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Serine-Specific Phosphorylation of Nicotinic Receptor Associated 43K Protein†

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ABSTRACT: In *Torpedo marmorata* electroplaque, an extrinsic membrane protein of apparent mass 43 000 daltons colocalizes with the cytoplasmic face of the nicotinic acetylcholine receptor (AChR) in approximately 1:1 stoichiometry. We show that this 43K protein can be phosphorylated in vitro by endogenous protein kinases present in AChR-rich membranes. The extent of 43K protein phosphorylation exceeds that of the subunits of the AChR, well-established substrates for enzymatic phosphorylation. We demonstrate that significant 43K phosphoprotein exists in vivo. The kinetics of phosphate incorporation mediated by endogenous kinases differed significantly from those of the AChR subunits, suggesting that different phosphorylation cascades are involved. Use of specific inhibitors of a variety of protein kinases indicated that endogenous cAMP-dependent protein kinase catalyzes phosphorylation of the 43K protein in vitro. All of the phosphate incorporated into 43K protein was accounted for by phosphoserine (0.65 mol/mol of 43K protein). Potential structural and functional consequences of 43K protein phosphorylation are discussed.

Efficient signal transmission in the nervous system relies upon the exquisite structural specialization of the chemical synapse. The machinery for release and subsequent degradation of neurotransmitter is closely aligned with the postsynaptic receptor. At the neuromuscular junction, nicotinic acetylcholine receptors (AChRs)[1] are apposed and immobilized in densely packed clusters (≈8000/μm^2; Fertuck & Salpeter, 1974) at the crests of postjunctional folds close to the sites of acetylcholine release. The mechanism whereby this structure develops, is maintained, and is regenerated after injury are the subject of intense inquiry [recent reviews: Bloch and Pumplin (1988) and Changeux et al. 1991)].

Concentrated at the motor endplate postsynaptic membrane are a number of proteins in addition to AChR. The most prominent among these proteins, termed 43K protein for its apparent molecular mass on SDS gels, colocalizes with the AChR in close association with the cytoplasmic face of receptor clusters (Froehner et al., 1981; Sealock, 1982; Nghiêm et al., 1983; Sealock et al., 1984; Kordeli et al., 1986, 1989; Bridgman et al., 1987). First discovered in a model system, the innervated face of the *Torpedo marmorata* electrocyte (Sobel et al., 1977), the function of 43K protein has yet to be elucidated.

Some evidence suggests that 43K protein is involved in the development and maintenance of postsynaptic cytoarchitecture [recent reviews: Changeux et al. (1991) and Froehner (1986)]. Removal of 43K protein by brief exposure to alkaline pH leads to increased lateral and rotational mobility of the AChR [see Froehner (1989) for references]. 43K protein is consistently found at the cytoplasmic face of mature AChR clusters; in the absence of receptor clusters, 43K protein cannot be detected (Kordeli et al., 1986; Bloch & Pumplin, 1988). In cultured myotubes, 43K protein coaggregates with the AChR during spontaneous and experimentally induced AChR aggregation (Burden, 1985; Bloch & Froehner, 1987; Peng & Froehner, 1985). AChRs reconstituted in *Xenopus* oocytes or in fibroblasts assemble into clusters when coexpressed with 43K protein (Froehner et al., 1990; Phillips et al., 1991).

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TCA, trichloroacetic acid; DTT, dithiothreitol.

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Together these observations suggest that 43K protein plays a role in the formation and/or stabilization of AChR clusters, perhaps by linking AChR to the cytoskeleton.

Protein phosphorylation modulates a number of the complex interactions between components of the cytoskeletal network [reviewed in Boivin (1988)]. Protein phosphorylation also plays a general role in the regulation of signal transduction systems, modulating the actions of neurotransmitter receptors, ion channels, and enzymes involved in neurotransmitter biosynthesis [reviewed in Greengard (1987) and Miles and Huganir (1988)]. *Torpedo* 43K protein is a substrate for endogenous protein kinases that copurify with AChR-rich membranes (Gordon et al., 1977, 1980; Saitoh & Changeux, 1980). In this paper, we extend these observations by characterizing the kinetics and stoichiometry of the 43K protein phosphorylation reaction. We demonstrate that phosphorylated 43K protein exists in vivo. We have delineated cAMP-dependent protein kinase as the specific kinase involved in 43K protein phosphorylation. We demonstrate that phosphoserine accounts for all phosphate incorporation. Phosphopeptide mapping suggests that the majority of 43K protein phosphorylation occurs at a single site. Finally, we discuss possible functional implications of these data.

Experimental Procedures

Materials. Live *T. marmorata* were obtained from the Institut de Biologie Marine (Arcachon, France). Catalytic subunit of cAMP-dependent protein kinase (bovine brain) was purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP and [^{125}I]-α-bungarotoxin were obtained from Amersham (Amersham France, Les Ulis). Tissue solubilizer was obtained from Koch-Light Limited (Haverhill, Suffolk, England). Centricon Microconcentrators were purchased from Amicon (W. R. Grace, Danvers, MA). Thin-layer cellulose plates (0.1-mm microcrystalline cellulose, TLC-Ready-Foils, F1440) were obtained from Schleicher and Schuell (Dassel, FRG).

Preparation of AChR-Rich Membranes. Nicotinic receptor rich membranes were prepared according to a protocol modified from Sobel et al. (1977). Briefly, freshly dissected electric organ tissue was minced and homogenized (Virtis homogenizer) as 150-g batches in 150 mL of buffer HBX (50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 1 mM EGTA, 5 units/mL aprotinin, 1 μg/mL pepstatin A, 1 mM PMSF, 2 μg/mL antipain, 2 μg/mL leupeptin) at 4 °C. After centrifugation (Beckman JA 10 rotor, 5K rpm, 10 min, 4 °C), the supernatant (S1) was collected and filtered through gauze. After the pellets were combined, rehomogenized (as above in 150-g batches), and centrifuged, the supernatant (S2) was filtered and combined with S1. Membranes were collected (Beckman JA 14 rotor, 12.5K rpm, 50 min, 4 °C), Potter homogenized in HBX, and adjusted to 35% sucrose (w/w) by addition of sucrose crystals. Nicotinic receptor rich membranes were purified on a discontinuous sucrose density gradient (25 mL of 43% sucrose, 40 mL of 35% sucrose, membrane suspension) at 40K rpm for 3 h (Beckman 45Ti rotor, 4 °C). Membranes at the 35%/43% interface were recovered and collected by centrifugation at 40K rpm. Purified membranes were washed two additional times (Potter homogenization in a large volume of HBX, centrifugation) to remove soluble creatine kinase. Typical membrane preparations exhibited 1–3 nmol of α-bungarotoxin binding sites/mg of protein, assayed according to Heidmann et al. (1983). Protein was assayed according to the method of Bradford (1976) with BSA as standard.

Lithium Diiodosalicylate Treatment. *T. marmorata* postsynaptic membranes (5 mg) were suspended in 10 mM sodium phosphate (pH 8.5), 1 mM EGTA, and 1 mM EDTA and made 10 mM in lithium diiodosalicylate (LIS). After incubation (1 h, 4 °C, gentle agitation), the suspension was centrifuged (9K rpm, 30 min, 4 °C) in a Sigma bench top centrifuge. The supernatant was filtered (Centricon 10) to concentrate the 43K protein enriched extract and to remove LIS salt. One-tenth of this extract was used in phosphorylation assays in the presence of dephosphorylated casein and dephosphorylated BSA as potential substrates.

Phosphorylation of Postsynaptic Membranes. Membrane vesicles were incubated in a phosphorylation buffer (0.05 M Tris-HCl, pH 6.8, 15 mM $MgCl_2$, 2 mM ouabain) with 10–200 μM [γ-^{32}P]ATP (3 Ci/mmol) at room temperature for 30 min (unless otherwise indicated). Under these conditions, no proteolysis of AChR subunits or 43K protein was detected. Where indicated, 50 nM–1 μM catalytic subunit of cAMP-dependent protein kinase (PKA) or 0.05% CHAPS was included in the reaction mixture. The phosphorylation reaction was stopped by the addition of $^1/_4$ volume buffer M (12.5% SDS, 50% glycerol, 33% 2-mercaptoethanol, and 1% bromophenol blue in 0.3 M Tris-HCl, pH 7.4) followed by incubation at 60 °C for 5 min. This mixture was subjected to SDS–polyacrylamide gel electrophoresis. After staining protein bands with 0.5% Coomassie Blue, the gel was destained and dried. Autoradiography was performed at −80 °C using preflashed Kodak X-OMAT AR film and an intensifying screen. The radioactive 43K band was identified in the dried gel and cut. The dried gel fragment was digested by agitation for 24 h in 10 mL of toluene, 0.3% (w/v) PPO, and 0.03% (w/v) POPOP with 10% (v/v) tissue solubilizer and 80 mM NH_4OH and counted in a liquid scintillation counter. Alternatively, ^{32}P incorporation in the dried gel piece was measured by Cerenkov counting. Stoichiometry calculations were based on 0.5 mol of 43K protein/mol of α-bungarotoxin binding site (LaRochelle & Froehner, 1986).

SDS–Polyacrylamide Gel Electrophoresis. One-dimensional SDS–polyacrylamide gel electrophoresis was carried out on 10% acrylamide and 0.05% *N,N'*-bis(methyleneacrylamide) gels (Laemmli, 1970) at 20–30 mA. Two-dimensional electrophoresis (O'Farrell, 1975) was performed as described (Nghiêm et al., 1983).

Purification of Phosphorylated 43K Protein. After one-dimensional electrophoresis, the band at 43 kDa was cut and minced into fine pieces. The gel pieces were rehydrated in 0.05 M NH_4HCO_3 and 0.1% SDS and then ground in a glass homogenizer. This suspension was made 5% in 2-mercaptoethanol, boiled for 5 min, and agitated overnight at 37 °C. After centrifugation at 10K rpm for 15 min in a bench top centrifuge, the supernatant was recovered. The pellet was resuspended in approximately half the volume of buffer originally added and agitated for 2 h (37 °C). After centrifugation as before, the supernatants were combined. One hundred micrograms of carrier protein (dephosphorylated casein) was added, mixed thoroughly, and then TCA precipitated (20% TCA, 4 h on ice). After centrifugation (10K rpm, 30 min, 4 °C), the supernatant was removed and the pellet washed in ethanol (−20 °C). After centrifugation again at 10K rpm for 5 min (4 °C), the pellet was washed with ethanol/ether (1:1) at −20 °C and recentrifuged.

Phosphoamino Acid Analysis. Briefly, purified phosphorylated 43K protein was hydrolyzed in 5.7 M HCl at 110 °C for 1.5 h and then evaporated over NaOH in a vacuum concentrator. The pellet was resuspended in 20–30 μL of pH 1.9 buffer [7.8% (v/v) acetic acid/2.2% (v/v) formic acid] containing phosphoserine, phosphothreonine, and phosphotyrosine

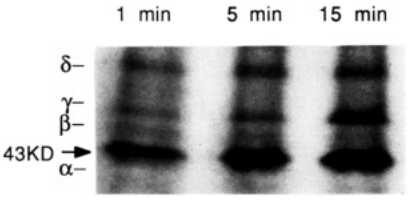

FIGURE 1: Autoradiogram of postsynaptic membranes phosphorylated by endogenous, copurifying protein kinases. Purified *T. marmorata* membranes were incubated in the presence of [γ-^{32}P]ATP and in the absence of exogenous kinase. Aliquots were removed at the time intervals indicated and prepared for one-dimensional SDS–PAGE.

as standards. This solution, along with a trace of phenol red, was spotted onto thin-layer cellulose plates. Electrophoresis at 750 V was performed in pH 1.9 buffer. The plate was dried and then electrophoresed in the second dimension in pH 3.5 buffer [5% (v/v) acetic acid/0.5% (v/v) pyridine]. Amino acids were revealed by spraying the dried plate with ninhydrin (1% in acetone).

CNBr Digestion of $^{32}PO_4$-Labeled 43K Protein. Purified radiolabeled 43K protein was resuspended as ≈1 mg/mL in 0.1 M Tris-HCl (pH 8.8), 4 mM EDTA, and 1% SDS. After reduction in the presence of 2 mM dithiothreitol (DTT) (1 h at 37 °C under N_2), the protein was carboxymethylated with 5 mM iodoacetic acid (IAA) (1 h at 37 °C under N_2). After a second cycle of reduction (1 mM DTT, 1 h at 37 °C) and carboxymethylation (2 mM IAA, 30 min at 37 °C), the protein was exposed overnight (37 °C under N_2) to 0.72 M DTT. The solution was desalted on a PD-10 column (Pharmacia), TCA precipitated, and resuspended in 70% formic acid and 4 mM tryptophan. 43K protein was digested (24 h, room temperature, under N_2, in the dark) in 60 mM CNBr. To ensure complete digestion, in two experiments the sample was again made 60 mM in CNBr and incubated as above.

Phosphopeptide Analysis. CNBr-digested 43K protein (150 pmol) was spotted onto a thin-layer cellulose plate. Electrophoresis (500 V) was performed in pH 3.5 buffer [7.8% (v/v) acetic acid/2.2% (v/v) formic acid] until the phenol red marker had migrated to the border of the thin-layer sheet. The plate was dried, and ascending chromatography was performed in the second dimension using 37.5% (v/v) 1-butanol, 25% (v/v) pyridine, and 7.5% (v/v) acetic acid. Radioactively labeled phosphopeptides were detected by autoradiography.

RESULTS

43K Phosphoprotein. After incubation of postsynaptic *Torpedo* membranes in the presence of [γ-^{32}P]ATP, several radiolabeled phosphoprotein bands were observed on one-dimensional SDS–PAGE (Figure 1). Prominent among the radioactive bands is one that comigrates with the 43K protein. In addition, bands at 55, 60, 65, and 95 kDa comigrating with the β, γ, and δ subunits of the AChR as well as the Na,K-ATPase were seen. Under basal assay conditions performed in the presence of copurifying protein kinases, the band at 43 kDa was the major phosphoprotein band.

Postsynaptic protein migrating at the level of 43 kDa serves as a substrate for PKA-mediated phosphorylation (see below). Exposure to purified PKA catalytic subunit led to marked increase in [^{32}P]phosphate incorporation in the 43-kDa band, ranging from 10- to 100-fold. Addition of 0.05% CHAPS produced only a slight increase in ^{32}P uptake, confirming that these right-side-out (Wennogle & Changeux, 1980) postsynaptic membrane vesicles are leaky to hydrophilic molecules and enzymes. As such, detergents were omitted from routine phosphorylation assays.

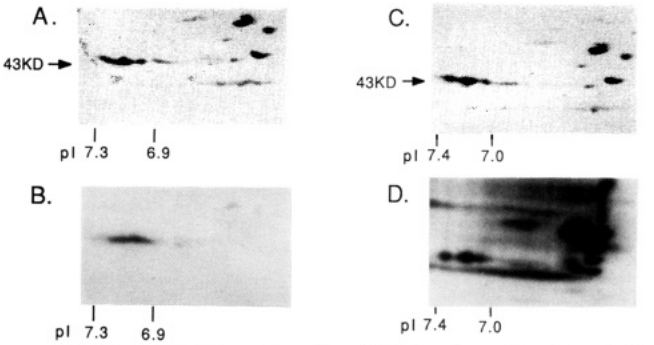

FIGURE 2: Panel A: Two-dimensional SDS–polyacrylamide gel of postsynaptic membranes phosphorylated in the presence of endogenous protein kinases that copurify with the membranes. This 2-D SDS gel is stained for protein with Coomassie Blue. Panel B: Autoradiographic image of the gel shown in panel A. Panel C: 2-D gel, stained for protein, of postsynaptic membranes phosphorylated in the presence of exogenously applied catalytic subunit of PKA. Panel D: Autoradiographic image of the gel shown in panel C.

Two-Dimensional Gels. In order to establish that the 43-kDa phosphoprotein band corresponds to 43K protein, two-dimensional gel electrophoresis was performed on postsynaptic membranes (Figure 2). As reported [e.g., Nghiêm et al. (1983) Porter and Froehner (1983), and Carr et al. (1987)], 43K protein is prominently observed as 3–4 isoelectric variants between p*I* 6.9 and 7.4. Under these conditions actin focuses at more acidic p*I*'s and at a slightly higher apparent molecular mass (45 kDa) (Porter & Froehner, 1983; Carr et al., 1987; our unpublished observations). No creatine kinase (CK) was detected with Coomassie stain (Figure 2, panels A and C) or with an anti-CK antibody (data not shown). Thus, under these electrophoresis conditions, 43K protein is the only protein that migrates at the level of 43 kDa.

Postsynaptic membranes were phosphorylated in the presence of endogenous protein kinases and subjected to 2-D SDS–PAGE. Autoradiographic images of the gels reveal a single group of 3–4 radiolabeled subspecies at the level of 43 kDa, extending between p*I* 6.9 and 7.3 (Figure 2, panel B), confirming that phosphorylated 43K protein accounts for the phosphoprotein band at 43 kDa apparent molecular mass. Superimposing the autoradiograms over the protein stained gels reveals a shift of 0.1–0.2 p*I* units such that the more basic protein stains are not radioactive. Further, the most acidic autoradiographic spot corresponds to a point where no Coomassie Blue is localized. These observations conform to the notion that phosphate incorporation leads to a more acidic isoelectric focusing point.

Two-dimensional gel separations were performed on membranes phosphorylated in the presence of exogenously applied catalytic subunit of protein kinase A (Figure 2, panel C). A similar pattern of charge heterogeneity was observed in the autoradiograms (Figure 2, panel D) along with a slight shift toward more acidic p*I*'s.

Thus, 2-D gels confirm that 43K phosphoprotein accounts for the radioactive band migrating at molecular mass 43 kDa. First, 43K protein is the sole component of the protein band migrating at molecular mass 43 kDa; creatine kinase is not detectable in our membranes, and actin migrates as a discrete band at 45 kDa. Second, radiolabeled phosphoprotein cofocuses with 43K protein as a characteristic triplet between p*I* 6.9 and 7.3.

Endogenous PKA Mediates 43K Protein Phosphorylation. To begin to characterize the functional significance of 43K protein phosphorylation, it was necessary to identify the kinase active in the enzymatic transfer of phosphate to 43K protein.

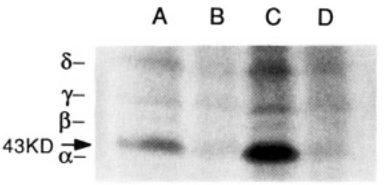

FIGURE 3: cAMP-dependent protein kinase phosphorylates 43K protein. Four standard phosphorylation assays (75 μg of postsynaptic membrane each) were conducted in parallel in the absence (lanes A and C) or presence (lanes B and D) of 1 μM protein kinase inhibitor. Proteins in lanes C and D were incubated in the presence of 10 nM catalytic subunit of PKA. After SDS–PAGE, the gel was dried and exposed overnight to Kodak X-OMAT AR film.

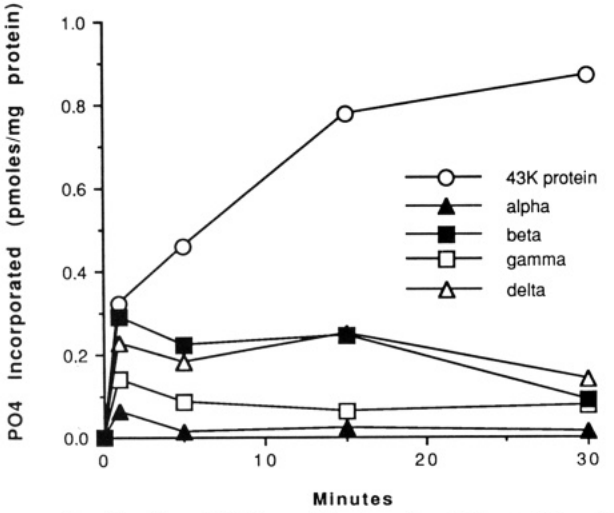

FIGURE 4: Kinetics of 43K protein phosphorylation. *Torpedo* postsynaptic membranes were incubated in the presence of [γ-^{32}P]ATP as described under Experimental Procedures. Aliquots were removed at intervals and prepared for SDS–PAGE. Bands from the dried gel corresponding to 43K protein as well as the AChR subunits were cut and counted.

As shown in Figure 3, protein kinase inhibitor (PKI), a specific antagonist of PKA catalytic subunit (McPherson et al., 1979), led to a marked decrease in [^{32}P]phosphate uptake in 43K protein (lane B). The enhanced phosphorylation of 43K protein (lane C) mediated by exogenous catalytic subunit of PKA was also eliminated in the presence of PKI (land D). In the presence only of copurifying endogenous kinases, the addition of cAMP did not lead to an increase in [^{32}P]phosphate incorporation (data not shown). Together, these data strongly suggest that the dissociated catalytic subunit of protein kinase A that copurifies with the postsynaptic membranes mediates the phosphorylation reaction.

No specific modulatory effects on phosphorylation of 43K protein were detected upon exposure to Ca^{2+} (0.5 mM)–calmodulin (1.5 μM), the calmodulin inhibitor trifluoperazine (10 μM), Mn^{2+} (2 mM, a modulator of tyrosine kinases), cGMP (10 μM), TPA (1 μM), or staurosporine, an inhibitor of protein kinase C (100–1000 ng/mL).

Phosphorylation Kinetics. Kinetic measurements reveal dissimilarity in the time course of ^{32}P incorporation between 43K protein and the AChR subunits (Figure 4). The covalent uptake of phosphate by 43K protein exceeds those of the AChR subunits, well-established physiological substrates for enzymatic phosphorylation. A monotonic rise in [^{32}P]phosphate incorporation by 43K protein was observed up to 30 min. In contrast, there was a substantial decrease in the phosphorylation of other phosphoproteins (migrating at the levels of the receptor subunits) after 2 min, consistent with the presence of protein phosphatases in the *Torpedo* electrocyte membranes

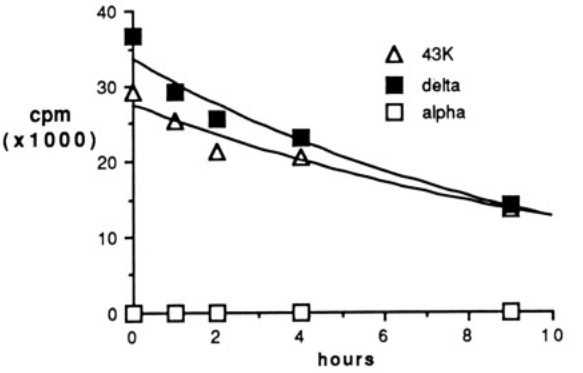

FIGURE 5: Dephosphorylation of 43K protein. *Torpedo* postsynaptic membranes (1.3 mg) were phosphorylated in the presence of the exogenous catalytic subunit of PKA. The membranes were washed and then resuspended in HBX. The suspension was made 10 mM in ADP and incubated at room temperature. At the times indicated, 35-μL aliquots were removed and prepared for SDS–PAGE. Subsequently, the presence of ^{32}P in 43K protein as well as α and δ subunits of AChR was measured as described.

(Teichberg & Changeux, 1977; Gordon et al., 1979). As reported (Saitoh & Changeux, 1980; Huganir & Greengard, 1983), AChR subunit phosphorylation at 20 °C peaks within the first few minutes. These results indicate that, under the present conditions, the steady state between phosphorylated and dephosphorylated forms of 43K protein favors 43K phosphoprotein (relative to the steady states of the receptor subunits where the dephosphorylated forms are favored).

In Vivo 43K Phosphoprotein. In order to address the issue of basal phosphate incorporation in postsynaptic proteins, we measured phosphorylation stoichiometry in two experiments. First, it was necessary to remove endogenous covalently bound phosphate. To do this, the PKA-catalyzed phosphorylation reaction was driven in the reverse direction in the absence of ATP and in the presence of Mg^{2+} and 10 mM ADP (Flockhart, 1983). This allowed us selectively to remove phosphate moieties at PKA phosphorylation sites (Figure 5).

Dephosphorylated membranes were thoroughly washed to remove ADP, resuspended in phosphorylation buffer, and used in standard phosphorylation assay conditions. Control membranes, treated in a similar way but in the absence of MgADP ("sham dephosphorylation"), were also subjected to phosphorylation assays under conditions designed to promote rapid and saturated phosphate incorporation. These data (see below) demonstrate that more radioactive phosphate can be incorporated in 43K protein after prior dephosphorylation, indicating the presence of covalent phosphate in purified 43K protein.

In the presence of exogenous PKA, 0.65 mol of [^{32}P]-phosphate was incorporated per mole of 43K protein from the dephosphorylated membranes (stoichiometry for phosphorylation of the γ AChR subunit under these conditions was 0.34). 43K protein phosphorylation stoichiometry in sham-dephosphorylated membranes was measured to be 0.22 mol of [^{32}P]phosphate/mol of 43K protein (0.09 mol of phosphate/mol of γ subunit). Thus, 43K protein serves as a good substrate for PKA. Further, as dephosphorylated membranes incorporated substantially more [^{32}P]phosphate than control membranes, these data indicate that a significant amount of 43K phosphoprotein exists in vivo. This is so even as no special efforts were taken to inhibit native phosphatases during the course of membrane isolation.

Nicotinic agonist (1 mM carbamylcholine) and antagonists (100 μM α-bungarotoxin, 100 μM erabutoxin B) had no effect on 43K or AChR subunit phosphorylation (data not shown).

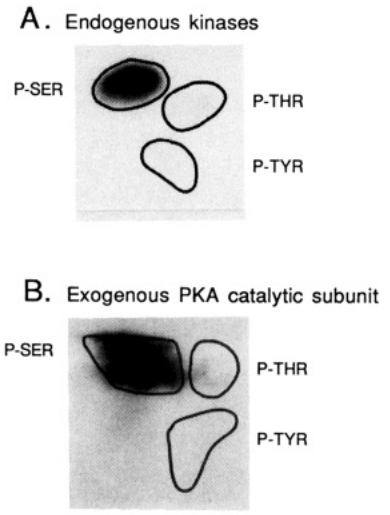

FIGURE 6: Phosphoamino acid analysis of 43K protein. Postsynaptic membranes (0.5 mg) were phosphorylated by endogenous protein kinases that copurify with the postsynaptic membranes (panel A) or in the presence of exogenously applied catalytic subunit of cAMP-dependent protein kinase (panel B). After one-dimensional SDS–PAGE, 43K protein was extracted and acid hydrolyzed. The suspension was electrophoresed on thin-layer cellulose plates along with nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine standards. The areas labeled by ninhydrin and corresponding to the final distribution of the amino acid markers are circled.

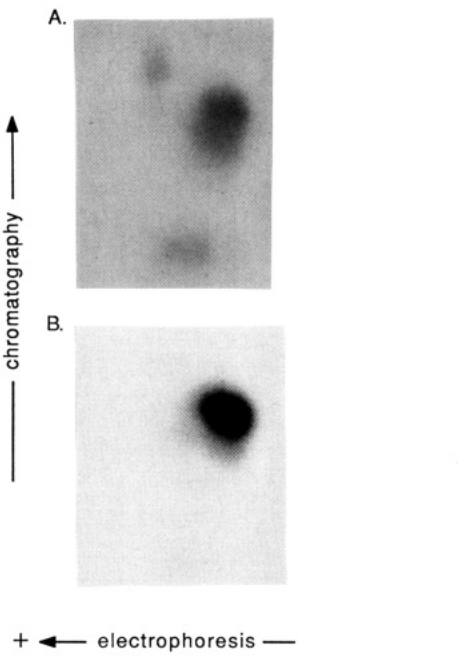

FIGURE 7: Phosphopeptide analysis of 43K protein. Membrane-bound 43K protein was phosphorylated in the presence of exogenous PKA catalytic subunit, and the purified radiolabeled protein was reduced, carboxymethylated, and subjected to CNBr cleavage. CNBr peptides were separated by high-voltage electrophoresis and thin-layer chromatography as described under Experimental Procedures (panel A). Covalent phosphate was removed from 43K protein by PKA-mediated dephosphorylation prior to in vitro phosphorylation, CNBr limit digestion, and two-dimensional separation (panel B). Again, most [^{32}P]phosphate localized to a single spot.

Is 43K Protein Itself a Protein Kinase? It has been reported that the 43K protein is capable of phosphorylating casein (Gordon & Milfay, 1986). To address this issue, postsynaptic membranes were treated with lithium diiodosalicylate (see Experimental Procedures) to extract peripheral membrane proteins. This treatment is more likely to preserve protein functional activity than most chaotropic agents (Klebe et al., 1980), and unlike most detergents, LIS is readily removed from solution. The 43K protein enriched fraction was first filtered to remove LIS and then incubated in standard phosphorylation buffer (1 h at 25 °C) with two readily phosphorylatable substrates (dephosphorylated BSA, dephosphorylated casein) in the presence of [γ-^{32}P]ATP (200 μM). In parallel, purified PKA catalytic subunit (50 units) was exposed to LIS, filtered, and incubated under phosphorylation assay conditions. Under these conditions, we were unable to demonstrate protein kinase activity for 43K protein. Specifically, no [^{32}P]phosphate incorporation was observed in BSA, 43K protein, or the casein polypeptide subunits. In contrast, LIS-treated PKA remained active under these conditions (data not shown).

Phosphoamino Acid Analysis. Contained within the amino acid sequence of 43K protein from *Torpedo californica* (Frail et al., 1987) there exist several sites for potential PKA-mediated phosphorylation. Among these consensus phosphorylation sites, six serines (201, 208, 260, 271, 405, 406) are potential phosphate acceptors. Also, two threonines (37, 83) are present within consensus PKA phosphorylation sites. The presence of both Lys and Arg residues N-terminal to serine 406 suggests that this serine is a preferred substrate for PKA-catalyzed phosphorylation (Kemp et al., 1977). Thus, we analyzed purified 43K phosphoprotein to determine the amino acid residue(s) radiolabeled by [^{32}P]phosphate.

Purified phosphorylated 43K protein was acid hydrolyzed and analyzed by electrophoresis on cellulose plates (Cooper et al., 1983). Radioactivity was detected only as phosphoserine (Figure 6, panel A). Radiolabeled phosphothreonine or phosphotyrosine was not detected, even upon overexposure of the autoradiograms for several weeks. Thus, 43K protein is phosphorylated by endogenous kinases exclusively on serine residue(s). Incubation in the presence of exogenously applied PKA led to a marked increase in [^{32}P]phosphate incorporation. Again, all phosphate was incorporated on serine residues (Figure 6, panel B); no other phosphoamino acids were detected. We conclude that all phosphate incorporated in 43K protein can be accounted for by phosphoserine, whether the active kinase was endogenous or exogenous PKA.

Phosphopeptide Mapping. We have shown that up to 0.65 mol of ^{32}P can be incorporated per mole of 43K protein. To begin to define site(s) of phosphate incorporation, we examined phosphopeptide maps of chemically cleaved 43K protein. Membrane-bound 43K protein was phosphorylated in the presence of exogenous PKA catalytic subunit, and the purified radiolabeled protein was reduced, carboxymethylated, and subjected to CNBr cleavage. Chemical digestion using CNBr was selected because the predicted peptides distinguish among potential phosphorylation sites (except between Ser-260 and Ser-271). Further, we have found that CNBr treatment is more likely to proceed to completion as compared with enzymatic methods. In five different experiments, digestion consistently generated a low molecular mass (<5-kDa) radioactive limit peptide (as judged by electrophoresis on 20% SDS–polyacrylamide gels), suggesting that the reaction was complete.

CNBr peptides were separated by high-voltage electrophoresis and thin-layer chromatography as described under Experimental Procedures. Consistently, most of the radioactivity (>80%) localized to a single location (Figure 7, panel A). Further exposure to CNBr for 24 h did not significantly change the pattern or location of radioactive phosphopeptide(s) in two-dimensional separations. In one experiment, 43K protein was dephosphorylated prior to exposure to exogenous PKA and [γ-^{32}P]ATP. Again, the majority of [^{32}-P]phosphate localized to a single spot in peptide maps (Figure 7, panel B),

suggesting that in vitro phosphorylation occurs at the same site(s) as that occupied by native phosphate. Together, these data provide suggestive evidence that the greater part of phosphate incorporation occurs at a single site.

Discussion

The 43K protein is a major constituent of the postsynaptic apparatus, present as one molecule per AChR in *Torpedo* electric organ (LaRochelle & Froehner, 1986). 43K protein has been detected at the neuromuscular junction (Froehner et al., 1981; Froehner, 1984) and at clusters of AChR in skeletal muscle cultures (Burden, 1985; Peng & Froehner, 1985; Bloch & Froehner, 1987) exhibiting substantial conservation of epitope and primary sequence characteristics between species (Baldwin et al., 1988; Frail et al., 1988; Froehner, 1989). 43K protein may serve as an intermediate piece between the cytoskeleton and the postsynaptic membrane [reviewed in Kordeli et al. (1989) and Froehner (1986)], and its function is under active investigation. In view of the modulatory role played by protein phosphorylation in cytoskeletal interactions and signal transduction systems, we have studied the phosphorylation of 43K protein.

This report is the first characterization of the 43K protein as a serine phosphoprotein. We have shown that the 43K protein can be phosphorylated in vitro to a significant stoichiometry. Under basal conditions, 43K protein incorporates [^{32}P]phosphate to a greater extent than the other protein bands in postsynaptic membranes.

Our experiments using specific inhibitors of protein kinases indicate that 43K protein is phosphorylated by endogenous PKA that copurifies with the postsynaptic membranes. Further, we demonstrate that substantial phosphate exists in the native 43K protein. This finding has been corroborated by the detection of phosphoserine in 43K protein extracts from *Torpedo* membranes by using polyclonal antisera directed against phosphoserine (Hill et al., unpublished observations). The issue of possible 43K protein phosphorylation in situ mediated by kinases other than those that copurify with postsynaptic membranes remains to be investigated. Taken together, these observations are consistent with the existence of a regulatory role for 43K protein phosphorylation in vivo.

Postsynaptic membranes from *T. californica* have been shown to contain PKA, protein kinase C, and Ca^{2+}/calmodulin-dependent kinase, and a tyrosine kinase [review: Miles and Huganir (1988)]. The AChR γ and δ subunits are known to be phosphorylated by endogenous PKA (Huganir & Greengard, 1983; Yee & Huganir, 1987). It is of interest that the time course of phosphate uptake by 43K protein is significantly different than that of the AChR subunits. Also, the steady-state level of phosphate incorporated in 43K protein markedly exceeds that of the AChR subunits. These findings implicate different phosphorylation/dephosphorylation reaction cascades and suggest that different phosphate-mediated regulatory mechanisms may exist. At present, we have no data to suggest that incorporated phosphate accounts for the variability in 43K protein p*I* on two-dimensional SDS–PAGE.

Phosphoamino acid analysis indicated that 43K protein is phosphorylated exclusively on serine residues. Following limit digestion with CNBr, the majority of radioactive phosphate localized to a single spot on two-dimensional separations. This suggests that phosphorylation occurs at one or a very few sites at significant stoichiometry. Sequencing experiments are underway to map these sites.

43K protein is strictly localized to the subsynaptic apparatus where it may play a role in the stabilization of AChR clusters. Synthesis of 43K protein mRNA in skeletal muscle is down-regulated by electrical activity (Baldwin et al., 1988; Frail et al., 1989; Froehner, 1989). A variety of agents that elevate cAMP levels have been shown to up-regulate the biosynthesis of certain postsynaptic proteins [reviewed in Laufer and Changeux (1989)]. Notable among these is calcitonin gene related peptide (CGRP), a factor that coexists with acetylcholine in several vertebrate motor systems (Hökfelt et al., 1986). It has been proposed that such anterograde factors released from the motor neuron stimulate AChR gene expression at subsynaptic nuclei [see Changeux et al. 1991)]. Associated with this process is the PKA-mediated phosphorylation of AChR (Miles et al., 1989). It is tempting to speculate that PKA-mediated phosphorylation also regulates the putative stabilizing role of 43K protein in the postsynaptic apparatus. Interestingly, the favored C-terminal PKA phosphorylation site is conserved in the primary sequence of 43K protein of mouse muscle (Frail et al., 1988).

The experiments described herein were performed by using native, receptor-associated 43K protein in intact membrane vesicles. Evidence for close association of 43K protein with cytoplasmic projections of AChR has been provided by freeze–fracture immunoelectron microscopy (Bridgman et al., 1987). X-ray diffraction studies have suggested that 43K protein may lie beneath the AChR ion channel (Toyoshima & Unwin, 1988) or just to one side, perhaps joining adjacent receptors (Mitra et al., 1989). These studies, together with observations made with bifunctional cross-linking reagents (Burden et al., 1983), suggest that significant portions of the native 43K protein are involved in (still putative) AChR interactions. Our observations have shown that a phosphorylation site accessible from the cytoplasm exists. It will be interesting to elucidate the functional significance of 43K protein phosphorylation as well as the mechanisms that regulate the enzymatic cascade.

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Registry No. Protein kinase, 9026-43-1; serine, 56-45-1.

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