ACTH and Synaptic Membrane Phosphorylation in Rat Brain

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ACTH AND BRAIN FUNCTION

In the last decades it has become increasingly clear that adrenocorticotropic hormone (ACTH) influences the behavior of both animal and man by a direct action on the nervous system. De Wied and his co-workers demonstrated that the N-terminus of the polypeptide hormone is responsible for the behavioral activity. Fragments of that region possess full behavioral and neurophysiological activity but lack the classical endocrine effects of ACTH. The term "neuropeptides" was chosen to designate those peptides that act directly on the nervous system. In an attempt to formulate a unifying theory for the behavioral action of ACTH, Bohus and De Wied suggested that the peptide influences motivation or attention, resulting in improved performance in a variety of behavioral tests (Bohus and De Wied, 1980).

In view of the possible clinical relevance of ACTH and congeners, more knowledge is required on the neurochemical mechanism of action. It is possible that the peptide is a neurotransmitter in a peptidergic synapse. Firing of the presynaptic nerve cell would result in the release of ACTH in the synaptic cleft. However, at present there is insufficient experimental support for the hypothesis that ACTH acts as a classical neurotransmitter in the CNS. Currently, the influence of ACTH-like neuropeptides on brain neuronal activity is best formulated as neurohormonal or neuromodulatory (Gispen et al., 1979). When ACTH acts as a neurohormone it reaches its target in the brain via the liquor or blood circulation. An example of such a mode of action may be trophic influence of the peptide on nervous tissue. This influence is evidenced by an enhanced protein synthesis (Dunn and Schotman, 1981) and cell repair (Bijlsma et al., 1981). The neuromodulatory action of the peptide involves its release in the vicinity of the synaptic cleft, and the modulation of synaptic efficacy.

Recent research into the mechanism of action of ACTH-like neuropeptides has therefore focussed on the molecular events that are known to be crucial for membrane function.

ACTH AND THE PHOSPHORYLATION OF SYNAPTIC MEMBRANE PROTEINS

Fragments of the synaptic plasma membrane are the richest known source of protein phosphorylating systems in mammalian tissues. This suggests that they may be involved in synaptic transmission. The hypothesis that a change in the state of phosphorylation of membrane protein may govern the ion permeability of the neural membrane, and thus may play a key role in determining the functional activity of the neuron has stimulated a great amount of

research. Originally, attention was focussed on the cyclic AMP (cAMP)-dependent phosphorylation. The cAMP-dependent phosphorylating system in synaptic membranes have been investigated in several laboratories and are discussed in great detail in several other chapters in this volume.

Other protein phosphorylating systems have been described in brain which are sensitive to Ca^{2+} but not to cAMP (Rodnight, 1980). Synaptic plasma membranes contain the so-called B-50 protein (Fig. 1), which is an acceptor protein that is phosphorylated in a Ca^{2+}-sensitive and cAMP-insensitive way. The phosphorylation of this protein is sensitive to ACTH and some of its behaviorally active fragments. Extensive studies have been performed on the extraction of this protein and of its protein kinase from the synaptic membrane and its purification and characterization.

Treatment of SPM with 0.5% Triton X-100 in 75 mM KCl solubilized 15% of the total B-50 protein kinase activity and preserved the sensitivity of the enzyme to $ACTH_{1-24}$. Column chromatography of the solubilized material over DEAE-cellulose pointed to the presence of multiple protein kinase activities, one of which was the ACTH-sensitive B-50 protein kinase (Zwiers et al., 1979). The column fractions containing the B-50 protein kinase were subjected to ammonium sulfate precipitation and a protein fraction (57–82% ammonium sulfate)

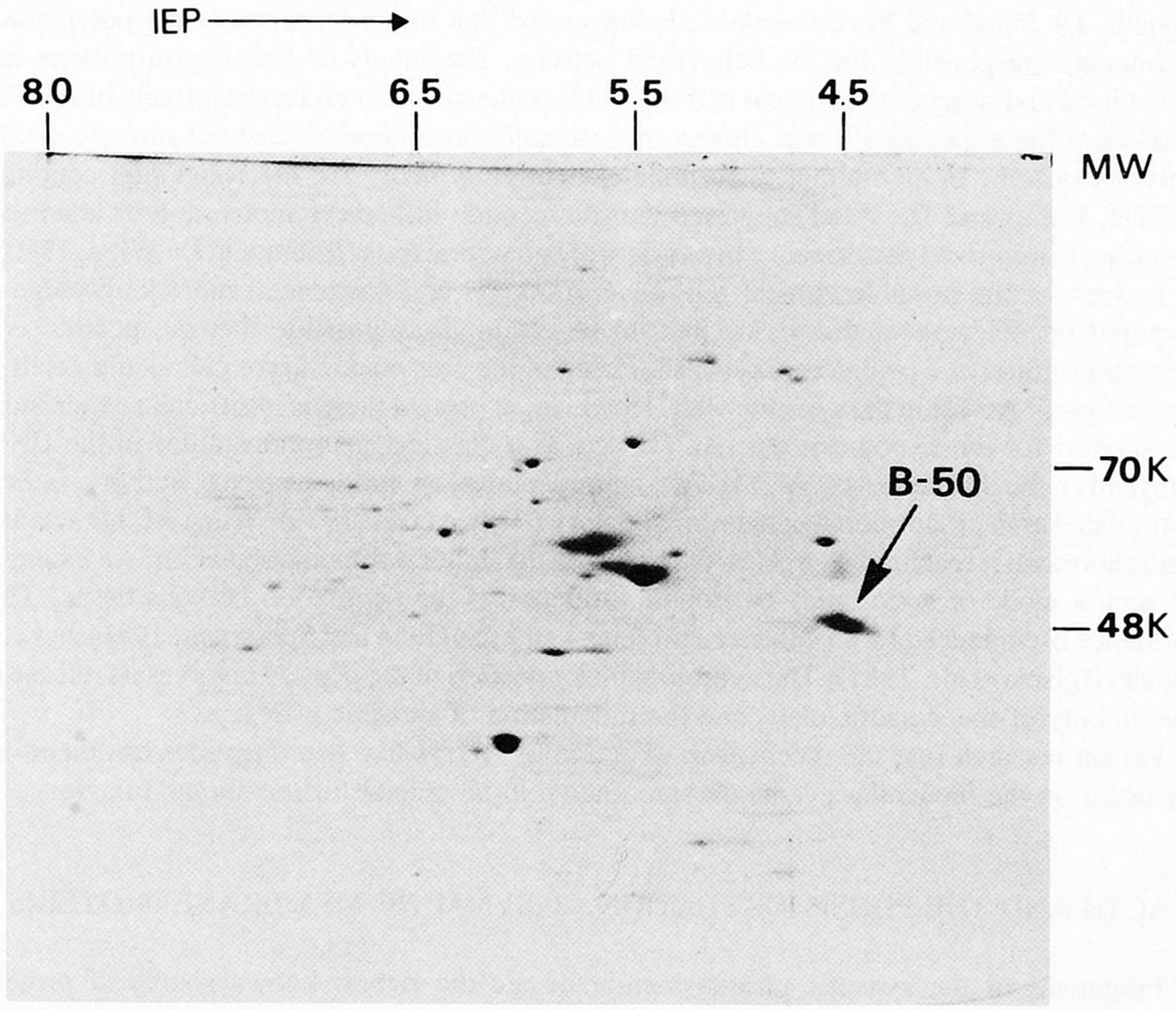

Fig. 1. Two-dimensional separation of synaptosomal plasma membrane proteins from rat brain cortex. Separation in the first dimension was performed with isoelectric focusing (pH 3–9) in polyacrylamide. The second dimension was a separation based on molecular weight in a SDS–polyacrylamide slab gel. B-50 appeared to be one of the most acidic proteins of SPM (IEP 4.5).

enriched in endogenous B-50 phosphorylation activity was obtained. The time course of the endogenous phosphorylation of B-50 in this fraction showed a linear incorporation with time for at least 10 min and a maximal incorporation of 0.65 mole P/mole B-50 was reached after 60 min. The inhibition of $ACTH_{1-24}$ of the protein kinase was dose-dependent; the half-maximal effective concentration 5×10^{-6} M, being 10–20 times lower as compared to intact SPM. The B-50 protein kinase required both magnesium and calcium for optimal activity (Gispen et al., 1979; Zwiers et al., 1980a). After two-dimensional electrophoresis on polyacrylamide slab gels, the B-50 protein kinase and the B-50 protein could be further identified, purified and characterized. The isoelectric point (IEP) of the kinase in 5.5 and the apparent molecular weight 70 K, whereas the IEP of the substrate protein B-50 is 4.5 and the apparent molecular weight 48 K. Amino acid analysis on μg quantities of purified kinase and B-50 protein revealed basic/acid amino acid ratios in agreement with the respective IEPs (Zwiers et al., 1980a).

Although the B-50 protein band as separated on SDS–polyacrylamide gels consists of more than one protein as was seen after two-dimensional separation, the major component is the phosphoprotein B-50 (Fig. 1) (Zwiers et al., 1980a). Therefore, it was decided to try to raise antibodies to the protein B-50 using B-50 material isolated from rat brain SPM and separated in one dimension on SDS–polyacrylamide slab gels (Oestreicher et al., 1979, 1981). The presence of specific antibodies to B-50 in rabbit antiserum was demonstrated by an immunoperoxide staining method. By use of this peroxidase–antiperoxidase (PAP) method, the immunohistochemical localization of B-50 was studied in sections of rat brain cerebellum and hippocampus. In agreement with the presumed synaptic origin of B-50, the antiserum reacted with tissue components in brain regions rich in synaptic contacts. In contrast, white matter and cell perikarya were virtually without immuno staining. The staining pattern was markedly similar to that found by others using synaptic antigens (Matus et al., 1976) and suggests that at the cellular level there is a restricted localization of the B-50 protein (i.e., synaptic region) but at the brain regional level the protein seems ubiquitous. In order to obtain insight in the question of whether the B-50 protein was specific for nervous tissue, we monitored the endogenous phosphorylation of proteins in three subcellular fractions ($1000 \times g$ and $150\,000 \times g$ supernatants; $150\,000 \times g$ pellet) obtained from seven different organs of the rat (brain, liver, lung, skeletal muscle, heart, spleen and adrenal cortex; Kristjansson et al., 1982). In agreement with Carstens and Weller (1979) the particulate fractions of brain displayed the highest phosphorylating activity. Furthermore, a relative enrichment of endogenous B-50 protein phosphorylation was observed when purified SPM was used. This observation is in line with previous studies indicating that in brain tissue the B-50 protein was mainly found in regions rich in synaptic projections (Oestreicher et al., 1981) and in the synaptic region predominantly in the presynaptic membranes (Sörensen et al., 1981). Endogenous phosphorylation of a 48 K protein, which could be inhibited by $ACTH_{1-24}$ was only observed in the $1000 \times g$ supernatant and the $150\,000 \times g$ particulate fraction from brain. The absence of a phosphorlated protein band with M_r 48 K (B-50) in the other organs studied per se is not convincing evidence that indeed the phosphate acceptor protein was not present. For, in the absence of the corresponding protein kinase, no phosphorylation of B-50 would be measured despite the fact that B-50 itself was present in the fractions studied. Therefore, we focussed on two-dimensional separation and immunochemical identification of the B-50 protein itself. A two-dimensional separation technique consisting of IEF followed by SDS–PAGE rendered data suggesting that B-50 is only present in those fractions where endogenous phosphorylation of this protein was observed, e.g., subcellular fractions from brain containing particulate material ($1000 \times g$ supernatant, $150\,000 \times g$ pellet; Kristjansson et al., 1982).

To further substantiate this tentative conclusion that B-50 is exclusively localized in brain particulate material, gel sections containing SDS–PAGE separated proteins obtained from the various subcellular fractions were incubated with the antiserum to B-50. In agreement with our two-dimensional PAGE results, the immunochemical procedure did not demonstrate the presence of B-50 in cell fractions of organs other than the particulate fraction of brain tissue (Kristjansson et al., 1982).

We studied the regional distribution of endogenous B-50 phosphorylating activity in brain tissue under conditions that measured the initial reaction velocity (Wiegant et al., 1978). The data suggest that there are indeed differences in endogenous phosphorylation reaction velocity in different brain regions. The highest value was found in SPM from septal origin, the lowest in SPM from medulla spinalis. It is of interest that in SPM of septal origin $ACTH_{1-24}$ is more effective in inhibiting B-50 protein kinase as compared to SPM from total brain (Fig. 2). The

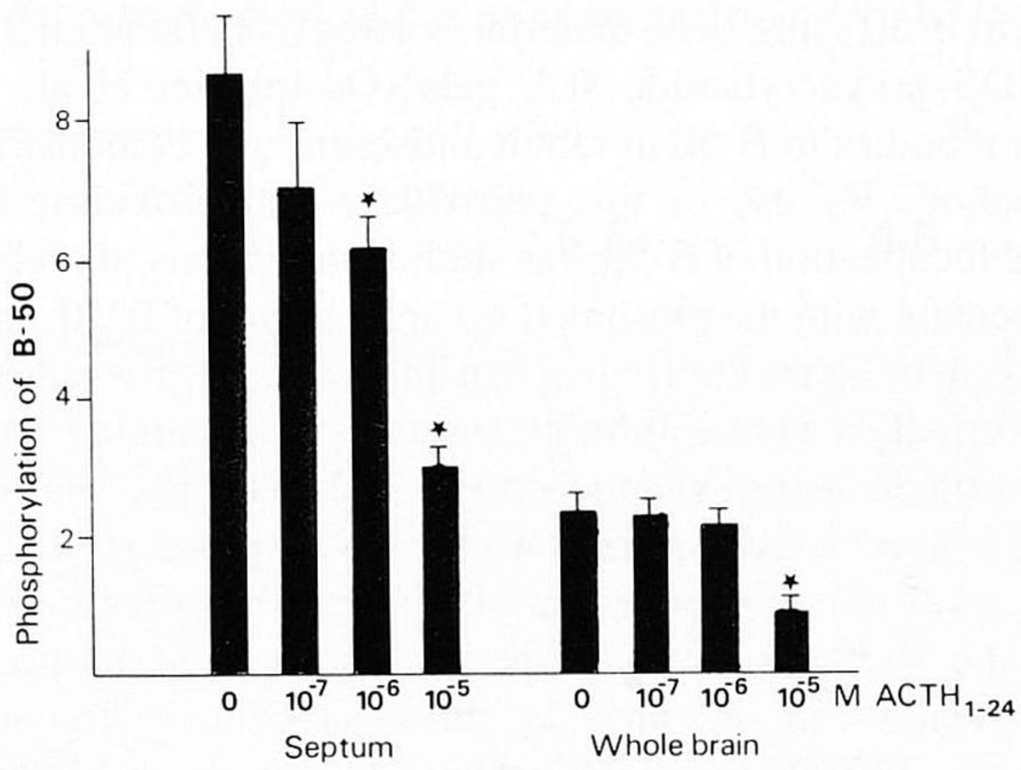

Fig. 2. Effect of $ACTH_{1-24}$ on phosphorylation of B-50 in SPM obtained from septum or from whole rat brain. Aliquots of 20 μg of SPM protein were phosphorylated in the presence of various concentrations of $ACTH_{1-24}$. Proteins were separated bt SDS–PAGE and the amount of ^{32}P incorporated into B-50 was determined by liquid scintillation counting. Incorporation is expressed as fmoles phosphate incorporated into B-50 per μg total SPM protein ($\pm$ S.E.M., $n = 4$). * Significantly different from control values.

septal area has previously been reported to be essential for the expression of some of the behavioral effects of ACTH-like peptides (Van Wimersma Greidanus et al., 1975). Also other studies point to a special relationship between ACTH and the septum: stereospecific uptake mechanism (Verhoef et al., 1977a,b); specific transport of pituitary ACTH into the septal complex (Mezey et al., 1978), ACTH containing nerve fiber projections (Krieger et al., 1980) and ACTH-induced increase in septal cAMP in vivo (Wiegant et al., 1979).

Thus, it may well be that the modulation of the degree of membrane phosphoprotein underlies some of the behavioral activities of ACTH. The specificity of the ACTH/B-50 protein kinase interaction being provided for by local delivery of peptidergic synapses in the brain.

SPECIFIC PROTEOLYTIC BREAKDOWN OF PROTEIN B-50

In the procedure to isolate and purifiy the ACTH-sensitive membrane B-50 substrate protein/protein kinase complex, dialysis of the protein fraction was one of the tools used. Davis and Ehrlich (1979) reported that after dialysis of SPM a general enhancement of endogenous protein phosphorylation could be observed. They suggested the presence of a dialysable phosphorylation inhibiting factor. Also, in our hands dialysis of the ASP_{57-82} protein fraction led to a marked enhancement of — in this case — only the phosphorylation of the B-50 protein. As the effect of prior dialysis was evident at 3 different purification stages we could rule out that the enhancement was caused by the removal of certain ions such as NaCl or ammonium sulfate. Next, the dialysate of ASP_{57-82} proteins obtained from 150 g rat brain tissue was lyophilized, resuspended in water and subjected to HPLC in a system developed to separate small molecular weight peptides (MW < 4000; Loeber et al., 1979). When tested in the endogenous phosphorylation assay only one peak displayed the inhibiting activity. Based on its sensitivity to pronase or acid hydrolysis and the nature of the separation system used, the factor was thought to be a small peptide. Indeed, amino acid analysis of the inhibiting fraction revealed the presence of a basic peptide of approximately 15 amino acids with a molecular weight in the order of 1650 (phosphorylation inhibiting peptide, PIP; Zwiers et al., 1980b). Furthermore, we reported preliminary evidence that during dialysis PIP occurred in the dialysate as the result of a specific proteolytic cleavage of the B-50 protein. In collaboration with the laboratory of Dr. Henry Mahler, we substantiated this working hypothesis that B-50 is subject to proteolysis yielding a small fragment PIP and a larger breakdown product B-60 (MW 46 K) (Zwiers et al., 1982). The ammonium sulfate precipitate containing the highest endogenous B-50 phosphorylating activity, also contains protease activity. When incubated in the absence of calcium a time-dependent decrease of the protein B-50 is observed with a concomi-

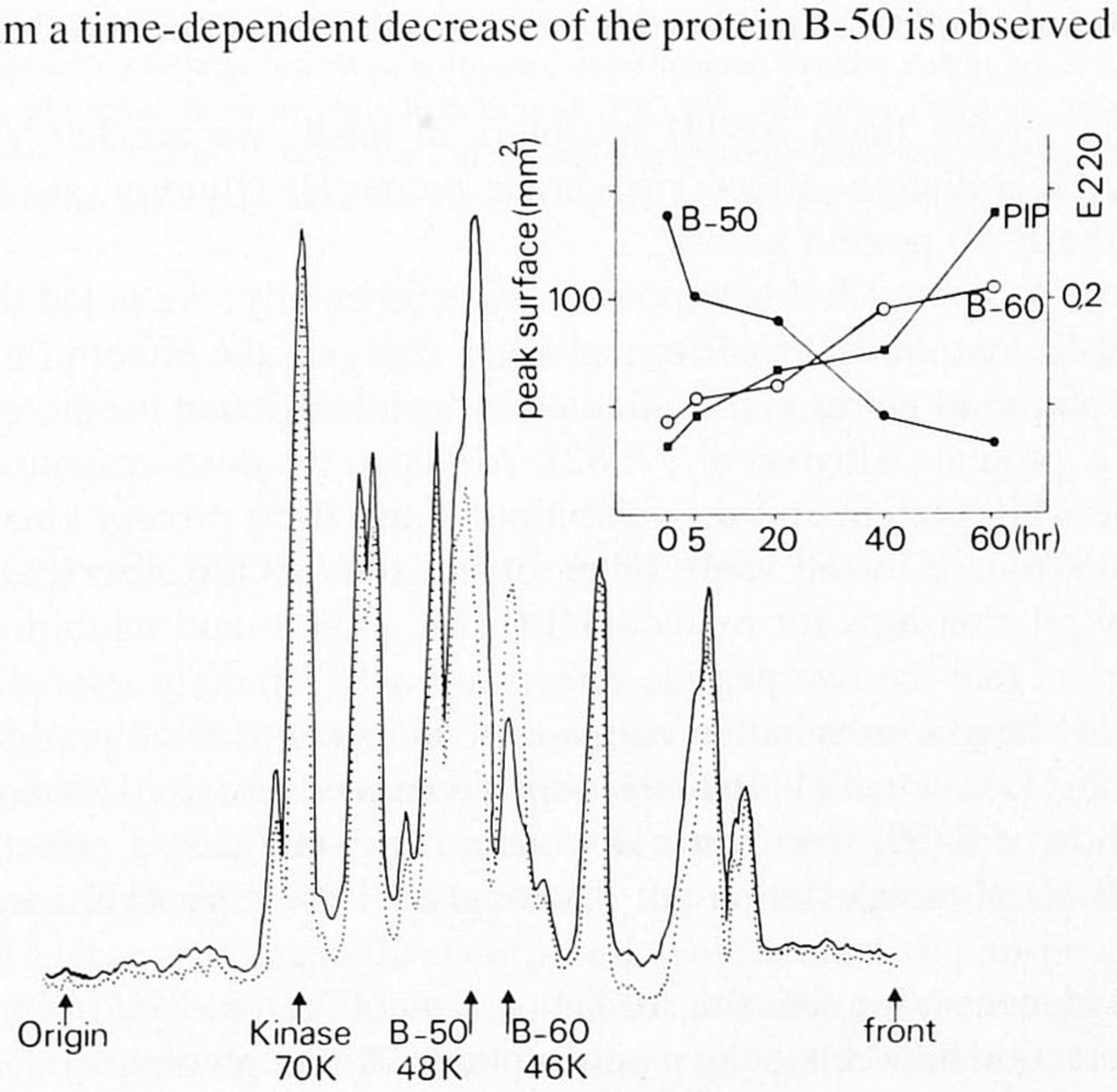

Fig. 3. Densitometric scans of Fast Green-stained protein profiles of ASP_{57-82} proteins after SDS–polyacrylamide slab gel electrophoresis before (————) and after (. . . . .) dialysis for 20 h at 4° C. Insert: Surface area of the peaks of B-50 and B-60 as a function of the dialysis time. The amount of PIP in the dialysate is indicated by the extinction at 220 nm of the PIP-containing fraction obtained by high pressure liquid chromatography.

tant appearance of phosphoprotein B-60 and PIP (Fig. 3). Addition of calcium and/or calmodulin enhances the protease activity whereas the substrate specificity is lost. Results of both isoelectric focussing and peptide mapping indicate that B-50 and B-60 are related proteins (Zwiers et al., 1982).

If this proteolysis is also taking place in vivo, this mechanism opens the possibility for specific proteolysis of B-50 in synaptic membranes, releasing a peptide (PIP) inhibiting the B-50 protein kinase and thus modulating the degree of B-50 phosphorylation.

HOW DOES ACTH INHIBIT THE PHOSPHORYLATION OF B-50?

As is discussed elsewhere (Aloyo et al., this volume), there is good reason to suppose that the B-50 kinase first isolated by Zwiers et al. (1980a) is identical to the protein kinase C isolated by Nishizuka and co-workers (this volume). The apparent ubiquitous localization of the C kinase, the restricted localization of B-50 and the fact that the B-50 protein kinase co-purified with the B-50 protein, suggest that in brain part of the B-50 kinase forms a complex with its membrane substrate protein B-50. By what mechanisms is this membrane complex affected by ACTH? As reviewed by Witter (1980) there is little evidence so far pointing to classical binding sites as assessed by radioligand assays. In relatively high concentrations ACTH and related peptides may interfere with the specific binding of dihydromorphin and naloxone to opiate receptors in SPM obtained from rat brain (Terenius, 1975; Terenius et al., 1975; Gispen et al., 1976). As preincubation with naloxone did not counteract the effect of ACTH on B-50 kinase (Zwiers et al., 1981) but did so of that of met-enkephalin on the phosphorylation of a 50 K protein in subcellular fractions of hippocampal tissue (Bär et al., 1980), it was concluded that occupation of opiate receptors was not involved in B-50 kinase inhibition.

At present, not having brain ACTH receptors in hand, we are left with the working hypothesis that ACTH directly affects membrane properties (fluidity, see Herskowitz, this volume) and/or the B-50 protein kinase.

In a first attempt to approach this hypothesis experimentally, we noted that incubation of SPM with ACTH-like peptides, as performed when studying the effect of the peptide on the endogenous phosphorylation, results, in addition to the inhibition of the protein kinase, also in a release of a 41 K protein (Aloyo et al., 1982). Although the dose–response relationship for the release of the 41 K protein and the inhibition of the B-50 protein kinase by $ACTH_{1-24}$ (Zwiers et al., 1976) are in the same order of magnitude, the discrepancy between the structure–activity relationships for extraction of 41 K protein and inhibition of B-50 phosphorylation suggests that the two peptide effects are only remotely related. Thus, although removal of the 41 K band results in the reduction of endogenous B-50 phosphorylation, there are sequences of ACTH such as (1–16)-NH_2 which do not release the 41 K band but do inhibit the phosphorylation of B-50. Even more disconcerting is the lack of effect of the sequence (11–24) on the B-50 phosphorylation yet it strongly releases the 41 K band (Aloyo et al., 1982).

Preliminary evidence shows that the $ACTH_{1-24}$ cannot be removed from the 41 K protein indicating an interaction between the two polypeptides. Although we are not certain about the biological significance of the release of 41 K, it is of sufficient interest for further research in view of the lack of success of finding binding of labeled ACTH to brain membranes (Witter, 1980). Thus, although in the $ACTH_{1-24}$ extract the presence of the 41 K protein was observed, at present it would appear that the release of this band is not the sole explanation for the

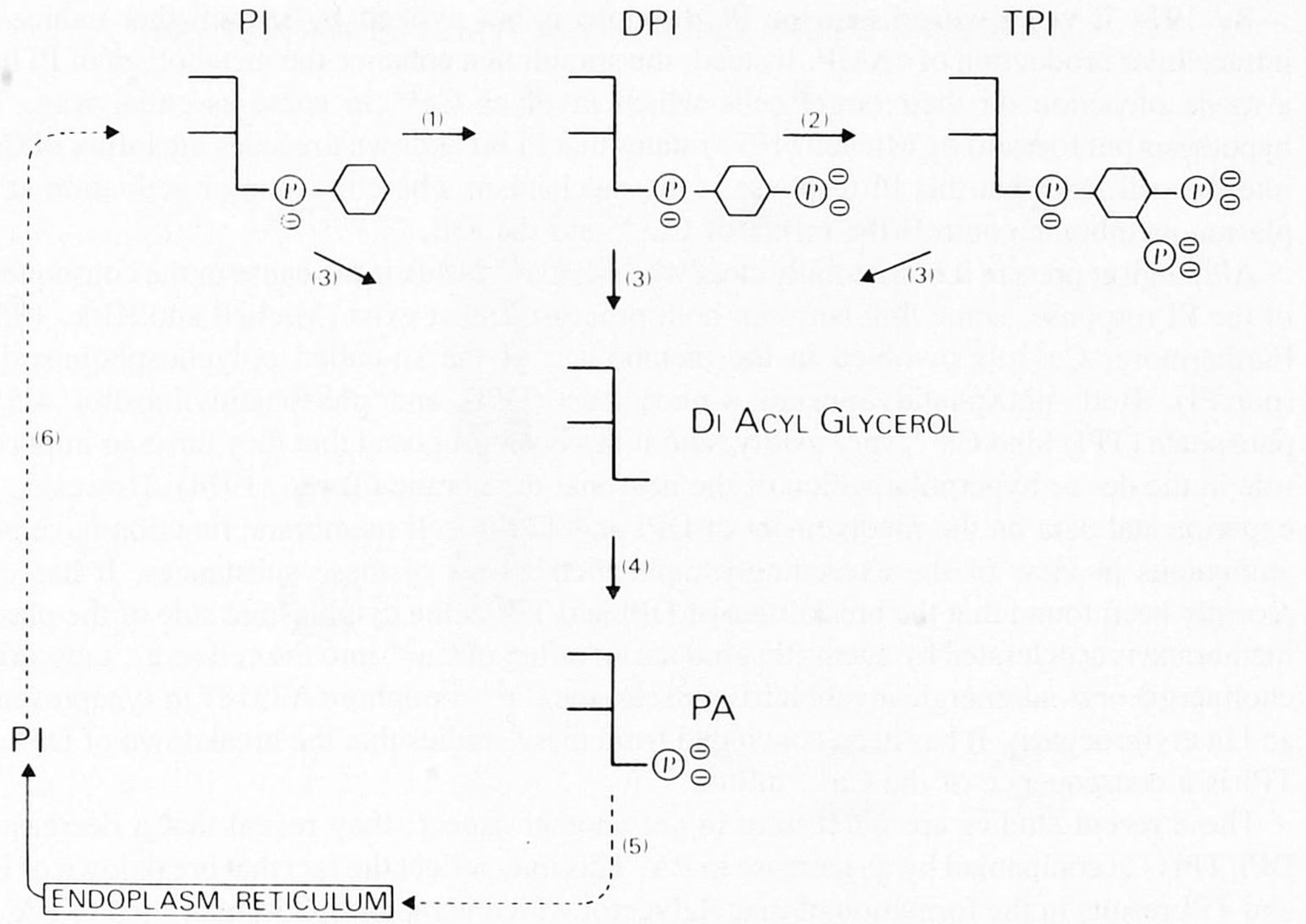

Fig. 4. Metabolism of polyphosphoinositides. Phosphatidyl-*myo*-inositol (PI) and its phosphorylated derivates phosphatidyl-*myo*-inositol 4-phosphate (DPI) and phosphatidyl-*myo*-inositol 4,5-diphosphate (TPI) are rapidly interconverted by lipid kinases and/or phosphomonoesterases (1 and 2). A polyPI-specific phosphodiesterase can hydrolyse the phosphodiester linkage in a phospholipase C-type manner, thereby yielding 1,2-diacylglycerol (3). This substance is rapidly phosphorylated to phosphatidic acid (PA) (4). All these reactions take place at the plasma membrane. Resynthesis of PI from PA takes place in the endoplasmic reticulum, and the transport of the lipid between plasma membrane and intracellular membrane is performed by phospholipid-exchanging proteins.

inhibition of B-50 protein kinase. It is entirely possible that the interaction with the 41 K protein relates to other known chemical effects of ACTH in the brain.

INOSITOL PHOSPHOLIPIDS AND THE METABOLISM OF Ca^{2+} AT THE CELL MEMBRANE

Not only phosphoproteins, but also phospholipids are involved in processes that are crucial for synaptic membrane function. The inositol phospholipids (Fig. 4) are implicated in receptor activation, Ca^{2+} entry through the membrane and membrane (de)polarization. The original interest in phosphatidylinositol (PI) arose from the observation that acetylcholine-treated pancreas showed a large increase in the incorporation of radioactive phosphate into phosphatidic acid (PA) and PI (Michell, 1975). It is now clear that the initial event in the so-called "PI response" is the breakdown of PI. The 1,2-diacylglycerol which is formed, is rapidly phosphorylated to PA. Because this structure is again the precursor of PI, the increase in [^{32}P]PA and [^{32}P]PI manifests a prior breakdown of PI. It has since been found that PI has a special role in the responses of cells to external stimuli such as hormones and neurotransmitters. It appears that the response can be elicited in virtually any type of cell.

By 1974 it was realized that the PI response is not evoked by stimuli that induce the intracellular production of cAMP. Instead, the stimuli that enhance the metabolism of PI have a mode of action on their target cells which involves Ca^{2+} in some essential way. The hypothesis put forward by Michell (1975) states that PI breakdown precedes the influx of Ca^{2+} into the cell, and that this PI response is the mechanism whereby receptor activation at the plasma membrane controls the influx of Ca^{2+} into the cell.

Although at present it is not totally clear whether Ca^{2+} influx is the cause or the consequence of the PI response, some link between both processes must exist (Michell and Kirk, 1981). Furthermore, Ca^{2+} is involved in the metabolism of the so-called polyphosphoinositides (polyPI). Both phosphatidylinositol 4-phosphate (DPI) and phosphatidylinositol 4,5-diphosphate (TPI) bind Ca^{2+} very avidly, and it has been proposed that they have an important role in the de- or hyperpolarization of the neuronal membrane (Torda, 1974). However, the experimental data on the involvement of DPI and TPI in cell membrane function have been ambiguous in view of the exceedingly rapid metabolism of these substances. It has only recently been found that the breakdown of DPI and TPI at the cytoplasmic side of the plasma membrane is accelerated by agents that induce an influx of Ca^{2+} into the cell (e.g., muscarinic cholinergic or α-adrenergic in rabbit iris muscle; the Ca^{2+} ionophore A23187 in synaptosomes and in erythrocytes). It has been concluded from these studies that the breakdown of DPI and TPI is a consequence of the Ca^{2+} influx.

These recent studies are interesting in yet another aspect: they reveal that a decrease in DPI/TPI is accompanied by an increase in PA. This may reflect the fact that breakdown of DPI and TPI results in the formation of diacylglycerol which is rapidly phosphorylated to PA. So the formation and the disappearance of the polyphosphoinositides may be different manifestations of the same phenomenon.

ACTH AND THE METABOLISM OF THE INOSITOL PHOSPHOLIPIDS

There were several reasons why we have started to investigate the possible relationship between protein phosphorylation and polyPI metabolism. In the first place, as described above, both processes were thought to serve a similar physiological function; secondly, because the B-50 protein phosphorylation was critically dependent on the presence of Ca^{2+}, whereas this ion is also critically involved in the interconversion of the polyPI.

The first demonstration that ACTH affects brain polyPI metabolism was in prelabeled synaptosomes (Jolles et al., 1979). However, the experimental approach used prevented conclusions as to the molecular mechanism involved. We have therefore turned to the approach that has been used for many years in the study of protein phosphorylation: ATP was used as the phosphate precursor, and brain subcellular fractions were incubated under hypotonic conditions for very short incubation times (seconds). It was found that indeed the phosphorylation of proteins and lipids could be measured at the same moment (Jolles et al., 1981a). Moreover, very specific effects of ACTH were monitored: the peptide inhibited the formation of phosphoprotein and PA and stimulated the formation of TPI (Fig. 5). (Note this inverse relationship between PA and TPI which is also described in the preceding paragraph.)

With regard to the molecular mechanisms involved it appeared that ACTH acted on the kinases for both protein (Zwiers et al., 1978) and lipid (Jolles et al., 1981a), but not on the respective phosphatases. Furthermore, the ion Ca^{2+} was of critical importance for both B-50 protein phosphorylation and TPI formation. These two processes were inversely related as B-50 phosphorylation was maximal in the presence of Ca^{2+} whereas TPI formation was

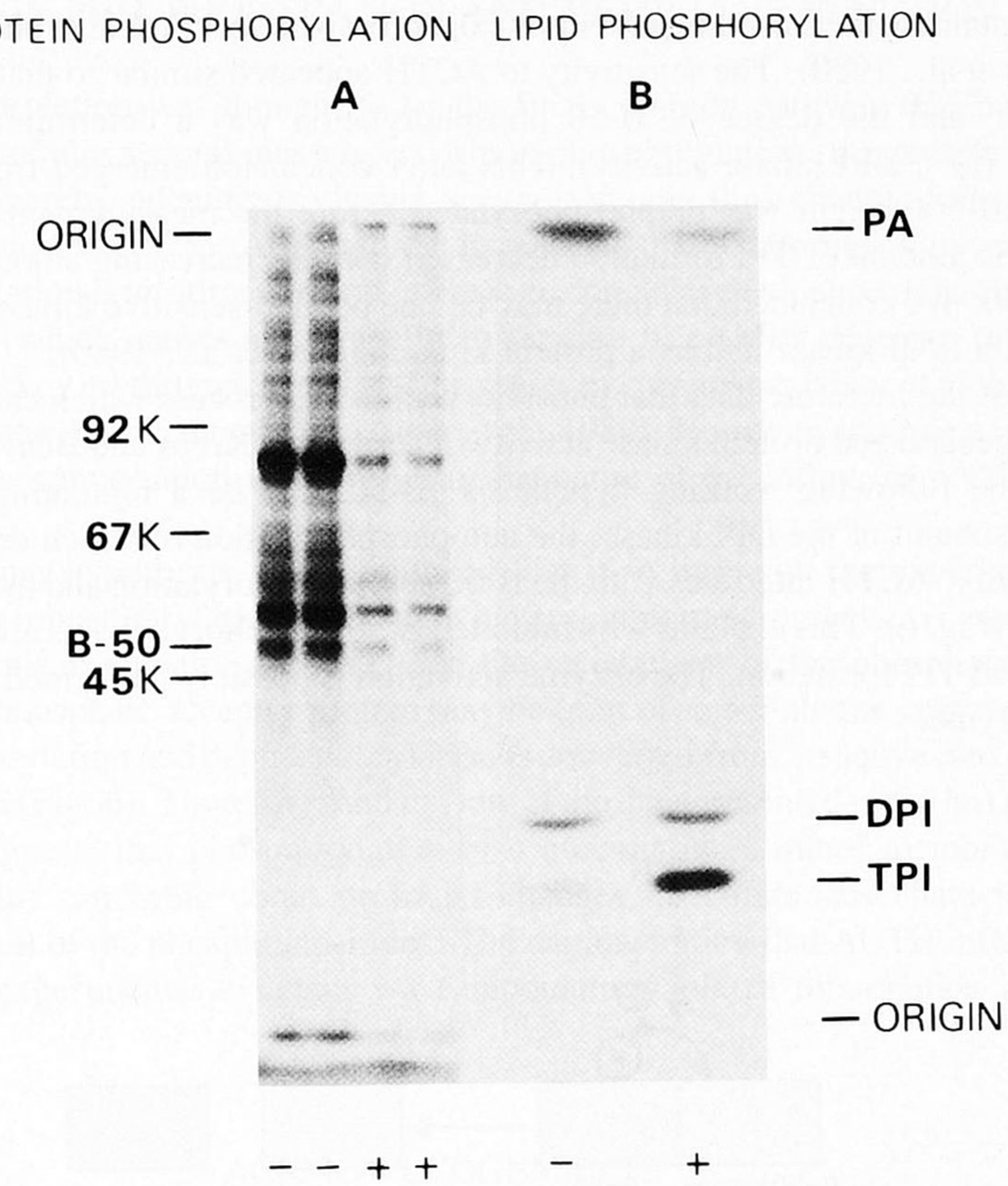

Fig. 5. Autoradiogram (A) after protein separation by polyacrylamide gel electrophoresis, showing the endogenous phosphorylation profile of the lysed P2 fraction obtained from the subcortical part of the rat brain tissue. The position of molecular weight marker proteins and of B-50 protein is indicated. Autoradiogram (B) after lipid extraction and thin layer chromatography. The position of the separated lipids is shown on the right. The absence (−) and presence (+) of 100 μM $ACTH_{1-24}$ in the incubations is indicated.

maximal in the absence of this ion. There were also other correlations between both processes. The dose–response relationship of ACTH was identical for the phosphorylation of the different membrane components. Furthermore, the same regions in the ACTH molecule were found to contain the site(s) that were important for B-50 phosphorylation, for TPI/PA formation and for behavioral activity in vivo (Zwiers et al., 1978; Gispen et al., 1979; Jolles et al., 1981b).

Taken together, the evidence provided in this paragraph suggested that a correlation might exist between B-50 protein phosphorylation and PA/TPI formation, and that in fact such a relationship might underly the mechanism of action of ACTH.

WHAT IS THE RELATIONSHIP BETWEEN PROTEIN AND LIPID PHOSPHORYLATION?

The hypothesis that lipid and protein phosphorylation might have to do with each other was advanced by selectively extracting the protein kinase complex from the membrane and testing its ability to use exogenously added DPI as lipid substrate. Indeed, this purified enzyme

fraction containing the substrate protein (B-50) and its kinase was able to phosphorylate DPI to TPI (Jolles et al., 1980). The sensitivity to ACTH appeared similar to that obtained in intact membranes, and the degree of B-50 phosphorylation was a determining factor for TPI production (i.e., DPI kinase activity). This latter conclusion emerged from experiments in which the B-50 protein was prephosphorylated before the measurement of its DPI kinase activity. The amount of TPI formation decreased with the increasing amount of prephosphorylated B-50. We concluded that there may be one peptide-sensitive kinase complex that can act both as a lipid kinase and as a protein kinase (Jolles et al., 1980).

In view of the literature data that phosphorylation is a process which can regulate enzyme activity in general and protein kinase activity in particular (Krebs and Beavo, 1979), we have proposed the following working hypothesis: B-50 may be a regulating factor to or the regulatory subunit of the DPI kinase, the autophosphorylation of which determines the lipid kinase activity. ACTH interferes with the B-50 autophosphorylation and thereby activates the DPI kinase (Fig. 6). This explains why inhibited B-50 phosphorylation is always accompanied by stimulated TPI formation. The enzyme activation is possibly performed by dissociation of the holoenzyme.

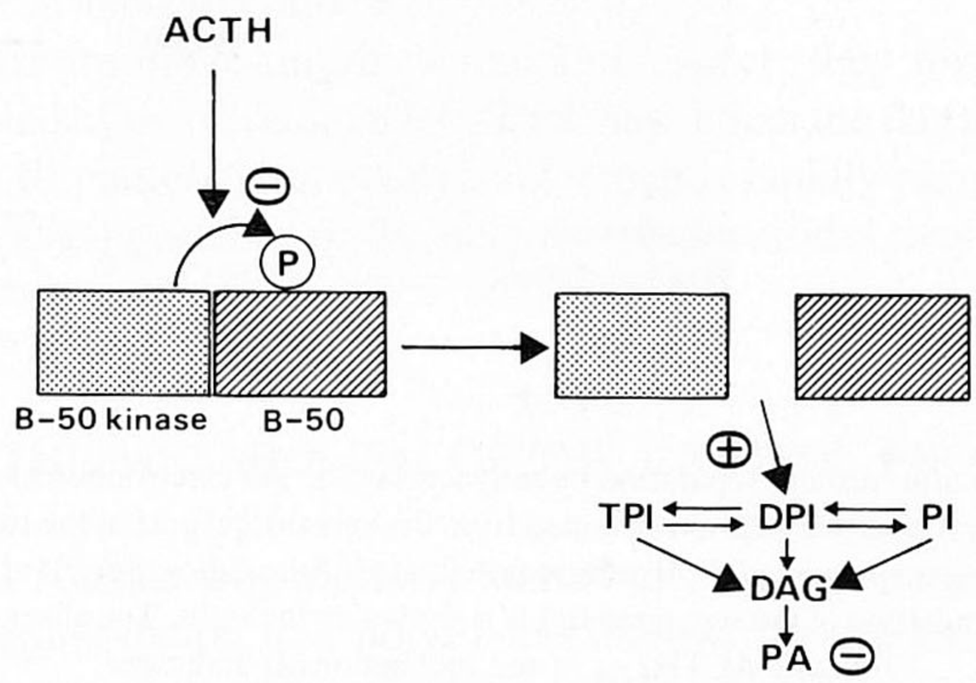

Fig. 6. Relationschip between ACTH-sensitive phosphorylation of protein and of lipid. In this working model B-50 in its phosphorylated form is bound to the B-50 kinase. By inhibiting the phosphorylation of B-50 ACTH induces the dissociation of the complex. The free B-50 acts as a modulator of the system that controls the phosphorylation state of the phosphoinositides.

It is of interest to note that the characteristics of the protein kinase used in our studies resemble those of the protein kinase which has been studied extensively at the Kobe University in Japan. These investigators described a multifunctional protein kinase in brain that was independent of cyclic nucleotides. Using histone as exogenous protein substrate they found that the kinase activity was absolutely dependent on the presence of Ca^{2+} and 1,2-diacylglycerol (Kishimoto et al., 1980). Because diglyceride is the breakdown product of PI, this suggests that this kinase may have another importance function in the PI response (see also Nishizuka and Aloyo et al., both this volume).

Taken together, these and our own studies support the notion that protein phosphorylation and polyphosphoinositide metablism are related. Now what does this tell us about cell membrane functioning and about the response of the membrane to extracellular agents such as neuropeptides?

PROTEIN PHOSPHORYLATION AND MEMBRANE FUNCTION

Protein phosphorylation was thought to be the final common pathway that mediates the effects of all intracellular second messengers. Upon phosphorylation, the protein conformation would alter, thereby opening or closing an ion pore and thus changing the membrane permeability (Greengard, 1978). Although the hypothesis that ion permeability is caused by protein phosphorylation is an attractive one, there is no conclusive evidence. It has been shown that isoproterenol (which causes an intracellular increase in cAMP) increases the influx of sodium ions into turkey erythrocytes, and also increases the phosphorylation of globin, a 230 K intrinsic protein of the plasma membrane (Alper et al., 1980). However, it is by no means clear whether protein phosphorylation is the result or the cause of the influx, or possibly only a correlate.

Therefore, another hypothesis was formulated that is in line with the experimental data discussed: protein phosphorylation, although a very important process, is not the final pathway. It is a link in a chain of events, and the metabolism of the phosphoinositides is another link. The phosphate acceptor protein may be (part of) a membrane enzyme which by reversible phosphorylation and dephosphorylation is converted from an active into an inactive state and vice versa (Fig. 6). Therefore, and in view of the data presented in the last paragraph, this hypothesis suggests that phosphorylation of a quantitatively minor membrane protein (e.g., B-50 protein) can bring about profound changes in membranes characteristics by changing the content of the phosphoinositides. The demonstration that ACTH influences this process provides experimental evidence for a modulatory role of this peptide in ongoing neurotransmission.

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