Corticotropin-(1–24)-tetracosapeptide affects protein phosphorylation and polyphosphoinositide metabolism in rat brain

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1. Effects of corticotropin-(1–24)-tetracosapeptide on the endogenous phosphorylation of proteins and lipids were studied in a membrane/cytosol fraction prepared from a lysed crude mitochondrial/synaptosomal fraction. 2. The labelling of proteins and lipids was monitored by incubation of the subcellular fraction for 10 s with $[\gamma^{-32}P]ATP$. 3. The phosphorylation of proteins was dose-dependently inhibited by the peptide (40% of control incubations at 100 μM-corticotropin). 4. Of the membrane phospholipids only phosphatidylinositol phosphate, phosphatidylinositol bisphosphate and phosphatidic acid became labelled. Corticotropin dose-dependently increased the formation of phosphatidylinositol bisphosphate and inhibited the production of phosphatidic acid (470% and 50% respectively of control incubations, at 100 μM of the peptide) and had no effect on phosphatidylinositol phosphate. 5. Phosphatase activity was observed to act on phosphatidylinositol bisphosphate, phosphatidylinositol phosphate and phosphoprotein but not on phosphatidic acid. 6. Corticotropin interacted with the kinases rather than with the phosphatases. 7. The formation of phosphatidylinositol bisphosphate and phosphatidic acid was maximal at 1–10 mM-Mg^{2+} in the absence of Ca^{2+}, and the production of phosphatidylinositol phosphate was maximal at 30 mM-Mg^{2+}. 8. The basal value of lipid phosphorylation decreased with increasing Ca^{2+} concentration. 9. Ca^{2+} abolished the effect of corticotropin on phosphatidylinositol bisphosphate formation (470%, 190% and 100% of control incubations at respectively 0, 0.1 and 1 mM-Ca^{2+}). 10. The data provide evidence that the effects of corticotropin on protein phosphorylation and on polyphosphoinositide metabolism in brain membranes are related.

The phosphorylation of membrane components is thought to be an important process in membrane function. Both phosphorylated proteins and a special class of phospholipids, the (poly)phosphoinositides, have been implicated in the regulation of membrane permeability and synaptic transmission in neurons (Michell, 1975; Williams & Rodnight, 1977; Greengard, 1978; Hawthorne & Pickard, 1979). An enhanced metabolism of phosphatidyl-*myo*-inositol was observed in various tissues after receptor activation by hormones and neurotransmitters that utilize Ca^{2+} as their intracellular second messenger (Michell, 1975). This ion is also implicated in the metabolism of the polyphosphoinositides (phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-bisphosphate). A rapid breakdown of phosphatidylinositol bisphosphate was seen on influx of Ca^{2+} into the cell (Abdel-Latif *et al.*, 1977,

1979; Griffin & Hawthorne, 1978). The relatively high content of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in brain tissue (Hawthorne & Pickard, 1979) and their rapid metabolism (Hawthorne & Kai, 1970) also suggest that these lipids may play an important role in brain cell membrane function.

It was found that corticotropin affects both protein phosphorylation and polyphosphoinositide metabolism (Jolles *et al.*, 1979, 1980*a,b*; Zwiers *et al.*, 1978). Corticotropin exerted a dose- and structure-dependent inhibitory effect on the phosphorylation of proteins present in synaptosomal plasma-membrane fractions from rat brain (Zwiers *et al.*, 1976, 1978), and also in adrenal cortex effects on protein phosphorylation have been obtained (Podesta *et al.*, 1979). The peptide affected the metabolism of the polyphosphoinositides in a

synaptosomal fraction from rat brain (Jolles et al., 1979). Also effects on phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in adrenal cortex have been reported after the administration of the peptide to rats in vivo (Farese et al., 1979). Recently, the corticotropin-sensitive protein kinase complex, containing the kinase and the substrate protein (B-50), was isolated in soluble form from rat brain synaptosomal plasma membranes (Zwiers et al., 1979, 1980). It was shown to possess phosphatidylinositol phosphate kinase activity, and the lipid phosphorylation seemed to be regulated by phosphorylation of the B-50 protein (Jolles et al., 1980a,b).

To clarify whether such a direct relation between protein phosphorylation and lipid phosphorylation has any physiological significance we have examined whether corticotropin has similar effects in a brain membrane preparation. A brain subcellular fraction containing both light membranes and cytosolic proteins was used, as many enzymes involved in the metabolism of the polyphosphoinositides are partially or completely soluble (e.g. 1,2-diacylglycerol kinase, Kanoh & Åkesson, 1978; phosphatidylinositol phosphate kinase, Kai et al., 1968; Shaikh & Palmer, 1977; Ca^{2+}-dependent phosphatidylinositol phosphodiesterase, Irvine et al., 1979; polyphosphoinositide phosphomono- and phosphodiesterase, Akhtar & Abdel-Latif, 1978). We report in the present paper that corticotropin not only affects protein phosphorylation and phosphatidylinositol bisphosphate formation but also phosphatidic acid formation in this membrane-cytosol fraction. More information was obtained on the mechanism of action of corticotropin and on the importance of Ca^{2+} for the effects of the hormone.

Materials and methods

Animals and brain dissection

Male rats (150 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). After decapitation the brain was rapidly removed. All subsequent operations were performed at 0–4°C. Limbic structures including the mesencephalon were dissected as described before (hippocampus, septum, basal ganglia, pyriform cortex, diencephalon, mesencephalon; Gispen et al., 1973).

Subcellular fractionation

The dissected material from one rat (0.3 g) was homogenized in 3 ml of homogenization medium (0.32 M-sucrose, 1 mM-EDTA, 10 mM-Tris/HCl, pH 7.4; Booth & Clark, 1978) by ten up-and-down strokes of a Potter–Elvehjem Teflon/glass homogenizer with a radial clearance of 0.125 mm, rotating at 700 rev./min. The subcellular fractiona-

tion was based on the method of Gray & Whittaker (1962). Briefly, the homogenate was centrifuged at 1000 g for 10 min. After centrifugation of the supernatant at 10000 g for 10 min, the resulting crude mitochondrial/synaptosomal pellet (P_2) was subjected to osmotic lysis. The pellet was resuspended in 10 vol. of aqua bidest and stirred for 10 min at 4°C. This suspension was centrifuged for 20 min at 10000 g to remove unlysed structures. The 10000 g supernatant of the lysed P_2 fraction (termed the membrane/cytosol fraction) was taken as the enzyme fraction.

Phosphorylation assay

Endogenous phosphorylating activity was assayed as described previously (Zwiers et al., 1976, 1978, 1979). Briefly, the incubations were performed under the following condition: 7.5 μM-ATP, 3 μCi of [γ-^{32}P]ATP (approx. 3000 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.), 5 μl of enzyme fraction (approx. 15 μg of protein), 50 mM-sodium acetate, 10 mM-magnesium acetate, pH 6.5, in a final volume of 25 μl at 30°C. The membrane samples were preincubated for 5 min, and the incubation was started by the addition of the ATP. Buffer solution plus or minus corticotropin-(1–24)-tetracosapeptide (Organon Int. BV, Oss, The Netherlands) was added 15 s before ATP. Lipid phosphorylation and protein phosphorylation were always studied in the same experiment. The lipid phosphorylation reaction was terminated by the addition of 2 ml of ice-cold chloroform/methanol/ 13 M-HCl (200:100:0.75, by vol.; Shaikh & Palmer, 1977). The protein phosphorylation reaction was terminated by the addition of a denaturing solution resulting in final concentrations of 62.5 mM-Tris/HCl, 2% sodium dodecyl sulphate, 10% glycerol, 0.001% Bromphenol Blue and 5% 2-mercaptoethanol. Phosphorus determination was performed by the method of Hess & Derr (1975) and protein was determined by the method of Lowry et al. (1951).

Lipid extraction and t.l.c.

After termination of the phosphorylation reaction carrier polyphosphoinositides (10 μg of phosphorus) were added, isolated as described by Dawson & Eichberg (1965), and the mixture was allowed to stand for 10 min at 0°C. By adding 0.375 ml of 0.6 M-HCl a biphasic system was formed. The upper phase was removed and the lower phase washed twice with 1 ml of chloroform/methanol/0.6 M-HCl (3:48:47, by vol.). The resulting lower phase was dried under N_2 at 30°C, and redissolved in 100 μl of chloroform/methanol/water (75:25:2, by vol.). Two portions (5 μl) were taken for the determination of radioactivity incorporated into total phospholipids, and one portion (5 μl) was taken for the

determination of total lipid phosphorus. The remaining part of the extract was again dried under N_2 at 30°C and redissolved in 20 μl of chloroform/methanol/water (75:25:2, by vol.). Phospholipids were separated by one-dimensional t.l.c. on silica gel 'High-Performance' thin-layer plates (layer thickness 25 μm; Merck).

Before use, the plates were impregnated with potassium oxalate, by development in methanol/water (2:3, v/v) containing 1% potassium oxalate, drying at room temperature and activation at 110°C for 15 min. Lipid extracts were applied to the plate in a volume of 15–20 μl by a Capilettor (Labora, Mannheim). Chromatograms were developed in a paper-lined chromatographic chamber with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.; Shaikh & Palmer, 1977). On each plate (10 cm × 20 cm) 18 samples were run simultaneously. This procedure yielded a quantitative separation of all labelled lipids. Recovery after t.l.c. was greater than 97%. The lipids were detected with I_2 vapour, and ^{32}P-labelled spots were detected by autoradiography on Kodak Royal X-Omat film (10–20 h). The spots were scraped from the plate and counted for radioactivity in a liquid-scintillation spectrometer. Membrane preparation, phosphorylation assay, lipid extraction and t.l.c. were always performed on the same day.

Separation of membrane proteins by polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on slab gels was performed by the method of Lugtenberg *et al.* (1975). The slab was 1.3 mm thick and consisted of a running gel (11% acrylamide, 9 cm) and a stacking gel (3% acrylamide, 2 cm). Routinely, 20 μl of the reaction mixture, containing approx. 20 μg of protein, was applied directly to the gel. On each slab 25 samples were run simultaneously. Electrophoresis was carried out at room temperature with a current of 35 mA (100–200 V)/gel. When the tracking dye (Bromphenol Blue) had run as far as 1 cm from the bottom of the gel (2½h), electrophoresis was terminated. The gel was stained for 10 min in a solution of 0.1% Fast Green FCF in destaining mixture [methanol/15% acetic acid (1:2, v/v)] and destained overnight. The gel was dried on Whatman 3M paper, and subjected to autoradiography as described above.

The radioactivity of individual protein bands was determined by cutting the bands from the gel and counting for radioactivity in a liquid-scintillation spectrometer.

Determination of radioactivity

^{32}P was determined by liquid-scintillation counting in a fluid prepared by mixing a commercially available scintillation fluid (Xylofluor; Baker) with Triton X-100 in the ratio 23:7 (v/v). Radioactivity in 0.2 ml of water was counted with 3.8 ml of this fluid in a Mark II liquid-scintillation spectrometer (Searle). Counting efficiency was 93%.

Results

The phosphorylation of phospholipids and phosphoproteins is sensitive to corticotropin-(1–24)-tetracosapeptide: time curve

The membrane/cytosol fraction was prepared as described in the Materials and methods section. This fraction was incubated under conditions in which effects of corticotropin-(1–24)-tetracosapeptide on protein phosphorylation have been obtained (Zwiers *et al.*, 1976, 1978, 1979). After incubation for 10 s in the presence of [γ-^{32}P]ATP (7.5 μM) a significant incorporation of label into proteins and phospholipids was observed. Fig. 1(*a*) shows that a large
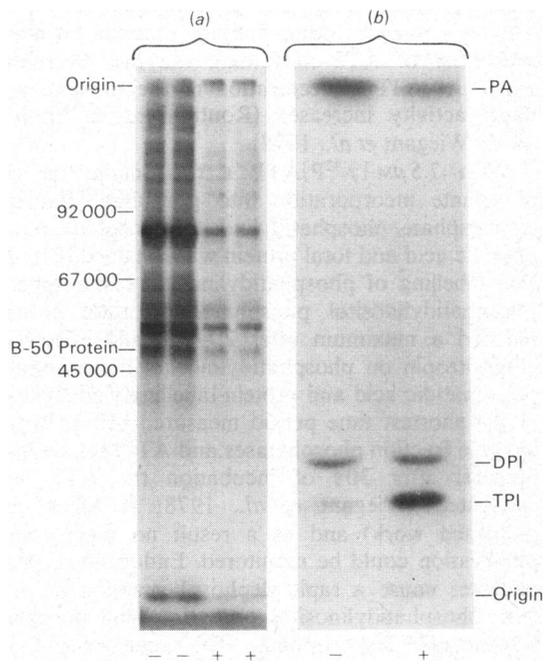
Fig. 1. *Autoradiographs (a) after protein separation by polyacrylamide-gel electrophoresis, showing the endogenous phosphorylation profile of the lysed P_2 fraction, and (b) after lipid extraction and t.l.c.*
Incubation (10 s) was carried out as described in the legend to Fig. 2. The position of molecular-weight-marker proteins and of B-50 protein is indicated. For (*b*) the position of the separated lipids is shown on the right. The absence (−) and presence (+) of 100 μM-corticotropin-(1–24)-tetracosapeptide in the incubation is indicated. Abbreviations used: PA, phosphatidic acid; DPI, phosphatidylinositol phosphate; TPI, phosphatidylinositol bisphosphate.

number of protein bands was labelled. The presence of corticotropin-(1–24)-tetracosapeptide (100 μM) resulted in an overall decrease of protein labelling (40% of control incubations). Among the affected proteins was the B-50 protein (mol.wt. 48000, isoelectric point 4.5), whose phosphorylation has been shown to be particularly sensitive to corticotropin in the purified synaptosomal plasma membranes (Zwiers et al., 1976, 1978). Of the many phospholipids present in this fraction, only phosphatidylinositol bisphosphate, phosphatidylinositol phosphate and phosphatidic acid became labelled (Fig. 1b). Corticotropin did not affect the labelling of phosphatidylinositol phosphate but the peptide stimulated the incorporation of ^{32}P into phosphatidylinositol bisphosphate and inhibited the incorporation into phosphatidic acid (470% and 50% of control incubations respectively at 100 μM of the peptide). When higher concentrations of ATP were used (200 μM, 1 mM), the basal value of lipid phosphorylation and protein phosphorylation increased but the effects of corticotropin decreased and even disappeared (J. Jolles, unpublished work). This corroborates other findings that the hormone sensitivity of ATP-consuming enzymes decreases when the ATP concentration in the medium and basal activity increases (Routtenberg & Ehrlich, 1975; Wiegant et al., 1978).

With 7.5 μM-[γ-^{32}P]ATP a time curve for the phosphate incorporation into phosphatidylinositol bisphosphate, phosphatidylinositol phosphate, phosphatidic acid and total protein was obtained (Fig. 2). The labelling of phosphatidylinositol bisphosphate, phosphatidylinositol phosphate and total protein reached a maximum after 20–30 s and effects of corticotropin on phosphatidylinositol bisphosphate, phosphatidic acid and protein labelling were evident at the shortest time period measured (10 s). In the enzyme fraction phosphatases and ATPases are also present; after 30 s of incubation the ATP was exhausted (Wiegant et al., 1978; J. Jolles, unpublished work) and as a result no more phosphorylation could be monitored. Endogenous phosphatases cause a rapid dephosphorylation of protein, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. The time course of phosphatidic acid labelling was different: the incorporation increased until 30 s, and no dephosphorylation was seen.

The effect of corticotropin-(1–24)-tetracosapeptide on phospholipid and protein phosphorylation: dose–response relation

The effect of different concentrations of corticotropin was tested at 10 s incubation time. Fig. 3 shows that the peptide dose-dependently influenced phosphatidylinositol bisphosphate and phosphatidic acid but not phosphatidylinositol phosphate label-
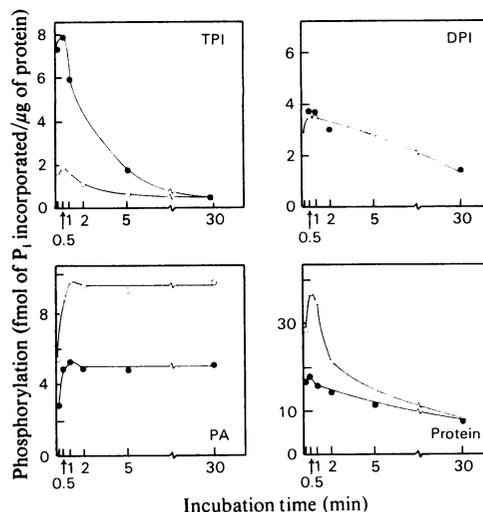
Fig. 2. Phosphorylation of lipids and proteins: time curve

A lysed P_2 fraction was incubated with [γ-^{32}P]ATP in the absence (O) and presence (●) of 100 μM-corticotropin-(1–24)-tetracosapeptide, as described in the Materials and methods section. The corticotropin was added at –15 s. The incubation time was varied from 10 s to 30 min. Results are expressed as fmol of phosphate incorporated/μg of protein incubated. Abbreviations used: TPI, phosphatidylinositol bisphosphate; DPI, phosphatidylinositol phosphate; PA, phosphatidic acid.
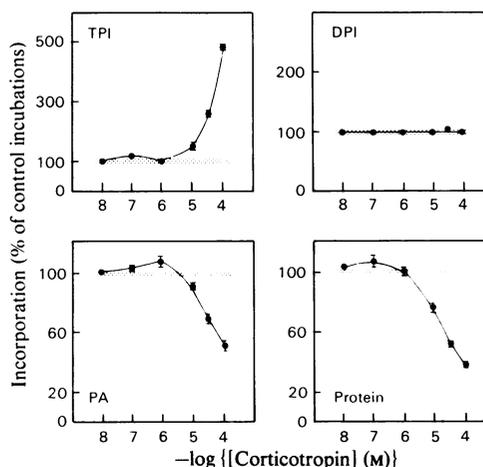
Fig. 3. The effect of corticotropin-(1–24)-tetracosapeptide on the phosphorylation of lipids and proteins: dose–response curve

A lysed P_2 fraction was incubated for 10 s with [γ-^{32}P]ATP in the absence and presence of various concentrations of corticotropin-(1–24)-tetracosapeptide (0.01–100 μM). Results are expressed as means ± S.E.M. (indicated by the bars). Abbreviations used: TPI, phosphatidylinositol bisphosphate; DPI, phosphatidylinositol phosphate; PA, phosphatidic acid. The stippled line indicates control incubations.

ling. The smallest dose of the hormone that was effective on phosphatidylinositol bisphosphate and phosphatidic acid labelling was $10\mu M$ (140% and 90% of control incubations respectively). The protein phosphorylation was also inhibited: at $10\mu M$-corticotropin the labelling was decreased to 77% of control incubations.

Does corticotropin affect phosphorylation or dephosphorylation?

From the experiments above it cannot be concluded whether the hormone affects phosphorylation or dephosphorylation. To discriminate between these two possible mechanisms, the following experiment was designed: the enzyme fraction was incubated with $[\gamma^{-32}P]ATP$ as described. The phosphorylation of proteins and lipids was followed with time and a net dephosphorylation was seen when the incubation period was longer than 30 s. Corticotropin-(1–24)-tetracosapeptide was added at $t = 30$ s, and no effect was found on the dephosphorylation of either lipids or proteins (Fig. 4). After 15 min of dephosphorylation a new portion of
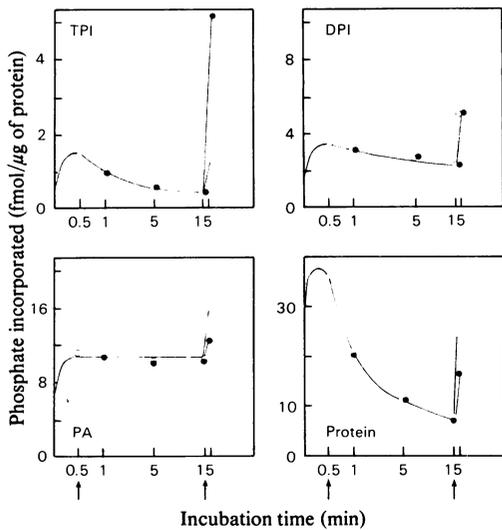
Fig. 4. *The effect of corticotropin-(1–24)-tetracosapeptide on kinase and phosphatase activity*
Incubations were performed as described in the legend to Fig. 2, but corticotropin was added at $t = 30$ s (first arrow). At $t = 15$ min a new portion of $[\gamma^{-32}P]ATP$ (final concentration $7.5\mu M$) was added (second arrow), and incubations were terminated after a further 10 s. Results are expressed as fmol of phosphate incorporated/μg of protein incubated. Abbreviations used: TPI, phosphatidylinositol bisphosphate; DPI, phosphatidylinositol phosphate; PA, phosphatidic acid. O, Control; ●, corticotropin treatment.
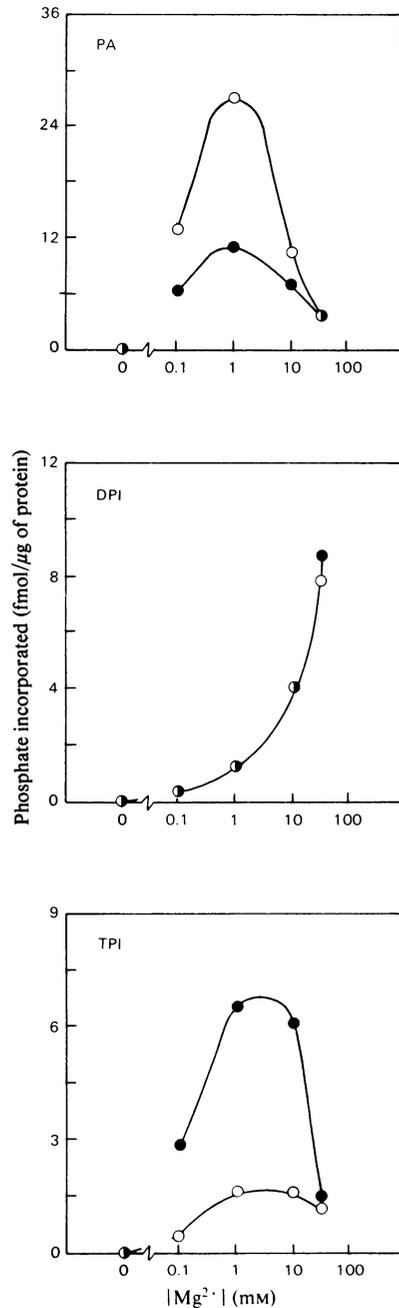
Fig. 5. *The influence of Mg^{2+} on lipid phosphorylation*
A lysed P_2 fraction was incubated as described in the legend to Fig. 2, for 10 s with $[\gamma^{-32}P]ATP$ in the absence (O) and presence (●) of $100\mu M$-corticotropin-(1–24)-tetracosapeptide. The Mg^{2+} concentration in the medium was varied from 0 mM in the presence of EDTA (1 mM), to 30 mM. The medium did not contain Ca^{2+}. Results are expressed as fmol of phosphate incorporated/μg of protein incubated. Abbreviations used: PA, phosphatidic acid; DPI, phosphatidylinositol phosphate; TPI, phosphatidylinositol bisphosphate.
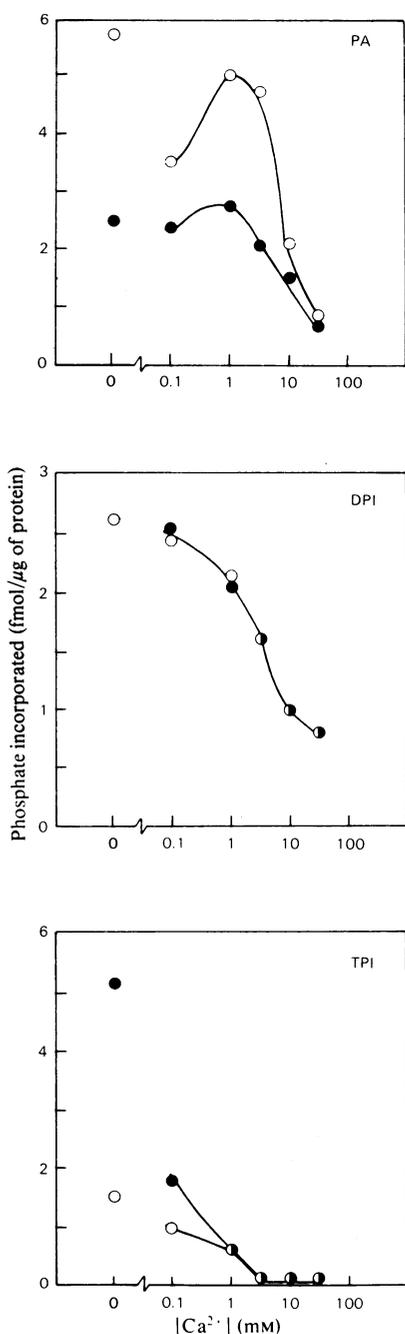
Fig. 6. *The influence of Ca^{2+} on lipid phosphorylation*
A lysed P_2 fraction was incubated, as described in the legend to Fig. 2, for 10 s with [γ-^{32}P]ATP in the absence (O) and presence (●) of 100 μM-corticotropin-(1–24)-tetracosapeptide. The Mg^{2+} concentration was 10 mM, and the Ca^{2+} concentration was varied from 0 mM, in the presence of 1 mM-EGTA, to 30 mM. Results are expressed as fmol of phosphate incorporated/μg of protein. Abbreviations used: PA, phosphatidic acid; DPI, phosphatidylinositol phosphate; TPI, phosphatidylinositol bisphosphate.

[γ-^{32}P]ATP was added (final concentration 7.5 μM) and the phosphorylation reaction was terminated after 10 s. Fig. 4 illustrates that the samples that had been treated with corticotropin showed an enhanced incorporation of phosphate into phosphatidylinositol bisphosphate and an inhibited labelling of phosphatidic acid and protein. These results are taken to indicate that corticotropin-(1–24)-tetracosapeptide acts on the lipid and protein kinases and not on the corresponding phosphatases. Morever, it is apparent that corticotropin still exerts its effects 15 min after its addition.

The influence of Mg^{2+} and Ca^{2+} on basal and hormone-influenced lipid phosphorylation

It is known that phosphatidylinositol bisphosphate formation and hydrolysis depend on Mg^{2+} and Ca^{2+} concentrations (Hawthorne & Kai, 1970; Griffin & Hawthorne, 1978). Therefore the influence of Mg^{2+} and Ca^{2+} on lipid phosphorylation was tested more fully. The membrane/cytosol fraction was prepared as described in the Materials and methods section, and incubated in sodium acetate buffer, containing increasing amounts of magnesium acetate without added Ca^{2+}. Fig. 5 shows that the enzymic reactions yielding phosphatidic acid, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate have a different requirement for Mg^{2+}; the production of phosphatidic acid and phosphatidylinositol bisphosphate was maximal at 1–10 mM, whereas the maximal phosphatidylinositol phosphate production was at 30 mM-Mg^{2+}. Corticotropin did not affect phosphatidylinositol phosphate labelling at any Mg^{2+} concentration tested, and the greatest corticotropin effects on phosphatidic acid and phosphatidylinositol bisphosphate labelling were obtained at 1–10 mM. The influence of Ca^{2+} was tested in sodium acetate buffer containing 10 mM-Mg^{2+}. The phosphorylation of phosphatidic acid, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate decreased with increasing Ca^{2+} concentration (Fig. 6). The effect of corticotropin on phosphatidylinositol bisphosphate production was especially sensitive to the presence of Ca^{2+} (450%, 190%, 100% at 0, 0.1 and 1 mM respectively). Similar results were obtained when the Ca^{2+} concentration was varied in medium containing 1.2 mM-Mg^{2+} (results not shown).

Discussion

The present paper describes the effects of corticotropin-(1–24)-tetracosapeptide on the protein phosphorylation and phospholipid phosphorylation in a lysed P_2 fraction from rat brain that contained both membranes and soluble enzymes. The limbic system was chosen as the starting material for the

tissue fractionation, as many behavioural and neurophysiological studies have shown that these brain structures are particularly sensitive to corticotropin-(1–24)-tetracosapeptide and congeners (Gispen et al., 1977). After incubation of the membrane/cytosol fraction with $[\gamma^{-32}P]ATP$ a rapid labelling of proteins and lipids was obtained (Figs. 1 and 2).

After longer time periods only net dephosphorylation could be measured. In the present conditions corticotropin had a rather general inhibitory effect on protein phosphorylation (Fig. 1), as was also found in Triton-solubilized material from synaptosomal plasma membranes (Zwiers et al., 1979), whereas in intact membranes few proteins (including the B-50 protein) were affected by the peptide (Zwiers et al., 1976, 1978). As the effect of corticotropin seems to be restricted to the protein kinases and thus does not include the corresponding phosphoprotein phosphatases (Fig. 4 of the present study; Zwiers et al., 1978), it seems likely that the peptide inhibits a protein kinase that is able to phosphorylate other proteins in a non-specific manner, when given access to them. The incorporation of phosphate into phosphatidylinositol phosphate and phosphatidylinositol bisphosphate followed the same time course as the protein phosphorylation, but phosphatidic acid labelling proceeded differently in that no breakdown of this phospholipid occurred even after long incubation periods (30 min). The inhibitory effect of corticotropin-(1–24)-tetracosapeptide on protein labelling was paralleled by a similar inhibition of phosphatidic acid labelling and by a stimulation of phosphatidylinositol bisphosphate formation (Fig. 3). It appeared that the peptide affected the lipid kinases but not the respective phosphatases (Fig. 4). Thus corticotropin inhibits the protein kinase(s) and 1,2-diacylglycerol kinase, and stimulates the phosphatidylinositol phosphate kinase. As effects of the peptide on both protein phosphorylation (Zwiers et al., 1976, 1978; Podesta et al., 1979) and polyphosphoinositide metabolism (Jolles et al., 1979; Farese et al., 1979) have been obtained, the results of the present study suggest that these processes may be related. Evidence in support of this notation was recently obtained (Jolles et al., 1980a,b); the corticotropin-sensitive protein kinase–B-50 protein complex was isolated in soluble form from rat brain synaptosomal plasma membranes. When this protein kinase complex was incubated in the presence of exogenously added phosphatidylinositol phosphate and ATP, phosphatidylinositol phosphate kinase activity was observed. Corticotropin dose-dependently inhibited the B-50 protein phosphorylation and stimulated the formation of phosphatidylinositol bisphosphate, as was also found in the present study. An inverse relation seems to exist between protein phos-

phorylation and lipid phosphorylation, as the state of phosphorylation of the B-50 protein was a determinant factor for phosphatidylinositol bisphosphate production (Jolles et al., 1980a,b). It may be that the B-50 protein is a subunit of the phosphatidylinositol phosphate kinase, the phosphorylation of which inhibits its activity. Stimulation of phosphatidylinositol bisphosphate formation by corticotropin can then be explained by inhibition of associated protein kinase activity. In favour of such a mechanism is the abundant evidence that enzyme activity can be regulated by phosphorylation (see Krebs & Beavo, 1979, for a review). Indeed autophosphorylation of the regulatory subunit was found to modulate protein kinase activity (Walter et al., 1977). Furthermore, a direct relation between protein phosphorylation and phosphatidic acid metabolism has more recently been found by others. Takai et al. (1979a) demonstrated that the activity of a Ca^{2+}-dependent protein kinase is linked to phosphatidylinositol, and especially to 1,2-diacylglycerol (Takai et al., 1979b).

It has been shown that Ca^{2+} has profound effects on phosphorylation (Greengard, 1978), phosphatidylinositol response (Michell, 1975) and polyphosphoinositide metabolism (Griffin & Hawthorne, 1978; Abdel-Latif et al., 1979). Indeed in the present study this ion was found to have a marked effect on both basal and corticotropin-affected lipid phosphorylation. Increasing the concentration of this ion above 1–3 mM decreased the production of labelled phosphatidic acid and phosphatidylinositol phosphate and completely abolished the formation of phosphatidylinositol bisphosphate. The stimulatory effect of corticotropin on phosphatidylinositol bisphosphate formation was very sensitive to the presence of Ca^{2+}; in medium containing 1 mM of this ion, phosphatidylinositol bisphosphate was slightly decreased but the effect of the peptide had disappeared altogether. Similarly, Griffin & Hawthorne (1978) showed that the metabolism of phosphatidylinositol bisphosphate is very sensitive to the presence of Ca^{2+}. In addition, the presence of Mg^{2+} was essential for kinase activity, and the production of phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate was maximal at 1 mM, 30 mM and 1–10 mM respectively. For 1,2-diacylglycerol kinase (Kanoh & Åkesson, 1978) and phosphatidylinositol kinase (Kai et al., 1966) a similar Mg^{2+} dependency has been described, but the optimal phosphatidylinositol phosphate kinase activity in the present study was lower than that reported by Kai et al. (1968) and by Shaikh & Palmer (1977).

These data place previously reported results on polyphosphoinositide metabolism in a synaptosomal fraction in another perspective (Jolles et al., 1979). The synaptosomal phospholipids were pre-

labelled with [^{32}P]P$_i$ and incubated with cortico-tropin-(1–24)-tetracosapeptide. The peptide appeared to have an inhibitory effect on the labelling of phosphatidic acid, phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in medium containing 1.2 mM-Mg^{2+} and 2.7 mM-Ca^{2+}. As discussed above, the high Ca^{2+} concentration may have prevented corticotropin to stimulate the formation of phosphatidylinositol bisphosphate. It is probable that the decreased labelling of phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate is a secondary consequence of the inhibited phosphatidic acid production as phosphatidic acid is the precursor of phosphatidylinositol and thus also of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. The finding that hormone effects on the inosities were seen after 10 but not after 5 min (Jolles et al., 1979) supports this notion.

The conflicting evidence from many previous studies on the effects of agonists on polyphospho-inositide metabolism may be due to the long incubation period employed. In these studies usually a P$_2$ fraction (crude synaptosomes) or purified synaptosomes were labelled with [^{32}P]P$_i$ in iso-osmotic medium for incubation periods ranging from 10 to 60 min. The incubation conditions employed in the present study (a lysed P$_2$ fraction containing membranes of synaptosomal origin and cytoplasmic enzymes, incubated for 10 s with [γ-^{32}P]-ATP in hypo-osmotic medium) may prove advantageous in view of the rapid metabolism of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. The enzymes involved in their metabolism are among the fastest acting known (Hawthorne & Kai, 1970). Accordingly, more than 50% of the monoesterified phosphate groups of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate are exchanged within 2 min (J. Jolles, unpublished work), making it difficult to interpret changes in polyphosphoinositide metabolism 10–30 min after agonist addition.

It remains to be established whether agonist-induced changes in polyphosphoinositide metabolism are related to the phosphatidylinositol response (Michell, 1975), but the fact that corticotropin simultaneously stimulates phosphatidylinositol bisphosphate production and inhibits the formation of phosphatidic acid (Fig. 3) suggests that this may indeed be the case. It is possible that the production of 1,2-diacylglycerol and hence of phosphatidic acid is changed by the phosphorylation of phosphatidylinositol to phosphatidylinositol phosphate and phosphatidylinositol bisphosphate, but more research is needed to substantiate this notion. For instance, it is not clear whether the phosphatidylinositol-specific phosphodiesterase (Irvine &

Dawson, 1979) can also cleave phosphatidylinositol phosphate and phosphatidylinositol bisphosphate.

The considerations on the important role of Ca^{2+} on protein phosphorylation, phosphatidylinositol response and phosphatidylinositol bisphosphate metabolism point to the possible physiological mechanisms involved. Phosphatidylinositol phosphate and especially phosphatidylinositol bisphosphate are very potent chelators of Ca^{2+} and Mg^{2+} and interact strongly with proteins (Dawson, 1965; Palmer & Dawson, 1969). Owing to the negative charges on their polar head group these lipids may carry the negative potential of the membrane (Torda, 1974). Thus the breakdown of the phosphoinositides or a change in the relative amounts of phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in the membrane may profoundly affect the conformation of membrane proteins, and change the potential of the membrane and its content of Ca^{2+} and Mg^{2+}. The influx of Ca^{2+} that frequently accompanies the phosphatidylinositol response (Michell, 1975, 1979) may be regulated by such mechanisms. In view of the presently reported effects of corticotropin on phosphoinositide metabolism it is of interest that this hormone has been shown to induce changes in membrane potential both in its adrenal target cell (Matthews & Saffran, 1973) and in the central nervous system (Urban, 1977), whereas corticotropin-(4–10)-heptapeptide hyperpolarizes the postsynaptic membrane from the ganglion superior cervicale (Wouters & van de Bercken, 1979).

In conclusion, our demonstration that protein phosphorylation and phosphatidylinositol bisphosphate formation are related in a purified enzyme preparation (Jolles et al., 1980a,b) and the present findings that corticotropin affects protein phosphorylation and phosphatidylinositol bisphosphate/phosphatidic acid formation in a membrane/cytosol fraction from brain, may point to the physiological mechanisms involved in the action of this behaviourally active neuropeptide.

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