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4-Aminopyridine Stimulates B-50 (GAP-43) Phosphorylation in Rat Synaptosomes

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Abstract. Recently, we have shown that stimulation of [^{3}H]-noradrenaline release from hippocampal slices by 4-aminopyridine (4-AP) is accompanied by an enhancement of the phosphorylation of B-50, a major presynaptic substrate of protein kinase C (PKC). PKC has been implicated in the regulation of transmitter release. In this study, we investigated the effects of 4-AP on B-50 phosphorylation in synaptosomes from rat brain and compared the effects of 4-AP with those of depolarization with K^+, in order to gain more insight into the mechanism of action of 4-AP. B-50 phosphorylation was stimulated by incubation with 4-AP for 2 minutes at concentrations ranging from 10 μM to 5 mM. 4-AP (100 μM) stimulated B-50 phosphorylation already within 15 seconds; longer incubations revealed a sustained increase in the presence of 4-AP. B-50 phosphorylation was also stimulated by depolarization with 30 mM K^+ for 15 seconds. The effects of both 4-AP or K^+ depolarization on B-50 phosphorylation were abolished at low extracellular Ca^{2+} concentrations. The increase in B-50 phosphorylation induced by 4-AP seemed to be dependent on the state of depolarization, since the effect of 4-AP was largest under nondepolarizing conditions. Comparing the effects of 4-AP and K^+ depolarization on B-50 phosphorylation suggests that a different mechanism of action is involved. These results indicate that the stimulation of B-50 phosphorylation by 4-AP in hippocampal slices can be attributed to a direct action of 4-AP on presynaptic terminals. In addition, our results support the hypothesis that B-50 phosphorylation by PKC is involved in Ca^{2+}-dependent transmitter release evoked by 4-AP.

4-AP is a drug inducing seizures (Szente and Baranyi, 1987), whereas application in vitro to hippocampal slices elicits epileptic bursting activity (Buckle and Haas, 1982; Voskuyl and Albus, 1985; Rutecki et al., 1987). Electrophysiological experiments have revealed that in excitable tissues 4-AP specifically blocks voltage-dependent K^+ channels known to carry the A current (Segal et al., 1984; Rogawski, 1985). These channels are thought to play an important role in neuronal activity by regulating spike frequency (Segal et al., 1984; Rogawski, 1985). Although the drug is widely applied as a blocker of K^+ channels in electrophysiological experiments, little information is available on its impact on biochemical processes inside the cell.

One of the most striking features of 4-AP is that the drug is capable of stimulating release of many neurotransmitters in the PNS and the CNS. In brain, stimulatory effects of 4-AP on Ca^{2+} dependent transmitter release have been reported both in slices (Löffelholz and Weide, 1982; Doležal and Tuçek, 1983; Foldes et al., 1988; Heemskerk et al., 1990) and in synaptosomes (Tapia and Sitges, 1982; Tibbs et al., 1989).

The release of many transmitters is thought to be regulated by Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (Augustine et al., 1987; Kaczmarek, 1987). Phorbol esters effective in activating PKC stimulate transmitter release (Allgaier et al., 1986; Versteeg and Florijn, 1986), whereas depolarization-evoked transmitter release can be attenuated by PKC inhibitors (Versteeg and Ulenkate, 1987; Hertting and Allgaier, 1988).

B-50 is a neuronal-tissue specific presynaptic phosphoprotein (Sörensen et al., 1981; Gispen et al., 1985; van Lookeren Campagne et al., 1989) with an acidic isoelectric point (4.5) and an apparent molecular weight of 48 kDa on 11% SDS–polyacrylamide gels (Zwiers et al., 1980). It is identical to F1 (Gispen et al., 1986; Benowitz and Routtenberg, 1987), which has been implicated in long-term potentiation, to the growth-associated protein GAP-43 (Nielander et al., 1987; Basi et al., 1987), and to the calmodulin-binding protein neuromodulin/P57 (Cimler et al., 1987; for review see Skene, 1989). The phosphorylation of B-50, a major pre-

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synaptic substrate of PKC (Zwiers et al., 1980; Gispen et al., 1986; De Graan et al., 1988, 1989), has been shown to be closely correlated with transmitter release (Dekker et al, 1989). In hippocampal slices phorbol esters stimulate B-50 phosphorylation as well as transmitter release (Dekker et al., 1989), whereas the phosphorylation of B-50 was also enhanced by depolarization with 30 mM K^+. The latter effect was dependent on the presence of extracellular Ca^{2+} and was attenuated by the PKC inhibitor polymyxin B (Dekker et al., 1989).

Recently, we have found that 4-AP potently stimulated [^{3}H]-noradrenaline release from hippocampal slices (Heemskerk et al., 1990). Concomitantly, 4-AP stimulated the phosphorylation of B-50 in these slices in a time- and concentration-dependent manner. Moreover, this effect of 4-AP was found to be PKC-mediated (Heemskerk et al., 1989a). We were interested resolving the mechanism by which 4-AP stimulates transmitter release. Depolarization of synaptosomes with 30 mM K^+ has been shown to increase PKC activity (Wu et al., 1982; Rodnight and Perrett, 1986; Diaz-Guerra et al., 1988; Wang et al., 1988) and B-50 phosphorylation (Dekker et al., 1990). Therefore, in this study we investigated the effects of 4-AP on the phosphorylation of B-50 in synaptosomes and compared these to the effects of K^+ depolarization on B-50 phosphorylation, in order to gain more insight into the (presynaptic) mechanism of action of 4-AP.

Materials and Methods

Isolation of Synaptosomes

Cerebrocortical synaptosomes were obtained from male Wistar rats (100–150 g; TNO, Zeist, NL) according to the method of Dunkley et al. (1988). Synaptosomes separated on discontinuous Percoll–sucrose gradients (fraction 4) were washed twice in 40 ml Krebs-Ringer buffer (KRB) [124 mM NaCl, 1.3 mM $MgSO_4$, 5 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, pH 7.4, thoroughly gassed with 95% O_2–5% CO_2 (carbogen)], and collected at 11,000*g* for 20 min using a SS-34 rotor. Subsequently, the pellet was resuspended in carbogenized KRB and kept on ice. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Phosphate Labeling

Synaptosomes (2 μg/μl) were incubated for 60 minutes under carbogen flow at 34°C, with 1 μCi/μl $^{32}P_i$ (carrier free, Amersham, UK) in KRB in order to label the endogenous ATP pool with ^{32}P, aliquoted (10 μl), and further incubated at 34°C (Dekker et al., 1990). Drugs were added (in a volume of 10 μl) at the times indicated in the text, maintaining a total incubation time of 10 minutes (unless stated otherwise in the text). The reaction was stopped by adding 10 μl of ice-cold concentrated denaturing stop solution (final concentrations: 62.5 mM Tris HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol, pH 6.5). Elevated K^+ concentrations in the buffer were always compensated by corresponding lower Na^+ concentrations, in order to avoid osmotic changes. In some experiments synaptosomes were diluted four times in Ca^{2+}-free KRB with EGTA after labeling (at 4 μg/μl) in normal KRB (with 2 mM Ca^{2+}), in order to reach low extracellular free Ca^{2+} conditions (final concentrations were: 0.5 mM Ca^{2+} and 2 mM EGTA, resulting in an estimated free Ca^{2+} concentration below 10^{-7}M).

Quantification and Expression of Data

The amount of phosphate incorporation in B-50 was determined after quantitative immunoprecipitation, SDS-PAGE, and autoradiography (De Graan et al., 1989). In order to reduce aspecific precipitation of labeled material, a preclearing step (Dekker et al., 1990) was performed (involving incubation with the *Staphylococcus aureus* membranes used for the immunoprecipitation) before immunoprecipitation with specific anti-B-50 antibodies was carried out. B-50 phosphorylation was quantified by densitometric scanning of the autoradiograms. Changes in B-50 phosphorylation were expressed as percent of parallel control incubations (control = 100%) in order to compare the results of separate experiments. A two-tailed Student's *t* test was used for statistical analysis of the results. Changes in total protein phosphorylation were assessed by SDS-PAGE (11% gel) followed by autoradiography or by precipitation of total proteins with trichloroacetic acid (TCA), as described by Schrama et al. (1984).

Results

4-AP-induced changes in presynaptic protein phosphorylation were investigated in [^{32}P]orthophosphate-labeled synaptosomes. 4-AP (100 μM) induced no overall changes in the phosphorylation pattern analyzed after separation by one-dimensional SDS-PAGE (Fig. 1A) nor in TCA-precipitable protein (results not shown), indicating that 4-AP had no general effect on the labeling of the ATP

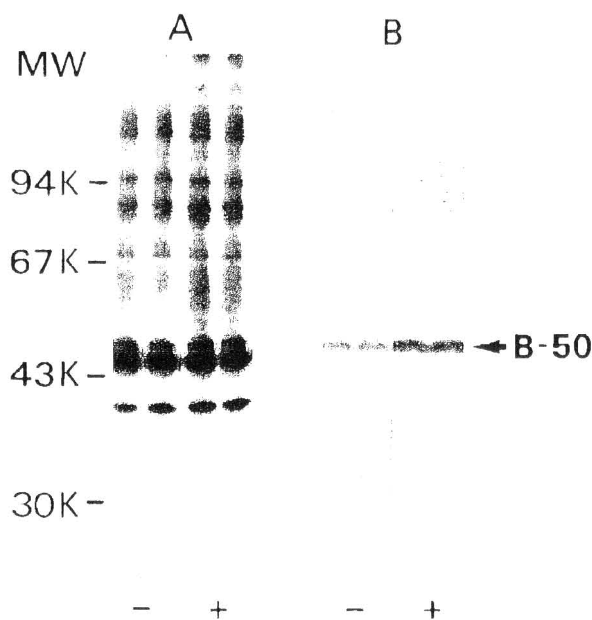

Fig. 1. Effect of 4-AP on the phosphorylation of B-50 in rat cerebrocortical synaptosomes. [^{32}P]orthophosphate-labeled synaptosomes were incubated for 2 minutes with buffer containing 100 μM 4-AP (+) or with control buffer (−). The ^{32}P incorporation in proteins was analyzed by 11% SDS-PAGE and autoradiography. Autoradiograms show ^{32}P incorporation in total proteins (A) and immunoprecipitated B-50 (B). N.B.: In (B) the autoradiogram was made by exposing the film for 63 hours using an intensifying screen, compared to direct exposure for 20 hours in (A); therefore the densities of lanes (A) are not directly comparable to those of lanes (B). Positions of molecular weight markers (MW, in kDa) are indicated on the left. The position of B-50 is indicated with an arrow on the right.

pool. No consistent changes because of 4-AP in individual major phosphobands were observed, except for an increase in the phosphorylation of a protein of 80–87 kDa in a number of experiments (Fig. 1A). In this study we were mainly interested in changes in B-50 phosphorylation, therefore we did not investigate any changes in phosphorylation of this 80 to 87-kDa protein in detail, although depolarization of synaptosomes with K^+ has been reported to enhance the phosphorylation of two proteins of a similar molecular weight (see Discussion).

Quantitative immunoprecipitation of B-50 and subsequent SDS-PAGE revealed an increase in B-50 phosphorylation 2 minutes after addition of 100 μM 4-AP (Fig. 1B). It is not possible to detect changes in B-50 phosphorylation directly after one-dimensional SDS-PAGE (N.B. in Fig. 1: the exposure times presented in panel B where much longer than those presented in panel A, as explained in the legend).

The 4-AP-induced increase in B-50 phosphorylation was already detectable after 15 seconds (Fig. 2) and reached its maximum after 2 minutes (142 ± 5% over control values). The phosphorylation of B-50 remained elevated for at least 15 minutes (121 ± 6%, compared to controls, $P < 0.01$). The magnitude of the increase in B-50 phosphorylation induced by treatment with 4-AP for 2 minutes depended on the 4-AP concentration applied (Fig. 3). The lowest concentration of 4-AP significantly stimulating B-50 phosphorylation was 10 μM, while 4-AP concentrations up to 5 mM (the highest concentration tested) stimulated B-50 phosphorylation even further.

Since stimulation of B-50 phosphorylation by depolarization with 30 mM K^+ is only observed in the presence of extracellular Ca^{2+} (Dekker et al., 1989, 1990), we investigated the effects of 4-AP on B-50 phosphorylation at low extracellular Ca^{2+} concentrations ($<10^{-7}$ M). [^{32}P]Orthophosphate-labeled synaptosomes were incubated for 10 minutes in KRB with Ca^{2+} (2 mM) or with low extracellular Ca^{2+} (with EGTA added), and 4-AP was added 2 minutes before the reaction was stopped. Parallel samples were depolarized with 30 mM K^+ for 15 seconds under these two Ca^{2+} conditions, before terminating the reaction. As can be seen in Fig. 4, B-50 phosphorylation in KRB with Ca^{2+} was stimulated by 100 μM 4-AP, as well as by depolarization. Chelation of extracellular calcium with EGTA decreased basal phosphorylation of B-50 to 82% ± 5 ($P < 0.05$). Under these conditions neither 4-AP

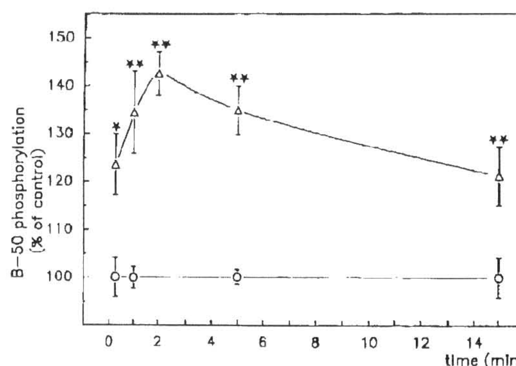

Fig. 2. Time course of the effect of 4-AP on B-50 phosphorylation. After incubation with 100 μM 4-AP (triangles) or control buffer (circles), B-50 phosphorylation was determined as described in the legend of Fig. 1. Data are expressed as percent (± SEM) of controls incubated for 5 min (= 100%). Significant differences are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$.

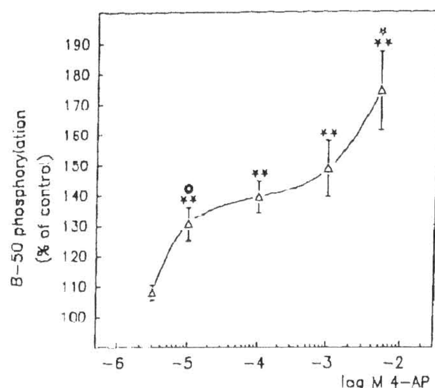

Fig. 3. Dose-response curve of 4-AP on the phosphorylation of B-50. B-50 phosphorylation was determined 2 minutes after addition of 4-AP as described in the legend of Fig. 1. Data are expressed as percent (± SEM) of B-50 phosphorylation in control incubations. Significant differences are indicated: $^{**}P < 0.01$. Differences between 10 μM 4-AP and 3 μM 4-AP (resp. 5 mM 4-AP) were significant: $^{\circ}P < 0.05$ (resp. $^{*}P < 0.01$).

nor K^+ depolarization significantly affected B-50 phosphorylation (Fig. 4).

As both K^+ depolarization and 4-AP stimulated B-50 phosphorylation in a Ca^{2+}-dependent manner, we investigated whether these stimulatory effects were additive (Fig. 5). Indeed, at half-maximal stimulation with 18 mM K^+, 100 μM 4-AP further stimulated B-50 phosphorylation (Fig. 5A). However, at maximal stimulation by 30 mM K^+, 100 μM 4-AP did nor further enhance B-50 phosphorylation (Fig. 5A). In Fig. 5B the time course of the effects of prolonged incubation with K^+ or 100 μM 4-AP on B-50 phosphorylation are shown. During incubation of synaptosomes with 30 mM K^+, B-50 phosphorylation is transiently enhanced with a maximum at 15 seconds and returns to control levels within 5 minutes (see also Dekker et al., 1990). In contrast, incubation in the presence of 100 μM 4-AP and 30 mM K^+ B-50 phosphorylation remained significantly elevated above control levels ($P < 0.05$) for at least 5 minutes.

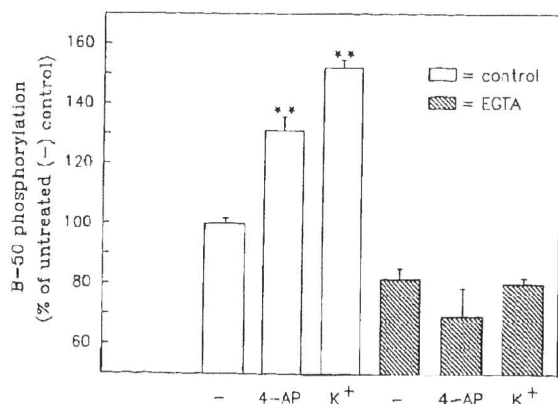

Fig. 4. Ca^{2+}-dependency of stimulation of B-50 phosphorylation by 4-AP or K^+ depolarization. B-50 phosphorylation was determined as described in the legend of Fig. 1, after incubation in KRB (−) or in the presence of 100 μM 4-AP (4-AP) or 30 mM K^+ (K^+). Data are expressed as percent (± SEM) of control incubations (2 mM $CaCl_2$) without 4-AP (5 mM K^+). Total incubation time in 2 mM $CaCl_2$ (control) or $\sim 10^{-7}$ M Ca^{2+} (EGTA) was 10 minutes. 4-AP was present for the last 2 minutes, 30 mM K^+ for the last 15 seconds of the incubation. Significant differences are indicated: $^{**}P < 0.01$ [compared to control incubations (2 mM $CaCl_2$)].

Discussion

We have reported that 4-AP stimulated transmitter release in hippocampal slices and concomitantly increased B-50 phosphorylation (Heemskerk et al., 1990). The changes in B-50 phosphorylation in synaptosomes induced by 4-AP described in this paper are similar to those induced by 4-AP in hippocampal slices, with respect to time as well as concentration dependency (Heemskerk et al., 1990). This indicates that the effects of 4-AP on the phosphorylation of B-50 in hippocampal slices can be attributed to a direct action of 4-AP on presynaptic terminals.

In some experiments we observed an increased phosphorylation of a 80 to 87-kDa band by 4-AP (Fig. 1). Although we did not investigate the identity of this phosphoband in detail, it could either be synapsin I or the 87-kDa substrate of PKC. Phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent kinase II has been shown to facilitate transmitter release (Llinás et al., 1985; Colbran et al., 1989), and the phosphorylation of both proteins was found to be enhanced upon depolarization of synaptosomes, possibly as a result of Ca^{2+} influx (Wu et al., 1982; Dunkley and Robinson, 1986; Rodnight and Perrett, 1986; Wang et al., 1988).

There appears to be a close correlation between the effects of 4-AP on B-50 phosphorylation and Ca^{2+}-dependent transmitter release in synaptosomes. It has been reported that 4-AP stimulates the release of several transmitters from synaptosomes in a Ca^{2+}-dependent manner (Tapia and Sitges, 1982; Tibbs et al., 1989). Interestingly, 4-AP enhanced spontaneous transmitter release but was not able to stimulate release under depolarizing conditions (Tapia and Sitges, 1982). 4-AP affected

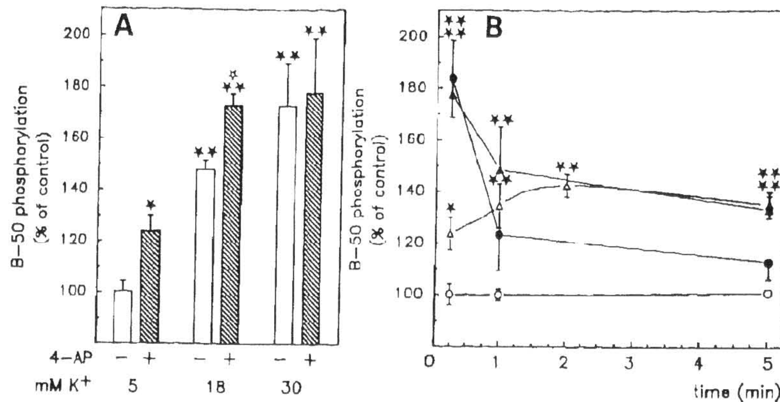

Fig. 5. Effects of 4-AP and K^+ depolarization on B-50 phosphorylation. B-50 phosphorylation was determined as described in the legend of Fig. 1. (A) Effects of incubation for 15 seconds in the presence of 18 or 30 mM K^+, with (+) or without (−) 100 μM 4-AP. Data are expressed as percent (± SEM) of control incubations (5 mM K^+) without 4-AP. Significant differences are indicated: *$P < 0.05$ and **$P < 0.01$ (compared to control incubation in the presence of 5 mM K^+) and ☆$P < 0.01$ (incubated with 4-AP in the presence of 18 mM K^+ compared to incubations in the presence of 18 mM K^+ alone). (B) Time course of B-50 phosphorylation after incubation in the presence (triangles) or in the absence (circles) of 100 μM 4-AP under normal (5 mM K^+, open symbols) or depolarized conditions (30 mM K^+, filled symbols). Data are expressed as percent (± SEM) of control incubations (5 mM K^+) without 4-AP. Significant differences are indicated: *$P < 0.05$ and **$P < 0.01$ (compared to parallel control incubations in the presence of 5 mM K^+).

transmitter release in those studies in the same dose range as B-50 phosphorylation (Fig. 3). Similar to its effects on transmitter release, 4-AP stimulates B-50 phosphorylation only under nondepolarizing conditions (Fig. 5) and in a Ca^{2+}-dependent manner (Fig. 4).

The Ca^{2+} dependency of the effects of 4-AP and K^+ suggests that Ca^{2+} influx might be involved in the stimulation of B-50 phosphorylation. Although depolarization with K^+ and 4-AP both stimulate Ca^{2+}-dependent release and increase B-50 phosphorylation, there are some important differences. Chemical depolarization of synaptosomes with K^+ stimulates B-50 phosphorylation within 15 seconds to a maximal level (Fig. 5) (Dekker et al., 1990), whereas stimulation of B-50 phosphorylation by 4-AP is much slower, reaching its maximal level not before 2 minutes (Fig. 2). Moreover, stimulation of B-50 phosphorylation in the presence of 30 mM K^+ seems to be a transient phenomenon, while 4-AP induces a sustained stimulation of B-50 phosphorylation. Since the effects of 4-AP and 30 mM K^+ were not additive, they are apparently not independent (see Fig. 5). This implies that one of the steps leading to B-50 phosphorylation was already maximally stimulated by 30 mM K^+. K^+ at 18 mM is known to stimulate Ca^{2+} entry and transmitter release from synaptosomes submaximally (Cotman et al., 1976; Nachshen and Blaustein, 1980; Nachshen, 1985). Stimulation of B-50 phosphorylation with 18 mM K^+ indeed allowed an additional stimulation by 4-AP to a level of B-50 phosphorylation that was not significantly different from that by 30 mM K^+.

Together with the results shown in Fig. 4, the data described above indicate that, although Ca^{2+} influx might be involved in the increase of B-50 phosphorylation, the effect of 4-AP on B-50 phosphorylation seems to be dependent on the state of depolarization. Many authors have suggested that 4-AP is able to enhance Ca^{2+} entry (Thesleff, 1980) either directly (Lundh and Thesleff, 1977; Rogawski and Barker, 1983; Agoston et al., 1983) or indirectly by blocking K^+ channels (Segal et al., 1984; Rogawski, 1985). However, only a few experiments have been described investigating the effects of 4-AP on Ca^{2+} entry directly. A stimulation of $^{45}Ca^{2+}$ uptake of synaptosomes by 4-AP or its structural analog 3,4-diaminopyridine was reported by some investigators (Agoston et al., 1983; Peterson and Gibson, 1983, 1985) but was not found by others (Tapia et al., 1985). Gibson and Manger (1988) reported that 4-AP caused, within seconds, an increase in the cytosolic free Ca^{2+} concentration in synaptosomes under nondepolarizing conditions, as determined with the fluorescent dye Fura-2. Recently, these results have been confirmed (Heemskerk et al., 1989b; Nicholls et al., 1989).

Taken together, the available data support an stimulatory effect of 4-AP on Ca^{2+} influx in synaptosomes under nondepolarizing conditions. Consequently, we hypothesize that 4-AP might enhance B-50 phosphorylation and transmitter release by stimulating Ca^{2+} influx.

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