Pre-incubation of subcellular fractions from rat cerebral cortex inactivates protein phosphorylation

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Pre-incubation of various subcellular fractions from rat brain caused a significant decrease in the phosphorylation of individual polypeptides. The rate and extent of this loss of labelling was not uniform and polypeptides whose phosphorylation was independent of cyclic AMP were primarily affected, whereas substrate availability remained unaltered. It is recommended that pre-incubation effects must be carefully monitored if valid conclusions are to be made about the physiological relevance of changes in protein phosphorylation observed *in vitro*.

Particulate fractions isolated from central-nervous-system tissue contain numerous phosphoproteins and protein kinases that label endogenous substrates *in vitro* (Greengard, 1978; Rodnight, 1979). To relate the behaviour of these enzymes and substrates *in vitro* to their role *in vivo* it is necessary to analyse fully how incubation conditions *in vitro* can alter the extent and nature of protein phosphorylation. One major variable that has been introduced into assays *in vitro* is pre-incubation of particulate fractions before estimation of protein phosphorylation activity. Thus the extent of the pre-incubation period used for brain particulate fractions varies from no pre-incubation (Dunkley *et al.*, 1976), to pre-incubation for 1 min (Lohmann *et al.*, 1980), 3 min (DeBlas *et al.*, 1979) or 5 min (Zwiers *et al.*, 1979). Pre-incubation has been used to alter the phosphorylation state of synaptic plasma-membrane proteins (Weller & Rodnight, 1971, 1973), but 'for unknown reasons such treatment leads to unacceptable losses of protein kinase activity' (Rodnight *et al.*, 1975). In the present study we have investigated the nature and extent of the loss of protein-phosphorylation activity consequent on pre-incubation of various subcellular fractions from rat cerebral cortex.

Experimental

Acrylamide, bisacrylamide, Trizma base, ATP and cyclic AMP were obtained from Sigma and low-molecular-weight standards were from Pharmacia.

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[γ -³²P]ATP was obtained from Amersham (PB132).

Subcellular fractions were isolated fresh each day from the cerebral cortex of adult wistar rats (Whittaker, 1972). The fractions were lysed by resuspension and homogenization in 30 mM-Tris buffer at pH 7.4 (approx. 10 mg of protein/ml), and after 15 min on ice the suspension was homogenized again, before being used for protein kinase assays or polyacrylamide gels.

Conditions for phosphorylation were adapted from those used previously (Dunkley *et al.*, 1976) and the standard incubation (total volume 1 ml) contained, finally, 30 mM-Tris/HCl (pH 7.4), 1 mM-MgSO₄, 100 μ M-[γ -³²P]ATP (2.5×10^5 c.p.m./nmol) and the subcellular fraction (approx. 1 mg of protein/ml). All components except the ATP and the subcellular fraction were added on ice, the tubes were then transferred to a 37°C water bath and rapidly stirred for 1 min before addition of the subcellular fraction (50 μ l) for various pre-incubation times. Incubations were started by the addition of 50 μ l of ATP and were terminated after 10 s by the addition of 2 ml of ice-cold 15% trichloroacetic acid. When cyclic AMP (final concentration 50 μ M) or Triton X-100 (final concentration 0.5%, v/v) was present it was added immediately before the ATP at the end of the pre-incubation period. Subsequent measurement of total protein phosphorylation ensured that only phosphate bound to serine or threonine residues on proteins was estimated (Rodnight *et al.*, 1975).

When the crude mitochondrial fraction was phosphorylated for analysis of substrates by polyacrylamide-gel electrophoresis and autoradiography, the incubation conditions were the same as

for the standard phosphorylation assay, except that the ATP concentration was $40\text{ }\mu\text{M}$ ($26 \times 10^6\text{ c.p.m./nmol}$), the incubation time was 30 s and the assay volume was $250\text{ }\mu\text{l}$. Reactions were stopped by the addition of sodium dodecyl sulphate and samples were applied to a gel with a gradient of 7.5–15% acrylamide (Dunkley & Anderson, 1979). Dried gels were exposed to Kodak X-Omat S film for up to 5 days and changes in the extent of phosphorylation of individual proteins are based on quantitative estimations using a Helena Densitometer.

Results

Pre-incubation caused a marked and progressive decrease in the level of total protein phosphoryla-

tion of the crude mitochondrial fraction when cyclic AMP was not added to the incubation (Table 1); the most rapid decrease occurred during the first 2 min of the pre-incubation and by 30 min over 60% of the total protein phosphorylation was lost. When cyclic AMP was added at the end of the pre-incubation period and immediately before the radiolabelled ATP the protein phosphorylation activity was decreased and after 30 min almost 40% of the total protein phosphorylation activity was lost. The absolute stimulation due to cyclic AMP remained essentially constant (see Table 1) and the observed decrease in total protein phosphorylation was therefore confined primarily to cyclic AMP-insensitive labelling. A decrease in total protein phosphorylation was also observed on pre-incubation of other preparations of brain tissue, including the

Table 1. *The effect of pre-incubation time and cyclic AMP on total protein-phosphorylation activity*

A crude mitochondrial fraction was pre-incubated for up to 30 min before reactions were initiated by adding $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Values are means for at least four separate experiments each performed in triplicate; s.e.m. values were all less than 12%. The absolute stimulation of phosphorylation due to cyclic AMP is indicated in parentheses.

Time (min)	Cyclic-AMP	Protein-phosphorylation activity (nmol of P/mg of protein per 10 s)		Stimulation (%)
		—	+	
0		0.25	0.28 (+0.03)	12
0.17		0.23	0.32 (+0.09)	39
1		0.19	0.26 (+0.07)	37
2		0.17	0.25 (+0.08)	47
5		0.14	0.22 (+0.08)	57
30		0.09	0.17 (+0.08)	89

Table 2. *The effect of pre-incubation time and cyclic AMP on phosphorylation of individual polypeptides*

Protein-phosphorylation activity remaining after 5 or 30 min pre-incubation was calculated relative to a sample run on the same gel that was not pre-incubated. Values are means for at least six gels from four separate experiments; s.e.m. values were all less than 18%. Phosphoproteins A–F are shown in Fig. 1 and were previously identified by Dunkley (1981).

Phospho-protein	Stimulation by cyclic AMP at 0 min (%)	Remaining protein-phosphorylation activity (%)	
		5 min	30 min
E	178	90	61
A	150	85	79
C	54	77	47
B	40	56	31
D	12	35	22
F	10	62	10

Fig. 1. *The effect of cyclic AMP and Triton X-100 on phosphoprotein profiles before and after pre-incubation*

After pre-incubation of a crude mitochondrial fraction for 0 or 5 min, and incubation in the presence or absence of Triton X-100 or cyclic-AMP, an autoradiograph was prepared. Positions of the molecular-weight standards ($\times 10^{-3}$) are shown to the left of the gels.

total brain homogenate, the P1 pellet myelin, synaptosomes and synaptic plasma membranes. The extent of the decrease in total protein phosphorylation varied considerably among these fractions and with synaptic plasma membranes a decrease of 38% was observed after 5 min pre-incubation, whereas for myelin the decrease was only 5%.

The extent and rate of the decrease in labelling of individual polypeptides on pre-incubation was not uniform and in general the polypeptides whose phosphorylation was most readily stimulated by cyclic AMP were the least affected by pre-incubation (Table 2; Fig. 1). After 30 min pre-incubation all of the phosphoproteins showed some decrease in extent of labelling. A decrease in labelling of individual polypeptides was still observed after 5 min pre-incubation and incubation in the presence of Triton X-100 (Fig. 1, lanes 2 and 3). Vesiculation or subtle changes in membrane proteins could not therefore be the cause of the loss of phosphorylation observed on pre-incubation, as Triton X-100 lyses membranes and solubilizes many membrane proteins. When cyclic AMP was added to the crude mitochondrial fraction a marked increase in the phosphorylation of a number of polypeptides was observed (Fig. 1, lanes 2 and 5), but there was no obvious decrease in the labelling of these polypeptides on pre-incubation for 5 min and incubation with Triton X-100 (Fig. 1, lanes 5 and 6). Quantitative analysis confirmed this finding, suggesting that the cyclic AMP-stimulated protein kinase enzymes and their substrates were not degraded or lost on pre-incubation. Neither cyclic AMP nor Triton X-100 had an effect on the polypeptide patterns of the crude mitochondrial fractions at any time during the pre-incubation.

Discussion

A decrease in protein phosphorylation such as that observed on pre-incubation of the crude mitochondrial fraction can occur only if there is a decrease in substrate availability and/or a loss of protein kinase activity. A decrease in substrate availability could be due to prior occupation of acceptor sites by unlabelled phosphate, proteolysis, dissociation of substrate from membranes or loss of acceptor function due to dephosphorylation beyond a critical level necessary for an appropriate tertiary structure. Loss of protein kinase activity could similarly be due to proteolysis, dissociation from membranes or loss of a bound activating factor.

A decrease in available acceptor sites on protein substrates is unlikely to be an explanation, as protein phosphatase activity would continue during pre-incubation in the absence of ATP (Weller & Rodnight, 1971) and would be expected to increase

the number of acceptor sites. No changes in polypeptide patterns were observed on pre-incubation, suggesting that proteolysis of protein substrates was not occurring. A decrease in ATP concentration is also unlikely to be an explanation as essentially the same results were obtained with 100 μ M- and 40 μ M-ATP (P. R. Dunkley & P. J. Robinson, unpublished work).

The phosphorylation of a number of polypeptides was stimulated by exogenous cyclic AMP when the crude mitochondria had not been pre-incubated. Pre-incubation did not decrease markedly the phosphorylation of these polypeptides when Triton X-100 and cyclic AMP were present in the incubation, suggesting that cyclic AMP-stimulated protein kinases are largely unaffected by pre-incubation. The constant level of stimulation by cyclic AMP of total protein phosphorylation is consistent with this conclusion. A decrease in activity of the Ca^{2+} -stimulated protein kinases (Schulman & Greengard, 1978) during pre-incubation could explain some of the loss of protein phosphorylation activity observed on pre-incubation. Such a decrease might be due to loss of Ca^{2+} bound to the protein kinase-substrate complex, or to a loss of calmodulin.

Incubation of tissue preparations before the start of an assay is a common procedure, but the conditions used for pre-incubation vary considerably, and this could in part explain why different laboratories, using essentially the same preparations, obtain markedly different phosphoprotein patterns and degrees of cyclic AMP stimulation. The pre-incubation changes in protein phosphorylation observed here are artefacts of the assay *in vitro* and are unlikely to relate directly to any physiological function. However, as many studies on the effects of drugs and other agents are being conducted after pre-incubation of subcellular fractions *in vitro*, it will be important to determine whether the effects of the drugs on protein phosphorylation are really of physiological importance or whether they simply reflect alterations in the extent of the loss of protein kinase activity that normally occurs on pre-incubation. The fact that pre-incubation affected a number of subcellular fractions in the same general way would suggest that pre-incubation of other tissues might also lead to similar decreases in protein phosphorylation. If this is a general phenomenon then it will be important to obtain a more complete understanding of the mechanism(s) involved.

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