B-50 PHOSPHORYLATION, PROTEIN KINASE C AND THE INDUCTION OF EXCESSIVE GROOMING BEHAVIOR IN THE RAT

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INTRODUCTION

Behaviorally active neuropeptides might affect synaptic plasticity by changing the degree of phosphorylation of synaptic proteins. Neuronal electrical activity and neurotransmission are accompanied by covalent modification of synaptic proteins through cyclic phosphorylation and dephosphorylation (c.f. Weller, 1979). Studies using behavioral paradigms similar to those used to measure the behavioral effects of melanocortins (ACTH/MSH) suggested that the acquisition of new information may be accompanied by changes in the degree of phosphorylation of synaptic phosphoproteins (Glassman et al., 1973). The original idea was to study the in vitro modulation of synaptic plasma membrane phosphorylation by $ACTH_{1-24}$ and its behaviorally active fragments and to compare the structural requirements of ACTH in this assay with those influencing the extinction of active avoidance behavior (Greven and De Wied, 1973). In our first study along this line, we noted that high concentrations of $ACTH_{1-24}$ indeed inhibited the endogenous phosphorylation of several phosphoproteins in rat brain synaptic membranes (Zwiers et al., 1976). These phosphoproteins were phosphorylated by a cyclic AMP-independent mechanism, at that time the most important phosphorylation system studied (Zwiers et al., 1976). Our next step was to investigate the nature of the affected substrate proteins and their corresponding kinase(s).

The first characterized substrate protein and its kinase, B-50 and B-50 kinase, were isolated in a complex from synaptosomal plasma membranes (Zwiers et al., 1980). The B-50 kinase has been shown to be very similar, if not identical to protein kinase C (Aloyo et al., 1982, 1983). In the present paper we review our current knowledge on B-50 and discuss the evidence that the degree of B-50 phosphorylation may modulate receptor-mediated polyphosphoinositide breakdown (Gispen et al., 1985a). The protein B-50 discussed in this paper is most likely identical to protein $\gamma 5$ of Rodnight (Rodnight, 1982, Gower and Rodnight, 1982), to p54 (Ca)p by Mahler (Mahler et al., 1982), to protein F_1 by Routtenberg (Routtenberg et al., 1985, Akers and Routtenberg, 1985, Gispen et al., 1986) and to pp46 described by Pfenninger (Katz et al., 1985, De Graan et al., 1985). Furthermore we will put emphasis on the possible role of kinase C and possibly B-50 phos-

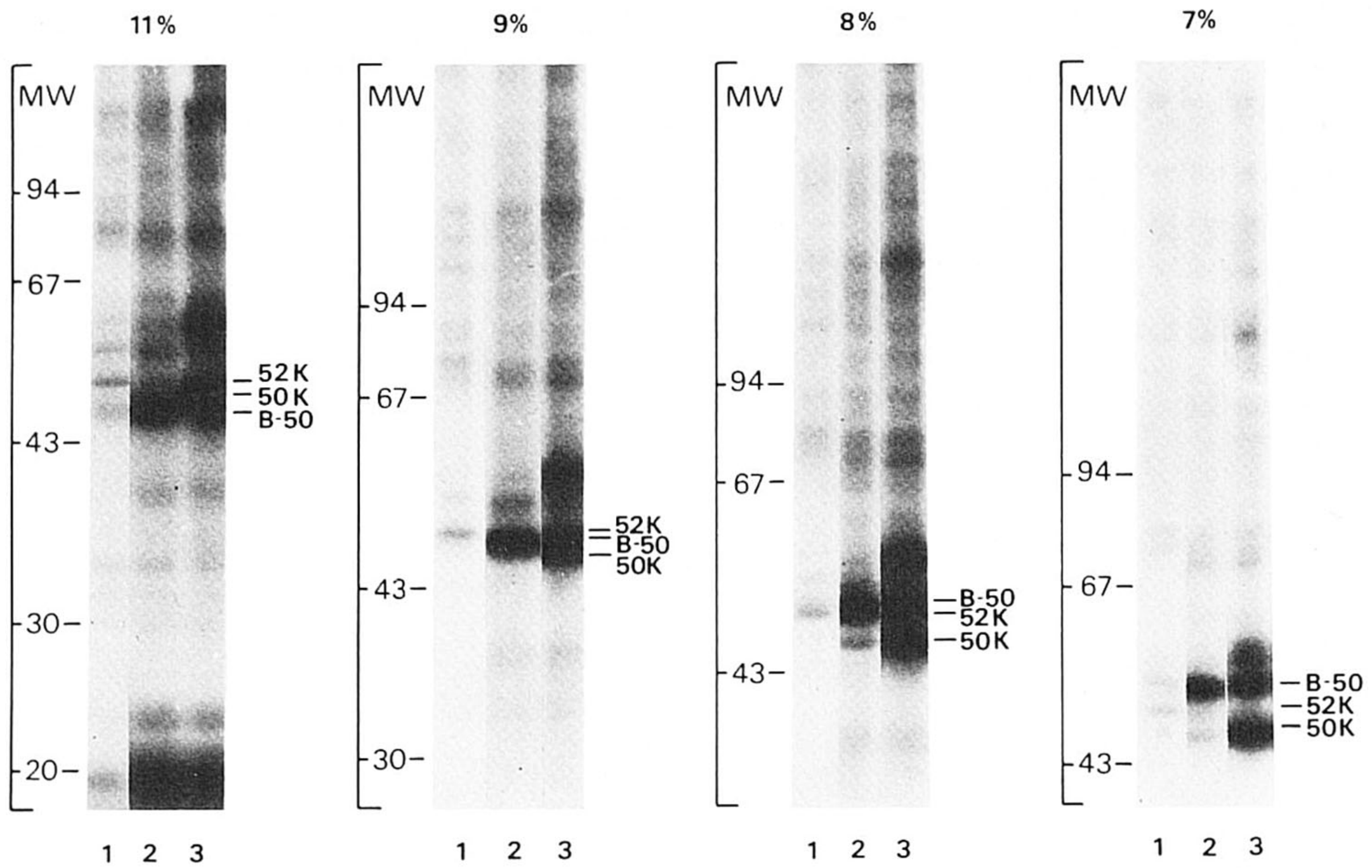

FIG. 1: SPM was phosphorylated in 10 mM Na-acetate, 10 mM Mg^{2+}-acetate, 0.5 mM EGTA (pH6.5) and varying Ca^{2+} concentrations for 15 sec at 30°C. The phosphorylation assay was carried out in the absence of Ca^{2+} (lanes 1), in the presence of 0.6 mM Ca^{2+} (lanes 2) and in the presence of 0.6 mM Ca^{2+} and 5U calmodulin in 25 µl (lanes 3). The proteins were separated on 4 different gels, containing 11% acrylamide and 0.2% bisacrylamide, and 9, 8 and 7% acrylamide containing the same ratio of acrylamide to bisacrylamide as the 11% gel. The numbers to the left indicate the position of the Pharmacia low molecular weight markers in kDa, the identity of the three phosphoproteins indicated to the right is explained in the text.

phorylation in some of the behavioral effects of ACTH.

Biochemical Characterization of B-50

Initial phosphorylation studies on B-50 have been performed in synaptosomal plasma membranes, and these membranes have been used as the source for the isolation and characterization of both B-50 and B-50 kinase. B-50 is an acidic (pI 4.5) 48 kDa protein that is intimately associated with the synaptic plasma membrane, since it can only be solubilized in the presence of detergent (Zwiers et al., 1979, 1980). The B-50 protein displays heterogeneity both in its relative molecular weight on SDS-PAGE and in its isoelectric point. Depending on the percentage crosslinking of the gel and on the percentage of acrylamide in the gel used, B-50 can be found between 43 and 54 kDa. The lower the cross-linking of the gel and/or the percentage acrylamide used, the higher molecular weight B-50 displays (Fig. 1, Gower and Rodnight, 1982). The 11% gel contained 0.2% bisacrylamide, and the ratio of acrylamide to bisacrylamide was kept constant for the lower percentage gels (Fig. 1). We have distinguished between calcium- and calmodulin-dependent phosphorylation by incubating SPM either in the presence of EGTA (0.5 mM EGTA, lanes 1), in the presence of calcium (0.5 mM EGTA and 0.6 mM Ca^{2+}, lanes 2), or in the presence of 5 U calmodulin (lanes 3). The 50 kDa phosphoprotein is the lower molecular weight autophosphorylated

subunit of the calcium/calmodulin-dependent protein kinase II (Schrama et al., 1986b) and the 52 kDa phosphoprotein is the major phosphorylated coated vesicle protein (Schrama et al., 1986b, De Graan et al., 1986b). These data indicate that the molecular weight of B-50 determined by SDS-PAGE will be higher, the lower the amount of acrylamide in the gel. This characteristic of the B-50 protein has added to the confusion on the nature of this protein in various laboratories.

Separation of purified B-50 on a narrow pH gradient (pH 5.0-3.5) in the first dimension and on SDS-PAGE in the second dimension, results in 4 distinctprotein spots (48 kDa on 11% acrylamide) which are partly interconvertible by extensive phosphorylation or dephosphorylation of B-50 (Zwiers et al., 1985). From these data the conclusion was drawn that B-50 contains at least 2 phosphorylatable sites, as confirmed by limited digestion of the protein with Staphylococcus aureus protease V-8 (Zwiers et al., 1985). The microheterogeneity displayed in isoelectric focussing is most probably not the result of the presence of a glycomoiety on B-50, since several methods failed to detect the presence of sugar residues (Zwiers et al., 1985).

Phosphorylation Characteristics of B-50

The phosphorylation of B-50 by its endogenous B-50 kinase in synaptosomal plasma membranes (SPM) is inhibited by ACTH and several of its congeners and this effect of ACTH was the result of inhibition of B-50 kinase rather than a stimulation of B-50 phosphatase (Zwiers et al., 1976, 1978). The activity of the kinase is not influenced by cyclic nucleotides (Zwiers et al., 1976), but is highly sensitive to calcium (Zwiers et al., 1980). Calmodulin in our hands, either purified from calf brain or obtained from a commerical source (Sigma, St. Louis, MO) does not stimulate nor inhibit the phosphorylation of B-50 tested at several calcium concentrations (Zwiers et al., 1980). This in contrast with the results of Gower et al. (1986), who showed that a small but significant stimulation of B-50 phosphorylation could be observed at 50-130 nM free calcium; a similar observation was made by the group of Pfenninger (Katz et al., 1985). Dunkley and co-workers (Dunkley et al., 1986) also showed that the phosphorylation of B-50 in synaptosomes with $^{32}P_i$ is calmodulin-sensitive. This discrepancy could be the result of another yet unidentified kinase phosphorylating B-50 present in crude brain extracts. It should also be beared in mind that inhibition of B-50 phosphorylation by calmodulin at low calcium concentrations could be the result of binding of calcium to calmodulin, thereby lowering the effective free calcium concentration (see also Gower et al., 1986).

The protein kinase phosphorylating B-50 is similar if not identical to protein kinase C (Aloyo et al. 1982, 1983). This conclusion was based on the following data: i) both kinases are cyclic nucleotide-independent, ii) out of a number of kinases, only B-50 kinase and protein kinase C were able to phosphorylate B-50, iii) exogenously added kinase C to native SPM preferably phosphorylated B-50, iv) both protein kinase C and B-50 kinase are sensitive to calcium, phospholipid, ACTH, chlorpromazine and can be activated by proteolytic breakdown by Ca^{2+}-dependent proteases, v) the peptide maps produced by Staphylococcus aureus protease V8 are identical (Aloyo et al., 1982, 1983).

Localization of B-50

Initial immunohistochemical localization of B-50 with antibodies raised against a rat brain membrane extract revealed its presence in neuropil areas of both cerebellum and hippocampus (Oestreicher et al., 1981), whereas it was absent from cell bodies in these regions. This pattern of distribution of B-50 immunoreactivity closely resembles that found by [^{3}H]-PDB (phorbol 12,13-dibyturate) binding in both cerebellum and hippocampus

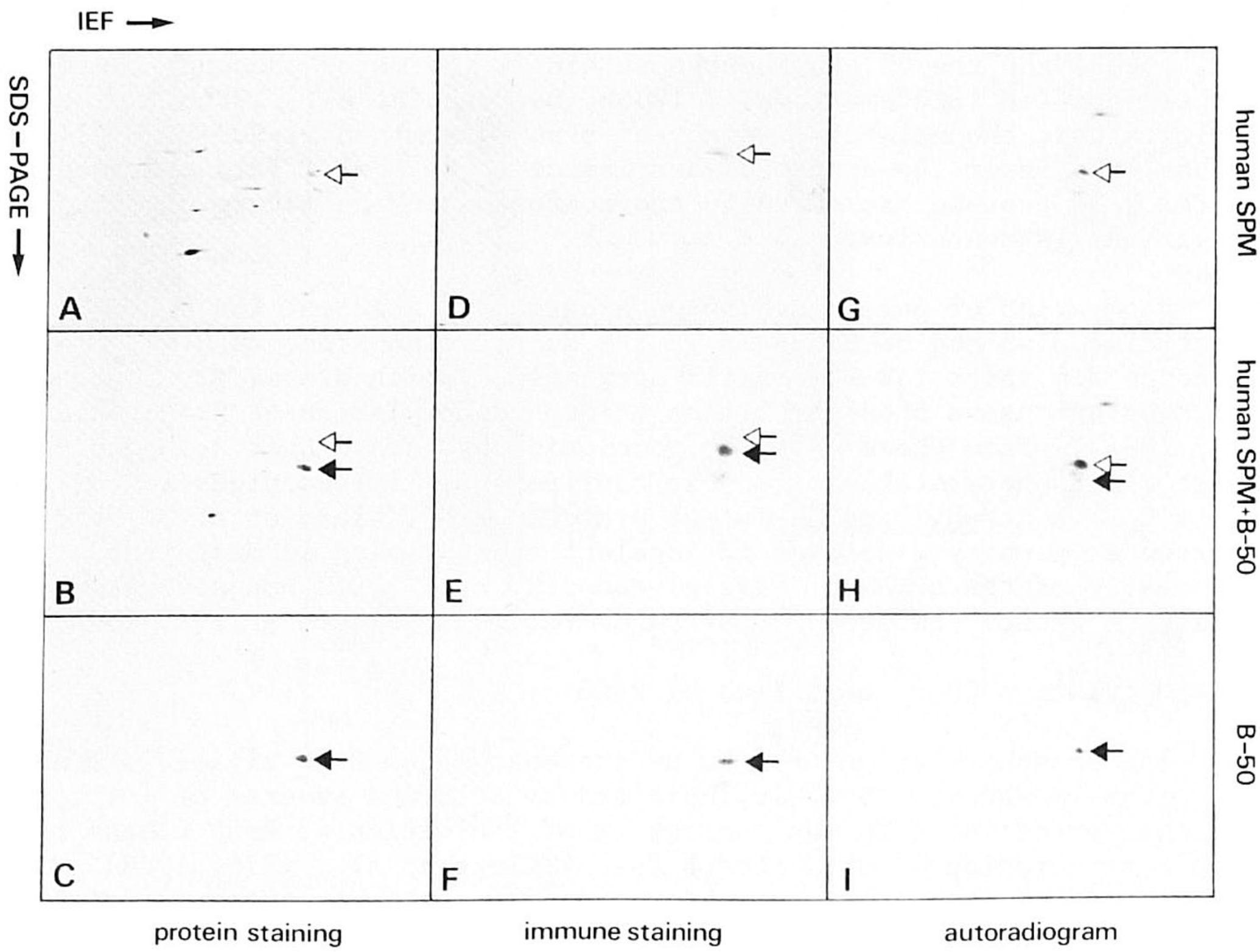

FIG. 2: Human SPM was phosphorylated under standard conditions (Kristjansson et al., 1982) and phosphorylated tracer B-50 was prepared as described by Oestreicher et al., (1986). Human SPM and/or rat B-50 were separated by isoelectric focussing (IEF) in the first dimension on a pH 9-4 gradient, followed by SDS-PAGE in the second dimension. The position of rat B-50 is indicated by a filled arrow head and the position of the human B-50-like protein by an open arrow head.

(Woley et al., 1986). The binding of PDB is most probably an indicator for the presence of protein kinase C, since this kinase is considered to be the receptor for phorbol diesters (Castagna et al., 1982, Kikkawa et al., 1983, Niedel et al., 1983). On the light microscopic level, this indicates that B-50 and its protein kinase are co-localized in neuron-enriched areas. Specific anti-B-50 immunoglobulines were purified from the crude antiserum by affinity chromatography on a Sepharose-4B column conjugated with purified B-50 (Oestreicher et al., 1983). With the purified anti-B-50-IgGs, B-50 could be demonstrated in brain homogenates of various mammals and birds, whereas no cross-reactivity was found in fish and amphibians (Oestreicher et al., 1984). Adult human brain also contains a B-50-like protein, as analyzed by Western blot of a two-dimensional polyacrylamide gel. Fig. 2 shows the migration of purified rat B-50 with human SPM after two-dimensional separation on isoelectric point and molecular weight (panels A-C, protein staining, panels G-1, autoradiogram) and the cross-reaction of the human B-50-like protein with anti-B-50 antiserum on a Western blot (panels D-F). The human B-50-like protein has a very similar isoelectric point to rat B-50, but the molecular weight is slightly higher, about 52 kDa (Fig. 2, see also De Graan et al., 1986a). Such species differences in M_r of B-50 have been reported earlier (Oestreicher et al., 1984).

In the rat, the B-50 protein could only be detected in brain and spi-

TABLE 1: B-50 levels in total homogenates (RIA) and endogenous B-50 phosphorylation in SPM of various rat brain regions.

Brain region	B-50 pg/μg tissue*	Endogenous B-50 phosphorylation in SPM**
Cortex cerebrum	43.2 ± 2.2	8.8 ± 0.4
Septum	84.4 ± 7.3	10.8 ± 0.2
Hippocampus	36.7 ± 2.4	7.2 ± 0.9
Periaquaductal gray	53.7 ± 5.1	n.d.
Cerebellum	17.6 ± 0.5	2.7 ± 0.1
Medulla spinalis	9.2 ± 1.1	0.4 ± 0.1

* n=9; Medulla spinalis n=6. ** Data obtained from Kristjansson et al. (1982). n.d.: not determined.

nal cord with the crude B-50 antiserum and endogenous B-50 phosphorylation (Kristjansson et al., 1982). Using the endogenous phosphorylation of B-50 in SPM prepared from several brain regions and two-dimensional analysis, a clear regional distribution of B-50 phosphorylating activity was observed. The order of decreasing endogenous phosphorylation activity was septum > hippocampus/neocortex > cerebellum > medullla spinalis (Kristjansson et al., 1982, Table 1). Recently, a radioimmunoassay (RIA) for B-50 was developed (Oestreicher et al., 1986) and using this more accurate method to quantify the regional distribution of B-50 we found that the distribution pattern was the same as that observed by Kristjansson et al., (1982, Table 1). These data support our earlier suggestion that the regional difference in endogenous B-50 phosphorylation reflects the content of B-50 rather than the activity of B-50 kinase (Kristjansson et al., 1982).

Ultrastructural localization of B-50 was assessed in cryosections of adult rat hippocampus with affinity-purified anti-B-50 lgGs and visualized with protein A coupled to gold particles. These studies revealed that B-50 is predominantly associated with the presynaptic terminals and that the postsynaptic protein A-gold staining was not different from that obtained with preimmune lgGs (Gispen et al., 1985b). This indicates that the B-50 protein is a presynaptic protein presumably associated with the inner surface of the plasma membrane (Gispen et al., 1985b, see also Sörensen et al., 1981).

B-50 Phosphorylation, Kinase C and Polyphosphoinositide Metabolism

Kinase C activation and B-50 phosphorylation

The nervous tissue specific protein B-50 is phosphorylated by protein kinase C (see previous section, Aloyo et al., 1982, 1983). Since this protein kinase can be stimulated by phorbol esters and diacylglycerol (Takai et al., 1979, Takai et al., 1985, Nishizuka, 1984), we studied the effects of these modulators of protein kinase C on B-50 phosphorylation in SPM either by endogenous B-50 kinase or by added purified protein kinase C. The synthetic short chain diacylglycerol, 1,2-dioctanoylglycerol (DOG) stimulated the phosphorylation of B-50 at concentrations of 100-300 μM in isolated synaptic plasma membranes, either in the absence or in the presence of exogenous protein kinase C. Comparable stimulation of protein kinase C was achieved with 1 μM DOG when histone was used as the substrate (Eichberg et al., 1986). Phorbol 12,13-dibutyrate (PDB) was a much more effective

stimulator of endogenous B-50 kinase activity than DOG. This phorbol diester stimulated the B-50 phosphorylation at 10 nM. Phorbol 12,13-diacetate (PAA) and phorbol 12-myristate 13-acetate (PMA) were effective at concentrations above 1 μM. The 4α-phorbol did not affect B-50 kinase at concentrations as high as 100 μM (De Graan et al., manuscript in preparation). In general both the effect of the short chain diacylglycerol DOG and of the tumor promoting phorbol diesters could only be detected at low calcium concentrations (300 nM) and were optimal when ionic conditions approximating intracellular concentrations in the brain were used (Eichberg et al., 1986). These data further support the hypothesis that B-50 kinase is indeed identical to protein kinase C.

B-50 phosphorylation and polyphosphoinositide (PPI) metabolism

In response to receptor activation by a variety of hormones, neurotransmitters and other extracellular messages, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphodiesteratic cleavage leads to the formation of two products, diacylglycerol (DAG) and D-myo-inositol 1,4,5-trisphosphate (IP_3) (Berridge and Irvine, 1984). DAG is considered to be the activator of protein kinase C (Nishizuka, 1984), while IP_3 is capable of mobilizing Ca^{2+} from most probably the endoplasmic reticulum (Streb et al., 1983). The simultaneous processes of protein phosphorylation and Ca^{2+} mobilization are thought to constitute synergistic effects, which are integral to a large number of cellular responses (Nishizuka, 1984, Kikkawa et al., 1986).

Some years ago we observed that ACTH, when added to isolated SPM, stimulated the phosphorylation of PIP_2 (Jolles et al., 1981, Table 2). In this same membrane preparation it was already shown that ACTH inhibited B-50 phosphorylation (Zwiers et al., 1976, 1978, Table 2). The same effects of ACTH were found in a membrane fraction prepared from human brain, where the peptide inhibited the phosphorylation of a B-50-like protein of 52 kDa (see Fig. 2) and stimulated PIP_2 phosphorylation (Table 1). Furthermore Jolles et al. (1980) demonstrated that the degree of phosphorylation of B-50 modulated the activity of PIP-kinase in SP_{55-80}, a fraction obtained from the

TABLE 2: Modulation of polyphosphoinositide (PPI) metabolism and B-50 phosphorylation in a crude rat and human plasma membrane fraction by $ACTH_{1-24}$

	Rat		Human	
	control	ACTH	control	ACTH
PIP_2	20.5±0.5	66.2±5.0*	15.4±1.8	47.0±1.0*
PIP	32.8±2.6	25.4±2.1	17.3±0.2	15.6±1.1
PI	9.8±1.3	7.2±1.3	5.6±1.1	8.3±1.6
PA	17.2±2.0	18.5±1.4	6.1±0.4	7.0±0.4
B-50	74.3±7.8	36.7±2.2*	27.3±1.0	10.1±0.4*

The incorporation of phosphate into the phospholipids is expressed as pmol/μg protein; the incorporation of phosphate into B-50 is expressed as peak height above background. The data are presented as mean ± S.E.M. (n=3). *2p<0.02, determined with Students t-test.

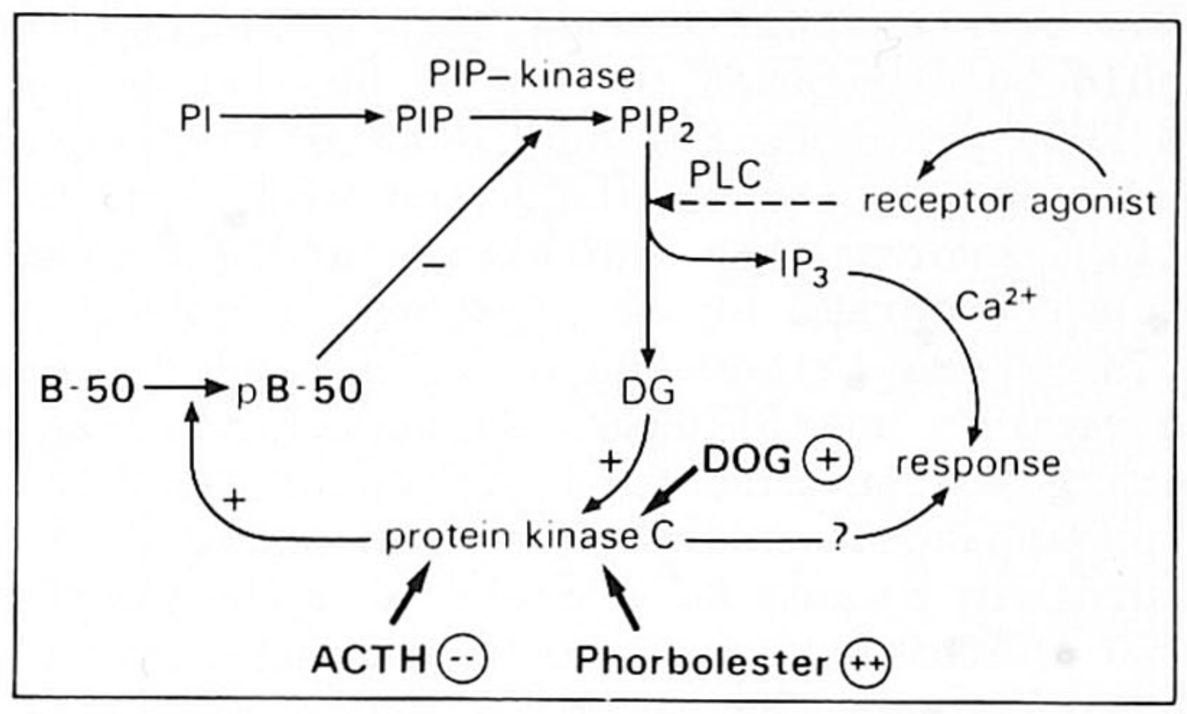

Fig. 3: Feedback control mechanism by B-50 phosphorylation in receptor-mediated hydrolysis of inositol phospholipids. For explanation of the scheme see text.

isolation procedure of B-50 (Zwiers et al., 1980). This fraction contains PIP-kinase activity as well as B-50 and its kinase, whereas phospholipase C is absent. Therefore, these observations were interpreted as a direct influence of the phosphoprotein B-50 on PIP-kinase activity. Again an inverse relation existed, as in SPM incubated with ACTH, between the degree of B-50 phosphorylation and PIP-kinase activity. Two further examples of this inverse relationship were demonstrated with dopamine in the hippocampal slice (Jork et al., 1984) and with anti-B-50 IgGs in SPM (Oestreicher et al., 1983).

PIP kinase (MW 45 kDa, IEP 5.8) has been purified from rat brain and was identified by means of specific immunostaining and reduction of enzyme activity due to interaction with affinity-purified anti-45 kDa protein antibodies (Van Dongen et al., 1984, 1986). Purified B-50 was phosphorylated with protein kinase C or dephosphorylated with alkaline phosphatase (Zwiers et al., 1985), after which both B-50 forms, the acidic phosphorylated form and the dephosphorylated basic form, were purified by flat-bed isoelectric focussing on a narrow pH gradient. When the effect of phosphorylated or dephosphorylated B-50 was tested on the activity of purified PIP kinase, the dephosphorylated form had no effect on PIP kinase activity, wherease the identical amount of phosphorylated B-50 substantially dimiished the formation of PIP_2. Non-specific protein-protein interactions were prevented by the presence of bovine serum albumin (Van Dongen et al., 1985).

These findings have led us to propose that B-50, B-50 kinase and PIP kinase exist together in a multi-molecular complex in the presynaptic plasma membrane and that the phosphorylation of B-50 exerts a regulatory effect on PIP kinase. This implies that the nervous tissue specific phosphoprotein B-50 may be part of a feedback control mechanism in the receptor-mediated hydrolysis of inositol phospholipids, by regulating the amount of PIP_2 available for phosphidiesteratic cleavage after receptor activation (Fig. 3). The hypothesis that phosphorylation of a kinase C substrate protein exerts a negative feedback function is supported by several groups and in several systems (see results from Vicentini et al., 1985, Labarca et al., 1984, Watson and Lapetina, 1985, Orellana et al., 1985, Leeb-Lundberg et al., 1985, Okano et al., 1985).

Recently we have been able to demonstrate that the inhibitory effect of protein kinase C on receptor-mediated polyphosphoinoisitide hydrolysis in hippocampal slices could be partially reversed by preincubation of the slice with $ACTH_{1-16}$. In a similar system as employed by Labarca et al.,

(1984) we were able to show that the carbachol-induced inositol phosphate (IP) formation could be inhibited by 50% by preincubation of the slice for 10 min. with 1 µM PDB, prior to the addition of 1 mM carbachol (Schrama et al., 1986a). Pre-preincubation of the slice with 5 x 10^{-5} M $ACTH_{1-16}$-NH_2 for 20 min partially reversed the inhibition of IP formation by PDB in response to carbachol (Schrama et al., 1986a). Incubation of the hippocampal slice with $ACTH_{1-16}$-NH_2 alone had no effect on IP formation. We have interpreted these results as follows. Carbachol stimulates the hydrolysis of PIP_2 after binding to the muscarinic receptor, resulting in the formation of inositol phosphates. Addition of PDB prior to the carbachol stimulation activates protein kinase C, thereby phosphorylating B-50 (Fig. 3). Phosphorylated B-50 inhibits the formation of PIP_2 by an interaction with PIP kinase, which will lower the amount of PIP_2 available for receptor-mediated hydrolysis. The reversal of the inhibition by PDB by pre-preincubation of the slice with ACTH is most probably due to inhibition of B-50 phosphorylation by the peptide, resulting in a less inhibited PIP kinase thereby leaving more PIP_2 available for hydrolysis. In human platelets PMA also inhibits the degradation of PIP_2 in response to thrombin. The inhibitor of protein kinase C 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) could reverse the inhibitory effect of PMA on phosphoinositide turnover and Ca^{2+}-mobilization (Tohmatsu et al., 1986). Concomitant with this finding these

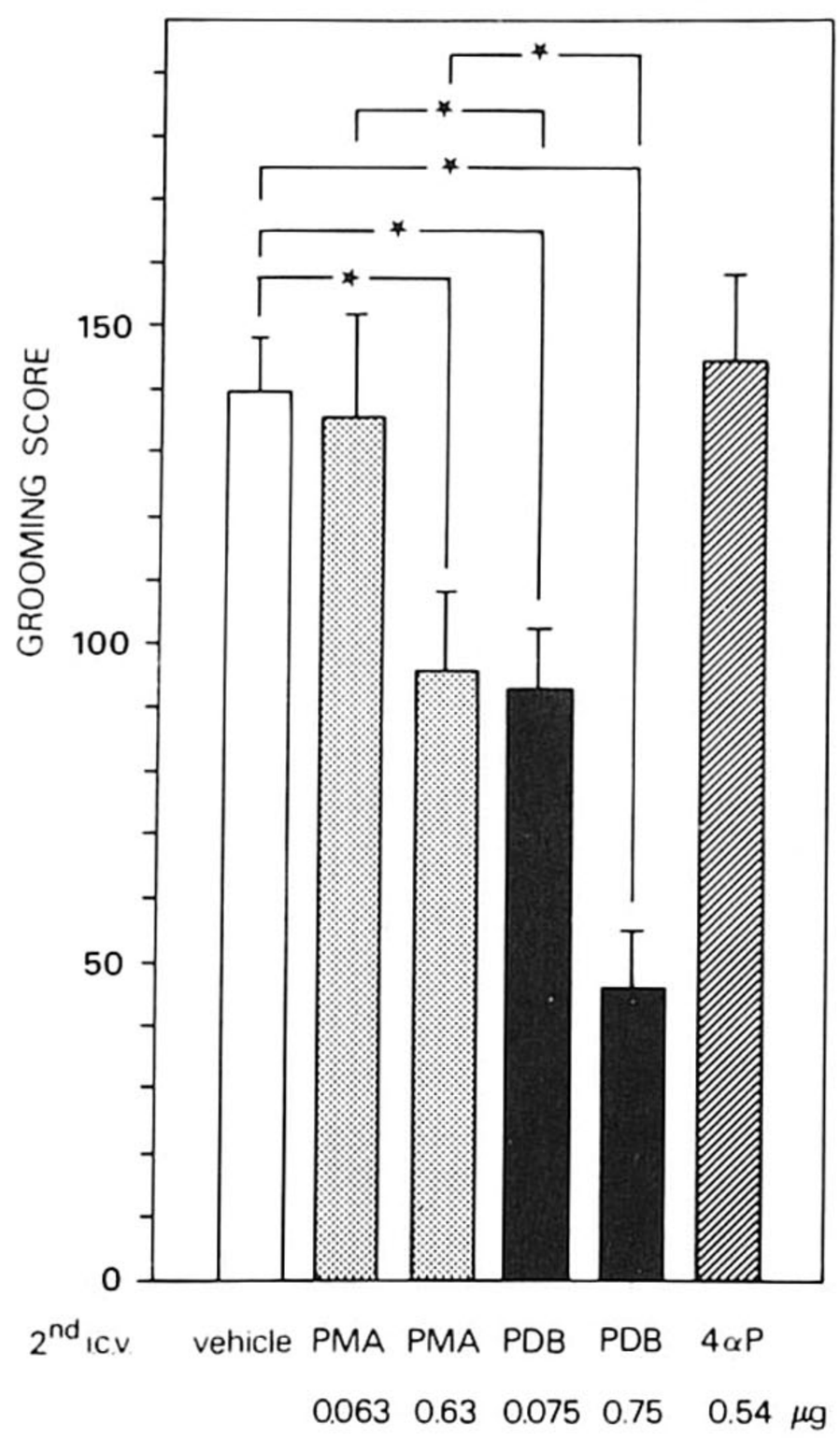

FIG. 4: Modulation of ACTH-induced excessive grooming by phorbol esters. All rats received an i.c.v. injection with 0.1 µg $ACTH_{1-24}$ in 3 µl, followed at t = 10 min by a second injection with either vehicle (0.5% ethanol in saline), phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDB) or 4α-phorbol (4αP) at the amount indicated in a volume of 3 µl. The bars represent the mean grooming score ± SEM (number of animals was at least 5 in each group). The asterisk indicates a significant difference at $p < 0.05$ determined with one-factor analysis of variance followed by a supplemental t-test.

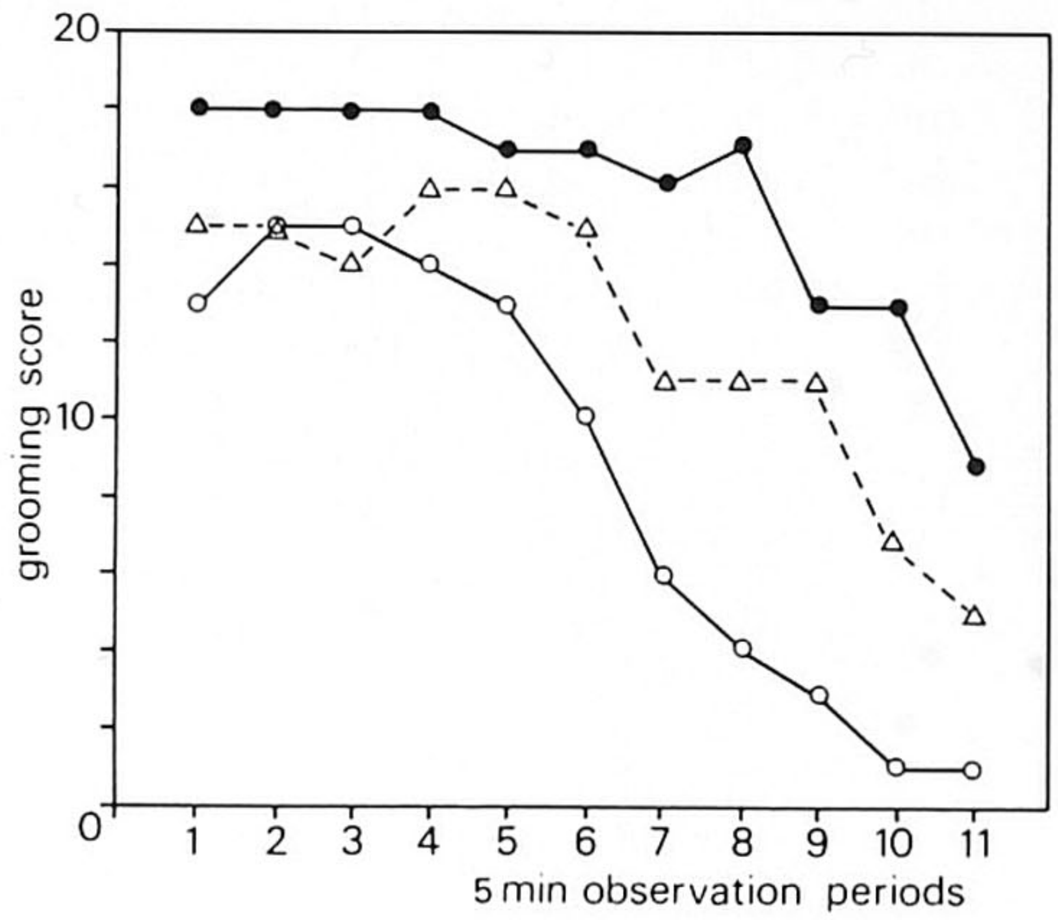

FIG. 5: Effect of DOG on ACTH-induced excessive grooming. Animals received a 1st i.c.v. injection with saline followed by vehicle (open circles), or a 1st injection with 0.1 μg $ACTH_{1-24}$ followed at 10 min by either vehicle (closed circles) or 10 μg DOG (open triangles). The mean grooming score over 5 min observation periods (maximal score 20, 5 rats per group) is given for a total grooming period of 55 min.

authors showed that the phosphorylation of the 20 kDa protein was reduced to the basal level after preincubation of the platelets with H7 before addition of PMA, and that the phosphorylation of the 40 kDa protein was only partially inhibited by pretreatment with H7. Whether one of these two phosphoproteins in the platelet may serve a similar feedback function as B-50 in nervous tissue remains to be proven.

Activators of Protein Kinase C and ACTH-Induced Excessive Grooming in the Rat

In a variety of studies we have used the peptide $ACTH_{1-24}$ as a tool to modulate the phosphorylation of B-50, irrespective of the question whether such modulation represents a physiological mechanism by which the peptide could affect the brain. The use of broken cell preparations and the relatively high peptide concentrations required to inhibit B-50 phosphorylation ($IC_{50} = 3 \times 10^{-6}$M) have cast some doubt as to the physiological importance of the inhibition of protein kinase C by $ACTH_{1-24}$ in rat brain membrane preparations. Recent experiments on the sensitivity of endogenous B-50 phosphorylation to $ACTH_{1-24}$ in SPM indicate that under special conditions $ACTH_{1-24}$ significantly inhibits B-50 phosphorylation at concentrations as low as 10^{-7}M.

Evidence is accumulating to suggest that the proposed mechanism of action of ACTH is of relevance to the induction of excessive grooming behavior (Gispen et al., 1975). First of all, it was observed that intracerebroventricular (i.c.v.) administration of $ACTH_{1-24}$ in rats followed by a post-hoc endogenous phosphorylation assay of SPM prepared from the brains of these rats, resulted in a dose- and time-dependent change in the phosphorylation of those phosphoproteins that were affected by ACTH when added to the phosphorylation assay in vitro (Zwiers et al., 1977). Thus, the effect of the peptide can be induced in the intact system and is not just an artefact of broken cell preparations.

Secondly, we have shown that the structural requirements of ACTH for

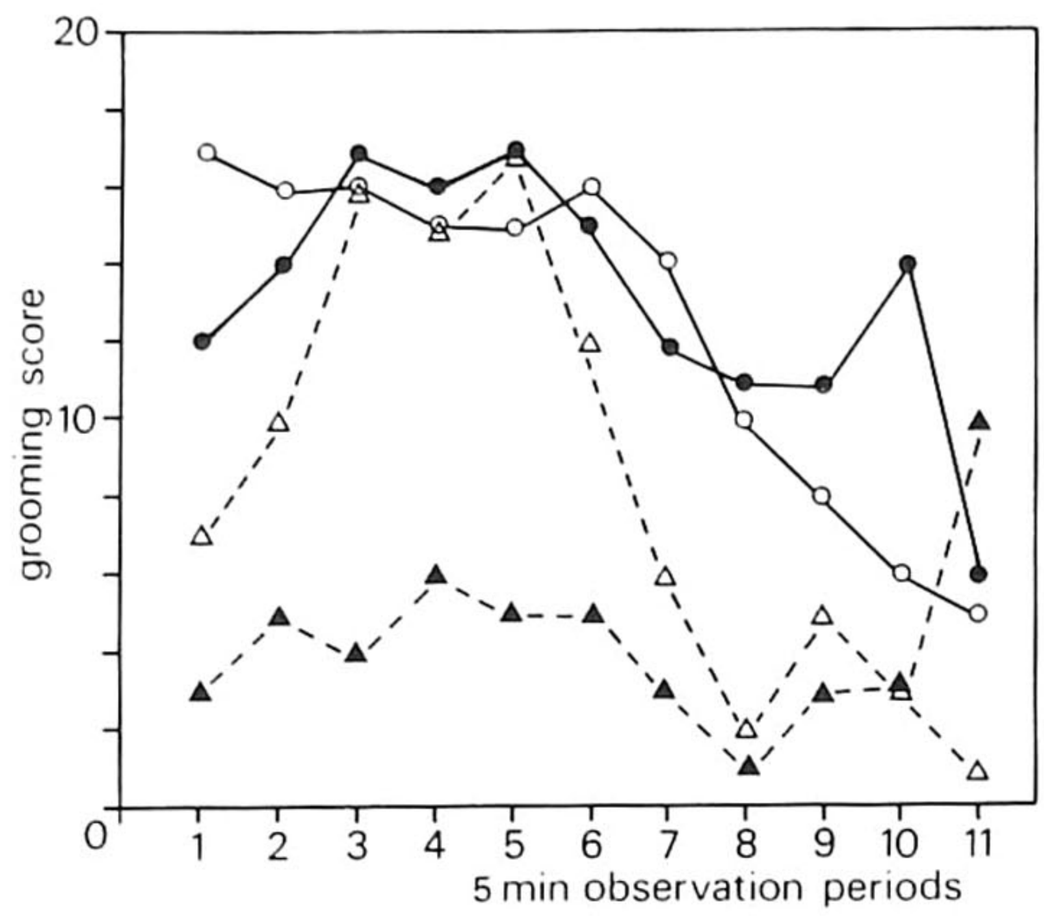

FIG. 6: Effect of phorbol esters on ACTH-induced excessive grooming. Animals received an i.c.v. injection with 0.1 μg ACTH followed at 10 min by a 2nd injection with vehicle (open circles), 0.63 μg PMA (open triangles), 0.75 μg PDB (filled triangles) or 0.54 μg 4αP (closed circles). For explanation of the grooming score see legend to Fig. 5 and text.

the in vitro inhibition of B-50 kinase in SPM are very similar to those required for the ACTH-induced excessive grooming behavior in the rat (Zwiers et al., 1978, Gispen et al., 1979). Furthermore, one of the regions which is also enriched in B-50 (see Table 1), the periaqueductal gray (PAG), is known to receive peptidergic terminals containing peptides from the pro-opiomelanocortin (POMC) family (Watson et al., 1978). The PAG is the primary target for the induction of excessive grooming behavior by ACTH-like peptides (Spruijt et al., 1986). Lesions placed in the dorsal part of the PAG, prevented the display of excessive grooming after i.c.v. injection of $ACTH_{1-24}$.

Thirdly, we have recently reported that treatment of rats with DOG suppresses ACTH-induced excessive grooming (Gispen et al., 1985c). Here, we will describe more detailed information on the inhibition of ACTH-induced grooming by both DOG and phorbol diesters.

One week prior to the grooming test, a polyethylene cannula was implanted in the third brain ventricle (Gispen and Isaacson, 1981). The rats received an i.c.v. injection of either saline or $ACTH_{1-24}$(0.1 μg/3.0 μl) at the beginning of the behavioral test. Ten min after the first injection the rats received a second i.c.v. injection with either 0.5% ethanol in saline (vehicle) or an injection with either DOG or phorbol diesters, both of which were dissolved in vehicle (total injection volume 3 μl). After this second injection the animals were placed individually in novel glass boxes and the observation of the rats started 10 min after the second injection and lasted for 55 min. Every 15th sec an observer scored whether the rats displayed elements of the grooming repertoire, yielding a maximal score of 220. The individual grooming scores were averaged per group and analyzed by one-factor of variance followed by an adjusted supplemental t-test. The results of such an experiment for 3 different phorbol esters are given in Fig. 4. In order to compare the relative potency of the 3 phorbol esters with DOG (Gispen et al., 1985c), equimolar amounts to 1 μg DOG were used. As can be seen from the data presented in Fig. 4, the non-active 4-α -phorbol did not reduce the grooming score compared to the second injection with vehicle. PDB was more effective in reducing the ACTH-induced excessive grooming than PMA was.

In order to gain better insight on the time course of inhibition of excessive grooming by the phorbol diesters and by DOG, the grooming score over 5 min time periods is given in Figs. 5 and 6. The inhibition of the grooming by DOG is already visible during the first observation times and persists over the whole observation period (Fig. 5). The inhibition by 0.75 μg PDB resembled that of DOG, but again it is shown that PDB is a much more potent inhibitor than DOG (Fig. 6). PMA did inhibit grooming behavior at the onset of the observation period after which no effect was observed during 15 min (Fig. 6). From the 5 min observation periods 6-11, PMA again inhibited the excessive grooming behavior. It thus seems that the major effect of the active phorbol diesters and DOG is observed in the dopamine-sensitive portion of the peptide-induced excessive grooming behavior (Isaacson et al., 1983). From Fig. 5 and 6 it is also clear that the order of potency for inhibition of grooming by kinase C activators is PDB > PMA > DOG > 4-α-phorbol. This order of relative potency for inhibition of grooming behavior is very similar to that found for the stimulation of B-50 phosphorylation in SPM (see above, Eichberg et al., 1986, De Graan et al., in preparation). These data support the notion that modulation of protein kinase C activity may be part of the molecular mechanism underlying ACTH-induced excessive grooming.

CONCLUDING REMARKS

In nervous tissue the inositol cascade seems to be controlled by the phosphorylation of a membrane-bound protein, B-50, by the calcium phospholipid-dependent protein kinase C. This hypothesis is supported by several findings using both biochemical and behavioral paradigms. In the hippocampal slice system, preincubation of the slice with ACTH seems to prevent the phorbol diester-induced inhibition of IP formation in response to carbachol. In the whole animal, phorbol diesters have the capacity to reduce ACTH-induced excessive grooming. It remains to be proven, whether in both systems PIP_2 breakdown after receptor activation is modulated by the degree of B-50 phosphorylation and also whether the degradation of PIP_2 itself is involved in the induction of excessive grooming behavior in the rat by ACTH. Very recently, Farese and coworkers have been able to demonstrate in rat adrenal cells that ACTH at certain concentrations can activate both the cAMP and the IP_3-Ca^{2+} intracellular signalling system (Farese et al., 1986). Very low concentrations of $ACTH_{1-24}$, between 10^{-14}-10^{-11} M, significantly stimulated the formation of inositol phosphates, with a concomitant increase in the intracellular calcium concentration, whereas at concentrations above 10^{-10} M cAMP production was very pronounced.

As mentioned above several groups have independently shown that stimulation of protein kinase C by either phorbol diesters or synthetic diacylglycerols partially prevents the rise in cytosolic calcium in response to receptor stimulation. One of the explanations for this action of protein kinase C is that it phosphorylates a specific substrate protein thereby regulating the amount of PIP_2 available for phosphodiesteratic cleavage. For those cells containing B-50, phosphorylation of this protein might indeed fulfill this role. On the other hand, evidence has been gathered that stimulation of protein kinase C by either diacylglycerol analogues or phorbol diesters attenuates the calcium current through voltage-sensitive calcium channels (Di Virgilio et al., 1986, Rane and Dunlap, 1986). It would be of particular interest to measure the activation of protein kinase C and/or to add either protein kinase C itself or antibodies directed against the kinase to establish its role in modulating these voltage-sensitive calcium-channels. This is especially necessary since DeRiemer et al. (1985a) showed exactly the opposite effect, namely an increase in calcium

current due to activated protein kinase C in Aplysia bag cells. Moreover these authors could also demonstrate an endogenous enzyme in these cells, that was activated by PMA and had properties very similar to mammalian protein kinase C (DeRiemer et al., 1985b). The endogenous substrate proteins phosphorylated by this protein kinase C-like enzyme are not yet identified and it is therefore not clear whether the modulation of calcium channels by protein kinase C involves the phosphorylation of the channel itself or whether the inhibition is due to a secondary effect of protein kinase C via the polyphosphoinositide metabolism.

In order to understand more about the role of protein kinase C in the modulation of excessive grooming behavior induced by ACTH in the rat, we will have to establish whether our biochemical observations in the hippocampal slice also apply to the whole animal. In view of the fact that the PAG is involved in the expression of grooming behavior and the fact that ACTH-terminals as well as B-50 protein are present in this structure, we believe that studies in the PAG will help us to understand how ACTH induces excessive grooming behavior and learn more about the role of phosphoprotein B-50 in the nervous system.

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