fraction was dissolved in 100 μl buffer A (10 mM Tris–HCl, 1 mM $CaCl_2$, 0.1 mM dithiothreitol (pH 7.4)) giving a final protein conc. ~5 μg/μl. This mixture was dialyzed overnight against 1 ml of the same buffer. The ASP 55–80% dialysate was subjected to high-pressure liquid chromatography (HPLC) on a reverse-phase μBondapak C_{18} column (0.39 × 30 cm; Waters Ass.) as detailed in [6]. Elu-

tion was performed using a 60 min linear gradient from 100% 0.01 M ammonium acetate (pH 4.15) to 100% methanol containing 1.5 ml acetic acid/liter. The flow rate was 2 ml/min and ultraviolet absorbance was measured continuously at 210 nm. Fractions were collected and evaporated at 60°C using a Büchler vortex evaporator. The dry material was dissolved in 150 μl aqua bidest. Aliquots of 5 μl were tested in the endogenous phosphorylation assay. The aqua bidest fraction containing the inhibiting activity is further referred to as HPLC-X.

2.4. *Characterization of the PIP*

(A) Acid hydrolysis: From the HPLC-X fraction aliquots of 15 μl were again lyophilized in glass tubes and dissolved in 50 μl distilled 6 N HCl. The tubes were evacuated under N_2 and subsequently sealed. Hydrolysis was done at 110°C for 24 h. After lyophilization, the hydrolysate was dissolved in 15 μl aqua bidest and 5 μl aliquots were tested in the endogenous phosphorylation assay.

(B) Pronase treatment: From the HPLC-X fraction aliquots of 15 μl were lyophilized and dissolved in 15 μl buffer A. These samples were incubated in the presence of 2 ng pronase (Sigma) at 37°C for 4 h. Subsequently, the samples were heated at 70°C for 20 min. This procedure inactivated the pronase completely as established in a pilot experiment. Aliquots of 5 μl were tested in the endogenous phosphorylation assay.

(C) Amino acid analysis: The amino acid composition of the PIP was determined by automated amino acid analysis (TSM, Technicon) using a stainless steel 18 × 0.21 cm column filled with Durrum DC-5A resin and fluorescence detection with *o*-phthaldehyde (Serva, Heidelberg) and 2-mercaptoethanol (detailed in [3]). Samples were run in duplicate; each sample was followed by a protein hydrolysate standard (Pierce Chemical Corp., IL) for comparison. Amino acid quantities as low as 5 pmol could be detected. The fluorescence detection procedure did not detect proline and cysteine.

2.5. *Excessive grooming test*

The induction of excessive grooming in rats was studied essentially as in [7]. In short, conscious rats received an intraventricular injection of 3 μl of either saline, vehicle (lyophilized HPLC-elution buffer residue dissolved in aqua bidest). $ACTH_{1-24}$ (0.3 μg/3 μl saline) or 3 μl of the HPLC-X fraction containing PIP (5 × concentrated as compared to those aliquots used in the phosphorylation assay). Rats were individually placed in glass observation boxes in a sound proof room. Using a 15 s time sampling procedure, the display of excessive grooming was recorded for a period of 50 min, beginning 15 min after the injection. The data are expressed as % of maximal possible grooming scores (50 × 4 = 200).

3. Results and discussion

As can be seen in the scans of autoradiograms, prepared after endogenous phosphorylation of proteins in the DEAE-pool, specifically the phosphorylation of B-50 was enhanced by prior dialysis (fig.1A). This effect was even more pronounced when the phosphorylation was studied in an ASP 55-80 fraction (a substantially purified B-50 preparation; fig.1B). In table 1, it is shown that dialysis increased the specific activity of B-50 at 3 different purification stages [2,3]. This could not be explained by the removal of certain ions such as ammonium sulphate or NaCl, because the various fractions differed in constituents. At this stage we postulated a dialysable inhibitory factor. Indeed, an aliquot of a concentrated dialysate (20 ×) reduced the endogenous phosphorylation of freshly prepared, dialysed ASP 55–80% material with 63% (without dialysate: 1263 cpm; with dialysate: 454 cpm incorporated into B-50). Next, the dialysates of 5 batches of ASP 55–80% material derived from 150 g brain tissue, were pooled, lyophilized and resuspended in1 ml aqua bidest. This material was subjected to HPLC in a system developed to separate small molecular weight peptides (<4000 mol. wt [6]). The chromatographic profile is shown in fig.2. Aliquots obtained from only one peak displayed inhibiting activity when tested in the endogenous phosphorylation assay. This fraction was used to further characterize the inhibiting principle. The material showed absorbance at both 220 and 260 nm (ratio 4:1) and was sensitive to acid hydrolysis and pronase treatment (table 2). This, together with the HPLC-elution characteristics strongly suggested that the inhibiting factor is a peptide with a relative small molecular weight and/or polar properties. To further investigate this possibility, a 15 μl aliquot of the HPLC-X fraction was subjected to acid hydro-

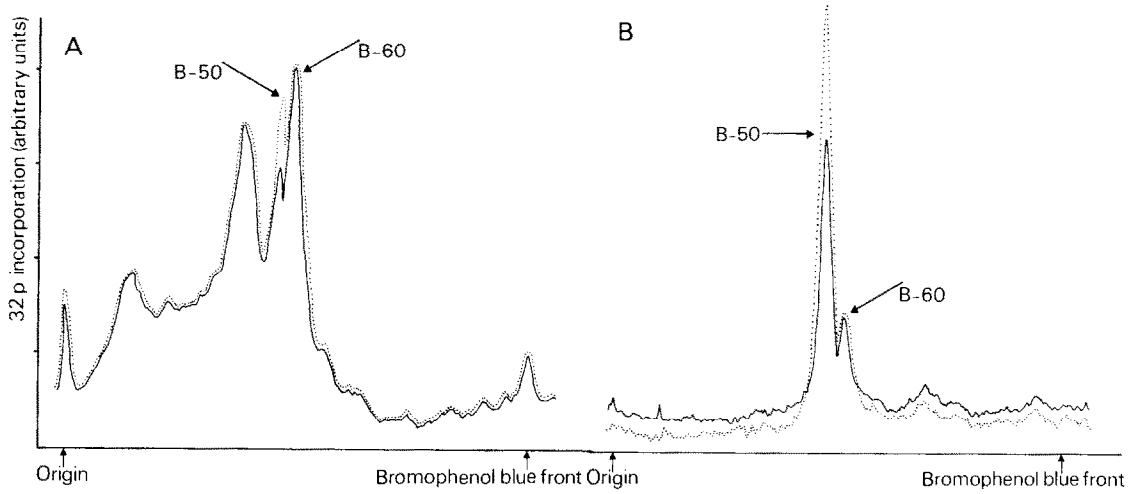

Fig.1. Densitometric scans. (A) Autoradiogram after SDS–polyacrylamide gel electrophoresis of endogenously phosphorylated proteins of DEAE-pool (see section 2); (B) Idem of ASP 55–80% proteins (see section 2). (——) before dialysis; (· · ·) after dialysis (20 h at 4°C).

Table 1
Effect of dialysis on endogenous B-50 protein kinase (spec. act., pmol . mg total protein^{-1}. min^{-1})

	Dialysis	
	Without	With
Triton X-100/KCl extract	0.7	1.8
DEAE-pool	2.7	3.2
ASP 55–80%	70.4	137.4

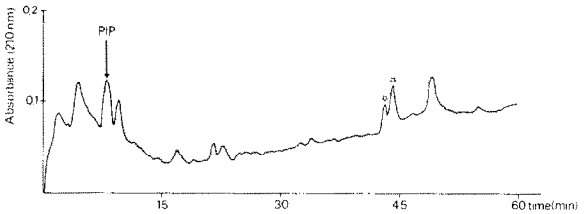

Fig.2. HPLC-profile of a concentrated dialysate of ASP 55–80% proteins. PIP, fraction containing phosphorylation inhibiting peptide. ☆, impurities from the mobile phase.

lysis, followed by amino acid analysis. Hydrolysis of the active HPLC-X fraction revealed the presence of a restricted number of amino acids (table 3). A relative high amount of basic amino acid residues was found, whereas neutral amino acids were virtually absent. The amino acid composition was calculated, based on the assumption that we are dealing with one single small peptide (table 3). The data suggest a chain of 15 amino acid residues in total, with a molecular weight in the order of 1650. Therefore, we refer to the inhibiting factor as the phosphorylation inhibiting peptide (PIP). The preliminary data on an inhibiting

Table 2
Characterization of the phosphorylation inhibiting factor

	Endogenous B-50 protein kinase specific activity in ASP 55–80% (pmol . mg total protein^{-1}. min^{-1})[a]
Control	140.0
+ 5 μl HPLC-X	35.6
+ 5 μl HPLC-X after acid hydrolysis	137.3
+ 5 μl HPLC-X after pronase treatment	130.3

[a] Determined in duplicate

Table 3
Amino acid composition of PIP

	pmol[a]	Residues/molecule[b]
Aspartate	33	1
Threonine[c]	43	1
Serine[c]	76	2
Glutamate	41	1
Glycine	88	2
Alanine	46	1
Valine	35	1
Methionine	–	–
Isoleucine	9	–
Leucine	–	–
Tyrosine	–	–
Phenylalanine	7	–
Lysine	93	2
Histidine	117	3
Arginine	35	1

[a] In HPLC sample (mean of 2 determinations)
[b] Assuming 40 pmol peptide in HPLC sample
[c] Corrections were made for degradation during hydrolysis by extrapolation to zero time hydrolysis

factor point to an inhibiting entity with a molecular weight in the same range [4]. Previously, for ACTH-fragments a marked correlation was found between this inhibition of phosphorylation of B-50 and the activity in the induction of excessive grooming [8,9]. Therefore, in a preliminary experiment we tested whether the PIP was also capable of inducing excessive grooming after intracerebroventricular application in rats. Indeed, like ACTH, the PIP induced display of excessive grooming (fig.3). Whereas in ACTH-induced grooming the predominant element of the displayed behavior is grooming itself [7], the PIP-induced grooming was typified by the high intensity of scratching. Clearly, the approximate amino acid composition of PIP is markedly different from that of $ACTH_{1-24}$. Yet, both peptides are similar in that they contain a high amount of basic amino acid residues. It is remarkable that both basic peptides ($ACTH_{1-24}$ and PIP) induce excessive grooming and inhibit membrane protein phosphorylation, supporting the notion that membrane protein phosphorylation may have a role in the onset of peptide-induced grooming behavior [9,10].

In view of the ample opportunity for proteolysis to occur during the dialysis, it is difficult to assess whether or not PIP is a natural free occurring neuropeptide. The data from fig.4 suggest that PIP can be

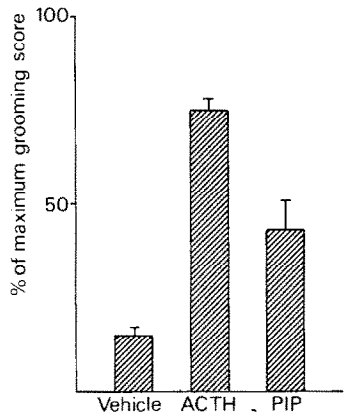

Fig.3. Induction of excessive grooming in the rat. ACTH: $ACTH_{1-24}$ (0.3 μg/3 μl); PIP: phosphorylation inhibiting peptide (0.1 μg/3 μl), n=4; Means ± SEM.

released from the B-50 substrate protein by enzymatic cleavage of B-50 into a protein with app. mol. wt 45 000–46 000 (B-60) and PIP. In this experiment, freshly prepared ASP 55-80 was dialyzed for 5, 20, 40 or 60 h. Samples of the dialyzed proteins were subjected to PAGE and the dialysates to HPLC, for identification of B-50, B-60 and PIP, respectively. Dialysis

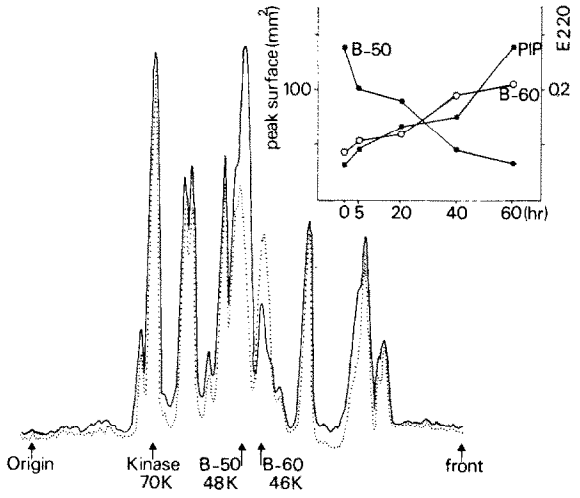

Fig.4. Densitometric scans of fast green-stained protein profile of ASP 55–80% after SDS–polyacrylamide slab gel electrophoresis before (——) and after (· · ·) 20 h dialysis at 4°C. Insert: Surface of the peaks B-50 and B-60 in the ASP 55–80% profile as a function of dialysis time. The extinction at 220 nm of the HPLC-fraction containing PIP as a function of the dialysis time of the ASP 55–80% fraction (see section 2).

led to the specific disappearance of B-50 and the specific accumulation of B-60, whereas the production of PIP closely followed that of B-60. Furthermore, preliminary data suggest that B-60 has an IEP of <4.5, thus being a protein more acidic than B-50 [3], in line with the release of a basic peptide from B-50. As a control ASP 55-80 was heated to 70°C for 20 min, followed by dialysis; under these conditions no breakdown of B-50 or the production of B-60 and PIP was observed, following dialysis (data not shown). In view of the treatment of the samples with SDS and β-mercaptoethanol prior to the electrophoresis it is unlikely that PIP is merely sticking to B-50 protein and subsequently released during dialysis. Whether PIP is split from B-50 under physiological conditions and what role phosphorylation of B-50 plays in the production of PIP is subject of further study. Also others reported on the instability of brain membrane phosphoproteins with an apparent molecular weight in the range of that of B-50 ([11], Rodnight, personal communication). In addition, we noted that endogenous phosphorylation and peptide sensitivity of rat SPM diminished upon storage at −20°C [1].

There is ample evidence pointing to a key role of membrane protein phosphorylation in receptor activation and neurotransmission [12] and that peptides may exert a modulatory role in those processes [9]. This report opens the possibility for an additional role of a specific proteolytic activity releasing a peptide inhibiting the B-50 protein kinase and thus modulating the degree of phosphorylation of the B-50 substrate protein in the synaptic membrane.

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