Synaptic Membrane Phosphorylation: Target for Neurotransmitters and Peptides

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INTRODUCTION

The biochemistry of brain tissue confronts us with an enormous heterogeneity of components. In certain brain areas (corpus striatum, thalamus, medulla oblongata) the various cell types are distributed heterogeneously and disorderly, but in other regions (cerebral cortex, cerebellum, hippocampus) a strict organization determines the arrangement and the connectivity of the neurons (Ramon y Cajal, 1952). Diversity in the functional activity of neural circuits appears to reflect important differences in the rate of metabolic processes (Siegel et al., 1976).

Neurotransmission, the process of communication between neurons, is well documented. Many aspects of neurotransmission have been described. Still, the dynamics of the molecular events are largely unresolved. Studies are in progress to find out which substances of the nervous system act as neurotransmitters, or as neuromodulators at the various synapses. Some 30 different substances are known or suspected to be transmitters in the brain. Each has a characteristic excitatory or inhibitory effect on neurons. These inquiries include questions about the role of peptides as a new class of chemical messengers in the brain. Other research is concerned with the mechanisms that are fundamental to the chemical signalling in nervous tissue. This aspect will be the subject of this chapter. We will discuss the possible role of membrane phosphorylation in interneuronal communication.

PROTEIN PHOSPHORYLATION

For detailed background information on the nature, metabolism and function of proteins which contain covalently bound phosphorus, the reader is referred to the excellent monograph on protein phosphorylation by Weller (1979). In short, it has been shown that certain amino acids such as serine and threonine can be phosphorylated in the polypeptide chain which is then converted into a so-called phosphoprotein. Phospho-serine is the most abundant protein-bound phosphorylated amino acid. The protein-bound phosphate has a rapid turnover in many tissues and species, the highest rate is observed in brain. The endogenous protein phosphorylating capacity is most enriched in the subcellular fraction of brain tissue that contains the synaptic plasma membranes.

The turnover of protein-bound phosphate is brought about by the catalytic activity of protein kinases and phosphoprotein phosphatase. The kinase facilitates the transfer of the γ-phosphate

group of ATP to the protein substrate and the phosphatase catalyses the removal of this protein-bound phosphate by hydrolysis.

A generally accepted concept is developed from many observations on the reversibility of phosphorylation and dephosphorylation of proteins and simultaneously occurring changes in properties of these proteins. This concept states that phosphorylation and dephosphorylation of a protein alter its stereo-conformation and so regulate its function (Weller, 1979). If the acceptor protein is an enzyme, the phosphorylation may change its catalytic activity. In other cases, for instance with structural proteins in the membrane, alterations in the degree of protein-bound phosphate of specific ion channel proteins is thought to regulate membrane permeability. Phosphorylation and dephosphorylation cycles are supposedly involved in the opening/closing mechanism of selective membrane pores. Since changes in membrane permeability are crucial to nerve cell function, this possibility has been and still is one of the major reasons for the study of phosphoproteins in nervous tissues (Greengard, 1978, 1979).

Recent evidence suggests that also in brain membranes phosphorylation of certain proteins serves to control enzymatic activity. By this effect the polarization state of the neuronal membranes may be regulated indirectly (Jolles et al., 1980b).

Since changes in ion permeability at the synapses and chemical transmission are closely linked, we will review the role of membrane phosphorylation occurring during certain types of neurotransmission. Firstly, we will describe the phosphorylation of receptor proteins of the nicotinic cholinergic synapse of the peripheral nervous system. Secondly, we consider the phosphorylation of synaptic proteins as modulated by second messengers. Finally, we will report on our own research on the modulatory influences of neuropeptides on membrane phosphorylation in the central nervous system.

PHOSPHORYLATION AND DEPHOSPHORYLATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR (= AChR)

Characteristics of the AChR

Acetylcholine released from nerve terminals interacts with the nicotinic receptor in the post synaptic membrane and so triggers fast responses (latency < ms, duration 30–100 ms) such as the contraction of skeletal muscle or the electrical activity in the electric organ of rays *(Torpedo)* and eels *(Electrophorus)*. Recently, the concept has developed (reviews by Gordon and Diamond, 1979, 1980) that the mode of action by which the nicotinic AChR transduces extracellular signals in the post synaptic cell may be regulated by a phosphorylation–dephosphorylation reaction of the polypeptide subunits of the receptor. We will restrict this discussion to phosphorylation and dephosphorylation processes associated with the nicotinic receptor of the electric organ.

In many studies on the function of the AChR the electric organ of rays and eels has been an useful model system. The electroplax is homogenously innervated by nicotinic cholinergic neurons. It is a rich source of AChR. AChR-enriched synaptic membranes purified from the electric organ of *Torpedo californica* are and have been employed in studies concerning the relationship between neurotransmission and membrane protein phosphorylation (Gordon and Diamond, 1980). Though protein phosphorylation of the AChR has been observed in several cholinergic tissues (Teichberg et al., 1977; Saitoh and Changeux, 1980; Schoffeniels and Dandrifosse, 1980) we will concentrate on the evidence derived from studies with AChR-enriched membranes isolated from *T. californica* (Gordon and Diamond, 1979, 1980).

The nicotinic AChR has been purified from *T. californica* and other electric fishes (Karlin et al., 1975). The receptor of *T. californica* is composed of 4 different subunits of apparent molecular weights of 65,000, 58,000, 52,000 and 40,000 daltons (Raftery et al., 1975; Karlin et al., 1975; Flanagan et al., 1976). The ratio between the subunits is 1:1:2:4. The 40,000-dalton polypeptide binds acetylcholine. The function of the other polypeptides is unknown (Gordon and Diamond, 1980). Biochemical characterization of the receptor has been described by Michaelson et al. (1974) and Vandlen et al. (1979).

Protein phosphorylation in AChR-enriched membranes

Membranes enriched in AChR have been purified from electroplax of *T. californica*. This fraction shows increased specific binding capacity for the cholinergic blocker, the *Naja naja siamensis* toxin (Duguid and Raftery, 1973) and was able to support cholinergic agonist-dependent cation transport (Popot et al., 1976).

Gordon et al. (1977a, 1980) demonstrated that incubation of AChR-enriched membranes with [γ-^{32}P]ATP in the presence of Mg^{2+} and K^+ ions resulted in phosphorylation of several protein bands. Three of the [^{32}P]-labeled polypeptides in the receptor containing membranes had nearly identical molecular weights (M_r: 65,000, 56,000 and 41,000 daltons) as the subunits of the purified AChR.

It turned out that K^+ ions at 100 mM produced a marked stimulation of the endogenous membrane phosphorylation, since in the absence of K^+ ions phosphorylation of only one major band of molecular weight 91,000 daltons was seen. Na^+ ions could not substitute for K^+ ions.

Optimal phosphorylation of the 65,000-dalton polypeptide in the AChR-enriched membranes occurred at Na^+ (1 mM) and K^+ (about 100 mM) ion concentrations very similar to the intracellular concentrations of these cations for *Torpedo*. Three other polypeptides of molecular weights 56,000, 41,000 and 36,000 daltons showed the same type of Na^+,K^+-dependent phosphorylation.

Furthermore, only in the presence of K^+ ions, did nicotinic ligands, like carbachol, tubocurare and decamethonium, inhibit the endogenous phosphorylation of the K^+-dependent bands. These ligands were inhibitory at concentrations at which they are known to react specifically with the AChR (Meunier et al., 1974).

In contrast, phosphorylation of the 91,000-dalton polypeptide is insensitive to cholinergic ligands and less affected by monovalent ions (Gordon et al., 1977a; Gordon and Diamond, 1979). This band is assumed to be a phosphorylated component of the Na-K-ATPase (Jean et al., 1975), which was present in variable amounts in these preparations. Receptor-enriched membranes contain only a few other proteins which are closely associated with the AChR.

The question whether all the endogenously phosphorylated polypeptides were indeed constituent subunits of the purified AChR was answered by examining the cross-reaction of the phosphorylated polypeptides with a specific goat antiserum to purified *Torpedo* AChR (Gordon et al., 1977a,b). By means of two-dimensional immunoelectrophoresis, Gordon et al. (1977b) established that only the 65,000-dalton component reacted with anti-AChR antibody. The immunoprecipitate of this polypeptide also contained the incorporated radioactive label. This result indicates that the membrane protein kinase present in AChR-enriched membranes was able to phosphorylate in situ one specific subunit of the receptor of *T. californica*. The finding that the component of a membrane receptor protein with known function has been identified as a substrate for an endogenous membrane protein kinase (cf. Teichberg et al., 1977) is an important step in correlating phosphorylation of membrane proteins with synaptic function.

The protein kinase activity

Protein phosphorylation in the receptor enriched membranes shows a dependence on divalent cations typical for protein kinases (Rubin and Rosen, 1975). It is noteworthy that the differential response to monovalent cations was not observed with other phosphorylating membrane preparations. Only ATP and not GTP could be used as a phosphate donor to support phosphorylation in the AChR-enriched membranes. cAMP and cGMP were ineffective as stimulators (Gordon et al., 1980). The endogenous protein kinase phosphorylated added substrates like casein and histone. K^+ and carbachol used in concentrations affecting the endogenous membrane phosphorylation were without effect on the phosphorylation of casein.

The phosphoprotein phosphatase activity

If protein phosphorylation of the AChR is an important regulatory event at the synapse, then dephosphorylation of the receptor proteins must also occur in situ. Gordon et al. (1979b) demonstrated the dephosphorylation of prelabeled phosphorylated AChR-enriched membranes in situ and also of exogenous substrate, prelabeled [^{32}P]casein, by these membranes. The membrane phosphoprotein phosphatase activity was inhibited by fluoride (10–100 mM), ATP (1 mM) and GTP (1 mM). The effect of the inhibitors and activators studied indicates that the enzyme had properties similar to other cAMP-independent protein phosphatases.

ATP binding sites

Gordon et al. (1979a) demonstrated with photoaffinity labeling experiments using arylazido-β-alanyl [^{32}P]ATP as an analog of ATP that 3 polypeptides with molecular weights 45,000, 55,000 and 100,000 daltons of the AChR-enriched membranes reacted with the photoaffinity label. These polypeptides were not identical to the subunits of the AChR which did not react in the membranes. The affinity reaction is specific. For instance, unlabeled ATP added at increasing concentrations inhibited the photoaffinity labeling of the membranes. Interaction of the ATP binding sites with the protein kinase activity was demonstrated by the finding that increasing concentrations of unlabeled ATP photoaffinity analog progressively inhibited endogenous phosphorylation in AChR-enriched membranes.

Conclusions

Phosphorylation and dephosphorylation of polypeptides of the AChR has been studied in membrane preparations of various cholinergic tissues (Teichberg et al., 1977; Teichberg and Changeux, 1977; Gordon et al., 1977a; Gordon and Diamond, 1979; Schoffeniels and Dandrifosse, 1980).

Gordon and Diamond (1979, 1980) showed that in AChR-enriched membranes of *T. californica* one subunit, the 65,000 polypeptide, of the AChR is phosphorylated and dephosphorylated in situ. This reversible phosphorylation–dephosphorylation cycle is modulated by the phosphate donor ATP, K^+ ions and nicotinic ligands and by the ATP binding polypeptides. Most likely, cholinergic ligands and K^+ do not influence the receptor phosphorylation by regulating the protein kinase activity. Instead, these agents appear to interact directly with the AChR to produce a conformational change (Changeux et al., 1975) and alter its availability as a substrate for phosphorylation. If this is the case the conformational state of the membrane-bound AChR could determine its degree of phosphorylation.

Gordon and Diamond (1979, 1980) speculate that the ATP binding polypeptides of molecular weights 45,000 and 55,000 daltons may be subunits of the endogenous protein kinase and phosphatase activities. They suggest further that since these enzyme activities have characteristics very similar to most membrane cAMP-independent protein kinases and phosphatases, the unique behavior of the membrane-bound phosphorylation of the AChR is determined by the receptor and its location in the postsynaptic membrane.

Phosphorylation and dephosphorylation influence structural properties of the AChR, such as its release from the membrane by detergents and its susceptibility to heat inactivation (Saitoh and Changeux, 1980). With AChR-enriched membranes changes in agonist induced Na^+ flux and "desensitization" of this response have been described (Sugiyama et al., 1976). How phosphorylation and dephosphorylation of the AChR affect receptor affinities for ligands as well as receptor mediated ion fluxes, are important questions. Answers to these problems may be useful for understanding the mode of action of the AChR. Very revealing might be studies that would correlate phosphorylation of the AChR in vivo with conductance changes during nerve activity.

SECOND MESSENGERS AND BRAIN PROTEIN PHOSPHORYLATION

The second messenger concept as formulated by Sutherland (1972) states that an extracellular effector (first messenger) activates a membrane mechanism resulting in the intracellular rise of a second messenger (for instance cAMP, calcium, etc.), which is responsible for the metabolic and physiological responses of the target cell to the extracellular effector. Here we consider the second messengers calcium and cAMP both being implicated at different levels in the process of chemical neurotransmission (Greengard, 1979; Rodnight, 1980).

Calcium

Phosphorylation of specific protein substrates has been implicated in the release of neurotransmitters. This release is a calcium-dependent process and indeed experiments were reported describing a calcium-dependent phosphorylation of membrane proteins in relation to neurotransmitter release (Katz and Miledi, 1967; Douglas, 1973; Redburn et al., 1976; DeLorenzo and Freedman, 1977; Krueger et al., 1977; Hershkowitz, 1978; Schulman and Greengard, 1978; DeLorenzo et al., 1979; Sieghart et al., 1979, 1980).

The calcium-dependent phosphorylation of synaptic plasma membrane (SPM) proteins is, for a major part, mediated by calmodulin. This protein (M_r 17,000 daltons, IEP 4.5) was first isolated by Lin et al. (1974) and it was characterized as the calcium-binding, heat stable inhibitor protein of the enzyme phosphodiesterase. However, over the past few years, it has become clear that calmodulin is involved in the regulation of several other enzyme systems, all of which are controlled by calcium (for review see Cheung, 1980). Such systems involve the activation of the membrane-bound calcium-stimulated ATPase (Jarrett and Penniston, 1978), the stimulation of calcium transport in the plasma membrane (Hinds et al., 1978), the regulation of myosin light chain kinase activity (Barylko et al., 1978), and that of skeletal muscle phosphorylase kinase (Cohen et al., 1978).

Calmodulin is widely distributed in brain tissue (Schulman and Greengard, 1978; Harper et al., 1980). Recently, a calcium and calmodulin-binding protein specific for nervous tissue has been described (Klee et al., 1979). This protein, calcineurin, inhibits the activation by calmodulin of several enzyme systems. Calcineurin is composed of two subunits with mole-

cular weights of 61,000 daltons (subunit A) and 15,000 daltons (subunit B). The B subunit, although not identical to calmodulin, binds 4 calcium ions per molecule. Thus, calcineurin A interacts with two calcium-binding factors, calmodulin and subunit B. From these data, a role for calcineurin in the regulation of free calcium concentrations in the nervous system has been suggested (Klee et al. 1979).

DeLorenzo (1980) outlined a model of the possible role of calmodulin in synaptic function. Following the depolarization-dependent entry of calcium into the presynaptic terminal, calcium is immediately bound to calmodulin near the membrane. This could then initiate a variety of processes: presynaptic protein phosphorylation, neurotransmitter release and vesicle–membrane interactions. Although the model does not exclude a postsynaptic calcium calmodulin-sensitive membrane protein phosphorylation to be of importance to synaptic function, it certainly emphasizes a presynaptic regulation.

cAMP

The studies of the groups of Rodnight and Greengard on cAMP-dependent protein phosphorylation in synaptic membranes revealed a possible role for cAMP and protein phosphorylation in synaptic transmission. Such membranes contain high levels of cAMP-dependent protein kinases (Maeno et al., 1971), substrate phosphoproteins (Johnson et al., 1972) and protein phosphatases (Maeno and Greengard, 1972). These 3 proteins are believed to exist as complexes in the brain membranes (Ueda et al., 1975), enabling a fast chemical response (phosphorylation–dephosphorylation) to different neuronal activities, with most likely the dephosphorylation as the rate limiting step (Zwiers et al., 1976; Ng and Matus, 1979a,b).

In synaptic membranes a number of substrate proteins are phosphorylated in a cAMP-dependent manner (Ueda et al., 1973; Ehrlich and Routtenberg, 1974; Zwiers et al., 1976; Mahler et al., 1977; DeBlas et al., 1979). Interestingly, cGMP has no effect on the phosphorylation of these membrane phosphoproteins. Up till now, clear effects of this cyclic nucleotide on brain protein phosphorylation were only found on a 23,000-dalton phosphoprotein, located in the cytosol fraction of the cerebellum of rats (Schlichter et al., 1978; Szmigielski and Guidotti, 1979). Two cAMP-dependent phosphorylated substrate proteins (protein I, 86,000 daltons and protein II, 52,000 daltons) have been the subject of an extensive study by the group of Greengard (Greengard, 1976, 1979). In 1977 the purification of both membrane-bound phosphoproteins (Ueda and Greengard, 1977) and a cAMP-dependent protein kinase (Uno et al., 1977) was reported. Protein I appears to consist of two polypeptides with respective molecular weights of 86,000 and 80,000 daltons, and isoelectric points of 10.3 and 10.2. The purified protein kinase is composed of an inhibitory regulatory subunit (R, M_r 52,000 daltons) and a catalytic subunit (C, M_r 40,000 daltons). The isoelectric point of the holoenzyme (RC) is 5.5. and differs from that of the R-subunit (4.8) and C-subunit (7.0). The activation of the kinase involves the binding of cAMP to the R-subunit, leading to dissociation of the holoenzyme: RC + cAMP → R-cAMP + C.

Protein II appears to be the R-subunit of the holoenzyme. Activation may involve autophosphorylation, which is also known to occur in cytosolic protein kinases (Krebs and Beavo, 1979).

In contrast to the apparent ubiquitous presence of the cAMP-dependent protein kinases (Rubin and Rosen, 1975; Krebs and Beavo, 1979; Rubin, 1979), the substrate protein I seems to be confined to the nervous system (Ueda and Greengard, 1977). It appears to be present only in neurones (Sieghart et al., 1978). Immunocytochemical studies (Bloom et al., 1979) indicate it to be present throughout the synaptic region, especially in synaptic vesicle membranes and

postsynaptic densities. Studies on subcellular fractions also point to such regional distribution in the neuron (Ueda et al., 1979). Furthermore, the appearance of protein I in the brain of developing rats and guinea pigs coincides with the formation of synaptic structures (Lohmann et al., 1978). These data are given in support of a function of protein I in the synaptic processes. The recent finding that the phosphorylation of protein I is calcium-dependent (via calmodulin) might be important. Evidence points to two different phosphorylation sites on the protein (Huttner and Greengard, 1979), one site being phosphorylated in the presence of cAMP, the other site in the presence of calcium-calmodulin. At present the physiological significance of such a regulation mechanism by two second messengers is difficult to assess.

In experiments with slices of rat cerebral cortex incubated in vitro, the phosphorylation of protein I was stimulated both by agents which increase intracellular cAMP and enhance calcium influx. In contrast, no effect of neurotransmitters was observed (Forn and Greengard, 1978). More recently, it was reported that incubation of slices obtained from rat facial nuclei in the presence of serotonin resulted in phosphorylation of protein I (Dolphin and Greengard, 1981). The authors conclude that a significant fraction of the protein I phosphorylated in response to serotonin is probably located in the presynaptic terminals that provide a facilitatory serotonergic input to the facial motor neurons (Dolphin and Greengard, 1981).

Although the degree of phosphorylation of protein I was originally considered to be a regulatory step in the depolarization of postsynaptic membranes (Nathanson and Greengard, 1977), the literature cited above opens the possibility of also presynaptic regulations. Thus, at present it seems difficult to properly assess the role of protein I in synaptic neurotransmission. Postsynaptically the degree of phosphorylation may relate to membrane permeability whereas presynaptically its association with the vesicle membrane may relate to the release mechanism of vesicular neurotransmitters.

MODULATION OF BRAIN MEMBRANE PHOSPHORYLATION BY ACTH

N-terminal peptides of ACTH (corticotropin) are known to influence specific behaviors in animal and man (Gispen et al., 1979) and to affect brain metabolism (Dunn and Gispen, 1977; Schotman et al., 1976) Zwiers et al. (1976) demonstrated that behaviorally active ACTH peptides cause a dose-dependent inhibition of the in vitro phosphorylation of at least 5 protein bands in SPM (Zwiers et al., 1976, 1978). The phosphoprotein bands affected by addition of $ACTH_{1-24}$ had molecular weights in the range of 15,000–48,000 daltons. Addition of cAMP in vitro stimulated the endogenous phosphorylation of protein bands of SPM with higher molecular weights, ranging from 54,000 to 75,000 daltons (Fig. 1). The observation that different protein bands were affected and that the effects were in opposite directions, indicates that the peptide acted without a mediating role of cAMP.

The question arose whether the neuropeptide inhibited the endogenous phosphorylation in SPM by interfering with the phosphorylation activity or by promoting the dephosphorylation of the phosphoproteins. Therefore the effect of $ACTH_{1-24}$ was studied in the following experiment (Fig. 2; Zwiers, 1979) SPM was incubated with [γ-^{32}P]ATP for a very short time (22 s) to allow the phosphorylation of the endogenous proteins with simultaneous exhaustion of the added ATP. Then, when net dephosphorylation was measured, ACTH was added. It was observed that the addition of ACTH did not change the rate of dephosphorylation. When, however, after the period of dephosphorylation (30 min) a new aliquot of [γ-^{32}P]ATP was added, then ACTH inhibited the newly started incorporation of phosphate into proteins (Zwiers et al., 1978). These results indicate that ACTH interacts with protein kinase(s) of SPM.

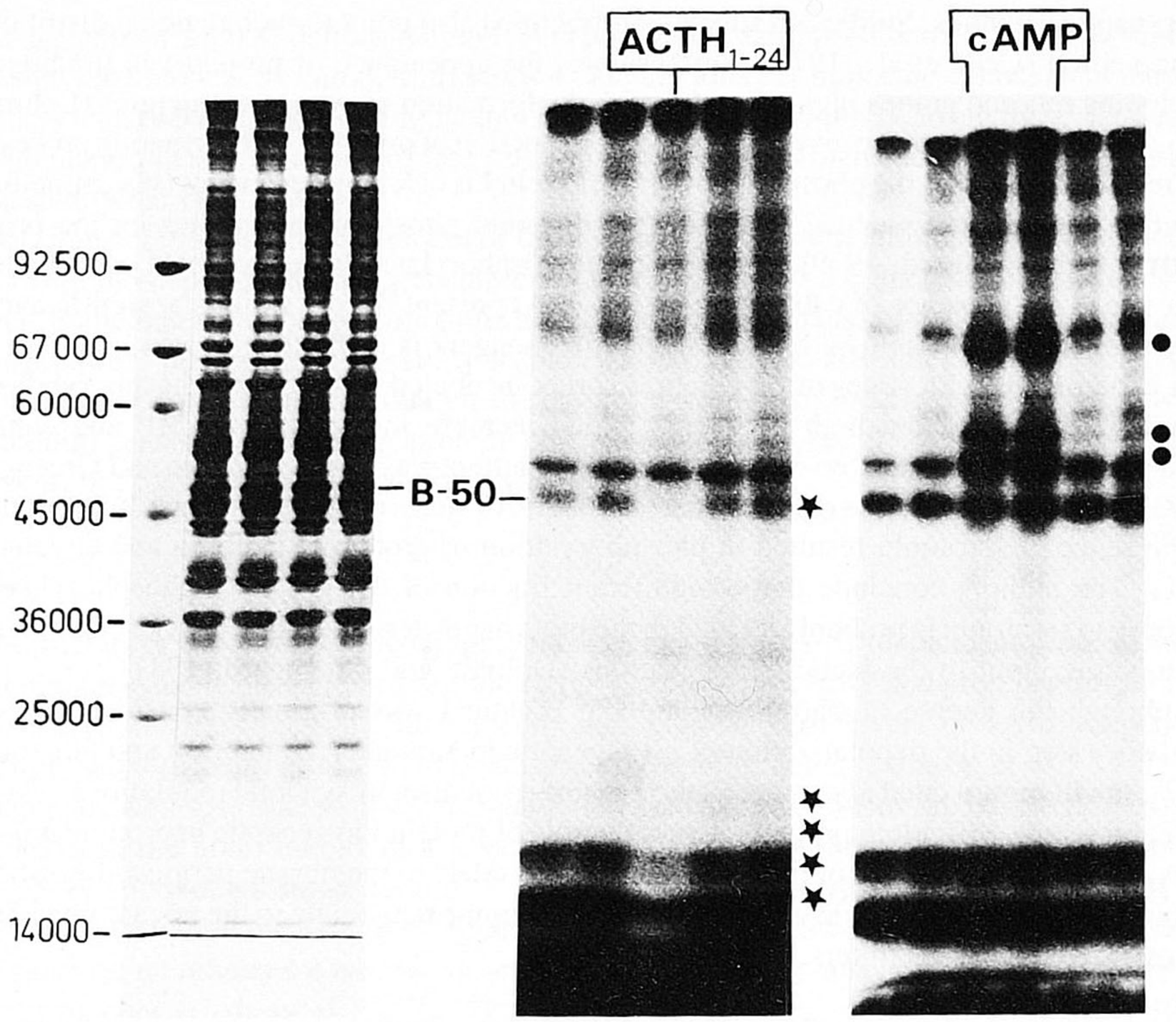

Fig. 1. Endogenous phosphorylation of synaptic plasma membrane proteins of rat brain. Left: protein pattern after separation by SDS-polyacrylamide gel electrophoresis. Right: corresponding autoradiographs, with the effects of $ACTH_{1-24}$ (*, 10^{-5} M) and cAMP (●, 5×10^{-6} M). (From Gispen and De Wied, 1980, with permission.)

The structure–activity relationship of the ACTH peptides in this system is rather complex. For, the inhibition of the endogenous phosphorylation of SPM is dependent not only on the primary structure of the peptide fragment used, but also on the protein band studied and the ratio of ATP and SPM protein concentration used during the reaction (Zwiers et al., 1978). The ability of $ACTH_{1-24}$ to inhibit phosphorylation of SPM protein bands such as the B-50 protein (M_r 48,000 daltons) is confined to the N-terminal part of the molecule. The shortest active sequence with the N-terminus intact is $ACTH_{1-13}$. The sequence $ACTH_{5-18}$ is as active as $ACTH_{1-16}$, and it is therefore concluded that the active site resides in the region $ACTH_{5-13}$. Possibly, C-terminal elongation of this sequence is necessary for expression of the activity since $ACTH_{5-16}$ was inactive but $ACTH_{5-18}$ active.

Interestingly, the effect of ACTH fragments on the phosphorylation of B-50 is very similar to that found for the induction of excessive grooming (Gispen et al., 1975; Gispen and Isaacson, 1980). The ACTH sequences 1–24, 1–16, 1–13, 5–18 and, to some extent, 5–16 induce the display of excessive grooming in the rat after intraventricular administration, whereas 1–10, 4–10, 11–24, 7–16 and the combination of 1–10 plus 11–24 are ineffecitve. It was of interest therefore, to see if in vivo intraventricular administration of a behavioral active ACTH fragment could result in subsequent changes in endogenous SPM phosphorylation in

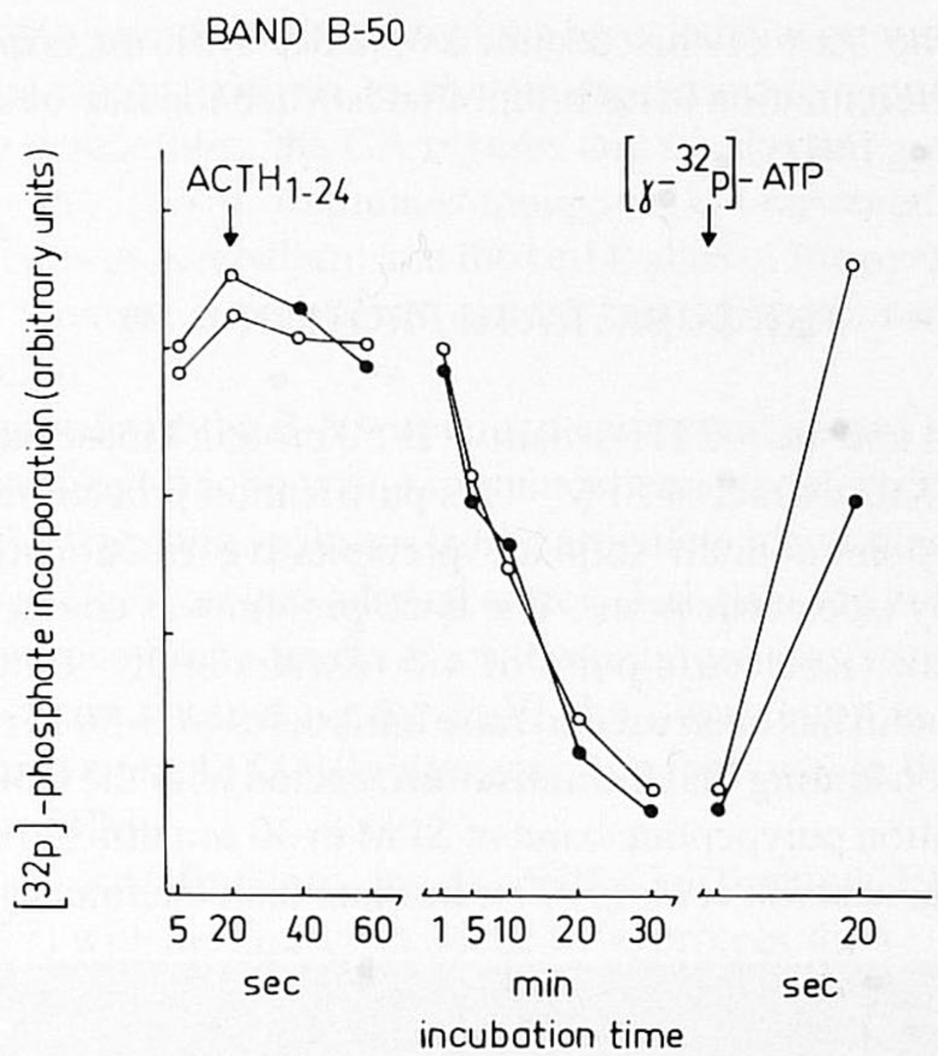

Fig. 2. Endogenous phosporylation of SPM protein band B-50 as a function of incubation time, measured by the densitometric method, and expressed in arbitrary units. The incubations were performed under standard conditions (7.5 μM ATP, 2.2 μg SPM/μl). At 22 s (arrow) either buffer (o——o) or $ACTH_{1-24}$, resulting in a concentration of 10^{-4} M (●——●) was added. At 30 min (arrow) [γ-^{32}P]ATP was added, to increase the ATP concentration with 7.5 μM. Results are representative of 3 separate experiments. (From Zwiers et al., (1978); with permission.)

vitro. Administration of $ACTH_{1-24}$ in rat and subsequent preparation of SPM after 30 min resulted in an increased amount of in vitro incorporated ^{32}P into the same 5 phosphoprotein bands (Zwiers et al., 1977) which also responded after in vitro administration of ACTH. There appears to be a U-shaped dose–response curve of phosphate incorporation into SPM protein bands between 30 and 3000 ng of injected $ACTH_{1-24}$. This effect of in vivo ACTH treatment on in vitro endogenous phosphorylation was also time-dependent, with maximal effect 30 min after the peptide injection (Zwiers et al., 1977).

Recently we studied the incorporation of [^{32}P]phosphate in brain membrane proteins in vivo in 8-day-old rats. Thirty minutes after intracisternal administration of labeled phosphate to infant rats, a membrane-enriched fraction isolated from the cerebral cortices consisted of numerous ^{32}P-labeled proteins. The incorporation of the radioactivity into protein was less than 5% of the total amount that was taken up by the lipids of the membranes.

Certain of these in vivo labeled protein bands have been characterized by comparison with the results obtained when neural membrane preparations, which were not labeled in vivo, were exposed to the neuromodulators $ACTH_{1-24}$ and cAMP in an in vitro endogenously phosphorylating system (cf. Berman et al., 1980; Mitrius et al., 1981). So we discovered that one of the protein bands that contained a relatively high level of the in vivo incorporated radioactive phosphate, had a molecular weight and isoelectric point identical to the B-50 protein of SPM (Oestreicher et al., 1981a; 1982).

From the investigations of Holmes and Rodnight (1981) one may deduce that the in vitro endogenous B-50 protein phosphorylating system is present at high activity in brain membranes of the neonate rat. The activity of this system, involving the protein kinase, the substrate and activity modulators such as calmodulin, appears to decline after 15 days of age to lower

levels at adulthood. The start of this decline coincides with an important developmental process of structural differentiation in rat brain, that is when the rate of synaptogenesis is high (Aghajanian and Bloom, 1967).

THE SUBSTRATE PROTEIN B-50

Both the B-50 protein and the ACTH-sensitive B-50 protein kinase have been purified from a 0.5% Triton–75 mM KCl extract of SPM. This purification procedure involved DEAE-cellulose chromatography, ammonium sulphate precipitative fractionation and finally electrophoretic separation by isoelectric point. The B-50 protein was characterized by amino acid composition and an acidic isoelectric point of 4.5 (Zwiers et al., 1980a).

The purified B-50 protein has been used to raise antibodies to B-50 in rabbits. The antiserum is characterized by demonstrating that the antiserum reacted with the isolated B-50 protein and only with the 48,000-dalton polypeptide band of SPM in 30 μm thin gel sections, produced by the procedure of Van Raamsdonk et al. (1977). Immunohistochemical studies of the distribu-

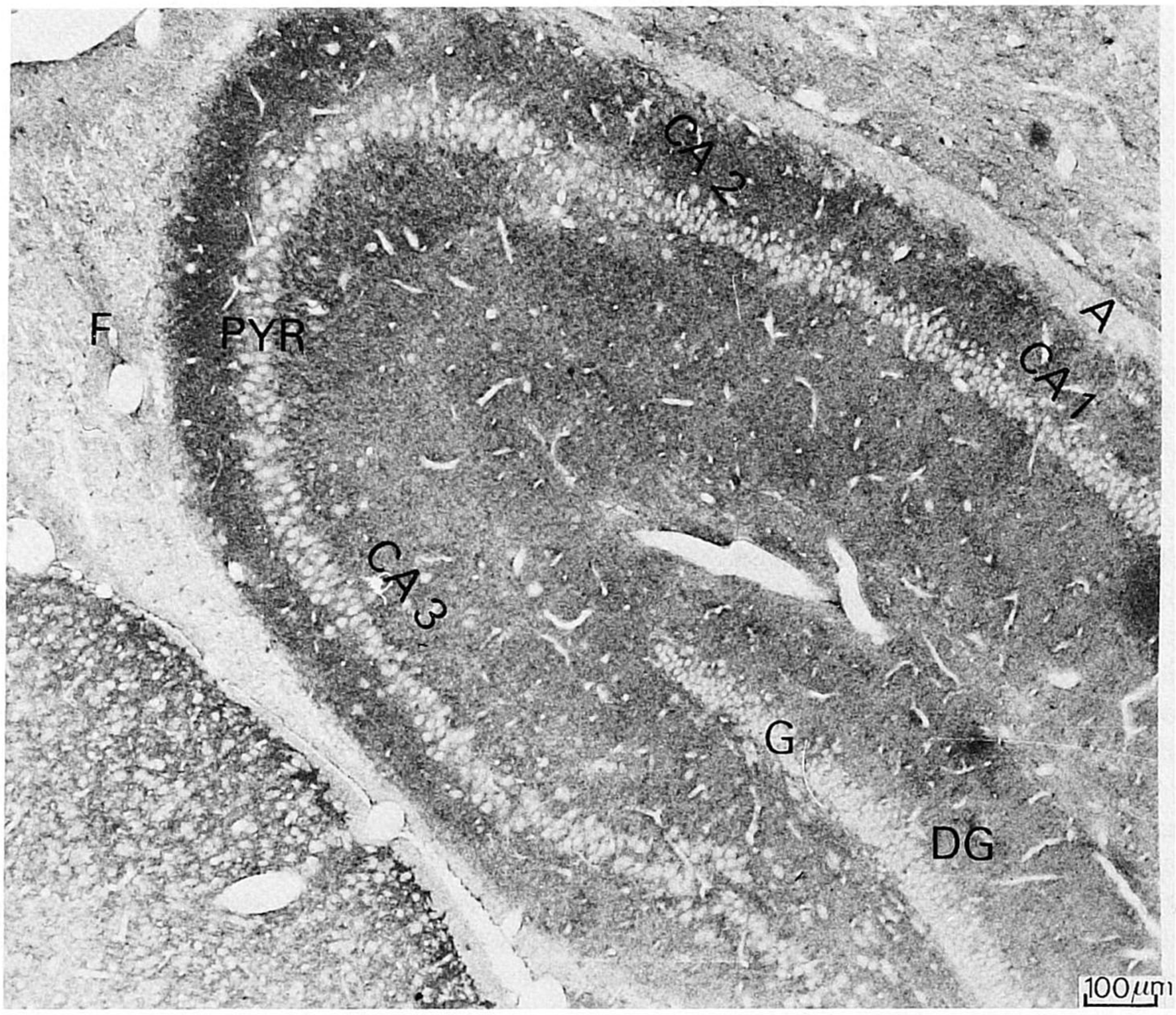

Fig. 3. Sagittal sections of rat hippocampus incubated with anti B-50 (1:400 dil.) and stained by the PAP method. Section cut approximately 2100 μm parasagittal. Note staining in CA1, CA2 and CA3 and dentate gyrus (DG). Little staining was found in the cell bodies of the pyramidal cells (PYR), in the granular cells (G), in the fimbria (F) and in alveus (A). (From Oestreicher et al., 1981b, with permission.)

tion of the B-50 protein in various rat brain regions revealed that immunoreactivity of the B-50 protein was present in regions known to contain numerous synaptic contacts, such as the molecular layer of the cerebellum, the CA regions and the dentate gyrus of the hippocampus (Fig. 3; Oestreicher et al., 1981b). Immunostaining was not observed in white matter and the perikarya of Purkinje cells of cerebellum and the cell bodies of the pyramidal and granule cells of the hippocampus. Control experiments carried out with preimmune serum showed no specific immunostaining.

Studies on the distribution of the B-50 protein in various rat tissues by means of two-dimensional analysis of solubilized fractions and immunochemical detection with anti-B-50 antiserum indicate that the B-50 protein is absent from particulate membrane material of tissues like heart, lung, spleen, muscle, liver and adrenal cortex (Kristjansson et al., 1982). When 20 μg protein from the crude membrane fractions of these tissues was incubated with [γ-^{32}P]ATP under conditions which are optimal for the B-50 phosphorylation in SPM, a labeled phosphoprotein of molecular weight 48,000 daltons was detected only in the membrane fraction of brain and of course in SPM.

The analysis of the same fractions by the SDS-Gel-Immuno-Peroxidase method (Van Raamsdonk et al., 1977) with the antiserum to the B-50 protein showed the presence of B-50 in brain particulate fraction and in SPM. Tests with equal amounts of protein of the membrane fractions of other tissues did not reveal immuno-reactive B-50. This suggests that the occurrence of the B-50 protein may be restricted to nervous tissue.

THE B-50 PROTEIN AND MEMBRANE PHOSPHOLIPID METABOLISM

Although evidence has been presented to suggest a regulatory role of phosphoproteins in synaptic transmission (see above), similar ideas were formulated with respect to a special class of membrane phospholipids, i.e. the (poly)phosphoinositides (Michell, 1979). The rapid metabolism of these phospholipids in various membranes led to search for their role in membrane function. Especially in brain membranes (poly)phosphoinositides are present in high amounts (Jolles et al., 1981). On the basis of a variety of studies, Michell (1975, 1979) concluded that the metabolism of (poly)phosphoinositides in the membrane may be closely connected to the influx of calcium into the cell. His hypothesis proposed that binding of a variety of agonists (hormones, neurotransmitters, etc.) to their respective receptors would initiate the hydrolysis of membrane (poly)phosphoinositides, followed by the influx of calcium. Calcium ions acting as second messenger would then activate specific metabolic pathways in the target cells. To investigate whether interaction of ACTH in the brain cell membranes would involve such a mechanism, Jolles et al. (1979) incubated synaptosomal fractions of rat brain, priorly labeled with inorganic [^{32}P]orthophosphate, in the presence of $ACTH_{1-24}$. Thereafter, the amount of label recovered in phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (DPI), phosphatidylinositol-4,5-diphosphate (TPI) and phosphatidic acid (PA) was measured. In a time- and dose-dependent manner the peptide decreased the amount of label incorporated in the polyphosphoinositides and PA. Most sensitive to the addition of the peptide were DPI and TPI. Recently, it was shown in a lysed synaptosomal fraction that the absence or presence of calcium determines the effect of the peptide (Jolles et al., 1981). Thus, the stimulating effect of ACTH can only be seen in the absence of calcium.

In an effort to relate these peptide-induced changes in polyphosphoinositide metabolism to the previously reported effect of the peptide on membrane phosphoproteins (see above), DPI and [γ-^{32}P]ATP were added to the ASP_{55-80} membrane-protein fraction containing the B-50

TABLE I

THE EFFECT OF $ACTH_{1-24}$ ON AND THE RELATION BETWEEN B-50 PROTEIN PHOSPHORYLATION AND TPI FORMATION*

The $ASP_{55-80\%}$ fraction (A) was incubated for 15 min in the presence of DPI and [γ-^{32}P]ATP. ACTH was added 15 s before the addition of DPI and ATP. The label incorporated into TPI and B-50 was measured.
The $ASP_{55-80\%}$ fraction (B) was incubated for various time periods (0, 10, 20, 30 and 40 min) in the presence of [γ-^{32}P]ATP. B-50 was the only labeled phosphoprotein and incorporation increased with time up to 40 min. DPI was added after this B-50 prephosphorylation period and incubation continued for another 5 min.

Treatment	*Incorporation (% of control)*	
	B-50	*TPI*
(A) $ACTH_{1-24}$ (10 μM)		
$ACTH_{1-24}$ (100 μM)		
(B) B-50 prephosphorylation, 10 min	–	83
20 min	–	68
30 min	–	54
40 min	–	31

* From Jolles et al. (1980b).

protein kinase/B-50 substrate protein complex (Jolles et al., 1980a,b, 1981). The only two labeled compounds appeared to be the B-50 protein and TPI. Apparently, this fraction also contains DPI kinase activity in addition to protein kinase activity. An inverse relationship exists between the degree of phosphorylation of B-50 and the amount of TPI produced. These and other findings strongly suggest that B-50 protein phosphorylation and membrane polyphosphoinositide metabolism are correlated events (Jolles et al., 1980a). Such a notion is underscored by data on the simultaneous effect of $ACTH_{1-24}$ on B-50 protein kinase and DPI kinase activity in the same ASP_{55-80} membrane protein fraction (Table I). Interestingly, a dose-dependent inhibition of B-50 phosphorylation was observed, again accompanied by a stimulation of TPI production (Jolles et al., 1980a,b).

The data with ACTH suggest that neuropeptides may modulate membrane characteristics by altering the degree of phosphorylation of certain proteins and lipids. Whether or not such changes are indeed related to concurrent changes in membrane permeability is still to be proven. Preliminary results suggest that ACTH in fact influences the uptake of calcium into intact synaptosomes under phosphorylating conditions (Gispen, unpublished). Also, the problem of specificity remains. The high concentrations used to demonstrate the inhibition of the B-50 protein kinase, the ubiquitous localization of the B-50 protein throughout the brain regions rich in projections and the restriction of ACTH to specific nerve fibers (Krieger et al., 1980) led us to propose that the specificity of the ACTH effect on brain membrane phosphorylation is provided for the local high supply of ACTH in peptidergic synapses (Zwiers et al., 1982).

We have used ACTH as an example as, to date, most is known about this neuropeptide and brain membrane phosphorylation. However, also other peptides have been shown to affect brain phosphoproteins, enkephalin (Davis and Ehrlich, 1979; Bär et al., 1980), PIP (Zwiers et al., 1980b), $dinorphin_{1-13}$ (Zwiers et al., 1981). Thus, it may well be that part of the modulating action of other neuropeptides may also involve changes in membrane phosphorylation.

CONCLUDING REMARKS

The literature of the past decade is a rich source for the demonstration that neurotransmitters and neuropeptides are able to modify the degree of phosphorylation of certain membrane proteins and lipids in peripheral tissues and the brain. The interaction of these messengers may be direct. For instance, the binding of acetylcholine to its nicotinic receptor affects the phosphorylation level of one of the subunits of the AChR. However, the molecular processes that link changes in receptor phosphorylation of the synapse with the depolarizing flow of sodium ions across the membrane are still obscure. In brain certain neurotransmitters may exert their effect by an indirect route, resulting in a change of the intracellular concentration of a second messenger. Like acetylcholine, monoamine transmitters are known to have more than one type of receptors in the nervous system. Some of these receptors are coupled to the cAMP and/or calcium system (Rodnight, 1980). The various second messengers then influence the phosphorylation of specific proteins and lipids leading finally to physiological responses of the target cells.

Neuropeptides show similarities to but also remarkable differences from the classical neurotransmitters in the way they influence synaptic membrane phosphorylation. The interaction of ACTH peptides with the B-50 protein kinase of synaptic plasma membranes of rat brain is assumed to occur by a direct effect of the neuropeptide on the membrane. This leads to changes in membrane properties as is suggested by simultaneous changes in calcium transport observed in synaptosomes isolated from rat brain (Gispen, in preparation).

Other neuropeptides like enkephalins may influence membrane protein phosphorylation by a mechanism which involves the opiate receptor (Davis and Ehrlich, 1979). Many of the neuropeptides found in mammalian brain have been demonstrated to be concentrated in terminals of particular groups of neurons. Several are known to be released from axon terminals by a calcium-dependent process. Recently, synaptic potentiation and the effect of enkephalins on the phosphorylation of specific proteins in hippocampal slices have been described (Lopes da Silva et al., 1982). In all these cases further investigation is needed to establish that the particular sequences of reactions participate directly in the chemical transmission.

Although in the last decade many reports have revealed changes in membrane phosphorylation in relation to interneuronal communication, little progress has been made in understanding the exact role of such changes in neurotransmission. Problems of causality, time, and order of events still remain to be addressed before definite statements can be made. Models and working hypotheses are abundantly present in current literature. Their positive influence is that they stimulate further research, their negative influence may be that some of them live a life of their own even in the absence of sufficient supportive data.

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DISCUSSION

S. MAZZARI: Is there any relationship between the B-50 protein kinase and the phospholipid-dependent, Ca^{2+}-stimulated protein kinase? Recently A. Kishimoto has postulated a correlation between the latter enzyme, with phosphatidylinositol turnover like you have suggested for the B-50 protein kinase.

W.H. GISPEN: Indeed, there is good reason to believe that the two enzymes are identical. Dr. Vincent Aloyo and Dr. Henk Zwiers recently reported that the two enzymes have similar molecular weights, isoelectric points and are affected by the same modulators (phosphatidyl serine, chlorpromazine, calcium and ACTH).

A. DOLPHIN: Would you like to speculate on why most of the protein phosphorylation systems that have been worked out have a pre- rather than a postsynaptic localization?

W.H. GISPEN: It is difficulet to assess whether this is caused by the experimental procedures used (e.g. work with light synaptosomal membranes, etc.), or of it is indeed an indication that the role of phosphorylation in neurotransmission primarily is presynaptic. More work is necessary to justify any speculation.

A. DOLPHIN: (1) Are B-50 protein and its kinase generally distributed presynaptically or is it present in specific types of terminals? (2) Have you looked for effects of ACTH on transmitter release?

W.H. GISPEN: The B-50 protein is primarily localized presynaptically. The B-50 protein kinase is likely to have a more general distribution and may even not be specific for nervous tissue. We have not looked for effects of ACTH in transmitter release. Yet there are numerous reports in the literature implying that peptides such as ACTH affect neurotransmission (Versteeg, *Pharmacol. Ther.*, 11, (1980) 535-557).

V. GALLO: Is the B-50 protein always present during brain development? Is it present at early stages in a different ("immature") form?

W.H. GISPEN: As far as we know, the B-50 protein is present neonatally as well as in adult or aged rat. The activity of its phosphorylation by the B-50 kinase seems to be higher in the young as compared to the old rat.

D.F. SWAAB: How good is the evidence that all the changes you described in, and related to phosphorylation are taking place in the neuronal compartment and not in glial cells?

W.H. GISPEN: Recent evidence of studies using different cell types suggests that the B-50 protein is not found in glial cells. Furthermore, the membrane isolation procedure used by Maler c.s. to pinpoint the subcellular localization, makes it highly unlikely that glial membranes would have accounted for the endogenous phosphorylation of B-50.

R.M. BUIJS: You demonstrated changes in the B-50 band which you discussed, but you also found changes in the D-20 band. Did you have any reasons to select only the B-50 band. Would D-20 be less interesting?

W.H. GISPEN: The B-50 band was chosen for further study as other laboratories had shown that a variety of experimental procedures could affect the phosphorylation of proteins with a molecular weight range of 40,000–60,000. Also, there is the hazard that the D-20 band with its low molecular weight is a product of rapid proteolysis during the preparation of the membrane.

R.M. BUIJS: Do you know which areas in the brain are involved in grooming? Would it be possible to detect in these areas the changes in phosphorylation you have spoken about?

W.H. GISPEN: At present we know only one area that induces excessive grooming upon local application, viz. the substantia nigra. Experiments are in progress to relate local changes in membrane phosphorylation to the elucidation of excessive grooming.

D.F. SWAAB: On several occasions you made a most subtle distinction in speaking about "neurotransmitters *and* neuropeptides". This formulation seems to exclude that neuropeptides may act as neurotransmitters. Did you mean to say this, and, if so, what is the *fundamental* difference between neuropeptides and the compounds you call neurotransmitters.

W.H. GISPEN: Neuropeptides may act as transmitters of information in a peptidergic synapse and thus, in a number of instances, may be considered to represent a certain class of neurotransmitters like other groups of substances (amino acids, amines, etc.). On the other hand, peptides may affect brain function also in a hormonal way, i.e., the brain cell may be a target for peptide action, also outside the synaptic region. Such an action is different from that of a neurotransmitter.

J. DREIFUSS: Would you mind saying a few words on membrane methylation processes as alternatives of membrane phosphorylation processes, which you so elegantly summarized in your lecture?

W.H. GISPEN: The work of Axelrod's laboratory emphasizes the potential of membrane methylation in a variety of biological signalling processes. I am not sure about the relative importance of the phosphorylation vs the methylation reactions in neuronal communication. At present the two lines of research to me both underscore the notion that local structural modifications of membrane proteins and lipids are involved in trans-membrane communication.

U. UNGERSTEDT: Is your "excessive grooming" clearly different from stereotyped behavior? Is so, can it really be linked to DA neurotransmission?

W.H. GISPEN: In my opinion, there is no doubt that the ACTH-induced excessive grooming is different from stereotyped behaviour as the peptide enhances the display of the total maintenance behavior repertoire without changing the composition of that behavior (Gispen and Isaacson, 1980). Nonetheless a variety of laboratories have now shown that ACTH-induced excessive grooming can be modulated by interfering with brain DA systems.

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