Presynaptic Mechanism of Action of 4-Aminopyridine: Changes in Intracellular Free Ca^{2+} Concentration and Its Relationship to B-50 (GAP-43) Phosphorylation

F. M. J. Heemskerk, L. H. Schrama, *W. E. J. M. Ghijsen, P. N. E. De Graan,
*F. H. Lopes da Silva, and W. H. Gispen

Division of Molecular Neurobiology, Rudolf Magnus Institute, Institute of Molecular Biology and Laboratory of Physiological Chemistry, University of Utrecht, Utrecht; and *Department of Experimental Zoology, University of Amsterdam, Amsterdam, The Netherlands

Abstract: Recently we have shown that 4-aminopyridine (4-AP), a drug known to enhance transmitter release, stimulates the phosphorylation of the protein kinase C substrate B-50 (GAP-43) in rat brain synaptosomes and that this effect is dependent on the presence of extracellular Ca^{2+} . Hence, we were interested in the relationship between changes induced by 4-AP in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and B-50 phosphorylation in synaptosomes. 4-AP (100 μM) elevates the $[\text{Ca}^{2+}]_i$ (as determined with fura-2) to approximately the same extent as depolarization with 30 mM K^+ (from an initial resting level of 240 nM to ~ 480 nM after treatment). However, the underlying mechanisms appear to be different: In the presence of 4-AP, depolarization with K^+ still evoked an increase in $[\text{Ca}^{2+}]_i$, which was additive to the elevation caused by 4-AP. Several Ca^{2+} channel antagonists

(CdCl_2 , LaCl_3 , and diphenylhydantoin) inhibited the increase in B-50 phosphorylation by 4-AP. It is interesting that the increase in $[\text{Ca}^{2+}]_i$ and the increase in B-50 phosphorylation by 4-AP were attenuated by tetrodotoxin, a finding pointing to a possible involvement of Na^+ channels in this action. These results suggest that 4-AP (indirectly) stimulates both Ca^{2+} influx and B-50 phosphorylation through voltage-dependent channels by a mechanism dependent on Na^+ channel activity. **Key Words:** Calcium—4-Aminopyridine—B-50/GAP-43/neuromodulin/ F_1 —Synaptosomes—Calcium channels—Tetrodotoxin. **Heemskerk F. M. J. et al.** Presynaptic mechanism of action of 4-aminopyridine: Changes in intracellular free Ca^{2+} concentration and its relationship to B-50 (GAP-43) phosphorylation. *J. Neurochem.* **56**, 1827–1835 (1991).

The neuron-specific protein B-50 [also known as GAP-43, F_1 , or neuromodulin (see Benowitz and Routtenberg, 1987; Skene, 1989)] is a major presynaptic substrate of the Ca^{2+} /phospholipid-dependent protein kinase C (PKC) (Aloyo et al., 1983; Gispen et al., 1985; Dekker et al., 1990a). PKC has been implicated in the modulation of many transmitter systems (Reichardt and Kelly, 1983; Nishizuka, 1986; Kaczmarek, 1987). It is conceivable that B-50 is involved in this modulatory action of PKC, as was proposed by Dekker et al. (1989a). Conditions that enhance B-50 phosphorylation by PKC, e.g., phorbol esters, K^+ depolarization, A23187, and 4-aminopyridine (4-AP), also stimulate neurotransmitter release. Depolarization

is known to stimulate Ca^{2+} influx in synaptosomes, e.g., by opening voltage-sensitive Ca^{2+} channels (VSCCs), and to stimulate PKC (Wang et al., 1988; Dekker et al., 1990a,b). Depolarization with 30 mM K^+ induced an increase in B-50 phosphorylation, which was dependent on the presence of extracellular Ca^{2+} and was attenuated by PKC inhibitors (Dekker et al., 1989a, 1990a,b). Moreover, antibodies to B-50, which inhibit PKC-mediated B-50 phosphorylation, inhibit Ca^{2+} -induced [^3H]noradrenaline release from permeated synaptosomes (Dekker et al., 1989b). Of the various kinase inhibitors tested in isolated synaptosomes (Dekker et al., 1990b), H-7 most specifically inhibited PKC-dependent phosphorylation, and calmi-

Received June 13, 1990; revised manuscript received September 30, 1990; accepted November 7, 1990.

Address correspondence and reprint requests to Dr. L. H. Schrama at Division of Molecular Neurobiology, Institute of Molecular Biology, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Abbreviations used: 4-AP, 4-aminopyridine; $[\text{Ca}^{2+}]_i$, intracellular

free Ca^{2+} concentration; DPH, diphenylhydantoin (phenytoin); fura-2 AM, fura-2 acetoxymethyl ester; KRB, Krebs–Ringer buffer; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PPI, polyphosphoinositide; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin; VSCC, voltage-sensitive calcium channel.

dazolium most specifically inhibited calmodulin-dependent phosphorylation; polymyxin B affected both kinase systems. Only polymyxin B effectively inhibited noradrenaline release in intact synaptosomes. Thus, PKC and calmodulin-dependent processes are important for the release event (Dekker et al., 1990b; Nichols et al., 1990).

Recently we reported that the convulsant drug 4-AP stimulates [^3H]noradrenaline release from hippocampal slices and concomitantly increased B-50 phosphorylation (Heemskerk et al., 1990a). The increase in B-50 phosphorylation in our studies (Heemskerk et al., 1989a) and the stimulation of transmitter release by 4-AP reported by others (Doležal and Tuček, 1983; Tibbs et al., 1989a) were inhibited by the Na^+ channel blocker tetrodotoxin (TTX). 4-AP is known to enhance spontaneous transmitter release from synaptosomes in a Ca^{2+} -dependent manner (Tapia and Sitges, 1982; Tibbs et al., 1989a). Because B-50 is a presynaptic protein, 4-AP might stimulate transmitter release by a voltage-dependent presynaptic mechanism in which B-50 is involved.

To investigate this hypothesis we studied the effects of 4-AP on B-50 phosphorylation in synaptosomes and compared these with the effects of K^+ depolarization. We showed that 4-AP is able to stimulate B-50 phosphorylation under nondepolarizing conditions, a finding indicating a direct effect of 4-AP on the presynaptic terminal (Heemskerk et al., 1990b). Depolarization with 30 mM K^+ caused a large, transient increase in phosphorylation, whereas 4-AP (100 μM) gradually stimulated B-50 phosphorylation in a more sustained manner (Heemskerk et al., 1990b). The stimulation of B-50 phosphorylation by 4-AP or depolarization was abolished when the extracellular Ca^{2+} concentration was lowered by chelation with EGTA (Heemskerk et al., 1990b). This indicates that enhanced PKC activity is a result of an increased Ca^{2+} influx. However, EGTA treatment of synaptosomes affects the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) as well (Ghijsen and Verhage, 1989; Tibbs et al., 1989a).

Although many authors have suggested that 4-AP could have an effect on Ca^{2+} entry either directly (Rogawski and Barker, 1983) or indirectly (Lundh, 1978; Thesleff, 1980), there have been only a few studies investigating directly the effects of aminopyridines on Ca^{2+} homeostasis or Ca^{2+} influx in presynaptic terminals. Using the fluorescent dye fura-2, two recent studies reported that 4-AP is able to elevate the Ca^{2+} concentration in synaptosomes (Gibson and Manger, 1988; Tibbs et al., 1989b). Studies in synaptosomes have shown that $^{45}\text{Ca}^{2+}$ uptake, under nondepolarizing conditions, was enhanced by 4-AP (Pasantes-Morales and Arzate, 1981; Tibbs et al., 1989b) or by the structurally related 3,4-diaminopyridine (Peterson and Gibson, 1983, 1984). However, it cannot be concluded from these studies whether Ca^{2+} influx through Ca^{2+} channels was affected by 4-AP, because intracellular

redistribution of $^{45}\text{Ca}^{2+}$ might have occurred during the relatively long incubations used (1–20 min) in these experiments. Other studies determining $^{45}\text{Ca}^{2+}$ uptake after seconds did not provide conclusive evidence either (Agoston et al., 1983; Tapia et al., 1985).

To study the mechanism by which 4-AP stimulates B-50 phosphorylation, we compared the effects of 4-AP treatment or depolarization with KCl on B-50 phosphorylation and $[\text{Ca}^{2+}]_i$ in synaptosomes loaded with fura-2. Part of this study has been presented in preliminary form (Heemskerk et al., 1989b).

MATERIALS AND METHODS

Materials

Percoll was obtained from Pharmacia (Uppsala, Sweden), and ionomycin was from Behring Diagnostics (La Jolla, CA, U.S.A.). Fura-2 and its acetoxymethyl ester (fura-2 AM) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Stock solutions of fura-2 and fura-2 AM were prepared in dimethyl sulfoxide. 4-AP was obtained from Merck (Darmstadt, F.R.G.), diphenylhydantoin (phenytoin; DPH) and TTX were from Sigma (St. Louis, U.S.A.), and [^{32}P]orthophosphate was from Amersham (Bucks, U.K.).

Isolation of synaptosomes

Cerebrocortical synaptosomes were isolated from male Wistar rats (weighing 100–200 g; TNO, Zeist, The Netherlands) according to the method of Dunkley et al. (1988). The whole isolation procedure was carried out at 4°C. After collection of synaptosomes from the discontinuous Percoll/sucrose gradients (fraction 4), they were washed twice in 40 ml of low- Ca^{2+} Krebs–Ringer buffer [KRB; 124 mM NaCl, 5 mM KCl, 1.3 mM MgSO_4 , 26 mM NaHCO_3 , 10 mM D-glucose, and 20 μM CaCl_2 (pH 7.4), thoroughly gassed with 95% O_2 /5% CO_2 (carbogen)] and collected at 11,000 g for 20 min in a Sorvall SS-34 rotor. Subsequently the pellet was resuspended in carbogenized KRB and kept on ice. The protein concentration was determined according to the technique of Bradford (1976) using bovine serum albumin as the standard.

$[\text{Ca}^{2+}]_i$ determination and processing of the data

$[\text{Ca}^{2+}]_i$ was determined as described previously (Verhage et al., 1988). In brief, synaptosomes (2–5 μg of protein/ μl) were loaded with fura-2 AM (4 μM) in low- Ca^{2+} KRB for 30 min at 30°C in the dark. After a 10-fold dilution in low- Ca^{2+} KRB (30°C) followed by another 10 min of incubation at 30°C, the incubation was terminated by centrifugation for 10 min at 11,000 g in a Sorvall SS-34 rotor. The pellet was resuspended in low- Ca^{2+} KRB (four times the loading volume) and kept in the dark on ice. Immediately before use, an aliquot was centrifuged in a BHG table centrifuge, and the pellet was resuspended in 2.5 ml of carbogenized KRB (containing 2 mM CaCl_2 at 37°C) and transferred to a Perkin-Elmer luminescence spectrofluorometer (model LS 5), equipped with a cuvette containing a magnetic stirrer and thermostatted at 37°C. The cuvette was capped during the measurements to keep the synaptosome suspension saturated with carbogen. The excitation wavelength was alternated between 336 and 380 nm. Fluorescence (determined at 510 nm) was automatically averaged over periods of 3 s.

Calibration was performed in every experiment according to the procedure of Grynkiewicz et al. (1985) by determination of R_{max} with ionomycin ($2 \mu M$) and R_{min} (using 0.1% Triton X-100, 12 mM EGTA, and 80 mM Tris) and using a K_D of $225 \mu M$ for fura-2 for calculation of the Ca^{2+} concentration. The calculated $[Ca^{2+}]_i$ was corrected for effects caused by the drugs by measuring autofluorescence of unloaded synaptosomes in the presence of these drugs. Drugs (or KRB as the control) were added at $t = 300$ s (or at $t = 240$ and 480 s in the case of two additions). $[Ca^{2+}]_i$ (in nM) was averaged over fixed intervals (of 175 s starting 60 s after every drug addition). In addition, the slope of the increase in $[Ca^{2+}]_i$ with time (in nM/s) was determined. The mean $[Ca^{2+}]_i$ was calculated at a time central to each period, i.e., at $t = 148$ s (period I), $t = 388$ s (period II), and $t = 628$ s (period III), in the case of two additions. The values thus obtained for periods II and III (expressing $[Ca^{2+}]_i$ levels after the first or second addition, respectively) were divided by their own basal (control) values at the respective times, extrapolated from the first period, to yield the percent changes. The data from three independent synaptosome preparations were averaged and expressed as mean $\pm$ SEM values.

Phosphate labeling, quantification, and expression of data

Phosphorylation of synaptosomes was performed according to the method described by Heemskerk et al. (1990b). In short, synaptosomes ($2 \mu g/\mu l$) were incubated for 60 min under carbogen flow at $34^\circ C$ with $1 \mu Ci/\mu l$ of $[^{32}P]$ -orthophosphate (carrier-free; Amersham, U.K.) in KRB (containing 2 mM $CaCl_2$) to label the endogenous ATP pool with ^{32}P , aliquoted ($10 \mu l$), and further incubated at $34^\circ C$. Drugs were added (in a volume of $10 \mu l$) at the times indicated, maintaining a total incubation time of 10 min (unless stated otherwise). The reaction was stopped by adding $10 \mu l$ of ice-cold concentrated denaturing stop solution. Elevated K^+ concentrations in the buffer were always compensated for by corresponding lower Na^+ concentrations to avoid osmotic changes.

The amount of ^{32}P incorporation in B-50 was determined after quantitative immunoprecipitation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and autoradiography (De Graan et al., 1989). To reduce aspecific precipitation of labeled material, a preclearing step was performed as described by Dekker et al. (1990a). Changes in B-50 phosphorylation were expressed as percentages of values in parallel control incubations to compare the results of separate experiments. A two-tailed Student's t test was used for statistical analysis of the results.

Polyphosphoinositide (PPI) turnover

Hippocampal slices (Schrama et al., 1986) were incubated in KRB containing calcium for 90 min in the presence of either $[^{32}P]$ orthophosphate ($100 \mu Ci/ml$) or $[^3H]$ inositol ($2 \mu Ci/ml$, 16 Ci/mmol; Amersham) under carbogen flow. Synaptosomes were labeled as described above with $[^3H]$ inositol ($25 \mu Ci/70 \mu g$ of protein). Where indicated, 10 mM LiCl (final concentration) was added 30 min before addition of 4-AP or KRB. Phospholipids were extracted and analyzed as described by Jolles et al. (1981). Inositol phosphate formation was determined as described by Schrama et al. (1986).

RESULTS

Effects of 4-AP or chemical depolarization

Synaptosomes isolated on Percoll/sucrose gradients and loaded with fura-2 showed a basal $[Ca^{2+}]_i$ at the start of the experiment, of 239 ± 30 nM. During the incubation at $37^\circ C$ this level slowly increased linearly to 363 ± 31 nM at 750 s (untreated control incubations, Figs. 1–5). After addition of 4-AP ($100 \mu M$), the $[Ca^{2+}]_i$ rose within a few seconds to 481 ± 29 nM at $t = 448$ s (Fig. 1A), corresponding to an increase of $158 \pm 9\%$. This effect of 4-AP increased with the increasing concentrations tested ($10 \mu M$, $50 \mu M$, and 1 mM; data not shown). Depolarization of the synaptosomes with 30 mM K^+ elevated the $[Ca^{2+}]_i$ to 474 ± 29 nM at $t = 448$ s (Fig. 1B), corresponding to an increase of $170 \pm 9\%$. In most experiments a transient elevation of $[Ca^{2+}]_i$ was observed in the first minute after addition of K^+ . We did not observe such initial transient elevations in any of the experiments using 4-AP. Addition of 4-AP and K^+ depolarization resulted in a larger increase of $[Ca^{2+}]_i$ in time (reflected by a larger slope) of 184 ± 6 and $256 \pm 54\%$, respectively.

4-AP caused a small increase of the autofluorescence (see Materials and Methods), which apparently con-

FIG. 1. Time course of the effect of (A) 4-AP ($100 \mu M$) or (B) depolarization (30 mM K^+) on the free $[Ca^{2+}]_i$ (determined with fura-2 and expressed in nM) in synaptosomes. As described in Materials and Methods, in every experiment R_{max} and R_{min} were determined, and corrections for possible effects on autofluorescence were made. KRB ($\circ$), 4-AP ($\bullet$ in A), or K^+ ($\bullet$ in B) was added at $t = 300$ s.

FIG. 2. Time course of the effect of 4-AP (100 μ M) and subsequent depolarization (with 30 mM K^+) on the free $[Ca^{2+}]_i$ (expressed in nM) in synaptosomes: KRB ($\circ$) or 4-AP and K^+ ($\bullet$). For details, see the legend of Fig. 1. At $t = 240$ s, 4-AP was added, and at $t = 480$ s, K^+ was added.

tributed $\sim 10\%$ to the calculated $[Ca^{2+}]_i$. Therefore, the calculated $[Ca^{2+}]_i$ were corrected accordingly. No changes were detected in fluorescence of free fura-2 acid (without synaptosomes) or in the background signal (buffer alone) due to 4-AP. K^+ -induced depolarization of the synaptosomes treated with 100 μ M 4-AP resulted in an additional rise of $[Ca^{2+}]_i$ above the level obtained with 4-AP alone. After an initial transient elevation the $[Ca^{2+}]_i$ at $t = 628$ s was 686 ± 59 nM (Fig. 2), corresponding to an increase to $180 \pm 8\%$ of the extrapolated basal level at $t = 628$ s.

To investigate B-50 phosphorylation under these conditions, $[^{32}P]$ orthophosphate-labeled synaptosomes were incubated for 2 min with 4-AP (100 μ M) or depolarized for 15 s with 30 mM K^+ . 4-AP and K^+ depolarization resulted in an increase in B-50 phosphorylation with KRB of 120 and 150–190%, respectively (Figs. 6 and 7).

FIG. 3. Sensitivity of the effect of 4-AP (100 μ M) to TTX (1 μ M) as determined by measurement of the free $[Ca^{2+}]_i$ (expressed in nM) in synaptosomes: KRB ($\circ$) or TTX and 4-AP ($\bullet$). For details, see the legend of Fig. 1. At $t = 240$ s, TTX was added, and at $t = 480$ s, 4-AP was added.

FIG. 4. Sensitivity of the effect of 4-AP (100 μ M) to TTX (1 μ M) as determined by measurement of the free $[Ca^{2+}]_i$ (expressed in nM) in synaptosomes: KRB ($\circ$) or 4-AP and TTX ($\bullet$). For details, see the legend of Fig. 1. At $t = 240$ s, 4-AP was added, and at $t = 480$ s, TTX was added.

Involvement of Ca^{2+} channels

We tested whether the stimulation of B-50 phosphorylation by 4-AP or depolarization was sensitive to the Ca^{2+} channel antagonists $CdCl_2$ (Miller, 1987; Guan et al., 1988; Tsien et al., 1988) and $LaCl_3$ (Nachshen and Blaustein, 1980; Okada et al., 1989). In the presence of cadmium (30–300 μ M $CdCl_2$), B-50 phosphorylation was no longer stimulated by 4-AP, as can be seen in Fig. 6. The response to depolarization was completely inhibited by 300 μ M $CdCl_2$, but 30 μ M was not sufficient to inhibit the depolarization-evoked B-50 phosphorylation. Basal B-50 phosphorylation was not affected by cadmium alone. The effect of 4-AP on B-50 phosphorylation was also completely abolished by another inorganic Ca^{2+} channel antagonist, $LaCl_3$ (2 μ M); however, in the presence of $LaCl_3$ alone, the basal B-50 phosphorylation was enhanced (Fig. 7). The drug DPH (250 μ M), known to block (T-type) Ca^{2+} channels as well as Na^+ channels (Willow et al., 1985;

FIG. 5. Effect of depolarization (30 mM K^+) on the $[Ca^{2+}]_i$ (expressed in nM) in synaptosomes in the presence of TTX: KRB ($\circ$) or TTX and K^+ ($\bullet$). For details, see the legend of Fig. 1. At $t = 240$ s, TTX was added, and at $t = 480$ s, K^+ was added.

FIG. 6. Effect of $CdCl_2$ on B-50 phosphorylation stimulated by 4-AP (2 min, 100 μM) or depolarization (15 s, 30 mM KCl). Changes in ^{32}P incorporation in B-50 were analyzed by immunoprecipitation, SDS-PAGE on 11% gels, and autoradiography. Data are expressed as percent changes [mean $\pm$ SEM (bars)] above values for the respective untreated or $CdCl_2$ -treated controls. There was no significant difference between untreated ($100 \pm 3\%$) and $CdCl_2$ -treated (30 μM , $108 \pm 4\%$; 300 μM , $110 \pm 6\%$) values. Significant differences are indicated: * $p < 0.05$, ** $p < 0.01$ ($n \geq 6$).

Yaari et al., 1987; Griffith and Taylor, 1988), attenuated the effect of 4-AP on B-50 phosphorylation without affecting basal B-50 phosphorylation (Fig. 7). At a concentration as low as 25 μM , DPH already significantly decreased the stimulation of B-50 phosphorylation by 4-AP (data not shown). However, even at 250 μM DPH did not diminish the effect of K^+ depolarization on B-50 phosphorylation (Fig. 7).

Because of their inherent fluorescence, we were unable to test the effect of these Ca^{2+} channel antagonists on the elevation of $[Ca^{2+}]_i$ evoked by 4-AP.

Involvement of Na^+ channels

To investigate the possible involvement of Na^+ channels in 4-AP- and K^+ -induced effects, we used the specific Na^+ channel blocker TTX. Addition of TTX (1 μM) to synaptosomes caused a small decrease of $[Ca^{2+}]_i$ (Fig. 3) to $92 \pm 2\%$ of the extrapolated basal level at $t = 628$ s; however, this effect of TTX was equal to the decrease of $[Ca^{2+}]_i$ found after addition of KRB. No changes in autofluorescence were observed with TTX. TTX seems to have a larger attenuating effect on the slope of $[Ca^{2+}]_i$ with time than was found after addition of KRB. In the presence of TTX the stimulatory effect of 4-AP on $[Ca^{2+}]_i$ was attenuated (compare Figs. 3 and 1A). In this condition 4-AP caused an increase in $[Ca^{2+}]_i$ of only $130 \pm 11\%$ relative to TTX. Alternatively, addition of TTX to the synaptosomes in the presence of 4-AP resulted in a decrease of $[Ca^{2+}]_i$ (Fig. 4) to a level at $t = 628$ s ($69 \pm 6\%$ of the $[Ca^{2+}]_i$ level in the presence of 4-AP) that was not significantly different from the level (Fig. 3) observed in the presence of 4-AP and TTX (122 ± 8 and $117 \pm 4\%$, respectively, relative to the extrapolated

basal values). TTX did not affect the slope of $[Ca^{2+}]_i$ with time in the presence of 4-AP. In contrast, the increase of $[Ca^{2+}]_i$ by K^+ depolarization was not prevented by TTX (compare Fig. 5 with Fig. 1B).

The presence of TTX (1 μM) for 10 min did not change basal B-50 phosphorylation significantly. However, the stimulation of B-50 phosphorylation by 4-AP was abolished in the presence of TTX, in contrast to the effect of depolarization with 30 mM K^+ (Fig. 8). Involvement of Na^+ channels in the effects of 4-AP was further suggested in experiments where the extracellular concentration of Na^+ was lowered from 124 to 44 mM by equimolar replacement with choline. Under these conditions B-50 phosphorylation was $126 \pm 8\%$ of the untreated control incubation after 10 min of incubation, whereas addition of 4-AP did not increase B-50 phosphorylation further. In contrast, depolarization with 30 mM K^+ stimulated B-50 phosphorylation to a level similar to that observed on depolarization in control KRB (data not shown).

Effect of 4-AP on PPI turnover

The ^{32}P -prelabeled hippocampal slices were incubated for 5 or 30 min with or without 10 μM , 100 μM , or 1 mM 4-AP. The incorporation of phosphate into phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4-monophosphate, phosphatidylinositol, phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine was determined by TLC followed by liquid scintillation counting. No change in the turnover of PPIs or any of the other phospholipids was observed. The 3H -prelabeled hippocampal slices were incubated for 5 or 30 min with 100 μM 4-AP. The accumulation of inositol

FIG. 7. Effect of $LaCl_3$ (2 μM) or DPH (250 μM) on B-50 phosphorylation stimulated by 4-AP (2 min, 100 μM) or depolarization (15 s, 30 mM KCl). B-50 phosphorylation was determined as described in the legend of Fig. 6. Data are expressed as percent changes [mean $\pm$ SEM (bars)] above values for the respective untreated or $LaCl_3$ - or DPH-treated controls. $LaCl_3$ -treated control values were higher ($139 \pm 18\%$) than untreated values ($100 \pm 2\%$). There was no significant difference between untreated ($100 \pm 2\%$) and DPH-treated ($98 \pm 3\%$) values. Significant differences are indicated: * $p < 0.05$, ** $p < 0.01$ ($n \geq 6$).

FIG. 8. Effect of TTX (1 μ M) on B-50 phosphorylation stimulated by 4-AP (2 min, 100 μ M) or depolarization (15 s, 30 mM KCl). B-50 phosphorylation was determined as described in the legend of Fig. 6. Data are expressed as percent changes [mean $\pm$ SEM (bars)] above values for the respective untreated or TTX-treated controls. There was no significant difference between untreated (100 $\pm$ 3%) and TTX-treated (107 $\pm$ 7%) values. Significant differences are indicated: * p < 0.05, ** p < 0.01 ($n \geq 13$).

phosphates was determined in the presence of LiCl. 4-AP did not induce any changes in the accumulation of inositol phosphates after 5 or 30 min of incubation with 4-AP. After labeling of synaptosomes with [3 H]inositol, the amount of incorporation of the precursor into the membrane PPIs was determined by TLC and liquid scintillation counting. The mean lipid incorporation was found to be 10⁴ dpm/70 μ g of synaptosomal protein, of which 78% was incorporated into phosphatidylinositol, 16% into phosphatidylinositol 4-monophosphate, and 6% into phosphatidylinositol 4,5-bisphosphate. Stimulation of the 3 H-prelabeled synaptosomes with 100 μ M 4-AP for 1, 5, or 30 min in the presence of LiCl did not result in any significant changes in the accumulation of inositol phosphates.

DISCUSSION

In this article changes in [Ca^{2+}]_i and B-50 phosphorylation in synaptosomes evoked by 4-AP or K⁺ depolarization are compared. 4-AP induced a concentration-dependent elevation of [Ca^{2+}]_i within seconds, and 100 μ M 4-AP elevated the [Ca^{2+}]_i to almost the same level as evoked by 30 mM K⁺ (481 and 474 nM, respectively). Although both treatments elevated the [Ca^{2+}]_i to the same extent, the underlying mechanisms may differ. In contrast to 4-AP, K⁺ depolarization induced an initial large and transient elevation of [Ca^{2+}]_i. K⁺ depolarization in the presence of 4-AP resulted in an additional rise of [Ca^{2+}]_i. Moreover, the attenuation of the effect of 4-AP on [Ca^{2+}]_i by TTX is in sharp contrast with its lack of effect on the increase in [Ca^{2+}]_i evoked by 30 mM K⁺. These data are in line with recent observations by Tibbs et al. (1989a,b) in guinea pig cortical synaptosomes. These authors also showed that TTX inhibits 4-AP-induced endogenous glutamate re-

lease but not K⁺-induced release (Tibbs et al., 1989b).

The observation that the increase in [Ca^{2+}]_i due to 4-AP could be lowered by TTX indicates that synaptosomes are capable of actively controlling their [Ca^{2+}]_i, even in the presence of 4-AP. Therefore, the increase of [Ca^{2+}]_i due to 4-AP is most likely the net result of influx and extrusion and not a passive accumulation of Ca^{2+} .

Theoretically, the elevation of [Ca^{2+}]_i by 4-AP could be the result of Ca^{2+} entry or by (inositol phosphate-mediated) release of Ca^{2+} from intracellular stores. However, because TTX is a specific blocker of Na⁺ channels (Moore et al., 1967; Ulbricht, 1974; Catterall, 1980), Ca^{2+} entry through voltage-sensitive channels in the plasma membrane triggered by repetitive spiking seems to be the more likely explanation. This is supported by the observation that 4-AP does not affect PPI turnover in hippocampal slices or cortical synaptosomes.

It has been calculated that 30 mM K⁺ depolarizes the synaptosomes by ~ 45 mV (Suszkiw et al., 1989), which will activate VSCCs without the involvement of Na⁺ channel activation (Adam-Vizi and Ligeti, 1986). In contrast to this large depolarization, even 1 mM 4-AP has been shown to cause a depolarization of at most 8–15 mV (Agoston et al., 1983; Tibbs et al., 1989a; McMahon et al., 1989). Our observation that the elevation of [Ca^{2+}]_i by 4-AP is sensitive to TTX indicates the need for Na⁺ channel activity for its action and is in close agreement with recent data reported by others (Nicholls et al., 1989; Tibbs et al., 1989b) and our results that partial replacement of extracellular Na⁺ with choline abolishes the stimulation by 4-AP of B-50 phosphorylation. In the experiments reported here, TTX was able to attenuate the effect of 4-AP even when 4-AP was already present, a finding suggesting that Na⁺ channels are continuously active in the presence of 4-AP. These results suggest a different activation pattern of Ca^{2+} channels in synaptosomes by 4-AP compared with that observed during prolonged depolarization with 30 mM K⁺.

Differences in underlying mechanisms between K⁺ depolarization and 4-AP were also evident from the effects of 4-AP or K⁺ depolarization on B-50 phosphorylation (Heemskerk et al., 1990b). Depolarization with K⁺ caused a fast, large, transient increase in B-50 phosphorylation that returned to the control level in 5 min. In contrast, 4-AP induced a slower and smaller but sustained increase in B-50 phosphorylation (Heemskerk et al., 1990b). Comparing the results of B-50 phosphorylation with those of [Ca^{2+}]_i might give more information on how B-50 phosphorylation is stimulated by 4-AP. The increase in [Ca^{2+}]_i due to 4-AP is faster than the stimulation of B-50 phosphorylation, an observation suggesting that the increase in [Ca^{2+}]_i precedes the enhancement of phosphorylation. On the other hand, this also indicates that B-50 phosphorylation does not seem to be regulated by the overall

$[Ca^{2+}]_i$ per se. If this was the case, then B-50 phosphorylation would have been enhanced by depolarization and 4-AP to the same extent and with almost similar kinetics as shown for their effects on $[Ca^{2+}]_i$. This is obviously not the case. A correlation between B-50 phosphorylation and the overall $[Ca^{2+}]_i$ would not explain the transient nature of B-50 phosphorylation during depolarization, nor would it explain the additional effects of depolarization on $[Ca^{2+}]_i$ in the presence of 4-AP, whereas they were not additive on B-50 phosphorylation (Heemskerk et al., 1990b). Moreover, in experiments with the Ca^{2+} ionophore A23187, inducing a large elevation of $[Ca^{2+}]_i$, a relatively small increase in B-50 phosphorylation was detected after only 5 min (Dekker et al., 1990a).

Alternatively, B-50 phosphorylation might be stimulated by local Ca^{2+} influx through ion channels. The observations that the effects of depolarization or 4-AP on B-50 phosphorylation are abolished by lowering the extracellular Ca^{2+} concentration with EGTA (Heemskerk et al., 1990b) and attenuated by several ion channel antagonists are consistent with this hypothesis.

The fast and large increase in B-50 phosphorylation on depolarization could reflect a large Ca^{2+} influx through VSCCs opened immediately after the application of 30 mM K^+ . In contrast, 4-AP might cause a continuous enhancement of Ca^{2+} cycling across the plasma membrane, possibly by repetitive depolarization and repolarization, as pointed out already by Tibbs et al. (1989b). 4-AP is known to block transient K^+ channels, which are important in controlling excitability at voltages near the resting membrane potential, thereby preventing small depolarizing stimuli from reaching threshold and triggering action potentials (Rogawski, 1985; Storm, 1988). A block of such a presynaptic K^+ current by 4-AP would result in the removal of this dampening effect on the membrane potential. This might lead to larger fluctuations, to repetitive opening of voltage-sensitive Na^+ channels, and subsequently to enhanced Ca^{2+} influx. If this is the case, stimulation by 4-AP is probably more resembling electrical stimulation than is K^+ depolarization, as proposed by Tibbs et al. (1989b). The lack of effect of the combined Na^+ channel/ Ca^{2+} channel antagonist DPH on depolarization-evoked B-50 phosphorylation might be explained by DPH acting primarily on Na^+ channels in our preparation. Thus, it seems that in synaptosomes the stimulation of B-50 phosphorylation by 4-AP is voltage dependent rather than receptor mediated. Because TTX inhibits 4-AP-induced B-50 phosphorylation in hippocampal slices (Heemskerk et al., 1989a) and synaptosomes and endogenous glutamate release from synaptosomes (Tibbs et al., 1989b), our results further stress the involvement of calcium-induced B-50 phosphorylation in neurotransmitter release.

Taken together, our results indicate the possibility that B-50 phosphorylation in mature neurons is asso-

ciated with Ca^{2+} influx through voltage-sensitive channels. This change in influx of Ca^{2+} caused by 4-AP appears to stimulate PKC-mediated B-50 phosphorylation without receptor-mediated PPI hydrolysis. This indicates that PKC is activated either by local high $[Ca^{2+}]_i$ directly or by diacylglycerol, which is generated from non-PPI sources, for instance, phosphatidylcholine.

There is some evidence for more than one type of Ca^{2+} channel in the presynaptic nerve terminal with different activation/inactivation characteristics (Turner and Goldin, 1985; Lemos and Nowycky, 1989; Martínez-Serrano et al., 1989); thus, it remains to be shown which of these are involved in the effects of 4-AP and K^+ depolarization. The use of $CdCl_2$, DPH, and $LaCl_3$ as relatively unspecific Ca^{2+} channel antagonists does not allow any speculation yet about which Ca^{2+} channel subtype might be involved.

Although B-50 phosphorylation appears to play an important role in the regulation of noradrenaline release (Dekker et al., 1989b, 1990a,b), other aspects of the B-50 molecule, such as its calmodulin binding property, may also be of importance (Andreasen et al., 1983; Schrama et al., 1989; De Graan et al., 1990). Further investigation of a relationship among 4-AP-enhanced Ca^{2+} channel activity, B-50 phosphorylation, and calmodulin binding might produce further insight into the role of B-50 in the molecular mechanism of transmitter release.

Acknowledgment: The authors wish to acknowledge the artwork of Ed Kluis and Paul van der Most. Moreover, we would like to thank Dr. Beate Oestreicher for the generous gift of the anti-B-50 antibody, Dr. Reinhard Jork for his contribution to determining the 4-AP effects on PPI turnover, and Dr. David Nicholls for valuable discussions. This research was supported by CLEO-TNO grant A66 of the Dutch Epilepsy Foundation.

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