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Evidence for a relationship between B-50 (GAP-43) and [^{3}H]noradrenaline release in rat brain synaptosomes

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Phosphorylation of the neuron-specific substrate of protein kinase C (PKC), B-50 (GAP-43), was studied parallel with noradrenaline release in rat brain synaptosomes. Both could be evoked by treating the synaptosomes with high K^+ or veratridine. Phorbol 12,13-dibutyrate enhanced depolarization-induced B-50 phosphorylation and noradrenaline release. To investigate the involvement of PKC-mediated B-50 phosphorylation in noradrenaline release, we applied a variety of kinase inhibitors. Prior to measuring the effects of these inhibitors in intact synaptosomes, we determined their effectivity and specificity in a membrane phosphorylation assay. H-7 most specifically inhibited PKC-dependent phosphorylation, whereas calmidazolium inhibited calmodulin-dependent phosphorylation. Polymyxin B affected both protein kinase systems. Only polymyxin B effectively inhibited noradrenaline release in the intact synaptosomes. We conclude that PKC as well as calmodulin-dependent processes are important for the release event. Data are discussed in view of the presumed function of B-50 as a calmodulin-binding protein.

B-50 (GAP-43); Protein kinase C; Synaptosome; Transmitter release; Noradrenaline

1. Introduction

Protein B-50 is a well-characterized neuron-specific substrate of protein kinase C (PKC) that is localized at the plasma membrane of presynaptic terminals (Zwiers et al., 1980; Sörensen et al., 1981; Kristjansson et al., 1982; Aloyo et al., 1983; Gispen et al., 1985; De Graan et al., 1988; 1989; Van Lookeren Campagne et al., 1989). B-50 is identical to GAP-43, a member of the growth-associated proteins, to P-57, a calmodulin-binding protein, and to F_1, a protein implicated in long-term potentiation (for review see Skene, 1989).

In hippocampal slices, a close correlation exists between the phosphorylation of B-50 and neurotransmitter release. K^+-induced depolarization of rat hippocampal slices enhances both B-50 phosphorylation and transmitter release in a Ca^{2+}-dependent way (Dekker et al., 1989c). Both processes are stimulated by treating the slices with phorbol ester, the degree of stimulation depending on the depolarization state (Nishizuka, 1984; Allgaier et al., 1986; Wakade et al., 1986; Versteeg and Florijn, 1987; Versteeg and Ulenkate, 1987; Dekker et al., 1989c). Depolarization-induced B-50 phosphorylation and depolarization-induced transmitter release can be inhibited by polymyxin B, a compound known as an inhibitor of PKC

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(Mazzei et al., 1982; Allgaier and Hertting, 1986; Versteeg and Ulenkate, 1987; Dekker et al., 1989c). Recently, we have obtained evidence for a crucial involvement of B-50 in transmitter release, as antibodies to B-50 completely inhibit Ca^{2+}-dependent release of noradrenaline (NA) from rat cortical synaptosomes (Dekker et al., 1989b).

Detailed analysis of K^+-induced B-50 phosphorylation in rat cortical synaptosomes (Dekker et al., 1989a) revealed that the stimulation of B-50 phosphorylation is transient, reaching a maximum within seconds and declining towards control levels within 1-2 min. As K^+-induced phosphorylation of B-50 is not detectable under low extracellular Ca^{2+} conditions and as the Ca^{2+} ionophore A23187 enhances B-50 phosphorylation, it is likely that influx of extracellular Ca^{2+} underlies the effect of K^+ depolarization on B-50 phosphorylation (Dekker et al., 1989a). This suggestion is supported by recent findings of Heemskerk et al. (1989), which show a correlation in synaptosomes between the levels of intracellular Ca^{2+} and the degree of phosphorylation of B-50. Depolarization-induced B-50 phosphorylation is mediated by PKC as it can be inhibited by several PKC inhibitors (Dekker et al., 1989a).

The transient nature of the K^+-induced increase in B-50 phosphorylation found in synaptosomes is in contrast to the persistent increase in rat hippocampal slices (Dekker et al., 1989a,c). The cause of the difference in these preparations is unknown. It may be generated by interference of glia (K^+ buffering) or neuronal interactions in hippocampal slices. However, the transient increase in B-50 phosphorylation in synaptosomes seems to correlate very well with the time dependency of transmitter release induced by high K^+ treatment (Cotman et al., 1976; Nicholls et al., 1987). In the present study, we investigated in detail B-50 phosphorylation and transmitter release in the isolated presynaptic terminal, using a pharmacological approach. [^{3}H]NA release and B-50 phosphorylation could be induced in rat brain synaptosomes by high K^+ and by the secretagogue veratridine. To establish the involvement of PKC in both processes, we applied phorbol esters and a variety of kinase inhibitors, the specificity of which towards different kinases was determined in rat brain synaptosomal plasma membranes (SPM).

2. Materials and methods

2.1. Materials

Male Wistar rats (TNO, Zeist, The Netherlands) of 120-150 g were used. Veratridine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), polymyxin B (7800 IU/mg), phorbol 12,13-dibutyrate (PDB), calmodulin and calmidazolium were purchased from Sigma (St. Louis, MO, U.S.A.), staurosporin from Fluka (Buchs, Switzerland) and ^{32}P-labelled orthophosphate ($^{32}P_i$; carrier free), [γ-^{32}P]-labelled ATP ([γ-^{32}P]ATP; 3000 Ci/mmol) and L-[7,8-^{3}H]-labelled Na ([^{3}H]NA; 34 Ci/mmol) from Amersham (U.K.). Staurosporin (1 mM), PDB (10 mM) and calmidazolium (50 mM) were dissolved as stock solutions in 100% dimethylsulfoxide (DMSO). DMSO did not affect either B-50 phosphorylation or transmitter release in the dilution used. H-7, W-7, calmodulin and polymyxin B were dissolved in buffer. Pansorbin was from Calbiochem (La Jolla, CA, U.S.A.). Anti-B-50 antiserum was prepared as described by Oestreicher et al. (1983).

2.2. ^{32}P labelling and treatment of synaptosomes

Synaptosomes were prepared from rat cortex or hippocampus as described by De Langen et al. (1979). In some experiments, synaptosomes isolated according to Dunkley et al. (1988) were used. In none of the experiments could a difference be detected between the synaptosome preparations. The synaptosomal preparation was diluted in buffer A containing (mM): NaCl 124; KCl 5; $MgSO_4$ 1.3; $CaCl_2$ 2; glucose 10; $NaHCO_3$ 26; saturated with CO_2 and O_2 (5 : 95%); pH 7.4 in a concentration of 1 mg protein/ml and labelled with 2 μCi/μg protein $^{32}P_i$ at 34°C for 50 min. At the end of this labelling period, synaptosomes were divided in 10-μl aliquots (10 μg protein) and the incubation was started at 34°C (t = 0). Total incubation time was 10 min for each sample. High

K^+ buffer (buffer A in which K^+ was substituted for Na^+ to keep the buffer isotonic) was added at t = 9.45 min. Veratridine was added at the time points indicated in the figures. Phorbol ester was added at t = 5 min and inhibitors were added at t = 0. The final incubation volume was 20 μl. The incubation was stopped after 10 min by the addition of 10 μl denaturing solution (buffer B; final concentrations: 62.5 mM Tris-HCl, pH 6.5; 2% sodium dodecyl sulfate (SDS); 10% glycerol; 5% 2-mercaptoethanol; 0.001% bromophenol blue).

2.3. Quantification of ^{32}P incorporation in B-50

Twenty microliters of SDS-solubilized synaptosomes (containing 6.7 μg total protein) were incubated at room temperature with the immunoadsorbent pansorbin (final concentration 0.5%) in 350 μl buffer C containing (mM): NaCl 200; EDTA 10; NaH_2PO_4 10; 0.5% nonidet P-40; pH 7.2. After 30 min, samples were centrifuged for 20 min at 5000 × *g*. Three hundred μl of the supernatant were added to 100 μl buffer C containing anti-B-50 antiserum (final dilution 1 : 200). Subsequently, each sample was submitted to B-50 immunoprecipitation as described by De Graan et al. (1989). Immunoprecipitates were analyzed by 11% SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Kristjansson et al., 1982). B-50 phosphorylation was quantified by densitometric scanning of the autoradiogram.

2.4. Synaptosomal plasma membrane phosphorylation

Subcellular fractionation and phosphorylation were performed according to Kristjansson et al. (1982). The phosphorylation reaction mixture (final volume 25 μl) contained 10 μg SPM protein, 7.5 μM ATP and 1 μCi [γ-^{32}P]ATP in buffer D containing (mM): Tris/HCl 10; $MgCl_2$ 10; $CaCl_2$ 0.1; pH 7.4. After a preincubation at 30 °C for 5 min, the phosphorylation reaction was started by the addition of ATP. The reaction was stopped after 15 s by adding 12.5 μl buffer B. All compounds tested were present at the start of the preincubation. Proteins were separated by 11% SDS-PAGE. Protein phosphorylation was determined by autoradiography of the gel and quantified by densitometric scanning of the autoradiogram (Kristjansson et al., 1982).

2.5. Neurotransmitter release

[^{3}H]NA release from rat hippocampal synaptosomes (isolated as described in De Langen et al., 1979) was measured in a continuous superfusion system (De Langen et al., 1979; Schoffelmeer et al., 1981; Versteeg and Ulenkate, 1987). Briefly, synaptosomes prelabelled with [^{3}H]NA (15 min; 5 μCi; De Langen et al., 1979) were superfused with Krebs-Ringer bicarbonate buffer containing (mM) NaCl 121; KCl 1.87; K_2HPO_4 1.17; $MgSO_4$ 1.17; $CaCl_2$ 1.22; $NaHCO_3$ 20; glucose 11.1; pH 7.4, continuously gassed with $CO_2 : O_2$, 5 : 95%, at 37 °C. After a 40-min superfusion period, three 15-min fractions were collected. In order to evoke [^{3}H]NA release, the K^+ concentration of the buffer was raised to 15 mM during the first 7.5 min of the second 15-min period (the Na^+ concentration was reduced to keep the buffer isotonic) or a 7.5-min superfusion with different veratridine concentrations was performed. PDB was added to the superfusion medium 20 min before (t = −20) the beginning of the collection of the first fraction. Inhibitors were added at t = −25 min. After the third fraction was collected, the radioactivity remaining in the tissue was extracted with 0.1 M HCl. Release of [^{3}H]NA was calculated and expressed as a fractional rate as described previously (Versteeg and Ulenkate, 1987).

2.6. Protein determination

Proteins were determined according to Bradford (1976) with bovine serum albumin as a standard.

3. Results

3.1. Effects of high K^+, veratridine and PDB on B-50 phosphorylation and [^{3}H]NA release

K^+ depolarization of synaptosomes enhanced both the phosphorylation of B-50 and the efflux of

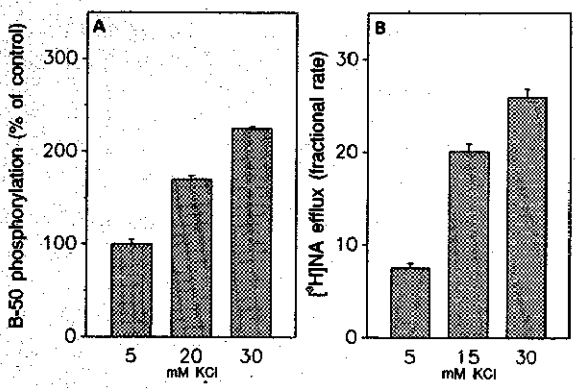

Fig. 1. Concentration dependency of the effect of K^+ on phosphorylation of B-50 (A) and release of [^{3}H]NA (B) in synaptosomes. Each value is the mean ± S.E.M. (n = 3 (A), n = 10-43 (B); data in panel A have been adapted from Dekker et al., 1989a).

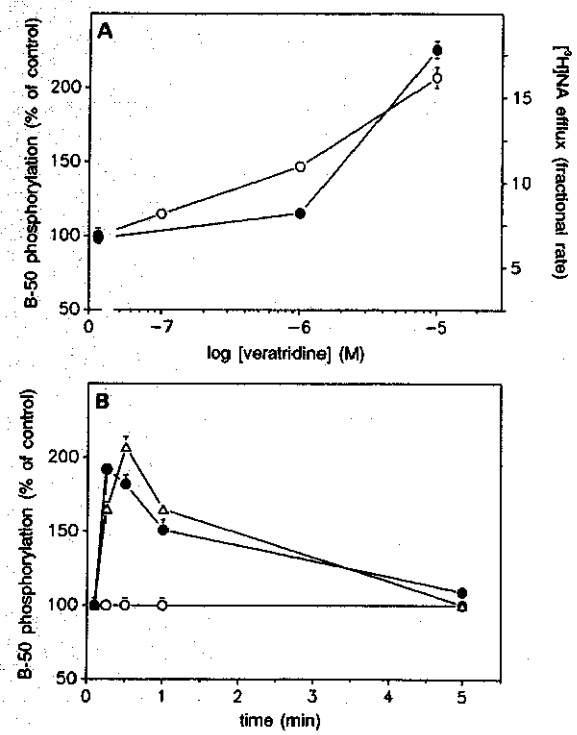

Fig. 2. (A) Concentration dependency of the effect of veratridine on phosphorylation of B-50 (filled circles) and on release of [^{3}H]NA (open circles) and (B) time dependency of the effect of 10^{-5} M veratridine (open triangles) and 10^{-4} M veratridine (filled circles) on phosphorylation of B-50 in synaptosomes. Open circles represent basal levels of B-50 phosphorylation at the various time points. Values are expressed as the means ± S.E.M. (B-50 phosphorylation, n = 3-4; [^{3}H]NA release, n = 8-16).

[^{3}H]NA in a concentration-dependent manner (figs. 1A,B). Treatment of the synaptosomes with the secretagogue veratridine also enhanced B-50 phosphorylation and [^{3}H]NA efflux in a concentration-dependent manner (fig. 2A). The effect of veratridine on both processes became apparent at concentrations of 10^{-6} M. As shown in fig. 2B, veratridine-induced phosphorylation of B-50 was transient. After a fast initial rise, it returned to basal levels within 5 min of incubation. The time dependency profile slightly differed for 10^{-4} M and 10^{-5} M veratridine (fig. 2B). Maximal stimulation of B-50 phosphorylation was obtained at 30 s of incubation with 10^{-5} M veratridine (207 ± 7% of control).

The effect of PDB on B-50 phosphorylation and [^{3}H]NA efflux is shown in fig. 3. Under basal conditions, PDB enhanced B-50 phosphorylation (200% of control), but had only small effects on [^{3}H]NA efflux (fractional rate enhanced from 6.08 ± 0.22 to 7.45 ± 0.41). Depolarization-induced efflux of [^{3}H]NA and phosphorylation of B-50 were enhanced to the same degree by PDB (380 and 359% of non-treated synaptosomes, respectively).

3.2. Specificity of PKC and calmodulin antagonists in synaptosomal plasma membranes

Before measurement of K^+-induced B-50 phosphorylation and release in response to various protein kinase inhibitors, the specificity and effectivity of these inhibitors were tested in a SPM phosphorylation assay. Phosphorylation of three phosphoproteins was quantified; the B-50 protein (a substrate of PKC), the 50 kDa subunit of calmodulin-dependent kinase type II (calmodulin-dependent autophosphorylation; De Graan et al., 1987) and the 52 kDa protein (of which the kinase has not yet been identified). The 50 kDa protein was only phosphorylated after addition of exogenous calmodulin (fig. 4). In the presence of exogenous calmodulin, the three proteins that have been quantified were main phosphoproteins in the SPM fraction (fig. 4). H-7 (Hidaka et al., 1984) inhibited the phosphorylation of B-50 in a concentration-dependent manner (figs. 4, 5A). At 10^{-4} M, phosphorylation of the 52 kDa protein was slightly inhibited (figs. 4, 5B). phosphorylation of

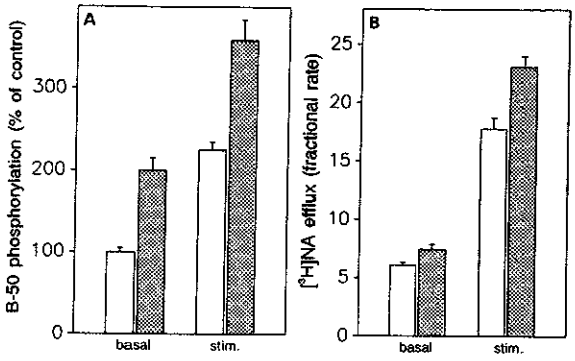

Fig. 3. Effects of 10^{-7} M PDB (dotted bars) on basal (basal) and K^+-induced (stim.) phosphorylation of B-50 (A) and release of [^{3}H]NA (B) in synaptosomes. Synaptosomes have been depolarized with 30 mM K^+ (A) or with 15 mM K^+ (B). Values are expressed as means ± S.E.M. (B-50 phosphorylation, n = 6; [^{3}H]NA release, n = 12).

the 50 kDa protein was not affected by H-7 (figs. 4, 5C). Staurosporin, 10^{-6} M (Tamaoki et al., 1986) inhibited phosphorylation of B-50 and the 50 kDa protein to the same extent (figs. 4, 5D,F). At 3×10^{-6} M, phosphorylation of other proteins was also inhibited (fig. 5E). Polymyxin B, 400 IU/ml (Mazzei et al., 1982), effectively inhibited phosphorylation of the 50 kDa protein (fig. 5I). Higher concentrations were necessary to inhibit phosphorylation of B-50 and the 52 kDa protein (figs. 4, 5G,H). Calmidazolium, 10^{-4} M (Gietzen et al., 1981) inhibited phosphorylation of the 50 kDa protein and had no effect on phosphorylation of the other two proteins (figs. 4, 5J-L). W-7, 10^{-4} M (Hidaka and Tanaka, 1983), inhibited the phosphorylation of the 50 kDa protein and slightly decreased the phosphorylation of B-50 and the 52 kDa protein (figs. 5M-O).

3.3. Effects of polymyxin B, H-7 and calmidazolium on B-50 phosphorylation and [^{3}H]NA release

In view of the specificity of the inhibitors in the SPM phosphorylation assay, polymyxin B, H-7 and calmidazolium were tested on K^+-induced B-50 phosphorylation and [^{3}H]NA release. Polymyxin B caused a concentration-dependent decrease in the depolarization-evoked release of [^{3}H]NA and B-50 phosphorylation (fig. 6). The release inhibition was evident at concentrations of 80 IU/ml and higher. At 2160 IU/ml polymyxin B, [^{3}H]NA release was 13.7 ± 1.9% of control K^+-evoked release. At this concentration, basal efflux of [^{3}H]NA was enhanced (129.5 ± 9.3% of control; table 1). Lower levels of polymyxin B did not affect basal [^{3}H]NA efflux. The inhibition of B-50 phosphorylation was evident at concentrations of 240 IU/ml and higher. At 2160 IU/ml polymyxin B, B-50 phosphorylation was 30% of control K^+-evoked phosphorylation. Polymyxin B did not affect basal B-50 phosphorylation.

H-7, 10^{-4} M, had no effect on depolarization-induced release of [^{3}H]NA, whereas B-50 phosphorylation was markedly inhibited (50% of con-

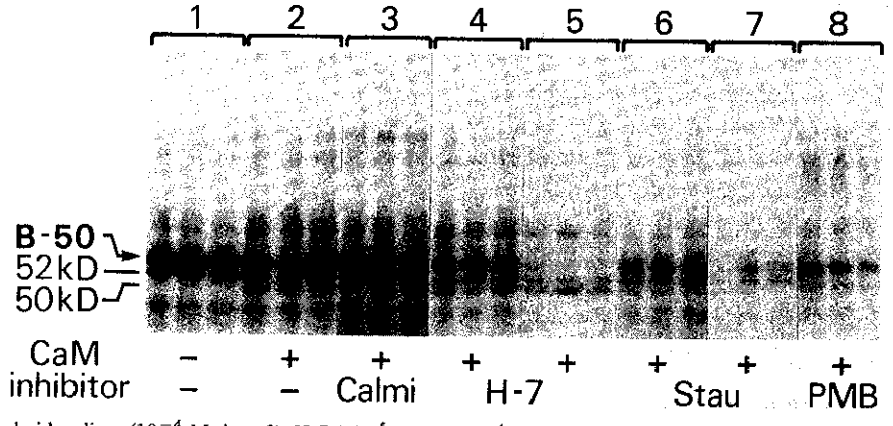

Fig. 4. Effects of calmidazolium (10^{-4} M; lane 3), H-7 (10^{-5} M and 10^{-4} M; lanes 4 and 5), staurosporin (10^{-6} M and 3×10^{-6} M; lanes 6 and 7) and polymyxin B (4000 IU/ml; lane 8) on phosphorylation of SPM proteins. Lane 1: control; lane 2: calmodulin-dependent phosphorylation induced by addition of 3 units of calmodulin per assay.

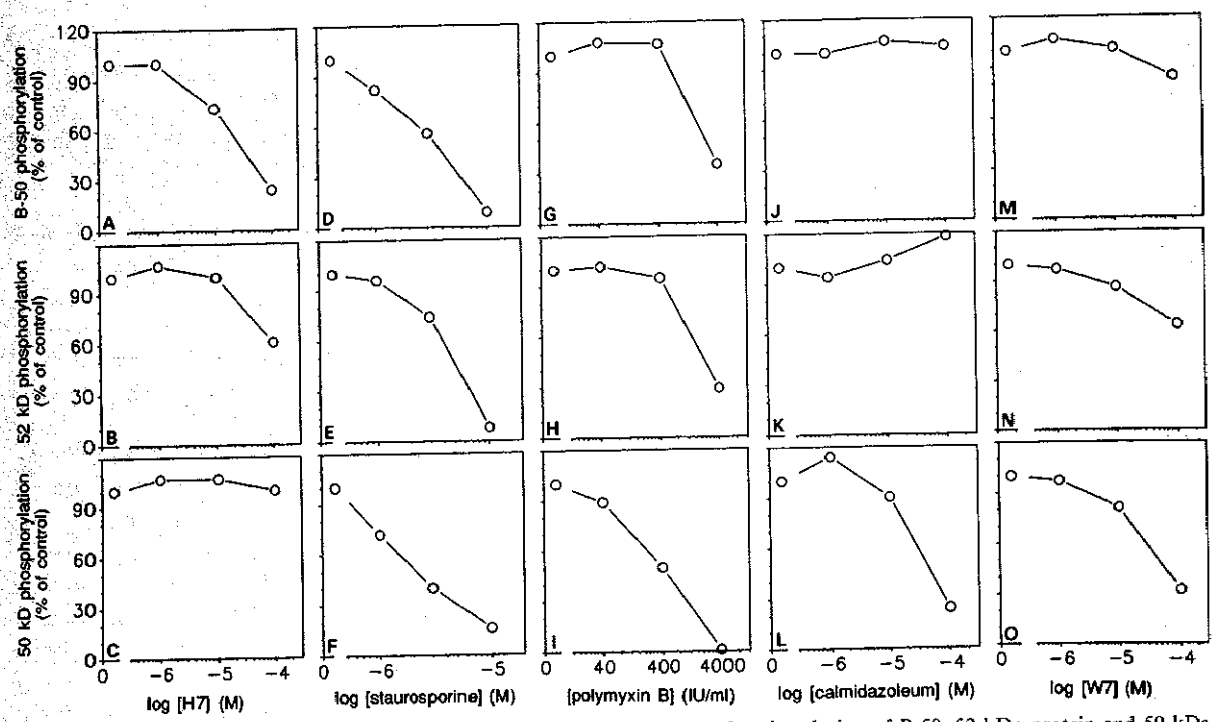

Fig. 5. Effects of H-7, staurosporin, polymyxin B, calmidazolium and W-7 on phosphorylation of B-50, 52 kDa protein and 50 kDa protein in SPM. Controls contained 3 units of calmodulin per assay. Phosphate incorporation into the various proteins was measured as peak height above background and was set at 100% for the untreated controls. Values are expressed as means of triplicate incubations. The same concentration dependencies were found in a second experiment.

trol; fig. 6). Calmidazolium, (10^{-4} M, inhibited the release of [^{3}H]NA (74.1 ± 5.4% of control) but had no effect on B-50 phosphorylation (fig. 6). Neither H-7 nor calmidazolium affected the basal efflux of [^{3}H]NA (table 1) and the basal phosphorylation of B-50 (not shown).

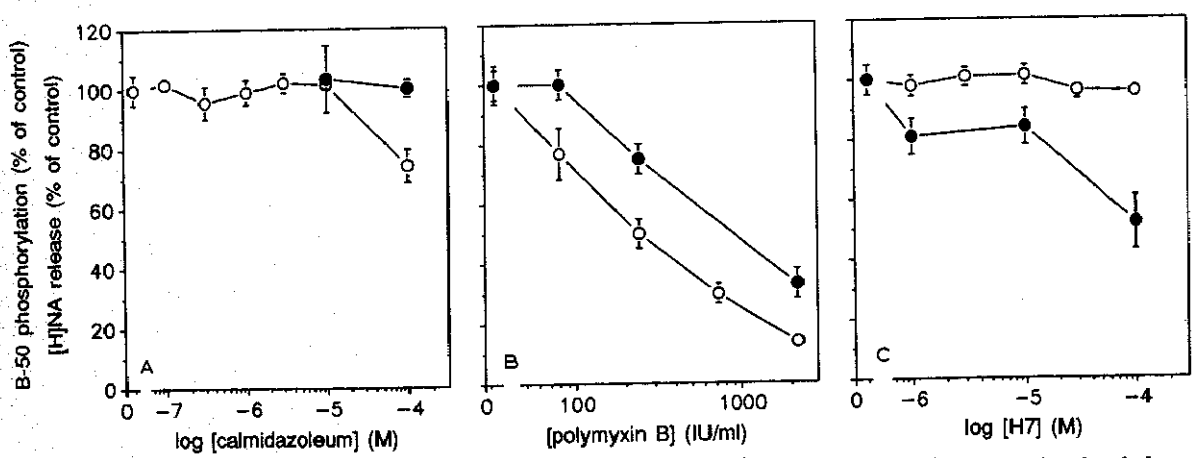

Fig. 6. Effects of increasing concentrations of H-7 (A), polymyxin B (B) and calmidazolium (C) on K^+-induced phosphorylation of B-50 (filled circles) and release of [^{3}H]NA (open circles). Each value is the mean ± S.E.M. (B-50 phosphorylation, n = 3-9; [^{3}H]NA release, n = 8-12; data on inhibition of B-50 phosphorylation by H-7 (C) were adapted from Dekker et al., 1989a).

TABLE 1

Effects of polymyxin B, H-7 and calmidazolium on basal efflux of [^{3}H]NA from rat brain synaptosomes. Each value is the mean ± S.E.M. of 8 (polymyxin B and calmidazolium) or 12 (H-7) observations obtained in two (polymyxin B and calmidazolium) or three (H-7) separate experiments. Absolute control values for the fractional rates of basal release (means ± S.E.M.) were 7.3 ± 0.3 (polymyxin B), 8.5 ± 0.3 (H-7) and 9.8 ± 0.8 (calmidazolium). ND, not determined.

Concentration		Basal efflux of [^{3}H]NA (% of control)		
Polymyxin B (IU/ml)	H-7/calmidazolium (M)	PMB	H-7	Calmidazolium
0	0	100.1 ± 6.5	100.0 ± 2.7	100.0 ± 8.2
80	10^{-6}	98.2 ± 6.3	90.6 ± 4.0	109.0 ± 5.0
240	3×10^{-6}	98.5 ± 4.4	95.9 ± 3.1	100.4 ± 8.3
720	10^{-5}	93.3 ± 5.6	92.3 ± 2.5	115.0 ± 4.3
2160	3×10^{-5}	129.5 ± 9.3	88.0 ± 4.2	ND
	10^{-4}		91.1 ± 3.3	101.0 ± 8.2

4. Discussion

Several lines of evidence suggest a correlation between phosphorylation of the PKC substrate B-50 and release of the neurotransmitter. K^+-induced depolarization of rat hippocampal slices induces a concomitant phosphorylation of B-50 and release of [^{3}H]D-Asp (Dekker et al., 1989c). The convulsant drug 4-aminopyridine induces release of NA from rat hippocampal slices parallel with a stimulation of B-50 phosphorylation (Heemskerk et al., 1989). Treatment of synaptosomes with high K^+ buffers induces a concentration-dependent release of [^{3}H]NA and phosphorylation of B-50 (fig. 1). The secretagogue veratridine also induces a concentration-dependent enhancement of [^{3}H]NA release and B-50 phosphorylation (fig. 2). The kinetics of high K^+- and veratridine-induced B-50 phosphorylation in synaptosomes are similar; both stimuli induce a transient enhancement of B-50 phosphorylation reaching a maximum within seconds of incubation and declining towards control levels after 1-2 min (fig. 2B; Dekker et al., 1989a). Such a kinetic profile coincides very well with the amount of transmitter released from synaptosomes per unit of time (Cotman et al., 1976; Nicholls et al., 1987), indicating that B-50 is phosphorylated during the process of transmitter extrusion. The instantaneous onset of B-50 phosphorylation in synaptosomes correlates well with rapid release of dopamine, NA and glutamate from synaptosomes (Drapeau and Blaustein, 1983; Daniell and Leslie, 1986; Nicholls et al., 1987).

Interference studies at the level of PKC, the kinase involved in K^+-induced B-50 phosphorylation (Dekker et al., 1989a), could shed light on the relationship between B-50 phosphorylation and neurotransmitter release. We have used PKC-stimulating phorbol esters and several known PKC inhibitors to manipulate neurotransmitter release and B-50 phosphorylation. Application of PDB shows a divergence of B-50 phosphorylation and [^{3}H]NA release as PDB enhances B-50 phosphorylation in non-depolarized synaptosomes, whereas [^{3}H]NA release is hardly affected (fig. 3). These results are in agreement with the effect of PDB on B-50 phosphorylation and neurotransmitter release in non-depolarized hippocampal slices (Versteeg and Florijn, 1987; Dekker et al., 1989c). Moreover, it has been reported that under low extracellular Ca^{2+} conditions, PDB induces B-50 phosphorylation in these slices, without affecting transmitter release (Dekker et al., 1989c). Like in depolarized hippocampal slices (Versteeg and Florijn, 1987; Dekker et al., 1989c), the magnitude of the effect of PDB on B-50 phosphorylation and on neurotransmitter release is similar in depolarized synaptosomes (fig. 3).

Prior to measuring the effects of various compounds supposed to inhibit PKC on NA release and B-50 phosphorylation, we determined the specificity and effectivity of these inhibitors in a SPM phosphorylation study. The inhibitors have

been tested on two Ca^{2+}-dependent kinases thought to be involved in transmitter release: on PKC by measuring B-50 phosphorylation, and on calmodulin kinase by measuring autophosphorylation of the 50 kDa subunit of calmodulin kinase type II (De Graan et al., 1987). It was found that polymyxin B, known as a PKC inhibitor (Mazzei et al., 1982; Kuo et al., 1984), more effectively antagonized calmodulin-dependent phosphorylation than PKC-dependent phosphorylation. Staurosporin, also known as a PKC inhibitor (Tamaoki et al., 1986), inhibited all protein phosphorylation systems in the same way. H-7 (Hidaka et al., 1984) was the most specific PKC inhibitor in the membrane phosphorylation assay. Furthermore, the two calmodulin antagonists, calmidazolium (Gietzen et al., 1981) and W-7 (Hidaka and Tanaka, 1983), efficiently inhibited calmodulin-dependent phosphorylation.

The present results show that the specificity of the inhibitors towards brain membrane protein kinases differs somewhat from that reported for that towards isolated kinases, particularly concerning the specificity of the PKC inhibitors. Each of these compounds has been proposed to inhibit PKC via a different mechanism. Polymyxin B probably acts by competing with the binding of the kinase to phosphatidylserine (Mazzei et al., 1982). The inhibition of PKC by H-7 occurs most likely at the catalytic site of the enzyme, probably by competing with ATP (Hidaka et al., 1984). Staurosporin does not compete with activators of PKC such as phosphatidylserine, Ca^{2+}, substrate, ATP or diacylglycerol (Tamaoki et al., 1986), but may have a direct effect on PKC. Recently, it has been reported that staurosporin had a stimulatory effect on the translocation of PKC (Wolf and Baggiolini, 1989). Of these three compounds, staurosporin is the most effective in inhibiting PKC in vitro ($IC_{50} = 2.7$ nM) (Tamaoki et al., 1986). However, as it has been reported that the cAMP-dependent protein kinase is inhibited with the same efficiency as PKC, the specificity of this inhibitor is questioned (Tamaoki et al., 1986). Also in the present study, staurosporin was not a specific inhibitor of PKC (fig. 5). In vitro H-7 inhibits PKC with an IC_{50} value of 6 μM (Hidaka et al., 1984). It affects myosin light chain kinase (a calmodulin-dependent kinase), cAMP- and cGMP-dependent protein kinases with IC_{50} values of approximately 97, 3.0 and 5.8 μM, respectively. This inhibition spectrum is in agreement with the present results (fig. 5). Polymyxin B seems to be the most specific PKC inhibitor described in the literature. It inhibits PKC with an IC_{50} value of 6-8 μM and myosin light chain kinase with an IC_{50} of 80-100 μM (Mazzei et al., 1982). It has no effect on cyclic nucleotide-dependent kinases (Mazzei et al., 1982). The present data show that polymyxin B inhibits the calmodulin-dependent kinase in the synaptic membrane more efficiently than PKC (fig. 5). Therefore, inhibition of a physiological process in the brain by polymyxin B, is not in all cases an exclusive argument for the involvement of only PKC in such a process.

We used polymyxin B (a mixed inhibitor of both PKC and calmodulin-dependent phosphorylation in synaptic membranes), H-7 (the most specific inhibitor of PKC-dependent phosphorylation) and calmidazolium (a very specific inhibitor of calmodulin-dependent phosphorylation) to compare their effects on K^+-induced B-50 phosphorylation and NA release in synaptosomes. Polymyxin B inhibited release as well as B-50 phosphorylation (fig. 6). However, release was more sensitive to polymyxin B-treatment than B-50 phosphorylation. The inhibition of B-50 phosphorylation by H-7 was not parallelled by an inhibition of [^{3}H]NA release (fig. 6). These results are in line with results of Daschmann et al. (1988) who showed that H-7 did not affect evoked release of [^{3}H]NA from rabbit hippocampal slices. Evoked release of [^{3}H]acetylcholine from these slices was reduced by H-7 by only 10%. Calmidazolium did not inhibit B-50 phosphorylation, whereas it inhibited [^{3}H]NA release by 25% (fig. 6).

The almost complete inhibition of release by polymyxin B gives rise to some interesting speculations. If the effect of polymyxin B on transmitter release were caused by its interaction with PKC (the most widely accepted hypothesis in the literature), it would be expected that the specific PKC inhibitor H-7 inhibits transmitter release in the same way as it inhibits B-50 phosphorylation. If on the other hand, the effect of polymyxin B on transmitter release is caused by an interaction

with calmodulin-dependent (phosphorylation) processes, it would be expected that the specific calmodulin-antagonist calmidazolium would inhibit transmitter release to a larger degree than found in the present study. Based on the efficiency of calmidazolium in the SPM phosphorylation assay, 80% inhibition of release by calmidazolium would be expected. Neither of the two predictions are supported by our data. Nevertheless, the 'mixed inhibitor' polymyxin B inhibits transmitter release and B-50 phosphorylation.

The present results could be explained by assuming that PKC-dependent and calmodulin-dependent processes interact with each other to regulate release of neurotransmitter. Interestingly, the B-50 protein has been co-identified with P-57, a calmodulin-binding protein (Cimler et al., 1987). In vitro P-57 binds calmodulin under low Ca^{2+} conditions and releases calmodulin under high Ca^{2+} conditions (Andreasen et al., 1983). Prephosphorylation of P-57 by PKC in vitro inhibits subsequent binding of calmodulin even under high Ca^{2+} conditions (Alexander et al., 1987). In vivo calmodulin may be dissociated from B-50 after a depolarization-induced rise in intracellular Ca^{2+}. Calmodulin may activate cellular processes that may be involved in transmitter release. Once calmodulin and B-50 have been dissociated, B-50 may be phosphorylated by PKC, thereby preventing reassociation of B-50 and calmodulin. From these data, it could be postulated that during transmitter release B-50 is the site of integration at the molecular level of signals involving PKC and calmodulin. Indeed, antibodies to B-50 (inhibiting PKC-mediated B-50 phosphorylation and presumably all molecular features of B-50) completely inhibit Ca^{2+}-dependent release of NA from rat cortical synaptosomes (Dekker et al., 1989b).

In conclusion, a complex interaction between PKC, B-50, B-50 phosphorylation and calmodulin seems to exist which may be very important for regulation of the process of transmitter release. However, the exact relationship between these different entities remains to be established.

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