

Depolarization-Induced Phosphorylation of the Protein Kinase C Substrate B-50 (GAP-43) in Rat Cortical Synaptosomes

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Abstract: We studied the molecular events underlying K^+ -induced phosphorylation of the neuron-specific protein kinase C substrate B-50. Rat cortical synaptosomes were prelabelled with ^{32}P -labelled orthophosphate. B-50 phosphorylation was measured by an immunoprecipitation assay. In this system, various phorbol esters, as well as a synthetic diacylglycerol derivative, enhance B-50 phosphorylation. K^+ depolarization induces a transient enhancement of B-50 phosphorylation, which is totally dependent on extracellular Ca^{2+} . Also, the application of the Ca^{2+} ionophore A23187 induces B-50 phosphorylation, but the magnitude and kinetics of A23187-induced B-50 phosphorylation differ from those induced by depolarization. The protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), *N*-(6-aminohexyl)-

5-chloro-1-naphthalenesulfonamide (W-7), and staurosporine antagonize K^+ - as well as PDB-induced B-50 phosphorylation, whereas trifluoperazine and calmidazolium are ineffective under both conditions. We suggest that elevation of the intracellular Ca^{2+} level after depolarization is a trigger for activation of protein kinase C, which subsequently phosphorylates its substrate B-50. This sequence of events could be of importance for the mechanism of depolarization-induced transmitter release. **Key Words:** B-50—GAP-43—Protein kinase C—Protein phosphorylation—Synaptosomes. **Dekker L. V. et al.** Depolarization-induced phosphorylation of the protein kinase C substrate B-50 (GAP-43) in rat cortical synaptosomes. *J. Neurochem.* **54**, 1645–1652 (1990).

Elevation of the concentration of intracellular Ca^{2+} is considered to be a major trigger for release of neurotransmitters in nerve cells (Zucker, 1987). The biochemical events responsible for transmission of the Ca^{2+} signal into vesicle fusion and transmitter release are poorly understood (Miller, 1989). Studies on neuronal, as well as on nonneuronal cells suggest that several proteins may be involved in transmitting the Ca^{2+} signal, including Ca^{2+} -binding proteins (Augustine et al., 1987), GTP-binding proteins (Howell et al., 1987), protein kinases, and their substrate proteins (Augustine et al., 1987). Apart from transmitting the Ca^{2+} signal, it is also possible that these proteins merely act as modulators of neurotransmitter release.

A large number of studies provided evidence for the involvement of the Ca^{2+} /phospholipid-dependent pro-

tein kinase C (PKC) in transmitter release. This evidence has been obtained mainly by studying the effects of agonists and antagonists of PKC in cultured cells, tissue slices, or synaptosomes on release of radiolabelled transmitter. Phorbol esters, which are known to activate PKC, stimulate neurotransmitter release (Allgaier et al., 1986; Versteeg and Florijn, 1987), and depolarization-induced neurotransmitter release can be attenuated by inhibitors of PKC or by down-regulation of PKC by phorbol esters (Allgaier and Hertting, 1986; Matthies et al., 1987; Versteeg and Ulenkate, 1987; Bartmann et al., 1989).

We have obtained evidence for the activation of PKC during transmitter release by measuring the degree of phosphorylation of an important endogenous substrate of this kinase, the B-50 protein (Zwiers et al., 1980;

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Abbreviations used: DOG, dioctanoylglycerol; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; PAGE, polyacrylamide gel electrophoresis; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SAC, *Staphylococcus aureus* membranes; SDS, sodium dodecyl sulfate; SPM, synaptosomal plasma membranes; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

De Graan et al., 1988b, 1989). K^+ depolarization of rat hippocampal slices enhances B-50 phosphorylation (Dekker et al., 1989a). This increase is dependent on extracellular Ca^{2+} and does not occur in the presence of the PKC inhibitor polymyxin B (Dekker et al., 1989a). B-50 is a neuron-specific protein (Kristjansson et al., 1982) the localization of which in the membrane of the presynaptic nerve terminal fits with a presumed role in the release process (Sørensen et al., 1981; Gispen et al., 1985; van Lookeren Campagne et al., 1989). However, the precise function of B-50, also called GAP-43, F1, or P-57 (see Skene, 1989), and its phosphorylation by PKC remain to be shown. It has been suggested that B-50 plays a role in the regulation of polyphosphoinositide metabolism (Gispen et al., 1986) and/or calmodulin binding (Andreasen et al., 1983). Furthermore, the protein has been implicated in the process of neuronal outgrowth and long-term potentiation (for reviews, see Benowitz and Routtenberg, 1987; Skene, 1989).

To investigate the molecular events at the presynaptic site underlying the depolarization-induced changes in B-50 phosphorylation in hippocampal slices, we studied B-50 phosphorylation in ^{32}P -labelled rat cortical synaptosomes. We show that K^+ depolarization induces a rapid and transient enhancement of B-50 phosphorylation, which is dependent on extracellular Ca^{2+} . K^+ -induced phosphorylation of B-50 can be inhibited by inhibitors of PKC. The trigger for B-50 phosphorylation seems to be influx of Ca^{2+} , because the Ca^{2+} ionophore A23187 partially mimics the effects of K^+ depolarization.

MATERIALS AND METHODS

Materials

Male Wistar rats (TNO, Zeist, The Netherlands) of approximately 120 g were used throughout the experiments. Phorbol 12,13-dibutyrate (PDB), phorbol 12-myristate, 13-acetate (PMA), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), calmidazolium, EGTA, and A23187 were purchased from Sigma (St. Louis, MO, U.S.A.). Dioctanoyl-glycerol (DOG) was purchased from Janssen (Beerse, Belgium), trifluoperazine (TFP) from Boehringer (Mannheim, F.R.G.), staurosporine from Fluka (Buchs, Switzerland), and ^{32}P -labelled orthophosphate ($^{32}P_i$, carrier-free) from Amersham (Buckinghamshire, U.K.). Staurosporine (1 mM), PDB (2 mM), PMA (2 mM), A23187 (2 mM), calmidazolium (50 mM), and DOG (100 mM) were dissolved as stock solutions in 100% dimethyl sulfoxide, which did not affect B-50 phosphorylation in the dilution used. H-7, W-7, and TFP were dissolved in buffer. *Staphylococcus aureus* membranes (SAC) were prepared from *S. aureus* Cowan I cells according to Kronvall (1973). Anti-B-50 antiserum was prepared as described by Oestreicher et al. (1983).

^{32}P labelling and treatment of synaptosomes

Synaptosomes were prepared from rat cortex as described by Dunkley et al. (1988) (fraction 4). The synaptosomal preparation was diluted in buffer A [124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 26 mM

$NaHCO_3$, saturated with CO_2 and O_2 (5:95), pH 7.4] at a concentration of 1 mg of protein/ml and labelled with 2 μCi of $^{32}P_i/\mu g$ of protein at 34°C for 50 min. At the end of this labelling period, synaptosomes were divided in 10- μl aliquots (10 μg of protein) and the incubation was started at 34°C ($t = 0$). Total incubation time was 10 min for each sample. PDB, PMA, and DOG were added at $t = 5$ min. High K^+ buffer (buffer A in which Na^+ was substituted by K^+ to retain osmotic pressure) was added at $t = 9.45$ min, except in the time dependency experiment, when it was added at various time points. EGTA was added at $t = 0$ to a final concentration of 6 or 12 mM. A23187 was present from $t = 0$ onwards, except in the time dependency experiment, when it was added at various time points. Protein kinase inhibitors were present from $t = 0$ onwards. The final incubation volume was 20 μl .

The incubation was stopped after 10 min by the addition of 10 μl of denaturing solution [buffer B; final concentrations: 62.5 mM Tris-HCl, pH 6.5, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue].

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

Prior to the immunoprecipitation, SDS-solubilized synaptosome samples were precleared with SAC to reduce non-specific precipitation: 20 μl of SDS-solubilized synaptosomes (containing 6.7 μg of total protein) were incubated at room temperature with SAC (final concentration 0.5%) in 350 μl of buffer C (200 mM NaCl, 10 mM EDTA, 10 mM NaH_2PO_4 , 0.5% Nonidet P-40, pH 7.2). After 30 min, samples were centrifuged for 20 min at 5,000 g. Three hundred microliters of the precleared supernatant was added to 100 μl of buffer C containing anti-B-50 antiserum (final dilution 1:200) and incubated overnight at 4°C. Subsequently, 50 μl of SAC were added (final concentration 0.5% in buffer C) and samples were incubated for 30 min at room temperature. After centrifugation (20 min at 5,000 g), the SAC pellet was resuspended in buffer B, boiled for 10 min, and analyzed by 11% SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography (De Graan et al., 1989). B-50 phosphorylation was quantified by densitometric scanning of the autoradiogram.

Other analyses

^{32}P incorporation into total synaptosomal proteins was analyzed by 11% SDS-PAGE and autoradiography (Kristjansson et al., 1982). Proteins were determined according to Bradford (1976) with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as a standard.

RESULTS

B-50 immunoprecipitation

In a previous article, we described that B-50 can be specifically and quantitatively immunoprecipitated from ^{32}P -labelled rat hippocampal slices (De Graan et al., 1989). Pilot studies revealed that synaptosomes can also be labelled with $^{32}P_i$. Stable labelling of the endogenous ATP pool in the synaptosomes is reached within 50 min (results not shown; Dunkley and Robinson, 1986). Analysis of ^{32}P -labelled synaptosomes by SDS-PAGE shows that three major and several minor protein bands are phosphorylated (Fig. 1, lane 1). The B-50 protein is not represented by any of the directly visible ^{32}P -labelled protein bands. However, it can be

detected by immunoprecipitation from the ^{32}P -labelled synaptosomes (Fig. 1, lane 3). Specific immunoprecipitation of B-50 requires a preclearing step of the samples with SAC to reduce nonspecific background (compare lanes 2 and 3 with lanes 4 and 5 in Fig. 1). This immunoprecipitation assay has been used to quantify B-50 phosphorylation in the synaptosomes.

Incubation of the synaptosomes for 5 min with the PKC activator PDB (10^{-6} M) enhances B-50 phosphorylation (Fig. 2A). PDB at 10^{-6} M has no substantial effect on any of the other phosphoprotein bands that are visible in the total homogenate after SDS-PAGE (Fig. 2A). The PDB-induced enhancement of B-50 phosphorylation is concentration-dependent (Fig. 2B). Significant stimulation is already detectable with 10^{-8} M PDB ($234 \pm 22\%$ as compared to control incubations). Incubation of the synaptosomes for 5 min with two other PKC activators, PMA and DOG, also enhances B-50 phosphorylation in a concentration-dependent way. PMA significantly stimulates B-50 phosphorylation at 10^{-7} M ($156 \pm 13\%$) and DOG at 10^{-4} M ($183 \pm 22\%$). No stimulation is detectable when synaptosomes are incubated in 10^{-6} M 4α -phorbol 12,13-didecanoate ($94 \pm 9\%$). These results show that, as described for rat hippocampal slices (De Graan et al., 1989), immunoprecipitation of B-50 from ^{32}P -labelled synaptosomes can be used to monitor B-50 phosphorylation in intact synaptosomes.

Effects of K^+ depolarization and A23187

Treatment of the synaptosomes with 30 mM K^+ enhances B-50 phosphorylation (Fig. 3A). Phosphorylation of several other proteins in the total homogenate is also affected by the K^+ treatment. Thirty millimolar K^+ reduces phosphorylation of a 90-kDa protein, which is probably P96 described by Robinson et al. (1987) (Fig. 3A). It enhances phosphorylation of a protein duplex of approximately 80 kDa which may represent synapsin Ia and Ib (Wang et al., 1988) (Fig. 3A). The

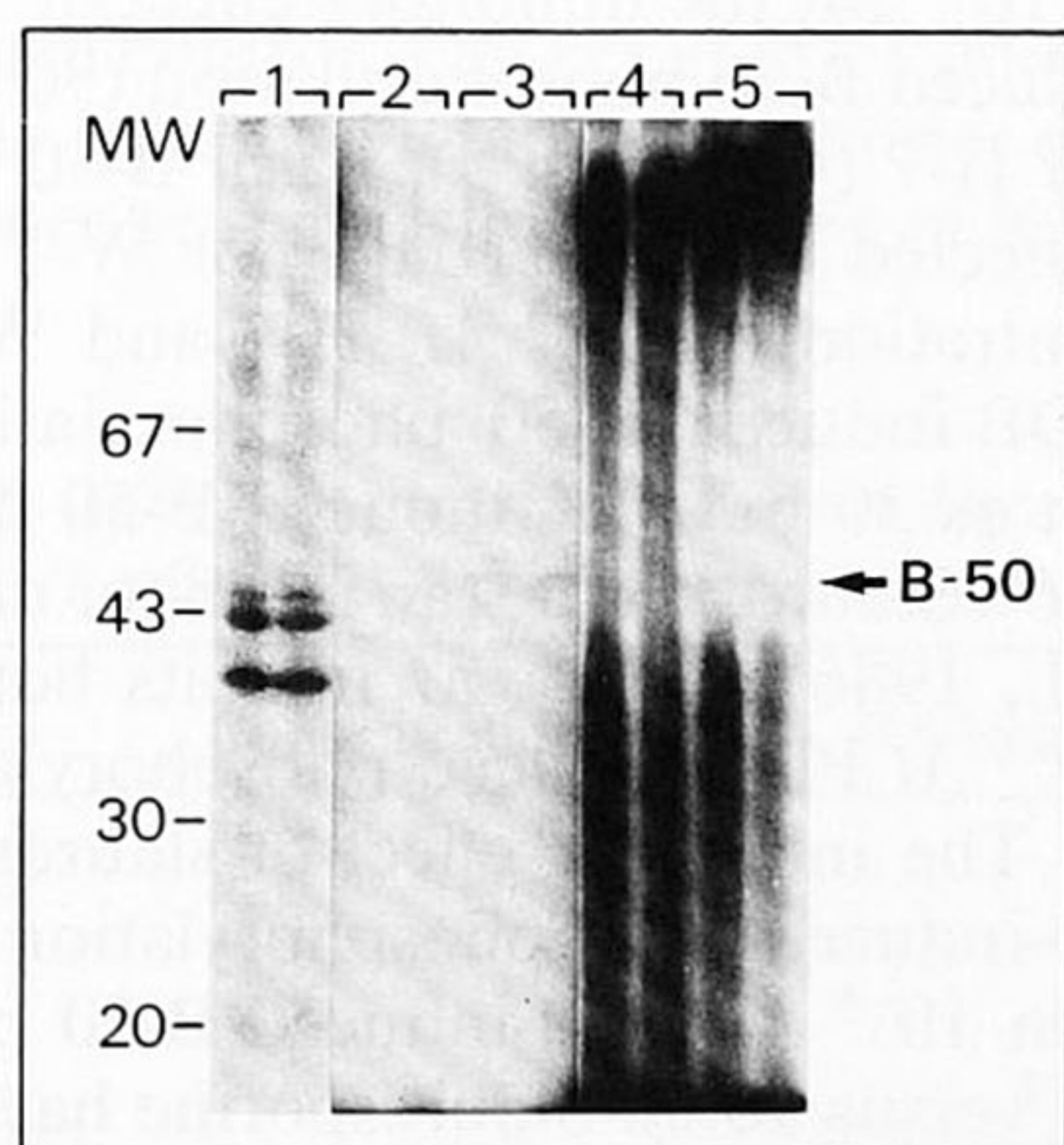


FIG. 1. Specificity of B-50 immunoprecipitation from ^{32}P -labelled synaptosomes. ^{32}P -labelled synaptosomes (lane 1) were directly subjected to immunoprecipitation with control (lane 4) and anti-B-50 antiserum (lane 5) or after preclearing (lanes 2 and 3).

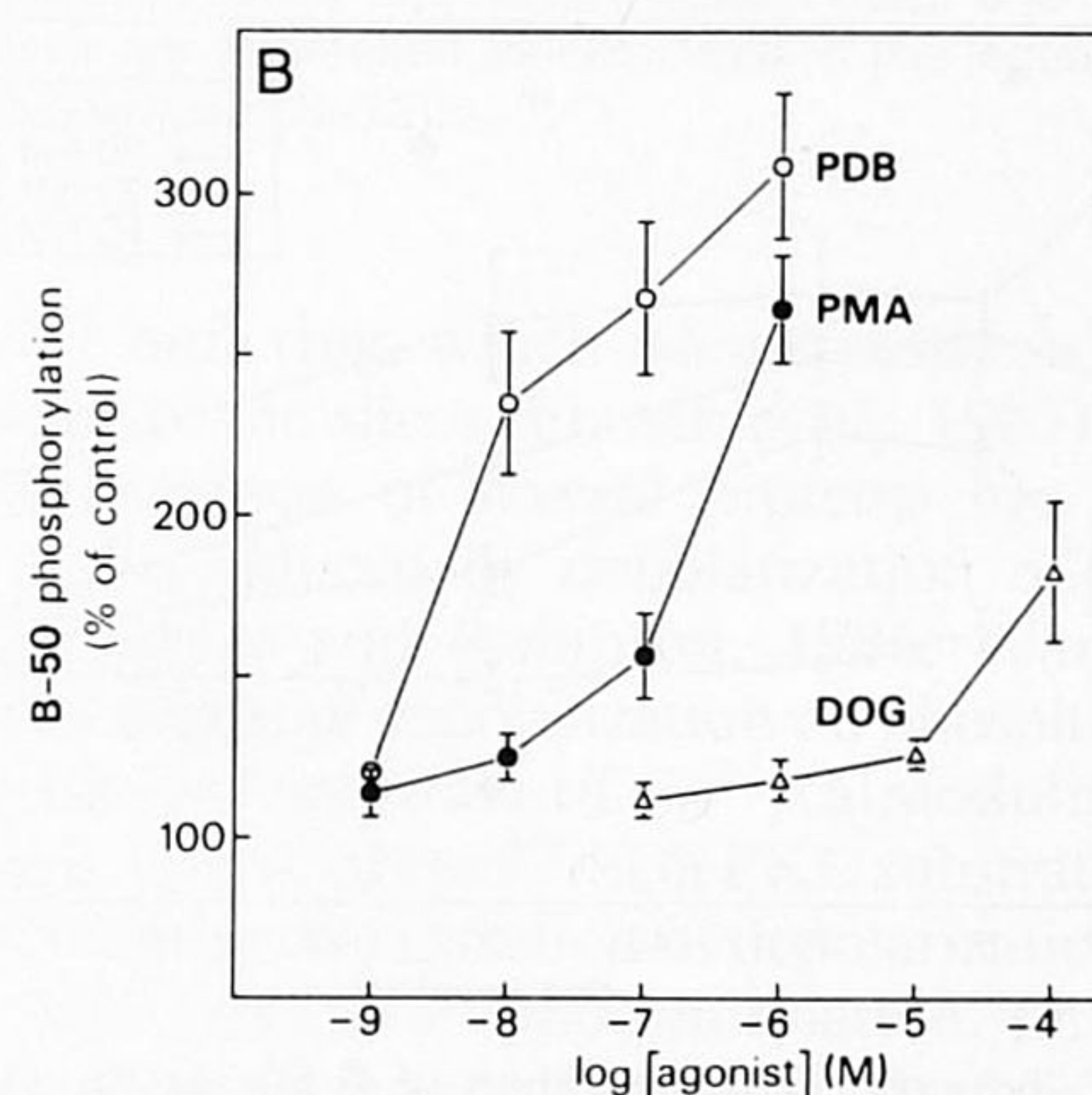
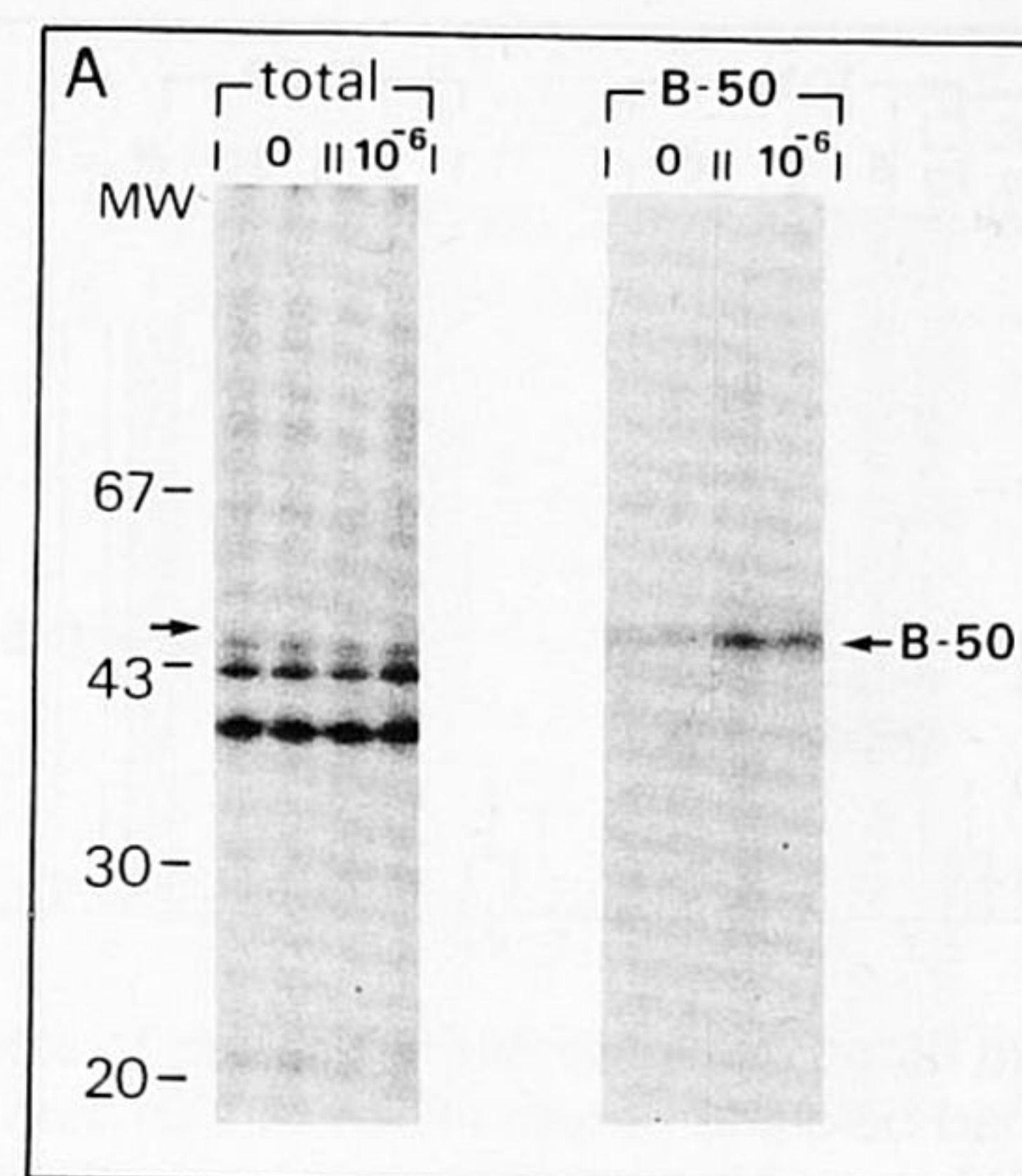


FIG. 2. Effects of PKC agonists on B-50 phosphorylation. **A:** An 11% SDS-PAGE analysis of ^{32}P -labelled synaptosomal proteins ("total") or B-50 immunoprecipitates ("B-50") obtained from synaptosomes treated for 5 min with 10^{-6} M PDB (" 10^{-6} ") or without PDB (" 0 "). **B:** Concentration dependency of the effects of PDB (○), PMA (●), and DOG (△) on B-50 phosphorylation. Values are expressed as percentage of control (means \pm SEM; $n = 4-12$).

enhancement of B-50 phosphorylation by K^+ depolarization is dependent on incubation time and K^+ concentration (Fig. 3B). After a fast initial rise, B-50 phosphorylation returns to control levels within 5 min. No changes in B-50 phosphorylation occur during the 10-min incubation in 5 mM K^+ . Maximal stimulation is obtained with 30 mM K^+ within 5 s.

We have two indications that an influx of Ca^{2+} underlies the effect of K^+ depolarization on B-50 phosphorylation. Firstly, no K^+ -induced enhancement of B-50 phosphorylation is detectable when a 6 or 12 mM concentration of the Ca^{2+} chelator EGTA is present during the depolarization (Table 1). Under these low extracellular Ca^{2+} conditions, 10^{-6} M PDB can still enhance B-50 phosphorylation. Secondly, treatment of synaptosomes with the Ca^{2+} ionophore A23187 for 5 min enhances B-50 phosphorylation in a concentration-dependent way, with maximal stimulation at 10^{-7} M ($34 \pm 2\%$ above control level; Fig. 4A). In contrast to K^+ treatment, A23187-induced B-50 phosphoryla-

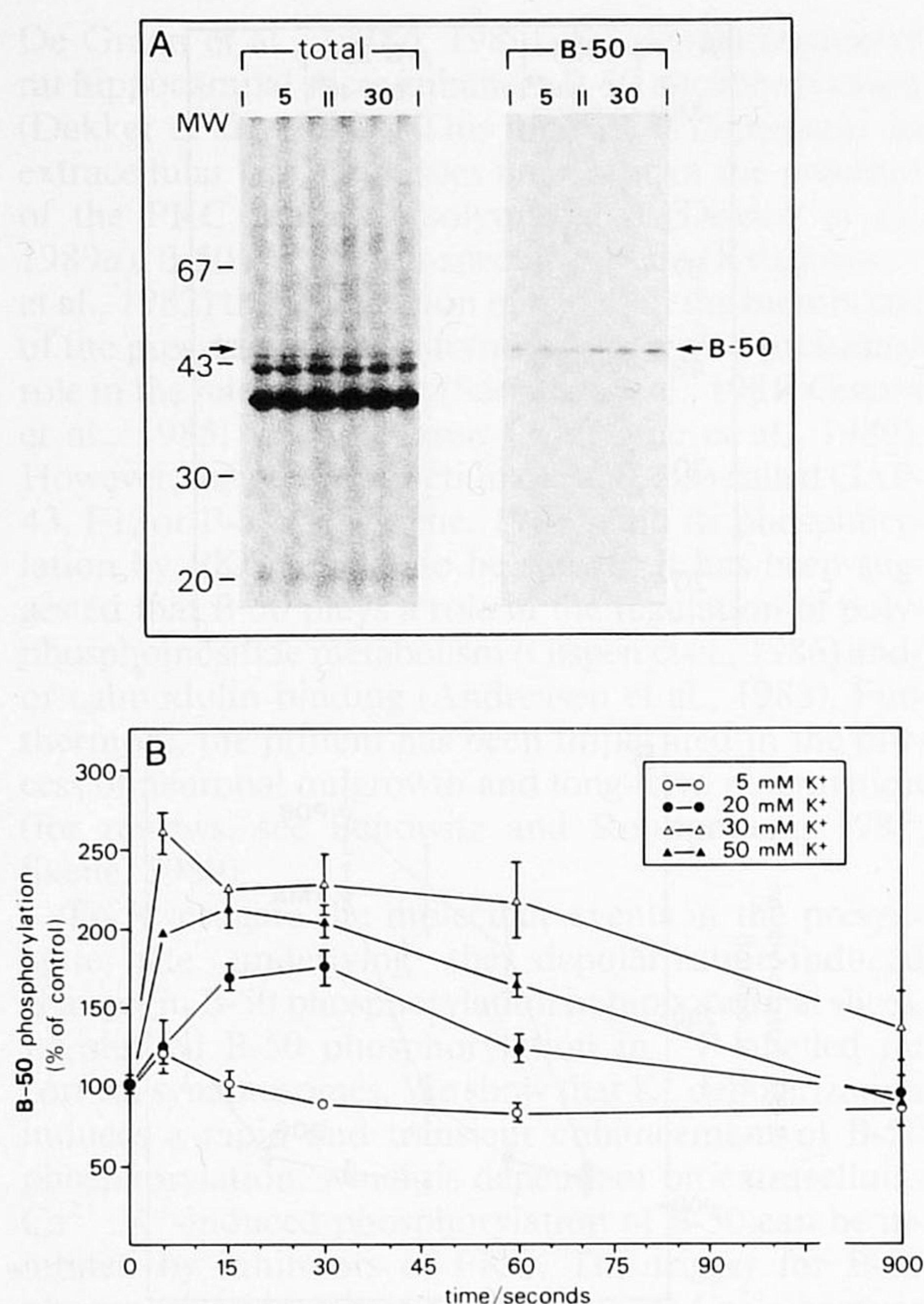


FIG. 3. K^+ -induced phosphorylation of B-50. **A:** An 11% SDS-PAGE analysis of ^{32}P -labelled synaptosomal proteins ("total") or B-50 immunoprecipitates ("B-50") obtained from synaptosomes treated for 15 s with 5 ("5") or 30 ("30") mM K^+ . **B:** Time and concentration dependency of K^+ -induced B-50 phosphorylation [5 mM K^+ (○); 20 mM K^+ (●); 30 mM K^+ (△); 50 mM K^+ (▲)]. Values are expressed as percentage of control (means \pm SEM; $n = 3$). Data are representative of two independent experiments. For experimental details, see Materials and Methods.

tion develops gradually, is first detectable after 1 min, and reaches its maximum after 5 min (Fig. 4B).

Effects of protein kinase inhibitors

In rat hippocampal slices, the PKC inhibitor polymyxin B inhibits K^+ -induced phosphorylation of B-50 (Dekker et al., 1989a). Several protein kinase inhibitors

TABLE 1. Effects of 30 mM K^+ (15 s) and 10^{-6} M PDB (5 min) on B-50 phosphorylation under normal and low extracellular Ca^{2+} conditions

EGTA (mM)	5 mM K^+	30 mM K^+	10^{-6} M PDB
—	100 \pm 4	152 \pm 3	210 \pm 4
6	98 \pm 6	99 \pm 5	205 \pm 10
12	83 \pm 2	83 \pm 2	176 \pm 9

Data are expressed as percentage of control (means \pm SEM; $n = 6$).

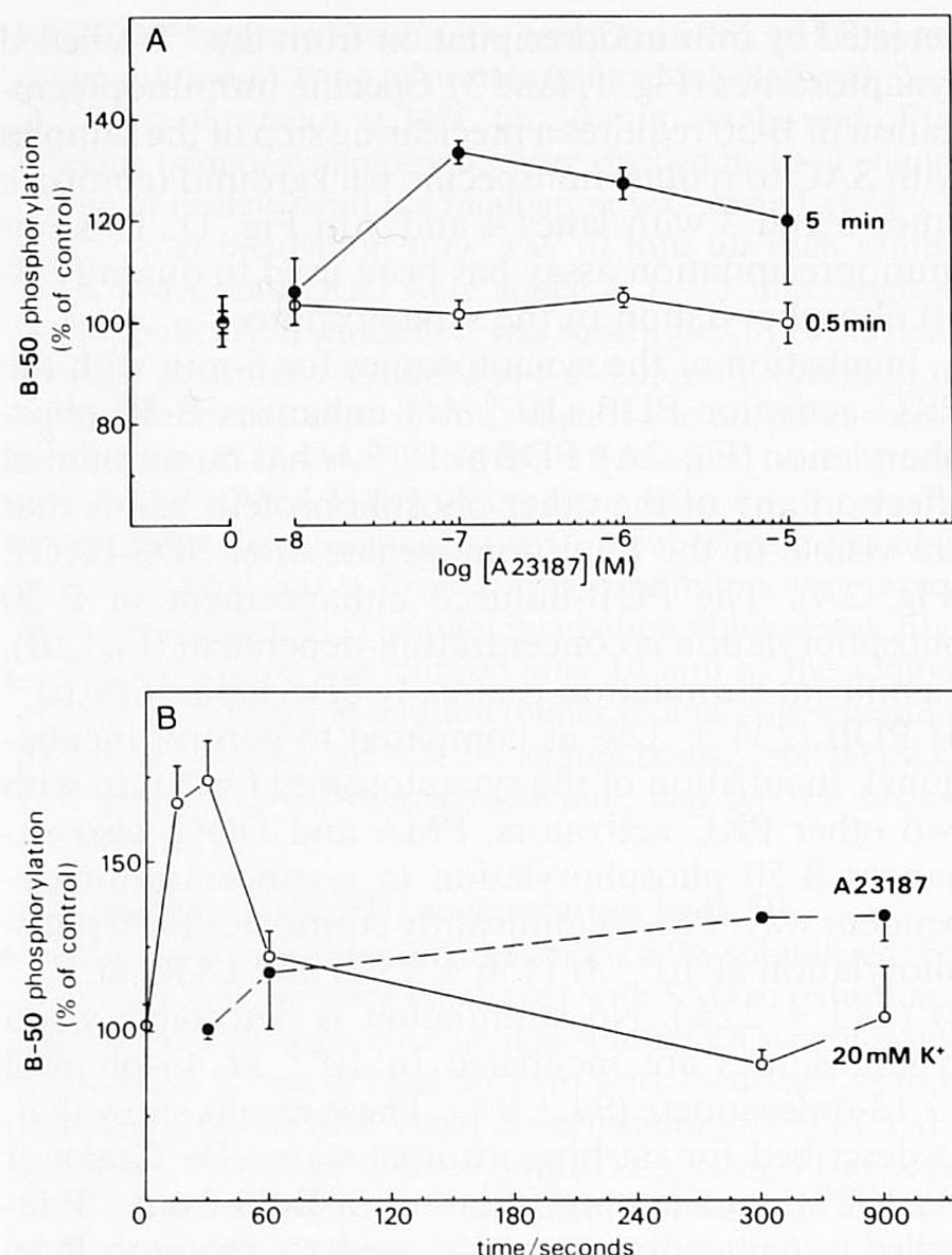


FIG. 4. **A:** Effect of A23187 on B-50 phosphorylation. Synaptosomes were treated with different concentrations of A23187 for 0.5 min (○) or 5 min (●). **B:** Comparison of time dependency of A23187- (●) and 20 mM K^+ -induced (○) phosphorylation of B-50. Values are expressed as percentage of control (means \pm SEM; $n = 3$).

were tested on B-50 phosphorylation in synaptosomes, and their effects were compared to PDB-induced B-50 phosphorylation. Both H-7 (a PKC inhibitor; Hidaka et al., 1984) and W-7 (a calmodulin antagonist; Hidaka and Tanaka, 1983) inhibit 30 mM K^+ -induced phosphorylation of B-50 in a concentration-dependent way (Fig. 5). At 10^{-4} M, the inhibitory effect of W-7 on 30 mM K^+ -induced B-50 phosphorylation (90%) is larger than that of H-7 (45%; Fig. 5). Basal B-50 phosphorylation is affected by neither H-7 nor W-7 (Table 2). At a concentration of 10^{-4} M, H-7 and W-7 inhibit 10^{-6} M PDB-induced B-50 phosphorylation to the same extent as 30 mM K^+ -induced B-50 phosphorylation (Fig. 6). Staurosporine (an inhibitor of PKC; Tamaoki et al., 1986) at 10^{-5} M inhibits both 30 mM K^+ - and 10^{-6} M PDB-induced phosphorylation of B-50 (Fig. 6). The inhibitory effect of staurosporine on 30 mM K^+ -induced B-50 phosphorylation is smaller than that on 10^{-6} M PDB-induced B-50 phosphorylation (50% versus 90%). Staurosporine has no detectable effect on basal B-50 phosphorylation (Table 2). The calmodulin inhibitors calmidazolium (10^{-5} M; Gietzen et al., 1981) and TFP (10^{-3} M; Levin and Weiss, 1978) have no effects on either 30 mM K^+ - or

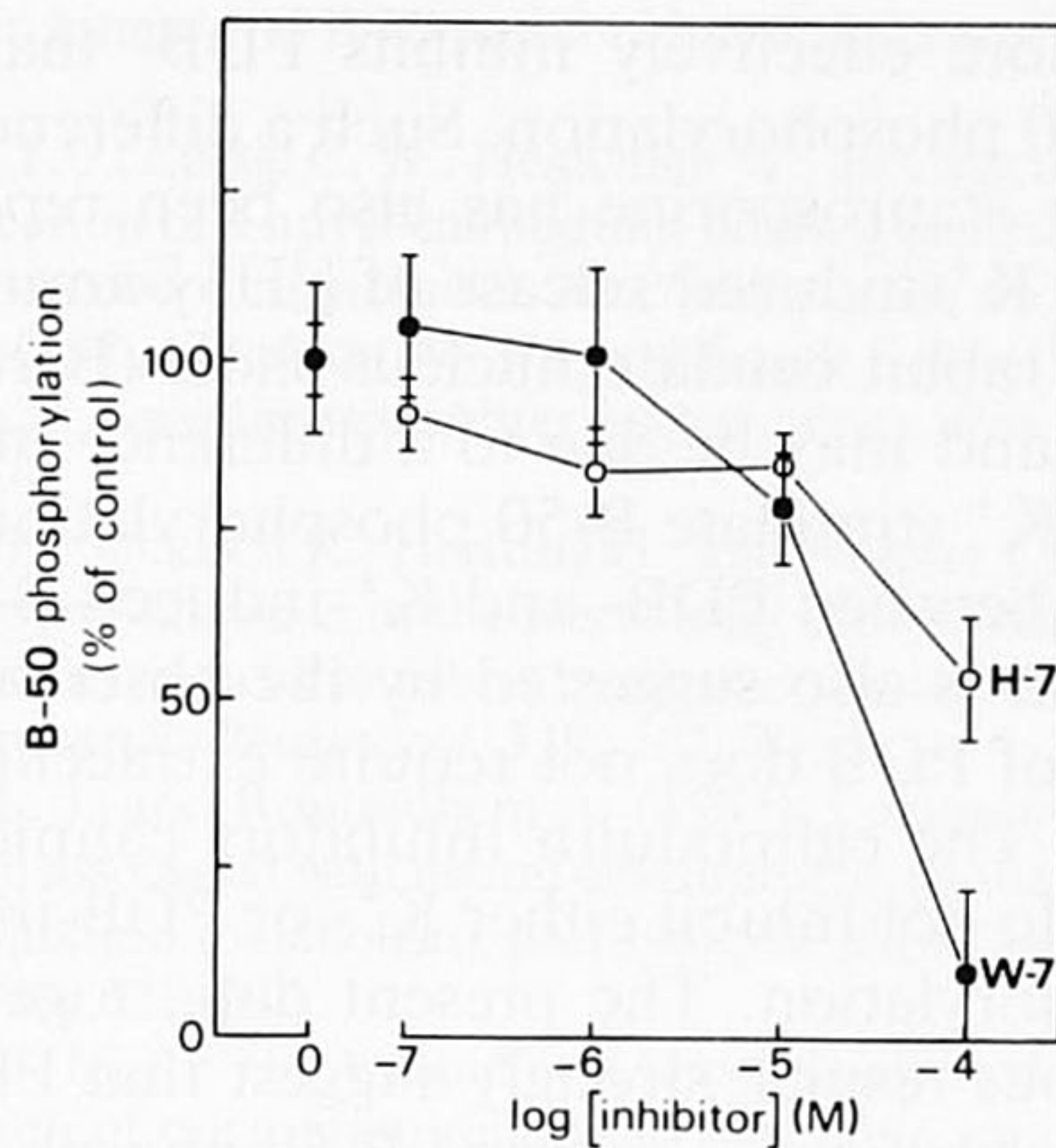


FIG. 5. Effects of H-7 (○) and W-7 (●) on B-50 phosphorylation induced by 15-s treatment with 30 mM K^+ . Values are expressed as B-50 phosphorylation at 30 mM K^+ (with different concentrations of inhibitor) minus basal B-50 phosphorylation. No effects of inhibitor were visible on basal B-50 phosphorylation (see Table 2). Thirty millimolar K^+ -induced B-50 phosphorylation was set at 100%. Data are expressed as means \pm SEM (H-7: $n = 9$; W-7: $n = 6$).

10^{-6} M PDB-induced B-50 phosphorylation (Fig. 6) or on basal B-50 phosphorylation (Table 2).

DISCUSSION

In this article, we show that K^+ depolarization of synaptosomes induces a rapid and transient Ca^{2+} -dependent stimulation of B-50 phosphorylation. Application of a number of protein kinase inhibitors indicates that K^+ -induced B-50 phosphorylation is dependent on activation of PKC. The time course of K^+ -induced B-50 phosphorylation in synaptosomes closely parallels that of transmitter release from this preparation (Cotman et al., 1976; Nicholls et al., 1987). The time dependency of depolarization-induced B-50 phosphorylation observed in synaptosomes differs from that previously observed in rat hippocampal slices. In slices, B-50 phosphorylation gradually increases during the 10 min of 30 mM K^+ treatment, with a maximum of 50% above control (Dekker et al., 1989a). This difference may be due to a rapid transmitter depletion in synaptosomes or to the slow diffusion of K^+ into slices

TABLE 2. Effects of protein kinase inhibitors on basal (5 mM K^+) B-50 phosphorylation

Inhibitor	Concentration (M)	B-50 phosphorylation
—	—	100
H-7	10^{-4}	97 ± 6
W-7	10^{-4}	87 ± 11
Staurosporine	10^{-5}	102 ± 26
Calmidazolium	10^{-5}	95 ± 11
TFP	10^{-3}	111 ± 4

Data are expressed as percentage of control (means \pm SEM; $n = 3-9$).

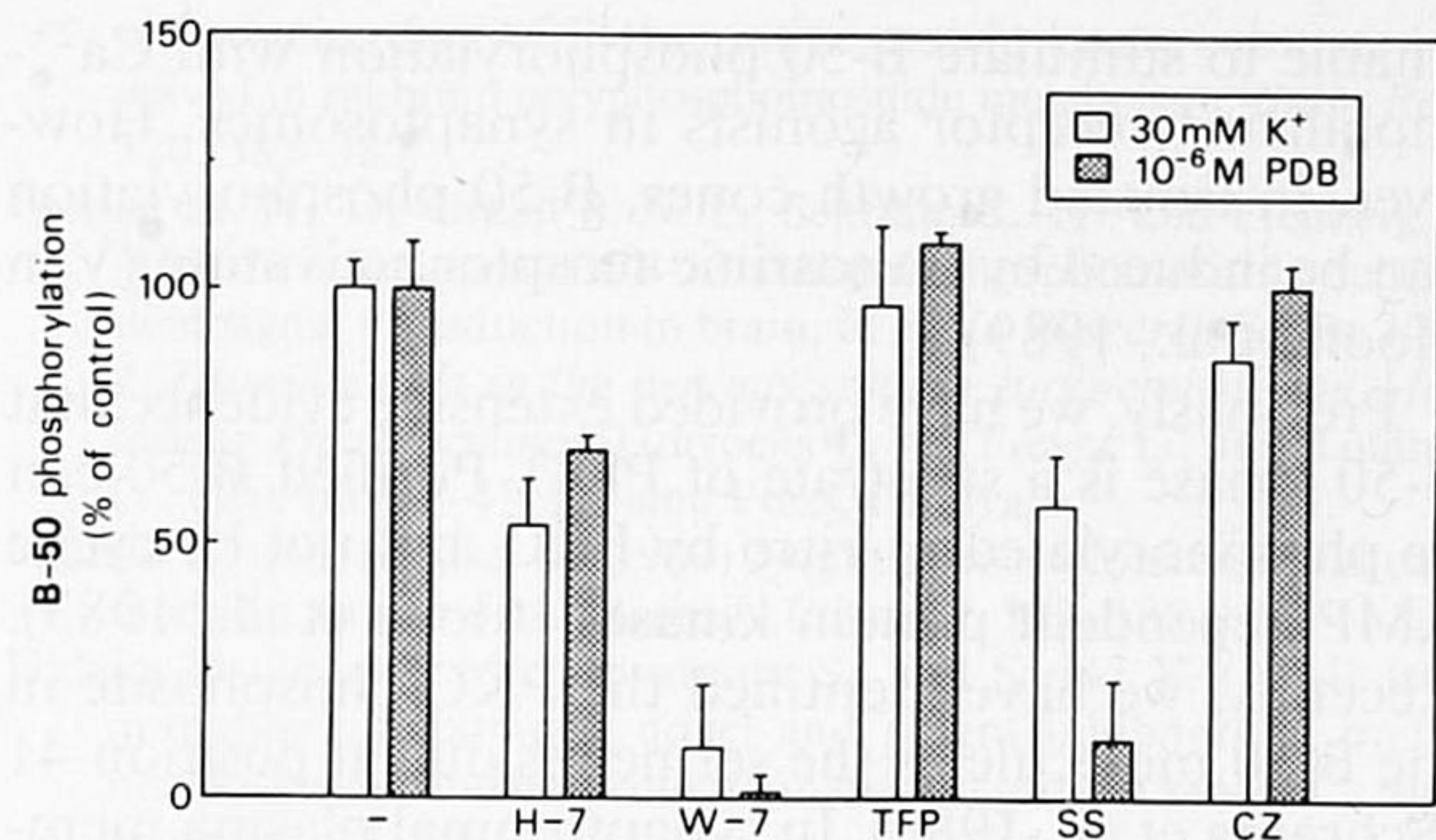


FIG. 6. Effects of protein kinase inhibitors on 30 mM K^+ -induced (open bars) and 10^{-6} M PDB-induced (stippled bars) phosphorylation of B-50. K^+ treatment lasted for 15 s, and PDB treatment for 5 min. Concentrations were 10^{-4} M for H-7 and W-7, 10^{-5} M for staurosporine (SS) and calmidazolium (CZ), and 10^{-3} M for TFP. Values are expressed as described in the legend of Fig. 5 (means \pm SEM; $n = 3-12$).

and to K^+ buffering, which occurs easily in the glial component of the slices (Franck et al., 1983).

Phosphorylation of several proteins has been reported to be induced by depolarization of synaptosomes (Dunkley and Robinson, 1986; Wang et al., 1988). The effects of depolarization on phosphorylation of synapsin I (a substrate of Ca^{2+} /calmodulin-dependent kinase II) and of the 87-kDa PKC substrate protein are dependent on the duration of depolarization (Wang et al., 1988). Like B-50 phosphorylation, phosphorylation of synapsin I is rapidly induced and decreases within 1 min of depolarization; also, phosphorylation of the 87-kDa protein occurs rapidly, but remains at its maximum level for at least 10 min. The discrepancy between the behavior of the two PKC substrate proteins, B-50 and the 87-kDa protein, may be due to differences in experimental protocol, but may also be caused by a functional difference.

The fact that depolarization-induced phosphorylation of B-50 is not detectable under low extracellular Ca^{2+} conditions and that the Ca^{2+} ionophore A23187 enhances B-50 phosphorylation indicates that influx of extracellular Ca^{2+} is underlying the effect of K^+ depolarization on B-50 phosphorylation. The concentrations of A23187 that are effective in stimulation of B-50 phosphorylation also effectively stimulate neurotransmitter release (Tanaka et al., 1984) and polyphosphoinositide metabolism (Kendall and Nahorski, 1984; Wei and Wang, 1987). The observed differences in kinetics and magnitude of high K^+ - and A23187-induced B-50 phosphorylation are most likely due to differences in the way these two compounds induce influx of extracellular Ca^{2+} . It may be that a local rise in Ca^{2+} at the site of initial Ca^{2+} entry evoked by K^+ depolarization (possibly voltage-dependent Ca^{2+} channels) is sufficient to increase B-50 phosphorylation. Thus, the more general rise induced by A23187 and sustained depolarization may be less effective in stimulating B-50 phosphorylation. So far, we have been

unable to stimulate B-50 phosphorylation with Ca^{2+} -mobilizing receptor agonists in synaptosomes. However, in isolated growth cones, B-50 phosphorylation can be induced by muscarinic receptor activation (Van Hooff et al., 1989).

Previously, we have provided extensive evidence that B-50 kinase is a substrate of PKC. Purified B-50 can be phosphorylated in vitro by PKC, but not by cyclic AMP-dependent protein kinases (Aloyo et al., 1983). Recently, we have identified the PKC phosphosite in the B-50 molecule as the serine residue at position 41 (Schrama et al., 1988). In synaptosomal plasma membranes (SPM) which have been heat-inactivated, B-50 is a major substrate for exogenously added PKC (De Graan et al., 1988a). The phorbol esters PDB and PMA increase B-50 phosphorylation in SPM (Eichberg et al., 1986; De Graan et al., 1988a, 1989) and in rat hippocampal slices (De Graan et al., 1988b, 1989; Dekker et al., 1989a). The PKC inhibitor polymyxin B inhibits B-50 phosphorylation in SPM (De Graan et al., 1989). Therefore, it is most likely that K^{+} -induced phosphorylation of B-50 is also mediated by PKC. In hippocampal slices, the PKC inhibitor polymyxin B indeed inhibits 30 mM K^{+} -induced phosphorylation of B-50 (Dekker et al., 1989a). To provide further evidence that PKC, rather than calmodulin-dependent protein kinases, is involved in K^{+} -induced phosphorylation of B-50 in synaptosomes, we applied several inhibitors of PKC and calmodulin-dependent kinases. We compared the effect of these inhibitors on K^{+} -induced B-50 phosphorylation with those on PDB-induced B-50 phosphorylation (which is certainly PKC-mediated). H-7 inhibits K^{+} - and PDB-induced B-50 phosphorylation to the same extent. The effective concentration is higher than that reported for inhibition of PKC in vitro (Hidaka et al., 1984). H-7, acting at the ATP-binding site of the kinase, inhibits PKC in vitro with an IC_{50} value of 6 μM (Hidaka et al., 1984). IC_{50} values for myosin light-chain kinase (a calmodulin-dependent kinase), cyclic AMP-dependent kinase, and cyclic GMP-dependent kinase are 97, 3.0, and 5.8 μM , respectively. The concentrations of H-7 inhibiting B-50 phosphorylation in the present study are the same as those reported to inhibit release of acetylcholine and serotonin from rabbit cortex slices (Daschmann et al., 1988). W-7 (Hidaka and Tanaka, 1983) also inhibits K^{+} - and PDB-induced B-50 phosphorylation to the same extent. Although W-7 is known as an inhibitor of calmodulin-dependent processes, the inhibition of the PDB-induced effect strongly indicates inhibition of PKC. Indeed, it has been reported that at high concentrations W-7 acts as an inhibitor of PKC (Hidaka and Tanaka, 1983). Staurosporine is a very effective inhibitor of PKC in vitro (Tamaoki et al., 1986). However, because it has been reported that the cyclic AMP-dependent kinase is inhibited with the same efficiency as PKC (Tamaoki et al., 1986), the specificity of this inhibitor is questioned. In the present study, stauro-

sporine more effectively inhibits PDB- than K^{+} -induced B-50 phosphorylation. Such a difference in sensitivity for staurosporine has also been reported for PDB- and K^{+} -induced release of [^3H] γ -aminobutyric acid from rabbit caudate nucleus slices (Bartmann et al., 1989) and may be due to a difference in the way PDB and K^{+} stimulate B-50 phosphorylation. Such a difference between PDB- and K^{+} -induced B-50 phosphorylation is also suggested by the observation that the effect of PDB does not require extracellular Ca^{2+} (Table 1). The calmodulin inhibitors calmidazolium and TFP do not inhibit either K^{+} - or PDB-induced B-50 phosphorylation. The present data, together with our previous results, strongly suggest that PKC is the kinase involved in K^{+} -induced B-50 phosphorylation.

We propose that PKC-mediated B-50 phosphorylation is induced by Ca^{2+} entering the synaptosome after the depolarizing stimulus. There are several possible mechanisms for the activation of PKC. Ca^{2+} may directly activate membrane-bound PKC or induce a translocation and subsequent activation of cytosolic PKC (Wakade et al., 1988). Alternatively, it may activate phospholipase C (Fisher and Agranoff, 1987), resulting in the production of diacylglycerol, which is generally regarded as the trigger for PKC activation. An effect of Ca^{2+} on a B-50 phosphatase cannot be ruled out.

Although the precise function of PKC-mediated B-50 phosphorylation has not yet been established, a close correlation exists between this process and transmitter release. Recently, we have obtained evidence for the crucial involvement of B-50 in transmitter release, because antibodies to B-50 inhibit Ca^{2+} -dependent release of noradrenaline in streptolysin-O-permeated synaptosomes (Dekker et al., 1989b). Future research will be aimed at providing more insight into the causal relationships between PKC-mediated B-50 phosphorylation and transmitter release.

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REFERENCES

- Allgaier C. and Hertting G. (1986) Polymyxin B, a selective inhibitor of protein kinase C, diminishes the release of noradrenaline and the enhancement of release caused by phorbol 12,13-dibutyrate. *Naunyn Schmiedeberg's Arch. Pharmacol.* **334**, 218-221.
- Allgaier C., Von Kügelgen O., and Hertting G. (1986) Enhancement of noradrenaline release by 12-O-tetradecanoyl phorbol-13-acetate, an activator of protein kinase C. *Eur. J. Pharmacol.* **129**, 389-392.
- Aloyo V. J., Zwiers H., and Gispen W. H. (1983) Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent

- protein kinase and B-50 protein kinase. *J. Neurochem.* **41**, 649–653.
- Andreasen T. J., Luetje C. W., Heideman W., and Storm D. R. (1983) Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry* **22**, 4615–4618.
- Augustine G. J., Charlton M. P., and Smith S. J. (1987) Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* **10**, 633–693.
- Bartmann P., Jackisch R., Hertting G., and Allgaier C. (1989) A role for protein kinase C in the electrically evoked release of [3 H] γ -aminobutyric acid in rabbit caudate nucleus. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**, 302–305.
- Benowitz L. I. and Routtenberg A. (1987) A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity. *Trends Neurosci.* **10**, 527–532.
- Bradford M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* **72**, 248–254.
- Cotman C. W., Haycock J. W., and White W. F. (1976) Stimulus-secretion coupling processes in brain: analysis of noradrenaline and gamma-aminobutyric acid. *J. Physiol. (Lond.)* **254**, 475–505.
- Daschmann B., Allgaier C., Nakov R., and Hertting G. (1988) Staurosporine counteracts the phorbol ester-induced enhancement of neurotransmitter release in hippocampus. *Arch. Int. Pharmacodyn. Ther.* **296**, 232–245.
- De Graan P. N. E., Dekker L. V., De Wit M., Schrama L. H., and Gispen W. H. (1988a) Modulation of B-50 phosphorylation and polyphosphoinositide metabolism in synaptic plasma membranes by protein kinase C, phorbol diesters and ACTH. *J. Recept. Res.* **8**, 345–361.
- De Graan P. N. E., Heemskerk F. M. J., Dekker L. V., Melchers B. P. C., Gianotti C., and Schrama L. H. (1988b) Phorbol esters induce long- and short-term enhancement of B-50/GAP-43 phosphorylation in hippocampal slices. *Neurosci. Res. Commun.* **3**, 175–182.
- De Graan P. N. E., Oestreicher A. B., Dekker L. V., Van der Voorn L., and Gispen W. H. (1989) Determination of changes in the phosphorylation state of the neuron specific protein kinase C substrate B-50 (GAP-43). *J. Neurochem.* **52**, 17–23.
- Dekker L. V., De Graan P. N. E., Versteeg D. H. G., Oestreicher A. B., and Gispen W. H. (1989a) Phosphorylation of B-50 (GAP-43) is correlated with neurotransmitter release in rat hippocampal slices. *J. Neurochem.* **52**, 24–30.
- Dekker L. V., De Graan P. N. E., Oestreicher A. B., Versteeg D. H. G., and Gispen W. H. (1989b) Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). *Nature* **342**, 74–76.
- Dunkley P. R. and Robinson P. J. (1986) Depolarization-dependent protein phosphorylation in synaptosomes: mechanisms and significance. *Prog. Brain Res.* **69**, 273–294.
- Dunkley P. R., Heath J. W., Harrison S. M., Jarvie P. E., Glenfield P. J., and Rostas J. A. P. (1988) A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. *Brain Res.* **441**, 59–71.
- Eichberg J., De Graan P. N. E., Schrama L. H., and Gispen W. H. (1986) Dioctanoylglycerol and phorbol diesters enhance phosphorylation of phosphoprotein B-50 in native synaptic plasma membranes. *Biochem. Biophys. Res. Commun.* **136**, 1007–1012.
- Fisher S. K. and Agranoff B. W. (1987) Receptor activation and inositol lipid hydrolysis in neural tissues. *J. Neurochem.* **48**, 999–1017.
- Franck G., Grisar T., and Moonen G. (1983) Glial and neuronal $\text{Na}^+ \text{K}^+$ pump. *Adv. Cell. Neurobiol.* **4**, 133–159.
- Gietzen K., Wüthrich A., and Bader H. (1981) R 24571: a new powerful inhibitor of red blood cell Ca^{++} -transport and of calmodulin-regulated functions. *Biochem. Biophys. Res. Commun.* **101**, 418–425.
- Gispen W. H., Leunissen J. L. M., Oestreicher A. B., Verkleij A. J., and Zwiers H. (1985) Presynaptic localization of B-50 phosphoprotein: the (ACTH)-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. *Brain Res.* **328**, 381–385.
- Gispen W. H., De Graan P. N. E., Schrama L. H., and Eichberg J. (1986) Phosphoprotein B-50 and polyphosphoinositide-dependent signal transduction in brain, in *Fidia Research Series, Vol. 4: Phospholipids in the nervous system: Biochemical and Molecular Pharmacology* (Horrocks L. A., Freysz L., and Tofano G., eds), pp. 31–41. Liviana Press, Padova.
- Hidaka H. and Tanaka T. (1983) Naphthalenesulfonamides as calmodulin antagonists. *Methods Enzymol.* **102**, 185–194.
- Hidaka H., Inagaki M., Kawamoto S., and Sasaki Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.
- Howell T. W., Cockcroft S., and Gomperts B. D. (1987) Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* **105**, 191–197.
- Kendall D. A. and Nahorski S. R. (1984) Inositol phospholipid hydrolysis in rat cerebral cortical slices. II. Calcium requirement. *J. Neurochem.* **42**, 1388–1394.
- Kristjansson G. I., Zwiers H., Oestreicher A. B., and Gispen W. H. (1982) Evidence that the synaptic phosphoprotein B-50 is localized exclusively in nerve tissue. *J. Neurochem.* **39**, 371–378.
- Kronvall G. (1973) A rapid slide-agglutination method for typing pneumococci by means of specific antibody absorbed to protein-A containing staphylococci. *J. Med. Microbiol.* **6**, 187–190.
- Levin R. M. and Weiss B. (1978) Specificity of the binding of trifluoperazine to the calcium-dependent activator of phosphodiesterase and to a series of other calcium-binding proteins. *Biochim. Biophys. Acta* **540**, 197–204.
- Matthies H. J. G., Palfrey H. C., Hirning L. D., and Miller R. J. (1987) Down regulation of protein kinase C in neuronal cells: effects on neurotransmitter release. *J. Neurosci.* **7**, 1198–1206.
- Miller R. J. (1989) Calcium signalling in neurons. *Trends Neurosci.* **11**, 415–419.
- Nicholls D. G., Sihra T. S., and Sanchez-Prieto J. (1987) Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem.* **49**, 50–57.
- Oestreicher A. B., Van Dongen C. J., Zwiers H., and Gispen W. H. (1983) Affinity-purified anti-B-50 protein antibody: interference with the function of phosphoprotein B-50 in synaptic plasma membranes. *J. Neurochem.* **41**, 331–340.
- Robinson P. J., Hauptschein R., Lovenberg W., and Dunkley P. R. (1987) Dephosphorylation of synaptosomal proteins P96 and P139 is regulated by both depolarization and calcium but not by a rise in cytosolic calcium alone. *J. Neurochem.* **48**, 187–195.
- Schrama L. H., De Graan P. N. E., Dekker L. V., Oestreicher A. B., Nielander H., Schotman P., and Gispen W. H. (1988) Functional significance and localization of phosphosite(s) in the neuron-specific protein B-50/GAP-43. *Soc. Neurosci. Abstr.* **14**, 197.15.
- Skene J. H. P. (1989) Axonal growth-associated proteins. *Annu. Rev. Neurosci.* **12**, 127–156.
- Sörensen R. G., Kleine L. P., and Mahler H. R. (1981) Presynaptic localization of phosphoprotein B-50. *Brain Res. Bull.* **7**, 57–61.
- Tamaoki T., Nomoto N., Takahashi I., Kato Y., Morimoto M., and Tomita P. (1986) Staurosporine, a potent inhibitor of phospholipid/ Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397–402.
- Tanaka C., Taniyama K., and Kusunoki M. (1984) A phorbol ester and A23187 act synergistically to release acetylcholine from the guinea pig ileum. *FEBS Lett.* **175**, 165–169.
- Van Hooff C. O. M., De Graan P. N. E., Oestreicher A. B., and Gispen W. H. (1989) Muscarinic receptor activation stimulates B-50/GAP-43 phosphorylation in isolated nerve growth cones. *J. Neurosci.* **9**, 3753–3759.
- Van Lookeren Campagne M., Oestreicher A. B., Van Bergen en Henegouwen P. M. P., and Gispen W. H. (1989) Ultrastructural

- immunocytochemical localization of B-50/GAP43, a protein kinase C substrate, in isolated presynaptic nerve terminals and neuronal growth cones. *J. Neurocytol.* **18**, 479-489.
- Versteeg D. H. G. and Florijn W. J. (1987) Phorbol 12,13-dibutyrate enhances electrically stimulated neuromessenger release from rat dorsal hippocampal slices in vitro. *Life Sci.* **40**, 1237-1243.
- Versteeg D. H. G. and Ulenkate H. J. L. M. (1987) Basal and electrically stimulated release of [3 H]noradrenaline and [3 H]dopamine from rat amygdala slices in vitro: effects of 4 β -phorbol 12,13-dibutyrate, 4 α -phorbol 12,13-didecanoate and polymyxin B. *Brain Res.* **416**, 343-348.
- Wakade A. R., Wakade T. D., Malhotra R. K., and Bhawe S. V. (1988) Excess K $^+$ and phorbol ester activate protein kinase C and support the survival of chick sympathetic neurons in culture. *J. Neurochem.* **51**, 975-983.
- Wang J. K. T., Walaas S. I., and Greengard P. (1988) Protein phosphorylation in nerve terminals: comparison of calcium/calmodulin-dependent and calmodulin/diacylglycerol-dependent systems. *J. Neurosci.* **8**, 281-288.
- Wei J.-W. and Wang E. K. (1987) Effects of calcium ionophore A23187 and calcium antagonists on 32 P $_i$ incorporation into polyphosphoinositides of rat cortical synaptosomes. *Int. J. Biochem.* **19**, 607-611.
- Zucker R. S. (1987) Neurotransmitter release and its modulation, in *Neuromodulation* (Kaczmarek L. K. and Levitan I. B., eds), pp. 243-263. Oxford University Press, New York.
- Zwiers H., Schotman P., and Gispen W. H. (1980) Purification and some characteristics of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. *J. Neurochem.* **34**, 1689-1699.