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Comparison of a 52-kDa Phosphoprotein from Synaptic Plasma Membranes Related to Long-Term Potentiation and the Major Coated Vesicle Phosphoprotein

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Abstract: In the in vitro hippocampal slice preparation a short tetanus induces long-term potentiation (LTP) and an increase in the post hoc phosphorylation of a 52-kDa protein in synaptosomal plasma membranes (SPM) prepared from these slices. This 52-kDa SPM phosphoprotein closely resembles the predominant phosphoprotein in coated vesicles, pp50, with respect to the insensitivity of its phosphorylation to Ca^{2+}/calmodulin and cyclic AMP. This resemblance prompted us to compare in rat brain the 52-kDa SPM protein with pp50 in isolated coated vesicles. Both proteins appear to be very similar on basis of the following criteria: (1) relative molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (2) peptide mapping, (3) phospho-amino acid content, and (4) isoelectric point. Since coated vesicles are thought to be involved in receptor-mediated endocytosis and membrane recycling, our data suggest that LTP-correlated changes in 52-kDa phosphorylation may reflect increased coated vesicle activity. **Key Words:** Long-term potentiation—Protein phosphorylation—Coated vesicles—Synaptosomal plasma membranes. **Schrama L. H. et al.** Comparison of a 52-kDa phosphoprotein from synaptic plasma membranes related to long-term potentiation and the major coated vesicle phosphoprotein. *J. Neurochem.* **47**, 1843–1848 (1986).

In the in vitro hippocampal slice preparation long-term potentiation (LTP) has been studied extensively in a number of monosynaptic subsystems (Andersen et al., 1971, 1980). In correlative studies on LTP and protein phosphorylation we have used the perforant path-granule cell subsystem to elicit and monitor LTP (Bär et al., 1980*a*, 1982; Lopes da Silva et al., 1982*a,b*). Tetanic stimulation of the perforant path increased the phosphorylation of a 52-kDa protein band as measured in a crude synaptosomal plasma membrane (SPM) fraction using a post hoc endogenous phosphorylation assay (Bär et al., 1980*a*, 1982). The phosphorylation of this protein band was also found to be sensitive to the opioid peptide enkephalin (Bär et al., 1980*b*). Initial biochemical characterization revealed that the phosphorylation of this 52-kDa protein was Ca^{2+}/calmodulin- and cyclic nucleotide-independent (Bär et al., 1982).

Brain coated vesicles contain a number of phosphoproteins in the range of 50–55 kDa (Pfeffer et al., 1983). The two highest molecular weight phosphoproteins, 54 and 56 kDa, have been identified as α and β tubulin, whereas the major phosphorylated protein is a 52-kDa protein, called pp50 (Pauloin et al., 1984). The phosphorylation of the latter protein is catalyzed by a casein kinase II-like enzyme (Kadota et al., 1984; Usami et al., 1984) and is not sensitive to modulation by the second messengers cyclic AMP, cyclic GMP, and calcium, or to modulation by the calcium binding protein calmodulin (Pauloin et al., 1982; Moskowitz et al., 1983). In search for the identity of the 52-kDa protein (De Graan et al., 1985) it was realized that the major phosphoprotein in brain coated vesicles, pp50, shares both molecular weight and phosphorylation characteristics with the 52-kDa protein in SPM (Kadota et al., 1982; Pauloin et al., 1982; Usami et al., 1982, 1984; Pfeffer et al., 1983; Moskowitz et al., 1983). In this study we compare the rat brain coated

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Abbreviations used: IEF, isoelectric focussing; IEP, isoelectric point; LTP, long-term potentiation; SAP, *Staphylococcus aureus* protease V8; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPM, synaptosomal plasma membranes.

vesicle phosphoprotein to the 52-kDa SPM phosphoprotein with respect to molecular weight, isoelectric point (IEP), phospho-amino acid composition, and peptide map.

MATERIALS AND METHODS

Preparation of a coated vesicle-enriched fraction

Coated vesicles were isolated according to the method of Pearse and Robinson (1984) with minor modifications. Frozen (−20°C) rat brains were thawed and homogenized in four volumes of isolation buffer (buffer A), consisting of 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.2; 150 m*M* NaCl; 1 m*M* EGTA; 0.5 m*M* $MgCl_2$; 0.02% (wt/vol) NaN_3; and 0.2 m*M* phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000 *g* for 30 min in a Sorvall SS-34 rotor at 4°C. To the resulting supernatant Triton X-100 was added to a final concentration of 1% (vol/vol) and the fraction was incubated for 15–25 min at room temperature until the turbidity in the solution had resolved. The Triton X-100-treated supernatant was centrifuged at 100,000 *g* for 60 min at 4°C in a Beckman SW 27 rotor and the resulting pellet was dissolved in a minimal volume (1 ml per five brains) of buffer A containing 1% (vol/vol) Triton X-100. The resuspended pellet was loaded onto a discontinuous sucrose gradient of 7.5 ml each 5, 10, 15, 20, and 25% (wt/vol) sucrose dissolved in buffer A. The gradient was centrifuged in a Beckman SW 27 rotor at 45,000 *g* for 60 min at 4°C. The 15–25% sucrose layers were collected and diluted three times in buffer B (10 m*M* Na-acetate, 10 m*M* Mg-acetate, 0.1 m*M* Ca-acetate, pH 6.5) and centrifuged in a Beckman SW 27 rotor at 100,000 *g* for 60 min at 4°C. The pellet was resuspended in buffer B to give a final concentration of 1 mg/ml protein. The yield of this coated vesicle-enriched fraction (15–25% sucrose layers) was 125 μg protein/brain.

Preparation of SPM and a vesicle-enriched fraction

SPM were isolated from fresh rat brain according to the method of Kristjansson et al. (1982). The final resulting SPM pellet was resuspended in buffer B to a protein concentration of 1 mg/ml. The 0.4 *M* vesicle-enriched layer of the sucrose gradient used to isolate SPM was also collected in buffer B and resuspended at a protein concentration of 1 mg/ml.

Protein phosphorylation assay

Endogenous protein phosphorylation was assayed as described previously by Zwiers et al. (1976) and Kristjansson et al. (1982). In brief, the incubations were as follows: the protein fraction (10 μg) was preincubated at 30°C for 5 min in buffer B, then 2 μCi [γ^{32}P]ATP (sp act 3,000 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.) and 7.5 μ*M* ATP were added to a final volume of 25 μl. The reaction was terminated by addition of 12.5 μl of a denaturing solution (Zwiers et al., 1976).

Isolation of the 52-kDa protein from coated vesicles and SPM

Phosphorylated coated vesicles and SPM were separated on an 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After staining and destaining of the gel an autoradiogram was made and the 52-kDa phosphoprotein was excised from the gel. The accuracy of the excision was verified by a second autoradiogram of the gel. The gel pieces were soaked for 20 min in buffer C [25 m*M* Tris, 192.5 m*M* glycine, 0.1% (wt/vol) SDS, pH 8.3] to remove excess methanol and glycine. The proteins were collected from the gel in buffer C in an Isco sample concentrator (Model 1750, Lincoln, NE, U.S.A.) at 3 W constant power (6 mA) for 2.5 h in a total volume of 200 μl. The purity of the preparations was verified by rerunning 20 μl on SDS-PAGE, staining, destaining, and autoradiography. The remainder of the fractions was dialyzed overnight against double-distilled water. The dialyzed fractions were dried and dissolved in 6 *M* HCl, 1 μ*M* thioglycolic acid for phospho-amino acid analysis or in 125 m*M* Tris-HCl, pH 7.4 for peptide mapping (see below).

Phospho-amino acid analysis

The purified fractions containing the isolated 52-kDa phosphoprotein from coated vesicles and SPM were each split into equal amounts. To one half of each fraction (100 μl), 10 μg of the phospho-amino acid standards phosphotyrosine, phosphothreonine, and phosphoserine (Sigma, St. Louis, MO, U.S.A.) were added. The preparations with and without standard were sealed in a glass capillary and incubated for 3 h at 110°C. The hydrolysates were dried under vacuum and redissolved in double-distilled water repeatedly, until the pH was neutral. The samples were applied to Whatman 540 paper (height 40 cm, width 20 cm) and run for 2.5 h at 3,000 V in 5% (vol/vol) acetic acid, 1 m*M* EDTA, pH 3.5 with pyridine. The amino acid standards were visualized with ninhydrin and marked with ^{32}P-containing ink before an autoradiogram was made.

Peptide mapping

The isolated 52-kDa phosphoprotein was incubated in 125 m*M* Tris-HCl with 50 ng *Staphylococcus aureus* protease V8 (SAP, Miles Laboratories, Slough, U.K.) in a total volume of 25 μl at 30°C for the times indicated. The reaction was stopped by addition of 12.5 μl of denaturing solution (Zwiers et al., 1976) and boiling for 10 min. The incubated samples were separated on a 15% polyacrylamide gel.

Two-dimensional gel electrophoresis

Isoelectric focussing (IEF) followed by SDS-PAGE was performed on a pH 3.5–10 IEF gel with phosphorylated coated vesicles and SPM according to the method of Zwiers et al. (1980). Nonequilibrium pH gradient gel electrophoresis was performed according to the method of O'Farrell et al. (1977), with 1 m*M* H_2SO_4 as anodic solution and 0.1 *M* NaOH as cathodic solution. The rest of the conditions were the same as those described for IEF–SDS-PAGE by Zwiers et al. (1980).

Protein determination

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

RESULTS

Relative molecular weight on SDS-PAGE

Freshly isolated SPM, vesicles, and coated vesicles were phosphorylated, separated on an 11% SDS-polyacrylamide gel, and submitted to autoradiography (Fig. 1). On the autoradiogram the 52-kDa bands in SPM (lanes 1) and in the vesicle-enriched fraction (lanes 2) show as a minor phosphoband, whereas the major phosphoprotein in coated vesicles (lanes 3)

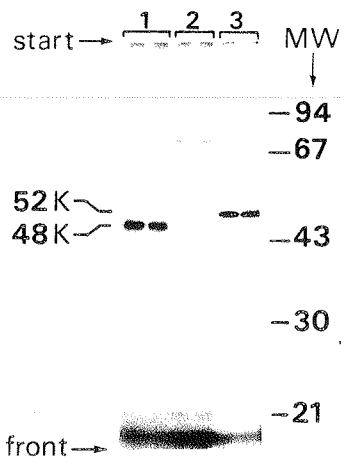

FIG. 1. Molecular weight determination on SDS-PAGE. Ten micrograms of phosphorylated SPM (lanes 1), vesicles (lanes 2), and coated vesicles (lanes 3) were separated on an 11% SDS-polyacrylamide gel. The numbers to the right of the figure indicate the position of the low-molecular-weight markers from Pharmacia (Uppsala, Sweden). The arrow to the left of the figure indicates the position of the 52-kDa phosphoprotein on the autoradiogram.

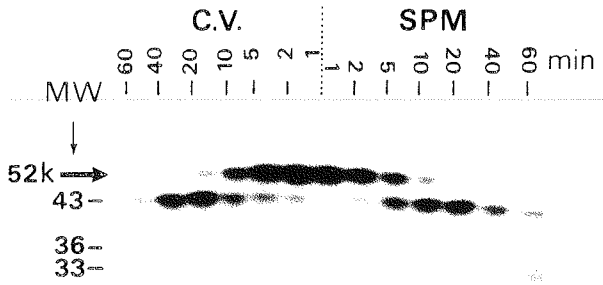

FIG. 2. Peptide mapping. Isolated phosphorylated 52-kDa protein from SPM and coated vesicles (C.V.) was incubated with 50 ng SAP for the times indicated at the top of the figure. The resulting polypeptides were separated on an 15% polyacrylamide gel. The numbers to the left of the figure indicate the relative molecular weights of the digestion products; the arrow points to the position of the original 52-kDa phosphoprotein.

is visible as a predominant band that runs at the same height as the 52-kDa phosphoproteins in lanes 1 and 2.

Peptide mapping

The 52-kDa phosphoprotein was isolated from both SPM and coated vesicles after phosphorylation of 250 μg of the respective fractions with 50 μCi [γ-^{32}P]ATP and 7.5 μM ATP for 30 s. The amount of ^{32}P incorporation in both 52-kDa proteins was determined and an equal amount of radioactive 52-kDa protein of the respective fractions was incubated in 25 μl with 50 ng SAP in 125 mM Tris-HCl, pH 7.4, at 30°C for 1, 2, 5, 10, 20, 40, and 60 min. The reaction was terminated with a denaturing solution (10 min, 100°C). The resulting polypeptides were separated on a 15% polyacrylamide gel. The autoradiogram of the gel (Fig. 2) shows the digestion of the 52-kDa protein after protease treatment of coated vesicles (left part of the figure) and SPM (right part of the figure). After 1 min of protease treatment only one digestion product (M_r 43 kDa, as judged from the molecular weight markers run alongside the gel) is visible in both preparations. After a 20–40-min protease treatment the original 52-kDa phosphoprotein in both preparations was completely digested and two further breakdown products of 33 and 20 kDa were formed.

Incubation of both 52-kDa phosphoproteins with inactivated SAP (10 min, 100°C) did not result in any breakdown of protein. Similar results with respect to time scale and digestion products were obtained when both 52-kDa phosphoproteins were incubated with chymotrypsin A4 (Boehringer, Mannheim, F.R.G.; results not shown). Thus, the digestion of both 52-kDa phosphoproteins with SAP resulted in identical peptide maps. Moreover, the time scale of the formation of digestion products was very similar for both 52-kDa proteins.

Phospho-amino acid analysis

The 52-kDa phosphoproteins isolated from coated vesicles and SPM were hydrolyzed with 6 M HCl and separated by high-voltage paper electrophoresis. Both 52-kDa proteins contained phosphoserine and phosphothreonine, whereas phosphotyrosine was absent (Fig. 3: coated vesicles, lane 1; SPM, lane 2). The phosphorylated products near the origin probably represent partially unhydrolyzed 52-kDa phosphoprotein. The ratios of phosphothreonine to phosphoserine were 7:1 for the coated vesicle and 1:1 for the

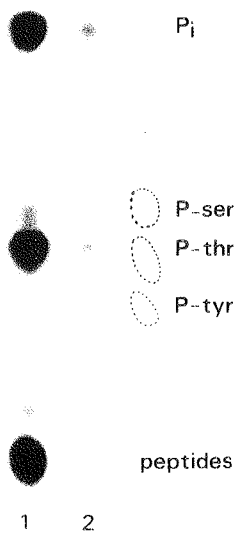

FIG. 3. Phospho-amino acid analysis. Isolated 52-kDa phosphoprotein from coated vesicles (lane 1) and SPM (lane 2) was hydrolyzed with 6 M HCl. The resulting products were separated by means of high-voltage paper electrophoresis. The position of reference nonradioactive phospho-amino acids was marked with radioactive ink and is indicated to the right of the figure.

SPM 52-kDa phosphoprotein as determined by liquid scintillation counting of the excised spots from the paper.

Two-dimensional gel electrophoresis

Coated vesicles and SPM were phosphorylated in the absence of calcium and in the presence of 1 m*M* EGTA, to suppress the calcium-dependent phosphorylation of B-50 in SPM, the major phosphoprotein in this region of the gel (Zwiers et al., 1980; see Fig. 1, lanes 1). The proteins were separated on a pH 10–3.5 gradient in the first and on 11% SDS-PAGE in the second dimension. The resulting autoradiogram (Fig. 4) shows the 52-kDa phosphoprotein in both coated vesicles (Fig. 4A) and SPM (Fig. 4B) as a homogeneous smear from the origin of the IEF gel (pH 9.0) to the middle (pH 6.5). The position of the 52-kDa phosphoprotein is indicated to the left of the figures by an arrow.

Comigration studies with a mixture of both phosphorylated fractions resulted in the same appearance of the 52-kDa phosphoprotein on the autoradiogram, whereas no extra phosphoprotein spots were visible (results not shown). Separation of both phosphorylated fractions by nonequilibrium pH gradient gel electrophoresis according to O'Farrell et al. (1977) did not improve the resolution of the 52-kDa phosphoprotein in the first dimension (results not shown). Isolated 52-kDa phosphoprotein from both sources was also run on both IEF and nonequilibrium pH gel electrophoresis. With this approach no improvement of resolution compared to the above described methods was achieved (data not shown).

DISCUSSION

LTP is a form of synaptic plasticity that is observed in many parts of the CNS when a short train of pulses at relative high frequency (a tetanus) is applied to a set of fibers (Schwartzkroin and Wester, 1975; Alger and Tyler, 1976; Andersen et al., 1980; Bliss and Dolphin, 1982). Tetanic stimulation of the perforant path of the hippocampal slice stimulated the phosphorylation of a 52-kDa protein in a crude synaptosomal plasma membrane fraction (Bär et al., 1980*a*, 1982; Tielen et al., 1982). The degree of phosphorylation of this 52-kDa protein was found to be highly correlated with the degree of LTP. Linear regression analysis of the degree of 52-kDa phosphorylation versus the height of the postsynaptic potential showed a semilogarithmic plot with a correlation coefficient of 0.71 ($p < 0.005$, Tielen et al., 1983).

In this article we present evidence that the LTP-related 52-kDa phosphoprotein in rat brain SPM is very similar to the 52-kDa protein in isolated rat brain coated vesicles. This conclusion is based on the following observations: (1) the molecular weight of the 52-kDa phosphoprotein in coated vesicles and SPM is identical when analyzed on an 11% polyacrylamide gel (Fig. 1); (2) digestion of isolated 52-kDa phosphoprotein from both sources with SAP (Fig. 2) or chymotrypsin A4 resulted in the same breakdown products as analyzed by SDS-PAGE on an 15% gel; (3) phospho-amino acid analysis revealed the same phosphorylated amino acids (i.e., threonine and serine) in both 52-kDa phosphoproteins (Fig. 3); (4) combined IEF with SDS-PAGE on an 11% gel indicated an identical behavior for both proteins, resulting in a smear between pH 9 and 6.5 (Fig. 4); (5) both phosphoproteins are phosphorylated by a Ca^{2+}/calmodulin- and cyclic nucleotide-independent protein kinase (Bär et al., 1982).

The data on phospho-amino acid analysis of the 52-kDa phosphoprotein in coated vesicles presented in this article show that the major phosphate acceptor amino acid is threonine. Direct comparison of these results with data from literature is difficult, since to the best of our knowledge no reports have been pre-

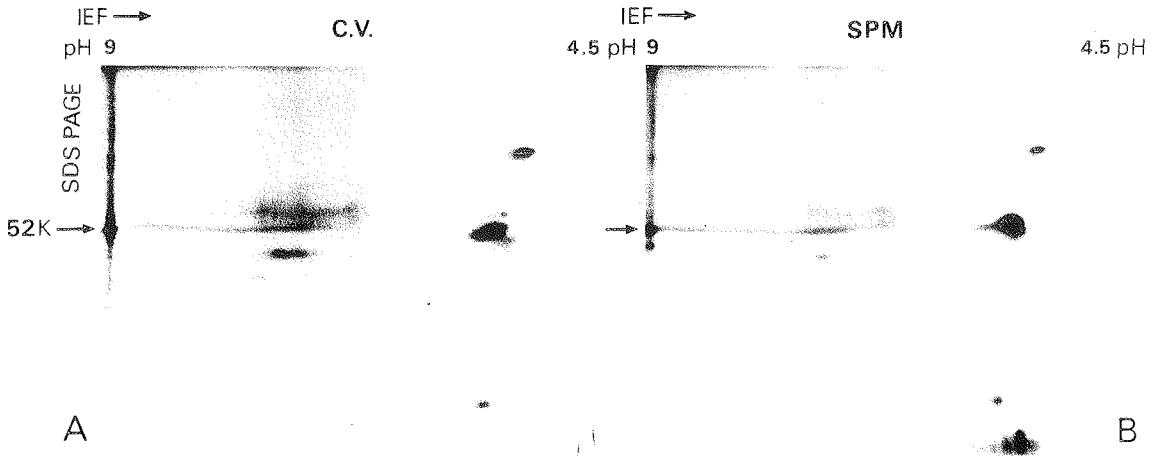

FIG. 4. IEF and SDS-PAGE. Phosphorylated coated vesicles (C.V., **A**) and SPM (**B**) were separated on an pH 3.5–10 isoelectric focussing gel followed by SDS-PAGE in the second dimension. The arrow indicates the position of the 52-kDa protein in both fractions.

sented on phospho-amino acid analysis of a purified 52-kDa preparation obtained from coated vesicles. Campbell and co-workers showed that in the coat fraction isolated from rat liver coated vesicles threonine was the major phosphate accepting amino acid, whereas serine was phosphorylated to a lesser extent (Campbell et al., 1984). Assuming that the 52-kDa protein was the major phosphoprotein in their coat fraction, their findings are in line with our results using isolated 52-kDa protein from rat brain coated vesicles. In the 52-kDa phosphoprotein isolated from SPM less phosphate is incorporated into threonine than in serine. The qualitative differences in the labeling of serine and threonine between 52-kDa protein from coated vesicles and from SPM are most likely due to differences in the initial phosphorylation state of the respective proteins, since the 52-kDa protein containing fractions were not dephosphorylated before phosphorylation with [γ-^{32}P]ATP.

Campbell et al. (1984) also tried to determine the IEP of their 53/51-kDa phosphoprotein(s) in coated vesicles from rat liver. The resolution they obtained on the IEF gel was as poor as that presented in this article and the IEP range was also pH 9–6.5. The major phosphorylated coated vesicle protein from bovine brain and liver was also found to have an IEP ranging from pH 9 to 6.5 (Pfeffer et al., 1983). IEF of the purified 52-kDa protein gave a similarly poor resolution.

Coated vesicles and coated pits have been implicated in a number of intracellular processes such as receptor-mediated endocytosis, secretory pathways, intracellular protein traffic, and presynaptic membrane recycling after transmitter release (Heuser and Reese, 1973; Goldstein et al., 1979; Kadota and Kadota, 1982). The precise role of the 52-kDa protein and its phosphorylation in the function of coated vesicles is still unclear. Preliminary data may suggest that phosphorylation of the 52-kDa protein promotes decoating of the coated vesicles (A. Pauloin, personal communication).

Previous results from our laboratory indicate that after application of a tetanus to a hippocampal slice the increase in 52-kDa phosphorylation is positively correlated with the degree of potentiation (Tielen et al., 1983). During LTP there is an increase in neurotransmitter release (Dolphin et al., 1982), which will result in an increase in the amount of membrane material at the presynaptic membrane. Furthermore, chemical depolarization of synaptosomes from cerebral cortex results in the appearance of new large vesicles and coated vesicles (Fried and Blaustein, 1978) after prolonged stimulation. Electrical stimulation of the nerves of isolated frog sartorius muscles transiently depleted synaptic vesicles (Heuser and Reese, 1973). The synaptic vesicle membrane was retrieved by coated vesicles and synaptic vesicles were recycled via intermediate cisternae (Heuser and Reese, 1973). The relative importance of coated vesicles in the process of membrane retrieval is still unclear, but the two examples presented above indicate a role for coated vesicles in membrane retrieval after increased neurotransmitter release. Thus we propose that the changes seen 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.

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