COMPLEXITY OF CYCLIC AMP-DEPENDENT PHOSPHOPROTEINS IN MEMBRANES FROM BRAIN TISSUE CONTAINING SYNAPSES

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1. Introduction

There is now considerable evidence that some of the effects of neurotransmitter substances on their receptor cells are mediated through the synthesis of adenosine 3′5′-monophosphate (cyclic AMP) (see [1]). A number of studies have further suggested that such effects might occur through modification of the state of phosphorylation of synaptic membrane proteins. In intact cell-containing preparations of nervous tissue, for example, protein phosphorylation is stimulated by various putative neurotransmitters [2–5]. This may be related to the phosphorylation of endogenous proteins in synaptic membrane fragments by intrinsic cyclic AMP-stimulated protein kinase activity [6–11]. Cyclic AMP-stimulated phosphorylation of at least four protein components was found in such preparations [9]. If any of these proteins were specifically involved in synaptic transmission it would be expected that they would only occur in tissue containing synapses. We have therefore investigated the distribution of phosphorylated proteins in crude membrane fractions from two regions of bovine cerbral cortex: grey matter, which contains neurons with synaptic contacts as well as other cells and white matter, which lacks synaptic contacts and consists predominantly of oligodendroglial cells.

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2. Methods

Fresh ox cerebra were obtained from the local slaughter-house. The cortical grey matter and white matter were carefully dissected and homogenised in 9 vol. 10% (w/w) sucrose. The homogenates were centrifuged at 800 × *g* for 20 min and the supernatants so obtained recentrifuged at 9000 × *g* for 20 min. The resulting post-mitochondrial supernatants were centrifuged at 100 000 × *g* for 1 h to obtain crude microsomal pellets. Before using for phosphorylation experiments the pellets were washed once with 5 mM Tris–HCl, pH 7.4, twice with the same buffer containing 150 mM NaCl, and three times with buffer without NaCl (approximately 10 ml buffer/mg protein in each wash). The resulting crude membrane fraction was resuspended in the same buffer and stored at −20°C at a protein concentration of 10 mg/ml.

Phosphorylation was carried out at 37°C for 20 s in a reaction mixture (final volume 200 μl) containing 5 μM [γ-^{32}P]ATP, ammonium salt (Amersham Buchler), 1 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.4, with or without 10 μM cyclic AMP. The reaction was initiated by addition of crude membrane protein (final concentration 0.5 mg/ml). The incorporation of ^{32}P was stopped with 20 μl of a solution containing 10 mM non-radioactive ATP (to dilute the specific radioactivity of the substrate) and 10 mM $CuCl_2$ (to inhibit subsequent dephosphorylation by intrinsic phosphoprotein phosphatase activity [6]). The phosphorylated membranes were concentrated by centrifugation at 100 000 × *g* for 1 h.

For electrophoresis the phosphorylated crude

membrane pellets were resuspended in 25 μl of deionised water by sonication, followed by addition of 25 μl of a solution containing 8% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) 2-mercaptoethanol, 4 mM Tris-HCl, pH 6.8. The membranes were solubilised by heating at 50°C for 90 min before adding 20 μl glycerol and 5 μl 0.05% bromophenol blue. Electrophoresis was performed on polyacrylamide disc-gels (7.5% acrylamide, 0.25% *N,N'*-methylene-bisacrylamide, 5 mm diameter) in the presence of 0.1% SDS using the discontinuous, Tris-buffered system described by Laemmli [12]. At the end of electrophoresis the gels were frozen at −70°C and cut into 1 mm slices at −15°C. Cerenkov radiation was measured in each slice after adding 10 ml water.

The total incorporation of ^{32}P into acid-insoluble material using the above incubation conditions, but in a final volume of 25 μl, was determined by pipetting 20 μl aliquots of reaction mixture onto 1.7 cm squares of Whatman 3MM chromatography paper [13] after inhibiting further incorporation of ^{32}P with 5 μl ATP/$CuCl_2$ solution as described above. The papers were washed three-times in a solution of 10% (w/v) trichloroacetic acid containing 1 M phosphoric acid (50 ml/sample/wash) to remove soluble phosphates, and Cerenkov radiation was measured in 10 ml of water.

Protein was measured by the method of Lowry et al. [14] using bovine serum albumin as standard.

3. Results

The distribution of ^{32}P among membrane components separated by polyacrylamide gel electrophoresis is shown in fig.1. The patterns of phosphorylation were quite different in crude microsomal fractions from grey matter and white matter. Twelve phosphoprotein fractions were obtained. All fractions showed cyclic AMP-stimulated phosphorylation which in fraction H was as much as eight-times the basal activity (table 1).

In contrast to the complex pattern of cyclic AMP-stimulated phosphorylation observed in crude microsomal preparations from grey matter, most of that in white matter was associated with fraction L (fig.1b).

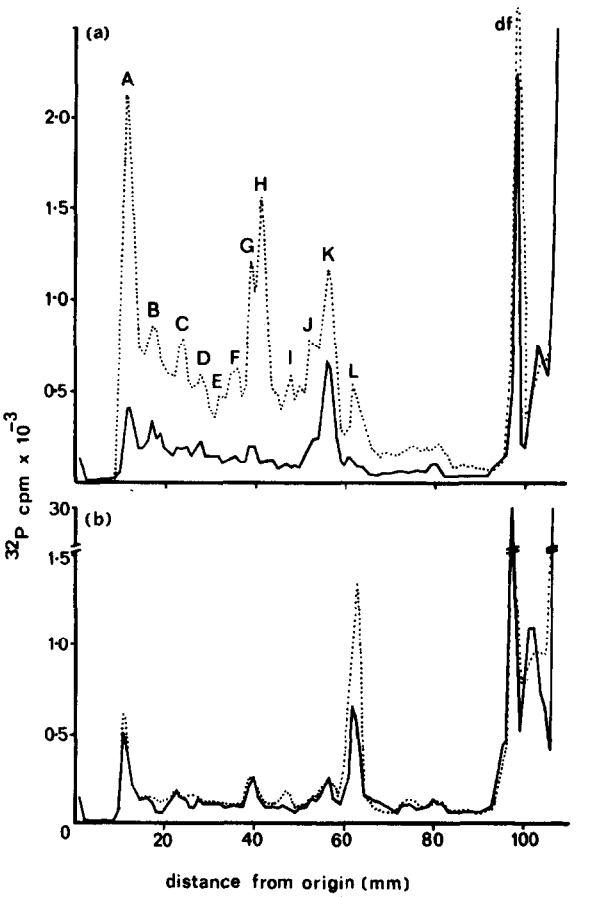

Fig.1. Distribution of [^{32}P]phosphoproteins after polyacrylamide gel electrophoresis of microsomal fractions labelled in the presence (dotted lines) or absence (solid lines) of 10 μM cyclic AMP. Crude microsomal membranes from bovine cerebral cortex were phosphorylated, subjected to electrophoresis and cut in 1 mm slices as described in Methods. In each case 100 μg of protein was applied to the gel. (a) Grey matter, (b) White matter. df = bromophenol blue dye front.

The basal and cyclic AMP-stimulated phosphorylation of this component were enriched in membranes from white matter by factors of 5.6 and 1.5 respectively (table 1). On the other hand, cyclic AMP-stimulated phosphorylation in fractions A–K was enriched by as much as 50-fold in membranes derived from grey matter. The basal phosphorylation in these fractions was similar in microsomal preparations from both tissues.

Table 1
Distribution of ^{32}P among phosphoproteins from grey matter and white matter

	(pmol ^{32}P/mg microsomal protein)					
	Grey matter		White matter		Ratio Grey : White	
Fraction	Basal	Cyclic AMP-stimulated	Basal	Cyclic AMP-stimulated	Basal	Cyclic AMP-stimulated
A	2.6	13.8	2.9	0.4	0.9	33
B	2.8	6.1				
C	1.4	5.8	1.1	0.1	1.3	50
D	1.5	3.1				
E	0.7	1.8				
F	0.7	3.4				
G	1.0	4.6	1.7	0.2	0.6	25
H	1.1	8.3				
I	0.7	5.0	1.0	0.6	0.7	8
J	1.3	3.2				
K	3.8	5.7	1.8	0.3	2.0	20
L	0.7	3.1	3.9	4.6	0.2	0.7
df	5.0	4.3	81.7	2.1	0.1	2
Total recovered[a]	32.4	72.0	116	6.0	0.3	12
Total incorporated[b]	26.9	63.8	111	7.2	0.2	9

[a] Calculated from gels
[b] Determined as trichloroacetic acid-insoluble ^{32}P (see Methods)

Values are given as pmol ^{32}P incorporated in 20 s/mg original membrane protein and are based on three determinations. Fractions correspond to those in fig.1. Where no values are shown the incorporation of ^{32}P was not statistically different from background radioactivity. Standard deviations were in all cases less than ± 20% of the mean. Cyclic AMP-stimulated incorporation is that measured in the presence of cyclic AMP minus basal incorporation.

4. Discussion

In this report we have shown that a crude microsomal fraction prepared from the grey matter of bovine cerebral cortex contains at least 12 proteins which can be phosphorylated in vitro by means of intrinsic, cyclic AMP-stimulated protein kinase activity. In contrast, a similar crude membrane preparation from bovine white matter lacked most of these cyclic AMP-stimulated components (Fractions A–K of fig.1a) but was enriched in the smallest protein component (fraction L). It is important to note, however, that the fact that two membrane preparations contain phosphoproteins having the same relative mobilities does not imply that the proteins are identical.

The higher concentration of fraction L in membranes from white matter raises the question as to the source of this material. It is unlikely to be derived from myelin since the phosphorylation of myelin proteins is unaffected by cyclic AMP [15]. Axolemma fragments are also an improbable source since membrane fractions prepared from peripheral nerves containing mainly axons and lacking synaptic structures are devoid of intrinsic protein kinase activity [7]. It is therefore most likely that fraction L is derived from oligodendroglial cells. The extent to which it might be specific to such cells remains to be determined. Phosphoproteins having similar molecular weights (approximately 50 000) have been described in erythrocyte membranes [16], in plasma membranes from skeletal muscle [17] and in membranes from a

number of other tissues [8]. Direct comparison of the results obtained from different studies is, however, difficult since various conditions for incubation of the membranes and subsequent electrophoresis have been used. Nevertheless, it is interesting to speculate that a membrane-bound protein such as that found in fraction L might mediate the effects of cyclic AMP in glial cells [18].

In general, very few cyclic AMP-dependent phosphoproteins are found in membranes from non-neuronal tissues (e.g. [8,16,17]). The complexity of such proteins in neuronal membranes is therefore exceptional. The presence of fractions A–K in crude microsomes from grey matter (containing synaptic contacts) in contrast to their absence in similar preparations from white matter (devoid of synapses) is consistent with a role for these proteins in synaptic transmission. Synaptic membrane fragments are indeed constituents of crude microsomal fractions from grey matter [19]. Further, it has been reported recently that the bulk of the endogenous cyclic AMP-stimulated protein phosphorylation system is found in subcellular fractions from brain which contain predominantly synaptic junctions [20]. These observations, together with the data presented here, suggest that synaptic junctions contain various substrates for their intrinsic, cyclic AMP-stimulated protein kinase. It is thus possible that cyclic AMP could regulate a number of events at the synaptic junction through the phosphorylation of specific membrane proteins. However, it is not yet clear whether such phosphorylatable proteins are located pre- or post-synaptically. Nor is it possible to say whether the multiplicity of substrates for cyclic AMP-stimulated protein kinase represents the homogeneous distribution of these proteins among all synapses or if specific proteins are phosphorylated at different synapses.

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