Journal of Neurochemistry
Raven Press, Ltd., New York

Phosphorylation of B-50 (GAP43) Is Correlated with Neurotransmitter Release in Rat Hippocampal Slices

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Abstract: Recent studies have demonstrated that phorbol diesters enhance the release of various neurotransmitters. It is generally accepted that activation of protein kinase C (PKC) is the mechanism by which phorbol diesters act on neurotransmitter release. The action of PKC in neurotransmitter release is very likely mediated by phosphorylation of substrate proteins localized in the presynaptic nerve terminal. An important presynaptic substrate of PKC is B-50. To investigate whether B-50 mediates the actions of PKC in neurotransmitter release, we have studied B-50 phosphorylation in intact rat hippocampal slices under conditions that stimulate or inhibit PKC and neurotransmitter release. The slices were labelled with [^{32}P]orthophosphate. After treatment, the slices were homogenized, B-50 was immunoprecipitated from the slice homogenate, and the incorporation of ^{32}P into B-50 was determined. Chemical depolarization (30 m*M* K^+) and the presence of phorbol diesters, conditions that stimulate neurotransmitter release, separately and in combination, also enhance B-50 phosphorylation. Polymyxin B, an inhibitor of PKC and neurotransmitter release, decreases concentration dependently the depolarization-induced stimulation of B-50 phosphorylation. The effects of depolarization are not detectable at low extracellular Ca^{2+} concentrations. It is concluded that in rat hippocampal slices B-50 may mediate the action of PKC in neurotransmitter release. **Key Words:** B-50/GAP43—Hippocampal slices—Neurotransmitter release—Protein kinase C—Protein phosphorylation. **Dekker L. V. et al.** Phosphorylation of B-50 (GAP43) is correlated with neurotransmitter release in rat hippocampal slices. *J. Neurochem.* **52,** 24–30 (1989).

Recent studies have demonstrated that phorbol diesters enhance the release of various neurotransmitters in a number of neuronal preparations (for review, see Kikkawa and Nishizuka, 1986). Stimulation of release occurs under basal conditions but is markedly enhanced when the tissue is depolarized electrically or chemically (Tanaka et al. 1984, 1986; Zurgil and Zisapel, 1985; Allgaier and Hertting, 1986; Allgaier et al., 1986; Versteeg and Florijn, 1986; Zurgil et al., 1986; Feuerstein et al., 1987; Nichols et al., 1987; Versteeg and Ulenkate, 1987).

It is generally accepted that activation of protein kinase C (PKC) is the mechanism by which phorbol diesters act on neurotransmitter release. This is substantiated by the fact that 4α-phorbol 12,13-didecanoate (4α-PDD), a phorbol diester that does not activate PKC (Castagna et al., 1982), does not stimulate release (Allgaier et al., 1986; Versteeg and Florijn, 1986) and that inhibitors of PKC such as polymyxin B and H-7 inhibit neurotransmitter release that is stimulated by electrical depolarization or phorbol esters (Allgaier and Hertting, 1986; Tanaka et al., 1986; Versteeg and Ulenkate, 1987).

The possible involvement of PKC in neurotransmitter release is not restricted to a certain type of transmitter or to a specific target organ or tissue preparation. It has been reported for norepinephrine (NE), serotonin (5-HT), acetylcholine (ACh) (Versteeg and Florijn, 1986), and glutamate (Malenka et al., 1987) in rat hippocampal slices; for 5-HT and NE in rabbit hippocampal slices (Allgaier et al., 1986; Feuerstein et al., 1987); for NE and dopamine (DA) in rat amygdala slices (Versteeg and Ulenkate, 1987); for ACh in guinea-pig caudate nucleus slices (Tanaka et al., 1986) and ileum

Received March 9, 1988; revised manuscript received June 6, 1988; accepted June 24, 1988.

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Abbreviations used: ACh, acetylcholine; [^{3}H]D-Asp, D-[2,3-^{3}H]aspartic acid; DA, dopamine; DAG, diacylglycerol; 5-HT, serotonin; LTP, long-term potentiation; NE, norepinephrine; PDB, 4β-phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PKC, protein kinase C; SAC, *Staphylococcus aureus* cell membrane(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

nerve ending preparations (Tanaka et al., 1984); for several of these transmitters in synaptosomes (Nichols et al., 1987), in cultured primary neurons (Zurgil et al., 1986) and in PC 12 cells (Pozzan et al., 1984).

The action of PKC is very likely mediated by phosphorylation of its substrate proteins. According to the mechanism of neurotransmitter release, these substrates must be localized in the presynaptic terminal. B-50 is an important presynaptic PKC substrate (Zwiers et al., 1980; Sörensen et al., 1981; Aloyo et al., 1983; Gispen et al., 1985). Recent data from several laboratories indicate that B-50 is identical to GAP43, F1, and pp46 (Gispen et al., 1986; Meiri et al., 1986; Benowitz and Routtenberg, 1987; Karns et al., 1987; Nielander et al., 1987; Rosenthal et al., 1987). In isolated synaptosomal plasma membranes B-50 is a PKC substrate because the phosphorylation of B-50 can be stimulated by phorbol diesters and by the addition of purified PKC (Eichberg et al., 1986; De Graan et al., 1988*a*, 1989) and can be inhibited by polymyxin B (De Graan et al., 1989). In rat hippocampal slices phorbol diesters enhance B-50 phosphorylation (De Graan et al., 1989), indicating that B-50 is a substrate of PKC in vivo.

To investigate whether B-50 mediates the actions of PKC in neurotransmitter release, we have studied B-50 phosphorylation in intact rat hippocampal slices under conditions that have been shown to stimulate or inhibit PKC and neurotransmitter release. Under a number of these conditions, we have made a direct comparison between B-50 phosphorylation and D-aspartate release. We show that chemical depolarization, phorbol diesters, and a combination thereof, enhance B-50 phosphorylation. The enhancement of B-50 phosphorylation can be suppressed by the PKC inhibitor polymyxin B. These phenomena are not observed at a low concentration of extracellular Ca^{2+}, a condition that prevents neurotransmitter release. Our results show a close correlation between B-50 phosphorylation and neurotransmitter release and provide new evidence for the involvement of PKC in the release process.

MATERIALS AND METHODS

Materials

Male Wistar rats (TNO, Zeist, The Netherlands) weighing 120–140 g were used in all experiments. *Staphylococcus aureus* cell membranes (SAC) were prepared from *S. aureus* cells according to Kronvall (1973). 4β-Phorbol 12,13-dibutyrate (PDB), 4α-PDD, and polymyxin B sulfate (7,800 U/mg) were purchased from Sigma (St. Louis, MO, U.S.A.). Radiolabelled orthophosphate ($^{32}P_i$; carrier-free) and D-[2,3-^{3}H]aspartic acid ([^{3}H]D-Asp; 25 Ci/mmol) were obtained from Amersham (Bucks, U.K.).

Preparation of hippocampal slices and ^{32}P-labelling

Hippocampal slices (400 μm) were prepared as described by Tielen et al. (1983). Three slices per tube were preincubated at 35°C in buffer A (124 m*M* NaCl, 5 m*M* KCl, 1.3 m*M* $MgSO_4$, 26 m*M* $NaHCO_3$, 10 m*M* D-Glucose, and 2 m*M* $CaCl_2$; pH 7.4; continuously gassed with 5% CO_2/95% O_2) for 30–50 min and subsequently incubated with $^{32}P_i$ according to De Graan et al. (1989).

Treatment schedule

Slices were subjected to a treatment schedule that was adapted from Versteeg and Florijn (1986). At t = 0, the ^{32}P labelling was started in an incubation volume of 900 μl. One hundred microliters of polymyxin B or control buffer (buffer A) was added at t = 85 min. One hundred microliters of PDB or control buffer was added at t = 90 min. At t = 120 min, medium was replaced by 1 ml 30 m*M* K^+ buffer (buffer A with 99 m*M* NaCl and 30 m*M* KCl) or control buffer without $^{32}P_i$. Polymyxin B or PDB was continuously present in this buffer, depending on its presence before the buffer change. Low Ca^{2+} experiments were performed with 1 m*M* EGTA instead of 2 m*M* $CaCl_2$ present from t = 120–130 min. At t = 130 min (unless otherwise indicated; see Results section) the slices were inactivated and homogenized according to De Graan et al., 1989. In several experiments the inactive phorbol diester 4α-PDD was used as a specificity control. In accordance with published data (Versteeg and Florijn, 1986; Versteeg and Ulenkate, 1987; De Graan et al., 1988*a*, 1989). 4α-PDD was ineffective for stimulation of B-50 phosphorylation and neurotransmitter release.

Preparation of slice homogenates

After incubation, the slices were washed twice in ice-cold buffer A containing 1.2 m*M* Na_2HPO_4, 100 m*M* NaF, 10 m*M* EDTA, and 5 m*M* EGTA (pH 7.4). Slices were collected and homogenized in 100 μl of double distilled water containing 100 m*M* NaF, 10 m*M* EDTA, and 5 m*M* EGTA (pH 7.4) by 10 up-and-down strokes in a Potter–Elvehjem tube with a Teflon pestle (clearance 50 μm). Immediately after homogenization, 80 μl of the homogenate was added to 40 μl three-times concentrated stopmix [final concentration 62.5 m*M* Tris-HCl pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue], mixed well, 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.

Determination of ^{32}P labelling of B-50

^{32}P Labelling of B-50 was determined by quantitative immunoprecipitation of B-50 from the slice homogenate as described previously (De Graan et al., 1989). Incubations were carried out in 400 μl buffer B [200 m*M* NaCl, 10 m*M* EDTA, 10 m*M* NaH_2PO_4, 0.5% (vol/vol) Nonidet P-40, and 0.1% (wt/vol) SDS; final pH 7.4] at 4°C for 18 h. Anti-B-50 antiserum 8502 raised according to Oestreicher et al. (1983), was present in a final dilution of 1:200. Routinely, 20 μg of homogenate protein was subjected to immunoprecipitation. SACs containing protein A were used to precipitate the immunoglobulins. Precipitates were collected by centrifugation and washed once in 200 μl buffer B. Finally the pellets were resuspended in 40 μl stopmix and boiled for 10 min. Total immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (De Graan et al., 1989). Quantification of ^{32}P incorporation into B-50 was performed by densitometric scanning of the autoradiogram (De Graan et al., 1989).

Protein determination and TCA precipitation

Proteins were measured according to Bradford (1976) using bovine serum albumin as standard. For TCA precipitation 20 μg of homogenate protein was spotted on 3MM Whatman

filter paper. Filters were washed with TCA, ethanol, and acetone (to remove phospholipids) according to Corbin and Reimann (1974) and counted in a Packard Model 2000CA liquid scintillation counter.

Expression of data

To calculate the specific phosphorylation of B-50 in each slice homogenate, incorporation of ^{32}P into B-50 as measured by densitometric scanning was divided by the incorporation of ^{32}P into TCA precipitable protein. Values of treated samples were expressed as percentage of control in each experiment, to permit the results of several experiments to be combined.

Neurotransmitter release studies

[^{3}H]D-Asp release from rat hippocampal slices was measured in a continuous superfusion system (Schoffelmeer et al., 1981; Versteeg and Ulenkate, 1987). Briefly, hippocampal slices prelabelled with [^{3}H]D-Asp (15 min; 5 μCi) were superfused with Krebs–Ringer bicarbonate buffer (121 m*M* NaCl, 1.87 m*M* KCl, 1.17 m*M* K_2HPO_4, 1.17 m*M* $MgSO_4$, 1.22 m*M* $CaCl_2$, 20 m*M* $NaHCO_3$, and 11.1 m*M* D-glucose; pH 7.4; continuously gassed with 5% CO_2/95% O_2) containing 10^{-6} *M* dihydrokainic acid at 37°C. After a 40-min superfusion period, three 15 min fractions were collected. In order to evoke [^{3}H]D-Asp release, the K^+ concentration of the buffer was raised to 30 m*M* during the first 10 min of the second 15 min period. (The Na^+ concentration was reduced to keep the buffer isotonic.) PDB was added to the superfusion medium 20 min before the beginning of the collection of the first fraction. For experiments at low Ca^{2+} conditions Ca^{2+} was omitted from the buffer and replaced by EGTA (1 m*M*). After the third fraction was collected, the radioactivity remaining in the tissue was extracted with 0.1 *M* HCl. Release of [^{3}H]D-Asp was calculated and expressed as a fractional rate as described previously (Versteeg and Ulenkate, 1987).

RESULTS

Effect of 30 m*M* K^+ depolarization

Incubation of rat hippocampal slices in 30 m*M* K^+ buffer for 10 min significantly enhanced B-50 phosphorylation from 100 ± 5% in control slices to 150 ± 5% in treated slices (Fig. 1A). No changes occurred in the incorporation of ^{32}P into TCA precipitable protein.

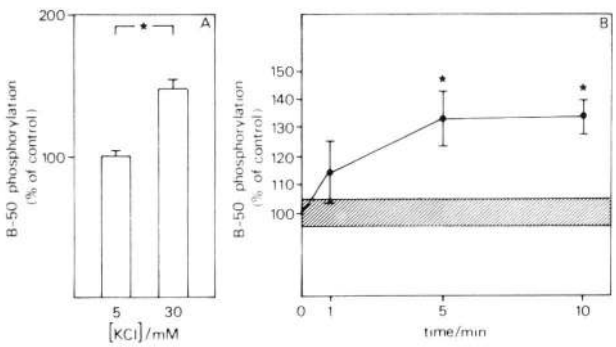

FIG. 1. A: Effect of 30 m*M* K^+ on B-50 phosphorylation in hippocampal slices. Slices were treated with 30 m*M* K^+ for 10 min. **B:** Time dependence of 30 m*M* K^+ stimulation of B-50 phosphorylation. Cross-hatched area represents B-50 phosphorylation in control slices. *2p < 0.05 [Student's *t* test: n = 21 (A); n = 6 (B)] when compared with 5 m*M* K^+-treated slices. Bars indicate SEM.

The 30 m*M* K^+-induced increase in B-50 phosphorylation is time dependent (Fig. 1B). After 1 min, B-50 phosphorylation was slightly but not significantly enhanced; it reached a plateau after 5 min. At 5 m*M* K^+, no time-dependent changes occurred in either B-50 phosphorylation (Fig. 1B) or in the incorporation of ^{32}P into total TCA precipitable protein.

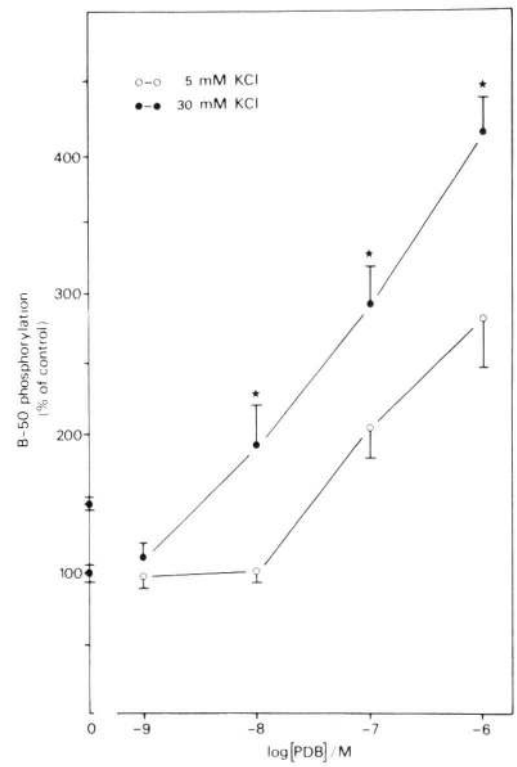

FIG. 2. Effect of different concentrations of PDB on B-50 phosphorylation in control (○) and 30 m*M* K^+-depolarized (●) hippocampal slices. Values were obtained in nine independent experiments and are expressed as percentage of control B-50 phosphorylation (5 m*M* KCl; no PDB), a value determined separately in each experiment. *2p < 0.05 (Student's *t* test) when compared with 5 m*M* K^+-treated slices. Bars indicate SEM.

Effect of PDB in control and 30 m*M* K^+-depolarized slices

Treatment of rat hippocampal slices with PDB in control buffer enhanced B-50 phosphorylation in a concentration-dependent way (Fig. 2). The PDB treatment lasted for 40 min, including 30 min in the presence of $^{32}P_i$ and 10 min after buffer change. Under these conditions, PDB does not affect the incorporation of ^{32}P into TCA precipitable protein. The lowest concentration producing a significant stimulation of B-50 phosphorylation was 10^{-7} *M* PDB (200 ± 10%). At 10^{-9} *M* PDB, a slight decrease in B-50 phosphorylation could be observed. This decrease was not statistically

significant, but was consistently observed in all experiments.

Incubation of the slices in 30 m*M* K^+ buffer during the last 10 min of the PDB treatment increased B-50 phosphorylation significantly compared with slices treated with 5 m*M* K^+ and PDB at 10^{-8}, 10^{-7}, and 10^{-6} *M* (Fig. 2). PDB does not affect ^{32}P incorporation in TCA precipitable protein of the slices at 30 m*M* K^+; 30 m*M* K^+ treatment of the slices did not alter total protein labelling at any of the PDB concentrations tested. The minimal PDB dose producing a significant increase in B-50 phosphorylation at 30 m*M* K^+ is 10^{-8} M (190 ± 30%). Under these conditions, 10^{-9} *M* PDB again induced a slight decrease in B-50 phosphorylation.

The 30 m*M* K^+-induced increase in PDB-stimulated B-50 phosphorylation is time dependent; it has the same characteristics as the 30 m*M* K^+-induced increase in B-50 phosphorylation in the absence of PDB. At 5 m*M* K^+, no time-dependent changes occurred in PDB-induced B-50 phosphorylation.

Effect of polymyxin B on 30 m*M* K^+-induced increase in B-50 phosphorylation

The increase in B-50 phosphorylation induced by incubating the slices in 30 m*M* K^+ buffer for 10 min (Fig. 1A) was not observed when the slices were treated with 1,000 U/ml polymyxin B (Fig. 3A). Total polymyxin B treatment lasted for 45 min, including a 35-min pretreatment in the presence of $^{32}P_i$ and a 10-min treatment during the 30 m*M* K^+ depolarization. The inhibitory effect of polymyxin B is concentration dependent (Fig. 3B). It did not occur at 10 U/ml and was almost maximal at 100 U/ml.

Under basal conditions, treatment of the slices with 1,000 U/ml polymyxin B for 45 min did not significantly inhibit B-50 phosphorylation (Fig. 3A). No changes in the incorporation of ^{32}P into total TCA-precipitable protein occurred upon treatment with polymyxin B at either 5 m*M* or 30 m*M* K^+.

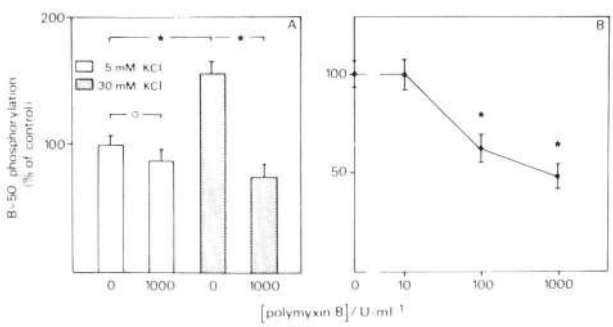

FIG. 3. A: Effect of 1,000 U/ml polymyxin B on B-50 phosphorylation in control (open columns) and depolarized slices (cross-hatched columns). **B:** Concentration dependence of the inhibitory effect of polymyxin B on B-50 phosphorylation in 30 m*M* K^+-depolarized slices. Values were obtained in four independent experiments and are expressed as percentage of control B-50 phosphorylation (no polymyxin B), a value determined separately in each experiment. *$2p < 0.001$ (Student's *t* test); °not significantly different. Bars indicate SEM.

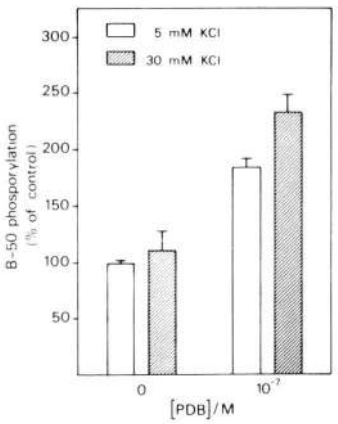

FIG. 4. Effect of 10^{-7} *M* PDB on B-50 phosphorylation in 5 and 30 m*M* K^+-treated slices at a low concentration of extracellular Ca^{2+} (1 m*M* EGTA). See Materials and Methods. Data represent mean ± SEM of six observations obtained in two independent experiments, and are expressed as percentage of control B-50 phosphorylation (1 m*M* EGTA, 5 m*M* KCl; and no PDB), a value that was determined separately in each experiment. Bars indicate SEM.

Effects of 30 m*M* K^+ are dependent on extracellular Ca^{2+}

Slices were treated with 30 m*M* K^+ buffer (see the first section in Results) except that 1 m*M* EGTA instead of 2 m*M* $CaCl_2$ was present during the 30 m*M* K^+ treatment. Under these conditions, B-50 phosphorylation is not stimulated by 30 m*M* K^+ treatment (Fig. 4; for comparison see Fig. 1A). One millimolar EGTA itself reduces B-50 phosphorylation to 57 ± 2% as compared with 2 m*M* $CaCl_2$ treated slices (not shown). This reduction is partly due to an enhancement of ^{32}P incorporation into the total TCA precipitable protein of the slices.

PDB stimulated B-50 phosphorylation at both 2 m*M* $CaCl_2$ and 1 m*M* EGTA by about twofold as compared with the corresponding control values (Fig. 4; for comparison see Fig. 2). The effect of 30 m*M* K^+ treatment on PDB-induced B-50 phosphorylation was smaller at 1 m*M* EGTA than at 2 m*M* $CaCl_2$, but it was still present (Fig. 4).

Parallel with our experiments on B-50 phosphorylation, we have studied the effect of PDB on 30 m*M* K^+-evoked neurotransmitter release under low and normal Ca^{2+} conditions (Table 1). Under normal Ca^{2+} conditions, 10^{-7} *M* PDB enhances the 30 m*M* K^+-evoked release of [^{3}H]D-Asp almost twofold. At low extracellular concentrations of Ca^{2+} (1 m*M* EGTA), [^{3}H]D-Asp release was reduced to approximately 20% of control levels. PDB at 10^{-7} *M* has no significant effect on [^{3}H]D-Asp release under these conditions. No effects on basal release of [^{3}H]D-Asp are associated with PDB or changes in the extracellular Ca^{2+} concentration.

TABLE 1. *Effect of 10^{-7} M PDB on 30 mM K^+-evoked release of [^{3}H]D-Asp from rat hippocampal slices at 1.22 mM $CaCl_2$ and 1 mM EGTA*

PDB concentration (M)	[^{3}H]D-Asp release in excess of basal release	
	1.22 mM $CaCl_2$	1 mM EGTA
0	3.49 ± 0.13 (100 ± 4)	0.67 ± 0.18 (19 ± 5)
10^{-7}	6.08 ± 0.26 (195 ± 7)	0.73 ± 0.17 (21 ± 5)

Values are expressed as the fractional rate of [^{3}H]D-Asp release in excess of basal release (mean ± SEM of six to seven observations obtained in two separate experiments).

Values in parentheses represent data calculated as the percentage of control ± SEM.

DISCUSSION

In the present article, we have provided evidence that conditions known to stimulate or inhibit neurotransmitter release also stimulate or inhibit the degree of B-50 phosphorylation: (1) Both B-50 phosphorylation and neurotransmitter release can be induced by 30 mM K^+ stimulation (Fig. 1A; Table 1). (2) PDB enhances both K^+-evoked neurotransmitter release (Table 1) and K^+-evoked B-50 phosphorylation (Fig. 2). The minimal effective dose and EC_{50} for PDB-induced B-50 phosphorylation are in close agreement with those reported for [^{3}H]NE, [^{14}C]ACh and [^{3}H]5-HT release in the hippocampus (Versteeg and Florijn, 1986). Under basal conditions, B-50 phosphorylation is stimulated by PDB (Fig. 2), whereas [^{3}H]D-Asp release is not affected. The effects of PDB on basal neurotransmitter release seem to depend on the brain region and transmitters studied. In rat hippocampal slices basal release of [^{3}H]NE and [^{14}C]ACh, but not that of [^{3}H]5-HT, is enhanced by PDB (Versteeg and Florijn, 1986). In rat amygdala slices PDB enhances the basal release of [^{3}H]NE and [^{3}H]DA (Versteeg and Ulenkate, 1987). (3) The depolarization-induced phosphorylation of B-50 can be attenuated by polymyxin B (Fig. 3). Under similar conditions, polymyxin B has been shown to attenuate depolarization-induced transmitter release from rabbit hippocampal slices (Allgaier and Hertting, 1986) and from rat amygdala slices (Versteeg and Ulenkate, 1987). (4) The stimulatory effects of 30 mM K^+ depolarization on B-50 phosphorylation in the presence or absence of PDB do not occur when neurotransmitter release is blocked by a low extracellular Ca^{2+} concentration (Fig. 4; Table 1). At such a concentration, PDB still stimulates B-50 phosphorylation (Fig. 4), whereas [^{3}H]D-Asp release is not affected (Table 1). This discrepancy may be due to an incomplete chelation of Ca^{2+} by EGTA in the tube incubation system used for the phosphorylation studies. Apart from indicating that B-50 phosphorylation and neurotransmitter release are closely correlated, our findings also provide new evidence that B-50 is a PKC substrate in intact neuronal preparations because B-50 phosphorylation can be enhanced by PDB (Fig. 2) and inhibited by polymyxin B (Fig. 3).

The stimulation of B-50 phosphorylation in response to the depolarizing stimuli is not due to a general stimulation of ^{32}P incorporation in the slice phosphoproteins, because (1) total TCA precipitable protein labelling of the slices does not change upon 30 mM K^+ treatment; (2) no changes occur in the degree of phosphorylation of individual protein bands after 30 mM K^+ treatment as determined by SDS-PAGE and autoradiography (not shown), indicating that the specific activity of the ^{32}P-labelled ATP pool does not change after depolarization; and (3) the results are expressed as the ratio of the incorporation of ^{32}P into B-50 to the incorporation of ^{32}P into total TCA precipitable protein and therefore represent specific phosphorylation of B-50 in the slices.

Several publications have reported on the effects of depolarization on protein phosphorylation in various experimental designs. In cultured primary neurons electrical and chemical depolarization enhance the phosphorylation of a 43-kilodalton protein (Zurgil and Zisapel, 1983) that has the same isoelectric point as tubulin on two-dimensional gels (Zurgil and Zisapel, 1984). Phosphorylation of this protein can be stimulated by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate in the presence but not in the absence of extracellular Ca^{2+} (Zurgil et al., 1986). Similarly, Rodnight and Perrett (1986) and Dunkley and Robinson (1986) have reported that in rat cortex synaptosomes phosphorylation of a 45-kilodalton protein is enhanced upon depolarization. High K^+ concentrations have also been reported to induce phosphorylation of a second PKC substrate in synaptosomes, the 87-kilodalton protein (Wu et al., 1982; Rodnight and Perrett, 1986).

The present data agree closely with recent hypotheses on the involvement of PKC in the mechanism of long-term potentiation (LTP). It has been shown that activation of PKC by phorbol diesters can induce LTP-like phenomena in rat hippocampal slices (Routtenberg et al., 1986; Malenka et al., 1987; De Graan et al., 1988*b*). Injection of PKC in CA1 hippocampal pyramidal neurons elicits features of LTP (Hu et al., 1987), and inhibition of PKC by polymyxin B prevents the maintenance of LTP (Lovinger et al., 1987; Reymann et al., 1988). One of the mechanisms underlying LTP is an enhanced release of neurotransmitter at the presynaptic site. PKC may be involved in LTP at the presynaptic site with B-50 as a mediator of its action because LTP-like phenomena in the hippocampus are correlated with B-50/F1 phosphorylation both in vitro (B-50; De Graan et al., 1988*b*) and in vivo (F1; Lovinger et al., 1985).

The observed changes in B-50 phosphorylation most likely occur at the presynaptic site. Earlier subcellular fractionation studies (Sörensen et al., 1981) and electron microscopic studies in the hippocampus (Gispen et al., 1985) have shown that B-50 is a presynaptic protein. Recent experiments in rat cortex synapto-

somes, which are presynaptic nerve endings, have revealed that the same changes in B-50 phosphorylation occur upon 30 m*M* K^+ stimulation (unpublished results), a finding that further indicates that a presynaptic mechanism is involved.

The present results are consistent with the hypothesis that 30 m*M* K^+ depolarization and phorbol diesters activate B-50 phosphorylation via the same PKC-mediated pathway. This would imply a depolarization-dependent generation of diacylglycerol (DAG), a mechanism that has emerged from various experimental designs. Breakdown of phosphatidylinositol (implicating generation of DAG) has been observed in synaptosomes after electrical (Pickard and Hawthorne, 1978) and high-K^+ stimulation (Wei and Wang, 1987). This breakdown most likely occurs through the activation of phospholipase C by incoming Ca^{2+} because the effect on phosphatidylinositol breakdown can be mimicked by the calcium ionophore A23187 (Griffin and Hawthorne, 1978; Wei and Wang, 1987). However, more direct effects of Ca^{2+} on PKC activity (with or without generation of DAG) cannot be excluded. Our results show a close correlation between B-50 phosphorylation and neurotransmitter release. A causal relationship between PKC activation and B-50 phosphorylation and neurotransmitter release remains to be established.

Acknowledgment: The authors wish to acknowledge the enthusiastic experimental assistance of Jac Hens and Henk Spierenburg and the artwork of Inge Hassink and Ed Kluis. LVD is supported by a MEDIGON grant (900-548-082) from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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