A POSSIBLE ROLE OF HIPPOCAMPAL PHOSPHOPROTEINS IN EPILEPTOGENESIS

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Introduction

Synapses in many parts of the brain show a certain degree of plasticity. Plasticity is the ability of a synaptic system to adapt its response to electrical stimuli as a function of previous experiences. Synaptic plasticity can be observed when a set of fibers is electrically stimulated by a series of brief pulses at relatively high frequencies, i.e. a tetanus. After such a tetanus, the corresponding synapses may present an enhancement in their responses to test stimuli for a period of time that can last from hours to even days. This phenomenon is called long-term potentiation (LTP). It may be considered as the physiological substrate for information storage in the brain (Bliss 1979; Bliss and Dolphin 1982; Lopes da Silva et al. 1982a,b; Eccles 1983; Voronin 1983; Teyler and Discenna 1984; Lynch and Baudry 1984).

A new dimension was added to the LTP research after the discovery that daily application of a tetanus to various regions of the brain may cause epileptiform activity and will eventually lead to generalized convulsions (Goddard et al. 1969). This experimental model is known as the kindling model of epilepsy.

Several lines of evidence point to a crucial role of calcium in epileptogenesis and the related phenomenon LTP. It has been proposed (Meldrum 1981, 1983) that an excessive influx of Ca^{2+} into selectively vulnarable cells, associated with paroxysmal activity during status epilepticus, overwhelms the capacity of the neuron to extrude or sequester Ca^{2+}, leading to an excessively high cytosolic Ca^{2+} concentration. Indeed convulsive drugs induce calcium accumulation in hippocampal neurons visualized by the oxalate-pyroantimonate technique (Griffiths et al. 1982). Moreover, we have demonstrated an increased CA^{2+}-permeability of hippocampal pyramidal cells after kindling using ion-selective micropipets (Wadman et al. 1985). An increased Ca^{2+}-influx has also been demonstrated concomitant with LTP (Baimbridge and Miller, 1981), and is thought to result in the activation of CA^{2+}-dependent proteases and the unmasking of glutamate receptors (Lynch and Baudry 1984).

The molecular mechanism(s) underlying epileptogenesis at the level of the synapse and the synaptic membrane are still largely unknown. Our research is directed toward the role of protein

phosphorylation in epileptogenesis and LTP. A general concept has developed from many observations that cyclic phosphorylation and dephosphorylation of proteins plays an important role in the regulation of ion permeability and synaptic transmission (for a review see Nestler and Greengard 1984). The early studies by Heald (1957, 1962) and Trevor and Rodnight (1965) demonstrated that in association with spike activity, serine residues of neuronal membrane proteins were phosphorylated (see also Reddington and Rodnight 1972). Since then, it has been shown that a variety of extracellular signals produce many of their diverse physiological responses by regulating the state of phosphorylation of especially membrane- or vesicle-bound substrate proteins (Oestreichter et al. 1982, Nestler and Greengard 1984).

However, the precise relation between neurotransmission and phosphorylation is not yet clear. One of the hypotheses is that the increase in intracellular CA^{2+} concentration, which is essential for neurotransmission, mediates Ca^{2+}-sensitive protein phosphorylation. Indeed, evidence from DeLorenzo and coworkers (DeLorenzo et al. 1982) suggests that the increase in intracellular Ca^{2+} stimulates the phosphorylation of cytoskeletal elements, possibly modifying cytoskeletal function and facilitating neurotransmission. Moreover, Wasterlain and Farber (1982, 1983) have shown that septal kindling results in dramatic changes in the Ca^{2+}/calmodulin sensitive phosphorylation of a 50 kDa protein.

We have studied two models for epileptogenesis in the transverse hippocampal slice system. This in vitro system has been widely used for the analysis of possible correlative changes in its electrophysiological and neurochemical properties. The hippocampal slice system combines well defined electrophysiological parameters (it contains an intact trisynaptic pathway) with a good accessability for biochemical and pharmalogical techniques. In this in vitro hippocampal slice system we induced (semi)permanent changes in electrophysiology (by applying a tetanus and the convulsant 4-aminopyridine, respectively) and studied concomitant correlative changes in protein phosphorylation.

Ltp, and the phosphorylation of a putative 52 kDa coated vesicle protein

Tetanic stimulation in the perforant path of rat hippocampal slices increased the degree of phosphorylation of a protein band with an apparent molecular weight of about 50 kDa (Bär et al. 1980). The increase in $[^{32}p]$-incorporation in the tetanized slices was 24% as compared to non-tetanized controls. Changes in other phosphoprotein bands were less consistent and reached

significance only in the 48 kDa protein, identified as the B-50 protein (Zwiers et al. 1980; see below). In further experiments it could be demonstrated that the increase in 50 kDa phosphorylation was dependent on the frequency of the tetanus. Application of 1 pulse per 4 s. for 15 min (225 pulses), a frequency which is ineffective in producing LTP, instead of 15 pulses per s. for 15 s., did not induce significant changes in 50 kDa or 48 kDa phosphorylation (Bär et al. 1980). In the absence of extracellular Ca^{2+}, a condition which inhibits neurotransmission and LTP production (Dunwiddie and Lynch 1979), no changes could be detected in either 50 kDa or 48 kDa phosphorylation, indicating that the observed changes relate to synaptic activity.

In a subsequent study (Bär et al. 1982) we investigated the characteristics of the 50 kDa band in more detail. The calculated apparent molecular weight using a high resolution separation system was 52 kDa. In fact the so-called 50 kDa band separated into a doublet with estimated molecular weights of 52 kDa and 50 kDa, respectively. Only the 52 kDa band was shown to be affected by tetanic stimulation (Bär et al. 1982).

Since the electrophysiological changes of the evoked response to single test stimuli after a tetanus may vary considerably in amplitude, we attempted to make a quantitative correlation between the changes in amplitude of the post-synaptic potential (PSP) and the population spike (PopSP) measured extracellularly, and the degree of phosphorylation of the 52 kDa band (Tielen et al. 1983). In this study each slice was tested for clear responses to test stimuli and for each slice a stimulus-responses relationship of the PSP was made, as well as a determination of the PopSP threshold. Two high frequency stimulations (50 pulses/s. for 2 s.) were given 5 min apart and 10 min after the last tetanus each slice was processed separately and assayed for endogenous protein phosphorylation. Paired control slices from the same hippocampus received test stimuli but no tetanus. The mean post hoc endogenous phosphorylation of the 52 kDa protein band was significantly increased in the tetanized group as compared to the control (+ 24%; $P < 0.05$). These data confirmed our earlier study (Bär, et al. 1980). In Fig. 1 the percentual change in 52 kDa phosphorylation is related to the change in postsynaptic potential per individual slice. A semi-logarithmic plot of these data fits a straight line with a correlation coefficient of 0.71 ($P < 0.005$). These data suggest that there may be a quantitative correlation between electrophysiological synaptic changes and synaptic membrane protein phosphorylation. However, the causality of this relationship remains to be determined.

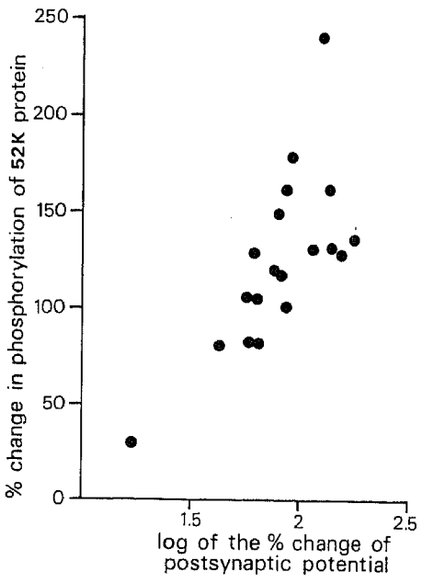

Fig. 1. Relationship between the postsynaptic potential and the endogenous phosphorylation of the 52 kDa protein, 15 min after tetanization of the perforant path fibers. Protein phosphorylation was assayed post hoc using [γ-^{32}P]-ATP in a crude synaptosomal/mitochondrial fraction (Tielen et al. 1983).

To further characterize the 52 kDa protein band we studied its subcellular localization (Bär et al. 1982). First of all we could show that the 52 kDa protein band is present in a crude synaptosomal plasma membrane fraction (t-SPM) prepared from tetanized slices and that the phosphorylation of the 52 kDa band in this t-SPM is stimulated as compared to controls (+ 30%; $P < 0.02$). A more purified synaptosomal plasma membrane fraction (as judged by electron microscopy), the so-called light synaptosomal plasma membrane fraction (l-SPM), was also found to be rich in 52 kDa phosphorylation (Fig. 2, lanes 1). Two-dimensional separation of l-SPM proteins revealed that the 52 kDa protein has an IEP of the IEF gel, thus producing a streak in the basic region of the gel. This phenomenon appears to be due to solubilization conditions (Schrama et al. in preparation) and is subject of further study.

The phosphorylation of the 52 kDa protein is not dependent on CA^{2+}/calmodulin. The [^{32}P]-incorporation into the 52 kDa protein in a crude mitochondrial/synaptosomal fraction is not affected when the Ca^{2+}-concentration during the phosphorylation assay is varied from 0-50 mM exogenous Ca^{2+} in the presence of 1 mM EGTA (Bär et al. 1982), conditions in which the 50 kDa protein and B-50 (48 kDa) show marked Ca^{2+} dependency. The in vitro endogenous phosphorylation of the 52 kDa protein is not affected by

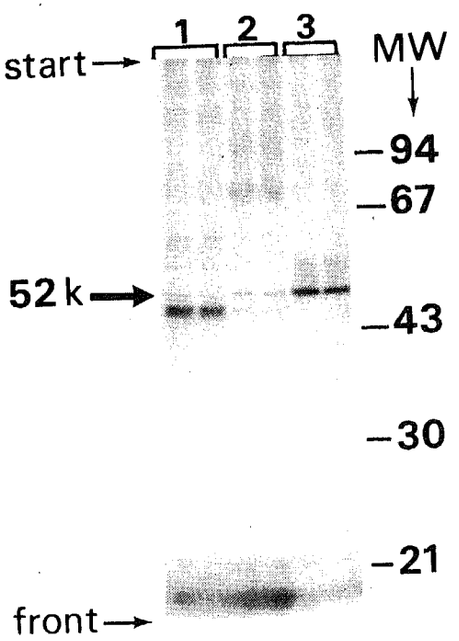

Fig. 2. Autoradiogram showing the phosphorylated 52 kDa protein in l-SPM (1), a vesicle-enriched fraction (2) and isolated coated vesicles (3) after protein separation on 11% SDS-polyacrylamide gels. The position of molecular weight standards (MW) is indicated at the right.

cAMP either. Similarly, we have found the 52 kDa phosphorylation in l-SPM to be Ca^{2+}- and cAMP-independent (De Graan unpublished observations).

Recently, the phosphorylation of a protein in the 50 kDa range in purified bovine brain coated vesicles has been described (Pauloin et al. 1982; Kadota et al. 1982; Pauloin and Jolles 1984). Our attention was drawn to this 50 kDa protein called pp50 since it shares with our 52 kDa protein its insensitivity to Ca^{2+}/calmodulin and cAMP (Pauloin et al. 1982; Bär et al. 1982). Subcellular localization studies show that 52 kDa phosphorylation can be detected in the l-SPM fraction from rat brain (Bär et al. 1982), and is very pronounced in a vesicle-enriched fraction prepared from synaptosomes (Fig. 2, lanes 2). Therefore, we isolated coated vesicles from rat brain according to the method of Pearse and Robinson (1984). The major phosphoprotein in this coated vesicle preparation was found to be a 52 kDA protein (when analyzed on 11% SDS-polyacrylamide gel),. which comigrates with our 52 kDa protein from l-SPM (Fig. 2, compare lanes 1 to lanes 3). Moreover, the phosphorylation of the 52 kDa coated vesicle pro-

tein was not affected by Ca^{2+} and calmodulin or cAMP. Preliminary data from two-dimensional separation systems and peptide mapping indicated that both 52 kDa proteins are related if not identical.

Coated vesicles are regarded as highly specialized subcellular organelles, which are involved in receptor-mediated endocytosis (for review see Goldstein et al. 1979). Receptor-mediated endocytosis is an important and general mechanism by which animal cells take up nutrients and regulatory proteins. Proteins which bind to plasma membrane receptors are rapidly internalized, by clustering of the receptors in specialized regions of the plasma membrane, called coated pits, that invaginate rapidly into the cell during endocytosis to form coated vesicles. Most data on coated pits and coated vesicles are derived from studies on the low density lipoprotein receptor system (Goldstein et al. 1979). In brain the synaptic vesicle membrane has been postulated to be selectively retrieved from the presynaptic membrane by receptor-mediated endocytosis (Heuser and Reese 1973). More recent data indicate indeed that coated pits and coated vesicles are involved in presynaptic membrane recycling (Heuser and Reese 1979; Kadota and Kadota 1982). Although the precise role of pp50 phosphorylation still remains unclear, its presence in various different coated vesicle species (Pauloin et al. 1984) suggests an important role in the coated-vesicle working mechanism, like regulating selective internalization, repeated membrane fusion and fission. As the 52 kDa protein in 1-SPM is related or identical to the pp50 protein, it may well be that the mechanism of LTP is linked to presynaptic coated vesicle function. Hence modulation of 52 kDa phosphorylation in response to tetanic stimulation may thus be related to an increase in presynaptic membrane renewal and an increased neurotransmisser turnover (see Fig. 5).

4-aminopyridine and modulation of Ca^{2+}/calmodulin-dependent protein phosphorylation

Several lines of evidence point to a crucial role of calcium in epileptogenesis and in general convulsant drugs, such as 4-aminopyridine, induce calcium accumulation in hippocampal neurons (Griffiths et al. 1982). The precise mechanism by which aminopyridines increase intracellular calcium is not yet identified, although it is now clear that the mechanism is distinct from that of the dihydropyridines, a group of calcium entry activators, e.g. Bay K 8644 (Greenberg et al. 1984). The convulsant 4-aminopyridine (4-AP) is thought to induce epileptoform activity by blocking the potassium channels associated with an influx of calcium (Llinas et al. 1976; Nicholson et al. 1976; Baranyi and Feher 1979; Pant et al. 1983). This influx of calcium results in an increase in synaptic transmitter release in the peripheral and

central nervous system, leading to prolongation of action potentials in unmyelinated nerve fibers and terminals (Thesleff 1980; Haas et al. 1983). The effects of 4-aminopyridine have been shown in both excitatory and inhibitory neurotransmission (Jankowska et al, 1977; Buckle and Haas, 1982; Van Harreveld 1984). These effects of the convulsant have been confirmed by morphological changes in 4-aminopyridine treated synapses (Tokunaga et al. 1979; Forsman and Elfvin 1983).

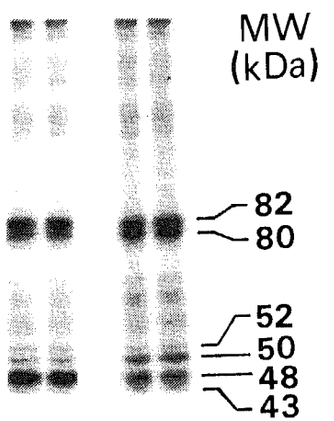

Fig. 3. Autoradiogram of a phosphorylated crude mitochondrial/synaptosomal fraction after incubation of hippocampal slices for 30 min in the absence (A) or presence (B) of 10^{-5} M 4-AP. Indicated are the estimated molecular weights of the six major phosphoproteins.

After treatment of hippocampal slices with 10^{-5} M 4-AP, a pronounced increase was found in the phosphorylation of the 50 kDa protein (Fig. 2). Quantification of the [^{32}P] incorporation after incubation with 10^{-5} M 4-AP (Fig. 3) revealed a stimulation of 86% of the 50 kDa phosphorylation, whereas a smaller but significant stimulation was found of the 80 kDa phosphorylation. The 48 kDa protein was inhibited by 4-AP with a maximal effect at 10^{-4} M of 20%. The 4-AP induced stimulation of 50 kDa phosphorylation was dose-dependent (Fig. 4), with a half maximal stimulation of 5×10^{-7} M. The effect of the convulsant on 80 kDa phosphorylation was only significant at 10^{-5}M. No effect could be detected

on the phosphorylation of other major phosphoproteins (82, 52 and 43 kDa) at any of the 4-AP concentrations tested.

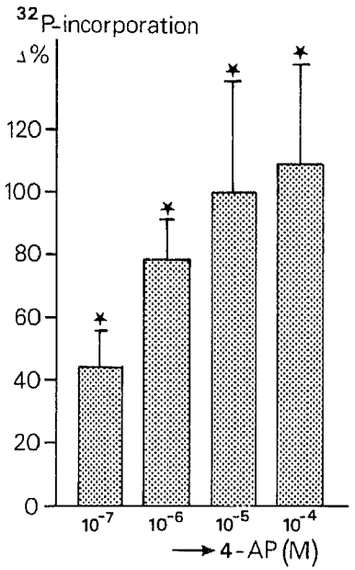

Fig. 4. Quantification of the effect of 10^{-5} M 4-AP as shown on the autoradiogram in Fig. 1 (n = 13). Quantification was performed by densitometric scanning of the autoradiogram and measured as peak height above background. The data are expressed as percentage of controls. Bars indicate SEM values. * = $2p = < 0.05$ as determined with Students t-test.

The conditions leading to enhancement of 50 kDa protein phosphorylation in the post hoc phosphorylation assay resemble those leading to spontaneous epileptic activity by 4-AP in hippocampal slices both with respect to dose- and time-dependency of the effect (Buckle and Haas 1982; Haas et al. 1983; Van Harreveld 1984).

The 50 kDa phosphoprotein band described here is the same as the one described earlier by Bär et al. (1982). The phosphorylation of this protein is not affected by cyclic AMP or tetanic stimulation, in sharp contrast to the 52 kDa protein also present in the crude mitochondrial/synaptosomal fraction (Bär et al. 1980; Lopes da Silva et al. 1982a,b; Tielen et al. 1983). The phosphorylation of this 50 kDa protein is, however, strongly dependent on the presence of calcium ions and the calcium binding protein calmodulin (Bär et al. 1982).

In another experimental model for epilepsy, the kindling model, a marked increase in ^{32}P incorporation into a 50 kDa hippocampal protein is reported (Wasterlain and Farber 1982). The phosphorylation of this protein and the effect of kindling are calmodulin-dependent (Wasterlain and Farber 1984). Biochemical characterization showed that the protein is probably the alpha or rho subunit of the calcium/calmodulin-dependent protein kinase II (Goldenring et al. in press). This protein kinase is a premominant brain phosphoprotein (Bennett et al. 1983), consisting of two subunits, with a characteristic distribution between the subunits over the brain (McGuinness et al. 1985). The major postsynaptic density protein has been identified as the alpha or rho subunit of brain calcium/calmodulin-dependent porotein kinase (Kennedy et al. 1983). Moreover, this protein kinase is associated with cytoskeletal components of post-synaptosomal fractions (Sahyoun et al. 1985).

The 50 kDa phosphoprotein which is affected by 4-AP treatment of hippocampal slices is most probably the autophosphorylated alpha or rho subunit of the brain calcium/calmodulin-dependent protein kinase, on basis of its molecular weight range and its calcium/calmodulin sensitivity (Bär et al. 1982). The precise mechanism of 4-AP action is not yet clear, but might involve the influx of calcium leading to a change in the autophosphorylation of the α-subunit of the calcium/calmodulin-dependent protein kinase. Autophosphorylation of the kinase probably modulates the activity of the enzyme, thus affecting cytosekeletal structure and synaptic transmission.

Concluding remarks

In recent years, several different mechanisms have been proposed to underly epiletogenesis. These include 1) an increase in the Ca^{2+}-permeability of the neruonal membrane, resulting in an elevation of cytosolic Ca^{2+} (Meldrum 1981, 1983; Griffith et al. 1982, Wadman et al. 1985), 2) an increase in extracellular K^+, possibly resulting from a reduced spatial buffering (Heineman et al. 1977, 1983), 3) a decrease in GABA-ergic inhibition (Meldrum 1983; Rondouin et al. 1983) and 4) blockage of Ca^{2+}-dependent K^+ channels (Galvan et al. 1982; Agoston et al. 1983). We have focussed our research on a possible role of phosphorylation processes in epileptogenesis with special emphasis on Ca^{2+}-dependent phosphorylation. Both in the LTP model and in the 4-AP model we have shown that characteristic electrophysiological changes are parallelled by changes in the phosphorylation of specific synaptic proteins.

In the LTP model, primarily the phosphorylation of a 52 kDa protein was affected, which appears to be identical to the pp50 protein in coated vesicles. This protein is thought to play a role in modulating coated vesicle function, i.e. receptor-mediated

endocytosis and membrane renewal (Fig. 5). In the 4-AP model, phosphorylation was confined to a 50 kDa protein, tentatively identified as the α-subunit of the Ca^{2+}/calmodulin-dependent protein kinase type II. The autophosphorylation of this kinase is thought to regulate kinase activity. The kinase is involved in the modulation of cytoskeletal function and synaptic transmission (Fig. 5).

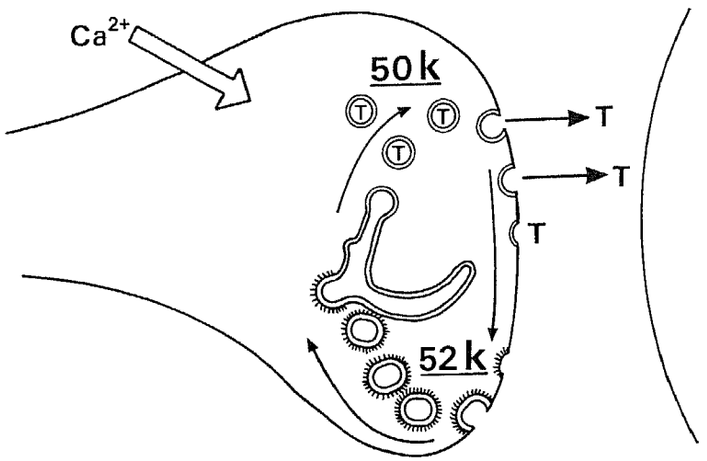

Fig. 5. Schematic representation of the putative role of the 52 kDa and 50 kDa protein in epileptogenesis. The 52 kDa protein is found in the synaptic plasma membrane and the coated vesicles. This protein is thought to play a modulatory role in receptor-mediated endocytosis and membrane renewal. The 50 kDa protein appears to be the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase type II. This kinase may play a regulatory role in cytoskeletal function and possible in neurotransmission.

(T) = neurotransmitter

In both the LTP and 4-AP model the phosphorylation of the nervous tissue specific, presynaptic protein B-50 (for review see Gispen et al. 1985) is affected. In the LTP model we initially described a rather variable increase in B-50 phosphorylation (Bär et al. 1982), but more recently Routtenberg and coworkers (Lovinger et al. 1985; Routtenberg and Lovinger 1985) have reported a selective increase in the phosphorylation of protein F_1 (which we believe to be identical to B-50) in an in vivo LTP model. Indeed, we have recently been able to demonstrate a good correlation in the hippocampal slice LTP model, between the degree of potentiation and the increase in B-50 phosphorylation (unpublished results). In the 4-AP model a consistant decrease in B-50 phosphorylation has been observed (see above). B-50 is thought to

play a role in the modulation of receptor-mediated breakdown of polyphosphoinositides (for a review see Gispen et al. 1985) and thus in the mobilization of Ca^{2+} as a second messenger.

Thus, we have reported changes in the degree of phosphorylation of 3 different neuronal proteins, which parallel changes seen in electrophysiological parameters of the neuronal network involved. We realize that correlative studies only provide indirect evidence for a role of protein phosphorylation in epileptogenesis. A causal relationship has yet to be established. The in vitro experimental models for epilepsy used in our studies are very suitable to investigate the effects of anti-epileptic drugs on protein phosphorylation. By doing so, we hope to contribute to the understanding of the molecular mechanisms underlying epileptogenesis and the mechanism of action of antiepileptic drugs.

Acknowledgement

This research was supported by CLEO-TNO (The Hague), The Netherlands, grant no's A 42 and A 47.

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