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4-Aminopyridine affects synaptosomal protein phosphorylation in rat hippocampal slices

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Rat brain hippocampal slices were incubated with or without the convulsant 4-aminopyridine (4-AP). From these slices a crude mitochondrial/synaptosomal membrane fraction was prepared and analyzed for endogenous protein phosphorylation. 4-AP (10^{-5} M) stimulated the phosphorylation of a 50 kDa protein by 86%. The phosphorylation of this 50 kDa protein is Ca^{2+}/calmodulin-dependent and we suggest that this protein is the lower molecular weight subunit of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II).

The molecular mechanisms underlying epileptogenesis at the level of the neuronal membrane are largely unknown. One of the experimental models to study epileptogenesis is the 4-aminopyridine model of epilepsy. The convulsant drug, 4-aminopyridine (4-AP), is known to block potassium channels associated with an influx of calcium[3,16,22,27]. Several lines of evidence point to a crucial role of calcium in epileptogenesis and the action of convulsant drugs[9,10,15,29,32]. Ca^{2+}-dependent phosphorylation of membrane proteins may be one of the processes underlying changes in synaptic plasticity found in epileptogenesis[8,33,34].

The transverse hippocampal slice system has been widely used to study the neural mechanisms underlying epileptogenesis and the related phenomenon of long-term potentiation (LTP). In this slice system, the degree of phosphorylation of several proteins has been reported to correlate with the degree of LTP[6,23,26,28]. This paper describes changes in the post hoc phosphorylation of proteins in a crude mitochondrial/synaptosomal fraction prepared from hippocampal slices treated with 4-AP.

Hippocampal slices (400 μm) were prepared from male inbred Wistar rats (TNO, Zeist, NL)[28]. The slices were incubated in 2 ml Krebs-Ringer buffer (KRB): 124 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, pH 7.4 equilibrated with 5% CO_2/95% O_2 at 34 °C. The slices were divided over glass tubes in such a way that each tube contained 3 slices from 3 different rats. After preincubation for 60 min at 34 °C, the medium was replaced by 2 ml KRB containing 4-AP (Sigma, St. Louis, MO, U.S.A.) at the concentrations indicated or by KRB alone. Incubation with or without the convulsant was continued for 30 min and the incubation was then stopped by replacing the medium with 3 ml ice-cold 0.32 M sucrose. Immediately after this washing step the 3 slices were homogenized in 300 μl 0.32 M sucrose in a Potter-Elvehjem tube with a Teflon pestle (7 strokes at 1400 rpm, clearance 0.250 mm). The homogenate was centrifuged for 10 min at 1000 *g* and the resulting supernatant was centrifuged at 10,000 *g*

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for 20 min to obtain a crude mitochondrial/synaptosomal fraction (P_2). The P_2 obtained from three slices was lysed in 120 μl ice-cold H_2O for 15 min and the ion concentration was adjusted to 10 mM sodium acetate, 10 mM magnesium-acetate, 0.1 mM calcium acetate (pH 6.5, buffer A) with a 10 times concentrated solution.

The phosphorylation assay was performed according to Zwiers et al.[36] as modified by Kristjansson et al.[13], with 10 μg protein (determined according to Lowry et al.[18]), 7.5 μM ATP, 2 μCi [γ-^{32}P]ATP (New England Nuclear, Boston, USA, spec. act. 3000 Ci·mmol^{-1}) in buffer A for 15 s at 30 °C in 25 μl. The reaction was terminated by addition of 12.5 μl denaturating solution[36]. The proteins were separated on 11% slab gels[36] at 30 mA and the front was run of the bottom of the gel for 30 min in order to improve the separation of the phosphoproteins in the 50 kDa range. The incorporation of phosphate into proteins was quantified by scanning of the autoradiogram according to Zwiers et al.[36] and expressed as peak height above background. Comparisons between experimental and control groups were only made per one gel and the results are expressed as percentage difference to control (100%). Statistical analysis was performed using Student's t-test.

The major phosphoproteins present in the crude mitochondrial/synaptosomal fraction are shown in Fig. 1 and have been designated on the right side of the figure according to their relative molecular weights. The most pronounced effect of incubation of slices with 10^{-5} M 4-AP is a stimulation of the phosphorylation of a 50 kDa protein. Quantification of the autoradiogram shown in Fig. 1 revealed that this stimulation of 50 kDa phosphorylation was 86% over control (Fig. 2). In addition there was a small yet significant stimulation of 80 kDa phosphorylation. The 4-AP-induced stimulation of 50 kDa phosphorylation was dose-dependent (Fig. 3) and was detectable at concentrations as low as 10^{-7} M. The effect of 4-AP

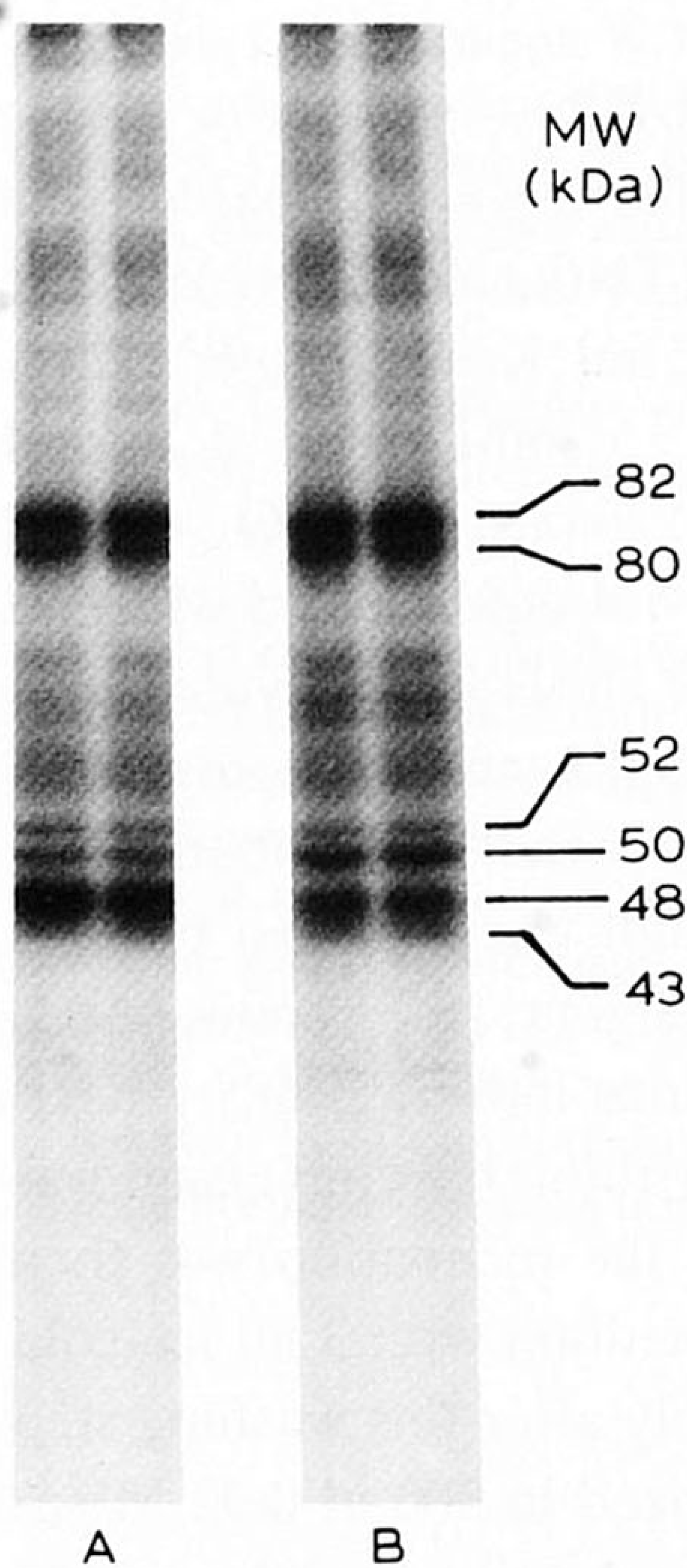

Fig. 1. Autoradiogram showing the effect of 4-AP treatment of hippocampal slices on in vitro endogenous protein phosphorylation. Slices were incubated without (A) or with (B) 10^{-5} M 4-AP for 30 min. From these slices a crude mitochondrial/synaptosomal (P_2) fraction was prepared and phosphorylated with [γ-^{32}P]ATP. Proteins were separated by 11% SDS-PAGE and subjected to autoradiography. Estimated molecular weights of the 6 major phosphoproteins are indicated.

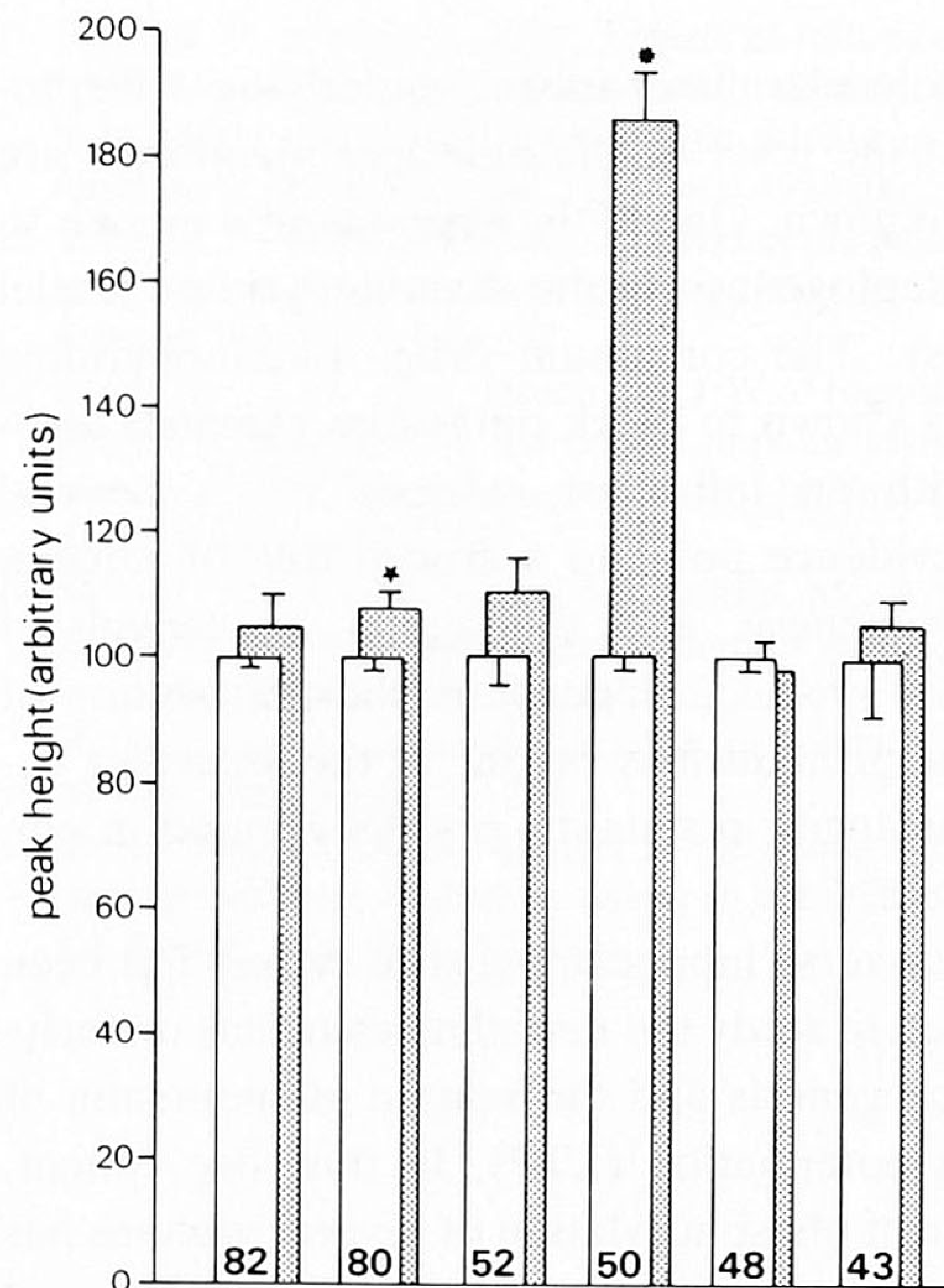

Fig. 2. Quantification of the effect of 10^{-5} M 4-AP as shown on the autoradiogram in Fig. 1 (n = 13). Quantification was performed by densitometric scanning of the autoradiogram and expressed as peak height above background. The data are expressed as percentage relative to control (100%). Indicated are: the mean and standard error of the mean (S.E.M., bars). ★, $2P < 0.05$, *, $2P < 0.001$, as determined with Student's t-test.

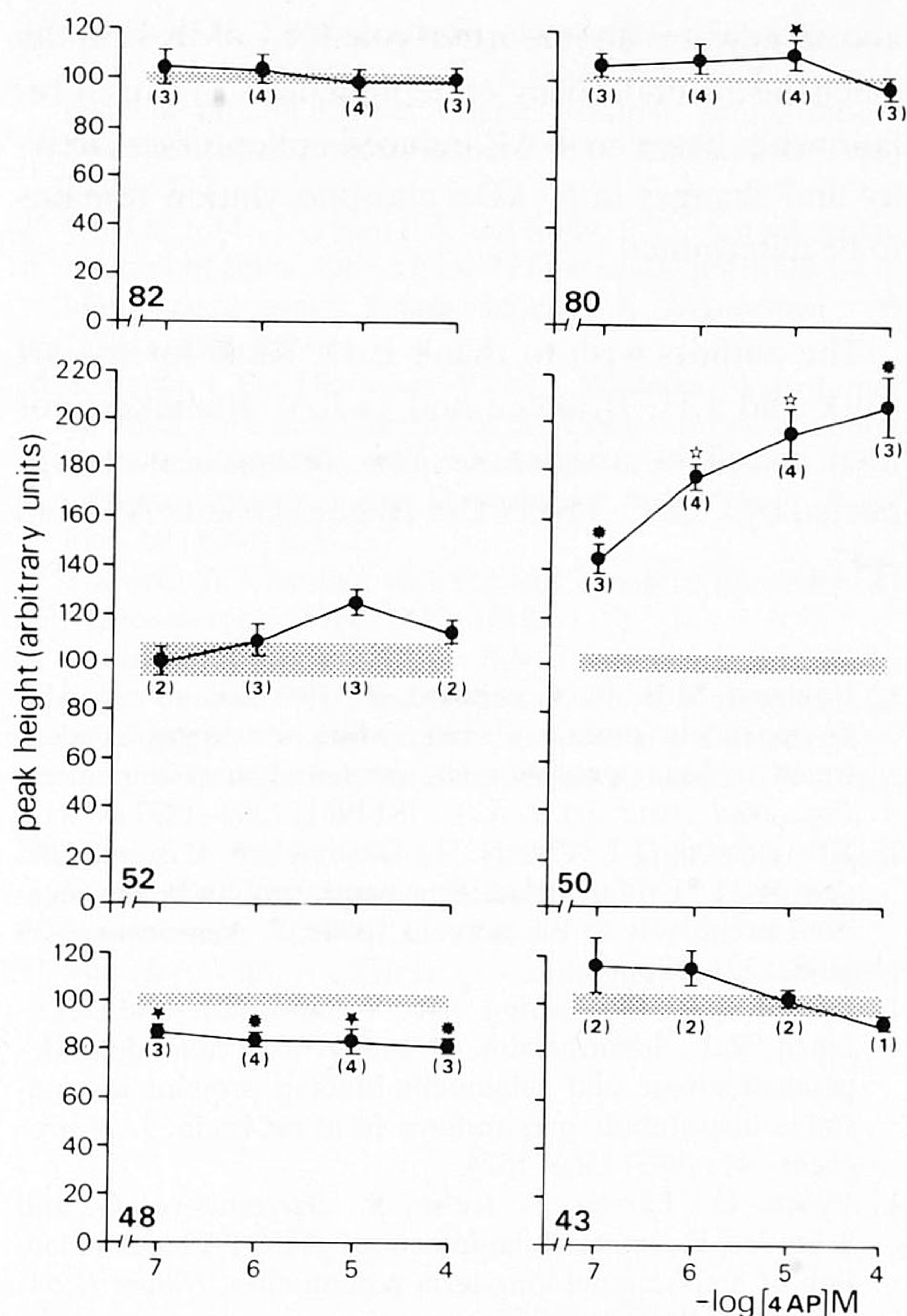

Fig. 3. Dose-dependency of the 4-AP effect on the phosphorylation of the 6 phosphoproteins indicated in Fig. 1. Quantification was performed as described in the legend of Fig. 2. Indicated are: the numbers in parentheses are the no. of determinations per concentration, the standard error of the mean (S.E.M., bars), the S.E.M. of the controls (shaded area) and the level of significance as determined by Student's *t*-test (★, $2P < 0.05$, *, $2P < 0.01$, ☆, $2P < 0.005$).

on the phosphorylation of the 48 kDa protein was variable, no effect was seen in the experiments shown in Fig. 2, whereas a small but significant inhibition of phosphorylation is shown in Fig. 3. The phosphorylation of the 80 kDa protein was only significantly stimulated at 10^{-5} M 4-AP (Figs. 2, 3). No effect could be detected on the phosphorylation of the other major phosphoproteins (82, 52 and 43 kDa) at any of the 4-AP concentrations tested. The stimulation of the 50 kDa protein phosphorylation could already be observed after a 5-min 4-AP treatment (data not shown).

The effects of 4-AP on protein phosphorylation were not observed after incubation of the P_2 fraction with 10^{-7} to 10^{-4} M 4-AP in vitro. Incubation of the P_2 fraction with 1 mM 4-AP resulted in a 40% decrease of 50 kDa phosphorylation (results not shown). Thus, stimulation of phosphorylation of the 50 kDa protein is probably not the consequence of a direct effect of 4-AP on the 50 kDa protein phosphorylation system.

The phosphorylation of the 50 kDa protein in the hippocampal P_2 fraction has been shown to be strongly Ca^{2+}/calmodulin-dependent[2,26]. Based on its relative molecular weight, its Ca^{2+}/calmodulin sensitivity and its subcellular distribution[26], the 4-AP-sensitive phosphoprotein is most probably the α or ϱ subunit of brain Ca^{2+}/calmodulin sensitive protein kinase type II (CaMK II)[4,12].

In another experimental model for epilepsy, the septal kindling model, a reduction of phosphate incorporation into a 50 kDa protein in hippocampal synaptosomal plasma membranes was observed[33]. The phosphorylation of this protein and the effect of kindling were calmodulin dependent[34]. Biochemical characterization showed that the protein is most likely the α or ϱ subunit of the CaMK II[8]. This protein kinase is a predominant brain phosphoprotein[4,10], consisting of two subunits. CaMK II has a number of substrate proteins in brain, such as cytoskeletal elements (tubulin and microtubule-associated protein-2)[7,14,24,35] and synapsin I[12]. The activity of the kinase towards synapsin I is stimulated by autophosphorylation[25]. The phosphorylation of synapsin I by CaMK II probably leads to the dissociation of synapsin I from synaptic vesicles, resulting in an enhancement of synaptic transmission[17], as elegantly shown in the squid giant axon after pressure injection of the kinase, thereby confirming the hypothesis that the phosphorylation of synapsin I by CaMK II may regulate vesicle function in presynaptic terminals[21].

In the mollusc, *Hermissenda*, incubation of the eyes and ganglia with $^{32}P_i$ in the presence of 4-AP resulted in a more than 85% reduction of phosphate incorporation into a 25 kDa protein[19]. This effect was found at a concentration range and time course similar to those required to block I_A in molluscan neurons[20]. This 25 kDa protein was found to be a substrate for cAMP- and Ca^{2+}-dependent protein kinases[20]. K^+ depolarization of *Hermissenda* neurons, which increases Ca^{2+} uptake and inactivates voltage-dependent K^+ currents, also reduces the phosphorylation of the 25 kDa protein[19]. Injection of rat brain

CaMK II into giant axons reduces I_A and I_C in *Hermissenda*[1]. From the combination of biochemical and biophysical data Neary and coworkers concluded that Ca^{2+}-stimulated protein phosphorylation can alter the activity of K^+ currents in molluscan neurons[20].

In our hippocampal slice studies, the dose- and time-dependency of the 4-AP effect on 50 kDa phosphorylation closely resemble those reported for 4-AP-induced epileptiform activity in this system[5,11,30,31]. Although the evidence summarized above indicates an important role for CaMK II in the mechanisms underlying epileptogenesis, a causal relationship between 4-AP-induced epileptiform activity and changes in 50 kDa phosphorylation remains to be determined.

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