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MODULATION OF B-50 PHOSPHORYLATION AND POLYPHOSPHOINOSITIDE METABOLISM IN SYNAPTIC PLASMA MEMBRANES BY PROTEIN KINASE C, PHORBOL DIESTERS AND ACTH

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ABSTRACT

One of the major phosphoproteins in synaptic plasma membranes (SPM) is the neuron-specific protein B-50 (M_r 48 kDa, IEP 4.5). Addition of purified protein kinase C (PKC) to native SPM increases B-50 phosphorylation. Exogenous PKC also phosphorylates B-50 in heat-inactivated SPM. Endogenous phosphorylation of B-50 in SPM is enhanced in a concentration-dependent manner by the tumor-promoting phorbol diesters 4β-phorbol 12-myristate,13-acetate, 4β-phorbol 12,13-dibutyrate (PDB) and 4β-phorbol 12,13-diacetate, with an EC_{50} of 7×10^{-8} M, 3×10^{-7} M and 10^{-6} M, respectively. This increase in the B-50 phosphorylation can be inhibited by $ACTH_{1-24}$. PDB (10^{-6} M) also stimulates B-50 phosphorylation by exogenous PKC in native and heat-inactivated SPM (204 and 712%, respectively).

The increase in B-50 phosphorylation induced by the addition of PKC to SPM is accompanied by a decrease in the [^{32}P]-incorporation into phosphatidylinositol 4,5-bisphosphate (PIP_2). These data support the hypothesis that in neuronal membranes the degree of B-50 phosphorylation exerts a negative control on receptor-mediated hydrolysis of PIP_2 in receptor systems coupled to phospholipase C.

INTRODUCTION

Protein kinase C (PKC) is a multi-functional regulatory enzyme phosphorylating multiple substrates (for reviews see 1-3). In neuronal systems the functional role of PKC and its substrate proteins is poorly understood. Recent studies by several laboratories indicate a role of PKC in neurotransmission (4-8), in feedback systems in transmembrane signal transduction (2,9) and in the regulation of ion channels (4,10-12). One of the major substrates of PKC in neuronal membranes is the nervous tissue-specific protein B-50 (M_r 48 kDa, IEP 4.5; 13). The endogenous kinase phosphorylating B-50 in synaptic plasma membranes (SPM) is indistinguishable from PKC (14,15) and can be stimulated by tumor-promoting phorbol diesters (16,17). Protein B-50 is a member of a family of proteins which are rapidly expressed during neurite outgrowth, i.e., the growth-associated proteins (GAPs; 18). The B-50 protein appears to be identical to GAP43 (19,20), GAP48 (21,22), pp46 (23,24) and F1 (25).

Endogenous activation of PKC is thought to be elicited by diacylglycerol formed upon receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). Detailed phosphorylation studies have revealed that in SPM a reciprocal relationship exists between the extent of B-50 phosphorylation and the degree of PIP_2 labelling (for a recent review see 17). For instance, specific reduction of the degree of B-50 phosphorylation by treatment of SPM with affinity-purified anti-B-50 IgGs (26) or ACTH (17,27) resulted in a concomitant increase in the [^{32}P]-incorporation into PIP_2. These findings have led us to propose that the degree of B-50 phosphorylation exerts a regulatory effect on the enzyme phosphatidylinositol 4-phosphate kinase (PIP-kinase), the rate-limiting enzyme in PIP_2 synthesis (see 17).

In this study we further characterize the phosphorylation of B-50 in SPM by PKC using phorbol diesters and ACTH. Furthermore, we provide new evidence for a reciprocal relationship between the degree of B-50 phosphorylation and PIP_2 labelling in SPM.

MATERIALS AND METHODS

Chemicals

4β-phorbol 12,13-dibutyrate (PDB), 4β-phorbol 12-myristate, 13-acetate (PMA), 4β-phorbol 12,13-diacetate (PDA), 4α-phorbol and 4α-phorbol 12,13-didecanoate (4α-PDD) were purchased from Sigma (USA). $ACTH_{1-24}$ was a gift from Organon International BV (Oss, NL). [$-^{32}P$]-ATP (spec.act. 3000 Ci/mmol) was obtained from Amersham (UK).

Phosphorylation Assay

SPM were prepared from male rats (140-150 g) of an inbred Wistar strain (TNO, Zeist, NL) and assayed for endogenous phosphorylating activity as described earlier (29). The phosphorylation reaction mixture contains 10 µg protein, 7.5 µM/2 µCi [$-^{32}P$]-ATP in buffer A (20 mM HEPES, pH 7.4, 10 mM Na^+-acetate, 10 mM Mg^{2+}-acetate, 80 mM KCl, 1 mM EGTA, 0.9 mM Ca^{2+}-acetate). When other buffer systems were used, their composition is given in the appropriate figure legends. After a 5 min preincubation at 30°C, the phosphorylation reaction was started by addition of ATP. $ACTH_{1-24}$ was added 15 s and phorbol diesters 75 s prior to the ATP. In some experiments 0.24 µg (2 µl) purified PKC (30) was added at the start of the preincubation. The final incubation volume was 25 µl. Inactivation of endogenous enzymes was performed by heating SPM proteins for 5 min at 100°C. The protein phosphorylation reaction was stopped after 15 s by addition of a SDS-denaturing solution (31) and lipid phosphorylation by addition of 2 ml ice-cold chloroform/methanol/12 N HCl (200:100:0.75; v/v; 27).

Quantitative and Qualitative Analysis of Phosphorylation

Proteins: denatured proteins were separated on SDS-polyacrylamide slab gels (acrylamide 11%, bisacrylamide 0.2%) as described earlier (13). After protein staining with Fast Green, gels were dried and subjected to autoradiography. [^{32}P]-incorporation into protein bands was determined by densitometric scanning of the autoradiograms and by liquid scintillation counting after excision

from the gel. Protein was determined as described by Lowry et al. (31) using bovine serum albumin as standard.

Lipids: lipids were extracted from the reaction mixture and quantitatively separated by TLC (27). Lipid spots on TLC plates were visualized with iodine vapor and by autoradiography. The [^{32}P]-labelled spots were scraped from the plates and radioactivity was measured by liquid scintillation counting.

RESULTS

After endogenous phosphorylation of SPM with [γ-^{32}P]-ATP under our assay conditions, B-50 is one of the major phosphoproteins (Fig. 1, lanes A). Addition of purified PKC to the SPM prior to the endogenous phosphorylation results in a large increase in the [^{32}P]-incorporation into B-50 (Fig. 1, lanes B). Not only B-50 phosphorylation, but also that of other PKC substrates with apparent molecular weights of 87, 20 and 18 kDa is increased. Phosphorylation of heat-inactivated SPM proteins with purified PKC results in the [^{32}P]-labelling of B-50 and those same 3 major PKC substrates (Fig. 1, lanes C). [^{32}P]-incorporation into B-50 and the other PKC substrates is highest after PKC-mediated phosphorylation of heat-inactivated SPM.

The degree of B-50 phosphorylation in SPM can also be enhanced by addition of PDB (Fig. 2). In the presence of 10^{-6} M PDB endogenous B-50 phosphorylation increases by about 80%. However, this increase can only be found in phosphorylation buffer A (Fig. 2), containing 80 mM K^+ and about 0.3 µM Ca^{2+}, concentrations which approximate intracellular conditions. Thus, the potency of 4 phorbol esters to enhance endogenous B-50 phosphorylation in SPM is tested in buffer A (Fig. 3). The most potent compound is PMA, inducing 50% stimulation at about 7 x 10^{-8} M. PDB induces 50% stimulation at about 3 x 10^{-7} M and PDA at 10^{-6} M (Fig. 3). PMA and PDB significantly enhance B-50 phosphorylation at concentrations as low as 10^{-8} M. A phorbol derivative known to be inactive with respect to PKC activation, 4α-phorbol, does not affect B-50 phosphorylation in concentrations up to 10^{-5} M (Fig.

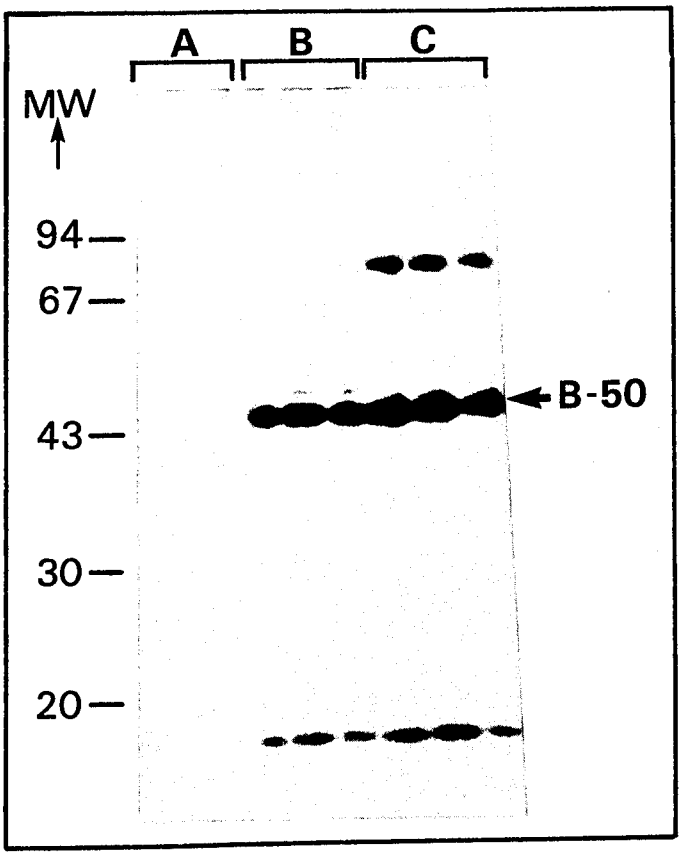

Fig. 1. Autoradiogram showing phosphorylated SPM proteins after separation on 11% SDS gels. SPM proteins (10 µg) were phosphorylated with [γ-^{32}P]-ATP in acetate buffer (pH 6.5, 10 mM Na^{+}-acetate, 10 mM Mg^{2+}-acetate and 0.1 mM Ca^{2+}-acetate) in the absence (lanes A) or presence of 2 µl purified PKC (lanes B) or with purified PKC after heat inactivation (lanes C). The position of the molecular weight markers is indicated on the left.

3). Similar results are obtained with the inactive phorbol diester 4α-PDD (results not shown). The increase in B-50 phosphorylation by PDB is inhibited by $ACTH_{1-24}$ (Fig. 4). $ACTH_{1-24}$ (3×10^{-5} M) inhibits endogenous B-50 phosphorylation by 46% and PDB-stimulated phosphorylation by 48%.

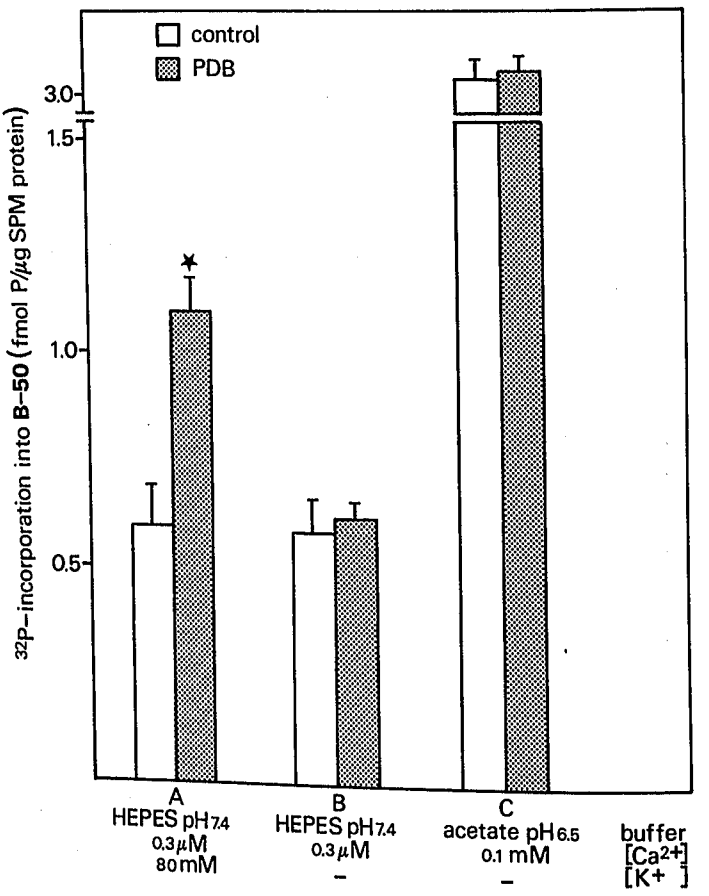

Fig. 2. Effect of 10^{-6} M PDB on endogenous B-50 phosphorylation in SPM. SPM proteins (10 µg) were phosphorylated with or without PDB in HEPES buffer (buffer A) with 80 mM K^+ (A, n = 12), without K^+ (B, n = 6) or in acetate buffer (C, n = 6; constitution see Fig. 1). The B-50 band was excised from the 11% SDS gel and analyzed for [^{32}P]-incorporation. Data are expressed as fmol P/µg SPM protein ± SEM. * significantly different from control ($p < 0.001$)

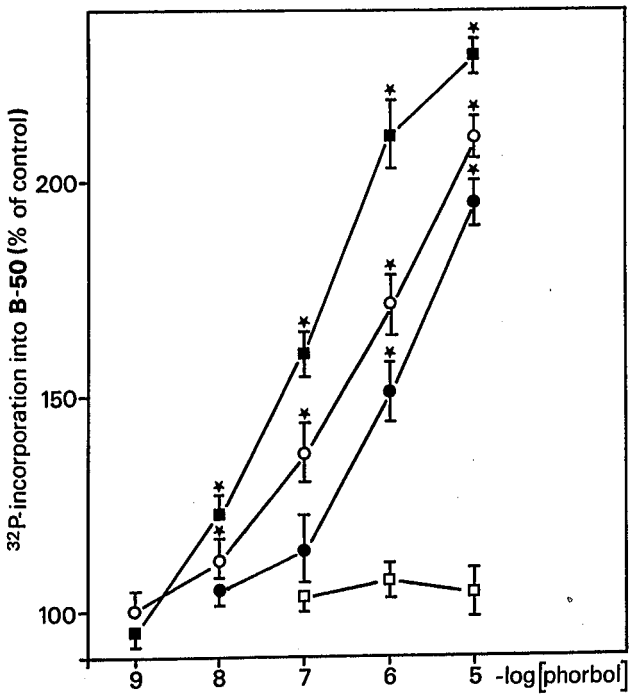

Fig. 3. Dose-response curves of the effect of PMA (■), PDB (○), PDA (●) and 4α-phorbol (□) on endogenous B-50 phosphorylation in SPM. Data are expressed as % of control ± SEM. * Significantly different from control ($p < 0.001$, $n = 9$).

Phorbol diesters not only stimulate endogenous B-50 phosphorylation in SPM (Figs. 3 and 5A), but also when purified PKC is added to native SPM (Fig. 5B) or when heat-inactivated SPM is phosphorylated by purified PKC (Fig. 5C). The addition of 10^{-7} M PDB to these 3 systems (A, B and C) results in an increase in B-50 phosphorylation of 37, 57 and 72%, respectively (Fig. 5). At 10^{-6} M PDB the increase in B-50 phosphorylation is 71, 204 and 712%, respectively.

The addition of PKC (without PDB) to native SPM in buffer A (HEPES, pH 7.4, 0.3 μM Ca^{2+}, 80 mM K^{+}) results in an increase in [^{32}P]-incorporation into B-50 of 48% (Fig. 5). Phosphorylation of

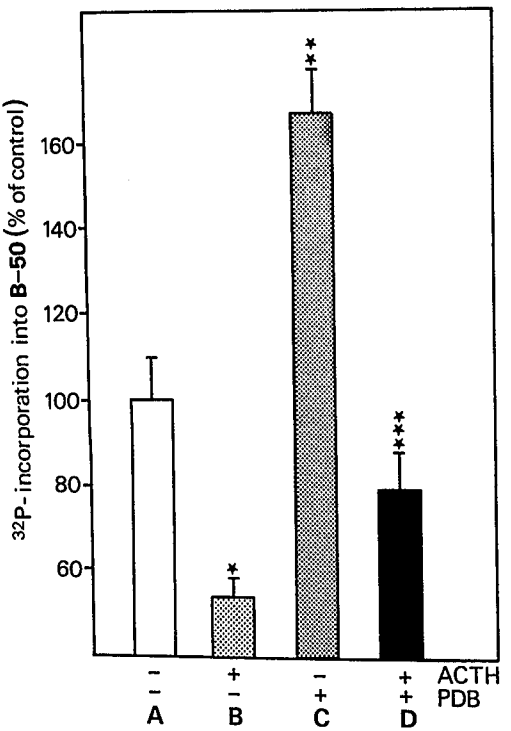

Fig. 4. $ACTH_{1-24}$-induced inhibition of endogenous and PDB-stimulated B-50 phosphorylation in SPM. SPM proteins were phosphorylated with and without 3×10^{-5} M $ACTH_{1-24}$ in the presence or absence of 10^{-6} M PDB (for details see Materials and Methods). Data are expressed as % of control ± SEM ($n = 6$). * Significantly different from control A ($p < 0.001$), ** significantly different from A and B ($p < 0.001$), *** significantly different from C ($p < 0.001$) and from A ($p < 0.05$).

B-50 by PKC in heat-inactivated SPM is 183% higher than in native SPM (Fig. 5). In acetate buffer (pH 6.5, 0.1 mM Ca^{2+}) the addition of PKC to native SPM induces a 173% increase in B-50 phosphorylation (Table 1). This acetate buffer is not suitable for the stimulation of endogenous B-50 phosphorylation by phorbol diesters (Fig. 2, Table 1), but has been used in studies on [^{32}P]-incorporation into PIP_2, because in buffer A PIP_2 labelling is virtually absent (P.N.E. De Graan, unpublished).

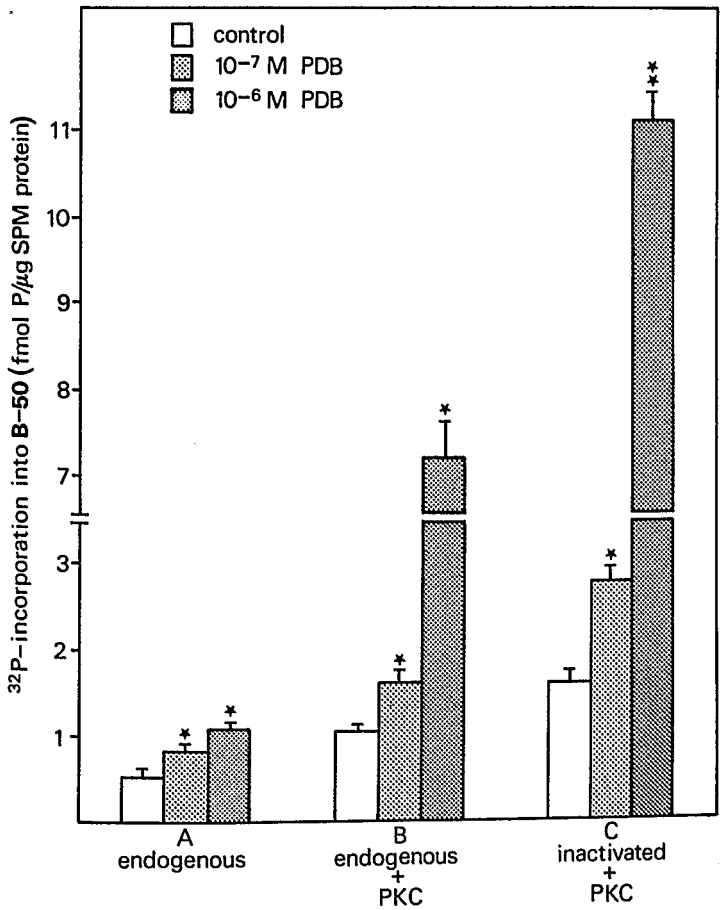

Fig. 5. Effect of PDB on B-50 phosphorylation in SPM in the absence (A) or presence of purified PKC (B) or in heat-inactivated SPM phosphorylated with purified PKC (C). (for details see Materials and Methods). Data are expressed as fmol P/µg protein ± SEM (n = 12). * Significantly different from control ($p < 0.001$).

In a number of paradigms we have shown that there is an inverse relationship between the degree of B-50 phosphorylation and the labelling of PIP_2 in SPM (reviewed in 17). For instance $ACTH_{1-24}$ inhibits endogenous B-50 phosphorylation by 62.5% and concomitantly induces a 69% increase in PIP_2 labelling (Table 1).

Table 1. Effects of purified PKC (2 µl), PDB (10^{-6} M) and $ACTH_{1-24}$ (3×10^{-5} M) on B-50 phosphorylation and PPI metabolism in SPM.

Addition	[^{32}P]-incorporation (fmol P/µg SPM protein)			
	PIP_2	PIP	PA	B-50
None	4.2 ± 0.4	8.9 ± 0.9	1.8 ± 0.2	5.6 ± 0.1
PKC	2.0 ± 0.3*	12.4 ± 1.2	0.5 ± 0.1*	15.3 ± 0.2*
PKC + PDB	1.8 ± 0.2*	11.9 ± 1.4	0.6 ± 0.1*	22.7 ± 0.3*
PDB	3.9 ± 0.2	8.0 ± 1.4	1.9 ± 0.3	6.1 ± 0.1
$ACTH_{1-24}$	7.1 ± 0.6*	8.4 ± 0.7	2.2 ± 0.3	2.1 ± 0.1*

Data are expressed as fmol P/µg SPM protein ± SEM. * Significantly different from control ($p \leq 0.001$, $n = 6$).

Therefore, we investigated whether such an inverse relationship exists in SPM between the PKC-induced increase in B-50 phosphorylation and the degree of PIP_2 labelling (Table 1). Indeed, the 173% increase in B-50 phosphorylation induced by PKC is accompanied by a 53% reduction in the PIP_2 labelling (Table 1). Addition of 10^{-6} M PDB and PKC further enhances the degree of B-50 labelling, but does not further decrease PIP_2 labelling (Table 1). Addition of 10^{-6} M PDB (without PKC) to native SPM does not stimulate B-50 phosphorylation in this acetate buffer and has no effect on PIP_2 labelling either (Table 1). PIP labelling is not significantly affected by the addition of PKC and/or PDB, whereas PA labelling is significantly decreased by the addition of PKC in the presence or absence of PDB, but not by PDB alone (Table 1).

DISCUSSION

The endogenous kinase phosphorylating the neuron-specific protein B-50 in SPM is PKC. The evidence for this conclusion can be summarized as follows: (a) The endogenous B-50 phosphorylation is Ca^{2+} dependent and cyclic nucleotide independent (17); (b) the major biochemical characteristics (including IEP, peptide map, phospholipid dependency) of the isolated B-50 kinase resemble

those of purified PKC (14,15); (c) B-50 is a major substrate of exogenous PKC in native and heat-inactivated SPM (this paper); (d) B-50 phosphorylation by endogenous and exogenous PKC is enhanced by phorbol diesters (this paper) and by the short chain diacylglycerol, 1,2-dioctanoylglycerol (16); (e) purified B-50 is a substrate to PKC (14,15); (f) treatment of intact neuronal tissue with phorbol diesters enhances B-50 phosphorylation (5,32,33).

Tumor-promoting phorbol diesters increase endogenous B-50 phosphorylation in SPM presumably through direct activation of PKC (35). The order of potency, PMA > PDB > PDA, is similar to that found in many systems. Interestingly, phorbol ester stimulation of B-50 phosphorylation in SPM requires 80 mM K^+, indicating that intracellular ionic conditions are essential. In a crude mitochondrial/synaptosomal fraction phorbol esters have been shown to stimulate F1 phosphorylation in the presence of detergent (35). It is not clear what the mechanism of activation of endogenous PKC is. Possibly phorbol esters lower the Ca^{2+} requirement for PKC activation or improve the B-50 SPM interaction for instance by a tighter binding of PKC to the membrane (36). Phorbol diesters also stimulate B-50 phosphorylation by exogenous PKC, presumably by inducing the translocation of PKC to the SPM (37). Such <u>in vitro</u> translocation of PKC has been shown in inside-out erythrocyte vesicles and in lysed synaptosomes (36). It remains to be shown whether endogenous and exogenous PKC phosphorylate B-50 at the same phosphorylation site. The fact that heat inactivation of SPM does not impair the effect of phorbol diesters suggests that membrane proteins are not essential for PKC binding or that PKC binding proteins are heat stable, like B-50 (26).

The function of the B-50 protein in neuronal membranes has not yet been resolved. In fetal rat brain and regenerating peripheral neurons B-50 has been implicated in the mechanism of neurite outgrowth (17, 19-24). In the central nervous system F1 (B-50) may be involved in membrane processes related to the phenomenon of long-term potentiation (38). We have suggested that B-50 is involved in the modulation of receptor-mediated PPI

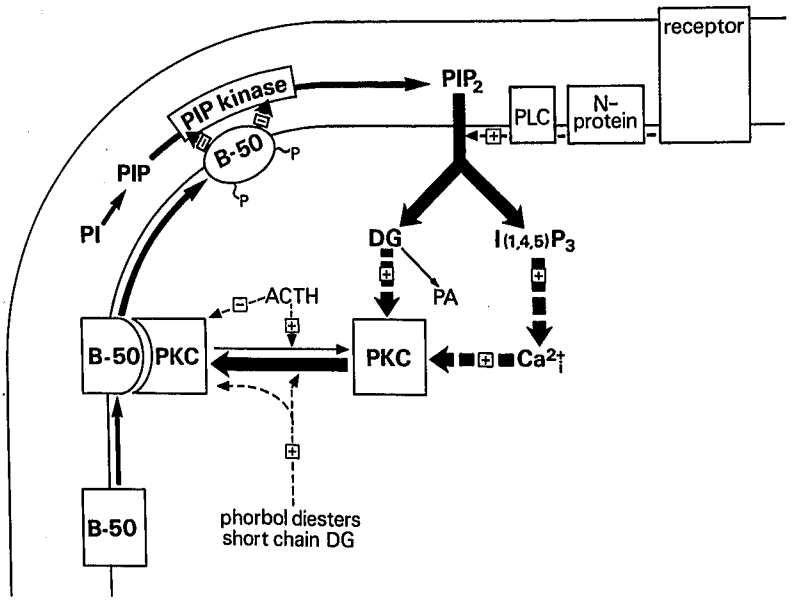

Fig. 6. Model describing the regulatory role of B-50 phosphorylation in receptor-mediated hydrolysis of PIP_2. DG, diacylglycerol; PLC, phospholipase C; N-protein, nucleotide binding protein; IP_3, inositol trisphosphate. For other abbreviations see text.

hydrolysis (see 17; Fig. 6). This hypothesis is based on the inverse relationship between the degree of B-50 phosphorylation and the [^{32}P]-incorporation found in a number of experimental approaches and can be summarized as follows: (a) pre-phosphorylation of B-50 in a partially purified B-50 preparation and SPM results in a decrease in PIP_2 labelling (27,39); b) inhibition of B-50 phosphorylation by $ACTH_{1-24}$ induces a concomitant increase in PIP_2 labelling, while the rate of loss of PIP_2 labelling is unaffected by ACTH (27); (c) affinity-purified anti-B-50 IgGs added to SPM specifically inhibit B-50 phosphorylation and simultaneously enhance [^{32}P]-incorporation into PIP_2 (26; (d) treatment of intact rat hippocampal slices with dopamine results in a decrease in the post hoc phosphorylation of B-50 and an

increase in PIP_2 labelling (40); (e) exogenous PKC enhances B-50 phosphorylation and inhibits PIP_2 labelling in SPM (this paper). We have proposed that the molecular mechanism underlying this inverse relationship is a direct modulatory effect of the degree of B-50 phosphorylation on the rate-limiting enzyme in PIP_2 synthesis, PIP-kinase, rather than a direct effect of PKC on other steps in the Ca^{2+}-mobilizing signal transduction pathway, for instance at the level of the receptor, the G-protein or the PLC. This suggestion is based on the fact that (i) mono-specific IgGs against B-50 specifically inhibit B-50 phosphorylation (no other PKC substrates are affected) and concomitantly enhance PIP_2 labelling in SPM, (ii) the inverse relationsip exists in a partially purified B-50 preparation, still containing the PIP-kinase and PKC, but no other PKC substrates (39), and (iii) in a semi-purified PIP-kinase system phospho-B-50 inhibits PIP-kinase, whereas dephospho-B-50 has no significant effect (41). Although we have shown in hippocampal slices that modulation of B-50 phosphorylation affects [^{3}H]-inositol phosphate production (42), more direct evidence for a feedback role of B-50 in physiological systems is required.

In search for the physiological function of B-50 we focus our research on biochemical parameters in the presynaptic terminal as B-50 has a presynaptic localization (43). Hence, we are interested in presynaptic receptor systems linked to Ca^{2+} mobilization and activation of PKC, possibly involved in presynaptic modulation of neurotransmitter release and/or long-term potentiation.

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