Synaptic Protein Phosphorylation and Long-term Potentiation

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INTRODUCTION

Plasticity of synaptic connections is one of the most intriguing and probably one of the most fundamental problems in neurobiology today. Within the context of this review synaptic plasticity is defined as the alteration of a synaptic response to a certain stimulus as a function of previous experience. The discovery of long-term potentiation (LTP) occurring after tetanic stimulation of mammalian hippocampal synaptic connection demonstrated that this form of plasticity may persist for many hours and days (Bliss and Lomo, 1973; Bliss and Gardner-Medwin, 1973). As the phenomenon of LTP appears to be restricted to the synapses being stimulated and is not transferred heterosynaptically (Andersen et al., 1977), neurochemists have tried to elucidate the molecular events involved in neurotransmission that would initiate and/or maintain LTP. In view of the importance of post-translational covalent modification of proteins to their respective function it is not surprising that a number of studies dealt with changes in the degree of phosphorylation of synaptic proteins in relation to their role in synaptic function (Nestler and Greengard, 1984).

The early studies by Heald (1957, 1962) already indicated that electrical stimulation of cerebral slices promoted the incorporation of phosphate into proteins. Since then it has been shown that a variety of extracellular signals produce many of their physiological responses by regulating the state of phosphorylation of synaptic membrane- or vesicle-bound proteins. In this review I shall briefly summarize our present knowledge of some of the synaptic proteins which phosphorylation is affected by tetanic stimulation or is linked to enhanced transmitter release.

PYRUVATE DEHYDROGENASE

In the first studies on LTP and protein phosphorylation it was

found that brief tetanic stimulation of Schaffer collaterals in rat hippocampal slices followed by a post hoc endogenous phosphorylation assay of a synaptic particulate fraction leads to a specific decrease in the phosphorylation of a 40 kDa protein (Browning *et al.*, 1979). Control experiments, in which calcium was omitted from the incubation medium or in which low frequency stimulation was applied, revealed no change in the endogenous phosphorylation of this protein band. Interestingly, the decrease in the post-hoc phosphorylation of the 40 kDa protein was only significant 2 and 5 min after tetanic stimulation and had already disappeared after 15 min (Browning *et al.*, 1982). In further work it was established that trifluoperazine a potent calmodulin inhibitor blocked the induction of LTP and the direct phosphorylation of the 40 kDa protein band (Finn *et al.*, 1980; Browning *et al.*, 1982). Studies on the identification of the 40 kDa protein revealed that the protein is highly enriched in the mitochondrial fraction of hippocampal synaptosomes and extensive neurochemical characterization led to the conclusion that the 40 kDa protein is identical to the α -subunit of pyruvate dehydrogenase, a mitochondrial enzyme involved in calcium sequestration (Browning *et al.*, 1982). Therefore, these authors hypothesize that the regulation of pyruvate dehydrogenase activity by phosphorylation of the α -subunit, resulting from the tetanic stimulation, would lead to a transient inhibition of mitochondrial calcium sequestration and thus temporarily elevating intracellular calcium affecting synaptic physiology. This short-term elevation in calcium could directly mediate the short-term potentiation seen after high frequency stimulation. However, these changes in calcium level could participate in the production of LTP only if they were to trigger cellular alterations of a more permanent nature (Browning *et al.*, 1982).

As Hoch *et al.* (1984) found only minor if any changes in the phosphorylation of the α -subunit of pyruvate dehydrogenase after tetanic stimulation of hippocampal slices, they suggest that the transient increase in calcium, known to occur after stimulation, is a reflection of an altered influx rather than an altered sequestration of calcium. More studies are needed to assess the precise role of sequestration in this respect.

B-50

From rat brain synaptic plasma membranes (SPM) and acidic phosphoprotein was purified and partially characterized (Zwiars *et al.*, 1980). This protein, named B-50, has a molecular weight of 48 kDa and an IEP of 4.5. The protein is localized in presynaptic terminals throughout the brain associated with vesicle and plasma membranes (Gispen *et al.*, 1985). As estimated by radioimmunoassay the rat hippocampus contains about 36 pg/ μ g tissue (Oestereicher *et al.*, 1986).

In a series of experiments we have shown that the kinase responsible for the phosphorylation of B-50 is identical to the cyclic nucleotide-insensitive, calcium- and phospholipid-requiring protein kinase C, originally isolated by Inoue *et al.* (1977; Aloyo *et al.*, 1982). This observation led us to explore what, if any, role this substrate protein of kinase C could play in the receptor-mediated events that are known to stimulate protein kinase C (Nishizuka, 1984). For, receptor-mediated activation of phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis by a phosphodiesterase (PLC) yields inositol triphosphate (IP₃) and diacyl-

glycerol (DG). IP₃ is thought to act as signal for the mobilization of calcium from internal stores, whereas DG is known to stimulate protein kinase C. Based on a variety of evidence it was proposed that an increase in the phosphorylation of B-50 inhibits the activity of phosphatidylinositol 4-phosphate (PIP) kinase converting PIP to PIP₂. Hence, it was suggested that this protein kinase C substrate could provide a negative feedback control in the receptor-mediated hydrolysis of PIP₂ (Gispen 1986; see Fig. 1). Stimulation of protein kinase C increases the degree of phosphorylation of B-50 and in turn decreases the activity of PIP kinase yielding less PIP₂ available for further receptor-mediated hydrolysis.

Fig. 1. Working model for the role of phosphoprotein B-50 in the feedback control of phosphatidylinositol 4,5-bisphosphate turnover (for explanation see text).

Recent data from Routtenberg's group point to an important role for protein F1 in LTP induced in rat hippocampus *in vivo* (Routtenberg *et al.*, 1985; Lovinger *et al.*, 1985). They showed that the post-hoc phosphorylation of F1 was related to the degree of potentiation after tetanic stimulation in the perforant path of the hippocampus and that the change in phosphorylation persisted over days after the tetanic stimulation. Since B-50 and F1 are most likely identical (Gispen and Routtenberg, in preparation), these results prompted us to reinvestigate the effects of tetanization on B-50 phosphorylation. For, in our initial publication on the effects of tetanic stimulation employing the hippocampal slice preparation followed by the post hoc phosphorylation assay, we showed a stimulation of B-50 phosphorylation ($p < 0.1$), however the B-50 protein responded in a more variable way than the 50/52 kDa protein (Bär *et al.*, 1980). After application of two tetanic stimulations of 100 pulses/sec for 2 sec, given 5 min apart, a time course of B-50 phosphorylation and LTP was made. The tetanized slices were processed to yield a crude SPM fraction at 10, 20, 35 and 65 min after application of the first tetanus. The correlation between the percentage stimulation of B-50 phosphorylation and the degree of potentiation of each individual slice was calculated at each time point. This correlation only reached significance 10 min after application of the first tetanus (Schrama *et al.*, 1986; see Fig. 2). Apparently, the more robust *in vivo* treatment as employed in the Lovinger study (Lovinger *et al.*, 1985) produces more pronounced effects on changes in the degree of phosphorylation of this protein. Nonetheless, our present data confirm our previous observation that also in

the hippocampal slice preparation the phosphorylation of B-50 is sensitive to tetanic stimulation. If the B-50 phosphorylation is affected by tetanic stimulation, then one may expect to find changes in inositol lipid metabolism as well.

Fig. 2. Correlation between the phosphorylation of B-50 and the degree of potentiation of a hippocampal slice.

In a recent study we examined the effects of tetanic stimulation of the perforant pathway in rat hippocampal slices on the metabolism of polyphosphoinositides (PPI) and phosphatidic acid (PA) (Bär *et al.*, 1984). The changes in PPI metabolism in relation to LTP were studied employing two different methods, both of which involved labeling of the slice with [32 P]-orthophosphate. Firstly, slices were prelabeled for 10 min, after which they were subjected to high frequency stimulation and were immediately processed. Secondly, the slices were first tetanized, after which the label was added 2 min to 4 hours after tetanization for a total time period of 10 min. After 10 min incubation almost all label was recovered in PIP₂, PIP, phosphatidylmyoinositol (PI) and PA, whereas the other phospholipids had only incorporated 4% of the total radioactivity in the phospholipid fraction. The results obtained with both methods were essentially similar and only the results obtained with the second method are discussed. Control incubations involved the application of a low frequency stimulus (1 pulse/4sec for 15 min), a stimulation frequency inducing neither LTP nor significant changes in any of the phospholipids studied. The time course for the formation of PIP₂ is shown in Fig. 3. At short intervals after tetanization a decrease of 20% was observed, whereas after longer time intervals there was a time-dependent increase in labeling of PIP₂ (Bär *et al.*, 1984). The results obtained for PA labeling were the same as for PIP₂ both with respect to magnitude and time dependency of the effect (data not shown; see Bär *et al.*, 1984).

It remains to be shown what the significance of these changes in the synaptic machinery for synaptic efficacy is. As B-50/F1 is present in growth cones (De Graan *et al.*, 1985; Pfenniger *et al.*, 1986). Routtenberg (1986) has proposed that the changes may reflect a synaptic trophic response to the tetanic stimulation. On the other hand, as the studies were carried out in the adult it may be that they merely reflect changes in presynaptic

Fig. 3. Labeling of PIP2 after tetanization of the hippocampal slice.

signal transduction leading to enhanced release of the transmitter (see also Bliss and Dolphin, 1982).

COATED VESICLE PROTEIN 52 kDa

In our initial studies using tetanic stimulation of the perforant path in rat hippocampal slices and recording the response in the dentate gyrus, we consistently observed changes in the phosphorylation of a 50 kDa protein band (Bär et al, 1980). In further studies it was shown that the phosphoprotein that responded to the tetanic stimulation was a 52 kDa protein present in membrane-enriched, non-mitochondrial subcellular fractions from rat hippocampus (Bär et al, 1982).

To establish as possible correlation between the changes in the degree of 52 kDa phosphorylation and the degree of potentiation, Tielen et al (1983) have studied the quantitative relationship between the degree of potentiation and the degree of 52 kDa protein phosphorylation in SPM. The percentual change in 52 kDa phosphorylation was therefore correlated with the change in amplitude of the EPSP per individual slice. A semi-logarithmic plot of these data fits a straight line with correlation coefficient of 0.71 ($p < 0.005$; Tielen et al, 1983, Fig. 4). These results suggest that there may be a quantitative correlation between electrophysiological synaptic changes and synaptic membrane protein phosphorylation for at least two phosphoproteins, e.g. B-50 and a 52 kDa phosphoprotein.

Fig. 4. Relationship between the postsynaptic potential and the endogenous phosphorylation of the 52 kDa protein, 15 min after tetanization of the perforant path fibers.

The next step in our studies on the relation between the phosphorylation of membrane-bound phosphoproteins and synaptic plasticity was the identification of the 52 kDa phosphoprotein. The *in vitro* phosphorylation of the 52 kDa protein was not dependent on calcium/calmodulin nor on cyclic nucleotides (Bär et al, 1982). Both the relative molecular weight and the phosphorylation characteristics of the 52 kDa protein resemble those of a phosphoprotein called pp50, which has been characterized in coated vesicles (Pauloin et al, 1982; Pauloin and Jolles, 1984; Kadota et al, 1982; Campbell et al, 1984). We have carried out a series of experiments to compare the 52 kDa phosphoprotein in SPM and pp50 in coated vesicles isolated from rat brain (Schrama et al, 1986; De Graan et al, this volume). The results obtained show that the 52 kDa phosphoprotein and pp50 are identical on basis of the following criteria: (i) relative molecular weight on 11% SDS-PAGE; (ii) insensitivity of phosphorylation to cyclic AMP, calcium and calmodulin; (iii) the phosphate accepting amino acids serine and threonine in both phosphoproteins; (iv) isoelectrical point range 9.0-6.5 for both proteins; (v) peptide mapping, the products formed and the time course for digestion by two different proteases were identical for both phosphoproteins. With respect to phospho-amino acid analysis and isoelectric focusing our results agree very well with those presented by Campbell et al, 1984). We believe that the 52 kDa phosphoprotein present in SPM and the pp50 protein from coated vesicles are similar if not identical.

The exact role of pp50 phosphorylation in the function of coated vesicles, both with respect to the coating/decoating process and to the coated vesicle transport mechanisms are still unclear. Preliminary data from Pauloin and Jolles suggest that the introduction of negatively charged phosphate groups into pp50 could

destabilize the interaction between certain coat proteins and the vesicle. This notion is substantiated by the fact that the activation of the pp50 kinase by clathrin light chains promotes the phosphorylation of pp50 (Pauloin and Jolles, 1984) and allows the dissociation of clathrin from the vesicles (Schmid *et al.*, 1984). It is tempting to speculate that the tetanic stimulation therefore may lead to changes either in receptor-mediated endocytosis or presynaptic membrane recycling after vesicles function (Pauloin and Jolles, 1986).

SYNAPSIN I

Another phosphoprotein which has been implicated in synaptic plasticity is synapsin I (previously named protein I), first identified in 1972 (see Nestler and Greengard, 1984). Synapsin I is a major endogenous substrate for cyclic AMP-dependent protein kinase and for calcium-dependent protein kinase in the presynaptic terminal. The available evidence to date suggests that synapsin I is part of the molecular machinery in nerve terminals that regulates neurotransmitter release. Synapsin I consists of two closely related proteins Ia and Ib with molecular weights of 86 kDa and 80 kDa, respectively, and an IEP of 10.3 resp. 10.2. The protein is present in virtually all nerve terminals and within the terminals primarily associated with synaptic vesicles, located at the cytoplasmic face of these vesicles. Several lines of evidence suggest that the phosphorylation of synapsin I is associated with the production of neurotransmitter release processes. Recently it was found that phosphorylation of synapsin I in the domain of the molecule that is associated with the vesicle membrane leads to dissociation of the protein and vesicle membranes. Hence it was suggested that in the resting state of the presynaptic terminal dephospho-synapsin I binds to the transmitter vesicle and thereby decreasing the availability of the vesicles to move towards and/or fuse with the presynaptic plasma membrane. In the stimulated state of the terminal, the phosphorylation of synapsin I in response to the stimuli, triggers the release of the molecule from the vesicle allowing the vesicle to attain a 'fusion-ready' state (Nestler and Greengard, 1986).

Although at present no studies have been carried out on the role of synapsin I in LTP, discussion of this protein in the present context cannot be omitted as direct evidence is accumulating for a role of synapsin I in phenomena in the squid axon that closely resemble LTP, i.e. increase in amplitude and rise of the postsynaptic potential (Llinas *et al.*, 1985). In these studies the authors injected synapsin I or calcium/calmodulin-dependent kinase II into the terminal region of squid giant axons and measured transmitter release and amplitude, rate of rise and latency of the potential generated in response to presynaptic depolarizing steps under voltage clamp conditions. Injection of purified dephosphorylated synapsin I was found to decrease the amplitude and rate of rise of the postsynaptic potential, whereas injection of phospho-synapsin I had no effect. In contrast, injection of purified calcium/calmodulin-dependent protein kinase II, presumably phosphorylating a synapsin I-like protein, into these nerve terminals increased the transmitter release and concomitantly decreases the latency of the postsynaptic potential and increased the amplitude and rise of the postsynaptic potential. These effects were observed in the absence of any detectable changes in presynaptic calcium current, suggesting a direct role of synapsin I in the enhanced release of the transmitter and the postsynaptic

potential (Llinas *et al.*, 1985). As these latter phenomena closely resemble those seen after tetanic stimulation of fiber systems in mammalian brain, it will be interesting to study possible changes in the degree of phosphorylation of synapsin I during LTP.

CONCLUSIONS

In the present paper I have discussed some of the evidence that points to an important role of synaptic protein phosphorylation in LTP and related electrophysiological phenomena. Although the localization of some of these proteins suggest a pre- rather than a postsynaptic molecular event, by no means is this to be taken as evidence for an exclusive presynaptic localization of the mechanisms underlying initiation and maintenance of LTP. In fact, a major line of research concerning the biochemistry of hippocampal LTP not involving protein phosphorylation as a key event, focusses on the unmasking of glutamate receptors at the postsynaptic dendritic spine (Lynch and Baudry, 1984). These authors suggest that brief tetanic stimulation causes a transient elevation of calcium levels in the spines activating the membrane-associated, calcium-sensitive protease calpain I. Calpain I subsequently degrades part of fodrin, a protein forming a network at the cytoplasmic face of the neuronal membrane. This degradation would be responsible for structural changes in the postsynaptic membrane, presumably unmasking glutamate receptors and inducing the morphological changes in the dendritic spines seen after tetanic stimulation (Lynch and Baudry, 1984). However, in view of the experiments from the group of Bliss showing an enhanced neurotransmitter release after tetanic stimulation (Bliss, this volume) treating his neurophysiological data in the context of a presynaptic locus of LTP, it is most likely that the mechanism of LTP onset and maintenance is far more complex than anticipated and that both pre- and postsynaptic events are part of the molecular mechanism underlying LTP. It may be that the phosphoproteins discussed in this present paper are mainly involved in processes directly linked to the release mechanism of the transmitter and therefore monitor a pre- rather than a postsynaptic event crucial for LTP. Clearly the present status of our knowledge is sketchy and any overview can only be a progress report. Future research should further unravel those chemical events that are causal to LTP and those that reflect the occurrence or that maintain LTP.

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