Complex Phosphorylation Activity in Neurosecretosomal Membranes Isolated from Ox Neurohypophyses

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Homogenates of neural lobes of bovine pituitary glands were fractionated on Ficoll gradients to yield purified neurosecretosomes (nerve endings). The neurosecretosomes were lysed in a hypo-osmotic buffer and the membranes were separated from the soluble components by centrifugation. On incubation with [γ - 32 P]ATP this membrane preparation showed an endogenous phosphorylation activity, which was studied by means of gel electrophoresis in the presence of sodium dodecyl sulphate, and subsequent autoradiography. The major part of the [32 P] P_i detected on the gel was shown to be incorporated into three protein bands, termed A, B and C, with minimal mol.wts. of 83 000, 59 000 and 47 000 respectively. The phosphorylation of these three proteins was studied under a variety of experimental conditions. The patterns obtained were partly similar. However, important individual differences were noted, particularly with respect to the effects of cyclic AMP, Mg^{2+} and Ca^{2+} . On the basis of these differences, it is suggested that in this system the phosphorylation activity is heterogeneous, bands A, B and C each reflecting the presence of a different site of phosphate turnover. The relationship of bands A, B and C to several of the previously described phosphoproteins in the brain is discussed.

The neurohypophysis contains nerve terminals of a homogeneous nature, which on stimulation release vasopressin and/or oxytocin by a Ca^{2+} -dependent mechanism. In these terminals, the presence of an intrinsic phosphorylation activity capable of phosphorylating several membrane proteins has been established (Treiman *et al.*, 1979a). Both adenylate cyclase and phosphodiesterase activities have been demonstrated in the system (Bonne *et al.*, 1977; Poirier *et al.*, 1977; Dartt *et al.*, 1979; Torp-Pedersen *et al.*, 1979) and a role for Ca^{2+} and calmodulin in controlling the concentrations of cyclic AMP in the neurohypophysis has been suggested (Dartt *et al.*, 1979).

It thus appears that analogous to the events in brain tissue with its complex and heterogeneous synaptic structures (Krueger *et al.*, 1977; Kelly *et al.*, 1979), the changes in the phosphorylation state of specific proteins in the neurosecretory nerve endings are likely to be important in the control of release of neurohypophysial hormones. In the

Abbreviations used: ATPase, ($Mg^{2+} + Na^+ + K^+$)-activated ouabain-sensitive ATP phosphohydrolase (EC 3.6.1.3); Tes, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulphonic acid.

present work we have attempted to obtain some knowledge about the characteristics of the phosphorylation system(s) that might be involved in such a control.

A preliminary account of some of these findings has been presented previously (Treiman *et al.*, 1979b).

Materials and Methods

Adenosine 5'-[γ - 32 P]triphosphate, triethylammonium salt (sp. radioactivity > 2000 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. It was supplied in 50% ethanol and was freeze-dried before mixing with non-radioactive ATP to give the labelled substrate of desired specific radioactivity. ATP (sodium salt), cyclic AMP (free acid) and histones (from calf thymus, mixed) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Synthetic [arginine]vasopressin for radioimmunoassay was a gift from Ferring A.B., Malmö, Sweden, and antiserum was kindly donated by J. Dencker from the Royal Pharmaceutical High School, Copenhagen. All other chemicals were of analytical grade.

Isolation of the neurosecretosomal membranes from ox neural lobes

The neural lobes were removed at the local slaughterhouse and transported to the laboratory on ice within 2 h of the animals' death.

Purified nerve endings were obtained by differential and density-gradient centrifugation, by using two discontinuous gradients of Ficoll in sucrose as described by Vilhardt *et al.* (1975). However, in addition to 0.3 M-sucrose employed in the original procedure, we included Tes (20 mM), pH 7.0, and EGTA (1 mM) in the medium used for homogenization and subsequent centrifugations.

The neurosecretosomes (fraction II 5; Vilhardt *et al.*, 1975) were lysed by mixing with 20 vol. of Tes (4 mM, pH 8.0)/EDTA (1 mM) and gently stirring in the cold for 1 h.

The lysate was centrifuged at $100\,000g_{\max}$ for 45 min to obtain the membrane pellet. This total membrane fraction, referred to as 'membranes', was stored for up to 3 weeks at -18°C in Tes (20 mM), pH 7.0, and used for all experiments in this work. Approx. 300 μg of membrane protein could be obtained from one neurohypophysis (mean wet wt. 0.156 g) by this procedure.

Assay for subcellular markers

[Arginine]vasopressin and ATPase activity were measured as described previously (Torp-Pedersen *et al.*, 1979). Lactate dehydrogenase (EC 1.1.1.27) activity was assayed by the method of Brooks & Olken (1965), and that of glutamate dehydrogenase (EC 1.4.1.2) as described by Schmidt (1970). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Assay for phosphorylation activity and electrophoresis

Before the phosphorylation assay, 50 μg of membrane protein was preincubated on ice for 5 min in the assay mixture, followed by 2 min at 30°C . The reaction was then started by adding [γ - ^{32}P]ATP. For the standard assay in a total volume of 50 μl , the final concentrations were: Tes (20 mM), pH 7.0; nitrilotriacetic acid (5 mM); theophylline (10 mM); 2-isobutyl-1-methylxanthine (1 mM); Mg^{2+} (free) (2.0 mM) (as MgCl_2); cyclic AMP (5 μM); [γ - ^{32}P]ATP (10 μM , sp. radioactivity 5–10 Ci/mmol). Except where indicated otherwise, the mixture was incubated at 30°C for 10 s with stirring, and the reaction was terminated by adding 50 μl of a solution containing Tris (40 mM), pH 7.8, sodium dodecyl sulphate (4%), β -mercaptoethanol (4%), glycerol (10%) and Bromophenol Blue (0.016%). After boiling for 3 min, this mixture was applied to a gel and electrophoresis was carried out as described previously (Treiman *et al.*, 1979a), except that a constant current of 8 mA and 16 mA per plate was

used for the passage of proteins through the stacking and separating gel respectively. After staining with Coomassie Blue the gels were dried and used for autoradiography. The minimal molecular weight of the phosphorylated components was determined after calibrating the gel system by means of marker proteins covering the molecular-weight range 130 000–25 700, as recommended by Weber & Osborn (1975).

Autoradiography

Kodak Royal X OMAT, XL 1 film was used, and a satisfactory image was usually obtained after 5 to 15 days of exposure. The film was developed according to the instructions of the manufacturer. The image was recorded and quantified by using a densitometer attachment to the Pye-Unicam SP. 1700 u.v. spectrophotometer and a chart recorder coupled to an integrator. To establish the relationship between the absorbance of the bands observed on the film and the amount of radioactivity on the gel, the system was calibrated by using a membrane preparation as source of kinase and histones as an exogenous substrate. In the gel system used the phosphorylated histones migrated at a dye front and well ahead of any major phosphorylated components of the membranes, providing distinct bands with little background after autoradiography. Phosphorylation assay was carried out as described above, with histones added to a final concentration of 1 mg/ml and the concentration of the membrane protein decreased to 0.2 mg/ml. A series of samples was run with various specific radioactivities for [γ - ^{32}P]ATP from 0.2 to 10 Ci/mmol. Samples containing 10 μg of histone each were applied to the gel in duplicate. The heights of the absorbance peaks corresponding to the histone bands were measured relative to the unexposed film background. From the duplicate samples, the histone bands were cut out and the radioactivity was measured in a scintillation counter as described previously (Treiman *et al.*, 1979a).

When the absorbance of the film bands was plotted against the counted activities, a straight regression line was obtained up to 1.0 absorbance unit ($r = 0.978$), whereas at 1.5 absorbance units there was a 36% deviation downward from linearity. Consequently, all exposure times and sample activities in the present work were adjusted so as to result in a film response within the linear range.

The straight regression line indicated that the response of the film was proportional to the total number of disintegrations accumulated over the time of exposure, and was not dependent on the initial value of radioactivity. To test this independently, we adjusted the autoradiography duration for samples of histones with various radioactivities so as to obtain a constant product of radioactivity and time,

thus resulting in a constant number of disintegrations in each exposure. [More accurately, the product kept constant was $A_0 \cdot I(t)$, where A_0 was the number of d.p.m. in a histone band at the beginning of autoradiography and $I(t)$ was the value of time integral for exposure duration of t days, defined as follows: $I(t) = \int_0^t e^{-k\tau} d\tau = k^{-1}(1 - e^{-kt})$. The radioactive decay constant (k) of ^{32}P was taken as 0.0485 (day^{-1}).]

Under these conditions, the absorbances of the bands did not differ over the range of 4 to 22 days.

Calculations

The measurement of the phosphorylation activity was obtained by integrating the absorbance readings above the background determined individually for each peak and converting them into pmol of [^{32}P]P_i incorporated per mg of protein by means of the linear relationship between absorbance and activity described above.

To take into account the chelating properties of the nitrilotriacetic acid and ATP, the total amounts of Mg^{2+} and Ca^{2+} to be added to obtain the desired free ion concentrations were calculated as described by Flodgaard & Torp-Pedersen (1978). The Ca^{2+} electrode described by Simon *et al.* (1978) was kindly provided by Professor W. Simon (Swiss Federal Institute of Technology, Zürich, Switzerland) and used to test the validity of the calculations.

Results

Check of the procedure for isolation of neurosecretosomal membranes

Since we were following (with only minor modifications mentioned previously) an already established procedure for isolation of neurosecretosomal membranes (Vilhardt *et al.*, 1975) rather than setting up an entirely new one, a limited testing of the distribution of subcellular markers was carried out to check that the material obtained in our hands corresponded to that originally described by Vilhardt and his co-workers. The relative specific activities (relative to the homogenate) in the neurosecretosomal fraction purified through the Ficoll were (means $\pm$ S.E.M., n): ATPase (plasma-membrane marker), 2.57 ± 0.73 , 3; [arginine]vasopressin (neurosecretory-granule marker), 0.96 (one experiment); glutamate dehydrogenase (mitochondrial marker), 2.00 ± 0.30 , 3; lactate dehydrogenase (cytoplasmic-content marker), 0.37 ± 0.03 , 3. The corresponding marker values quoted in the original procedure were 3.34, 1.07, 1.40 (succinate dehydrogenase, taken as the mitochondrial marker) and 0.83. [Neither standard errors nor numbers of experiments were given in the paper by Vilhardt *et*

al. (1975).] The comparison of these two sets of values shows a satisfactory agreement between the two procedures. The lower relative specific activity of lactate dehydrogenase found in our experiments probably indicates that the neurosecretosomes as prepared by us had lost more of their cytoplasmic content than those of Vilhardt *et al.* (1975). This would be of minor importance, however, since the neurosecretosomes in this work were solely used as a source of membranes.

The conclusion from the marker analyses was supported by electron-microscopic examination of the neurosecretosomal fraction, showing mainly intact nerve endings, with only a scant presence of other material (nondescript membranes, elements of rough endoplasmic reticulum or nuclei).

The resolution of the phosphorylated material on the gel

Fig. 1(a) shows that about 30 bands could be discerned after staining of the gel with Coomassie Blue. After the autoradiography of the phosphorylated material, about 10 radioactive bands could be detected. However, under the conditions of incubation employed in this work, the only bands constantly measurable were those termed A, B and C (Figs. 1b and 1c), with estimated minimal mol.wts. of 83000, 59000, and 47000 respectively. The various treatments applied to the preparation to test the nature of the bands and the character of the associated radioactivity, as described previously (Treiman *et al.*, 1979a), confirmed them to be proteins and suggested that the [^{32}P]P_i was attached by an ester bond to one or several hydroxyamino acid residues on the peptide chain. As may be seen in Fig. 1(b), band A consisted of two incompletely separated components. Under the conditions employed, these components always showed a parallel variation in [^{32}P]P_i incorporated. Therefore, the total area under the double peak was taken for the measurement of band A phosphorylation.

The effect of varying the incubation conditions on the phosphorylation of bands A, B and C

To facilitate the comparison of the behaviour of the three bands, the data are presented as percentages of the activity in the standard (control) assay. The absolute amounts incorporated under the standard conditions into the peaks A, B and C respectively were 0.587 ± 0.074 , 0.299 ± 0.027 and 0.091 ± 0.012 (means $\pm$ S.E.M., $n = 12$) pmol of [^{32}P]P_i per mg of total protein applied to the gel, the errors indicating the variation between independent batches of membranes.

Throughout this paper the amounts of phosphorylation activity measured at any time point result from the net differences between the velocity

Fig. 1. Distribution of protein and radioactivity on the gel after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the [^{32}P]P_i-labelled proteins from the neurosecretosomal membranes

(a) Shows the absorbance profile of the Coomassie Blue-stained material; (b) and (c) show the absorbance profiles of the autoradiograms obtained after electrophoresis of the material incubated with [γ - ^{32}P]ATP in the presence (b) and absence (c) of 5 μM -cyclic AMP. The details of the incubation, electrophoresis and autoradiography are given in the Materials and Methods section. A, B, and C denote the bands with minimal mol.wts. of 83 000, 59 000 and 47 000. The position of marker proteins is indicated below the lowest profile by their respective molecular weights. The markers used were (in order of decreasing molecular weight): β -galactosidase, phosphorylase α , bovine serum albumin, pyruvate kinase, ovalbumin, lactate dehydrogenase and chymotrypsinogen.

of phosphorylation and that of dephosphorylation. Therefore, no attempt has been made to use the presented data for estimating the kinetic parameters of the kinase(s) responsible for the phosphorylation of the individual components described here.

Time. The time course of the phosphorylation of the bands A, B and C is shown in Fig. 2.

Fig. 2. The time course of the phosphorylation of bands A (a), B (b) and C (c) under various assay conditions. All the values are expressed as percentages of the value obtained after 10s of incubation in the standard assay mixture described in the Materials and Methods section. The means for two determinations are shown, each performed in duplicate on an independent membrane preparation. Symbols: O, standard assay; ●, 10 mM-F⁻ added; ▲, 1 mM free Ca²⁺ added.

In the standard reaction mixture maximum labelling was reached at the 20s incubation time (approx. 180–200% of the control value for all the bands). After that, all the bands underwent dephosphorylation, demonstrating the presence of protein phosphatase activity in the system. In the presence of 10mM-F⁻, the dephosphorylation of proteins A and C was reversed, both peaks increasing in 5 min to an activity 300–350% of the control maximum at 20s. F⁻ also enhanced the phosphorylation of peak B. However, at 20s this peak reached its maximum activity, close to 150% of the value at 20s without F⁻, remaining at this value until 5 min. In view of our previous finding of an inhibition by Ca²⁺ of the cyclic AMP-dependent component of band A phosphorylation in this system (Treiman *et al.*, 1979a), it was also of interest to test the effect of this ion on the time course of the phosphorylation reaction. It may be seen in Fig. 2 that 1 mM-Ca²⁺ decreased the maximal amount of phosphorylation of bands A, B, and C, and the initial phosphorylation velocity of bands A and B. The point of maximum activity for all these bands was shifted to 10s.

To be able to estimate the extent to which the time course of the phosphorylation reactions was affected by the change in the [γ -³²P]ATP supply to the system, the rates of [γ -³²P]ATP hydrolysis were measured under the respective sets of conditions. Fig. 3 shows that in the standard assay about 50% of [γ -³²P]ATP was hydrolysed after 20s, and less than 20% was still present after 2min. Ca²⁺ did not

affect this time course. In contrast, in the presence of F⁻, the amount of [γ -³²P]ATP present after 20s was about 80%.

ATP. Fig. 4 demonstrates that the [γ -³²P]ATP concentration of 10 μ M used in the control conditions was far from saturating, the concentration dependence of all three activities being within the error of determination linear up to that point. In the range 25–100 μ M- $[\gamma$ -³²P]ATP, proteins A and C behaved similarly, reaching activities about 360% of their respective control values, with values of the [γ -³²P]ATP concentration for half-maximal effect being 18 and 23 μ M respectively. On the other hand, the maximal activity of peak B only slightly exceeded 200% of the control value, the half-maximal increase occurring at 11 μ M- $[\gamma$ -³²P]ATP.

Cyclic AMP. The membrane preparation used for this study also contained quite marked phosphodiesterase activity (Torp-Pedersen *et al.*, 1979). Inclusion of 10mM-theophylline and 1mM-2-isobutyl-1-methylxanthine into the assay mixture was necessary to ensure the 95% inhibition of cyclic AMP breakdown.

Fig. 5 shows that a striking heterogeneity exists in the response pattern of the three bands to cyclic AMP. Bands A and B both responded with an increase in the activity with increasing concentration of cyclic AMP, the maximal stimulation

Fig. 3. The time course of the [γ -³²P]ATP hydrolysis under various assay conditions

The values shown are percentages of the initial [γ -³²P]ATP content in the reaction mixture. The points represent means for two determinations, each performed in triplicate on an independent membrane preparation. The measurement of the [³²P]P_i released was as specified in the Materials and Methods section for the determination of ATPase activity. Symbols: O, standard assay; ●, 10mM-F⁻ added; ▲, 1mM free Ca²⁺ added.

Fig. 4. Dependence of the amount of phosphorylation of bands A, B and C on the concentration of [γ -³²P]ATP. All the values are expressed as percentages of the value obtained in the standard assay, as described in the Materials and Methods section. The points represent means for four duplicate determinations, each carried out on an independent batch of membranes. The bars show standard errors and are included if they exceed the symbols. O, ● and Δ refer to bands A, B, and C respectively.

Fig. 5. Dependence of the amount of phosphorylation of bands A, B and C on the concentration of cyclic AMP. The values are expressed as percentages of the amount obtained in the standard assay, as described in the Materials and Methods section. The points are means for four duplicate determinations, each carried out on the separate batch of membranes. The bars indicate standard errors and are shown if greater than the symbols. $\circ$, $\bullet$ and $\triangle$ refer to bands A, B and C respectively.

occurring at $1 \mu\text{M}$ -cyclic AMP. However, whereas in the absence of cyclic AMP the amount of phosphorylation of band A was 8% of the maximal value, this was 50% in the case of band B. The values for half-maximal stimulation were 0.1 and $0.06 \mu\text{M}$ respectively. The picture presented by band C was different. The activity with $0.01 \mu\text{M}$ -cyclic AMP was maximal and about 140% of the activity in the absence of this compound. The activity then declined to about 100% at $1 \mu\text{M}$ -cyclic AMP.

Mg^{2+} and Ca^{2+} . The effects of various concentrations of Mg^{2+} and Ca^{2+} are shown in Fig. 6. The three bands again responded with a differential pattern. Thus, although phosphorylation of all three peaks was absolutely dependent on the presence of Mg^{2+} in the assay, only peaks A and B exhibited a biphasic dependence, with the activity decreasing to some 20–30% after reaching a maximum at 2.0 mM - Mg^{2+} . In contrast, peak C activity was at a maximum with 3 mM - Mg^{2+} and did not show any decline at higher concentrations of this ion. Also, whereas the values for half-maximal phosphorylation of bands A and B were 0.55 and 0.65 mM respectively, this was 0.25 mM for band C.

Additional points of difference between the phosphoproteins considered became apparent when the relationship between the effects of Ca^{2+} and Mg^{2+} on the system was examined. Fig. 6 shows that when 1 mM - Ca^{2+} was added to the system, there was an inhibition of labelling of peaks A and B

Fig. 6. The effects of varying the concentration of free Mg^{2+} in the absence and presence of 1 mM - Ca^{2+} on the amount of phosphorylation of bands A (a), B (b) and C (c).

The values are percentages of the activity obtained under standard assay conditions (as described in the Materials and Methods section). $\circ$ and $\bullet$ refer to the absence and presence of 1 mM free Ca^{2+} in the assay respectively. The points with standard errors indicated by bars are means for four duplicate determinations, each from the separate membrane batch. The free concentrations of Mg^{2+} and Ca^{2+} were obtained as described in the Materials and Methods section.

throughout the whole range of Mg^{2+} concentrations, with no effect in the case of peak C. The pattern of inhibition was different in peaks A and B. The maximal activity of peak A in the presence of 1 mM - Ca^{2+} was about 60% of the control value, the Mg^{2+} concentration for the half-maximal effect being shifted to 1.0 mM . In the case of peak B, the maximum activity was close to 80% of the control, whereas no change in the Mg^{2+} concentration for half-maximal stimulation could be noted. Furthermore, whereas Ca^{2+} was not able to support the phosphorylation of peak A in the absence of Mg^{2+} ,

Fig. 7. The effects of varying the concentration of free Ca^{2+} on the amount of phosphorylation of bands A, B and C

The values are given as percentages of those obtained in the absence of Ca^{2+} (standard assay). Symbols $\circ$, $\bullet$ and $\triangle$ refer to bands A, B and C respectively, and represent the means for four duplicate determinations, each carried out on a separate batch of membranes. Standard errors are indicated by bars. The free concentrations of Ca^{2+} were obtained as described in the Materials and Methods section.

about 8% of the control activity was obtained in the case of peak B with 1 mM- Ca^{2+} alone.

When the effects of varying the concentration of Ca^{2+} alone were examined, the results shown in Fig. 7 were obtained. It can be seen that Ca^{2+} inhibited the phosphorylation of peaks A and B throughout the whole concentration range tested, peak A being the most sensitive. In contrast, at 0.25 mM- Ca^{2+} there was a stimulation of the phosphorylation of peak C of about 30%. When the concentration of Ca^{2+} was raised above that point, this effect was reversed, inhibition of peak C phosphorylation being achieved at 2.5 mM- Ca^{2+} .

Discussion

A general point emerging from the results reported in the present paper is that of the complexity of the labelling pattern obtained when membranes from purified ox neurohypophysis nerve terminals are incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the distribution of the radioactivity is followed by gel electrophoresis

and autoradiography. Out of the total of approx. ten radioactive bands identifiable, three bands, comprising a major part of the total $[\text{P}^{32}]\text{P}_i$ incorporated, displayed a level of activity permitting quantitative measurements. These three components were shown to be peptides with minimal mol.wts. of 83 000, 59 000 and 47 000 respectively.

The complex nature of the intrinsic phosphorylating activity in this system became further evident when the individual behaviour of each of the three bands was considered under various conditions. Among the conditions tested, varying the concentrations of cyclic AMP, Mg^{2+} and Ca^{2+} served most clearly to demonstrate the individual nature of the responses of proteins A, B and C. The main points of difference revealed by these conditions are summarized in Table 1.

As already pointed out, only net differences between the phosphorylation and dephosphorylation velocities of the individual bands were measured under all assay conditions in the present work. Therefore, the principal importance of our data is to indicate the degree of heterogeneity of the system rather than to provide unambiguous characteristics of the kinase(s) and phosphatase(s) involved with respect to the conditions tested. However, the data do allow certain tentative conclusions to be drawn. (1) The fact that the time course of phosphorylation of all the three bands in the standard assay conditions was approximately linear up to 20s (Fig. 2), as was the dependence of the phosphorylation level on the substrate concentration in the range 2.5–10 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 4), indicates that the extent of phosphorylation measured at the 10s time point predominantly reflected the kinase activity. This is also supported by the relatively slow dephosphorylation, as judged by the last part of the slope of the time-course curve for the standard assay shown in Fig. 2. (2) It follows from the above considerations and from the effect of F^- on the rate of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis (Fig. 3) that the enhancement of the activity level of each of the bands after addition of 10 mM- F^- was at least during the first 20s mainly due to the increased availability of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for phosphorylation. However, it should be noted that from 120s on, the concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was in the presence of F^- not greater than one-half of its value at 20s in the standard assay (Fig. 3), a point where the rate of dephosphorylation became greater than that of phosphorylation for bands A, B, and C. Thus at 120s and in the presence of F^- , based on the ATP dependence of the kinase velocity, one would expect it to be less than the velocity of phosphatase and the slope of the time course to be declining for bands A, B and C. Fig. 2 shows, however, that in this time range bands A and C continue to increase their degree of phosphorylation, band B staying at a

Table 1. *Individual responses of proteins A, B and C to various experimental conditions*

The phosphorylation value with respect to each of the three bands was measured and the [^{32}P]P_i incorporation expressed as a percentage of the value obtained under standard incubation conditions, as described in the Materials and Methods section.

Band ...	A (mol.wt. 83 000)		B (mol.wt. 59 000)		C (mol.wt. 47 000)	
	-Ca ²⁺	Ca ²⁺ (1.0 mM)	-Ca ²⁺	Ca ²⁺ (1.0 mM)	-Ca ²⁺	Ca ²⁺ (1.0 mM)
Mg ²⁺						
Maximal value (%)	100	58	100	82	110	110
Concn. (mM)	2	3	2	3	3	3
Concn. for half-maximal value (mM)	0.55	1.0	0.65	0.65	0.25	0.25
Ca ²⁺ free in presence of 2.0 mM-free Mg ²⁺ (mM for 50% inhibition)		0.88		1.7	Inhibition less than 50% in the Ca ²⁺ concentration range tested	
Activity with no cyclic AMP (%)		8		50		108
Maximal value with cyclic AMP (%)		92		92		142
Cyclic AMP (μM)		1		0.5		0.01
Concn. for half-maximal value (μM)		0.1		0.06		<0.01

plateau. Therefore, we conclude that at least in the case of bands A and C the action of F⁻ was not confined to increasing the availability of ATP for the kinase(s) involved. The additional action might be either a stimulatory effect on the kinase activity, an inhibition of the phosphatase, or both. In brain, the ability of F⁻ to inhibit protein phosphatase activity has indeed been demonstrated (Maeno & Greengard, 1972). (3) The data in Figs. 2 and 7 point to an inhibitory action of Ca²⁺ on the velocity of phosphorylation of bands A and B. The fact that the onset of net dephosphorylation of these bands, as well as band C, was shifted by Ca²⁺ from 20 to 10 s might be due to the changed balance of the phosphorylating and dephosphorylating velocities after kinase inhibition, but it could also partly result from a direct stimulatory action by Ca²⁺ on the phosphatase activity. This would especially seem to be the case with band C, where no effect of 1 mM-Ca²⁺ could be noted at 10 s, with marked decline in the net phosphorylation throughout the remaining time of the assay.

The differential action of Ca²⁺ on the phosphorylation of bands A, B and C found in this work should be seen in extension of our previous observations of the inhibition by Ca²⁺ of the phosphorylation of band A (Treiman *et al.*, 1979a). Under the improved conditions of membrane preparation and assay in the present work, we were not able to detect any marked effects of Ca²⁺ in the 'physiological' concentration range (0.1–10 μM). It has been documented (Schulman & Greengard, 1978; DeLorenzo *et al.*, 1979) that some of the actions of Ca²⁺ on the phosphorylation systems may require the presence of calmodulin, a Ca²⁺-binding

protein that can be reversibly bound to cell membranes, and which is also present in the neurohypophysis (Russell & Thorn, 1977). Further work will be needed to clarify whether the apparent lack of effect of Ca²⁺ in the 'physiological' concentration range on the phosphorylation of protein bands A, B and C reported here may be ascribed to the fact that the membranes in the present work were prepared by osmotically lysing the secretosomes in the presence of 1 mM-EDTA, a treatment likely to result in removal of most of the calmodulin bound to the membranes (Schulman & Greengard, 1978).

On the basis of the predominantly unique responses of each of the three bands under the variety of testing conditions described above, it is suggested that the phosphoproteins A, B and C reflect the presence in the system of at least three different types of [^{32}P]P_i-transfer reactions.

Although it is not possible at present to account for this heterogeneity, we should like to point out that the membrane preparation used for these studies included contributions from the plasma membranes, as well as the membranes of the secretory granules, the mitochondria and other subcellular membrane components present in the secretosomes (possibly including smooth endoplasmic reticulum, which has recently been demonstrated in the neurohypophyseal axons of rats by Alonso & Assenmacher, 1979).

Although there is one report on the presence of synapses involving neurosecretory terminals in the neurohypophysis (Bargmann, 1965), such structures seem to be very rare. It would be unlikely that they contribute to any sizeable extent to the phosphorylation systems demonstrated here. At the

same time, the secretosomes used as a source of membranes constituted quite a homogeneous fraction, due both to the relative uniformity of neuronal components in the neurohypophysis (Morris *et al.*, 1978) (barring any differences between oxytocic and vasopressic neurons) and to the high degree of purification of the secretosomal fraction in the procedure of Vilhardt *et al.* (1975). Thus in this system one might expect the heterogeneity to reside at the subcellular, rather than the cellular level.

It should also be realized that the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis technique in the present work could lead to the underestimation of the actual heterogeneity of the system. More powerful tools, as for example two-dimensional electrophoresis, might in principle resolve any of the phosphorylated bands into several components. To the extent that this in fact would apply to any of the bands A, B and C, the various characteristics of their responses as described here would merely represent the superimposition of the individual patterns of their respective subcomponents.

In brain, the intrinsic phosphorylation of several membrane proteins from microsomal and synaptosomal fractions has been shown in a number of studies (reviewed by Rubin & Rosen, 1975; Williams & Rodnight, 1977). In synaptosomal membranes, Ueda & Greengard (1977) have described a cyclic AMP-stimulated phosphorylation of a protein (protein I) composed of two polypeptides with minimal mol.wts. of 86 000 and 80 000 respectively. Kelly *et al.* (1979) have shown a cyclic AMP-stimulated phosphorylation of proteins with mol.wts. of 85 000 and 82 000 in a preparation of synaptic junctions from rat brain. It is possible that these brain phosphoproteins and their enzyme systems correspond to the system responsible for phosphorylation of band A in the neurohypophysis.

To the extent that the neurosecretosomes represent a modification of presynaptic terminals, this possibility is consistent with the hypothesis (Greengard, 1976), supported by later studies (Krueger *et al.*, 1977; Uno *et al.*, 1977; Ueda & Greengard, 1977) specifying the preferential location of the protein I-related enzyme system at the synaptic structures.

The incorporation of [^{32}P]P_i into the regulatory subunit of the type II protein kinase by an autophosphorylation reaction is stimulated by cyclic AMP (Rosen & Erlichman, 1975). Recently, Rubin *et al.* (1979) showed that this subunit was present in ox brain membranes and had a minimal mol.wt. of 55 000, equal to the regulatory subunit of the cytosol type II protein kinase. In addition, a decrease in the electrophoretic mobility in the Tris/glycine/sodium dodecyl sulphate buffer system, also used in the present study, of the phosphorylated regulatory

subunit of this enzyme has been described (Hofmann *et al.*, 1975). Thus, despite the apparent difference in the molecular weights, it cannot be excluded that band B in our system corresponds to the regulatory subunit of the type II protein kinase.

The 47 000-mol.wt. band (C) described in the present work was the one affected least by changes in the concentration of cyclic AMP and not at all by 5 μM -cyclic AMP. A phosphoprotein with a minimal mol.wt. of 47 000, enriched in the synaptosomal fraction of rat cerebral cortex, was described by Ehrlich *et al.* (1977). This protein showed only a slight increase of [^{32}P]P_i incorporation in the presence of 5 μM -cyclic AMP. Also, Zwiers *et al.* (1976) reported that a 48 000-mol.wt. polypeptide was endogenously phosphorylated in rat synaptic plasma membranes, with no effect of 5 μM -cyclic AMP on this reaction.

Ca²⁺ at a concentration of 0.25 mM stimulated phosphorylation of band C by about 30% (Fig. 6). Recently (Ehrlich, 1979), Ca²⁺ has been reported to stimulate phosphorylation of the 47 000-mol.wt. protein described by Ehrlich *et al.* (1977), which was mentioned above. Also, Hershkowitz (1978) and Hofstein & Hershkowitz (1979) have described an actin-like phosphoprotein having a mol.wt. of 41 000–43 000 and present in a crude synaptosomal fraction from rat cerebral cortex. The phosphorylation was stimulated by Ca²⁺. In addition to the apparent difference in the molecular weight, other features of this protein distinguishing it from the 47 000-mol.wt. peptide described by us in the neurohypophysis include: (1) Mg²⁺ was not necessary for its phosphorylation; (2) inhibition of phosphorylation was noted at an Mg²⁺ concentration greater than 1 mM; (3) stimulation by Ca²⁺ occurred in the range from 0.1 to several millimolar. In view of the differences in the assay and electrophoresis conditions employed in the various studies, it is difficult at present to resolve to what extent the protein C described in this work corresponds to any of the brain phosphoproteins mentioned above.

In summary we have shown that the intrinsic phosphorylation of the three major receptor peptides in membranes from the ox neurosecretosomes seems to reflect the occurrence of at least three phosphate-turnover processes with characteristic properties.

Further work is required to clarify the nature of these neurohypophysial phosphopeptides and also to establish to what extent there is a correspondence between these and some of the apparently similar proteins in synaptic regions in the brain.

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