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Changes in Membrane Phosphorylation Correlated with Long-Lasting Potentiation in Rat Hippocampal Slices

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INTRODUCTION

Long-term potentiation is a well known phenomenon which has been used as an experimental model for plasticity at the synaptic level. Long-term potentiation (LTP) is characterized by the fact that a synaptic response to stimulation of the corresponding afferents with a pulse, becomes enhanced after the application of a train of pulses to the same afferents; this potentiation can last for many minutes, even hours as shown in many preparations (for review see Spencer and April, 1970). LTP is considered to be an analogue of a class of plastic phenomena in the nervous system which underlie learning processes. The latter imply of course behavioural modification which certainly involves more complex stimulus patterns than LTP. Nevertheless, it can be assumed that a study of the cellular processes underlying LTP may give some clues regarding the basic changes in neuronal activity accompanying learning processes.

HYPOTHESES CONCERNING LTP

Several hypotheses have been advanced to explain LTP. Both presynaptic and postsynaptic processes have been implied. At the *presynaptic level* LTP may be caused by an increase in the amount of transmitter released, for example, induced by an enhancement of $[Ca^{2+}]_i$ or a modification of the local turnover of transmitter. At the *postsynaptic level* LTP may be related to a modification of receptor properties, to a change in the spike-generating capacity of postsynaptic neurons, and/or a change in input resistance of the dendrites of the postsynaptic neurons. These different possibilities are, of course, not mutually exclusive. Elsewhere, we have discussed that several of the factors indicated above, probably work in a complementary way in producing LTP (Lopes da Silva et al., 1982).

INFLUENCE OF NEURONAL STIMULATION ON NEUROCHEMICAL PROCESSES

All the possibilities listed above imply changes in neuronal membrane processes. Therefore, we have been trying to elucidate whether it is possible to understand the changes occurring at the membrane level after the electrical stimulation and which may help clarify the basic

processes in the development of LTP. The influence of electrical stimulation upon biochemical processes within neurons has been investigated in relation to different subsystems: macromolecular synthesis, enzyme and receptor induction, etc. Although macromolecular synthesis has been implied in mediating the function of memory traces (Dunn, 1976) it is not likely that it is responsible for the rapid changes occurring in LTP. Indeed, it has been shown by Schwartz et al. (1971) that blocking protein synthesis does not alter the post-tetanic facilitation of the monosynaptic response in neurons in the abdominal ganglion of Aplysia. Thus, although plastic changes taking place in the course of days may depend on protein synthesis, phenomena such as LTP which is established in a few seconds are not dependent on such biochemical changes. Enzyme induction has received some attention as a possible biochemical mechanism for explaining long-term effects of neural activity. Indeed, electrical stimulation of the pre-ganglionic input to the rat superior cervical ganglion lasting 10–90 min increases tyrosine hydroxylase (TH) activity in the ganglion (Zigmond and Bowers, 1981). TH is responsible for the synthesis of DOPA from tyrosine. Whether such a mechanism plays a role in LTP induced by stimuli within the physiological range is not yet clear.

Recently, Baudry and Lynch (1980) suggested that LTP elicited in hippocampal slices may depend on the exposure of additional glutamate receptors in the dendrites of hippocampal neurons; they proposed a multiple-stage hypothesis for LTP: (a) the tetanus would cause an increase in intracellular Ca^{2+}; (b) this would lead to the activation of a membrane-bound protease which (c) would expose glutamate receptors. This hypothesis is attractive since it does not imply new protein synthesis and it could account for the speed with which LTP is established. Additional experimental evidence, however,is needed in order to establish this hypothesis on a firm basis.

Another interesting biochemical phenomenon which may constitue a basis for explaining LTP is the formation of the covalent bond between a phosphate group and a protein amino acid residue (serine, threonine), since this is a very fast reaction which takes place in seconds and can have an important influence on protein structure and enzyme activity (Weller, 1979). This comprises the phenomenon of protein phosphorylation. It is known (Heald, 1957, 1962) that electrical stimulation of brain cortex slices leads to changes in protein phosphorylation. Moreover, Forn and Greengard (1978) have shown that depolarization of cortical slices using high concentrations of K^+ or veratridine is correlated with the degree of phosphorylation of a specific neuronal membrane protein. These new facts led us (Bär et al., 1980a) and others (Browning et al., 1979a) to investigate whether the degree of synaptic protein phosphorylation was related to LTP.

EXPERIMENTAL EVIDENCE ON THE RELATION BETWEEN LTP AND MEMBRANE PROTEIN PHOSPHORYLATION

The two initial studies on this topic were those of Browning et al. (1979a) and ours (Bär et al., 1980a). It is useful to discuss here the common aspects of those investigations and also the main differences. In both studies slices of hippocampus were used since in this preparation it is relatively easy to combine physiological and biochemical measurements. In the transversal slice of the hippocampus (Fig. 1) several sub-systems may be distinguished (Lopes da Silva and Arnolds, 1978); the main ones are the following: the perforant path → granule cell synapses, the mossy fibres (axons of granule cells) → CA3 pyramidal neurons, the Schaffer collaterals (collaterals of CA3 axons) → CA1 pyramidal neurons. Browning et al. (1979a) and Bär et al: (1980a) used two different sub-systems for their investigations: the former studied

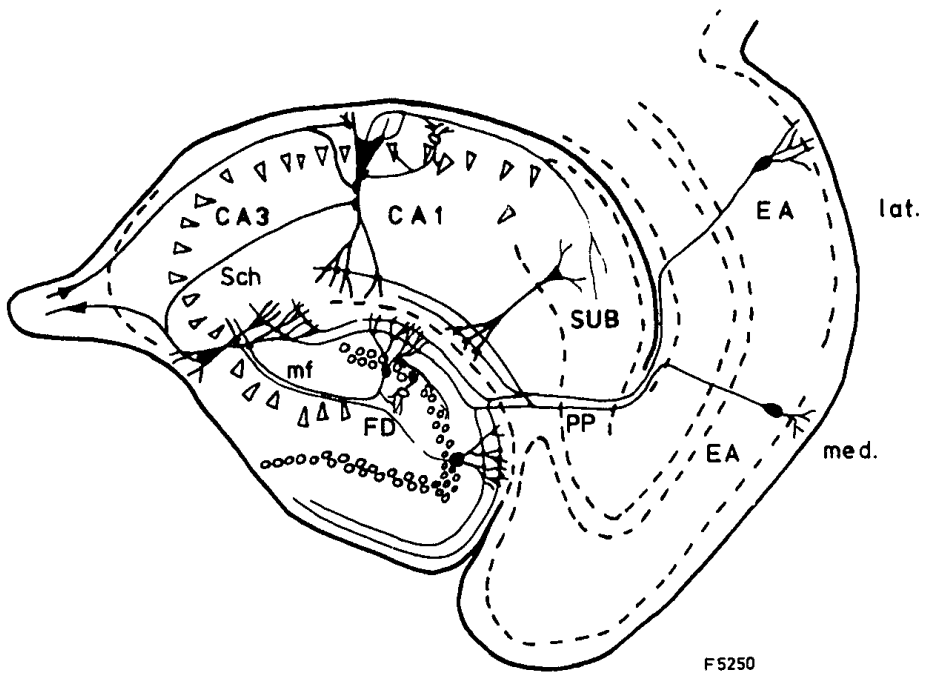

Fig. 1. Diagram of a section of the hippocampal region of a rat. Different fields and sub-fields are indicated. Two sub-systems, which we discussed in the text should be singled out; the Schaffer collaterals (Sch) projecting to the apical dendrites of sub-field Ca1 and the perforant path fibres (PP) projecting to the granular cells of the fascia dentata (FD).

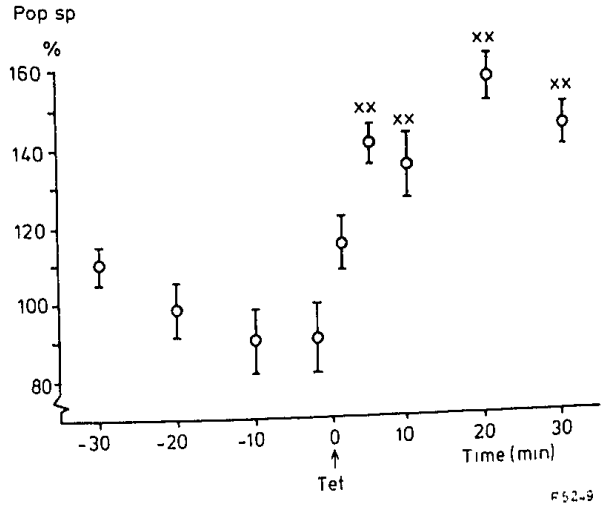

Fig. 2. Plot of the mean amplitude and the corresponding S.E.M. of the population spike (open circles) and the extracellularly recorded EPSP (closed circles) recorded from the fascia dentata and elicited by a short stimulus applied to the perforant path (5 slices). Note the time scale is logarithmic in order to emphasize the initial brief depression phase. The potentiation of both responses can be seen. "Controls" are values obtained before the tetanus. (Adapted from Tielen et al., 1982).

LTP of the synapses of the Schaffer collaterals → CA1 pyramidal neurons, whereas the latter used the perforant path → granule cell synapses in the dentate area (Fig. 2). In both systems LTP was found, although there were differences in some aspects which confirm results obtained previously (Alger and Teyler, 1976; Dudek et al., 1976; Schwartzkroin, 1975). The

main difference appears to be that LTP in CA1 is established almost immediately after the cessation of the tetanus whereas in the dentate area LTP appears clearly after a brief phase of depression, i.e. about 8 or 10 min after the tetanus (Tielen et al., 1982). In both cases, however, it may persist for hours. The way the slices were stimulated was also not exactly the same; Browning et al. (1979a) used a tetanus consisting of 100 pulses/sec for 1 sec, whereas Bär et al. (1980) used 15 pulses/sec for 15 sec. It is appropriate to note that in both hippocampal sub-systems the same synaptic transmitters are present: glutamate and aspartate (DiLauro et al., 1981; Storm-Mathisen, 1977). An important difference between the two studies, however, is the fact that Browning et al. (1979a) took the slices 2 min after the application of the tetanus for biochemical analysis, whereas Bär et al. (1980a) left them in the incubation bath for 15 min after the tetanus and only then proceeded with the biochemical analysis. The reason for this delay was the fact that LTP in the dentate was established in most slices only 8 or 10 min after the tetanus. The biochemical assay was essentially the same but the following differences should be pointed out: in the study of Browning et al. Mg^{2+} concentration was 1 mM, ATP was 50 μM and EGTA (125 μM) was included, whereas in that of Bär et al. 10 mM for Mg^{2+} and 7.5 μM for ATP were used and no EGTA was added. Undoubtedly, this may have some influence on the endogenous phosphorylation of the hippocampal acceptor proteins. In both studies, however, the so-called post-hoc phosphorylation assay (Routtenberg et al., 1975) was used. In this way one determines post hoc the capacity of the protein to accept phosphate; this capacity will be larger if the protein, when entering the assay, has more sites available than if the sites are already occupied. This means that a protein that in the post-hoc assay shows strong phosphorylation was rather depleted from phosphate in vivo, and vice versa.

The main results of the two studies differed in that Browning et al. put all the emphasis on the decrease of phosphate incorporation of a protein with a molecular weight around 40 000 (40 K) present in a crude mitochondrial fraction and only mention an increase of phosphorylation of two other major phosphoprotein bands: one at 53 K and the other at 27 K. In the study of Bär et al., also using a mitochondrial fraction, the main finding was an increase in phosphorylation of a 50 K band whereas the 40 K band only showed a slight decrease of phosphorylation which did not reach a significant level. The most likely reason which may account for these differences is the fact that Browning et al. (this volume) have shown that the increase in phosphorylation of the 40 K band is of short duration; it reaches again control values around 10 min after the tetanus. This is probably the reason why Bär et al. did not find significant changes in this band since they analysed the slices only after 15 min. Nevertheless it should be stressed that in both studies a decrease in post-hoc phosphorylation of a 50–53 K band was observed, although it was clearer in the study of Bär et al. It is possible that here also the factor time may account for the difference in degree of phosphorylation of the 50–53 K band, but at the moment there is no further evidence to support such an explanation.

Since these original observations, progress has been achieved in identifying these protein bands more precisely. Browning et al. (1979b) found that the 40 K band was phosphorylated in vitro by phosphorylase kinase and on the basis of molecular weight and proteolytic fingerprinting they recently reported (Browning et al., 1981) that this band is the α subunit of the enzyme pyruvate dehydrogenase (PDH). It is also most interesting to note in this connection that phosphorylation of the same protein band was shown by Routtenberg et al. (1975) in vivo to be responsive to behavioural training; later they determined that the protein band in question (40–43 K or band F2) was also the α subunit of PDH (Morgan and Routtenberg, 1980). Furthermore, Morgan and Routtenberg (1981) have shown that an increase in post-hoc assayed phosphorylation of the protein band 40 K was correlated with a decrease of PDH enzyme activity.

Our own recent work revealed that the 50 K protein band in fact consists of two proteins with different biochemical properties and subcellular localization (Bär et al., 1981, 1982). Only one of the proteins is sensitive to electrical treatment and present in synaptosomal plasma membranes. By direct comparison of the 50 K phosphoprotein band of a crude mitochondrial/synaptosomal (P2) fraction and of a fraction enriched in synaptic membranes on the same gel, and by analysis of the autoradiograms using a slit width of 10 μm instead of 100 μm it became apparent that the 50 K band could be separated into two components with M_r 50 K and 52 K. They run very close together and the 52 K band does not always appear as a distinct band, owing to the high incorporation of label into the nearby 50K band; often the 50K band which in

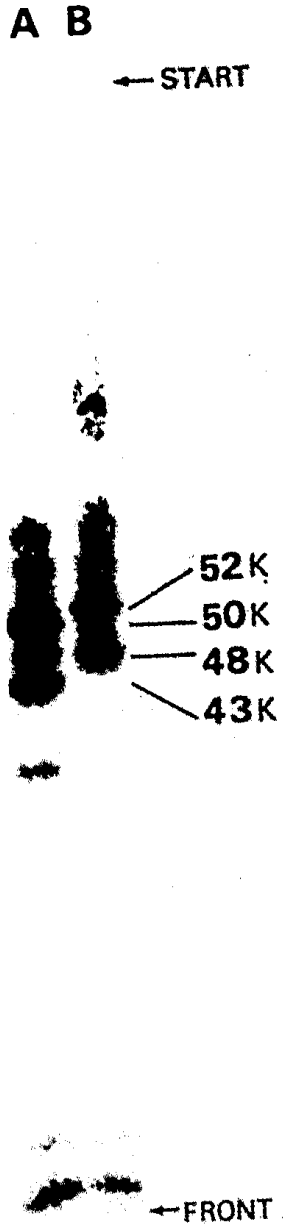

Fig. 3. Autoradiogram of separated proteins, phosphorylated in a crude mitochondrial/synaptosomal fraction (A) or in a purified synaptic membrane fraction (B) of rat hippocampal tissue. The numbers denote the position and apparent molecular weight of the phosphoproteins discussed in the text.

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 phosphoproteins in more detail we prepared the following fractions of a rat hippocampal homogenate: a crude synaptosomal/mitochondrial fraction (P2), and a synaptosomal membrane–enriched fraction (SPM). These fractions were assayed for endogenous phosphorylation and attention was focussed on bands in the 40–55 K region.

In the P2 fraction (Fig. 3, lane A) all four 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. The 48 K and 43 K phosphoprotein bands are also seen in other studies on brain phosphoproteins. The 48 K protein is phosphorylated by a calcium-sensitive protein kinase and is identical to the B-50 protein (M_r 48 K, IEP 4.5). This protein was purified (Zwiers et al., 1979, 1980) and may correspond to γ-5 (Rodnight, 1979), F1 (Ehrlich and Routtenberg, 1974) and is identical to p54p (Ca) (Sörensen et al., 1981). It was shown to be specific for nervous tissue (Kristjansson et al., 1982) and to have a synaptic localization throughout the brain (Oestreicher et al., 1981), presumably restricted to presynaptic membranes (Sörensen et al., 1981). Its phosphorylation is inhibited by ACTH but not by methionine-enkephaline (Zwiers et al., 1976, 1980).

The 43 K protein most likely is similar to the band F2 (Routtenberg et al., 1975), which is recently characterized as the α subunit of pyruvate dehydrogenase (see above). Indeed, in our bands this phosphoprotein was only found in fractions containing mitochondria.

After an osmotic shock of this crude fraction the light synaptic membranes were isolated by sucrose gradient centrifugation (Fig. 3, lane B) as described by Zwiers et al. (1976). The SPM shows a clear enrichment of phosphorylation of 48 K and 52 K. Thus, we concluded that the endogenous phosphorylation of the 50 K band takes place in material that sediments with the mitochondria whereas that of the 52 K band is associated with the SPM (Bär et al., 1982).

Subsequently, the proteins present in the P2 and SPM were separated two-dimensionally as described by Zwiers et al. (1979). Identification of the 50 K and 52 K proteins after autoradiography was 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; (3) MW markers, as well as a phosphorylated SPM fraction were always applied to the same SDS gel on which the second dimension separation was run. The 50 K protein appears to be heterogenous 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. It has been shown that the 50 K protein is strongly calcium-dependent in its phosphorylation and in the presence of calmodulin the calcium optimum is shifted towards lower concentrations of the divalent ion (Bär et al., 1982). In contrast, the endogenous phosphorylation of the 52 K protein is not sensitive to calcium. The IEP, M_r and its insensitivity to calcium suggest that the 52 K protein is similar to p54p (Mahler et al., this volume). 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 (Bär et al., 1981). 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 Bär et al., 1980b). Furthermore, it is possible that the former protein corresponds to a band in the region of 50 K, sensitive to depolarizing conditions (Krueger et al., 1977) and a band sensitive to diphenylhydantoin, an anticonvulsant (DeLorenzo and Freedman, 1977).

As reported before, high frequent stimulation (15 pulses/sec) during 15 sec applied to the

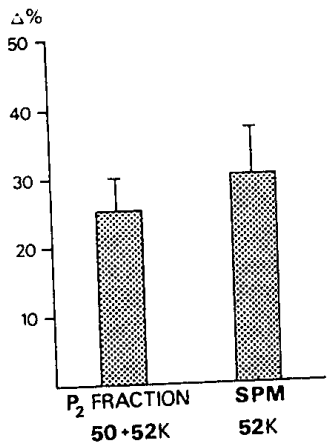

Fig. 4. Increase in phosphorylation of the 50–52 K proteins in different subcellular fractions in a post-hoc assay after high frequent stimulation of the hippocampal slice (n = 6) as compared to controls (n = 6).

perforant path fibres of the hippocampal slice, resulted primarily in an increased phosphorylation of a protein in a post-hoc assay using the P2 fraction (data given in Fig. 4). We demonstrated that 50 K and 52 K proteins behave differently after a tetanic stimulation. In the mitochondrial fraction, the 50 K protein did not show any change in phosphorylation after the tetanus, whereas the 52 K protein in the SPM showed a significant increase in ^{32}P incorporation of 30%. We therefore concluded that the increase of 24% after tetanus observed earlier (Bär et al., 1980a) in a P2 fraction is attributable to the 52 K component of the 50 K band (Bär et al., 1981, 1982).

CONCLUDING REMARKS

In conclusion, these data confirm and extend 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 (Bär et al., 1980a). The localization studies 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 et al. (this volume) and Lynch and Schubert (1980) (energy metabolism and calcium in mitochondria), tetanic stimulation may indeed bring about changes in protein phosphorylation in synaptic membranes. Besides these phenomena we have found (Bär, 1982) that a tetanus provokes also a change in phosphoinositide metabolism, namely the hydrolysis of membrane-bound (poly) phosphoinositides (PI, DPI, TPI) yielding diacylglycerol (dAG) which may play a role in the fusion of synaptic vesicles and hence in transmitter release and may change membrane fluidity and thus membrane permeability. Also, dAG is phosphorylated to PA which may function as Ca^{2+} ionophore (Harris et al., 1981; Salmon and Honeyman, 1980); in this way an influx of Ca^{2+} can take place and cytosolic Ca^{2+} may rise approximately 1000-fold. This increase would be maintained by a decreased activity of a mitochondrial enzyme pyruvate dehydrogenase which Browning et al. (this volume) showed to be affected by the tetanus. At the present moment we can only

hypothesize that the change in phosphorylation of the 52 K protein may result also in an altered sensitivity of the postsynaptic membrane, towards the neurotransmitter. In order to determine the exact role of this protein in the process of LTP it is necessary to find its exact location and to characterize it in more detail. Such characterization will certainly give insight in the synaptic processes responsible for LTP.

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