Phosphorylation of Synaptic-Membrane Proteins from Ox Cerebral Cortex *in vitro*

PARTITION OF SUBSTRATES AND PROTEIN KINASE ACTIVITIES WITH TRITON X-100

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Synaptic-membrane fragments from ox cerebral cortex contain basal and cyclic AMP-stimulated protein kinase activity catalysing the phosphorylation of endogenous substrates. Extraction of membrane fragments with Triton X-100 solubilized less than 20% of the kinase activity and left the major part of the endogenous substrates in the insoluble fraction.

Synaptic-membrane fragments contain bound protein kinase activity which catalyses the transfer of ^{32}P from [γ - ^{32}P]ATP to hydroxyl groups of serine (and to a lesser extent threonine) residues in membrane proteins (Weller & Rodnight, 1971, 1973; Johnson *et al.*, 1971; Ueda *et al.*, 1973). A number of endogenous proteins are phosphorylated, but only in a few cases is phosphorylation stimulated by cyclic AMP (Ueda *et al.*, 1973; Ehrlich & Routtenberg, 1974; Routtenberg & Ehrlich, 1975; Rodnight, 1975). Synaptic-membrane fragments also contain protein phosphatase activity (Weller & Rodnight, 1971; Maeno & Greengard, 1972). These and other findings have led to proposals that cyclic AMP-mediated phosphorylation of protein in the post-synaptic membrane may be involved in the modulation of synaptic transmission (for recent reviews see Rodnight, 1975; Greengard, 1976).

The detergent Triton X-100 has been used to obtain from crude preparations of synaptic-membrane fragments a fraction enriched in so-called 'junctional complexes', consisting of fragments of apposed pre- and post-synaptic membranes (Cotman & Taylor, 1972; Davis & Bloom, 1973). Depending on the conditions used, Triton X-100 solubilizes 20–60% of the membrane protein, but leaves the junctional complexes in the insoluble fraction. In earlier work we found that the greater part of the basal and cyclic AMP-stimulated protein kinase activity of membrane fragments is not solubilized by Triton X-100 (Weller & Rodnight, 1973); however, the soluble fraction, studied in detail by Ueda *et al.* (1975), does contain protein kinase and phosphatase activities together with certain of their acceptor proteins. No information is available on the distribution of the various proteins phosphorylated in the presence or the absence of cyclic AMP in the Triton X-100-soluble and -insoluble fractions. The present study makes this comparison by using polyacrylamide-gel

electrophoresis to separate labelled proteins. In some experiments intact membrane fragments were labelled and then treated with Triton X-100; in others, fractionation with detergent was done first, and protein phosphorylation studied in the resulting soluble and insoluble fractions.

Materials and Methods

Materials

Most of these were obtained from the sources stated by Alexander & Rodnight (1974). Triton X-100 ('Purified' batch no. 50800) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Protein was determined by the method of Miller (1959), by using bovine plasma albumin as standard.

Synaptic-membrane preparations

These were made from ox cerebral cortex essentially by the procedure of Rodnight *et al.* (1969). Preparations (10 mg/ml) were stored at -20°C in 4 mM-imidazole/HCl buffer (pH 7.4) and used within 3 months.

Phosphorylation of membrane proteins

Small-scale experiments were done at 37°C under the same general conditions recommended by Rodnight *et al.* (1975), except that the reaction tubes were vigorously agitated with a Microid flash shaker (A. Gallenkamp and Co. Ltd., Technico House, London EC2P 2ER, U.K.). The mixture (final volume 165 μl) contained 30 mM-Tris/HCl (pH 7.4 at 37°C), 1 mM-MgSO₄, 9.7 μM -[^{32}P]ATP (14×10^6 c.p.m./nmol), with or without 50 μM -cyclic AMP. The reaction was started by adding 200 μg (20–40 μl) of protein and terminated after 10 s with

35 μ l of 'solubilizing buffer' (final pH 8.7 at 18–24°C). The latter contained 286 mM-Tris/HCl, 266 mM-glycine, 5.7% (w/v) sodium dodecyl sulphate, 5.7% (v/v) 2-mercaptoethanol, 28.5% (w/v) sucrose and 0.03% Bromophenol Blue.

In large-scale experiments, designed to provide sufficient labelled material for subsequent extraction with Triton X-100, 2 mg of membrane protein was incubated for 1 min under the same conditions, except that the ATP concentration was 12.5 μ M (2×10^6 c.p.m./nmol). The reaction was terminated by transferring the samples to ice, diluting with 4 ml of ice-cold 4 mM-imidazole/HCl (pH 7.4) containing 0.5 mM-EDTA ('imidazole buffer') and centrifuging at 2600 g for 15 min at 4°C [EDTA was included in the diluting buffer to minimize protein dephosphorylation during centrifugation (see Weller & Rodnight, 1971)]. The supernatants were discarded and the pellets, comprising over 95% of the original protein, were homogenized in detergent as indicated below.

Fractionation of membrane protein with Triton X-100

Unlabelled membrane fragments (10 mg of protein) were homogenized by hand in a glass test-tube homogenizer at 4°C, in 2 ml of either 0.25 or 0.5% Triton X-100 (v/v, final concns.) containing 4 mM-imidazole/HCl (pH 7.4). Certain homogenates also contained 0.2 mM-dithiothreitol. In experiments using 0.5% Triton X-100, homogenates were immediately centrifuged at 106000 g for 1 h at 4°C (Weller & Rodnight, 1973); with the lower concentration of detergent the procedure of Ueda *et al.* (1975) was followed and centrifugation was delayed for 30 min. The supernatants were concentrated to 50–100 μ l by centrifuging in 'Centriflo' filters at 2000 g for 30 min, at 4°C (see Alexander & Rodnight, 1974). Pellets were resuspended by homogenizing in 250 μ l of the same detergent solution used for extraction. No attempt was made to remove Triton X-100 from the supernatants or pellets before determining kinase activity, since, in intact membrane fragments, the low final concentration was found to have no effect on protein phosphorylation in any gel region.

In the case of pre-labelled fragments the protein pellets (see above) were homogenized in 2 ml of 0.25 or 0.5% Triton X-100 by the same procedure, with the following differences introduced to discourage dephosphorylation: the detergent solution contained 0.5 mM-EDTA and centrifugation was for 30 min instead of 1 h. Subsequent treatment of the homogenates also followed that used for unlabelled material, except that the supernatants were centrifugally washed twice in the Centriflo filters with 4 ml of 'imidazole buffer' before final concentration to 50–100 μ l. The labelled pellets were resuspended in 'imidazole buffer', centrifuged at 2610 g for 30 min at 4°C and finally resuspended in 250 μ l of buffer. For

electrophoresis the supernatant and pellet residues were treated with a solution (final pH 8.7 at 18–24°C) containing 61 mM-Tris/HCl, 58 mM-glycine, 1.14% (w/v) sodium dodecyl sulphate, 5.8% (w/v) sucrose, 0.91% (v/v) 2-mercaptoethanol and 0.05% Bromophenol Blue such that the final ratio of sodium dodecyl sulphate/protein (w/w) was 11.4 and the protein concentration was 1 mg/ml.

Polyacrylamide-gel electrophoresis

A discontinuous system operated at 12°C was used. All pH values refer to this temperature. The upper buffer (pH 9.3) contained 53 mM-Tris/HCl, 50 mM-glycine and 0.1% sodium dodecyl sulphate; 2-mercaptoethanol to a concentration of 0.1% (v/v) was added immediately before use. The lower buffer was 10 mM-Tris/HCl (pH 8.6). The gel mixture consisted of 10% (w/v) acrylamide, 0.3% (w/v) *NN'*-methylenebisacrylamide, 0.1% sodium dodecyl sulphate, 0.05% ammonium persulphate, 0.07% (v/v) *NNN'*-tetramethylethylenediamine and 42 mM-Tris/HCl, pH 9.3. Gels were set in glass tubes (11 cm $\times$ 0.6 cm), overlaid with 0.1% sodium dodecyl sulphate and left at 18–24°C for 2 h. They were stored in buffer at 4°C for at least 16 h before use. In all experiments 100 μ g of protein in 100 μ l was applied to the gels. The starting current was 0.125 mA/gel and this was gradually increased to 3 mA/gel over the next 2 h. When the dye front reached the bottom of the gels (4–5 h) they were removed from the tubes and stained in 0.5% Naphthalene Black in methanol/acetic acid/water (4:1:6, by vol.) for 1 h. The same solvent was used for destaining. Protein distribution was recorded diagrammatically; the gels were frozen with solid CO₂ and cut into 2.5 mm slices with a multiple-blade cutter. Each section was suspended in 5 ml of 0.5 M-NaOH in scintillation vials and radioactivity determined by recording Čerenkov radiation.

Estimation of molecular weights

Approximate molecular weights for certain of the protein bands in the gels were determined from a plot of the logarithm of mol.wt. versus distance migrated, obtained with the following standards: γ -globulin (160000), bovine plasma albumin (67000), ovalbumin (45000), chymotrypsin (25000), trypsin inhibitor (21500) and lysozyme (14100).

Expression of results

Radioactivity in each gel section was corrected for background and radioactive decay and converted into pmol of phosphate. Since it was impossible to ensure that single sections did not contain more than one phosphoprotein, only regions of the gel showing

Fig. 1. Polyacrylamide-gel electrophoresis of ^{32}P -labelled synaptic-membrane fragments

Radioactivity profiles are from individual gels and are typical of results obtained in other experiments, mean values of which are quoted in Table 1. For details of experimental procedures see the Materials and Methods section. (a) Intact membrane fragments. (b) Membrane fragments extracted with 0.5% Triton X-100 after labelling with ^{32}P ATP: $\circ$, insoluble fraction; $\bullet$, soluble fraction. (c) Membrane fragments separated into insoluble and soluble fractions with Triton X-100 before incubation with ^{32}P ATP; symbols are as in (b). Horizontal bars mark the regions α , β , γ , δ and ϵ referred to in the text. The protein patterns are diagrammatically illustrated above the radioactivity profiles; in (b) and (c), (i) refers to the soluble fraction and (ii) to the insoluble fraction.

obvious peaks of radioactivity were considered; these were designated α , β , γ , δ and ϵ (see Fig. 1). The amount of phosphate transferred to these regions was determined from the sum of the contents in the corresponding sections. The actual number of sections included in each region varied from experiment to experiment; therefore to estimate reproducibility mean values for each region from a series of experiments were calculated (see Table 1). For the purpose of calculating recoveries, total protein kinase activity was taken as the sum of the phosphate transferred to protein in the five regions.

Results and Discussion

Intact membrane fragments

Phosphorylation was significantly ($P < 0.01$; Student's t test) stimulated by cyclic AMP only in regions α , β and γ ; in region β stimulation was almost 400% (Table 1). The phosphoprotein(s) in region γ (mol. wt. range 56×10^3 – 67×10^3) appears to be a major membrane component, whereas those in regions α (98×10^3 – 110×10^3), β (76×10^3 – 85×10^3), δ (33×10^3 – 37×10^3), and ϵ (12.5×10^3 – 13.5×10^3) are minor constituents (Fig. 1 and below). Region ϵ includes the anodal front up to the position of the dye. The identity of the major phosphoprotein is unknown; from its position both tubulin (Lagnado *et al.*, 1975; H. Holmes, unpublished work) and the regulatory subunit of a cyclic AMP-stimulated protein kinase (Malkinson *et al.*, 1975) are possible. Comparison of results in this field is difficult, since membrane preparations and the conditions used for phosphorylation and gel electrophoresis differ greatly. However, it is possible that regions β and γ correspond to proteins I and II respectively of Ueda *et al.* (1973), and that regions β , γ , δ and ϵ include proteins D, E and F, G and H respectively of Routtenberg & Ehrlich (1975).

In the protein pattern illustrated in Fig 1(a) protein bands invariably seen are numbered 1–15; about 15 other bands, less consistently seen, are not indicated. (In the Triton X-100 fractions, bands that appear to correspond on the basis of migration distance are given the same numbers, but this does not imply that identity has been established.) Radioautography (H. Holmes & R. Rodnight, unpublished work) showed that outside the regions α – ϵ only one band was phosphorylated in a cyclic AMP-stimulated reaction. In region α bands 2 and 3, and another minor component, were labelled. In region β the major protein phosphorylated was a minor component migrating between bands 5 and 6, which were also labelled to a lesser extent. In region γ bands 7 and 8 were phosphorylated, with the bulk of the label in band 8. In region δ bands 12 and 13 were phosphorylated, with most of the label in band 13.

Table 1. Distribution of [32 P]phosphate in polyacrylamide gels of synaptic-membrane fragments

Region (Fig. 1)	...	$10^2 \times$ Phosphate transferred (pmol)									
		Cyclic AMP (50 μ M) present					Cyclic AMP absent				
		α	β	γ	δ	ϵ	α	β	γ	δ	ϵ
(a)	(9)	93 $\pm$ 7	83 $\pm$ 5	85 $\pm$ 4	25 $\pm$ 2	36 $\pm$ 6	51 $\pm$ 1	21 $\pm$ 4	54 $\pm$ 4	27 $\pm$ 3	27 $\pm$ 12
(b), soluble	(8*)	25 $\pm$ 7	15 $\pm$ 4	47 $\pm$ 4	10 $\pm$ 1	29 $\pm$ 3	10 $\pm$ 3	8 $\pm$ 1	25 $\pm$ 2	7 $\pm$ 3	16 $\pm$ 7
(b), insoluble	(8*)	90 $\pm$ 19	116 $\pm$ 15	103 $\pm$ 15	28 $\pm$ 3	34 $\pm$ 8	45 $\pm$ 6	17 $\pm$ 7	44 $\pm$ 3	24 $\pm$ 2	15 $\pm$ 2
(c), soluble	(8)	18 $\pm$ 6	38 $\pm$ 4	75 $\pm$ 5	15 $\pm$ 1	27 $\pm$ 4	8 $\pm$ 1	6 $\pm$ 2	37 $\pm$ 2	5 $\pm$ 1	7 $\pm$ 2
(c), insoluble	(8)	67 $\pm$ 9	70 $\pm$ 5	83 $\pm$ 9	26 $\pm$ 2	15 $\pm$ 4	38 $\pm$ 2	14 $\pm$ 6	65 $\pm$ 3	37 $\pm$ 2	16 $\pm$ 3

* Only four gels are represented in the series without cyclic AMP.

The values quoted are the means $\pm$ s.e.m.; the numbers of gels obtained from at least four separate experiments are indicated in parentheses. Samples (a), (b) and (c) are the same as those defined in the legend of Fig. 1.

Extraction of labelled fragments with Triton X-100

Solubilization of membrane protein with Triton X-100 depends on the source of the fragments, ionic strength and pH of the buffer used, temperature, presence of Ca^{2+} and the detergent/protein ratio (Cotman *et al.*, 1971). In 12 extractions, soluble and insoluble fractions comprised $20.2 \pm 1.2\%$ and $57.0 \pm 1.8\%$ respectively of the original protein. This higher yield of insoluble protein must be remembered when interpreting the results shown in Fig. 1 and Table 1, as equal quantities of protein were applied to all gels.

The radioactivity profile in gels from the soluble fraction, labelled with or without cyclic AMP, indicated a low ^{32}P content localized mainly in region γ . The possibility that the low recovery was partly due to dephosphorylation occurring during fractionation with detergent cannot be entirely excluded, although experiments designed to investigate this suggested that it was not the case. For example, the same low profile was obtained whether or not EDTA [which inhibits protein dephosphorylation (Weller & Rodnight, 1971)] was included in the Triton X-100 solution used to extract the labelled membrane fragments. In the insoluble fraction, labelled with or without cyclic AMP, the radioactivity profile was not significantly different from that in intact fragments (Table 1). Therefore except for the phosphoprotein in region γ , it appears that the bulk of the substrate proteins for the protein kinases in synaptic-membrane fragments are insoluble in Triton X-100.

Labelling with membrane protein after extraction with Triton X-100

With unlabelled material extracted with 0.5% Triton X-100 the recovery of protein in eight extractions was again higher in the insoluble fraction ($56.3 \pm 1.8\%$, compared with $25.3 \pm 2.4\%$ in the soluble fraction). Phosphorylation of equal amounts of the two fractions yielded profiles shown in Fig. 1(c). Recovery of basal and cyclic AMP-stimulated kinase activity was 84 and 77% respectively of the total activity in the five regions displayed in profiles of intact phosphorylated fragments.

With regard to the soluble fraction, present results confirm previous work (Weller & Rodnight, 1973; Ueda *et al.*, 1975), showing that both protein kinase activity and associated substrates are to some extent solubilized by Triton X-100. Particularly noteworthy is the observation that without cyclic AMP only the phosphoprotein in region γ was phosphorylated, whereas with cyclic AMP present some phosphorylation of proteins in regions β and γ occurred (Fig. 1c). The latter result differs from the work of Ueda *et al.* (1975), who reported cyclic AMP-stimulated phosphorylation of only one protein in their Triton X-100-soluble fraction. This discrepancy was apparently

not due to their use of a lower concentration (0.25%) of Triton X-100 for extraction or to the inclusion of dithiothreitol, since in our hands these conditions made no difference to the radioactivity profiles in either the soluble or insoluble fractions. However, as pointed out above (see also Rodnight *et al.*, 1975), there are major differences in source of membrane protein, in conditions of phosphorylation and in gel electrophoresis between the present study and that of Ueda *et al.* (1975), who also give no information on their protein/detergent ratio.

The ^{32}P profile of the insoluble fraction was similar to that obtained by using intact membranes. Without cyclic AMP, phosphorylation of proteins in region α was significantly lower ($P < 0.001$), whereas in regions γ and δ it was significantly ($P < 0.05$) increased. Cyclic AMP-stimulated phosphorylation was lower than control ($P < 0.05$) in regions α , β and ϵ , but these differences were not marked.

Of the recovered kinase activity 87 and 77% respectively of the basal and stimulated components were present in the insoluble fraction. As the profiles for [^{32}P]protein for insoluble fractions prepared before or after phosphorylation were very similar, it appears that this higher recovery of labelled protein in the insoluble fraction was due to retention of acceptor proteins rather than increased enzyme activity as a result of Triton X-100 treatment. The retention of cyclic AMP dependency in the insoluble fraction indicates that the regulatory subunit of the membrane-bound protein kinase is not completely extracted with Triton X-100, in contrast with the situation for erythrocyte membranes (Rubin, 1975).

Thus it appears that the bulk of the acceptor proteins and cyclic AMP-stimulated protein kinase activity in synaptic-membrane fragments are located in a Triton X-100-insoluble fraction. Further work is required to establish which, if any, of these proteins are associated with the synaptic junction. To this end quantitative radioautography should prove a more powerful tool than the less discriminating approach used in the present work.

Note Added in Proof (Received 18 June 1976)

Since submission of this paper relevant results on the extraction of substrates and protein kinase activity with Triton X-100 from synaptic-membrane fragments have been published by Weller & Morgan (1976).

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