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Activation of protein kinase C by 4-aminopyridine dependent on Na^+ channel activity in rat hippocampal slices

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The convulsant drug 4-aminopyridine (4-AP) stimulates the phosphorylation of the neuron-specific presynaptic protein B-50 in hippocampal slices. This effect could be attenuated by the protein kinase C (PKC) inhibitor staurosporine. Moreover, the endogenous phosphorylation of B-50 was found to be restricted to the 15 kDa *Staphylococcus aureus* protease fragment of B-50, known to contain the PKC acceptor site. The effect of 4-AP on B-50 phosphorylation was sensitive to the Na^+ channel blocker tetrodotoxin. These results indicate that 4-AP stimulates PKC activity in hippocampal slices by a mechanism dependent on Na^+ channel activity.

4-Aminopyridine (4-AP) is known to produce convulsions in man and can be used as an experimental model in studies in vitro. In brain slices in vitro, 4-AP induces spontaneous epileptic bursting activity [15] along with a considerable stimulation of transmitter release, which is attenuated by the Na^+ channel blocker tetrodotoxin (TTX) [6]. However, the molecular mechanisms by which 4-AP stimulates transmitter release are not known.

We recently reported that stimulation of [^{3}H]noradrenaline release in hippocampal slices by 4-AP is accompanied by an increase of the phosphorylation of B-50 [8]. B-50 is a neuron-specific, presynaptic, protein with an apparent molecular weight of 48 kDa on SDS-polyacrylamide gels, and is a major substrate of protein kinase C (PKC) in presynaptic nerve terminals [7]. B-50 has been shown to be identical to growth-associated protein GAP-43, as well as to protein F1, which is implicated in neuronal plasticity, and to the calmodulin binding protein neuromodulin [18]. PKC is implicated in the modulation of transmitter release [10]. Phorbol esters effective in activat-

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ing PKC (such as 4β-phorbol 12,13-dibutyrate (PDB)), stimulate transmitter release, whereas depolarization induced transmitter release can be inhibited by PKC inhibitors (such as staurosporine) [9]. Phosphorylation of B-50 in hippocampal slices by PKC, was shown to be closely related with transmitter release [5]. In this paper we report of experiments in which we investigated whether the 4-AP induced B-50 phosphorylation involves the activation of PKC.

In all experiments male Wistar rats (TNO, Zeist, NL), weighing 100–140 g, were used. 4-AP was obtained from Merck (Darmstadt, F.R.G.); TTX, staurosporine and PDB were from Sigma (St. Louis, U.S.A.), *Staphylococcus aureus* V8 protease (SAP) from ICN Immuno-Biologicals (Lisle, IL, U.S.A.), $[^{32}P]$orthophosphate (carrier-free), $[\gamma\text{-}^{32}P]$ATP (3000 Ci/mmol) and ^{14}C-labeled molecular weight marker-proteins were from Amersham (Bucks, U.K.). After decapitation of the rats, the brains were rapidly removed and put in ice-cold Krebs–Ringer buffer (KRB) (in mM: NaCl 124, KCl 5, $MgSO_4$ 1.3, $NaHCO_3$ 26, D-glucose 10, $CaCl_2$ 2, pH 7.4, thoroughly gassed with 95% O_2/5% CO_2). Hippocampi were carefully removed and slices of 400 μm were cut. Three slices per tube were preincubated in 2 ml of KRB for 30–60 min at 34°C and labeled during 90 min in 900 μl carbogenized KRB in the presence of 100 μCi $[^{32}P]$orthophosphate per tube. Subsequently drugs were added and the incubation was terminated at the times indicated in the text. Samples were processed as described by [4]. Phosphorylation of B-50 was determined by immunoprecipitation and subsequent autoradiography of SDS-polyacrylamide gels and expressed as mean ± S.E.M. of B-50 phosphorylation in untreated control incubations (without drugs = 100%) [4]. A two-tailed Student's *t*-test was used for statistical analysis of the results.

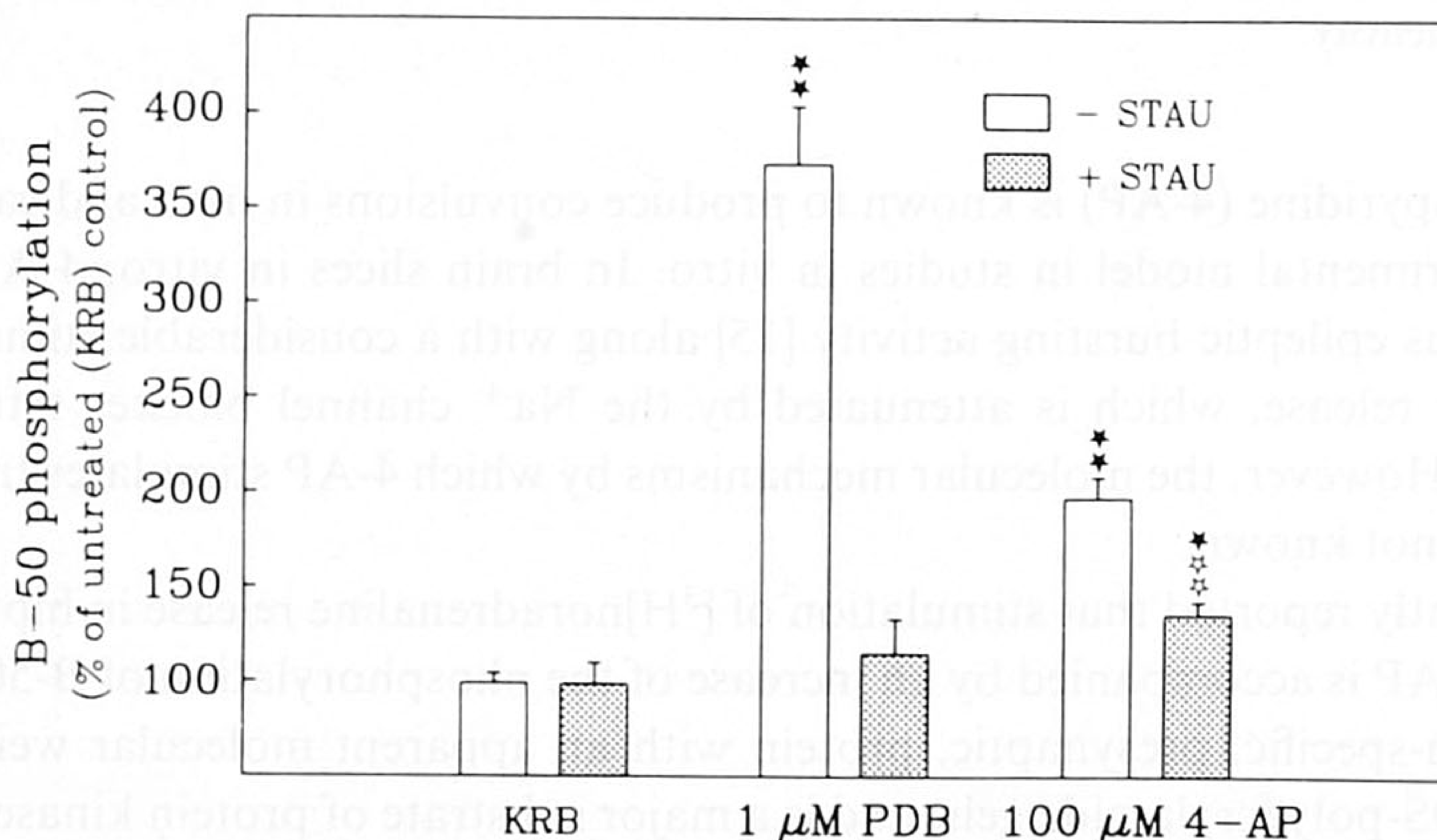

Fig. 1. Effect of staurosporine (STAU) on B-50 phosphorylation stimulated by PDB or 4-AP. Hippocampal slices were incubated, either in the presence (shaded columns) or in the absence (open columns) of STAU (10 μM) for 50 min. During the last 20 min of this incubation PDB (1 μM) or 4-AP (100 μM) or KRB were present. B-50 phosphorylation was determined as described in the text and data were expressed as mean ± S.E.M. of B-50 phosphorylation in untreated (KRB) control incubations (without STAU) (= 100%). Significant differences are indicated as: ★$2P<0.05$ (4-AP vs KRB samples in the presence of STAU); ★★$2P<0.01$ (4-AP or PDB vs KRB samples in the absence of STAU); ☆☆$2P<0.01$ (4-AP samples in the presence vs in the absence of STAU).

Synaptosomal plasma membranes (SPM) were phosphorylated with exogenous PKC as previously described [4]. In addition, B-50 was isolated by immunoprecipitation from homogenates of hippocampal slices, labeled with [^{32}P]orthophosphate and incubated for 20 min in the presence of either PDB (1 μM), 4-AP (100 μM) or KRB. After SDS-polyacrylamide gel electrophoresis (11%) the B-50 band from the three gels was excised, suspended in buffer B (25 mM Tris, 192.5 mM glycine, 0.1% SDS, pH 8.6) and concentrated separately by electrophoresis (for 3 h at 3 mW) in an ISCO Sample Concentrator (model 1750) to 200 μl fractions containing B-50. The isolated and concentrated B-50 fractions were digested with SAP (20 ng/μl) for 0, 5 and for 180 min at 30°C using the conditions described in [13]. The digestion was terminated by boiling the samples for 10 min. Subsequently the digestion products were separated on SDS-polyacrylamide gels (15%) and visualized by autoradiography, using colored, ^{14}C-labeled molecular weight markers as reference.

Addition of 4-AP (100 μM) increased the degree of B-50 phosphorylation in hippocampal slices to 197 ± 11% of control (Fig. 1). Staurosporine (10 μM) did not affect the basal phosphorylation of B-50, but inhibited the stimulation of B-50 phosphorylation by PDB (Fig. 1). The increase of B-50 phosphorylation by 4-AP as attenuated by staurosporine, resulting in a phosphorylation of 136% ± 7, which was significantly lower ($P < 0.01$) than the phosphorylation evoked by 4-AP in the absence of staurosporine (Fig. 1).

To find out which part of the B-50 molecule was labeled, hippocampal slices were incubated with [^{32}P]orthophosphate and stimulated with PDB (1 μM) or 4-AP (100 μM) for 20 min. B-50 was immunoprecipitated, isolated from the gel and digested with SAP for 5 or 180 min. As a control for the digestion procedure SPM was phosphorylated with exogenous PKC, B-50 was immunoprecipitated and digested under the same conditions (see Fig. 2). The autoradiogram of the gel revealed the formation

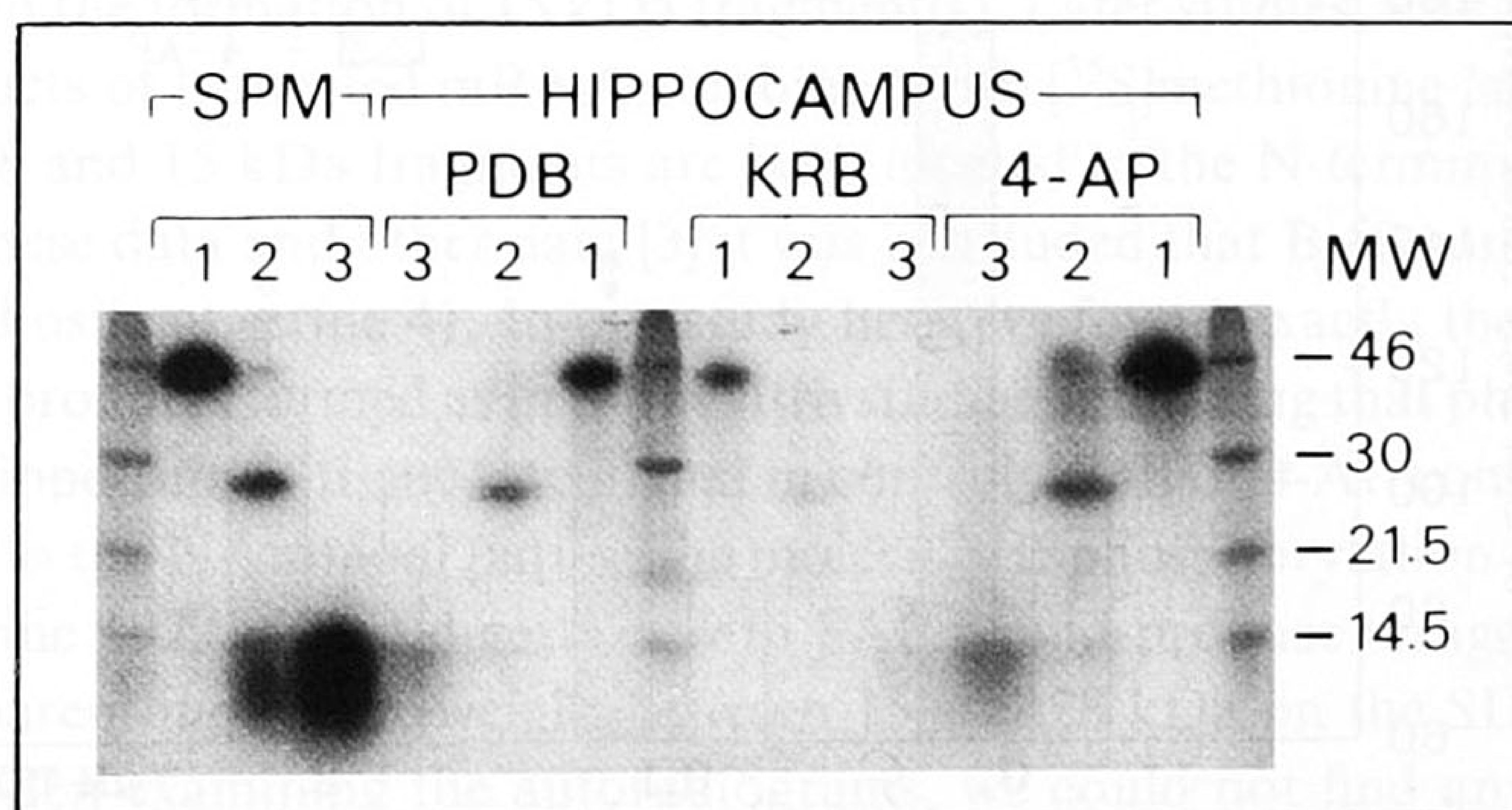

Fig. 2. Phosphopeptide mapping of phosphorylated B-50 isolated from hippocampal slices treated for 20 min with PDB (1 μM), 4-AP (100 μM) or KRB. In addition SPM was phosphorylated with exogenous purified PKC. At the left, in the middle and at the right of this autoradiogram radioactive molecular weight markers are shown; the weight of these markers is indicated (in kDa) at the right. B-50 was isolated from material from these 4 conditions as described in the text, and digested with SAP for 5 min (lanes 2) or for 180 min (lanes 3). Undigested B-50 (48 kDa) is visible in lanes 1 for each condition.

of radioactive phosphate containing SAP digestion products. The starting material for each digestion is shown in lanes of Fig. 2 marked as lane 1. Partial digestion of B-50 isolated from SPM for 5 min resulted in the formation of the two major phosphoprotein bands described for B-50, e.g. 28 and 15 kDa (Fig. 2: SPM, lane 2). After extensive digestion for 180 min both B-50 and the 28 kDa fragment disappeared, leaving radioactivity, which was visible only in fragment(s) of approximately 15 kDa (Fig. 2: SPM, lane 3). Digestion of B-50 isolated from untreated hippocampal slices resulted in the formation of the same products as observed during digestion of B-50 isolated from SPM (Fig. 2: KRB, lanes 1-3). Digestion of B-50 from slices treated either with PDB or 4-AP followed the same time-course: in the first 5 min of incubation with SAP, the 28 kDa was formed, which was further degraded to a 15 kDa phosphoprotein band after 180 min of incubation. No other radioactive phosphate containing proteins were detected on the autoradiogram. The phosphate incorporation in B-50 isolated from 4-AP- or PDB-treated slices is higher than that from control slices and these differences are conserved in the respective fragments formed during the digestion: e.g. the phosphate incorporation in the 28 and 15 kDa fragment(s) from 4-AP-treated slices is higher than the phosphate incorporated in those from untreated (KRB) control slices (Fig. 2).

B-50 phosphorylation in slices incubated in the presence of TTX did not differ significantly from untreated slices (Fig. 3). However, the increase in B-50 phosphorylation induced by 4-AP (100 μM for 20 min) was sensitive to TTX (Fig. 3).

In this study we investigated a possible involvement of PKC in the 4-AP-mediated effects in hippocampal slices. We observed that the stimulation of B-50 phosphorylation induced by 4-AP was attenuated by the PKC inhibitor staurosporine. Staurospo-

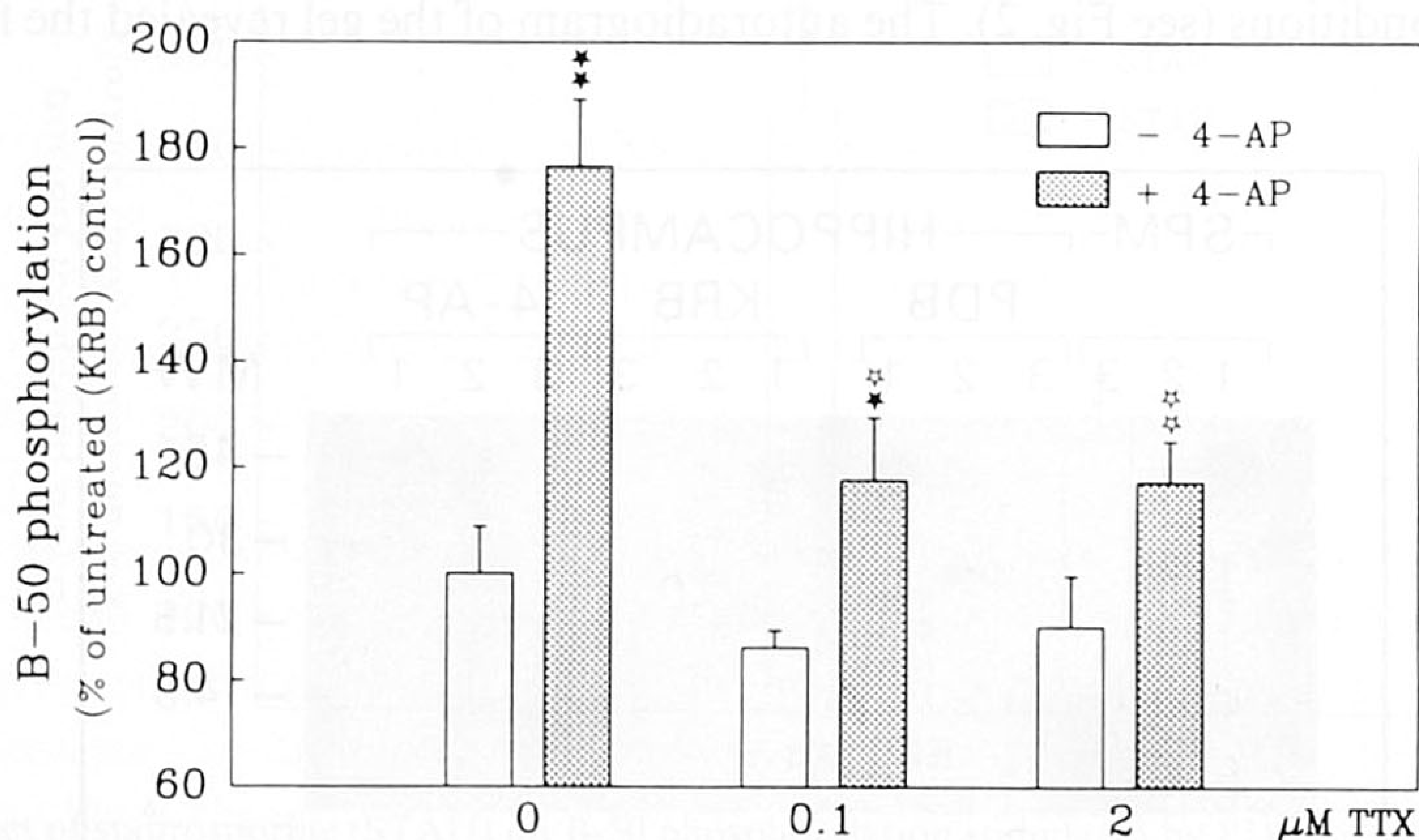

Fig. 3. Effect of TTX on B-50 phosphorylation stimulated by 4-AP. Hippocampal slices were incubated for 13 min in KRB containing 0.1 or 2 μM TTX or without TTX (0); 4-AP (100 μM) (shaded columns) was present during the last 5 min of incubation. B-50 phosphorylation was determined as described in the text and data were expressed as mean $\pm$ S.E.M. of B-50 phosphorylation in untreated control incubations (without TTX) (= 100%). Significant differences are indicated as: ★$2P<0.05$ and ★★$2P<0.01$ (4-AP vs respective control samples); ☆$2P<0.05$ and ☆☆$2P<0.01$ (4-AP samples in the presence vs in the absence of TTX).

rine however, did not affect total protein nor basal B-50 phosphorylation, probably due to low phosphate-turnover, since B-50 phosphorylation in slices is stable after approximately 1 h of incubation with [^{32}P]orthophosphate (unpublished results). Although staurosporine is the most potent PKC inhibitor currently known, it has been reported to affect cGMP-and cAMP-dependent kinases to some extent as well [19]. Therefore we felt that additional evidence was needed to establish that 4-AP in fact stimulates PKC activity. Among a number of protein kinases examined in vitro, only PKC was able to phosphorylate B-50 [1, 4]. Recently, the cDNA sequence of B-50 was determined [11], which revealed that the protein consisted of 226 amino acid residues, corresponding to a molecular weight of 23.6 kDa. Because of its anomalous behavior in SDS-polyacrylamide gels B-50 migrates at a rate corresponding to an apparent molecular weight of approximately 48 kDa. Acid hydrolysis of amino acids has shown that B-50 is phosphorylated only at serine residues (ref. 2 and unpublished results from our laboratory). Based on the sequence data and consensus PKC phosphorylation sites, a few putative phosphorylation sites can be predicted. Possible phosphorylation sites for PKC have been located at serine residues 41, 110 and 122 of which serine 41 is the likely one [11]. In addition, serine 41 is the only serine conserved in sequences from human, rat and mouse. As far as we have been able to establish, there is only one published study showing that, at least in vitro, it is possible to phosphorylate B-50 by a kinase other than PKC [14]. This in vitro phosphorylation by a second messenger independent kinase (casein kinase II), was predicted on basis of the consensus sequence for this kinase to occur at serine 192 [14].

Digestion with SAP of B-50 phosphorylated in vitro with purified PKC, has been applied recently to study the localization of PKC phosphorylation site(s) [13]. It was found that with limited digestion, only one phosphorylated polypeptide (of 28 kDa) was formed. Extensive digestion with SAP resulted in the further breakdown of this fragment and the formation of 15 kDa fragment(s). Later studies, using *in vitro* translation products of truncated mRNA combined with [^{35}S]methionine labeling showed that these 28 and 15 kDa fragments are both located at the N-terminus of B-50 [12, 16]. From these data and other data [3] it was concluded that B-50 contains only one PKC phosphosite at serine 41. In our study here, we found exactly the same pattern of digestion products formed as in the in vitro studies, indicating that phosphorylation of B-50 in hippocampal tissue (incubated in control, PDB or 4-AP containing buffer) is restricted to the N-terminal part of the molecule. If phosphorylation of B-50 would occur at serine 192, limited digestion with SAP would produce a digestion product with an apparent molecular weight between 15 and 28 kDa on the SDS-polyacrylamide gel. When examining the autoradiograms, we could not find any evidence for the existence of such a phosphorylated polypeptide. These results further support that B-50 is only phosphorylated by PKC. Together with the inhibition by staurosporine we described above, these results suggest involvement of PKC in the effect of 4-AP on B-50 phosphorylation.

Our observation that in the presence of TTX the effect of 4-AP on B-50 phosphorylation was attenuated, indicates that Na^+ channel activity is necessary for a stimu-

lation of B-50 phosphorylation by 4-AP. This result further supports the correlation between B-50 phosphorylation and transmitter release stimulated by 4-AP [8]. Attenuation of the 4-AP effect by TTX does not answer the question whether 4-AP has a direct effect on presynaptic terminals or more indirectly via activation of neurons making synapses upon other terminals. By inducing spontaneous bursting activity in hippocampal slices, 4-AP could stimulate B-50 phosphorylation indirectly by activation of the neuronal circuitry contained in the hippocampal slice. In recent experiments, however, we found that 4-AP is also able to stimulate B-50 phosphorylation in synaptosomes (unpublished results), suggesting a direct effect of 4-AP on presynaptic terminal membranes. From electrophysiological experiments it is known that 4-AP augments Ca^{2+} entry in neurons, probably by blocking K^+ channels [17]. Ca^{2+} entry, evoked by depolarization in synaptosomes, stimulates PKC activity and B-50 phosphorylation (ref. 20 and unpublished results from our laboratory). Therefore, knowing that 4-AP stimulates transmitter release in a Ca^{2+} dependent manner [6], we can speculate about the mechanism of action of 4-AP: Ca^{2+} entry, stimulated by 4-AP by a mechanism involving Na^+ channel activity, could enhance PKC activity and B-50 phosphorylation, resulting in increased transmitter release.

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