THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 10, Issue of March 11, pp. 7564-7570, 1994
Printed in U.S.A.

Synaptic Vesicles Isolated from ^{32}P-Prelabeled Synaptosomes Contain a Phosphoprotein of Apparent M_r 65,000 (pp65), a Possible Substrate for PKC*

(Received for publication, August 3, 1993)

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In the present study, we have investigated the subcellular localization of pp65, a synaptosomal phosphoprotein of apparent M_r 65,000. The results obtained strongly support that pp65 is localized to synaptic vesicles. The solubility properties of pp65, especially its partitioning into the detergent phase of Triton X-114, indicated that it is tightly associated with the membrane of synaptic vesicles. pp65 is multiply phosphorylated exclusively on serine. By studying the decay of labeled phosphate following incubation of ^{32}P-prelabeled synaptosomes in the presence of cold inorganic phosphate, we have found that pp65 shows an unusually high turnover of phosphate. Exposure of synaptosomes to 1 μM phorbol 12-myristate 13-acetate prior to prelabeling with $^{32}P_i$ led to a reduction in the steady state phosphorylation of pp65, and tryptic/chymotryptic mapping was shown to selectively affect phosphopeptide 4. Identical results were obtained following incubation of synaptosomes with the protein kinase C (PKC) inhibitor, GF 109203 X. These results indicated that one of the protein kinases involved in steady state phosphorylation of pp65 is PKC-dependent or is PKC itself. Several characteristics of pp65 reported in the present study suggest a regulatory role in nerve terminal function.

Current views on the molecular events underlying the process of exocytosis propose that a defined population of synaptic vesicles docked at active zones along the plasma membrane can mediate the early release of neurotransmitter (Thomas and Almers, 1992; Kelly, 1993). It is believed that the interaction between proteins of the submembrane cytoskeleton and synaptic vesicles may be important in the regulation of neurotransmitter release by holding synaptic vesicles at the plasma membrane ready for exocytosis and that protein phosphorylation/dephosphorylation might be involved in the modulation of that interaction (Robinson, 1992b; Greengard *et al.*, 1993). One experimental approach to identify phosphoproteins potentially involved in the regulation of exocytosis is to analyze the changes in the phosphorylation state of nerve terminal proteins in response to stimuli that induce or modulate the release of neurotransmitter. Isolated nerve terminals (synaptosomes) have been widely used in studies on the mechanisms of neurotransmitter release. Although synaptosomes are heterogeneous with respect to transmitter content, they are homogeneous in terms of bioenergetic parameters and possess all the components involved in coupling between calcium entry through voltage-sensitive calcium channels and neurotransmitter release (for a review, see McMahon and Nicholls (1991)). In this preparation, the phosphorylation state of the nerve terminal protein synapsin I, B-50 (neuromodulin), and P96 has been shown to be associated with the regulation of neurotransmitter release (Nichols *et al.*, 1990; Dekker *et al.*, 1989a; Sihra *et al.*, 1992).

We have previously reported that a synaptosomal phosphoprotein of an apparent M_r in SDS-PAGE[1] of 65,000 (pp65) undergoes a transient dephosphorylation following depolarization of synaptosomes under conditions in which calcium influx is limited (Gómez-Puertas *et al.*, 1991). In this work, we have set up methods to study the subsynaptosomal localization of pp65 while preserving ^{32}P labeling of the protein. Our results provide evidence that pp65 is localized to synaptic vesicles, showing solubility properties that indicate a tight association with the membrane vesicle. The turnover of phosphate of pp65 is very high in resting intact synaptosomes, and one of the protein kinases involved in the phosphorylation/dephosphorylation process is PKC-dependent or is PKC itself.

EXPERIMENTAL PROCEDURES

Preparation of Synaptosomes—Synaptosomes from whole rat brain were prepared according to the method of Dunkley *et al.* (1986b), using a discontinuous Percoll gradient as modified by Wang *et al.* (1989).

Protein Phosphorylation—Synaptosomes (2 mg/ml) were labeled with [^{32}P]orthophosphate at 2 mCi/ml for 30 min at 37 °C under an oxygen atmosphere in basal medium containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM $NaHCO_3$, pH 7.4, 1 mM $MgCl_2$, 100 mM glucose, and 0.5 mM $CaCl_2$. Phosphorylated proteins were subjected to SDS-PAGE as described previously (Gómez-Puertas *et al.*, 1991).

Subsynaptosomal Fractionation and Synaptic Vesicles Isolation—2 mg of ^{32}P-prelabeled synaptosomes were lysed at 4 °C for 20 min in 1 ml of hypo-osmotic buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 8.1, 50 mM NaF, 5 mM pyrophosphate-tetrasodium salt, 5 mM EDTA, and 40 mM *N*-ethylmaleimide). The lysate was centrifuged at 47,000 × *g* for 20 min and the supernatant subjected to centrifugation at 200,000 × *g* for 2 h. The pellet from this centrifugation (crude synaptic vesicles) was resuspended in 0.3 ml of chromatography buffer (0.3 M glycine, 5 mM HEPES-KOH, pH 7.4, 40 mM *N*-ethylmaleimide) and loaded onto a controlled pore glass column (CPG-3000, 1 × 120 cm, flow rate 0.3 ml/min, fraction volume 1.2 ml), collecting the purified synaptic vesicles from the first peak after the void volume peak, essentially as described by Hell *et al.* (1988).

Solubilization of Proteins from Synaptic Vesicles—CPG-purified synaptic vesicles (4 μg of protein) were incubated for 1 h at 4 °C in solubilization buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 150 mM NaCl, 2 mM

* This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología and from Boëhringer-Ingelheim, S.A., and by an institutional grant from the Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a fellowship from the Ministerio de Educación y Ciencia.

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[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CPG, controlled pore glass; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

EDTA; final volume 100 µl) containing various salts and detergents (1% (w/v) Triton X-100, 1 M KCl, 1% (w/v) sodium deoxicholate or 0.1 M Na_2CO_3, pH 11), as described by Huttner *et al.* (1983). After solubilization, the vesicles were subjected to centrifugation at 200,000 × *g* for 2 h in a Beckman TLA 100.3 rotor, and the pellets, containing the nonextracted proteins, were analyzed by SDS-PAGE. Temperature-induced phase separation in Triton X-114 was performed according to Bordier (1981) with the modifications described by Hooper and Bashir (1991); 4 µg of CPG-purified synaptic vesicles were resuspended in 200 µl of 10 mM Tris-HCl, pH 7.4, 2% (w/v) Triton X-114. After 5 min at 4 °C, the suspension was centrifuged at 200,000 × *g* for 2 h at 4 °C. The pellet represented the Triton X-114-insoluble fraction. The supernatant, or soluble fraction, was layered onto 0.3 ml of 0.6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% (w/v) Triton X-114, incubated at 30 °C for 3 min, and centrifuged at 3,000 × *g* for 3 min at room temperature. Then, the upper phase was removed, incubated at 4 °C for 5 min in the presence of 0.5% (w/v) Triton X-114, layered again onto the gradient, and centrifuged at 3,000 × *g* for 3 min at room temperature. The upper aqueous phase of the sucrose gradient was incubated with 2% (w/v) Triton X-114 at 4 °C for 5 min and then at 30 °C for 3 min and centrifuged at 3,000 × *g* for 3 min at room temperature; the bottom detergent phase was discarded, and the upper phase was used as the final aqueous phase. The detergent drop present at the bottom of the sucrose gradient was the final detergent phase. The Triton X-114-insoluble, aqueous, and detergent phases were resuspended in an SDS solution and subjected to SDS-PAGE.

Phosphopeptide and Phosphoamino Acid Analysis—Analysis of the phosphorylated peptides and amino acids was performed essentially as described by Boyle *et al.* (1991), using trypsin and chymotrypsin (two additions of 20 µg/protease during an incubation of 24 h at 37 °C). Phosphopeptides were separated by thin layer electrophoresis using cellulose TLC plates and 5% (v/v) acetic acid, 0.5% (v/v) pyridine, pH 3.5, as buffer (1,000 V, 90 min), followed by ascending chromatography for 8–10 h using a mobile phase composed of *n*-butanol:pyridine:acetic acid:water in the proportion 15:10:3:12 (v/v). Acid hydrolysis of the protein prior to phosphoamino acid analysis was performed in 6 N HCl at 110 °C for 1 h (partial acid hydrolysis) or 2 h (total acid hydrolysis). Phosphoamino acids were separated by electrophoresis using the buffer described above for 1 h at 1,000 V, and migration was determined by ninhydrin staining of standards.

Gel Electrophoresis and Western Blotting—SDS-PAGE was performed as previously described (Gómez-Puertas *et al.*, 1991). Western blotting was performed following Harlow and Lane (1988), using 25 mM Tris, 190 mM glycine, 20% methanol as transfer buffer. Washed nitrocellulose sheets were incubated with a commercial anti-synaptophysin antibody (Boehringer Mannheim), and the immunoreaction products were visualized in an x-ray film using the enhanced chemiluminiscence kit from Amersham Corp.

Determination of PKC Activity—PKC activity in synaptosomal lysates and soluble and particulate fractions was determined following Oda *et al.* (1991) and Aguilera *et al.* (1993). In brief, synaptosomes (2 mg/ml) were lysed at 4 °C for 30 min. The lysate was centrifuged for 1 h at 100,000 × *g* to obtain the soluble and particulate fractions. Partial enrichment of PKC activity was achieved by DE52 chromatography according to Aguilera *et al.* (1993). PKC was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 in the presence of 1.5 mM $CaCl_2$, 40 µg/ml phosphatidylserine, and 4 µg/ml 1-oleoyl-2-acetyl-glycerol as described by Oda *et al.* (1991).

Other Methods—Other enzyme assays used were as follows: rotenone-insensitive NADPH-cytochrome *c* reductase (Vermilion and Coon, 1978), 5′-nucleotidase (Ipata, 1968), and cytochrome *c* oxidase (Warton and Zagaloff, 1967). Protein content was determined according to Lowry *et al.* (1951). Quantitation of autoradiograms and of the chemiluminiscence fixed in x-ray films was performed in a 300 A computing densitometer from Molecular Dynamics. Results are expressed as the mean ± S.E., and statistical significance was analyzed using Student's *t* test.

Materials—GF 109203 X was a generous gift of Dr. Kirilovsky from Glaxo (Cedex, France). Tosylphenylalanyl chloromethyl ketone-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ). Chymotrypsin, leupeptin, and monoclonal antibody to synaptophysin were purchased from Boehringer Mannheim. [^{32}P]Orthophosphate in aqueous solution was from DuPont NEN. [γ-^{32}P]ATP was from Amersham International (United Kingdom). DE52 was obtained from Whatman BioSystems Ltd. (Maidstone, UK). Cellulose TLC plates were from Merck (Darmstadt, Germany). *N*-Ethylmaleimide, PMA, ATP, phosphatidylserine, 1-oleoyl-2-acetyl-glycerol, Percoll, and controlled pore glass beads (3,000 Å pore size, glyceryl-coated) were obtained from Sigma. All other chemicals were of the highest grade available.

RESULTS AND DISCUSSION

Subsynaptosomal Localization of pp65—The initial objective of this work was to investigate the subcellular localization of pp65. We set up a subfractionation protocol aimed at maintaining the nerve terminal phosphoproteins in their phosphorylated state, which was based in the procedure described by Hell *et al.* (1988). Briefly, ^{32}P-prelabeled synaptosomes were hypoosmotic lysed and centrifuged at 47,000 × *g* to pellet intact synaptosomes, mitochondria, and large membrane fragments. The supernatant was then centrifuged at 200,000 × *g* for 2 h. The pellet (crude synaptic vesicles) was chromatographed on a CPG column as the final step in the purification of synaptic vesicles. The different fractions obtained were analyzed by SDS-PAGE and ^{32}P autoradiography. It is worth noting that hypo-osmotic lysis of synaptosomes in the presence of the usual phosphatase inhibitors (EGTA, NaF, $NaPP_i$, orthovanadate) or a combination of phosphatase and protease inhibitors systematically resulted in an immediate loss of pp65 (results not shown). Since no phosphorylated bands of lower molecular weight appeared in the synaptosomal lysate after the hypoosmotic shock, loss of the pp65 band could be explained by dephosphorylation of the protein. The protective effect of sulfhydryl reagents during *in vivo* phosphorylation in *Drosophila* was noted by Buxbaum and Dudai (1987), and, therefore, we tested their potential protection in our system. Thus, inclusion in the lysis buffer of 40 mM *N*-ethylmaleimide prevented pp65 loss, preserving the phosphoprotein pattern shown by intact synaptosomes (Fig. 1*A*, *SP* and *LYS*). *p*-hydroximercurybenzoate and iodoacetamide were also effective (results not shown). Fractionation of the synaptosomal lysate by differential centrifugation to obtain synaptosomal cytosol, synaptic plasma membranes, and crude synaptic vesicles revealed a clear enrichment of the 65-kDa phosphoprotein band in the crude synaptic vesicle fraction (Fig. 1*A*, *cSV*). To investigate whether pp65 was present in synaptic vesicles or in contaminating membranes, ^{32}P-labeled synaptic vesicles were purified by chromatography of the crude synaptic vesicle fraction on CPG beads. The elution profile for total protein, synaptophysin (synaptic vesicle), rotenone-insensitive NADPH-cytochrome *c* reductase (endoplasmic reticulum), 5′-nucleotidase (plasma membrane), and cytochrome *c* oxidase (mitochondrial membrane) from CPG-chromatography is shown in Fig. 1*B*. The two peaks corresponding to synaptic vesicles (*Peak II*) and larger membrane vesicles (*Peak I*) (Hell *et al.*, 1988) were clearly defined. Fractions collected as Peak I and Peak II were analyzed by autoradiography for the presence of the 65-kDa phosphoprotein band. As shown in Fig. 1*B*, most of radioactive phosphoproteins comigrated with Peak II, among them the phosphoprotein of 65 kDa. These results strongly suggested that pp65 is localized to synaptic vesicles.

It could be argued that the presence of a 65-kDa phosphorylated band in the 47,000 × *g* pellet (Fig. 1*A*, *SPM*) does not exclude the localization of pp65 in a fraction other than synaptic vesicles, *i.e.* mitochondria or plasma membrane. However, (i) a proportion of hypotonically lysed synaptosomes appear to reseal after lysis (Adam-Vizi and Marchbanks, 1983), and the majority of synaptic vesicles remain entrapped within the lysed synaptosome (Wilkinson and Nicholls, 1989); and (ii) comparison of the 65 kDa-associated radioactivity with the amount of synaptophysin among the fractions obtained throughout the subfractionation protocol showed that the distribution of pp65 closely follows that of synaptophysin (Fig. 1*C*). Therefore, the presence of the pp65 band and synaptophysin in the 47,000 × *g* pellet is most probably due to the presence of synaptic vesicles .

Comparative Analysis of the 65-kDa Synaptic Vesicle Phosphoprotein and pp65—To further show that the 65-kDa phos-

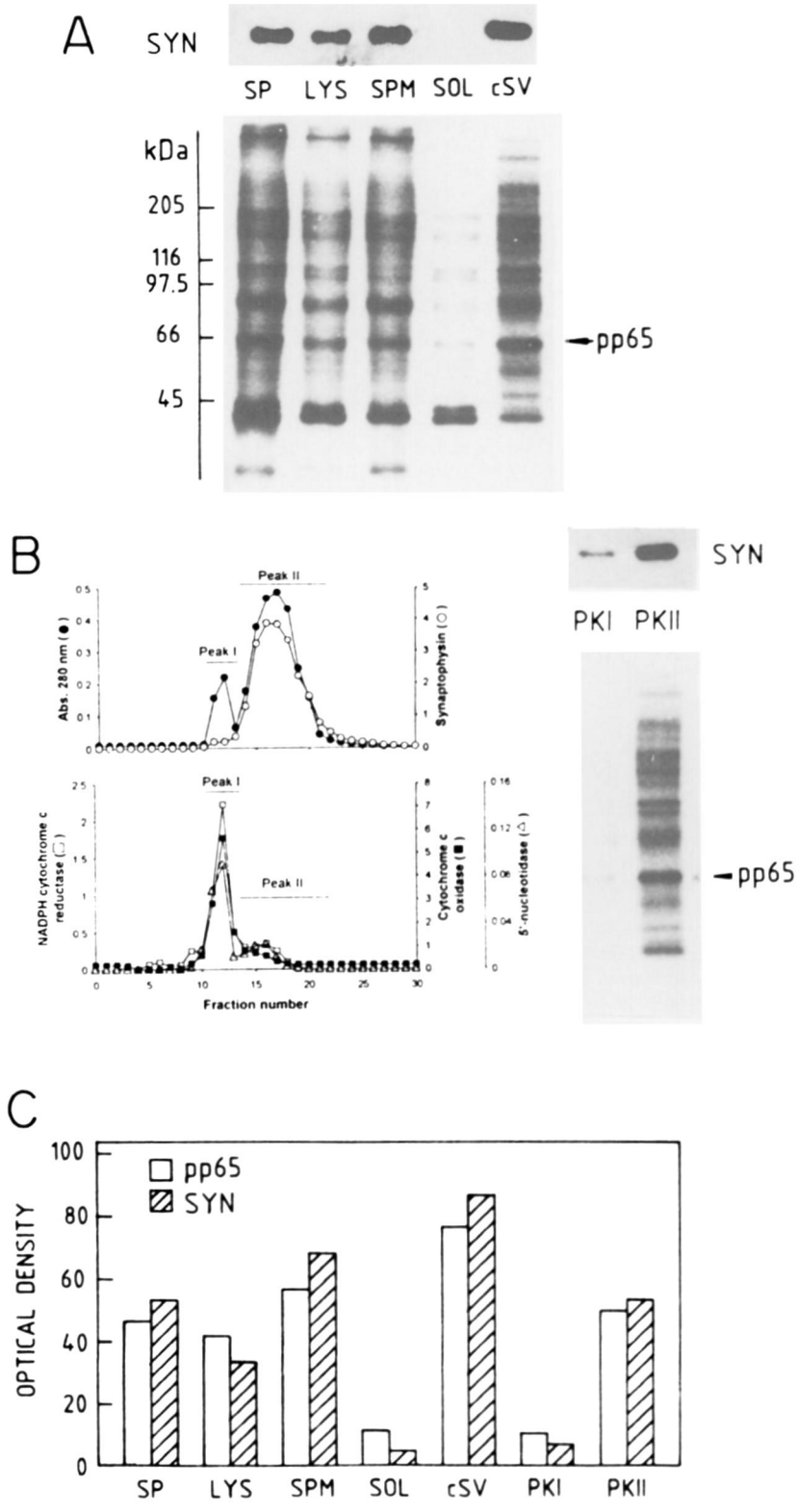

FIG. 1. **Subfractionation of ^{32}P-prelabeled synaptosomes.** Synaptosomes were prelabeled with [^{32}P]orthophosphate, lysed, and separated into subcellular fractions as described under "Experimental Procedures." *A*, ^{32}P autoradiography and Western blot of synaptophysin (*SYN*). *SP*, intact synaptosomes; *LYS*, synaptosomal lysate; *SPM*, synaptic plasma membranes; *SOL*, soluble fraction; *cSV*, crude synaptic vesicles. The positions of molecular mass standards are shown on the *left* in kDa. pp65 is indicated by an *arrow*. *B*, elution profile from the CPG column for total protein (absorbance at 280 nm), anti-synaptophysin immunoreactivity (optical density in arbitrary units), rotenone-insensitive NADPH-cytochrome *c* reductase (nanomoles of cytochrome *c* reduced × min^{-1}), 5′-nucleotidase (negative increment of absorbance at 265 nm × min^{-1}), and cytochrome *c* oxidase (nanomoles of cytochrome *c* oxidized × min^{-1}). ^{32}P autoradiography and Western blot of synaptophysin (*SYN*) from the fractions collected as peak I (*PKI*) and peak II (*PKII*) are shown. *C*, comparative analysis of the relative amounts of synaptophysin (*striped bars*) and pp65 (*white bars*) present in the different subsynaptosomal fractions. Optical density from the pp65 and synaptophysin bands is represented in arbitrary units.

phoprotein present in synaptic vesicles and that shown in synaptosomes are the same protein, we analyzed the phosphopeptide maps obtained after tryptic/chymotryptic digestion of the excised 65-kDa bands from ^{32}P-prelabeled synaptosomes and purified synaptic vesicles (Fig. 2*A*). As shown, the phosphopeptide maps were virtually identical, supporting that the two phosphoproteins are the same entity. Moreover, tryptic/chymotryptic digestions rendered four phosphopeptides, suggesting the existence of multiple phosphorylation sites on pp65.

To identify the phosphorylated residue(s) in pp65, the 65-kDa band from ^{32}P-prelabeled synaptosomes and purified synaptic vesicles was partially (to preserve Tyr(P) residues) or totally hydrolyzed, and the resulting phosphoamino acids were analyzed. Fig. 2*B* shows that most of the radioactivity comigrates with phosphoserine both in synaptosomes and synaptic vesicles. These results indicated that the only phosphorylated residue present in pp65 is serine and excluded a selective loss of label in a different residue (Thr(P) or Tyr(P)) during the isolation of synaptic vesicles from ^{32}P-prelabeled synaptosomes.

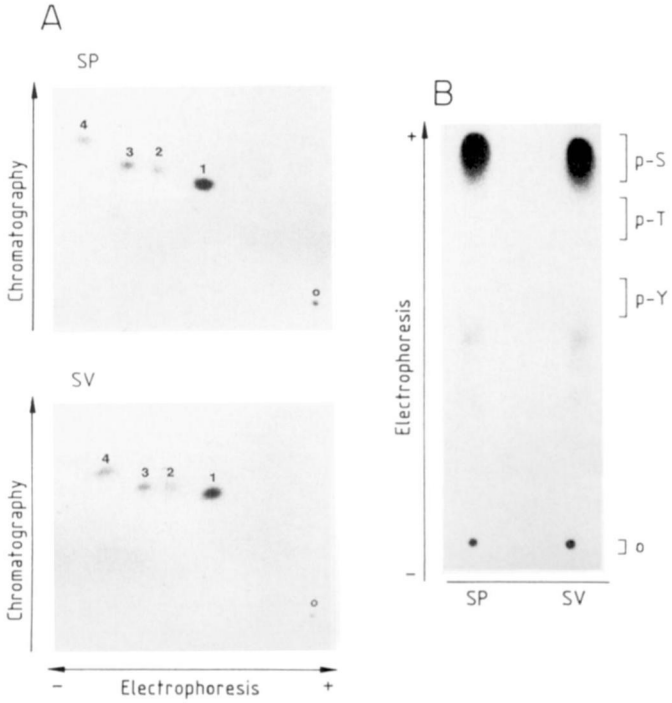

FIG. 2. **Comparative analysis of phosphopeptide and phosphoamino acid patterns of pp65 from ^{32}P-prelabeled synaptosomes and purified synaptic vesicles.** pp65 bands from ^{32}P-prelabeled synaptosomes and purified synaptic vesicles were excised from the gel and proteins extracted for phosphopeptide or phosphoamino acid analysis as described under "Experimental Procedures." *A*, representative autoradiograms of the phosphopeptide maps obtained following thin layer electrophoresis and chromatography of the tryptic/chymotryptic digests of pp65 from synaptosomes (*SP*) and synaptic vesicles (*SV*). The positions of the application points are indicated (○). The four phosphopeptides are *numbered* in order of decreasing electronegativity and hydrophilicity. *B*, autoradiogram of the phosphoamino acid pattern obtained following acid hydrolysis and thin layer electrophoresis of pp65 from synaptosomes (*SP*) and synaptic vesicles (*SV*). The positions of the phosphoamino acid standards (*p-S*, phosphoserine; *p-T*, phosphothreonine; *p-Y*, phosphotyrosine) and the origin (○) are indicated.

Solubilization and Temperature-induced Phase Separation of the 65-kDa Phosphoprotein Present in Synaptic Vesicles—To characterize the nature of the association of pp65 with the membrane vesicle, synaptic vesicles purified from ^{32}P-prelabeled synaptosomes were incubated for 1 h on ice under the conditions indicated in Fig. 3, and soluble and insoluble fractions were separated by centrifugation at 200,000 × *g* for 2 h. Insoluble fractions were subjected to SDS-PAGE followed by ^{32}P autoradiography and Western blot analysis with anti-synaptophysin antibodies. This was included to control for solubilization of an integral membrane protein (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985). Triton X-100 treatment was the most effective in solubilizing pp65 in a manner comparable to synaptophysin (Fig. 3*A*). Although 1 M KCl appeared to dissociate pp65 from the vesicles, synaptophysin was also affected, indicating an incomplete precipitation of synaptic vesicles due to the high salt concentration. Deoxycholate hardly dissociated either pp65 or synaptophysin, and the basic pH value did not show a specific effect on pp65, since a general decrease in the phosphorylation level of most phosphoproteins was observed. On the other hand, the solubility characteristics showed by pp65 were clearly different from the phosphoband of 75–80 kDa that probably corresponds to synapsin I, a peripheral membrane protein (Huttner *et al.*, 1983) (compare *lanes TX100* and *KCl* in Fig. 3*A*).

Since these results suggested a tight association of pp65 with the synaptic vesicle membrane, we studied the partition properties of pp65 following temperature-induced phase separation of ^{32}P-labeled synaptic vesicle proteins in Triton X-114 (Bordier, 1981) with the modifications described by Hooper and Bashir (1991). Fig. 3*B* shows that the 65-kDa phosphorylated band partitioned predominantly into the detergent phase, as did synaptophysin.

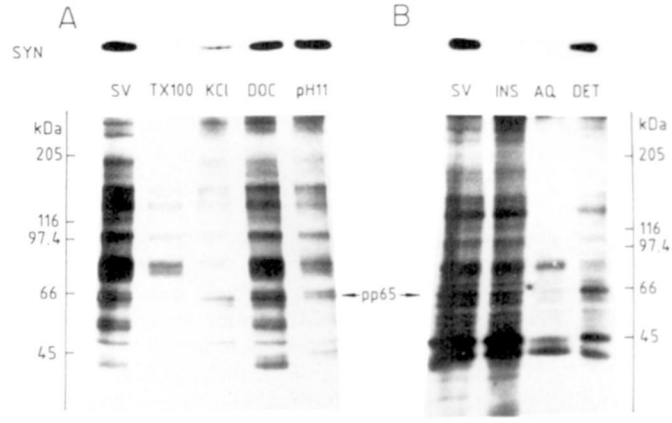

FIG. 3. **Solubilization properties of pp65 from purified synaptic vesicles.** *A*, synaptic vesicles purified from ^{32}P-prelabeled synaptosomes were incubated for 1 h at 4 °C in the presence of various membrane protein perturbants and centrifuged at 200,000 × *g* for 2 h. The pellets, containing the nonsolubilized proteins, were subjected to SDS-PAGE followed by ^{32}P autoradiography and Western blot analysis of synaptophysin (*SYN*). *SV*, control, nontreated vesicles; *TX100*, 1% (w/v) Triton X-100; *KCl*, 1 M KCl; *DOC*, 1% (w/v) sodium deoxicholate; *pH11*, 0.1 M Na_2CO_3, pH 11. pp65 is indicated by an *arrow*. *B*, synaptic vesicles purified from ^{32}P-prelabeled synaptosomes were subjected to phase partitioning in Triton X-114 as described under "Experimental Procedures." The figure shows a representative ^{32}P autoradiogram and Western blot of synaptophysin (*SYN*). *SV*, control of nontreated vesicles; *INS*, Triton X-114-insoluble fraction; *AQ*, aqueous phase; *DET*, detergent phase. pp65 is indicated by an *arrow*. *SV* and *INS lanes* were overexposed in order to visualize the phosphorylated bands present in *AQ* and *DET lanes*.

From the results described, we conclude that pp65 is localized to synaptic vesicles. Several lines of evidence support this conclusion as follows: (i) the 65-kDa phosphoproteins present in ^{32}P-prelabeled synaptosomes and in synaptic vesicles isolated from them rendered identical phosphopeptide maps after tryptic/chymotryptic digestion, (ii) the distribution of synaptophysin in the different fractions obtained in the subfractionation experiments is paralleled by that of the 65-kDa phosphoprotein, and (iii) the solubility properties shown by the 65-kDa phosphoprotein present in synaptic vesicles indicate a tight association with the synaptic vesicle membrane, ruling out an artefactual association promoted by the lysis and subfractionation of synaptosomes. To our knowledge, only DeLorenzo *et al.* (1979) have analyzed the overall phosphoprotein pattern of synaptic vesicles isolated from ^{32}P-prelabeled synaptosomes. The results reported by DeLorenzo *et al.* (1979) bear no resemblance to the results we are presenting now. Indeed, they did not find a major 65-kDa phosphoband, probably because the procedure they used for isolation of synaptic vesicles differs notably from ours, especially regarding protection against phosphatase activity.

The similarity in solubility properties of pp65 and synaptophysin does not necessarily mean that pp65 is an integral membrane protein, since some types of covalent modifications can confer hydrophobic type behavior. A recent example of a hydrophobic modification responsible for the partitioning into the detergent phase of Triton X-114 is the polyisoprenylation of the carboxyl-terminal sequence of rab 3A, a GTP-binding protein attached to the membrane of synaptic vesicles (Fischer v. Mollard *et al.*, 1990; Johnston *et al.*, 1991). Therefore, whether the tight association of pp65 with the synaptic vesicle membrane is

due to a covalent modification or a transmembrane peptide cannot be solved at present.

Phosphate Groups Present in pp65 Undergo a High Turnover Rate, and Endogenous PKC Activity Is Partially Responsible for the Basal Phosphorylation of pp65 in Intact Synaptosomes—There are very few reports on pp65, probably because, although it is one of the mayor phosphoproteins labeled in intact synaptosomes, it does not respond to most stimuli used to activate protein kinases and phosphatases in this preparation. ^{32}P incorporation into pp65 is very rapid when compared with other synaptosomal phosphoproteins, *i.e.* synapsin I or P96, a fact long ago reported, and attributed to a possible mitochondrial location that would provide an immediate access to newly labeled [^{32}P]ATP (Robinson and Dunkley, 1983). Because our experimental evidence indicated that pp65 is not localized to mitochondria (Bogónez *et al.*, 1992) and is tightly bound to synaptic vesicles (present results), an alternative possibility that could account for the rapid phosphorylation of pp65 was that the turnover of phosphate was unusually high. To investigate this hypothesis, synaptosomes were prelabeled with $^{32}P_i$ for 45 min, washed twice to eliminate $^{32}P_i$, resuspended in basal medium containing nonradioactive phosphate (KH_2PO_4, 1.2 mM), and incubated at 37 °C. Samples were collected at different times and processed for SDS-PAGE and autoradiography. Fig. 4 shows ^{32}P remaining in pp65 along the incubation time in nonradioactive medium. For comparison, the behavior of other synaptosomal phosphoproteins was also analyzed. ^{32}P present in P96, SPI, and P42 did not significantly decrease with time during the incubation in cold medium. On the contrary, the behavior of pp65 was notably different; the radioactivity present in pp65 decreased with time, the loss of ^{32}P after 10 min being about 80% of the initial content (*i.e.* that at time 0 of incubation in nonradioactive medium). These results indicated that the turnover of phosphate in pp65 was very rapid relative to that shown by the other phosphoproteins included in this study.

We next tried to identify the possible protein phosphatases and kinases responsible for phosphate turnover in pp65. To determine the identity of the phosphatases acting on pp65, we tested the effect of 1 μM okadaic acid on pp65 phosphorylation level both during prelabeling of synaptosomes with $^{32}P_i$ and during experiments of ^{32}P decay in cold medium. However, we did not find any change in the level of pp65 phosphorylation with respect to controls (in the absence of okadaic acid) in any of these conditions. Since okadaic acid preferentially inhibits protein phosphatase 1 and protein phosphatase 2A (Bialojan and Takai, 1988), these results suggested that none of these phosphatases is responsible for pp65 dephosphorylation in resting synaptosomes.

We also investigated the potential role of the Ca^{2+} and phospholipid-dependent PKC on pp65 phosphorylation. We have done this by means of two independent approaches as follows: (i) by activating PKC by phorbol esters in synaptosomes depolarized with high potassium or 4-aminopyridine, and (ii) by down-regulation of PKC following prolonged exposure to phorbol esters or by inhibition of PKC by the competitive inhibitor GF 109203 X (Toullec *et al.*, 1991) in resting conditions.

Depolarization of synaptosomes in the presence of PMA did not cause any change in pp65 phosphorylation level (results not shown). In agreement with earlier reports (Díaz-Guerra *et al.*, 1988; Oda *et al.*, 1991), incubation of synaptosomes with 1 μM PMA for 20 min induced down-regulation of PKC (Table I). PMA pretreatment resulted in an almost complete loss of PKC activity in the soluble fraction and a substantial decrease in that associated with the particulate fraction, down to 11% of the total PKC activity present in lysates from control synaptosomes. Longer incubation with PMA, up to 40 min, gave identical results (not shown). Pretreatment of synaptosomes with PMA for 20 min, prior to labeling with $^{32}P_i$, led to a reduction in ^{32}P incorporation into pp65, down to 60% of that present in control synaptosomes after 30 min of labeling with $^{32}P_i$ (Fig. 5, *A* and *B*). This reduction was observed at all times of incuba-

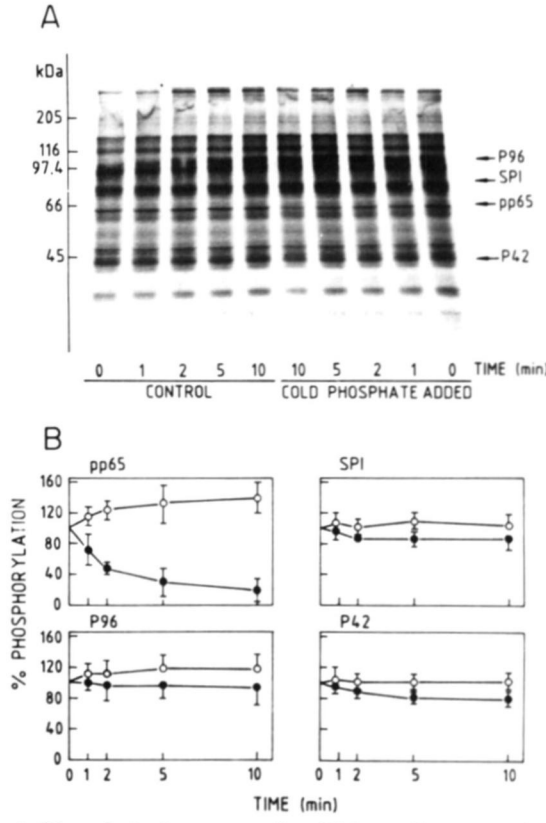

FIG. 4. **Phosphate turnover of pp65 in resting synaptosomes.** Synaptosomes were prelabeled with [^{32}P]orthophosphate for 45 min at 37 °C, washed three times by low speed (3,000 × *g*, 30 s) centrifugation at 37 °C, and then incubated in the presence of 1.2 mM nonradioactive KPO_4H_2 (1.2 mM KCl in control samples). Incubations were stopped with an SDS solution, and the samples were analyzed by SDS-PAGE and autoradiography. *A*, autoradiogram of the synaptosomal phosphoproteins at different times of incubation in the absence (*CONTROL*) or presence (*COLD PHOSPHATE ADDED*) of nonradioactive KPO_4H_2. The positions of pp65, P96, synapsin I (*SPI*), and P42 are indicated. *B*, time course of phosphorylation of pp65, P96, SPI, and P42 following control (○) or cold phosphate (●) incubation. Phosphorylation is expressed as a percentage of the ^{32}P content at time 0 of incubation in nonradioactive medium. Data represent the mean ± S.E. of four independent experiments.

TABLE I
Effect of PMA pretreatment on PKC activity

Synaptosomes were preincubated in the presence or absence of 1 μM PMA for 20 min at 37 °C and washed three times in basal medium by centrifugation at 3,000 × *g* for 30 s at 37 °C. Soluble and particulate fractions were obtained as described under "Experimental Procedures." PKC activity is defined as that measured in the presence of calcium, phosphatidylserine, and 1-oleoyl-2-acetyl-glycerol minus that measured in the presence of EGTA and is expressed in picomoles of ^{32}P incorporated into histone H1/min/mg of lysate protein. The results represent the mean ± S.E. of four independent experiments performed in triplicate. Values in parentheses are percentages of the PKC activity present in lysates from control synaptosomes.

	PKC activity		
	Lysate	Soluble fraction	Particulate fraction
	pmol × min⁻¹ × mg⁻¹		
Control	126.9 ± 8.4 (100%)	47.9 ± 2.5 (38%)	75.3 ± 5.1 (59%)
PMA pretreatment	14.6 ± 1.9 (12%)	1.3 ± 1.0 (1%)	13.7 ± 2.0 (11%)

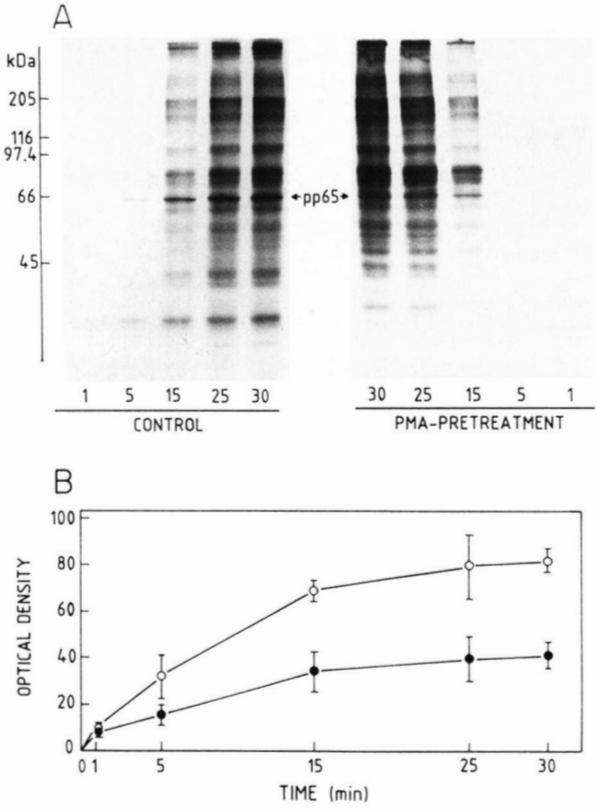

FIG. 5. **Time course of pp65 phosphorylation after pretreatment with 1 μM PMA.** Synaptosomes were preincubated for 20 min in the presence or absence of 1 μM PMA at 37 °C, washed three times by low speed (3,000 × *g*, 30 s) centrifugation at 37 °C, and then incubated with [^{32}P]orthophosphate. *A*, autoradiogram of the synaptosomal phosphoproteins at different times during the labeling with [^{32}P]orthophosphate after control or PMA pretreatment. pp65 is indicated by an *arrow*. *B*, quantitation of the time course of pp65 phosphorylation in control (○) and PMA-pretreated (●) synaptosomes. Phosphorylation is expressed as optical density, in arbitrary units, from the pp65 band. Data represent the mean ± S.E. of three independent experiments.

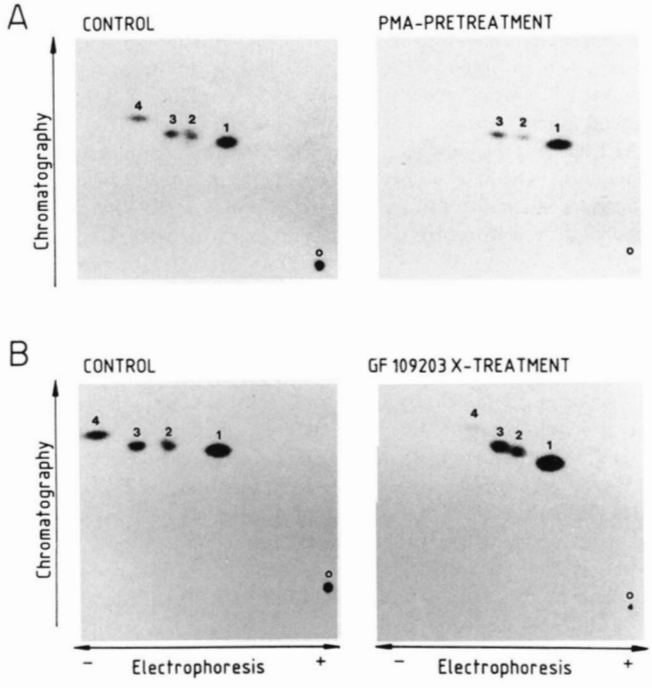

FIG. 6. **Phosphopeptide maps from pp65 following PMA pretreatment and GF 109203 X incubation.** *A*, synaptosomes were preincubated in the absence (*CONTROL*) or presence (*PMA-PRETREATMENT*) of 1 μM PMA and then incubated with [^{32}P]orthophosphate, as described in the legend to Fig. 5. After SDS-PAGE and autoradiography, the pp65 bands were excised, trypsin/chymotrypsin-digested, and analyzed by thin layer electrophoresis and chromatography as indicated in the legend to Fig. 2. The positions of the application points are indicated (*o*). Phosphopeptides are numbered as in Fig. 2. *B*, synaptosomes were prelabeled with ^{32}P[P]orthophosphate during 45 min at 37 °C and then incubated in the absence (*CONTROL*) or presence (*GF 109203 X-TREATMENT*) of 5 μM GF 109203 X for 10 min. Samples were processed for phosphopeptide analysis as described above. Experiments in *A* and *B* were performed twice with identical results.

tion with $^{32}P_i$ and reached a maximum effect from 15 min onwards (Fig. 5*B*). This was not due to a generalized effect of the PMA pretreatment, since, with a few exceptions, no significant changes in phosphate incorporation with time were observed in other phosphoproteins (Fig. 5*A*). Similar results were obtained when ^{32}P-prelabeled synaptosomes were incubated in the presence of GF 109203 X, although the extent of the decrease in ^{32}P labeling of pp65 was variable (results not shown). To investigate whether the reduction in pp65 phosphorylation level evoked by PMA and GF 109203 X involved a preferential loss of some of the phosphorylated residues, tryptic/chymotryptic peptide mapping of pp65 from PMA-pretreated, GF 109203 X-treated, and control synaptosomes was performed (Fig. 6). Interestingly, both PMA pretreatment and GF 109203 X addition elicited a selective effect on the phosphopeptide 4 that was almost undetectable following both treatments. Thus, it appears that PKC or a PKC-dependent protein kinase is responsible for the phosphorylation of pp65, specifically at phosphopeptide 4, under steady state conditions in resting intact synaptosomes. The lack of changes in the other pp65 phosphopeptides in PMA-pretreated synaptosomes indicates that additional protein kinases must be involved in the steady state phosphorylation of pp65.

The observation that the turnover of phosphate in pp65 is high with respect to other phosphoproteins suggests that the phosphorylation of this protein may be involved in a highly regulated process. Indeed, analysis of the mechanisms that lead to ultrasensitivity in regulation of biological processes leads to the conclusion that a reversible covalent system would produce ultrasensitivity if either or both of the converter enzymes, for example, the kinase or the phosphatase, were saturated with respect to its substrate (zero-order ultrasensitivity); if both of the converter enzymes are operating in the first order instead of the zero order region of the Michaelis-Menten equation, no such ultrasensitivity is observed (Koshland, 1987). Although our data do not confirm whether the phosphorylation or dephosphorylation of pp65 are zero-order processes, the fact that the turnover of phosphate, under steady state conditions, is much higher than that of other phosphoproteins, suggests that this may be so.

In their studies of transmitter release at neuromuscular junctions, Abdul-Ghani *et al.* (1991) have found evidence for the existence of key regulatory protein(s) associated with the presynaptic terminals that also undergo continuous phosphorylation and dephosphorylation. Okadaic acid addition enhanced neurotransmitter release, suggesting that these processes are involved in modulation of exocytosis. Since we have found that pp65 is located to synaptic vesicles, it has a rapid phosphate turnover rate, and it undergoes rapid and reversible changes in the phosphorylation state in a process matching neurotransmitter release (Gómez-Puertas *et al.*, 1991), it is possible that pp65 is one of the central nervous system counterparts of the regulatory protein(s) in neuromuscular junctions discussed in Abdul-Ghani *et al.* (1991). At present, we have no clues about the identity of pp65 phosphatase, except that it is okadaic acid-resistant. On the other hand, our results clearly indicate that PKC is probably one of the pp65 kinases involved in its steady state phosphorylation.

There is an increasing number of reports on the involvement of PKC in the regulation of neurotransmitter release (for a review, see Robinson (1992b)). Phorbol ester potentiation of K^+-evoked neurotransmitter release is paralleled by the enhanced phosphorylation of B-50 (Dekker *et al.*, 1989b) and MARCKS (myristoylated alanine-rich C kinase substrate) (Nichols *et al.*, 1987), the only known proteins whose phosphorylation is specifically stimulated in response to the addition of phorbol esters in resting synaptosomes (Wu *et al.*, 1982; Rodnight and Perret, 1986; Dunkley *et al.*, 1986a). However, Robinson (1992a) has recently shown that dephosphin (P96), a phorbol ester-unresponsive phosphoprotein that is an *in vitro* substrate for PKC in synaptosol (Robinson, 1991a) and synaptic vesicles (Robinson, 1991b), is phosphorylated following repolarization of intact synaptosomes by a PKC-mediated process, concluding that PMA stimulation of protein phosphorylation should not be used as the sole criterion for deducing an involvement of PKC (Robinson, 1992a). Our results show that pp65 is also a phorbol ester-unresponsive PKC substrate in resting intact synaptosomes.

In summary, a number of characteristics of pp65 reported in the present study make this phosphoprotein a candidate to play a regulatory role in nerve terminal function. It is tightly associated with the membrane of the synaptic vesicle, it shows a high turnover of phosphate under resting conditions, and one of the protein kinases responsible for its steady state phosphorylation *in situ* is PKC-dependent or is PKC itself.

Acknowledgment—We thank Dr. Antonio Andrés for helpful discussions in the development of this work.

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