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Determination of Changes in the Phosphorylation State of the Neuron-Specific Protein Kinase C Substrate B-50 (GAP43) by Quantitative Immunoprecipitation

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Abstract: To determine changes in the degree of phosphorylation of the protein kinase C substrate B-50 in vivo, a quantitative immunoprecipitation assay for B-50 (GAP43, F1, pp46) was developed. B-50 was phosphorylated in intact hippocampal slices with $^{32}P_i$ or in synaptosomal plasma membranes with [γ-^{32}P]ATP. Phosphorylated B-50 was immunoprecipitated from slice homogenates or synaptosomal plasma membranes using polyclonal anti–B-50 antiserum. Proteins in the immunoprecipitate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the incorporation of ^{32}P into B-50 was quantified by densitometric scanning of the autoradiogram. Only a single 48-kilodalton phosphoband was detectable in the immunoprecipitate, but this band was absent when preimmune serum was used. The B-50 immunoprecipitation assay was quantitative under the following condition chosen, as (1) recovery of purified ^{32}P-labelled B-50 added to slice homogenates or synaptosomal plasma membranes was >95%; and (2) modulation of B-50 phosphorylation in synaptosomal plasma membranes with adrenocorticotrophic hormone, polymyxin B, or purified protein kinase C in the presence of phorbol diester resulted in EC_{50} values identical to those obtained without immunoprecipitation. With this immunoprecipitation assay we found that treatment of hippocampal slices with 4β-phorbol 12,13-dibutyrate stimulated B-50 phosphorylation, whereas 4α-phorbol 12,13-didecanoate was inactive. Thus, we conclude that the B-50 immunoprecipitation assay is suitable to monitor changes in B-50 phosphorylation in intact neuronal tissue. **Key Words:** B-50—GAP43—Protein kinase C—Phosphorylation in vivo—Rat brain—Immunoprecipitation. **De Graan P. N. E. et al.** Determination of changes in the phosphorylation state of the neuron-specific protein kinase C substrate B-50 (GAP43) by quantitative immunoprecipitation. *J. Neurochem.* **52,** 17–23 (1989).

The neuron-specific phosphoprotein B-50 (apparent molecular mass, 48 kilodaltons; IEP 4.5; Zwiers et al., 1980) is one of the major substrates of protein kinase C (PKC) in synaptosomal plasma membranes (SPM; Aloyo et al., 1983; Eichberg et al., 1986; De Graan et al., 1988) and in the membranes of nerve growth cones isolated from fetal rat brain (De Graan et al., 1985; Van Hooff et al., 1988). The B-50 protein is identical to the growth-associated protein GAP43 (Karns et al., 1987; Nielander et al., 1987), to F1 (Gispen et al., 1986; Chan et al., 1986), and to pp46 (Meiri et al., 1986). The physiological role of B-50 and its phosphorylation by PKC are still unknown, but B-50 has been implicated in the modulation of transmembrane signal transduction in receptor systems operating through polyphosphoinositide hydrolysis (Gispen et al., 1985*b*), in the molecular mechanisms underlying long-term potentiation (F1, Routtenberg, 1986), and in the mechanism of neurite outgrowth (GAP43, Skene and Willard, 1981; B-50, Verhaagen et al., 1986; Zwiers et al., 1987). In neurons, PKC is thought to be involved in neurotransmitter release (Nishizuka, 1986) and the regulation of ion channels (Kaczmarek, 1987).

To investigate the physiological role of B-50 phosphorylation by PKC, a technique is required to measure changes in the degree of B-50 phosphorylation in vivo.

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Abbreviations used: ACTH, adrenocorticotrophic hormone; 1-D, one-dimensional; 2-D, two dimensional; GAP43, growth-associated protein 43; IP, immunoprecipitation assay; 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; SPM, synaptosomal plasma membrane(s); TCA, trichloroacetic acid.

Until now, changes in B-50 phosphorylation in vivo were estimated with a post-hoc in vitro phosphorylation approach (Jork et al., 1984; De Graan et al., 1987; F1, Routtenberg and Lovinger, 1985). This in vitro assay measures the degree of B-50 phosphorylation after homogenization (and subcellular fractionation) using endogenous PKC and [γ-^{32}P]ATP. The degree of B-50 phosphorylation in vitro is thought to represent the number of nonphosphorylated sites in vivo. This post-hoc design assumes that no changes in B-50 phosphorylation occur during sample preparation and that changes in the endogenous PKC activity do not affect the number of sites phosphorylated in vitro (for a detailed discussion, see Routtenberg, 1982). However, as it has been shown that PKC activation involves translocation of PKC to, or tighter association with the plasma membrane (Kraft and Anderson, 1983), the last assumption is no longer valid.

Therefore, we have developed a phosphorylation assay to measure changes in the degree of B-50 phosphorylation in vivo. This assay involves in situ ^{32}P labelling of B-50 by incubation of tissue in $^{32}P_i$ and subsequent immunoprecipitation of B-50 with polyclonal anti–B-50 antiserum. Immunoprecipitation conditions were optimized to allow quantitative precipitation of B-50 from phosphorylated SPM and hippocampal slice homogenates. With this new method we show that B-50 phosphorylation in intact hippocampal slices is mediated by PKC.

MATERIALS AND METHODS

Chemicals

4β-Phorbol 12,13-dibutyrate (PDB), 4α-phorbol 12,13-didecanoate (4α-PDD), and polymyxin B sulfate (7,800 U/mg) were purchased from Sigma (St. Louis, MO, U.S.A.). [γ-^{32}P]ATP (sp. act. 3,000 Ci/mmol) and $^{32}P_i$ (carrier-free) were obtained from Amersham (Bucks, U.K.). Adrenocorticotrophic hormone (ACTH1-24) was a gift from Organon International BV (Oss, The Netherlands). Formaldehyde-inactivated *Staphylococcus aureus* cell membranes (SAC) were kindly provided by Dr. W. H. Jansen (RIVM, Bilthoven, The Netherlands).

Slice and SPM preparation

In all experiments, male rats (140–150 g) of an inbred Wistar strain (TNO, Zeist, The Netherlands) were used. SPM were prepared from forebrains (Kristjansson et al., 1982) and resuspended at 1 mg/ml in phosphorylation buffer (buffer A: 10 m*M* Tris/HCl, 10 m*M* MgCl, and 0.1 m*M* $CaCl_2$; pH 7.4).

Hippocampi were dissected within 2 min of decapitation. Transverse hippocampal slices (400 μm) were prepared at 4°C as described by Tielen et al. (1983). Slices (three per tube) were preincubated for 30 min in 2 ml of phosphate-free Krebs–Ringer buffer (buffer B: 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) equilibrated with 5% CO_2/95% O_2 at 34°C.

Protein phosphorylation

SPM. The phosphorylation assay was performed according to Zwiers et al. (1976) as modified by Kristjansson et al. (1982). The phosphorylation reaction mixture (final volume, 25 μl) contained 10 μg SPM protein, 7.5 μ*M* ATP, and 2 μCi [γ-^{32}P]ATP in buffer A. After a preincubation at 30°C for 5 min, the phosphorylation reaction was started by the addition of ATP. The phosphorylation reaction was stopped after 15 s by adding 12.5 μl sodium dodecyl sulfate (SDS)-denaturing solution (Zwiers et al., 1976). ACTH1-24 was added 15 s and the phorbol diesters, 75 s before the addition of ATP. In some experiments, endogenous enzymes were inactivated prior to the phosphorylation assay by heating SPM proteins for 5 min at 100°C. In these experiments, 0.24 μg (2 μl) of purified PKC (Kikkawa et al., 1986) was added at the start of the preincubation.

Hippocampal slices. After preincubation of the slices for 30 min, buffer B was replaced with 0.9 ml of buffer B containing 100 μCi $^{32}P_i$ per tube. The slices were incubated for 90 min, then 100 μl buffer B with or without phorbol diester was added. The incubation was terminated after 30 min by two quick washes of the slices in ice-cold buffer B containing 1.2 m*M* K_2HPO_4, 100 m*M* NaF, 10 m*M* EDTA, and 5 m*M* EGTA. The three slices in each tube were pooled and homogenized in 100 μl H_2O containing 100 m*M* NaF, 10 m*M* EDTA and 5 m*M* EGTA (adjusted to pH 7.4) by 10 up-and-down strokes in a Potter–Elvehjem tube with a Teflon pestle (clearance, 50 μm). Immediately after homogenization, 40 μl of SDS-denaturing solution (final concentrations: 62.5 m*M* Tris-HCl, pH 6.5; 2% SDS; 10% glycerol; 0.001% bromophenol blue; 5% 2-mercaptoethanol; Zwiers et al., 1976) was added to 80 μl of the homogenate. The denatured homogenates were stored at −20°C to be used in the immunoprecipitation assay or to analyze ^{32}P incorporation into total homogenate protein by 11% SDS-polyacrylamide gel electrophoresis (PAGE) according to Zwiers et al. (1976). The remainder of each homogenate was used to determine protein (Bradford, 1976) and to determine ^{32}P incorporation into total protein by trichloroacetic acid (TCA) precipitation. For TCA precipitation, 20 μg homogenate protein was spotted on Whatman 3MM filter paper. Filters were washed with 10% TCA, alcohol, and acetone (to remove phospholipids) according to Corbin and Reimann (1974). Phosphopeptide mapping was performed according to Zwiers et al. (1985).

Immunoprecipitation of ^{32}P-labelled B-50

The immunoprecipitation assay was developed after a procedure used in the radioimmunoassay of B-50 (Oestreicher et al., 1986). Denatured phosphorylated SPM or hippocampal homogenates were adjusted to 1 μg protein/20 μl and 30 μg protein/20 μl, respectively, with dilute denaturing solution (1:2 vol/vol). Subsequently, 280 μl buffer C (200 m*M* NaCl, 10 m*M* EDTA, 10 m*M* Na_2HPO_4 and 0.5% Nonidet P-40) and 100 μl anti–B-50 antiserum (8502, diluted 1:50 in buffer C) were added, resulting in a final SDS concentration of 0.1% in a total volume of 400 μl. The antibodies were allowed to bind overnight at 4°C. Formaldehyde-inactivated SAC were washed twice with buffer C. To bind the anti–B-50 antibodies, 50 μl of a 4% SAC suspension in buffer C was added and incubated for 30 min at room temperature. After centrifugation for 10 min at 4,800 *g* in a Sorvall HS4 rotor, the SAC pellet was resuspended in 30 μl dilute denaturing solution (1:2 vol/vol) and the samples were boiled for 10 min. ^{32}P incorporation into immunoprecipitates and total protein were analyzed by 11% SDS-PAGE according to Zwiers et al. (1976) followed by autoradiography and densitometric scanning of the autoradiogram (Wiegant et al., 1978) or liquid scintillation counting of the excised B-50 band.

RESULTS

Immunoprecipitation of B-50 from SPM

Endogenous phosphorylation of SPM with [γ-^{32}P]ATP resulted in the phosphorylation of a number of phosphoprotein bands; on the autoradiogram the most prominent band after one-dimensional (1-D) SDS-PAGE is B-50 (Fig. 1A). Thus, in SPM, the degree of B-50 phosphorylation can be quantified directly without immunoprecipitation (Zwiers et al., 1976; Eichberg et al., 1986). After in vivo phosphorylation of brain tissue with $^{32}P_i$, B-50 is only a minor phosphoprotein that is not detectable after 1-D protein separation without immunoprecipitation (see Fig. 5). Therefore, only phosphorylated SPM is suitable to optimize conditions for the immunoprecipitation of B-50 and to validate the immunoprecipitation assay because it allows quantification of B-50 phosphorylation with and without immunoprecipitation. Incubation of the solubilized phosphorylated SPM proteins with polyclonal anti–B-50 antiserum in the presence of detergent, followed by precipitation of the antibody–antigen complex with SAC membranes, resulted in specific immunoprecipitation of B-50 (Fig. 1A). Only after long exposure times were other phosphobands detectable in the immunoprecipitate; these same faint bands were found in the immunopellet after incubation with preimmune serum. Immunoprecipitation of phosphorylated B-50 was optimal at a 1:200 dilution of the antiserum (Fig. 1A and B). Higher concentrations of antibody reduced the recovery of ^{32}P-labelled B-50, probably due to the interference of serum proteins. Thus, in all further assays antiserum was diluted 1:200. At this dilution, 82.4 ± 6.1% (n = 8) of the radioactivity present in the B-50 band in SPM was recovered after immunoprecipitation. The recovery after immunoprecipitation of purified ^{32}P-labelled B-50 added to nonphosphorylated SPM was 93.3 ± 4.3% (n = 8). The efficiency of precipitation of purified ^{32}P-labelled B-50 was not affected by the amount of SPM protein, to a maximum of 2.5 μg per assay (Fig. 2). The percentage of B-50 immunoprecipitable from SPM was independent of the amount of SPM protein up to 1.5 μg per assay (Fig. 2). In all further experiments, we used 1 μg of SPM protein per assay.

In the next series of experiments, we modulated the degree of B-50 phosphorylation in SPM and compared the results obtained with the immunoprecipitation assay (IP assay) with those obtained without immunoprecipitation (direct assay). Endogenous B-50 phosphorylation in SPM can be inhibited by the peptide hormone ACTH1-24 (Zwiers et al., 1976; Fig. 3A). The inhibition of B-50 phosphorylation as measured by the IP assay showed an EC_{50} of $5.1 \pm 0.3 \times 10^{-6}$ *M*, a value indistinguishable from the EC_{50} determined by the direct assay (Fig. 3A). With both assays, the lowest concentration of ACTH1-24 that produced a significant inhibition was 3×10^{-7} *M*. Endogenous B-50 phosphorylation can also be inhibited by the antibiotic polymyxin B (Fig. 3B), a relatively selective inhibitor of PKC (Mazzei et al., 1982; Kuo et al., 1984) that has been used in intact hippocampal slices (Allgaier and Herrting, 1986). Polymyxin B inhibited B-50 phosphorylation at an EC_{50} of 34.5 ± 9.1 U/assay and 29.4 ± 6.4 U/assay, as measured by the direct and the IP assay, respectively. In both assays, the lowest concentration producing a significant inhibition by polymyxin B was 10 U/assay. Thus, the sensitivity of both assays to inhibition of B-50 phosphorylation is identical.

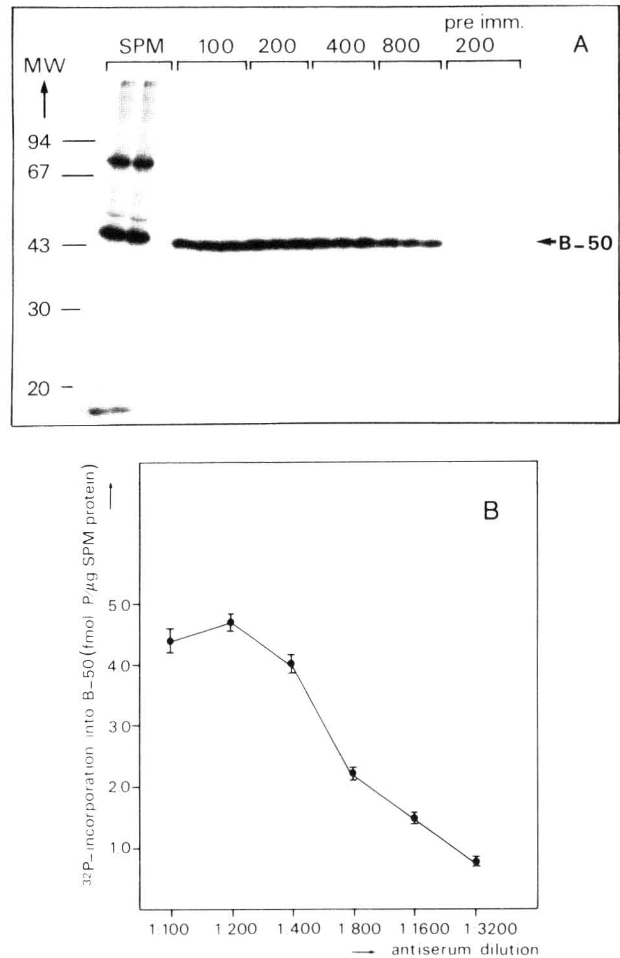

FIG. 1. Immunoprecipitation of B-50 from phosphorylated SPM with different concentrations of anti–B-50 serum. **A:** Autoradiogram showing ^{32}P incorporation into B-50 with and without immunoprecipitation. After endogenous phosphorylation of SPM with [γ-^{32}P]ATP, 1 μg of SPM protein was analyzed directly for ^{32}P incorporation into B-50 on 11% SDS-PAGE (SPM lanes) or after subsequent immunoprecipitation with anti–B-50 serum 8502 (dilutions, 1:100–1:800) or with preimmune serum (pre imm. lanes; dilution, 1:200). MW: positions of molecular weight markers. **B:** Quantification of the antiserum dilution curve in A. ^{32}P incorporation into B-50 was measured by liquid scintillation counting of the excised B-50 band and expressed in fmol P/μg SPM protein.

B-50 phosphorylation by purified PKC in heat-inactivated SPM is enhanced almost tenfold in the presence of 10^{-6} *M* tumor-promoting phorbol diesters (De Graan et al., 1988). Here we tested whether such large

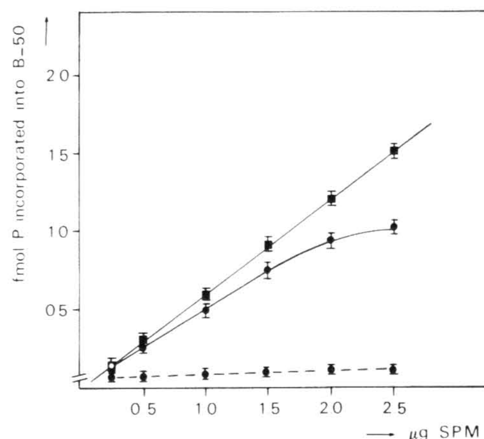

FIG. 2. Relationship between the ^{32}P incorporation into B-50 and the SPM protein concentration used in the immunoprecipitation assay. ^{32}P incorporation into the B-50 band was measured after 11% SDS-PAGE without (■ — ■) and with (● — ●) immunoprecipitation. (● - - - ●): immunoprecipitation of purified ^{32}P-labelled B-50 at different SPM protein concentrations.

differences in the degree of B-50 phosphorylation can be quantified by the immunoprecipitation method (Fig. 4). Up to concentrations of 10^{-6} *M* PDB, no significant differences could be detected between the direct and the IP assays. At 10^{-5} *M* PDB, the direct method appears to be slightly more sensitive (Fig. 4), but this is due to the difficulty of quantifying such a highly phosphorylated band by the direct method. With both methods, 10^{-8} *M* PDB is the lowest concentration exerting significant effects on B-50 phosphorylation. Having established that the IP method is quantitative under the conditions chosen, we applied this assay to quantify the degree of B-50 phosphorylation in intact hippocampal slices.

Hippocampal slices

Incubation of hippocampal slices with ^{32}P-labelled orthophosphate for 90 min resulted in the radiolabelling of many protein bands (Fig. 5). The B-50 phosphoprotein could not be detected after 1-D separation of the homogenate proteins (Fig. 5, lanes total). Two-dimensional (2-D) separation of slice homogenate proteins revealed a phosphoprotein with an apparent molecular mass of 48 kilodaltons and an IEP of 4.5, comigrating with purified ^{32}P-labelled B-50 (results not shown). Limited digestion of the phosphorylated protein with *Staphylococcus aureus* protease revealed two phosphopeptides with apparent molecular masses of 28 and 15 kilodaltons. This phosphopeptide map is identical to that obtained with purified B-50 (Zwiers et al., 1985). Phosphoamino acid analysis of the phosphoprotein and of purified B-50 reveals only ^{32}P incorporation into phosphoserine (results not shown). Thus, we conclude that the phosphorylated protein is identical to B-50.

Immunoprecipitation of B-50 from hippocampal slice homogenates with polyclonal anti–B-50 antiserum resulted in a single phosphoprotein band in the immunoprecipitate (Fig. 5, lanes 3), and it was identified as B-50 by 1-D and 2-D gel electrophoresis and comigration with purified B-50. This immunoprecipitation is specific, since precipitation in the absence of anti–B-50 antiserum (Fig. 5, lanes 1) or with preimmune serum (Fig. 5, lanes 2) did not result in the detection of any phosphoproteins in the precipitate. The amount of B-50 immunoprecipitated is linear with the amount of protein in the slice homogenate up to 30 μg per

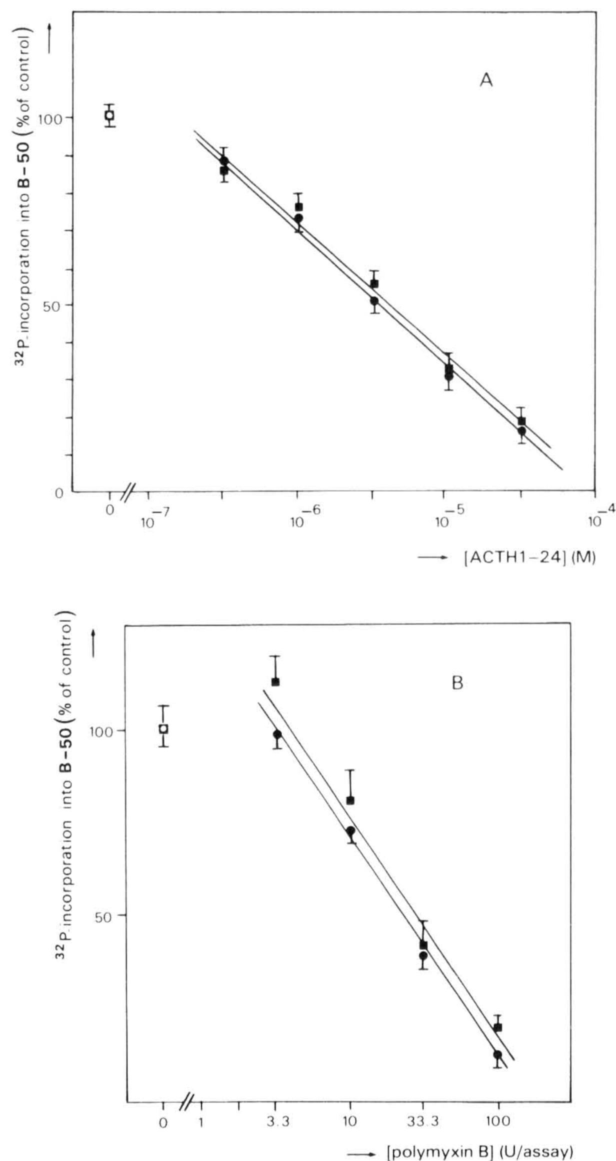

FIG. 3. Quantification of the inhibitory effects of ACTH1-24 (**A**) and polymyxin B (**B**) on endogenous B-50 phosphorylation in SPM after 11% SDS-PAGE without (■ — ■) and with (● — ●) immunoprecipitation (see Materials and Methods). Each point represents at least six determinations. Bars indicate SEM.

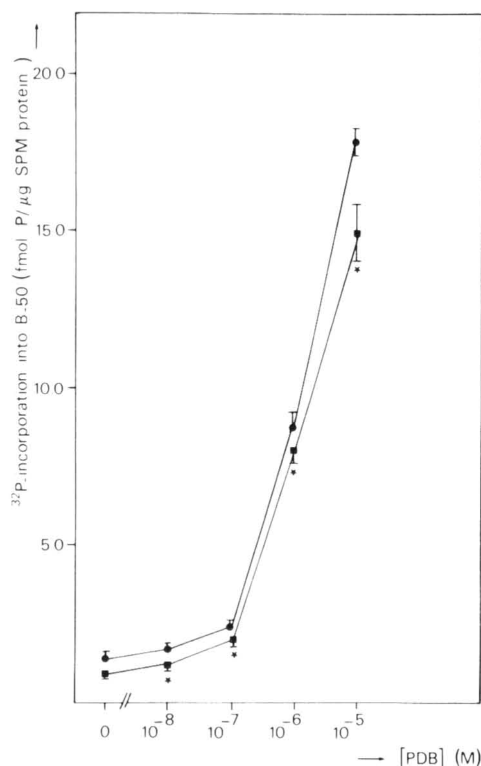

FIG. 4. Quantification of the stimulatory effect of PDB (10^{-6} *M*) on PKC-mediated B-50 phosphorylation in heat-inactivated SPM. SPM protein was heat-inactivated and phosphorylated with purified PKC in the presence of different PDB concentrations. ^{32}P incorporation into the B-50 band was quantified after 11% SDS-PAGE without (● — ●) or with (■ — ■) immunoprecipitation. $^{*}p < 0.05$ when compared with controls without PDB (n = 6). Bars indicate SEM.

assay. Routinely, we use 10 µg per assay. Under these immunoprecipitation conditions, purified ^{32}P-labelled B-50 is precipitated with 95 ± 3% efficiency. The efficiency of precipitation is not affected by protein concentrations up to 40 µg per assay (results not shown).

Treatment of hippocampal slices with 10^{-6} *M* PDB for 30 min strongly enhances the degree of B-50 phosphorylation (Fig. 5, lanes 4). A 30-min incubation with 10^{-6} *M* 4α-PDD, a phorbol diester known to be inactive in stimulating PKC, does not significantly affect B-50 phosphorylation. The phorbol diester treatment does not affect ^{32}P incorporation into total protein, as judged by TCA precipitation and the phosphoprotein pattern of the slice homogenates after 11% SDS-PAGE.

DISCUSSION

The B-50 protein, which is identical to GAP43, F1, and pp46, has been implicated in a number of physiological processes in neurons: the modulation of receptor-mediated signal transduction, neurite outgrowth, and long-term potentiation (for references see the introductory section). B-50 is one of the few well-characterized neuronal substrates of PKC, an enzyme that is thought to play an important role in the regulation of ion channels (Kaczmarek, 1987) and neurotransmitter release (Nishizuka, 1986). This implies that modulation of B-50 phosphorylation may be an important pathway in PKC-mediated physiological effects.

Measuring the degree of B-50 phosphorylation in vivo after $^{32}P_i$ labelling is difficult because the incorporation of ^{32}P-labelled phosphate into membrane proteins is low. In vivo ^{32}P-labelling of B-50 or B-50–like proteins has been reported after intracisternal injection of $^{32}P_i$ into young rats (Oestreicher et al., 1982), local injection of $^{32}P_i$ into adult rat brain (F1: Mitrius et al., 1981; B-50: Rodnight et al., 1986), injection of $^{32}P_i$ into the eye in goldfish (Larrivee and Grafstein, 1987), and in primary cultured rat neurons (Burgess et al., 1986). Labelling of B-50 with ^{32}P after $^{32}P_i$ incubation has also been shown in brain slices (Rodnight et al., 1986; Dekker et al., 1987) and in synaptosomes (Dunkley and Robinson, 1986; Rodnight et al., 1986). In most of these in vivo labelling studies, 2-D separation systems were used to resolve the minor phospho-spot of B-50 from other phosphoproteins. In this study, we report that incubation of hippocampal slices with $^{32}P_i$ for 90 min results in B-50 labelling that can be detected and quantified easily after immunoprecipitation followed by 1-D SDS-PAGE. Immunoprecipitation from slice homogenates is specific, as only a single phosphoband can be detected. Under the conditions chosen,

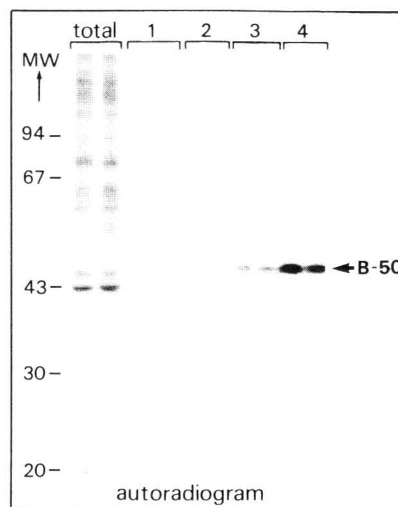

FIG. 5. Immunoprecipitation of phosphorylated B-50 from $^{32}P_i$-labelled hippocampal slices. Hippocampal slices were prelabelled for 90 min with $^{32}P_i$ and further labelled for 30 min in the presence (lanes 4) or absence (lanes 1–3) of 10^{-6} *M* PDB. Slices were homogenized, and B-50 was immunoprecipitated from the homogenates (see Materials and Methods). Total homogenate protein (lanes total; 1.5 µg) and the immunoprecipitates were analyzed on 11% SDS-PAGE. Immunoprecipitation was performed without anti–B-50 serum (lanes 1), with preimmune serum (lanes 2), and with anti–B-50 serum 8502 (dilution, 1:200). MW: positions of the molecular weight markers.

the immunoprecipitation assay is quantitative because (1) the amount of immunoprecipitable ^{32}P-labelled B-50 is linear with the amount of homogenate protein (range 1–30 μg); (2) the efficiency of precipitation of purified ^{32}P-labelled B-50 is about 95%, irrespective of the amount of homogenate protein (range 1–30 μg); and (3) quantification of the modulation of B-50 phosphorylation in SPM by ACTH, polymyxin B, or phorbol diesters in combination with purified PKC, results in EC_{50} values indistinguishable from those obtained by direct measurement of ^{32}P incorporation into the B-50 band after 1-D SDS-PAGE. Thus, the immunoprecipitation assay is a suitable method to monitor changes in B-50 phosphorylation in vivo, and may be a valuable tool to assess PKC activity in vivo, with endogenous substrate.

PKC activity in many cell types can be stimulated by tumor-promoting phorbol diesters (Nishizuka, 1986). In synaptosomes labelled with $^{32}P_i$, phorbol diesters were shown to stimulate phosphorylation of an 87-kilodalton PKC substrate as measured by ^{32}P incorporation into identified proteolytic phosphopeptide fragments (Nichols et al., 1987). In primary cultured neurons, phosphorylation of a 47-kilodalton phosphoprotein (IEP 4.8) was reported to increase upon phorbol diester treatment, as visualized by autoradiography after 2-D protein separation (Burgess et al., 1986). In this article we have shown that phorbol diesters enhance the degree of B-50 phosphorylation in intact hippocampal slices. 4α-PDD, a phorbol diester that does not stimulate PKC, has no effect on B-50 phosphorylation. These data confirm earlier results from in vitro studies with SPM (Aloyo et al., 1983; Eichberg et al., 1986; De Graan et al., 1988) and show that B-50 is a physiological substrate of PKC.

As B-50 is a neuron-specific PKC substrate localized predominantly in the presynaptic membrane (Gispen et al., 1985*a*), it is likely that B-50 phosphorylation is involved in PKC-mediated presynaptic events, such as neurotransmitter release. Modulation of neurotransmitter release has been described with both PKC agonists and antagonists (Allgaier and Herrting, 1986; Zurgil et al., 1986; Nichols et al., 1987; Versteeg and Ulenkate, 1987). Therefore, it will be of interest to apply the immunoprecipitation assay described in this article to study a possible role of B-50 phosphorylation in neurotransmitter release.

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