

# Characterization of a 52 kDa Phosphoprotein Possibly Related to Long-term Potentiation

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## ABSTRACT

Tetanyzation of afferent fibers in the transverse hippocampal slice, results in long-term potentiation (LTP) and a concomitant increase in the degree of phosphorylation of a 52 kDa synaptosomal protein. The *in vitro* phosphorylation of this 52 kDa protein is calcium/calmodulin and cyclic nucleotide independent. This 52 kDa protein, which was originally described in synaptosomal membrane fractions, resembles the major coated vesicle phosphoprotein with regard to its molecular weight (MW) and phosphorylation characteristics. In this study we compare the 52 kDa proteins from synaptosomal plasma membranes (SPM) and coated vesicles. The two proteins appear to be identical on basis of the following criteria: (i) relative MW, (ii) peptide map, and (iii) isoelectric point. Coated vesicles are thought to be involved in receptor-mediated endocytosis and membrane recycling. We suggest that the changes in 52 kDa phosphorylation, concomitant with LTP, may reflect an increase in membrane recycling through coated vesicles.

## KEYWORDS

Long-term potentiation, protein phosphorylation, hippocampal slices, coated vesicles.

## INTRODUCTION

Long-term potentiation (LTP) is a form of synaptic plasticity, which can be elicited by a short chain of electrical pulses (50-100 Hz stimulation for 1-2 sec at moderate intensity). Because of the long duration of the LTP and the near physiological properties of the stimulation required to evoke it, this form of plasticity is often considered as a model for information storage in the brain (for reviews see Lopes da Silva *et al.*, 1982; Voronin, 1983; Eccles, 1983; Teyler and Discenna, 1984; Lynch and Baudry, 1984).

After the first demonstration of LTP in the hippocampus (Bliss and Lomo, 1973) many studies have been devoted to the elucidation of the molecular mechanism underlying this phenomenon. Owing to

the lamellar structure of the hippocampus, transverse hippocampal slices contain a relatively intact trisynaptic pathway (Andersen *et al.*, 1971). We have used this hippocampal slice system to monitor LTP-induced changes in synaptosomal protein phosphorylation, an important posttranslational modification of proteins involved in neurotransmission (see Gispen, this volume).

Tetanic stimulation of perforant path fibers, resulting in LTP, affected the degree of phosphorylation of a 52 kDa protein band, measured with a post hoc phosphorylation assay in crude synaptosomal plasma membranes (SPM) prepared from tetanized slices (Bär-*et al.*, 1980, 1982). Since the electrophysiological changes in the evoked response to single test stimuli after a tetanus may vary considerably in amplitude, we attempted to establish a quantitative correlation between the changes in amplitude of the post-synaptic potential (PSP) measured extracellularly, and the degree of phosphorylation of the 52 kDa band (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 ( $p < 0.005$ ). These data suggest that there may be a quantitative correlation between LTP and 52 kDa phosphorylation.

In search for the identity of the 52 kDa protein we noted that brain coated vesicles contain a major 52 kDa phosphoprotein (Kadota *et al.*, 1982; Moskowitz *et al.*, 1983; Pauloin *et al.*, 1983) that shares the phosphorylation characteristics of the 52 kDa protein in SPM: its phosphorylation is insensitive to calcium/calmodulin or cyclic nucleotides. In this study we compare the 52 kDa phosphoprotein in rat brain coated vesicle with the 52 kDa protein in SPM and suggest that the two proteins are similar if not identical.

#### MATERIALS AND METHODS

SPM were isolated from fresh rat brain according to Kristjansson *et al.* (1982). SPM were resuspended in buffer B (1 mg protein/ml). Protein was determined according to Lowry *et al.* (1951) using BSA as a standard.

Coated vesicles were isolated according to Pearce and Robinson (1984) with minor modifications. Frozen ( $-20^{\circ}\text{C}$ ) rat brains were thawed and homogenized in 4 vol buffer A (10 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 1 mM EGTA, 0.5 mM  $\text{MgCl}_2$ , 0.02 % (w/v)  $\text{NaN}_3$  and 0.2 mM PMSF). The homogenate was centrifuged at  $20,000 \times g$  for 30 min. The supernatant was incubated for 15-25 min at room temperature after addition of SDS (1 %, v/v, final concentration) and centrifuged at  $100,000 \times g$  for 60 min. The pellet was resuspended in buffer A containing 1 % (v/v) Triton X-100 and loaded onto a discontinuous sucrose gradient of 7.5 ml each 5, 10, 15, 20 and 25 % (w/v) sucrose dissolved in buffer A. The gradient was centrifuged at  $45,000 \times g$  for 60 min. The 15-25 % sucrose layers were combined, diluted 3 times in buffer B (10 mM Na acetate, 10 mM Mg acetate, 0.1 mM Ca acetate, pH 6.5) and centrifuged at  $100,000 \times g$  for 60 min. The pellet was resuspended in buffer B (1 mg protein/ml).

Endogenous protein phosphorylation was assayed as described previously (Kristjansson *et al.*, 1982; Zwiers *et al.*, 1976).

Phosphorylated coated vesicle and SPM proteins were separated on an 11 % SDS-PAGE gel (Zwiers *et al.*, 1976) and the 52 kDa phospho-band was excised from the gel. The gel pieces were equilibrated for 20 min in buffer C (25 mM Tris, 192.5 mM glycine, 0.1 % (w/v)

SDS, pH 8.3). The 52 kDa protein was extracted in buffer C in an ISCO sample concentrator (model 1750, Lincoln, USA), dialyzed against double-distilled water overnight, and freeze dried.

For peptide mapping the phosphorylated 52 kDa protein was incubated in 125 mM Tris-HCl with 50 ng Staphylococcus aureus protease V8 (SAP, Miles Laboratories, Slough, UK) in a total volume of 25  $\mu$ l at 30  $^{\circ}$ C for the times indicated. The reaction was stopped by addition of 12.5  $\mu$ l denaturing solution (Zwiers *et al.*, 1976) and boiling for 10 min. The incubated samples were separated on a 15 % polyacrylamide gel.

Two dimensional separation of phosphorylated proteins was accomplished by isoelectric focussing (IEF) on pH 3.5-10.0 IEF gels followed by 11 % SDS-PAGE (Zwiers *et al.*, 1980).

#### RESULTS AND DISCUSSION

The 52 kDa phosphoprotein in SPM, which is affected by tetanic stimulation (Bär *et al.*, 1982) comigrates on 11 % SDS-PAGE gels with the major phosphoprotein in coated vesicles (De Graan *et al.*, 1986; see also Fig. 1). Two-dimensional separation of phosphorylated CV and SPM proteins (Fig. 1) reveals both 52 kDa proteins as a homogenous smear from pH 9.0 to pH 6.5. The use of other two-dimensional separation systems did not improve the resolution in the IEF direction. Similar problems with the determination of the IEP were reported for the major phosphoprotein in coated vesicles from rat liver (Campbell *et al.*, 1984) and bovine brain and liver (Pfeffer *et al.*, 1983).

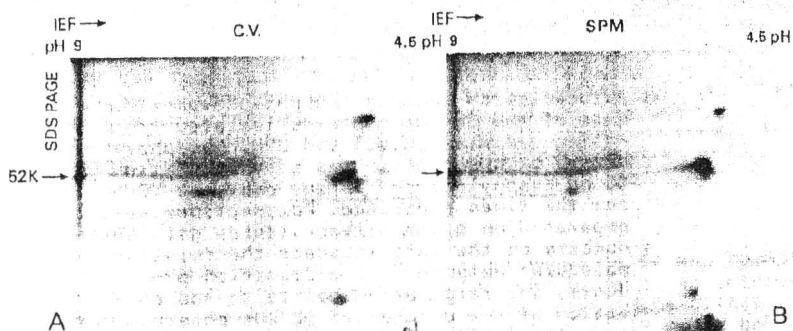


Fig 1. Autoradiogram showing two-dimensional separation of phosphorylated coated vesicle (C.V., panel A) and SPM (panel B) proteins. The first dimension was IEF on a 3.5-10 pH gradient, the second dimension was 11 % SDS-PAGE. The position of the 52 kDa proteins is indicated by the arrows on the left. Coated vesicle and SPM proteins were phosphorylated in buffer B (see Materials and Methods) without  $\text{Ca}^{2+}$  containing 1 mM EGTA to suppress  $\text{Ca}^{2+}$ -dependent B-50 phosphorylation (Zwiers *et al.*, 1980).

For the peptide mapping experiments equal amounts of purified P-labelled 52 kDa from coated vesicles and SPM were treated for different times with SAP. The generated polypeptides were separated on 15% SDS-PAGE gels. An autoradiogram of such a 52 kDa digest is shown in Fig. 2. Already after 1 min treatment 43 kDa fragments are no longer visible and 2 additional fragments of 33 and 20 kDa are formed. Thus, the phospho-peptide map as well as the time course for digestion of the 52 kDa proteins are very similar

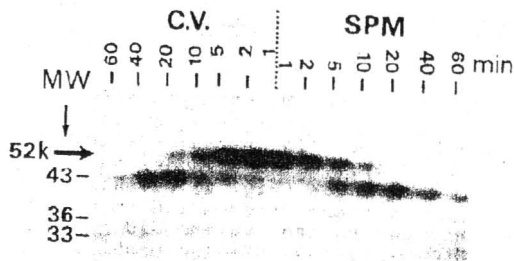


Fig. 2. Autoradiogram showing the phospho-peptide maps of the 52 kDa proteins isolated from coated vesicles (C.V.) and SPM. Phosphorylated 52 kDa protein was incubated with 50 ng *Staphylococcus aureus* protease V8 for the times indicated. Polypeptides were separated on a 15% polyacrylamide gel. The numbers on the left indicate the relative molecular weights of the digestion products, the large arrow points to the position of the undigested 52 kDa phospho-protein.

Based on the following criteria we conclude that the 52 kDa proteins in SPM and coated vesicles are similar if not identical; (i) estimated MW on 11% SDS gels is 52 kDa, (ii) phospho-peptide maps show 3 fragments with estimated MWs of 43, 33 and 20 kDa, (iii) phosphorylated amino acids are serine and threonine (results not shown), (iv) IEP ranges between 9.0 and 6.5, (v) 52 kDa phosphorylation is insensitive to modulation by cyclic nucleotides, calcium or calmodulin (Bär *et al.*, 1982; Pauloin *et al.*, 1982).

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, 1979; Kado- ta and Kadota, 1982; Miller and Heuser, 1984). Thus, enhanced

transmitter release after tetanization (Bliss *et al*, 1982) might lead to an increase in coated pit and coated vesicle activity, to retrieve excess presynaptic membrane material (Fig. 3). The coated vesicle is thought to fuse with large intracellular

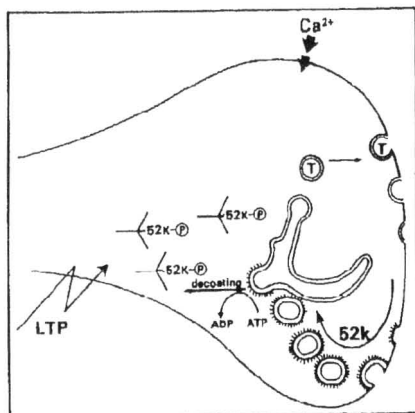


Fig. 3. Hypothesis concerning the role of 52 kDa phosphorylation in coated vesicle-mediated membrane recycling and LTP.

cisternae, whereby the coat is removed from the coated vesicle (Heuser and Reese, 1973; Miller and Heuser, 1984). Preliminary data from Pauloin and Jolles (1986) suggest that the phosphorylation of pp50 could destabilize the interaction between certain coat proteins and the vesicle (Fig. 3). This notion 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 Jolles, 1984) and induces the dissociation of clathrin from the vesicles (Schmid *et al*, 1984). However, at this moment the precise role of pp50 phosphorylation in coated vesicle function is still unclear.

#### CONCLUSION

In our LTP studies we have observed an increase in 52 kDa (pp50) phosphorylation in SPM. We believe this protein to be an intrinsic SPM protein, probably present in coated pits. More direct evidence for a role of 52 kDa phosphorylation in LTP could be obtained from studies on 52 kDa phosphorylation in coated vesicles prepared from tetanized hippocampal slices. However, the low yield of coated vesicles from brain tissue precludes this approach. Studies with 52 kDa antibodies may provide a useful alternative to study the role of 52 kDa phosphorylation in LTP.

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