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4-Aminopyridine Stimulates B-50 (GAP43) Phosphorylation and [^{3}H]Noradrenaline Release in Rat Hippocampal Slices

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Abstract: In situ phosphorylation of the presynaptic protein kinase C substrate B-50 was investigated in rat hippocampal slices incubated with the convulsant drug 4-aminopyridine (4-AP). Phosphorylation of B-50 was significantly enhanced 1 min after the addition of 4-AP (100 μM). This increase by 4-AP was concentration dependent (estimated EC_{50} 30–50 μM). Concomitant with the changes in B-50 phosphorylation, 4-AP also dose-dependently stimulated [^{3}H]noradrenaline ([^{3}H]NA) release from the slices. 4-AP stimulated [^{3}H]NA release within 5 min to seven times the control level. The B-50 phosphorylation induced by 4-AP remained elevated after removal of the convulsant, this in contrast to B-50 phosphorylation induced by depolarization with K^+. A similar persistent increase was observed for [^{3}H]NA release after a 5-min incubation period with 4-AP. These results give more insight into the molecular mechanisms underlying 4-AP-induced epileptogenesis and provide further evidence for the correlation between B-50 phosphorylation and neurotransmitter release in the hippocampal slice. **Key Words:** 4-Aminopyridine—Noradrenaline—B-50/GAP43/neuromodulin—Protein phosphorylation—Protein kinase C—Hippocampal slices. **Heemskerk F. M. J. et al.** 4-Aminopyridine stimulates B-50 (GAP43) phosphorylation and [^{3}H]noradrenaline release in rat hippocampal slices. *J. Neurochem.* **54,** 863–869 (1990).

4-Aminopyridine (4-AP) induces tonic-clonic seizures in vivo (Pasantes-Morales and Arzate, 1981; Szente and Baranyi, 1987) and spontaneous epileptic bursting activity in hippocampal slices in vitro (Buckle and Haas, 1982; Galvan et al., 1982; Voskuyl and Albus, 1985; Rutecki et al., 1987). In vitro electrophysiological experiments have shown that 4-AP affects neuronal excitability mainly by blocking K^+ channels (Segal et al., 1984; Kirsch et al., 1986), an effect that is probably associated with an influx of Ca^{2+} (Thesleff, 1980; Rogawski and Barker, 1983). Although many data about the electrophysiological effects of 4-AP are available now, little is known about the molecular mechanisms underlying 4-AP-induced epileptogenesis.

Protein phosphorylation is implicated in modulation of ion channel function and synaptic transmission (Nestler and Greengard, 1984; Llinás et al., 1985; Nishizuka, 1986; Kaczmarek, 1987). Previously we have investigated the effects of 4-AP on Ca^{2+}-dependent protein phosphorylation in vitro. Hippocampal slices were incubated with 4-AP, synaptosomal plasma membranes were isolated, and phosphorylation of the membrane proteins was studied in vitro. We showed that incubation of the slices with 4-AP resulted in changes in protein phosphorylation as determined with this post-hoc assay (De Graan et al., 1987). The observed changes were not due to direct effects of 4-AP on protein phosphorylation, since those effects are observed only at much higher concentrations of 4-AP ($\geq$1 mM) (Heemskerk et al., 1987). Therefore, changes in protein phosphorylation determined with this post-hoc assay must be due to effects of 4-AP during incubation of the slices. 4-AP induced small but variable changes in the phosphorylation of a 48-kilodalton

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Abbreviations used: 4-AP, 4-aminopyridine; KRB, Krebs-Ringer buffer; NA, noradrenaline; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

(kDa) protein (B-50) (Schrama et al., 1986; De Graan et al., 1987). Major changes were found in the phosphorylation of a 50-kDa protein, most likely the autophosphorylating α-subunit of the Ca^{2+}/calmodulin-dependent protein kinase II (Schrama et al., 1986; De Graan et al., 1987). This kinase phosphorylates, among other substrates, synapsin I, the phosphorylation of which is implicated in the regulation of synaptic vesicle release (Kennedy et al., 1983; Llinás et al., 1985).

Neurotransmitter release is also modulated by the activity of the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (Reichardt and Kelly, 1983; Nishizuka, 1986; Kaczmarek, 1987). One of the best-characterized substrates of PKC in the presynaptic nerve terminal is B-50 (Gispen et al., 1985; Benowitz and Routtenberg, 1987). B-50 is equivalent to GAP43, F1, and the calmodulin-binding protein neuromodulin (Gispen et al., 1986; Basi et al., 1987; Cimler et al., 1987; Nielander et al., 1987; Rosenthal et al., 1987).

Recently, a new technique was developed in our laboratory, using specific immunoprecipitation, to study changes in in situ B-50 phosphorylation (De Graan et al., 1989). Using this technique, Dekker et al. (1989) showed a correlation between changes in B-50 phosphorylation and K^+ depolarization–evoked neurotransmitter release from hippocampal slices.

Several studies have shown that 4-AP stimulates neurotransmitter release in cerebrocortical and striatal tissue, both in vitro (Thesleff, 1980; Löffelholz and Weide, 1982; Tapia and Sitges, 1982; Doležal and Tuček, 1983; Tapia et al., 1985; Tibbs et al., 1989) and in vivo (Damsma et al., 1988).

The aim of this study was to investigate whether 4-AP stimulates B-50 phosphorylation and whether this stimulation is correlated with neurotransmitter release in the hippocampus.

MATERIALS AND METHODS

Materials

In all experiments male Wistar rats (TNO; Zeist, The Netherlands), weighing 100–140 g, were used. 4-AP was obtained from Merck (Darmstadt, F.R.G.), and radiolabeled orthophosphate ($^{32}P_i$, carrier-free) and [7,8-^{3}H]noradrenaline ([^{3}H]NA; 32 Ci/mmol) were obtained from Amersham (Bucks, U.K.).

Phosphate labeling

Hippocampi were carefully removed from brains of male Wistar rats. Slices of 400 μm were cut manually as described previously (Tielen et al., 1983). Three slices per tube were preincubated at 34°C in Krebs-Ringer buffer (KRB) (124 m*M* NaCl, 5 m*M* KCl, 1.3 m*M* $MgSO_4$, 26 m*M* $NaHCO_3$, 10 m*M* D-glucose, 2 m*M* $CaCl_2$, pH 7.4, continuously gassed with 95% O_2/5% CO_2) for 30–60 min.

After a labeling period of 90 min with 100 μCi $^{32}P_i$ in 900 μl KRB [according to the method described by De Graan et al. (1989)], 4-AP was added in 100 μl KRB. In one series of experiments, 4-AP was removed after 5 min of incubation by rapidly washing the slices twice in 2 ml of KRB and the incubation was continued in 1 ml KRB containing 100 μCi $^{32}P_i$.

The incubation was stopped at the timepoints indicated in the text, and the slices were homogenized in the presence of inhibitors, as described previously (De Graan et al., 1989). Part of the homogenate was added to a denaturing solution containing sodium dodecyl sulfate (SDS) [as described by De Graan et al. (1989)] and stored on ice before B-50 immunoprecipitation. The remainder of the homogenate was saved on ice for protein determination and trichloroacetic acid (TCA) precipitation (see below).

Quantification and expression of data

Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as the standard. ^{32}P incorporation into total protein was determined by precipitation with TCA and subsequent removal of labeled phospholipids with ethanol/acetone washes and counted in a liquid scintillation counter (Packard model 2000CA) (Schrama et al., 1984) or by separation by SDS polyacrylamide gel electrophoresis (PAGE). B-50 was quantitatively immunoprecipitated as described previously (De Graan et al., 1989) and ^{32}P incorporation was quantified by densitometric scanning of the autoradiograms after separation by SDS-PAGE (Zwiers et al., 1976; Wiegant et al., 1978).

^{32}P incorporation into B-50 was corrected for changes in the ^{32}P incorporation in total protein precipitated by TCA to obtain a measure of the specific changes in B-50 phosphorylation and to correct for variability in metabolic activities between slices in different tubes. Values of treated samples were expressed as percentages (±SEM) of control (=100%) in each experiment to combine and compare the results of several experiments. Student's *t* test and the nonparametric Mann-Whitney U test were used for statistical analysis of the results. Changing K^+ concentrations in the buffer were always compensated for by correspondingly lower Na^+ concentrations to avoid osmotic changes.

Neurotransmitter release

Neurotransmitter release was measured in a continuous superfusion system (Stoof et al., 1980; Schoffelmeer et al., 1981; Versteeg and Ulenkate, 1987). In short, after dissection, the prepared hippocampal slices were preincubated for 10 min at 37°C in a metabolic shaker under a 95% O_2/5% CO_2 atmosphere. After a 15-min incubation in the presence of 5 μM [^{3}H]NA, the slices were gently washed twice with fresh KRB. Approximately 5 mg of the labeled tissue was transferred to each of the 24 perfusion chambers (vol 250 μl) maintained at 37°C and superfused at a rate of 250 $\mu l \cdot min^{-1}$.

After an initial 45 min of superfusion, fractions of 5 min were collected (t = −15 to −10, −10 to −5, −5 to 0 min, etc., until t = 25–30 min). After the first three fractions (prestimulus fractions), superfusion buffer was changed to buffer with or without 4-AP. At the end of the superfusion period (t = 30 min), the radioactivity remaining in the tissue was extracted in 0.1 *M* HCl. The radioactivity in the collected fractions and in the tissue was determined by liquid scintillation counting.

The release of ^{3}H radioactivity in each fraction was expressed as the fractional rate of the total amount of radioactivity calculated to be present in the tissue at the time the particular fraction was collected. Each value is the mean (±SEM) of eight observations.

RESULTS

Phosphorylation of B-50 in hippocampal slices was stimulated by 4-AP, as determined by quantitative im-

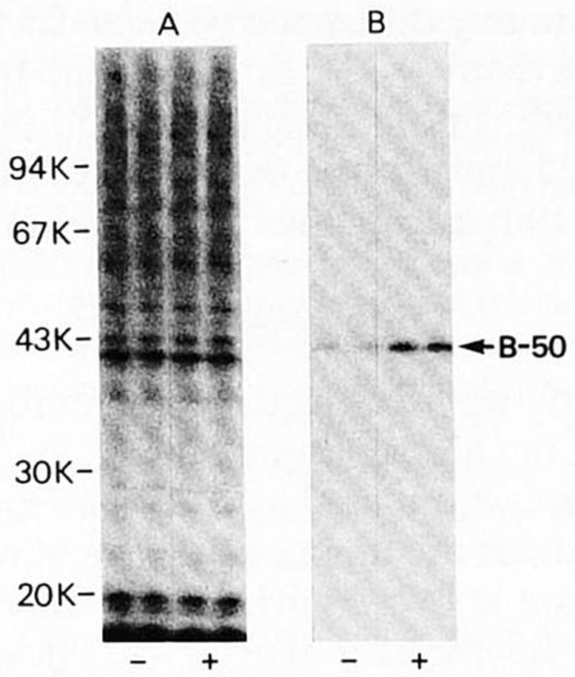

FIG. 1. Effect of 4-AP on in situ protein phosphorylation. Hippocampal slices were incubated with control KRB (−) or 100 μM 4-AP–containing KRB (+) for 5 min and in situ protein phosphorylation was analyzed by SDS-PAGE (11% gel) and autoradiography. Autoradiograms show ^{32}P incorporation in total proteins (**A**) and immunoprecipitated B-50 (**B**). Positions of molecular mass markers (values in kDa) are indicated to the left of the figure. The position of B-50 is indicated to the right of the figure with an arrow.

munoprecipitation of B-50 (Fig. 1B). 4-AP did not change total protein phosphate incorporation in general, as judged by liquid scintillation counting of TCA precipitates. One-dimensional SDS-PAGE did not reveal changes in ^{32}P incorporation in individual major proteins due to 4-AP (Fig. 1A). Therefore, changes in phosphate content of immunoprecipitated B-50 reflected specific differences in in situ phosphorylation of B-50. Incubation with 100 μM 4-AP stimulated B-50 phosphorylation within 1 min (technically the shortest measuring point possible) to 134 ± 10% (Fig. 2). The effect of 4-AP was concentration dependent (Fig. 3). The minimal effective concentration of 30 μM increased B-50 phosphorylation in 5 min to 128 ± 3%, whereas maximal stimulation required >1 mM

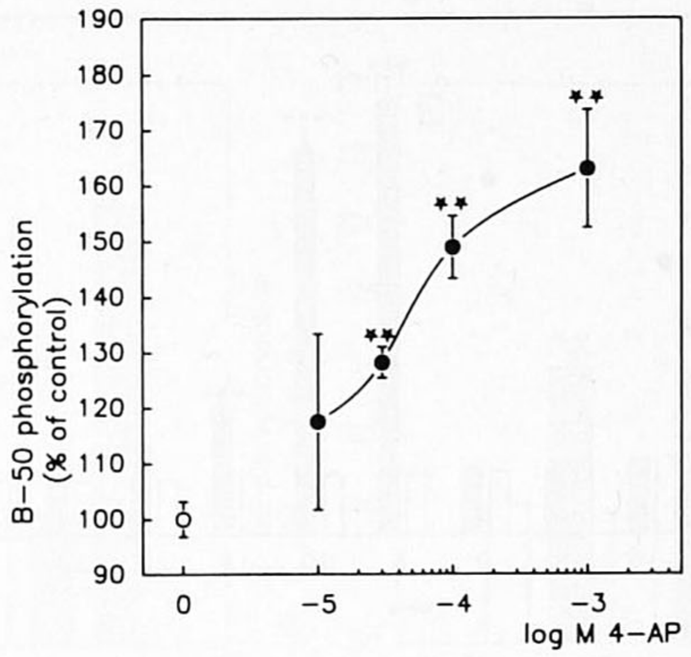

FIG. 3. Dose-response curve of 4-AP effect on the in situ phosphorylation of B-50. Hippocampal slices were incubated for 5 min with different concentrations of 4-AP and B-50 phosphorylation was determined as described in the legend to Fig. 2. Significant differences between 4-AP-treated samples and controls are indicated: $^{**}2p < 0.01$.

4-AP. Therefore, the estimated EC_{50} value is ~30–50 μM 4-AP.

Hippocampal slices, labeled with [^{3}H]NA and superfused with KRB, released label steadily during the experiment at a low rate (spontaneous release) as shown in Fig. 4. Superfusion of the hippocampal slices with 100 μM 4-AP induced release of [^{3}H]NA with maximal values reached within 10 min after addition of 4-AP (Fig. 4). [^{3}H]NA release remained elevated in the presence of 4-AP throughout the experiment, although the fractional rates after 30 min were somewhat lower than shortly after addition of 4-AP. Superfusion of the slices with 4-AP (100 μM) for only 5 min, followed by superfusion with control buffer, showed that the increase in release of [^{3}H]NA was reversible, as it decreased slowly to basal levels (Fig. 5). Remarkably even at 20 min after switching to control buffer (fractions t = 20–25 min), the release of [^{3}H]NA was still significantly

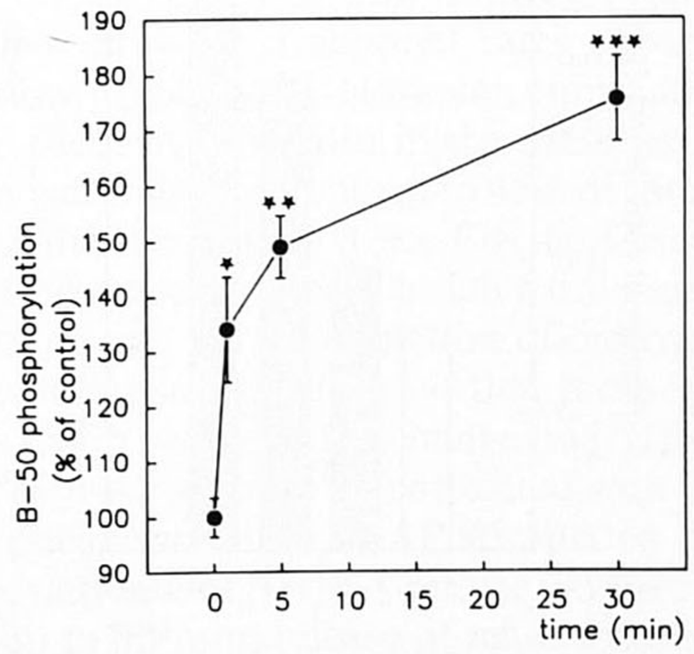

FIG. 2. Effect of 4-AP on in situ phosphorylation of B-50: time course of B-50 phosphorylation in hippocampal slices in the presence of 100 μM 4-AP. B-50 phosphorylation was determined as described in Materials and Methods and data were expressed as % ± SEM of B-50 phosphorylation after incubation in control buffer for 5 min. Significant differences between 4-AP-treated samples and controls are indicated: $^{*}2p < 0.05$, $^{**}2p < 0.01$, $^{***}2p < 0.001$.

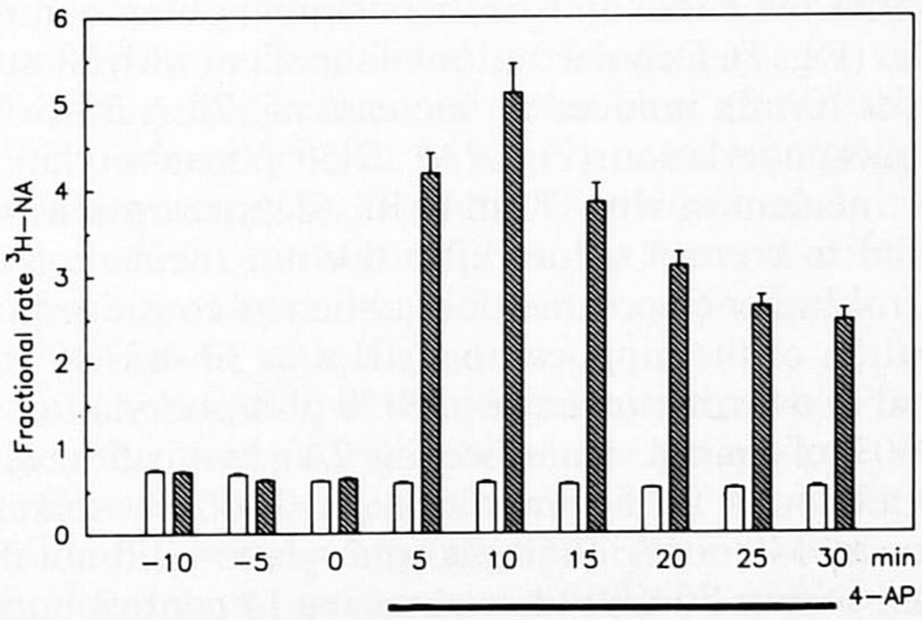

FIG. 4. Time course of [^{3}H]NA release from rat hippocampal slices in the presence or absence of 100 μM 4-AP. Fractional rates of 5-min fractions were determined as described in Materials and Methods and were expressed as means ± SEM. (Hatched bars), 100 μM 4-AP; (open bars), control buffer. The horizontal line beneath the abscissa indicates the period of superfusion with buffer containing 4-AP. During this period with 4-AP, fractional rates were all significantly higher than control fractions ($2p < 0.001$).

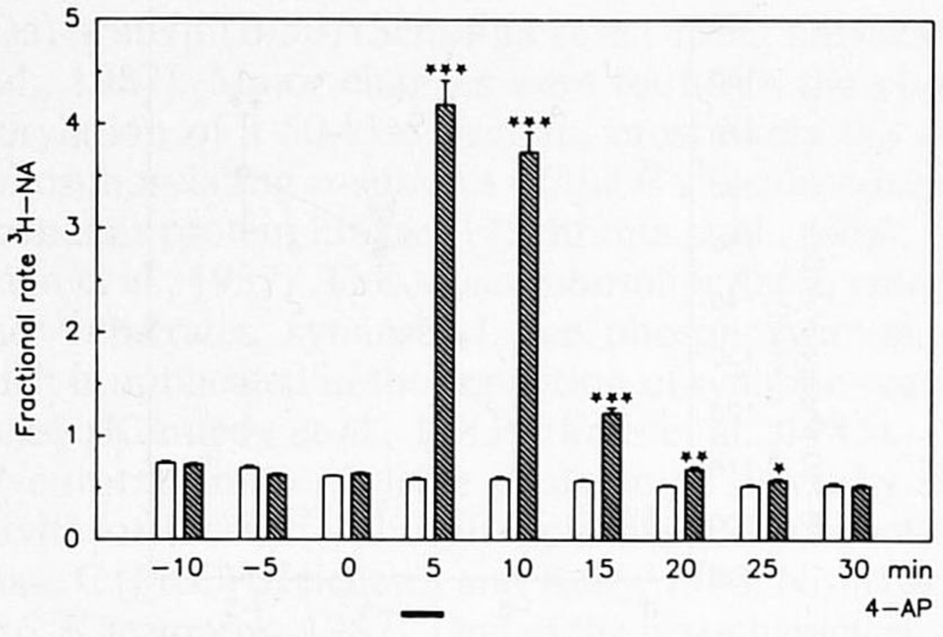

FIG. 5. Time course of [^{3}H]NA release from hippocampal slices after 5-min incubation with 100 μM 4-AP, followed by incubation in control buffer. The horizontal line beneath the abscissa indicates the period of superfusion with buffer containing 4-AP. The amount of [^{3}H]NA release was determined and expressed as in Fig. 4. Significant differences between control (open bars) and 4-AP (hatched bars) fractions are indicated: *$2p < 0.05$, **$2p < 0.01$, ***$2p < 0.001$.

enhanced above basal levels ($2p < 0.05$, Mann-Whitney U test; see Fig. 5). 4-AP stimulated release of [^{3}H]NA in a concentration-dependent manner, as was observed for the stimulation of B-50 phosphorylation (Fig. 6). The lowest concentration of 4-AP tested (10 μM) stimulated transmitter release in 5 min already up to 160 ± 7%. Because of the exponential increase in transmitter release induced by 4-AP, resulting in an unknown maximal stimulation, estimation of the exact EC_{50} value was difficult.

To correlate the effect of 4-AP on neurotransmitter release with the degree of B-50 phosphorylation and to compare this to the effects of depolarization, slices were incubated with 100 μM 4-AP or 30 mM K^+, followed by incubation in control buffer with new $^{32}P_i$ added to avoid changes in specific activity of endogenously labeled ATP. The amount of phosphate incorporated in B-50 in control slices was not altered by washing the slices and further incubation in control buffer (Fig. 7). Depolarization of the slices with 30 mM K^+ for 10 min induced an increase of 129 ± 5% in B-50 phosphorylation (Fig. 7A). B-50 phosphorylation after incubation with 30 mM K^+ (depolarization) returned to control values after 30-min incubation in control buffer (repolarization), whereas continued incubation of the hippocampal slices in 30 mM K^+ resulted in a further increase in B-50 phosphorylation to ~200% of control values (see Fig. 7A). Incubation with 100 μM 4-AP for 5 min stimulated B-50 phosphorylation to 149 ± 6%. B-50 phosphorylation did not decrease within 30 min after changing to control buffer (Fig. 7B). Incubation during 30 min in the continuous presence of 4-AP further stimulated B-50 phosphorylation to 175 ± 8% (Fig. 7B).

As can be seen in Fig. 7, the periods of incubation with 4-AP were not equal to those of depolarization. However, there is no difference in B-50 phosphorylation between 5 and 10 min of incubation with 4-AP; neither is there any difference between 25 and 30 min of incubation with 4-AP, as is evident from Fig. 2. Similarly, there is no difference in B-50 phosphorylation between 5 and 10 min or between 25 and 30 min of depolarization (see Dekker et al., 1989).

DISCUSSION

In this study we investigated changes in B-50 phosphorylation in hippocampal slices in relation to [^{3}H]NA release, stimulated by 4-AP or depolarization. Depolarization of the hippocampal slices with 30 mM K^+, a condition known to evoke Ca^{2+}-dependent neurotransmitter release, stimulated B-50 phosphorylation within 5 min (see Fig. 7A and Dekker et al., 1989). After prolonged depolarization, B-50 phosphorylation was enhanced even more, while upon repolarization, B-50 phosphorylation returned to control levels (Fig. 7A). Depolarization with K^+ is known to stimulate Ca^{2+} entry and to enhance PKC activity (Wu et al., 1982; Robinson et al., 1984; Diaz-Guerra et al., 1988; Wang et al., 1988). Moreover, it has been shown that the stimulation of B-50 phosphorylation by depolarization was dependent on extracellular Ca^{2+} and could be attenuated by a PKC inhibitor (polymyxin B; Dekker et al., 1989). Therefore, it has been hypothesized that depolarization with K^+ induces Ca^{2+} entry, increasing B-50 phosphorylation by stimulating PKC activity (Dekker et al., 1989). The observation that B-50 phosphorylation in hippocampal slices is increased by depolarization in a reversible manner (Fig. 7) supports the hypothesis that B-50 phosphorylation is stimulated by depolarization-induced Ca^{2+} influx.

We report here that 4-AP potently stimulates in situ phosphorylation of B-50 in hippocampal slices. Con-

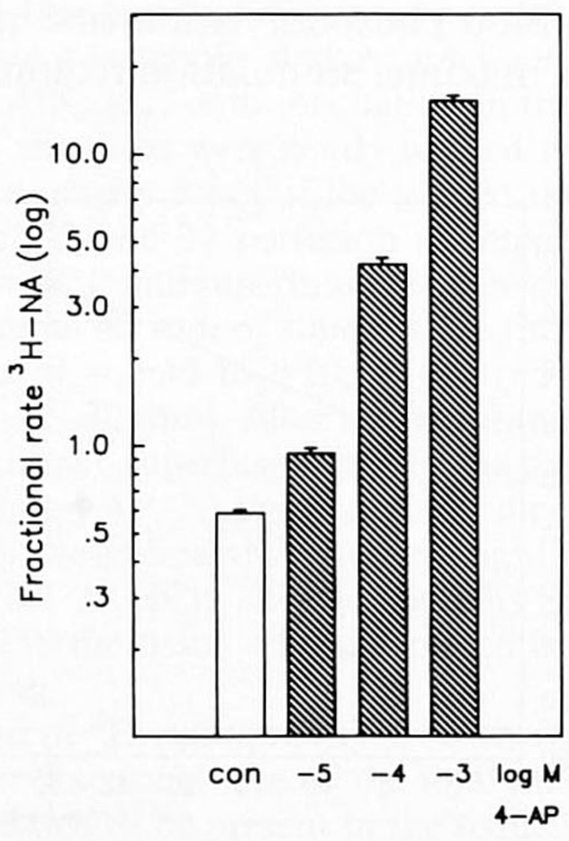

FIG. 6. Effect of different concentrations of 4-AP on [^{3}H]NA release from hippocampal slices. [^{3}H]NA release (fractional rates as in Figs. 4 and 5) was determined after 5 min of incubation with 4-AP and expressed in log values at the ordinate. Control value for fractional rates (mean ± SEM) at this timepoint was 0.59 ± 0.01. All three concentrations of 4-AP differed significantly from control values, as determined by Student's t test ($2p < 0.001$).

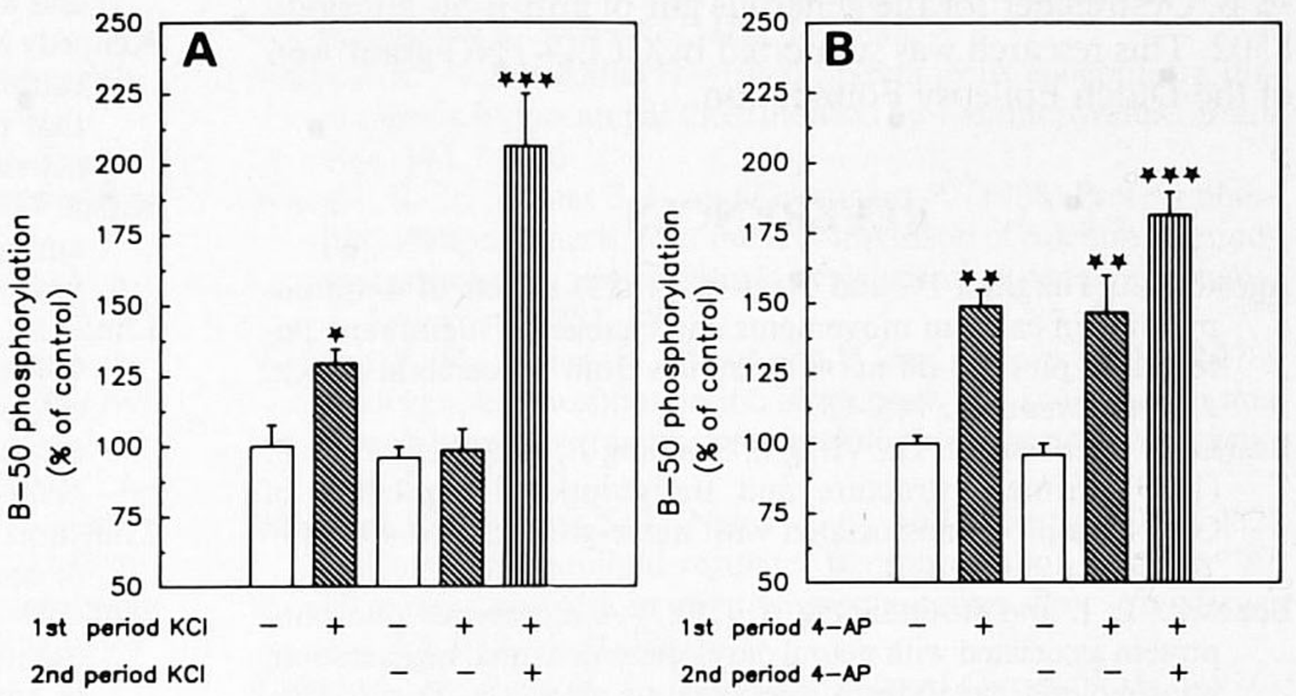

FIG. 7. Effect of brief and prolonged incubations of hippocampal slices with 100 μM 4-AP or during depolarization (30 mM K^+) on in situ phosphorylation of B-50. B-50 phosphorylation was determined as described in the legend to Fig. 2. **A:** Effect of incubating slices with control (5 mM K^+) buffer (−) or depolarization (30 mM K^+) buffer (+) during a first period of 10 min. B-50 phosphorylation was determined after this first period or after washing the slices followed by a second period of incubation for 30 min in control buffer (−) or depolarization buffer (+), as described in Materials and Methods. **B:** Effect of incubating slices with control buffer (−) or buffer containing 100 μM 4-AP (+) during a first period of 5 min. B-50 phosphorylation was determined after this first period or after washing the slices followed by a second period of incubation for 25 min in control buffer (−) or buffer containing 4-AP (+), as described in Materials and Methods. Significant differences are indicated: *$2p < 0.05$, **$2p < 0.01$, ***$2p < 0.001$.

centrations as low as 30 μM 4-AP were able to stimulate B-50 phosphorylation within minutes. This indicates that the variability in effects of 4-AP on B-50 phosphorylation that we found in our previous study using the post-hoc assay was due to the method used (De Graan et al., 1987).

Doses of 4-AP similar to those affecting B-50 phosphorylation in this study have been reported by others to affect neurotransmitter release in vitro (Thesleff, 1980; Löffelholz and Weide, 1982; Doležal and Tuček, 1983; Tapia and Sitges, 1985). In central nervous tissue, 4-AP has been described to stimulate acetylcholine release in rat striatal slices (Doležal and Tuček, 1983) and [^{14}C]glutamate and γ-[^{3}H]aminobutyric acid release from mouse brain synaptosomes (Tapia and Sitges, 1982; Tapia et al., 1985).

Therefore, we investigated the effects of 4-AP on transmitter release in the hippocampus. We found that 4-AP was a potent stimulator of [^{3}H]NA release in these slices, with low doses of 4-AP (<100 μM) stimulating [^{3}H]NA release within 5 min. During prolonged superfusion with 4-AP, fractional rates apparently decreased slowly (see Fig. 4). However, cumulative release from the slices over 30 min in the presence of 1 mM 4-AP, for example, could be up to 45% of total [^{3}H]NA present in the slices at $t = 0$ (see Fig. 4). Therefore, this apparent decrease in release at later timepoints could be attributed to a partial depletion of radioactively labeled neurotransmitter stores at that moment.

In this report we show that changes in [^{3}H]NA release induced by 4-AP seem to be correlated with the degree of B-50 phosphorylation. 4-AP stimulated both B-50 phosphorylation and [^{3}H]NA release within 5 min. For periods up to 30 min, release of neurotransmitter was twice the basal level or even higher, while after 30-min incubation in the presence of 4-AP, B-50 phosphorylation was still stimulated to a large extent. Stimulations of [^{3}H]NA release and of B-50 phosphorylation were dependent on the concentration of 4-AP, within the same dose range.

The effects of 4-AP on B-50 phosphorylation and [^{3}H]NA release seemed to be longer lasting compared to depolarization with K^+ (see Figs. 4 and 7). The discrepancy between B-50 phosphorylation and [^{3}H]NA release at $t = 30$ min after 5-min incubation with 4-AP might be due to the difference in the methods used to measure B-50 phosphorylation and release, respectively. We cannot exclude the possibility of a small amount of residual 4-AP stimulating B-50 phosphorylation locally, although the procedure used for washing the slices should theoretically decrease 4-AP to well below the effective concentration. Alternatively, it can be speculated that this long-lasting effect is due to a sustained activation of PKC. This might be an interesting hypothesis, in view of the induction of prolonged spontaneous activity in hippocampal slices after washing out of 4-AP (Buckle and Haas, 1982; Rutecki et al., 1987).

It has been suggested that 4-AP can enhance Ca^{2+} entry (Agoston et al., 1983; Rogawski and Barker, 1983; Muller and Lynch, 1989), but probably not directly by depolarization (Tapia et al., 1985; Tibbs et al., 1989). Electrophysiological experiments have shown that 4-AP is a potent blocker of K^+ channels regulating the spike frequency (Segal et al., 1984; Kirsch et al., 1986; Storm, 1988), indicating that 4-AP could stimulate transmitter release in a different way, as compared to depolarization with K^+.

In conclusion, our results indicate that 4-AP stimulates the phosphorylation of the presynaptic PKC substrate B-50 in hippocampal slices. Moreover, the effects of 4-AP on B-50 phosphorylation seem to be correlated with the increase of [^{3}H]NA release in the presence of 4-AP, suggesting a possible role of PKC in 4-AP-evoked transmitter release. For the first time, this offers more insight into the molecular mechanisms underlying epileptogenesis in this experimental model.

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