THE ROLE OF PROTEIN PHOSPHORYLATION IN LONG-TERM POTENTIATION

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INTRODUCTION

Long-term potentiation (LTP) is a phenomenon in which long-lasting changes in synaptic efficacy are induced by brief stimuli. It is often used as a model to investigate the mechanisms of information storage in the brain. The molecular events underlying LTP appear to occur in three phases: the initiation, development and maintenance of LTP. It is becoming clear that activation of the N-methyl-D-aspartate (NMDA) receptor-channel complex is important for the initiation and the development of certain types of LTP (for review see Collingridge and Bliss, 1987). However, there is no consensus on the molecular events responsible for the maintenance of LTP.

Most likely three mechanisms contribute to the maintenance of LTP: (i) a sustained increase in the release of excitatory amino acids (L-glutamate and L-aspartate) from the presynaptic terminal (Skrede and Malthe-Sörensen, 1981; Dolphin et al., 1982; Errington et al., 1987); (ii) an increase in the number of functional neurotransmitter receptors at the postsynaptic membrane (Lynch and Baudry, 1984); (iii) changes in synaptic morphology (Chang and Greenough, 1984).

Several lines of evidence point to a crucial role for Ca^{2+} during LTP. Extracellular Ca^{2+} is required for the induction of LTP, since lowering the extracellular Ca^{2+} concentration prevents LTP (Dunwiddie and Lynch, 1979). Moreover, a temporary increase in the extracellular Ca^{2+} concentration induces LTP-like effects, which can be blocked by specific NMDA antagonists (Turner et al., 1982; Bliss et al., 1987). The site of Ca^{2+} action presumably is intracellular, since injection of EGTA prevents LTP (Lynch et al., 1983). A role for Ca^{2+} in the maintenance of LTP is substantiated by the observation that there is a long-lasting increase in $^{45}Ca^{2+}$ uptake (Agoston and Kuhnt, 1986) and intracellular ionic Ca^{2+} concentration in synaptosomes prepared from potentiated hippocampal tissue (Lynch et al., 1987). Although these and other studies clearly establish the importance of Ca^{2+} for LTP, it is not known what the intracellular site(s) of action of Ca^{2+} is (are).

Many effects of the intracellular messenger Ca^{2+} in the brain are mediated through Ca^{2+}-dependent protein kinases, such as the Ca^{2+}/calmodulin-dependent protein kinase type II (CaMK II) and the Ca^{2+}/phospholipid-dependent protein kinase C (PKC; Nairn et al., 1985). The role of these two kinases in neurotransmitter release has recently attracted much attention (Kaczmarek, 1987). In the present paper we focus on the role of protein phosphorylation in neurotransmitter release during LTP-like phenomena. First we will summarize evidence for the role of a 52 kDa coated vesicle phosphoprotein in LTP. In the second part we introduce the neuron-specific PKC substrate protein B-50 (GAP-43, F1) and discuss the role of PKC and B-50 in LTP.

ROLE OF A 52 kDa COATED VESICLE PHOSPHOPROTEIN IN LTP

A brief tetanus (15 pulses/sec, 15 sec), applied to the perforant path-granule cell subsystem of hippocampal slices, induces significant changes in the phosphorylation of a 52 kDa protein and slight but non-

significant changes in B-50 phosphorylation (Bär et al., 1980; Lopes da Silva et al., 1982). The degree of phosphorylation of the two protein bands is unaffected when the frequency of stimulation is reduced and the duration increased, a condition not leading to LTP (Bär et al., 1980). Tetanization of the perforant path in the absence of extracellular Ca^{2+}, thereby preventing LTP and neurotransmission (Dunwiddie and Lynch, 1979), fails to generate changes in phosphorylation of the 52 kDa protein, indicating a relationship between the observed changes in protein phosphorylation and synaptic transmission.

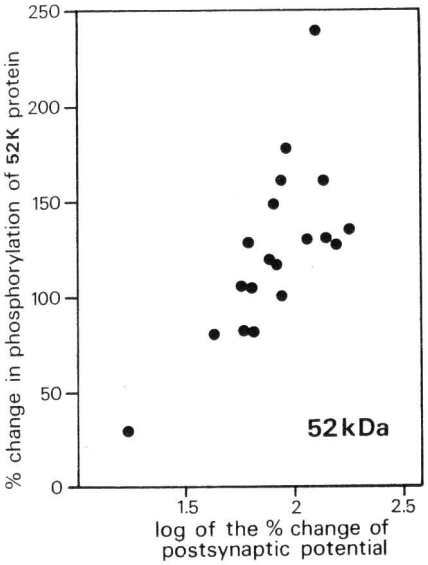

Fig. 1. Correlation between the degree of 52 kDa protein phosphorylation in hippocampal SPM employing a post-hoc phosphorylation assay 10 min after tetanization of the perforant path (15 pulses/sec, 15 sec) and the amplitude of the post-synaptic potential recorded at the granular cell layer of the fascia dentata.

Most of our initial studies were devoted to the 52 kDa protein since this protein displays the most consistent changes in post hoc protein phosphorylation following tetanization. We attempted to establish a quantitative correlation between the changes in amplitude of the post-synaptic potential (PSP) measured extracellularly, and the extent of phosphorylation of the 52 kDa protein (Tielen et al., 1983). A semi-logarithmic plot of the percentual change in 52 kDa phosphorylation versus the

change in amplitude of the PSP per individual slice, fits a straight line with a correlation coefficient of 0.71 ($p < 0.005$; Fig. 1). These data indicate that there may be a quantitative correlation between LTP and 52 kDa phosphorylation.

Brain coated vesicles contain a major 52 kDa phosphoprotein (Pauloin et al., 1982) sharing phosphorylation properties with the 52 kDa protein in synaptic plasma membranes (SPM): its phosphorylation is insensitive to cyclic-AMP, cyclic-GMP and Ca^{2+} or to the Ca^{2+}-binding protein calmodulin (Bär et al., 1982; Pauloin et al., 1984). The data summarized in Table I show that the 52 kDa protein in rat brain SPM and in coated vesicles have similar biochemical characteristics (for further details see Schrama et al., 1986[a]) and that a polyclonal antibody directed against pp50 isolated from calf brain coated vesicles cross-reacts with the 52 kDa phosphoprotein in rat brain SPM (Schrama, Pauloin and Jollès, unpublished results). Therefore, we conclude that the 52 kDa protein and pp50 are identical.

Table I. Comparison between the 52 kDa phosphoprotein in SPM and pp50 in coated vesicles.

		52 kDa	pp50
Molecular weight on 11% SDS-PAGE		52 kDa	52 kDa
Sensitivity of phospho-rylation to:	cAMP	0*	0
	calcium	0	0
	calmodulin	0	0
Phosphate acceptor amino acids		Ser, Thr	Ser, Thr
Isoelectric point range		9.0-6.5	9.0-6.5
Peptides formed after mapping with S. aureus protease V8		43,33,20 kDa	43,33,20 kDa
Immunostaining with pp50 antibody+		+	+

*= phosphorylation not sensitive to modulator
+= immunoreaction with the polyclonal pp50 antibody was determined on blots of SPM using an alkaline phosphatase-based staining procedure.

Coated vesicles and coated pits have been implicated in a number of intracellular processes, including presynaptic membrane recycling after transmitter release (Heuser and Rees, 1973; Kadota and Kadota, 1982; Miller and Heuser, 1984). Thus, enhanced transmitter release after tetanization (Bliss and Dolphin, 1982) may lead to an increase in coated pit and coated vesicle activity to retrieve excess presynaptic membrane material (Fig. 2).

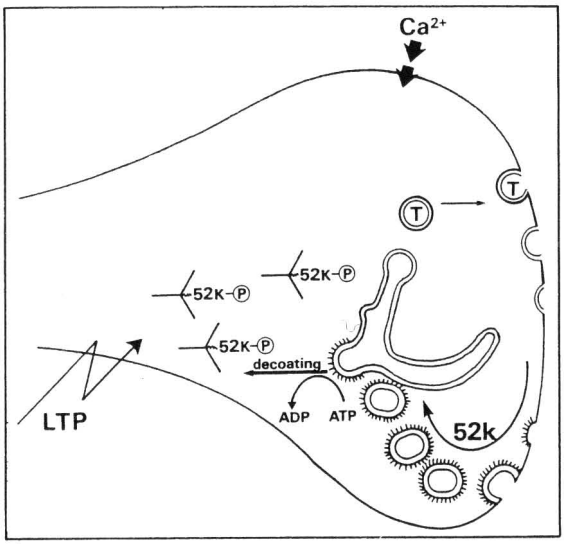

Fig. 2. Suggested role for the phosphorylation of the 52 kDa protein in coated vesicle-mediated membrane recycling and LTP.

The coated vesicle is thought to fuse with large intracellular cisternae, whereby the coat is removed from the coated vesicle (Heuser and Reese, 1973; Miller and Heuser, 1984). Preliminary data from Pauloin and Jollès (1986) suggest that the phosphorylation of pp50 can destabilize the interaction between certain coat proteins and the vesicle (Fig. 2). This idea is substantiated by the fact that the activation of pp50 kinase by the light chains of the coat protein clathrin, promotes pp50 phosphorylation (Pauloin and Jollès, 1984) and induces the dissociation of clathrin from the vesicles (Schmid et al., 1984). Therefore we propose that the changes in 52 kDa protein phosphorylation after LTP might reflect an increase in membrane retrieval concomitant with an increase in neurotransmitter release as a result of LTP.

NEURON-SPECIFIC PKC SUBSTRATE B-50

A second, well-characterized synaptosomal protein, of which the phosphorylation has been shown to be affected by tetanization is B-50 (for historical summary of research on B-50 see Table II).

Table II. Summary of research on B-50.

1976	B-50 phosphorylation sensitive to ACTH
1980	Purification of B-50: MW 48 kDa, IEP 4.5
1980	Purification of B-50 kinase
1980	Inverse relation B-50 phosphorylation PIP_2 labeling
1982	B-50 kinase = Protein kinase C
1982	B-50 is nervous tissue specific
1985	Microheterogeneity of B-50
1985	B-50 is mainly presynapticly localized (EM)
1985	Feedback role of B-50 in PPI respons
1985	B-50 in nerve growth cones
1986	B-50 levels increase during nerve regeneration
1986	B-50 = F_1 = GAP43 = pp46
1987	cDNA sequence GAP43, B-50 and F1

Under appropriate conditions it undergoes marked phosphorylation when SPM are incubated with [γ-^{32}P]-ATP (Zwiers et al., 1976). B-50 is a 48 kDa, acidic protein (IEP 4.5) that appears to be intimately associated with the plasma membrane, since it can only be solubilized in the presence of detergent (Zwiers et al., 1980). In view of its hydrophilic nature it is not likely to be an intrinsic membrane protein (Nielander et al., 1987). The purified protein displays microheterogeneity upon isoelectric focussing in a narrow pH gradient (pH 3.5-5.0): it resolves into four distinct protein spots, which are partially interconvertible by exhaustive phosphorylation or dephosphorylation (Zwiers et al., 1985). This indicates that B-50 contains more than one phosphorylation site, a notion which is supported by the fact that the primary structure of B-50 indicates three potential phosphorylation sites (Nielander et al., 1987).

Neonatal rat brain has been shown to be extremely rich in endogenous B-50 phosphorylation (Oestreicher et al., 1982). B-50 is associated with subcellular fractions enriched in growth cones (De Graan et al., 1985) and present in high amounts in outgrowing neurites in neonatal brain (Oestreicher and Gispen, 1986), in crushed peripheral neurons (Verhaagen et al., 1986) and in dorsal root ganglia in primary culture. Biochemical, immunological and recent genetical studies show that B-50 is identical to GAP43 (Karns et al., 1987; Nielander et al., 1987), pp46 (Meiri et al., 1986) and

F1 (Gispen et al., 1986; Rosenthal et al., 1987).

The endogenous kinase phosphorylating B-50 in SPM has been shown to be indistinguishable from the Ca^{2+}/phospholipid dependent PKC (Aloyo et al., 1982,1983). Of several kinases tested only PKC is able to phosphorylate purified B-50. In SPM B-50 phosphorylation can be stimulated by active phorbol diesters and by the membrane permeable diacylglycerol derivative dioctanoyl glycerol (Eichberg et al., 1986; De Graan et al., 1988), and inhibited by the PKC inhibitor polymixin B (De Graan et al., in preparation). Moreover, addition of purified PKC to SPM results in a marked increase in B-50 phosphorylation. Phosphorylation of heat-inactivated SPM with purified PKC results in an even stronger increase in B-50 phosphorylation (De Graan et al., 1988). From these data it is clear that the endogenous kinase phosphorylating B-50 in SPM is PKC.

In order to be able to measure the degree of B-50 phosphorylation *in vivo*, we developed a quantitative immunoprecipitation assay for B-50 (De Graan et al., in preparation). Intact hippocampal slices were loaded with [^{32}P]-orthophosphate and subsequently B-50 was immunoprecipitated from the solubilized slice homogenates with a polyclonal anti-B-50 antiserum. Immunoprecipitation of B-50 from a phosphorylated crude synaptosomal preparation (Fig. 3, lane 1) with crude antiserum results in a single phosphoband in the immunoprecipitate (lane 2), which disappears when an excess of cold B-50 was added during the immunoprecipitation (lane 3). Similar results are obtained when affinity-purified IgGs are used in the absence (lane 4) or presence (lane 5) of an excess of cold B-50.

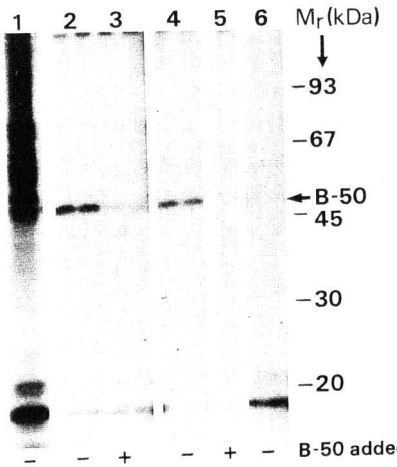

Fig. 3. SDS-PAGE of immunoprecipitates of [^{32}P]-labeled crude synaptosomal membranes. 1: total protein subjected to immunoprecipitation. 2-3: anti B-50 antiserum. 4-5: anti-B-50 IgGs. 6: affinity column effluent.

With this immunoprecipitation assay we have shown that active phorbol diesters stimulate the degree of B-50 phosphorylation in hippocampal slices (De Graan et al., in preparation). Since it is known that phorbol diesters stimulate neurotransmitter release and can mimic certain forms of LTP, we used the hippocampal slice system to study a possible involvement of B-50 in LTP.

ROLE OF PKC AND B-50 (F1/GAP43) IN LTP

Initial experiments designed to investigate the effect of tetanization of hippocampal slices on the phosphorylation of synaptic phosphoproteins revealed a non-significant increase (19%, $2p < 0.1$) in the degree of B-50 phosphorylation (Bär et al., 1980). After tetanization in vivo, Lovinger et al. (1985) reported an increase in F1 phosphorylation in the CA1 region measured with a post hoc in vitro assay. In their system the degree of F1 phosphorylation seems to be correlated with the degree of potentiation measured in vivo and the increase in F1 phosphorylation is maintained for at least 3 days. Concomitant with the increase in F1 phosphorylation they measured a tetanization-induced translocation of PKC activity from the cytosol to the plasma membrane (Akers et al., 1986). We have shown that tetanization of hippocampal slices under more standardized conditions results in an increase in B-50 phosphorylation which is correlated with the degree of potentiation (Schrama et al., 1986[b]).

Recently, it has been shown in the hippocampus that phorbol diesters induce LTP-like phenomena (Malenka et al., 1986; Hu et al., 1987) and prolong tetanus-induced LTP (Routtenberg et al., 1986). The involvement of PKC in the molecular mechanism of LTP is further substantiated by the observation that polymixin B, a relatively selective inhibitor of PKC, prevents the maintenance of LTP (Reymann et al., 1988) and by the observation that intracellular injection of PKC induces LTP-like phenomena (Hu et al., 1987). We confirmed that short treatment of hippocampal slices with phorbol diesters induced persistent LTP-like phenomena and we could show that concomitant with these changes in electrophysiology, an increase in the degree of phosphorylation of the PKC substrate B-50 occurs, which is not reversed upon removal of the phorbol diester (De Graan et al., in preparation).

It is not clear what the role of PKC-mediated phosphorylation in LTP is. One possibility is that PKC is involved in the mechanism of neurotransmitter release (for reviews see Nishizuka, 1986; Kaczmarek, 1987). Phorbol

diesters have been shown to enhance neurotransmitter release in a variety of systems (Allgaier et al., 1986; Zurgil et al., 1986; Nichols et al., 1987; Versteeg and Ulenkate, 1987) including the hippocampus (Versteeg and Florijn, 1986; Malenka et al., 1987). We have recently shown that the increase in neurotransmitter release in the hippocampus is correlated with the degree of phosphorylation of B-50 (Dekker et al., in preparation). K^+-induced neurotransmitter release is parallelled by an increase in B-50 phosphorylation (Fig. 4a). This increase can be inhibited by the PKC inhibitor polymixin B (Fig. 4a), whereas the B-50 phosphorylation under normal K^+ was not affected. Under similar conditions polymixin B attenuates electrically stimulated neurotransmitter release in rat amygdala slices, whereas basal release is unaffected (Versteeg and Ulenkate, 1987). Moreover, in hippocampal slices stimulation of B-50 phosphorylation and neurotransmitter release by phorbol diesters is much more pronounced under K^+-stimulated than under basal conditions (Fig. 4b; Versteeg and Florijn, 1986).

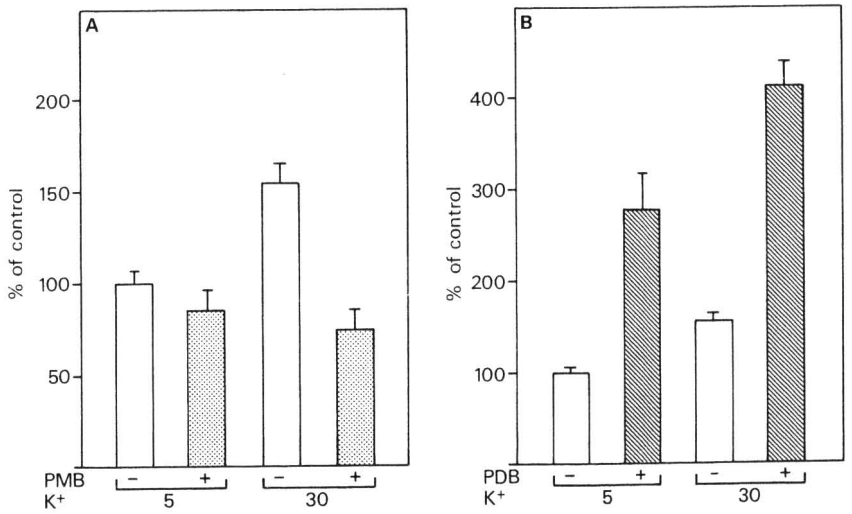

Fig. 4. Effect of polymyxin B (panel A) and PDB (panel B) on B-50 phosphorylation in K^+-depolarized and non-depolarized rat hippocampal slices. For experimental details see Dekker et al. (in preparation).

It is not clear how PKC is activated during LTP or under high potassium conditions. In general, PKC is thought to be activated by diacylglycerol which is generated together with inositol trisphosphate by the receptor-

mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (for recent review see Nishisuka, 1986). In a number of systems we have shown that there is an inverse relationship between the degree of B-50 phosphorylation and the labeling of PIP_2 (for a recent review see De Graan et al., 1986). Based on this evidence we have proposed that B-50 phosphorylation plays an important modulatory role in the synthesis of PIP_2 in the plasma membrane (Gispen et al., 1985). In line with this hypothesis we have shown in a semi-purified system that phosphorylated B-50 inhibits PIP_2 labeling (Van Dongen et al., 1985). Thus, B-50 phosphorylation seems to exert a negative feedback control on receptor-mediated PIP_2 hydrolysis.

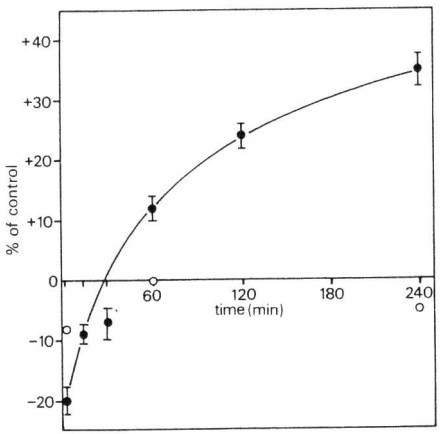

Fig. 5. Changes in PIP_2 labeling in rat hippocampal slices after tetanic stimulation of perforant path fibers. X-axis gives interval between application of tetanus and start of 10 min incubation with labeled phosphate. Data are expressed as % of control. Bars indicate the SEM. For experimental details see Bär et al. (1984).

LTP-induced stimulation of hippocampal slices induces a time-dependent change in PIP_2 labeling: at short intervals after tetanization a decrease of about 20% is observed; after longer time intervals there is a gradual increase in PIP_2 labeling (Fig. 5, filled symbols). A low stimulation frequency, which does not produce LTP, does not affect PIP_2 labeling (Fig. 5, open symbols; Bär et al., 1984). So, we have evidence that both protein and lipid phosphorylation change during LTP. However, at this moment it is not clear whether there is a causal relationship between these two phenomena.

CONCLUDING REMARKS

In the present paper we reviewed the significance of two presumably presynaptic phosphoproteins in neurotransmitter release during LTP-like phenomena in rat hippocampal slices. LTP-related changes in synaptic

transmission are accompanied by changes in the degree of phosphorylation of the 52 kDa coated vesicle protein (pp50) and the B-50 (GAP43, F1) protein present in vesicle and presynaptic membranes. In keeping with the literature, we assume that the phosphostate of the 52 kDa protein somehow is involved in presynaptic membrane retrieval following exocytosis. The B-50 protein may be involved in presynaptic modulation of transmembrane signal transduction, in the release process of certain transmitters or in presynaptic plasticity as such. At present it is unclear what the nature of the relationship between the observed neurochemical changes is with respect to LTP. They may reflect only a temporal correlate of enhanced neurotransmitter release. Further work is in progress to delineate the function of this protein in more detail.

ACKNOWLEDGEMENT

This research was supported by CLEO-TNO grants A47 and A66, and by MEDIGON grant 900-548-072 of the Dutch Organization for the Advancement of Pure Research (ZWO). The authors wish to thank Drs. A. Pauloin and J. Jolles for the gift of the polyclonal antibody directed against pp50 from calf brain coated vesicles, Drs. B. Melchers, F.H. Lopes da Silva and D.H.G. Versteeg for helpful advise.

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