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Membrane Phosphoproteins of Rat Hippocampus: Sensitivity to Tetanic Stimulation and Enkephalin

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Hippocampal slices are electrically stimulated in the perforant path with a pulse-train, which can lead to long-term potentiation (LTP). Of the thus stimulated slices, subcellular fractions are prepared and used in an endogenous protein phosphorylation assay. A phosphoprotein band which was reported earlier to be sensitive to electric stimulation as well as to methionine-enkephalin is now further analyzed: it consists of two phosphoproteins only slightly differing in molecular weight: 50,000 M_r (50 K) and 52,000 M_r (52 K), but having distinct biochemical properties and subcellular localization. Their IEP is dissimilar (3.5–4.3 and 5.3, respectively), they display different sensitivity towards calcium when tested in the phosphorylation assay, but are both cAMP-independently phosphorylated. Only one of them responds to tetanic stimulation with an increased phosphorylation post hoc. This protein, the 52 K component, is localized in synaptic membranes. Moreover, this protein also responds to incubation of slices with methionine-enkephalin. The phosphorylation of the 50 K component is not influenced by electric stimulation, nor by incubations with neuropeptides; its phosphorylation takes place in material sedimenting with the mitochondrial cell fractions and is strongly calcium- and calmodulin-dependent.

INTRODUCTION

In correlative studies on electrical and neurochemical parameters the transversal slice of the hippocampus is suitable to use as it combines good responses to electric stimuli with a relatively good accessibility for the study of biochemical parameters. Under the appropriate conditions these slices can be maintained in vitro up to 32 h[44,48,51]. Owing to the lamellar organization of the hippocampus[2,3,24], the slices still have a virtually intact trisynaptic pathway (perforant path: granular cells — mossy fibers: CA3 pyramidal cells — Schaffer collaterals: CA1 pyramidal cells; for review see ref. 30). Several groups have described the generation of long-term potentiation (LTP) by tetanic stimulation in one of the three monosynaptic pathways of the hippocampal slice[1,15,43]. Presumably the LTP is accompanied by swelling of dendritic spines, which may underly the increased synaptic efficacy[18]. In addition, Lee et al.[29] reported that the number of shaft synapses increased after stimulation of slices. Recently, the effect of tetanic stimulation and the subsequent development of LTP on protein phophorylation, measured in a post hoc endogenous phosphorylation assay, was studied. Browning et al.[10] observed that after high frequent stimulation in the Schaffer collaterals one protein band in particular (M_r 40,000) incorporated less phosphate when a fraction prepared from the treated slices was assayed afterwards in a phosphorylation assay. Later they reported that this band was phosphorylated in vitro by phosphorylase kinase[9] and on basis of studies on MW and proteolytic fingerprinting they recently reported[8] that the 40 K protein probably is the α-subunit of pyruvate dehydrogenase. It appeared that the phosphorylation of this enzyme in the brain is also responsive to behavioral experience[42]. Using an experimental set up comparable but not identical to that used by Browning et al.[10], we reported that

another protein band (M_r 50,000) was also responsive to tetanic stimulation. The so-called 50 K band shows an enhanced phosphorylation after stimulation[6] and is sensitive to methionine-enkephalin[5]. As discussed elsewhere[6,31], there is reason to believe that the interval between tetanic stimulation and homogenization of the slices is one of the major factors determining the difference in effects mentioned in the studies of Browning[10] and ourselves[6]. Browning finds that the maximum effect on the 40,000 M_r band occurs 2–5 min after the tetanus; after 10–15 min the effect has disappeared (personal communication). In our studies we wait 15 min before homogenizing the slices[6].

In this report we present data indicating that the 50 K protein band in fact consists of two proteins with different biochemical properties and subcellular localization. Only one of the proteins is sensitive to electrical treatment and present in fractions enriched in synaptosomal plasma membranes.

MATERIALS AND METHODS

Dissection and slice preparation

Male Wistar rats of an inbred strain (TNO, Zeist, The Netherlands) weighing 120–150 g, were used. After decapitation, the brains were dissected and the hippocampi obtained within 3 min after death. They were kept in a Krebs–Ringer buffer (NaCl 124 mM; KCl 5 mM, KH_2PO_4 1.24 mM; $MgSO_4$ 1.3 mM, $CaCl_2$ 2.0 mM, $NaHCO_3$ 26 mM, glucsoe 10 mM, pH 7.4). Before use, the buffer had been equilibrated with a mixture of CO_2 and O_2 (5%/95%). Subsequently, hippocampal slices were cut (300–400 μm thickness) by hand, with a special multiple slice cutter. The slices were then put in a perspex chamber and kept under a continuous flow of humidified CO_2/O_2 (20–40 liter/h), immersed in Krebs–Ringer buffer at 36 °C as described before[6].

Tetanic stimulation

After a preincubation of 60 min in the perspex chamber, a stimulating electrode was placed in the perforant path and a glass recording electrode in the stratum moleculare/stratum granulosum of the fascia dentata. In each slice a stimulus/response relationship was determined as described earlier[6]. The minimal current still giving rise to a population spike in the recorded area was chosen as stimulating current. Subsequently, a series of pulses was given using the same stimulation electrode during 15 s (15 pulses/s).

Tissue fractionation

Slices were taken out of the incubation chamber 15 min after stimulation and immediately homogenized in 100 μl of an ice-cold sucrose solution (0.32 M). Slices that were not stimulated but further equally treated and obtained from the same animal were used as controls. All procedures were performed at 0–4 °C, unless stated otherwise.

Homogenization was performed in a small Potter–Elvehjem device (volume 100 μl) with a motor-driven pestle made of Teflon (700 rpm). Seven strokes were sufficient to give a homogeneous suspension. Homogenates of 5 slices were pooled and spun down at 1000 *g* for 10 min. Thus a pellet was obtained containing mainly unbroken cells, nuclei and debris. The supernatant was carefully sucked off and spun again at 10,000 × *g* for 20 min. This pellet (P2) contains synaptosomes, mitochondria and membrane fragments. In some experiments this pellet was used in the endogenous protein phosphorylation assay. For further analysis the P2 pellet was lysed with 9 vols. of distilled water for 15 min. The lysate (60 μl) then was layered on top of a small discontinuous gradient (0.4 M sucrose 1.3 ml; 1.0 M sucrose, 2.2 ml) and spun down at 100,000 *g* for 80 min, according to the method of De Robertis et al.[12,13]. Material floating on top of the 1.0 M layer was collected, diluted with an acetate buffer (sodium acetate 50 mM, magnesium acetate 10 mM, pH 6.5) and collected by centrifugation (100,000 *g* for 20 min). The thus washed membranes were resuspended in the sodium/magnesium acetate buffer resulting in a protein concentration of approximately 1 μg/μl. This fraction is called t-SPM (see also Fig. 1).

When intact hippocampi were used to prepare a SPM fraction, the procedure described by Terenius[46] and Zwiers et al.[52] was followed: after lysis of the P2 pellet, the suspension was spun down at 10,000 *g* (10 min). The supernatant, called P2-lys-sup, containing light plasma membrane fragments, was layered on top of a gradient (0.4 M sucrose, 8 ml; 1.0 M sucrose, 8 ml; 1.2 M sucrose, 8 ml). After

centrifugation (100,000 *g* maximally, 80 min), material floating on the 1.0 M layer was collected, washed and suspended as described above. This fraction, diluted with buffer to 1 μg/μl protein, was called L-SPM (see also Fig. 1).

Peptide incubation

After cutting the slices, they were allowed to preincubate during 60 min in test tubes with 1 ml of Krebs–Ringer buffer, as described before[5]. A continuous gas flow provided oxygen and gentle movement of the slices. The temperature was kept constant at 29 °C. Ten min before adding methionine-enkephalin (final concentration 10^{-5} M, dissolved in 100 μl buffer) the buffer was replaced by fresh buffer to which bacitracin (30 μg/μl) was added. Incubation with peptide took 60 min at 36 °C and continuous O_2/CO_2 flow. These incubations were stopped by washing the slices twice with an ice-cold sucrose solution (0.32 M) and immediate homogenization subsequently, as described above.

Phosphorylation assay

Subcellular fractions prepared from hippocampal slices or from total hippocampi were phosphorylated as described before[5]. In short: aliquots of SPM or P2 material (ca. 20 μg protein) were incubated with labeled ATP. Unlabeled ATP was present yielding a total concentration of 7.5 μm ATP (incubation volume 25 μl). When substances such as cAMP were tested they were added 15 s before starting the phosphorylation with ATP. After 15 s the incubations were stopped by adding a protein-denaturing mixture containing SDS and β-mercaptoethanol. The total resulting solution was applied to slab gels (polyacrylamide, 11 %, 10 cm long, 1.0 mm thick) in slots with a volume of 50 μl. Proteins were separated during a run of 2–3 h (current 35 mA, voltage 80–120 V). After fixing and staining the thus separated proteins in a mixture of methanol: water:acetic acid (40:60:10, by volume) with 0.1 % Fast Green, the gels were destained overnight, dried and used for autoradiography.

Two-dimensional analysis of proteins

Proteins from L-SPM and the P2 fraction were separated in the first dimension by isoelectric focussing (IEF) in polyacrylamide slab gels. The procedure for casting and running the IEF gel was similar to that described by Zwiers et al.[53], except that the gel contained 8.5 M urea and 0.5 % Triton X-100. Before applying to the gel, the protein samples (100 μg protein, volume 20 μl) were phosphorylated (see above) and the reaction was stopped by freezing the samples in liquid nitrogen.

Membrane-bound proteins were dissolved by adding urea and Triton X-100 resulting in a final concentration of respectively 8.5 M and 0.5 % (v/v). The mixtures were shaken vigorously for 1 min on a Vortex mixer and subsequently ampholines (pH range 3.5–10.0) and sucrose were added (final concentration 2.5 % (w/v) and 5 % (w/v) resp.). The IEF gel was run overnight at 200 V. Individual tracks were excised and incubated for 2 min in 8 ml of the following wash solution: 62.5 mM Tris-HCl, pH 6.8; 2 % SDS; 10 % glycerol; 0.01 % bromophenol-blue and 5 % 2-mercaptoethanol. The washed tracks were run in the second dimension on SDS-polyacrylamide slab gels as described before[53]. Gels were stained for protein, dried, and subjected to autoradiography.

Preparation of calmodulin

Calmodulin was prepared from calf brain by the method of Wallace and Cheung[50].

Protein concentration was determined by the method of Lowry et al.[32].

Quantitation and statistics

Incorporation of label into proteins was quantified in two ways. A scan was made of the autoradiograms, using a set up with higher resolving power as compared with earlier reports (Zeiss PMQII coupled to a Zeiss scanning densitometer, slit width 0.01 mm, wave length 550 nm). Peak heights above background were measured according to Ueda et al.[49]. In some instances phosphoproteins were cut out of gels, guided by the autoradiograph, and counted for radioactivity (in a Xylofluor/Triton X-100 mixture, 23:7, by volume).

Statistical evaluation consisted of Student's *t*-test, two-tailed for non-paired data, if necessary preceded by one-way analysis of variance.

RESULTS

Localization studies

In earlier reports we reported changes in endogenous phosphorylation of a 50,000 M_r protein band as a function of preceding tetanic stimulation[6,47]. By direct comparison of the phosphoprotein of a P2 and a SPM fraction on the same gel, and by analysis of the autoradiograms using a slit width of 10 μm instead of 100 μm[5,6], it became apparent that the 50 K band could be separated into two components with M_r 50,000 and 52,000. They run very close together and the 52 K band does not always appear as a distinct band, due to the high incorporation of label into the nearby 50 K band (see Fig. 2, lane C) and often the 50 K band which in a P2 fraction is phosphorylated predominantly with respect to the 52 K band, overlaps the 52 K on the autoradiogram. In order to study the localization of these two proteins we prepared the following fractions of a rat hippocampal homogenate (see Fig. 1): a crude synaptosomal fraction (P2), a fraction enriched in light membranes and the contents of synaptosomes (P2-lys-sup), two synaptosomal membrane enriched fractions, (L-SPM and t-SPM) and a fraction enriched in mitochondria (P-mito). These fractions were assayed for endogenous phosphorylation and attention was focussed on bands in the 40–55,000 M_r region.

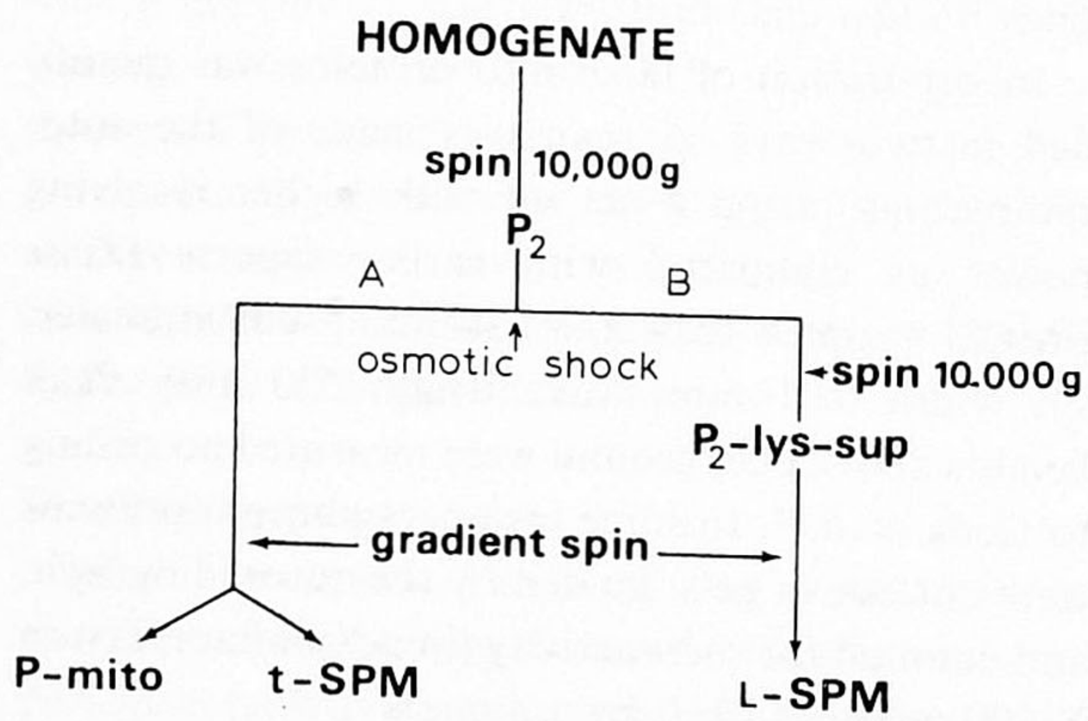

Fig. 1. Fractionation scheme; starting from slices or whole hippocampal tissue several fractions were prepared. Route (A) was used when up to 4 slices were homogenized, route (B) when more slices were pooled, or when whole hippocampi were used.

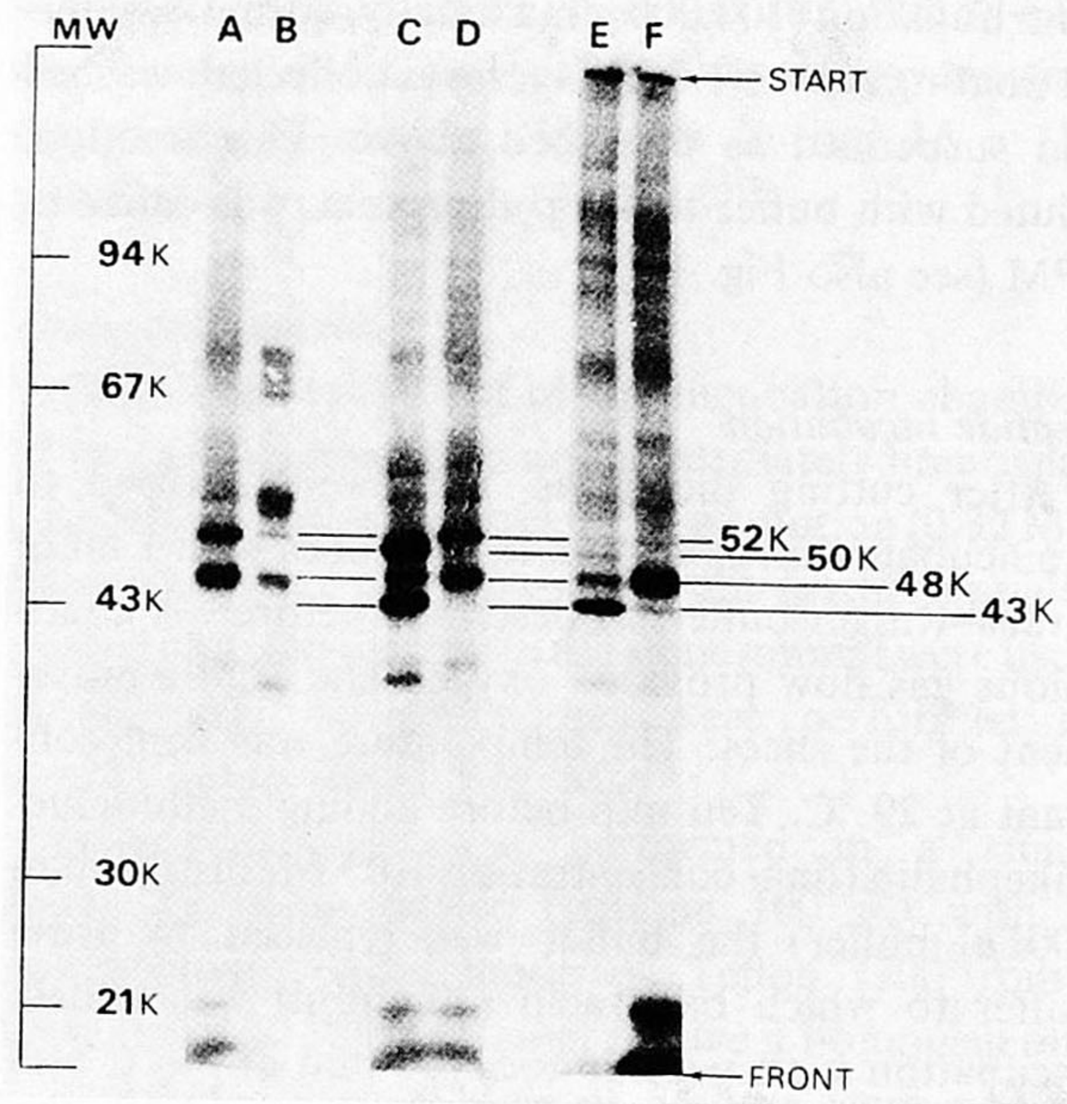

Fig. 2. Autoradiographs of phosphorylated subcellular fractions obtained from rat hippocampal tissue. Lane A, L-SPM; lane B, P2-lys-sup; Lane C; P2; lane D, L-SPM; lane E, P-mito; lane F, t-SPM. Positions of the MW marker proteins are indicated on the left.

In the P2 fraction (Fig. 2, lane C) all 4 major bands in this region are present: 43 K, 48 K, 50 K and 52 K. In this fraction, which still contains mitochondria, 43 K and 50 K are predominantly phosphorylated. After an osmotic shock of this fraction unbroken vesicles and mitochondria are removed by sedimentation. The phosphorylation profile of the resulting P2-lys-sup (Fig. 2, lane B) shows that 43 K and 50 K are no longer present. When this fraction is further separated on a discontinuous sucrose gradient, the material floating on top of the 1.0 M layer, L-SPM, shows a clear enrichment of 48 K and 52 K (Fig. 2, lanes A and D). We also prepared such a gradient fraction (t-SPM) from shocked P2, still containing intact and mitochondrial material. The t-SPM fraction, though less pure than L-SPM, was used when only few slices of rat hippocampus were fractionated after electrical treatment. Such a fractionation procedure yields t-SPM, as well as a pellet enriched in mitochondria (P-mito). It should be kept in mind that although this procedure increases yield it also results in less pure material. As can be seen from Fig. 2, lane E, the P-mito fraction like the P2 contains all four major bands, 43 K being predominantly phosphorylated, whereas in the membrane

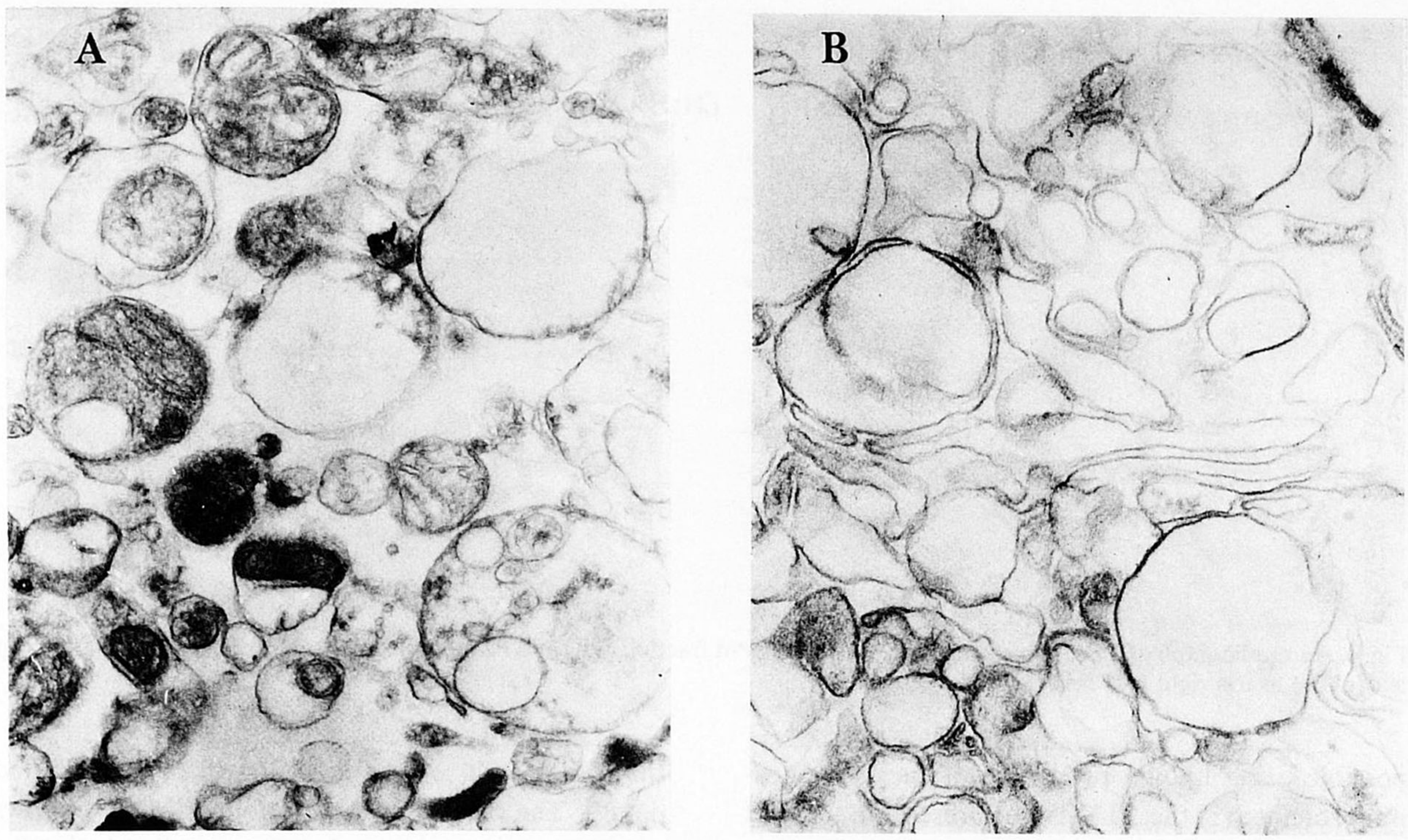

Fig. 3. Electron-microscopic pictures of two subcellular fractions prepared from rat hippocampus. A: P2 fraction. B: L-SPM fraction. × 44000.

fraction (t-SPM, Fig. 2, lane F) again 48 K and 52 K are enriched. Some 43 K phosphorylation is observable.

Fig. 3 shows electron microscopic pictures of the two extreme fractions used: the unpure P2 fraction (Fig. 3A) and the most enriched SPM fraction, the L-SPM (Fig. 3B). The P2 fraction shows intact synaptosomes with mitochondria, whereas in the L-SPM picture the empty synaptosomal structures are obvious. Furthermore, this and other pictures show virtually no myelin or mitochondrial contamination of the L-SPM preparation.

TABLE I

Effect of tetanic stimulation on endogenous phosphorylation assayed in a P2 fraction (A), a t-SPM fraction (B) and a P-mito fraction (C)

Mol. wt.	*(A) P2*		*(B) t-SPM*		*(C) P-mito*	
	△%	*P**	*△%*	*P*	*△%*	*P*
50 K	**		not present		+ 4	n.s.
52 K	+24	<0.01	+30	<0.02	+24	<0.05

* Student's *t*-test was used, two-tailed.
** Data taken from ref. 6; 50 K and 52 K were in that study not quantitatively separated.

Tetanic stimulation

As reported before, high frequent stimulation (15 pulses/s) during 15 s applied to the perforant path fibers of the hippocampal slice, resulted primarily in an increased phosphorylation of a 50 K band in a post hoc assay (ref. 6, data given in Table I). We now demonstrate that 50 K and 52 K, components of the 50 K band, behave differently after a tetanic stimulation. The 50 K protein did not show any change in phosphorylation in a P-mito fraction (see Table I). However, the 52 K protein, both in a P-mito fraction and in the t-SPM showed a significant increase in ^{32}P-incorporation after stimulation of 24% and 30%, respectively (see Table I). We therefore conclude that the increase of 24% after tetanus observed earlier[6] in a P2 fraction is attributable to the 52 K component of the 50 K band.

Two-dimensional gel electrophoresis and determination of IEP

The proteins present in the P2 and L-SPM were separated two-dimensionally as described in the Materials and Methods section. Identification of the 50 K and 52 K proteins after autoradiography was

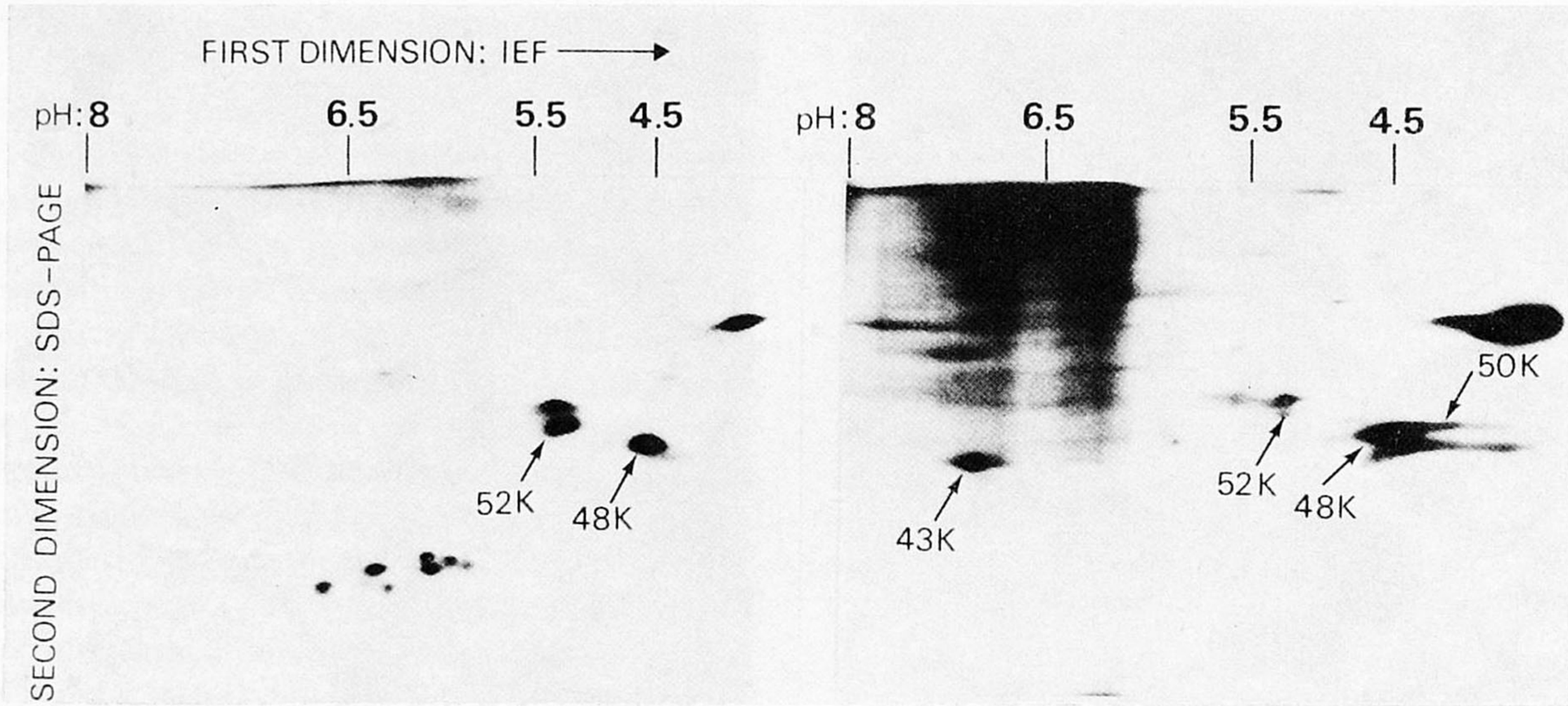

Fig. 4. Autoradiograph of a two-dimensionally separated L-SPM fraction (left) and P2 fraction (right). The pH-gradient is from top left (pH 8) to top right (pH 3.5).

possible using known properties of the two phosphoproteins: (1) the 50 K is not present in an SPM fraction; (2) the two different proteins show a different Ca^{2+}-dependency (see next section); (3) MW-markers, as well as phosphorylated SPM fraction were always applied to the same SDS gel on which the second dimension separation was run. As Fig. 4 (left) shows, the 50 K protein appears to be heterogeneous after isoelectric focussing. The IEP of the components ranges from 3.5 to 4.3. The 52 K protein is present as a minor phosphoprotein, with an IEP of 5.3. In Fig. 4 (left) the autoradiogram of phosphorylated and two-dimensionally separated L-SPM proteins is shown. The 50 K protein is not present in this fraction, whereas the 52 K protein is one of the major phosphoproteins.

Calcium, calmodulin and cAMP

In a series of experiments the sensitivity of the phosphoproteins in the P2 fraction to various modulators was tested. The sensitivity to calcium was measured in the presence of 10 mM magnesium and 1 mM EGTA; the calcium concentration was varied from 0 to 50 mM.

The amount of labeled phosphate, incorporated into 4 proteins as a function of the calcium concentration is depicted in Fig. 5. The 48 K (Fig. 5B) and the 50 K (Fig. 5C) protein show a clear optimum, both at 2–3 mM calcium. The calcium sensitivity of the 48 K protein is in agreement with earlier reported data[53]. The 52 K phosphoprotein (Fig. 5D) shows no Ca-dependency over the range studied here. The 43 K protein displays a biphasic sensitivity to calcium: a stimulation at low, and an inhibition at high concentrations of calcium (Fig. 5A). In all curves the incorporation is indicated when no EGTA, or divalent cation is added exogenously.

The influence of calmodulin of the endogenous phosphorylation of the 50 K and 52 K protein is measured in a P2 fraction in the presence of EGTA (1 mM) and varying calcium concentrations. Fig. 6 shows that the phosphorylation of the 43 K protein is stimulated with ca. 30% at all calcium concentrations. The 48 K proteins shows a shift in its optimal calcium concentration to a somewhat lower value, a phenomenon displayed by the 50 K protein in a more pronounced fashion: app. 45% more phosphate is incorporated (as established by cutting the proteins from the gel and liquid scintillation counting of the cut gel parts), and the optimal calcium concentration in the presence of calmodulin is shifted to a lower value (8×10^{-3} versus 10^{-3} with calmodulin).

When studied in a P2 fraction, cAMP does not seem to influence the phosphorylation of the 50 K or 52 K proteins. Over a dose range of 10^{-4}–10^{-8} M of cAMP no changes in endogenous phosphorylation of these proteins were observed (data not shown).

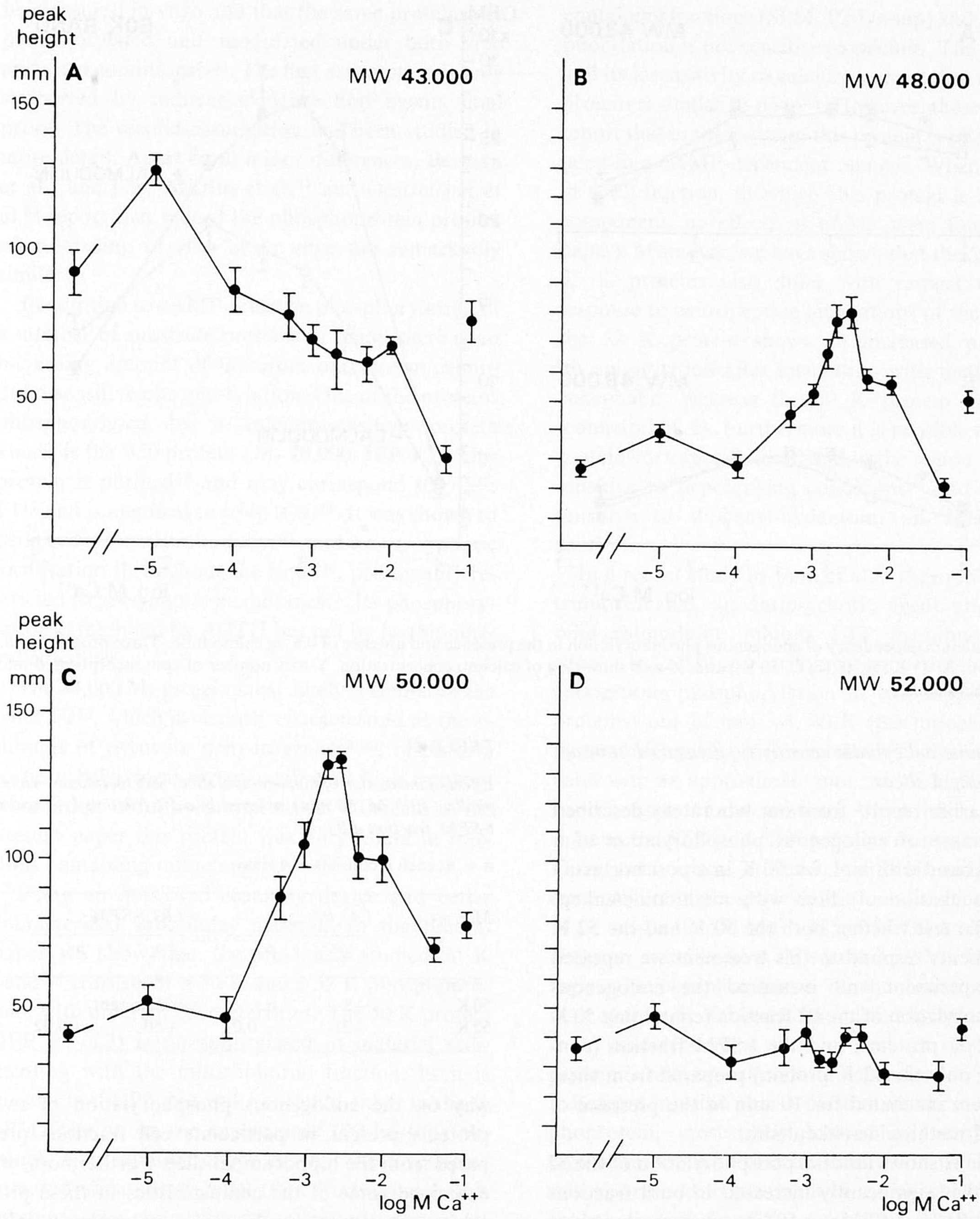

Fig. 5. Calcium dependency of endogenous phosphorylation for four proteins: A, 43 K; B, 48 K; C, 50 K; D, 52 K. On the X-axis, the log of calcium concentration is given, on the Y-axis the peak height (above background) in mm measured after scanning autoradiographs. Single points (right side of each curve) represent the peak height when no exogenous Ca nor chelator is added ('standard' phosphorylation conditions). Means ± S.E.M. (bars); n is at least 4.

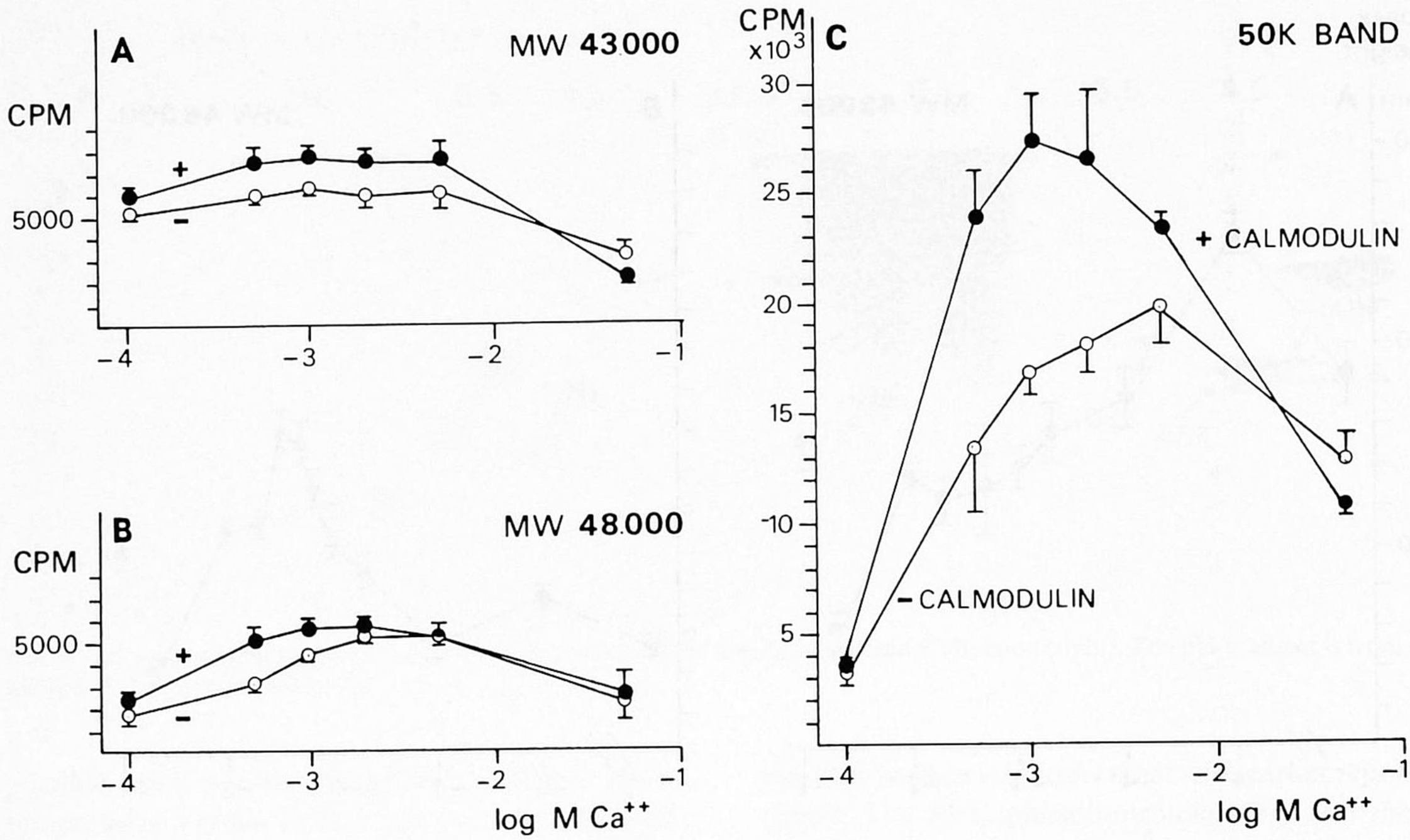

Fig. 6. Calcium dependency of endogenous phosphorylation in the presence and absence of 0.6 μg calmodulin. Three protein bands are shown. A, 43 K; B, 48 K; C, 50 K band. X-axis shows log of calcium concentration, Y-axis number of cpm incorporated into the protein bands.

Methionine-enkephalin sensitivity, measured in intact hippocampal slices

An earlier report[5] from our laboratory described the increase of endogenous phosphorylation of a protein band with mol. wt. 50 K in a post hoc assay after incubation of slices with methionine-enkephalin. To test whether both the 50 K and the 52 K components respond to this treatment we repeated that experiment and measured the endogenous phosphorylation of the P2 fraction (containing 50 K and 52 K proteins) and the t-SPM fraction (containing only the 52 K protein) prepared from slices that were incubated for 10 min in the presence of 10^{-5} M methionine-enkephalin.

Table II shows that the phosphorylation of the 52 K band is significantly increased in both fractions (P2: +21%; t-SPM: +50%), whereas the phosphorylation of the 50 K protein is not affected.

TABLE II

Effect of incubation of hippocampal slices with methionine-enkephalin (10^{-5} M, 60 min) assayed in a P2 fraction (A) and a t-SPM fraction (B)

n = 6; each n consists of 4 slices.

Mol. wt.	*(A) P2*		*(B) t-SPM*	
	△%	*P*	△%	*P*
50 K	−8	n.s.	not present	
52 K	+21	<0.01	+50	<0.02

DISCUSSION

In this report we dealt with the effect of high frequent stimulation of a hippocampal synaptic pathway on the endogenous phosphorylation of two proteins present in particulate cell fractions prepared from the hippocampal slice. Furthermore, we examined some of the characteristics of these proteins: subcellular localization, molecular weight, IEP and the effects of various conditions (calcium, enkephalin, cAMP, calmodulin) on their phosphorylation in vitro.

The post hoc approach we used in this study and many others[4,10,14,16,26,54] assumes that in vivo changes in endogenous protein phosphorylation can

be measured in vitro and that the same proteins are phosphorylated and modulated under both vivo and vitro conditions[5,54]. The first assumption is only supported by indirect evidence and awaits final proof. The second assumption has been studied in more detail. Apart from minor differences, Berman et al.[7] and later Mitrius et al.[37] and Oestreicher et al.[39] report that indeed the phosphoprotein profiles after labeling in vivo or in vitro are remarkably similar.

In addition to cAMP-sensitive phosphorylation of a number of substrate proteins in brain, there is an increasing amount of literature on calcium/calmodulin-sensitive phosphorylation. One of the proteins phosphorylated by a calcium-sensitive protein kinase is the B50 protein (M_r 48,000, IEP 4.5). This protein is purified[53] and may correspond to γ-5[25], F1[17] and is identical to p54p (Ca)[34]. It was shown to be specific for nervous tissue[21] and has a synaptic localisation throughout the brain[40], presumably restricted to presynaptic membranes[45]. Its phosphorylation is inhibited by ACTH but not by methionine-enkephalin[52].

The 43,000 M_r protein most likely is similar to the band F2[42], which is recently characterized as the α-subunit of pyruvate dehydrogenase[9,38], responsive to both behavioral experience[41] and high frequent stimulation of hippocampal tissue[10]. Indeed, in the present paper this protein was only found in fractions containing mitochondria.

Using an improved scanning device and better characterized subcellular material, in the present paper we show that the previously studied 50 K band[5,6] consists of a 50 K and a 52 K phosphoprotein, with different characteristics. The 50 K protein (IEP 3.5–4.3) is phosphorylated in material sedimenting with the mitochondrial fraction, as it is present in the P2, but was not found either in the L-SPM fraction or the supernatant (P2-lys-sup) obtained after osmotic shock of the crude synaptosomal/mitochondrial pellet (P2). Furthermore, this protein is strongly calciumdependent in its phosphorylation and in the presence of calmodulin the calcium optimum is shifted towards lower concentrations of the divalent cation.

The other protein found in the 50 K band has a slightly higher mol. wt. (52 K), a less acidic IEP (5.3), is found predominantly in plasma membrane containing fractions (SPM, P2-lys-sup) and its phosphorylation is not sensitive to calcium. The IEP, M_r and its insensitivity to calcium suggest that the 52 K protein is similar to p54p[35]. However, these authors report that in their system this protein is phosphorylated in a cAMP-dependent manner. When studied in a P2 fraction, in which this protein is a minor component, no effects of cAMP were found (this paper). Moreover, we have shown that the 50 K and 52 K proteins also differ with respect to their response to neuropeptide incubations of slices: only the 52 K protein shows an increased post hoc phosphorylation after incubation with methionine-enkephalin, whereas the 50 K protein does not (compare ref. 5). Furthermore it is possible that this protein corresponds to a band in the region of 50 K sensitive to depolarising conditions[28] and a band sensitive to diphenyl-hydantoin, an anticonvulsant[11].

In a recent study of Finn et al.[19] they report that trifluoperazine, an antipsychotic agent known to bind calmodulin, inhibits LTP in hippocampal slices. In addition, trifluoperazine inhibits the endogenous phosphorylation of two hippocampal proteins: one of mol. wt. 40 K (the mitochondrial pyruvate dehydrogenase α-subunit[9,38]), the other is a band with an approximate mol. wt. of 51,000. This band consists of two proteins, which might be the same as the 50 K and 52 K proteins mentioned here. Direct comparison, however, is difficult, as they use quite different phosphorylation conditions (1 mM Mg^{2+} versus 10 mM Mg^{2+}, 125 μM EGTA versus no chelator, and 50 μM ATP versus 7.5 μM). Interestingly, the phosphorylation of the lower component of their 51 K band is stimulated by calmodulin in a dose-dependent fashion, just as our 50 K protein is calcium/calmodulin-dependent.

Bearing in mind that a plasma membrane phosphoprotein, most likely located in the synaptic region, responds to electric stimulation (ref. 6 and this report) and to treatment of the intact slice with enkephalin (ref. 5 and this report) one is tempted to speculate on a functional correlation. Combining these data with the fact that: (1) methionine-enkephalin is present in the hippocampus in intrinsic peptidergic neurons[20,27], which are believed to modulate other hippocampal neurons, and (2) the idea that enkephalins affect neuronal circuits in the

hippocampus[22,23,36], it might be postulated that long-term potentiation at least partly involves enkephalinergic neurons[22,23] and that the 52 K protein is involved in the development and/or origin of this phenomenon.

The present study confirms and extends our observations of the change in protein phosphorylation after tetanic stimulation of the perforant path. The data show that the effects are confined to a 52 K protein (IEP 5.3), whose phosphorylation occurs in a calcium-independent manner. It is worth mentioning that the change in phosphorylation brought about by the tetanic stimulation itself is absolutely dependent on the presence of calcium[6]. The localization studies as carried out with various subcellular fractions point to a synaptic membrane rather than a mitochondrial origin of the 52 K phosphoprotein. Thus, in addition to the metabolic correlates discussed by Browning[9] and Lynch and Schubert[33] (energy metabolism and calcium in mitochondria), tetanic stimulation may indeed bring about changes in protein phosphorylation in synaptic membranes. Whether such changes are merely correlates or whether they indeed relate to the altered synaptic efficacy brought about by the stimulation is subject to further research.

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