

**4-AMINOPYRIDINE INHIBITS SYNAPTOSOMAL PLASMA MEMBRANE PROTEIN
PHOSPHORYLATION IN VITRO: EFFECT OF THE SELECTIVE
NMDA-ANTAGONIST 2-AMINO-5-PHOSPHONOVALERATE**

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Phosphorylation of synaptosomal plasma membranes from rat hippocampus in the presence of the convulsant drug 4-aminopyridine resulted in the inhibition of the phosphorylation of the nervous tissue specific protein kinase C substrate protein B-50 (48 kDa) and the α -subunit of calcium/calmodulin-dependent protein kinase II (50 kDa). Preincubation of SPM with 2-amino-5-phosphonovalerate prevents the inhibition of B-50 phosphorylation by 4-aminopyridine, but had no effect on the inhibition of 50 kDa phosphorylation. 2-Amino-5-phosphonovalerate is known to be a specific N-methyl-D-aspartate antagonist and has anti-epileptic activity in vitro and in vivo. Several other anti-epileptic drugs tested did not influence the 4-aminopyridine-induced inhibition of protein phosphorylation. © 1987 Academic Press, Inc.

The molecular mechanisms underlying epileptogenesis are still largely unknown. One of the experimental models we have been employing to study the changes in the phosphorylation of membrane proteins during epileptogenesis is based on the convulsant action of the potassium channel blocker 4-aminopyridine (4-AP,1,2). The blockade of K^+ -channels with 4-AP is associated with an influx of Ca^{2+} and subsequently with the release of neurotransmitters from the presynaptic terminal (3,4). Moreover, recently it has been shown that 4-AP facilitates long-term potentiation in the hippocampal slice (5,6), a process which seems to be dependent on activation of N-methyl-D-aspartate (NMDA) receptors, since it can be blocked by specific NMDA antagonists (7-10).

Recently, we have shown that incubation of the hippocampal slice with 4-AP, followed by post-hoc phosphorylation of a crude mitochondrial/synaptosomal fraction prepared from these slices resulted in a dose-dependent increase in the phosphorylation of a 50 kDa protein, which was tentatively identified as the α -subunit of calcium/calmodulin-dependent protein kinase II (CaMK II,1,2). In the present paper we show that the phosphorylation of the presynaptic membrane protein B-50 as well as the 50 kDa protein are inhibited

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by 4-AP in synaptosomal plasma membranes (SPM) and that the inhibition of B-50 by 4-AP can be specifically reversed by the NMDA-antagonist 2-amino-5-phosphonovalerate (APV,11).

Materials and Methods

The following chemicals were obtained from Sigma Chemicals (St. Louis, MI, USA): 4-AP, APV, bicuculline, valproate, diazepam, carbamazepine, diphenylhydantoin (DPH), picrotoxin, calmodulin (10^4 units/0.28 mg protein) and phenylmethylsulfonylfluoride (PMSF). Pentobarbital (Narcovet) was obtained from Apharmo (Arnhem, NL); 5,5-diethylbarbituric acid from Merck (Darmstadt, FRG); [γ - 32 P]ATP (spec. act. 3000 Ci/mmol) from Amersham (Slough, UK), low molecular weight markers from Pharmacia (Uppsala, S) and X-ray films XomatRP from Kodak (Rochester, NJ, USA). Synaptosomal plasma membranes (SPM) were isolated from hippocampal tissue from male inbred Wistar rats (TNO, Zeist, NL) according to the method of Kristjansson et al. (12). Phosphorylation of SPM proteins was carried out at 30°C in a final volume of 25 μ l in 10 mM Na-acetate, 10 mM Mg-acetate, 100 μ M Ca-acetate, 100 μ M PMSF (pH 6.5 with acetic acid) and 5 U calmodulin, containing 10 μ g protein determined according to the method of Bradford (13). After preincubation (for details see legends of figures) the phosphorylation reaction was started by the addition of 1-2 μ Ci labelled ATP (final ATP concentration 7.5 μ M) and terminated after 15 sec by the addition of 12.5 μ l of a denaturing solution (14). Proteins were separated by SDS-polyacrylamide gel electrophoresis (composition of the running gel: 7% acrylamide and 0.127% methylene bisacrylamide,15) at 30 mA. The incorporation of phosphate into proteins was determined by densitometric scanning of the autoradiograms from the dried gels (16,17).

Results

Incubation of SPM with 1 mM 4-AP for 5 min resulted in the inhibition of phosphorylation of two proteins, the nervous tissue specific protein B-50 (48 kDa) and the autophosphorylating α -subunit of CaMK II (50 kDa) (Fig.1, compare lanes A and B). The inhibition of phosphorylation of B-50 could be coun-

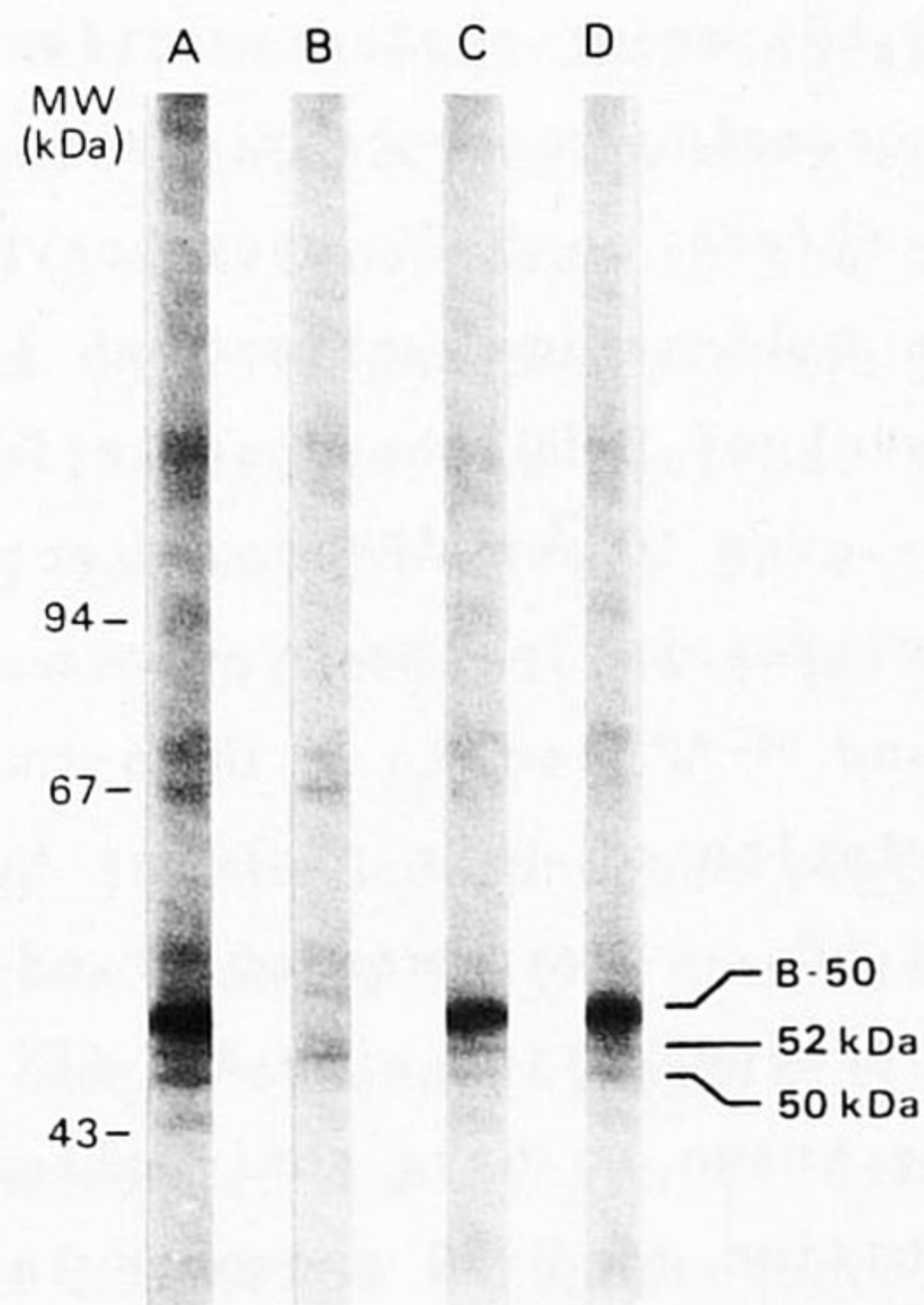


Fig.1. The autoradiograms shown were obtained from incubation of SPM under the following conditions. A: control, B: 5 min 4-AP, C: 5 min APV followed by 5 min 4-AP, D: 5 min APV. The final concentrations of APV and 4-AP used were 1 mM.

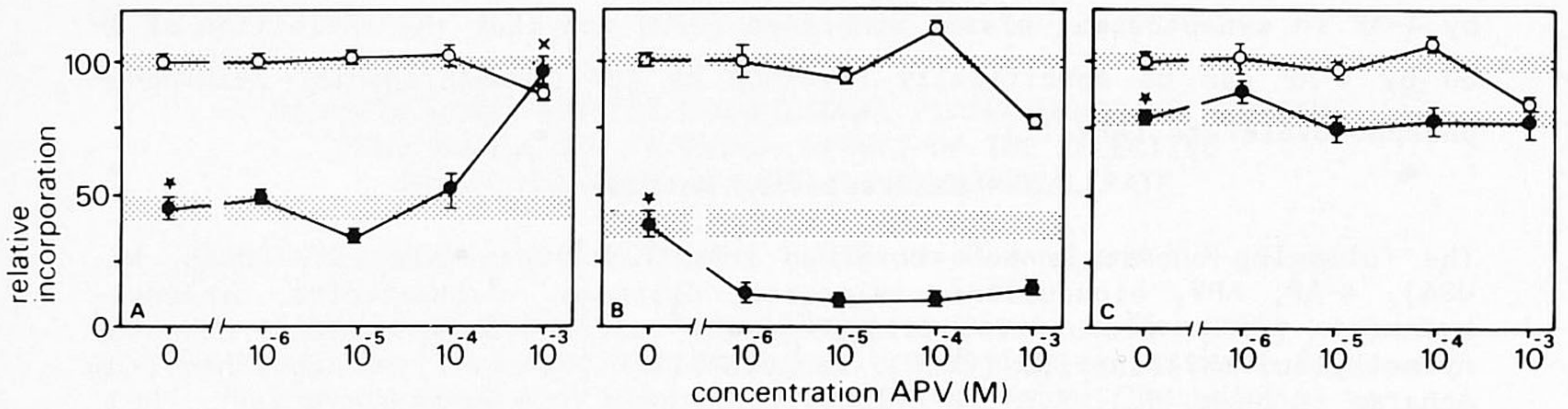


Fig.2. A concentration effect curve for APV was obtained in the presence (●) or absence (o) of 1 mM 4-AP. SPM was preincubated for 5 min with APV followed by 5 min with 4-AP. The amount of phosphate incorporated was quantified by densitometric scanning of the autoradiogram and is expressed as % incorporation to control (= 100%, no APV or 4-AP present). A: B-50 protein (48 kDa); B: α -subunit of CaMK II (50 kDa); C: 52 kDa major coated vesicle protein. *: Indicates a significant difference between control and 4-AP incubation ($2p < 0.025$, Students t-test); x: Indicates a significant difference between 4-AP and APV/4-AP incubation ($2p < 0.005$, Students t-test).

teracted by preincubation of SPM with the specific NMDA-antagonist APV when added 5 min before the addition of 4-AP (Fig.1, lane C). APV itself had no effect on the phosphorylation of any of the proteins present in SPM (Fig.1, lane D). Reversal of the addition of 4-AP and APV revealed that the antagonistic effect of APV was no longer present (results not shown). The inhibition of the 50 kDa protein phosphorylation was not antagonized by any of the APV concentrations tested.

A concentration-effect curve for the reversing effect of APV on the inhibition induced by 4-AP is shown in Fig.2 for 3 known phosphoproteins in SPM (B-50, CaMK II and the 52 kDa major coated vesicle phosphoprotein, 18). Preincubation of SPM with increasing concentrations of APV before addition of 1 mM 4-AP showed that the effect on B-50 phosphorylation was dose-dependent (Fig.2A). The approximate half-maximal effect of APV was found at $5 \cdot 10^{-4}$ M and at 10^{-3} M APV the level of B-50 phosphorylation was comparable to control. In contrast to B-50, even 10^{-3} M APV could not alter the level of phosphorylation of the 50 kDa protein in the presence of 4-AP (Fig.2B). In fact the combination of APV and 4-AP led to a dose-independent not significant decrease in the phosphorylation of this protein. Only a small inhibition of phosphorylation of the 52 kDa protein was observed in the presence of 4-AP, which was not affected by preincubation with APV (Fig.2C).

Other drugs which are known to have anti-epileptic activity were tested on the 4-AP induced inhibition of B-50 phosphorylation in SPM. None of the tested drugs counteracted the inhibition of B-50 phosphorylation by 4-AP (Table 1). Pentobarbital and DPH inhibited the phosphorylation of B-50 by reducing the phosphorylation all of proteins detectable in SPM. The GABA-re-

Table 1. Effect of several anti-epileptic drugs on the inhibition of B-50 phosphorylation by 4-aminopyridine

	Control	1 mM 4-AP
Control	100 ± 2	
APV	90 ± 2	+ ^a
Pentobarbital*	49 ± 4	0
Barbituric acid	99 ± 2	0
Valproate	104 ± 4	0
Diazepam	100 ± 1	0
Carbamezepine	98 ± 1	0
DPH*	15 ± 2	0

All anti-epileptic drugs were tested at 1 mM.

^a Indicates antagonism of 4-AP inhibition on B-50 phosphorylation.

* These compounds reduced the overall phosphorylation of proteins in SPM.

ceptor antagonists picrotoxin and bicuculline, which are also used in experimental models to study epileptogenesis, tested at a concentration of 1 mM, did neither alter the overall phosphorylation of proteins in SPM nor did they alter the phosphorylation of B-50 under the phosphorylation conditions used (results not shown).

Discussion

Our findings describe for the first time the inhibition of phosphorylation of the membrane-bound, nervous tissue specific phosphoprotein B-50 (12,19) in SPM by the potassium channel blocker 4-aminopyridine. Moreover, the inhibition of B-50 phosphorylation by 4-AP could be specifically reversed by the specific NMDA-receptor antagonist 2-amino-5-phosphonovalerate, whereas the inhibition phosphorylation of the α -subunit of CaMK II (50 kDa) by 4-AP was not sensitive to APV.

Previously we have shown that post-hoc phosphorylation of the 50 kDa protein was stimulated by 4-AP, when membranes isolated from hippocampal slices incubated with 4-AP were phosphorylated with radiolabelled ATP (2). In that report we also showed a small decrease in the degree of phosphorylation of B-50. Since the effects on B-50 phosphorylation were very small and difficult to interpret due to the use of the post-hoc phosphorylation assay, we investigated in this study the direct effect of 4-AP on the phosphorylation of purified synaptic plasma membrane proteins prepared from hippocampal tissue.

The decrease observed in the autophosphorylation of the α -subunit of CaMK II by 4-AP might reflect changes in the catalytic activity of this kinase, which phosphorylates, among others, synapsin I (20,21). Recently it has been shown that the activation or inhibition of CaMK II by autophosphorylation is determined by the amount of phosphate incorporated and thus by the amount of ATP employed to phosphorylate the kinase (22). At the concentration of ATP used in this study (7.5 μ M), the activity of the kinase is most probably inhibited by autophosphorylation, as deduced from the experiments of

Lou et al. (22). Autophosphorylation of CaMK II results in a loss of calcium sensitivity of the kinase (23). This implicates that in SPM the inhibition of phosphorylation by 4-AP results in an activation of CaMK II. 4-AP is known to stimulate the release of neurotransmitters from the presynaptic terminal (3). Since the release of neurotransmitters from their vesicles is stimulated by phosphorylation of synapsin I, which is a substrate protein for CaMK II (21, 24,25), the observed inhibition of autophosphorylation by 4-AP could be one of the biochemical effects of 4-AP leading to enhanced neurotransmission. The inhibition of phosphorylation of CaMK II by 4-AP could not be counteracted by APV and thus most probably does not involve the activation of the NMDA receptor.

The phosphorylation of the kinase C substrate protein B-50 was also inhibited by 4-AP, and this inhibition could be reversed by preincubation with APV. Reversal of the addition of 4-AP and APV did not neutralize the inhibitory effect of 4-AP on B-50 phosphorylation. This implicates that 4-AP interaction with SPM cannot be superseded by APV, but that binding of APV to the NMDA receptor prevents the inhibitory action of 4-AP on the phosphorylation of B-50. Whether or not the effects of 4-AP are the direct result of interaction with the NMDA receptor is remains to be proven. The inhibition of B-50 phosphorylation by 4-AP is most likely not the result of a direct effect of 4-AP with the protein kinase C - B-50 phosphorylation system, since the phosphorylation of purified B-50 with purified protein kinase C was not affected by 1 mM 4-AP (results not shown).

The reversal of the inhibition by 4-AP could not be shown by other anti-epileptic drug affecting GABAergic neurotransmission (e.g. pentobarbital, barbituric acid, carbamezepine and diazepam). Moreover, GABA receptor antagonists (picrotoxin and bicuculline) which are also employed in experimental models for epileptogenesis, did not change the degree of phosphorylation of any of proteins studied. This indicates that the changes in phosphorylation of membrane proteins in hippocampal SPM by the convulsant 4-AP is associated with the glutamate receptor and not with the GABA receptor.

Recently it has been shown that the activation of the NMDA receptor is also involved in the induction of long-term potentiation (LTP) in the hippocampus (7-10). During LTP the phosphorylation of B-50, which is identical to protein F1 of the Routtenberg group (26), is increased as a result of activation of protein kinase C (1,27-29). Protein kinase C activation by phorbol diesters without tetanic stimulation also induces LTP (30). B-50 is one of the major substrates for protein kinase C in the presynaptic membrane (31, 32) and is thus the most likely membrane phosphoprotein involved in LTP. The findings described in this paper add further evidence for such an involvement and support the participation of B-50/F1 phosphorylation and NMDA receptor activation during LTP and epileptogenesis.

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