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SYNAPTIC MEMBRANE PROTEINS AS SUBSTRATES FOR CYCLIC AMP-STIMULATED PROTEIN PHOSPHORYLATION IN VARIOUS REGIONS OF RAT BRAIN

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Summary

Synaptosomal plasma membranes from mammalian brain contain protein kinase activity which phosphorylates endogenous membrane proteins and is stimulated by cyclic AMP. Using polyacrylamide gel electrophoresis it was shown that at least ten proteins in the synaptosomal plasma membrane fraction could be phosphorylated by endogenous cyclic AMP-stimulated protein kinase activity. The number of proteins whose phosphorylation was stimulated by cyclic AMP was strongly influenced by the pH and Mg^{2+} concentration used in the phosphorylation reaction. A complex pattern of cyclic AMP-stimulated protein phosphorylation was obtained only with synaptosomal plasma membranes and a crude microsomal fraction. Mitochondrial and myelin fractions exhibited no cyclic AMP-stimulated protein kinase activity. Investigation of the distribution of substrates for cyclic AMP-stimulated phosphorylation among various brain regions failed to reveal any regional differences.

Introduction

A large body of evidence has been obtained in recent years indicating that a number of putative neurotransmitter substances can stimulate the intracellular production of cyclic AMP in their receptor cells and thereby regulate the cyclic AMP-dependent phosphorylation of neuronal proteins [1,2]. Thus, for instance, incubation of brain slices in the presence of various biogenic amines leads to an increase in both cyclic AMP content [3,4] and protein phosphorylation [5]. Of particular interest in this connection is the finding that synaptosomal plasma

membrane preparations contain a membrane-bound, cyclic AMP-stimulated protein kinase (EC 2.7.1.37) which is capable of phosphorylating a number of endogenous membrane proteins [6]. This suggests that cyclic AMP produced at the synapse could stimulate the phosphorylation of synaptic proteins resulting in a modification of membrane properties. In this report we demonstrate the presence of at least ten substrates for the endogenous cyclic AMP-stimulated protein kinase of synaptosomal plasma membranes from rat brain. Further, we describe their distribution among membranes derived from various brain regions in an attempt to determine whether regional differences in specific neurotransmitter systems might be reflected in the pattern of protein phosphorylation.

Methods

Male Wistar rats (250—300 g) were stunned by a blow on the back of the neck and decapitated. The whole cerebrum was removed and homogenised in 9 vols. of 10% (w/w) sucrose. The homogenate was fractionated by differential centrifugation to yield a crude nuclear pellet ($800 \times g$, 20 min), crude mitochondrial pellet ($9000 \times g$, 20 min) and a crude microsomal pellet (P_3; $100\,000 \times g$, 60 min). The crude mitochondrial pellet was subjected to osmotic shock and fractionated further by flotation density gradient centrifugation [7] to yield three fractions: P_2A (crude myelin), P_2B (synaptosomal plasma membranes) and P_2C (mitochondria). Before the membrane fractions were used for phosphorylation studies they were washed once with 5 mM Tris-HCl, pH 7.4, twice with the same buffer containing 150 mM NaCl and twice with buffer without NaCl (approximately 10 ml of buffer/mg of protein in each wash). Washing with salt did not have any effect upon the pattern obtained after electrophoresis of membrane phosphoproteins provided that the NaCl, which is an inhibitor of endogenous protein kinase activity [8], was washed out. Nevertheless, it was included as a precaution against artefacts which might arise due to nonspecific binding of soluble protein kinase to the membranes. The washed pellets were suspended in 5 mM Tris-HCl, pH 7.4, and stored at —20° C at a protein concentration of 10 mg/ml.

For studies on the regional distribution of membrane phosphoproteins in brain, P_2B fractions were prepared from cortical grey matter, corpus striatum and cerebellum.

Unless otherwise stated, membrane phosphorylation, electrophoresis and the determination of radioactivity were performed as previously described [9]. The nomenclature used to designate the various phosphoprotein fractions is based upon that previously used in the case of crude microsomes from bovine brain [9].

Cross-contamination of the P_2B and P_3 fractions was assessed using the following enzyme markers: Adenylate cyclase (EC 4.6.1.1) incubations were performed in 50 mM Tris/maleate, pH 7.0, 0.25 mM ATP, 1 mM $MgSO_4$, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase (EC 2.7.3.2), 0.1 mM papaverine-HCl. Cyclic AMP produced in the reaction was determined by radioimmunoassay (Cyclic AMP Assay Kit, NEN Chemicals). NADPH-cytochrome *c* reductase (EC 1.6.2.4) was measured spectrophotometrically [10]. Protein concentra-

tions used were: adenylate cyclase, 0.1—0.3 mg/ml; NADPH-cytochrome *c* reductase, 0.1—0.4 mg/ml.

Results

Protein phosphorylation in subcellular fractions from whole cerebrum

Various particulate subcellular fractions were labelled with ^{32}P by incubation in the presence of [γ-^{32}P]ATP and solubilized with sodium dodecyl sulphate. The separation of the ^{32}P-labelled protein components by polyacrylamide gel electrophoresis is shown in Figs. 1 and 2. It is evident that both the P_3 (Fig. 1c) and the P_2B (Fig. 2a) fractions contain a number of proteins which can be phosphorylated by means of endogenous protein kinase activity. The pattern of [^{32}P]phosphoproteins was virtually identical in these two fractions and the phosphorylation of all ^{32}P-labelled zones except that migrating with the dye front (df) was stimulated by cyclic AMP. By comparison with the relative mobilities of reference proteins the following molecular weights for the major ^{32}P-labelled components were obtained: (A) 230 000; (B) 180 000; (C) 160 000; (D) 135 000; (G) 90 000; (H) 86 000; (I) 70 000; (J) 55 000; (K) 50 000; (L) 47 000. An identical phosphorylation pattern was also obtained with synaptosomal plasma membranes prepared from intact synaptosomes by the method of Whittaker and Barker [11].

In contrast, the fractions, crude myelin and mitochondria, showed a much simpler pattern of phosphorylated proteins. In the case of myelin, the only distinct radioactive peak migrated with the dye front (df; Fig. 1a). This might represent the myelin basic proteins which have low molecular weights (approx. 13 000 and 16 700 [12]) and which can be phosphorylated by the endogenous protein kinase of myelin membranes [13,14]. The mitochondrial fractions (Fig. 1b) contained in addition to the dye front two major phosphorylated zones (M and N, approximate molecular weights 39 000 and 34 000, respectively) together with a trace of ^{32}P-labelled material corresponding in relative mobility to the phosphoproteins observed in the synaptosomal plasma membrane and P_3 fractions. In neither the crude myelin nor the mitochondrial fraction was phosphorylation stimulated by cyclic AMP.

The relatively low speed of centrifugation used to obtain the crude mitochondrial fraction suggested the likelihood that the P_3 fraction contained small synaptosomes and plasma membrane fragments and that these might contribute the complex phosphorylation pattern of the 'crude microsomal' fraction. This would be in agreement with the observation that cyclic AMP-stimulated protein kinase activity sediments in a 'heavy microsomal' fraction [15]. Using enzyme markers, we therefore assessed the contribution of plasma membrane to the P_3 fraction, as well as that of microsomal membranes to our synaptosomal plasma membrane fraction. The results are presented in Table I. Fraction P_2B (synaptosomal plasma membranes) showed a four fold enrichment of the plasma membrane component, adenylate cyclase [16], compared with the homogenate but was relatively low in the microsomal marker, NADPH-cytochrome *c* reductase [10]. The specific activity of the latter was only a quarter of that in fraction P_3. However, the P_3 fraction was also enriched in adenylate cyclase compared with the homogenate. These results indicate that

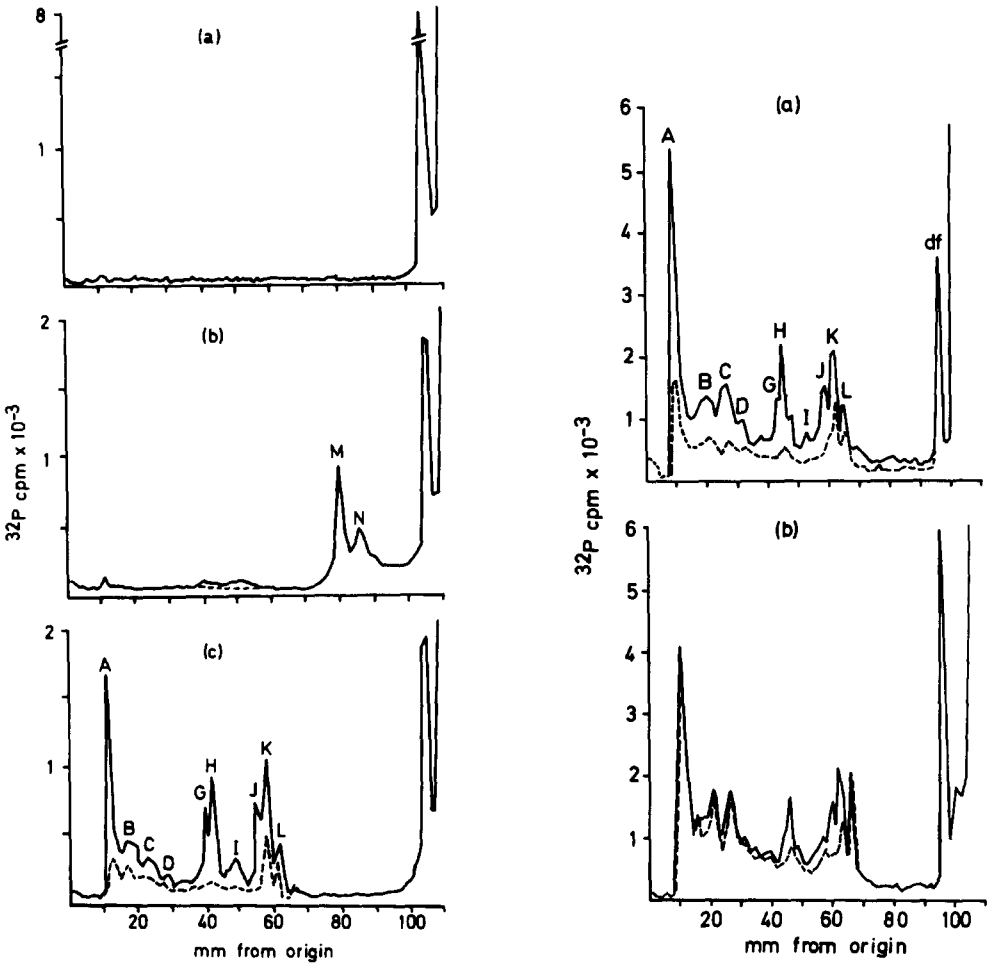

Fig. 1. Incorporation of ^{32}P into proteins in subcellular fractions from whole cerebrum by endogenous protein kinase activity. After incubation with [γ-^{32}P]ATP the subcellular fractions were solubilized and separated by polyacrylamide gel electrophoresis. Gels were sliced into 1 mm sections and ^{32}P radioactivity was determined. (a) P_2A (crude myelin); (b) P_2C (mitochondria); (c) P_3 (crude microsomes). - - - - - -, no cyclic AMP; ———, 10 μM cyclic AMP.

Fig. 2. Incorporation of ^{32}P into synaptosomal plasma membrane fraction by endogenous protein kinase activity. Incubation of the membranes was performed in (a) 5 μM [γ-^{32}P]ATP, 1 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4 [9], or (b) 5 μM [γ-^{32}P]ATP, 10 mM $MgSO_4$, 50 mM sodium acetate, pH 6.0 [14]. - - - - - -, no cyclic AMP; ———, 10 μM cyclic AMP.

our P_3 fraction indeed contains a considerable amount of plasma membrane which could contribute to the complex phosphorylation pattern in that fraction.

The complex pattern of substrates labelled by endogenous cyclic AMP-stimulated protein kinase activity in our synaptosomal plasma membrane fraction is in agreement with data obtained by other workers using similar conditions [17] but differs from the relatively simple pattern originally reported by Greengard's group [18] who found that cyclic AMP stimulated the phos-

TABLE I

DISTRIBUTION OF ADENYLATE CYCLASE AND NADPH-CYTOCHROME *c* REDUCTASE BETWEEN FRACTIONS P_2B (synaptosomal plasma membranes) AND P_3

The enzymes were determined as described in the text. Relative specific activity (RSA) is defined as the ratio of the specific activity in a given fraction to that of the homogenate. Results are from a typical experiment.

	Total activity (nmol/min)	Adenylate cyclase		NADPH-cytochrome *c* reductase		
		Specific activity (pmol/min per mg protein)	RSA	Total activity (μmol/min)	Specific activity (nmol/min per mg protein)	RSA
Homogenate	137	195	1	6.30	9.0	1
P_2B	7.1	898	4.6	0.06	7.7	0.85
P_3	44.3	528	2.7	2.42	28.9	3.2

phorylation of only two protein fractions. We therefore reexamined the phosphorylation of synaptosomal plasma membranes under the incubation conditions used by the latter authors. A synaptosomal plasma membrane fraction from rat cerebrum was incubated with 5 μM [γ-^{32}P]ATP for 20 s at 37°C in one of the following solutions: (a) 1 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4 [9] or (b) 10 mM $MgSO_4$, 50 mM sodium acetate, pH 6.0 [18]. After solubilization of the ^{32}P-labelled membranes with sodium dodecyl sulphate electrophoresis was performed as previously described [9]. The results are presented in Fig. 2. In contrast to the complex pattern of stimulation by cyclic AMP shown in Fig. 2a, the stimulation of phosphorylation by cyclic AMP under the conditions of Ueda et al. [18] was largely restricted to fractions G, H, J and K (Fig. 2b). The approximate molecular weights of these fractions correspond roughly to those of protein I (G and H) and protein II (J and K) of Ueda et al. [18]. The lack of stimulation by cyclic AMP of the phosphorylation of the other fractions seems to be due to an increase in basal phosphorylation rather than to an inhibition of cyclic AMP-stimulated protein kinase activity. These results clearly demonstrate that the choice of incubation conditions can have a profound effect upon the apparent complexity of substrates for cyclic AMP-stimulated protein kinase activity in synaptosomal plasma membrane preparations and suggests the presence of more than one type of protein phosphorylation system having different sensitivities to changes in pH and Mg^{2+} concentration. However, this does not necessarily imply the presence of more than one membrane-bound protein kinase but could represent a modification of substrate conformation leading to an alteration of enzyme-substrate interaction and full expression of enzyme activity even in the absence of cyclic AMP. Alternatively, the balance between protein kinase and phosphoprotein phosphatase activities might be changed such that the phosphatase becomes rate limiting and stimulation of protein kinase activity by cyclic AMP cannot lead to a net change in ^{32}P incorporation [8,19,20]. Sufficient data are not available at present to distinguish between these various possible explanations.

Distribution of phosphoproteins in synaptosomal plasma membranes from different brain regions

The complexity of the cyclic AMP-stimulated phosphorylation system in synaptosomal plasma membrane fractions led us to consider whether the observed heterogeneity of phosphoproteins resulted from the presence of a number of phosphorylated components at all synapses or if specific phosphoproteins might be associated with certain types of synapses. Specific phosphoproteins might, for instance, be associated with synapses characterised by the presence of specific neurotransmitter receptors, by proteins involved in the control of specific ion fluxes, or by cell recognition proteins. Such a correlation with a certain type of synapse might be expected to be reflected in differences in the regional distribution of specific phosphoproteins in brain. However, no differences were observed between the [^{32}P]phosphoprotein patterns obtained with synaptosomal membrane fractions from cerebral cortex, corpus striatum and cerebellum. Further, quantitative evaluation of the incorporation of ^{32}P into the individual protein zones showed no statistically significant differences either in the absence or presence of cyclic AMP (Table II). It is therefore unlikely that any of the major phosphoproteins separated under our conditions are associated specifically with synapses characteristic of these brain regions.

TABLE II

INCORPORATION OF ^{32}P INTO THE MAJOR PHOSPHOPROTEIN FRACTIONS OF SYNAPTOSOMAL PLASMA MEMBRANES FROM DIFFERENT REGIONS OF RAT BRAIN

cpm were measured in the slices of gel constituting fractions (A—L) after labelling with or without 10 μM cyclic AMP (see Figs. 1 and 2). The results are derived from six determinations and are expressed as pmol of ^{32}P incorporated/20 s per mg of protein together with standard deviations.

Fraction	Cyclic AMP	Cerebral cortex	Cerebellum	Corpus striatum
A	—	5.9 ± 2.8	5.1 ± 1.5	7.4 ± 2.5
	+	20.2 ± 3.4	18.2 ± 3.2	19.0 ± 1.6
B	—	4.9 ± 1.2	4.3 ± 1.1	5.0 ± 0.9
	+	10.1 ± 2.4	10.1 ± 1.9	10.8 ± 2.4
C	—	4.2 ± 2.5	3.8 ± 2.4	3.8 ± 2.3
	+	10.2 ± 3.4	8.2 ± 1.2	8.2 ± 1.5
D	—	3.0 ± 2.0	2.2 ± 2.0	2.1 ± 2.0
	+	7.5 ± 2.5	6.9 ± 2.8	10.2 ± 3.0
G	—	2.0 ± 1.8	0.5 ± 0.4	2.7 ± 1.7
	+	7.0 ± 3.0	6.5 ± 1.9	6.8 ± 1.4
H	—	4.2 ± 1.5	3.8 ± 1.6	3.8 ± 1.8
	+	17.9 ± 3.2	17.9 ± 1.4	17.0 ± 1.5
I	—	2.2 ± 1.4	2.9 ± 1.3	2.3 ± 0.9
	+	9.7 ± 3.2	11.7 ± 3.5	10.3 ± 2.2
J	—	2.4 ± 1.9	2.4 ± 2.3	3.2 ± 1.9
	+	14,7 ± 2.1	13.9 ± 2.2	13.4 ± 2.1
K	—	8.1 ± 2.7	10.0 ± 1.3	8.1 ± 1.3
	+	19.8 ± 1.3	20.4 ± 1.8	19.7 ± 1.2
L	—	3.9 ± 2.8	2.8 ± 1.9	4.0 ± 1.0
	+	9.0 ± 2.0	9.9 ± 2.0	9.9 ± 1.8

Discussion

The results reported in this paper clearly demonstrate the presence in synaptosomal plasma membrane preparations of a number of substrates for endogenous, cyclic AMP-stimulated protein kinase activity. In our hands, excellent resolution of ten major phosphoprotein fractions having approximate molecular weights of 47 000 and above was obtained. This compares very favourably with the resolution obtained by Dunkley et al. [17] using an autoradiographic method to determine protein labelling. Direct comparison of the results obtained by different authors in this field are difficult due to differences in methodology. However, on the basis of approximate molecular weights our fractions may be compared with those of other workers as summarised in Table III. In all cases cyclic AMP-stimulated phosphorylation of proteins corresponding to our fractions G, H, J and K were reported. Dunkley et al. [17] further obtained ^{32}P-labelled phosphoproteins with characteristics similar to our proteins A—D and the dye front. The fraction designated δ by those authors, which was not affected by cyclic AMP, corresponds in approximate molecular weight to zones M and N of our mitochondrial fraction and might therefore indicate the presence of mitochondria in the synaptosomal plasma membranes used in their studies.

A complex distribution of ^{32}P-labelled proteins was also observed in a P_3 (crude microsomal) fraction isolated from whole rat cerebrum. The phosphoprotein pattern of this fraction differed little from that of a similar fraction from bovine cerebral cortex [9] and was identical to that of the synaptosomal plasma membrane fraction. The presence of a considerable amount of plasma

TABLE III

PROBABLE RELATIONSHIPS BETWEEN PROTEIN SUBSTRATES FOR CYCLIC AMP-STIMULATED PHOSPHORYLATION REPORTED BY VARIOUS AUTHORS

Comparisons are based upon approximate molecular weights and the sensitivity of phosphorylation to cyclic AMP. df, dye front.

Our designation	Approximate molecular weight ($\times 10^{-3}$)	Dunkley et al. [17]	Ehrlich et al. [14]	Ueda et al. [18]
A	230			
B	180	α		
C	160			
D	135			
G	90	β	D	I
H	86			
I	70	γ	E	II
J	55			
K	50			
L	47		F	
M	39	δ		
N	34			
df	—	ϵ	H	

membrane in our fraction P_3 suggests that the phosphorylation pattern in that fraction might be derived, at least in part, from plasma membrane-associated structures. This conclusion would be consistent with a number of other observations. Thus, cyclic AMP-stimulated protein kinase activity has also been shown to sediment in a 'heavy microsomal' fraction, probably consisting of small synaptosomes and plasma membranes [15]. In addition, synaptosomal and 'microsomal' cyclic AMP-stimulated protein kinase are similar in being both insoluble in Triton X-100 [21]. Further, a possible synaptic origin of the complex phosphorylation pattern is suggested by the absence of such a pattern in a similar P_3 fraction from brain tissue lacking synapses [9]. The converse possibility, that some of the phosphoproteins in the synaptosomal plasma membane fraction were derived from microsomal contamination is unlikely on the basis of the distribution of NADPH-cytochrome *c* reductase. This microsomal marker enzyme was enriched approximately four fold in fraction P_3 compared to the synaptosomal plasma membrane fractions. Any phosphoproteins characteristic of microsomal membranes would therefore be enriched by a factor of four in fraction P_3. However, no evidence was obtained for the enrichment of additional specific phosphoproteins in that fraction. It is therefore not possible to ascribe any of the phosphoproteins of the synaptosomal plasma membrane fraction to contamination by microsomal membranes. We have also shown in this paper that none of the major phosphoproteins of our synaptosomal plasma membrane fraction are derived from contamination with components of the mitochondrial or myelin fractions. It may therefore be reasonably concluded that the complexity of substrates for membrane-bound, cyclic AMP-stimulated protein kinase activity is primarily associated with structures characteristic of the synaptosomal plasma membrane fraction.

In a number of studies, attempts have been made to determine the localisation of the cyclic AMP-stimulated protein phosphorylation system within the synaptosomal plasma membrane fraction. The main strategy has been to use detergents to solubilize the bulk of the synaptosomal plasma membrane leaving a residue containing predominantly synaptic complexes [22]. Thus it has been found that most of the cyclic AMP-stimulated protein kinase activity remains insoluble in Triton X-100 [21]. In addition, the analysis of ^{32}P-labelled phosphoproteins after extraction of phosphorylated membranes with various detergents has demonstrated that the main substrates for cyclic AMP-stimulated protein kinase activity are also concentrated in the fraction that is not solubilized by detergents [17]. It therefore seems likely that the cyclic AMP-stimulated phosphorylation systems of synaptosomal plasma membrane fractions is derived from synaptic complexes and, conversely, that this fraction contains a number of potential protein substrates.

The presence of a number of substrates for synaptic cyclic AMP-stimulated protein kinase activity suggests that cyclic AMP could regulate a number of events at the synaptic junction through the phosphorylation of specific proteins. The nature of these proteins is unknown. Possible candidates would be proteins associated with neurotransmitter receptors, proteins controlling ion fluxes, membrane-bound enzymes, cell recognition proteins or proteins involved in maintaining synaptic structure. Evidence also exists that membrane-bound tubulin is phosphorylated, although the function of this protein

in the synapse is unknown [23]. The lack of any measurable difference in the phosphorylation of specific proteins in various brain regions reported here suggests that, whatever the nature and function of these phosphoproteins, they are most probably general components of synaptic structures and are not associated only with synapses that might be characteristic of the regions studied (e.g. with dopaminergic synapses which occur in high concentrations in the corpus striatum).

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