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Biochemistry

Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase

(phosphotyrosine/phosphorylation sites/phosphoprotein/ion channel)

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ABSTRACT **Postsynaptic membranes from the electric organ of *Torpedo californica*, rich in the nicotinic acetylcholine receptor, were shown to contain an endogenous tyrosine protein kinase. This endogenous kinase phosphorylated three major proteins with molecular masses corresponding to 50 kDa, 60 kDa, and 65 kDa. The phosphorylation of these three proteins occurred exclusively on tyrosine residues under the experimental conditions used and was abolished by 0.1% Nonidet P-40 and stimulated by Mn^{2+}. The 50-kDa, 60-kDa, and 65-kDa phosphoproteins were demonstrated to be the β, γ, and δ subunits, respectively, of the nicotinic acetylcholine receptor by purification of the phosphorylated receptor using affinity chromatography. The endogenous tyrosine kinase specifically phosphorylated the β, γ, and δ subunits rapidly to a final stoichiometry of $\approx$0.5 mol of phosphate per mol of subunit. Two-dimensional phosphopeptide mapping of the phosphorylated β, γ, and δ subunits, after limit proteolysis with trypsin or thermolysin, indicated that each subunit was phosphorylated on a single site. Locations are proposed for the amino acid residues phosphorylated on the receptor by the tyrosine-specific protein kinase and by two other protein kinases (cAMP-dependent protein kinase and protein kinase C) which phosphorylate the receptor.**

Protein phosphorylation has been widely accepted as one of the principal regulatory mechanisms in the control of biological processes (1). It has recently been suggested that phosphorylation of membrane receptors plays an important role in the regulation of signal transduction systems. The nicotinic acetylcholine receptor (2, 3), the β-adrenergic receptor (4), the epidermal growth factor (EGF) receptor (5), the insulin receptor (6), and the platelet-derived growth factor (PDGF) receptor (7) have been demonstrated to be phosphorylated by endogenous protein kinases. The nicotinic acetylcholine receptor is phosphorylated on serine residues (8, 9), whereas the EGF receptor, the insulin receptor, and the PDGF receptor are phosphorylated on tyrosine (10–12), serine, and threonine residues (13–15). In fact, it appears that the EGF (5), insulin (16), and PDGF (7) receptors themselves are tyrosine-specific protein kinases that are stimulated by their respective physiological ligands.

In view of the potential physiological significance of phosphorylation of membrane receptors, we have carried out a detailed investigation of the phosphorylation of the nicotinic acetylcholine receptor (9, 17). This receptor, a neurotransmitter-regulated ion channel, is one of the most well-characterized membrane receptors and has served as a model system for the investigation of the structure, function, and regulation of membrane receptors (18). The purified receptor is a 255-kDa polypeptide complex, which consists of four subunits, α (40 kDa), β (50 kDa), γ (60 kDa), and δ (65 kDa), in a stoichiometry of $\alpha_2\beta\gamma\delta$ (19). This pentameric polypeptide complex is functionally complete and displays all of the known biological properties of the nicotinic receptor when it is reconstituted into phospholipid vesicles (for review, see ref. 20; refs. 21 and 22). The purified receptor, isolated in the presence of phosphatase inhibitors, has been reported to contain one phosphoserine on the α subunit, one phosphoserine on the β subunit, two phosphoserines on the γ subunit, and five phosphoserines on the δ subunit (8).

Postsynaptic membranes isolated from *Torpedo californica* contain endogenous protein kinases that phosphorylate the nicotinic receptor (2, 3, 23) as well as protein phosphatases that dephosphorylate the receptor (24). These postsynaptic membranes have been shown to contain three endogenous protein kinases: a cAMP-dependent protein kinase (17, 25), a calcium/calmodulin-dependent protein kinase (17, 25, 26), and a calcium/phospholipid-dependent protein kinase (protein kinase C) (9). The cAMP-dependent protein kinase phosphorylates the γ and δ subunits of the receptor on serine residues (9, 17, 25), whereas protein kinase C phosphorylates the δ and α subunits of the receptor on serine residues (ref. 9 and unpublished results). The calcium/calmodulin-dependent protein kinase phosphorylates proteins that comigrate on $NaDodSO_4$/polyacrylamide gels with the subunits of the receptor (17, 25, 26), although these proteins have been demonstrated to be distinct from the receptor subunits (17, 25). We now report that isolated postsynaptic membranes from *T. californica* also contain a very active tyrosine protein kinase that phosphorylates the nicotinic acetylcholine receptor rapidly and specifically on the β, γ, and δ subunits.

MATERIALS AND METHODS

Materials. Live *T. californica* were obtained from Winkler Enterprises (San Pedro, CA). Nonidet P-40 (NP-40), ouabain, phosphotyrosine, phosphoserine, phosphothreonine, benzamidine, cholic acid, TPCK-trypsin, and [5-valine]angiotension II were obtained from Sigma. Sodium vanadate was obtained from Fisher. Leupeptin, antipain, and chymostatin were obtained from Chemicon (Los Angeles, CA). ^{125}I-labeled α-bungarotoxin and [γ-^{32}P]ATP were obtained from New England Nuclear. The affinity resin was synthesized (19) by reacting bromoacetylcholine (27) with reduced Affi-Gel 401 (Bio-Rad). Protein Kinase Inhibitor (PKI), the specific peptide inhibitor of cAMP-dependent protein kinase, was purified from skeletal muscle by a modification of the procedure of McPherson *et al.* (28).

Preparation of Postsynaptic Membranes. Postsynaptic membranes were prepared from freshly dissected electric organ of *T. californica* by the method of Sobel *et al.* (29), as modified (21) with the following exceptions: (*i*) the minced organs were homogenized four times, 30 sec each, in a Waring blender in 20 mM Tris·HCl, pH 7.4/10 mM EGTA/5 mM

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Abbreviations: NP-40, Nonidet P-40; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKI, Protein Kinase Inhibitor.

EDTA/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/10 mM benzamidine/10 μg of pepstatin per ml/10 μg of leupeptin per ml/10 μg of antipain per ml/10 μg of chymostatin per ml/10 units of Trasylol per ml; and (*ii*) the membranes were not sonicated before loading onto the discontinuous sucrose gradient. (Preparing membranes in the presence of a high concentration of divalent cation chelators and without sonication produces a membrane preparation that is not tightly sealed and therefore does not require solubilization by detergents to allow access of [γ-^{32}P]ATP and PKI to the protein kinases on the intracellular side of the membranes [17, 30].) The material at the 37.5%/41.5% (wt/wt) sucrose interface of the discontinuous gradient, which had an α-bungarotoxin binding activity (31) of 1.5–2.0 nmol/mg of protein, was used for all experiments.

Phosphorylation Assays. Endogenous phosphorylation was measured under standard conditions by incubating 20–30 μg of membrane protein in 20 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/2 mM $MnCl_2$/1 mM ouabain/1 mM sodium vanadate/0.5 mM EGTA/0.5 mM EDTA/10 mM 2-mercaptoethanol/100 nM PKI/10 μg of leupeptin per ml/10 μg of antipain per ml/50–200 μM [γ-^{32}P]ATP (≈10^3 cpm/pmol), in a final volume of 100 μl. (Ouabain and sodium vanadate were present in the assay mixture in order to inhibit endogenous ATPases; however, substantial hydrolysis of ATP occurred even in the presence of these substances.) The reaction was initiated with [γ-^{32}P]ATP and carried out for the indicated times at 30°C. The reaction was stopped by the addition of 50 μl of 6% $NaDodSO_4$/375 mM Tris·HCl, pH 6.8/30% glycerol (wt/vol)/15% 2-mercaptoethanol/0.05% bromphenol blue. The samples were then subjected to electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels as described by Laemmli (32). The gels were stained with Coomassie blue, dried, and subjected to autoradiography on Kodak X-Omat film. The incorporation of ^{32}P was quantitated by cutting out the appropriate gel pieces and placing them directly in Liquiscint and counting the radioactivity by liquid scintillation spectrophotometry.

Phosphoamino Acid Analysis. Appropriate dried gel pieces were washed with 3× 10 ml of 25% methanol/10% acetic acid, followed by 2× 10 ml of 50% methanol. The gel pieces were then lyophilized and the phosphorylated proteins were digested at 37°C for 20 hr in 1.0 ml of 50 mM NH_4HCO_3 containing 0.15 mg of trypsin. The gel pieces were removed and rinsed with 0.5 ml of 50 mM NH_4HCO_3. The wash and the digest, which together always contained >90% of the incorporated ^{32}P, were combined, lyophilized, and resuspended in 6 M HCl and hydrolyzed under vacuum for 1.5 hr at 105°C (33). The acid-hydrolyzed peptides were then dried and resuspended in formic acid/acetic acid/H_2O, 1:10:89 (vol/vol), pH 1.9, and spotted 4 cm from the ends of 20 × 20 cm cellulose thin-layer plates (34). Phosphotyrosine, phosphoserine, and phosphothreonine standards (2 mg/ml) with a trace of phenol red were also spotted at the origin of each plate. Electrophoresis was carried out in the same pH 1.9 buffer at 500 V until the phenol red had moved 12 cm. The plates were dried and subjected to electrophoresis at 500 V in the second dimension in acetic acid/pyridine/H_2O, 19:1:89 (vol/vol), pH 3.5, until the phenol red had moved 6 cm. The cellulose plates were then dried, developed with 1% ninhydrin in acetone to detect the internal phosphoamino acid standards, and subjected to autoradiography to identify the ^{32}P-labeled phosphoamino acids.

Two-Dimensional Phosphopeptide Maps. Gel pieces containing the various phosphorylated proteins were washed, digested with trypsin (0.15 mg/ml) or thermolysin (0.3 mg/ml), and lyophilized as described above. The lyophilized peptides were resuspended in pyridine/acetic acid/H_2O, 1:10:89 (vol/vol), pH 3.5, and spotted on 20 × 20 cm cellulose thin-layer plates as described above. The cellulose plates were subjected to electrophoresis until the phenol red migrated 12 cm. The plates were dried and subjected to ascending chromatography in the second dimension in pyridine/butanol/acetic acid/H_2O, 15:10:3:12 (vol/vol). The plates were then dried and autoradiographed.

Protein Determinations. Protein was measured by a minor modification of the procedure of Lowry *et al.* (35) with bovine serum albumin as standard.

RESULTS

Endogenous Mn^{2+}-Stimulated Protein Phosphorylation. The isolated postsynaptic membrane preparation from the electric organ of *T. californica* contains seven major polypeptide bands with molecular masses corresponding to 40 kDa, 43 kDa, 50 kDa, 55 kDa, 60 kDa, 65 kDa, and 95 kDa. Four of these polypeptide bands comigrate on $NaDodSO_4$/polyacrylamide gels with the α (40 kDa), β (50 kDa), γ (60 kDa), and δ (65 kDa) subunits of the nicotinic acetylcholine receptor, which comprises ≈25% of the membrane protein. Endogenous protein phosphorylation was examined in this preparation using PKI to inhibit endogenous cAMP-dependent protein kinase and EGTA to inhibit endogenous calcium-dependent protein kinases (Fig. 1). In agreement with our previous results (17), very little protein phosphorylation was observed in the presence of the nonionic detergent NP-40 (Fig. 1, lane 1). However, when the phosphorylation reaction was carried out in the absence of NP-40, a detectable phosphorylation of six proteins with molecular masses corresponding to 50 kDa, 55 kDa, 60 kDa, 65 kDa, 85 kDa, and 95 kDa was observed (Fig. 1, lane 2). Moreover, the phosphorylation of the 50-kDa, 60-kDa, and 65-kDa proteins was markedly stimulated by the addition of 2 mM Mn^{2+} (Fig. 1, lane 3). In the presence of Mn^{2+}, the phosphorylation of the 50-kDa, 60-kDa, and 65-kDa proteins was completely inhibited by low concentrations of each of several nonionic, ionic, and zwitterionic detergents examined (data not shown). The Mn^{2+}-dependent phosphorylation of the 50-kDa, 60-kDa, and 65-kDa proteins was not affected by the presence or ab-

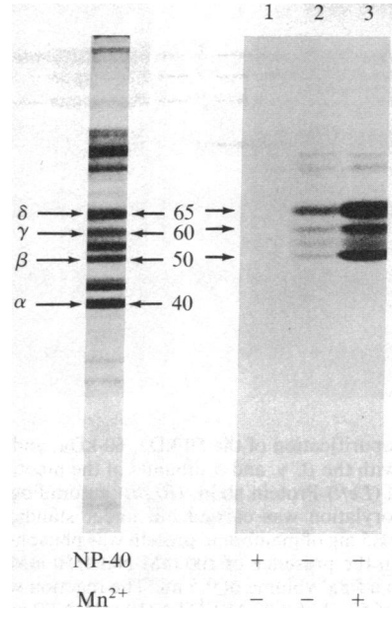

FIG. 1. Endogenous protein phosphorylation in membrane preparation rich in the acetylcholine receptor. (*Left*) Protein stain; (*Right*) autoradiogram. Endogenous phosphorylation was carried out under standard conditions for 2.5 min with 50 μM [γ-^{32}P]ATP in the presence and absence of 0.1% NP-40 and Mn^{2+}, as indicated. Size markers are in kDa.

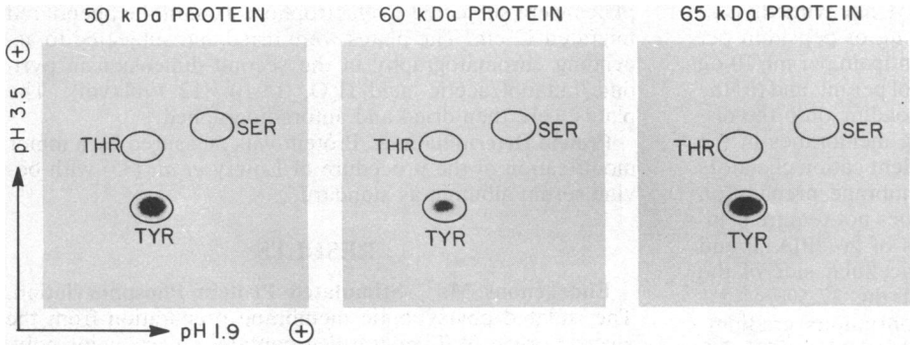

FIG. 2. Phosphoamino acid analysis of the 50-kDa, 60-kDa, and 65-kDa phosphoproteins phosphorylated by the endogenous Mn^{2+}-stimulated protein kinase. Endogenous phosphorylation was carried out under standard conditions for 30 min with 50 μM [γ-^{32}P]ATP. Two-dimensional thin-layer electrophoresis of the acid-hydrolyzed (33) phosphoproteins was carried out as described (34). Circles indicate positions of standard phosphothreonine (THR), phosphoserine (SER), and phosphotyrosine (TYR).

sence of PKI, EGTA, or trifluoroperazine nor was it affected by cAMP, calcium, or phospholipid (data not shown).

Phosphoamino Acid Analysis. Since stimulation of protein phosphorylation by Mn^{2+} is a known characteristic of tyrosine protein kinases (5–7), we analyzed the 50-kDa, 60-kDa, and 65-kDa phosphoproteins for phosphoamino acid content (33, 34). In the presence of PKI, EGTA, and Mn^{2+}, all three of these polypeptides are phosphorylated exclusively on tyrosine residues (Fig. 2).

Identification of the 50-kDa, 60-kDa, and 65-kDa Phosphoproteins as Subunits of the Acetylcholine Receptor. The 50-kDa, 60-kDa, and 65-kDa phosphoproteins comigrated on $NaDodSO_4$/polyacrylamide gels with the β, γ, and δ subunits of the nicotinic acetylcholine receptor. We therefore tested whether these phosphoproteins were the receptor subunits. For this purpose, we incubated the membrane preparation under standard phosphorylation conditions and stopped the reaction under nondenaturing conditions (i.e., by the addition of EDTA and unlabeled ATP). The phosphorylated membrane preparation was then extracted with 1% sodium cholate as described (21). The detergent extract, which contained 70–80% of the membrane protein and 70–80% of the acetylcholine receptor, was then incubated with an acetylcholine affinity resin in order to purify the nicotinic acetylcholine receptor (21). Under these conditions, ≈70% of the receptor in the extract is adsorbed to the affinity resin. The resin was then washed and the purified receptor was eluted with carbamoylcholine. When the different fractions from the purification were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis for phosphoprotein content, the 50-kDa, 60-kDa, and 65-kDa phosphoproteins were found to copurify with the nicotinic acetylcholine receptor, indicating that these phosphoproteins were the β, γ, and δ subunits of the receptor (Fig. 3). In contrast, the 55-kDa, 85-kDa, and 95-kDa phosphoproteins did not bind to the affinity resin.

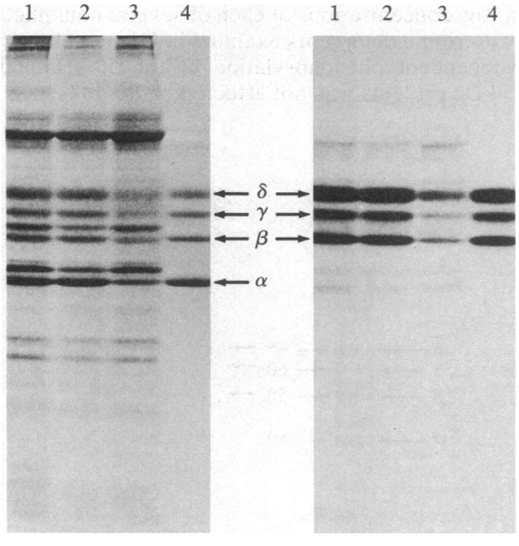

FIG. 3. Copurification of the 50-kDa, 60-kDa, and 65-kDa phosphoproteins with the β, γ, and δ subunits of the nicotinic acetylcholine receptor. (*Left*) Protein stain; (*Right*) autoradiogram. Endogenous phosphorylation was carried out under standard conditions, except that ≈1.5 mg of membrane protein was phosphorylated for 30 min at 30°C in the presence of 100 mM NaCl/50 mM KCl/100 μM [γ-^{32}P]ATP in a final volume of 0.5 ml. The reaction was stopped by the addition of 50 μl of 0.20 M EDTA/10 mM ATP and the mixture was placed on ice. The phosphorylated membranes were extracted with 1% sodium cholate and purified on a 0.25-ml acetylcholine affinity column (21). Aliquots of phosphorylated membranes (lanes 1), cholate extract applied to the affinity resin (lanes 2), flow-through from the affinity resin (lanes 3), and purified acetylcholine receptor (lanes 4) were analyzed for phosphoprotein content by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography.

Rate and Stoichiometry of Phosphorylation of β, γ, and δ Subunits of the Acetylcholine Receptor. The three subunits were phosphorylated by the endogenous tyrosine kinase at roughly similar rates, although the initial rate of phosphorylation of the δ subunit was always slightly faster than that of the β and γ subunits (Fig. 4). The phosphorylation of the β, γ, and δ subunits by the endogenous tyrosine kinase was five to six times faster than the rate of phosphorylation of the γ and δ subunits by the endogenous cAMP-dependent protein kinase (data not shown). The phosphorylation of the subunits by the tyrosine kinase leveled off at a final stoichiometry of 0.40–0.53 mol of phosphate per mol of subunit after 60 min (Fig. 4). This plateau is attributable at least in part to the inactivation of the endogenous tyrosine kinase by the extended period of incubation of the membrane preparation at 30°C (not shown).

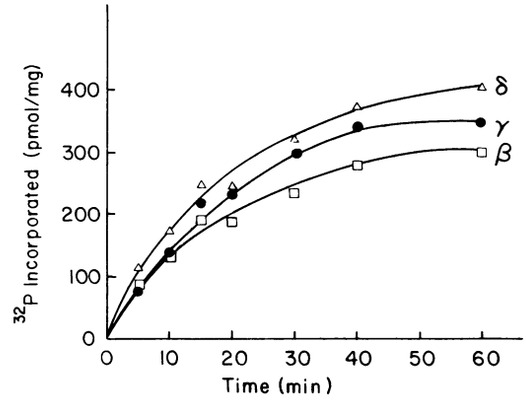

FIG. 4. Time course of endogenous tyrosine phosphorylation of the β, γ, and δ subunits of the acetylcholine receptor. Endogenous phosphorylation was carried out for the indicated times under standard conditions with 200 μM [γ-^{32}P]ATP. The final stoichiometry of phosphorylation of the β, γ, and δ subunits was 0.4, 0.45, and 0.53 mol of phosphate per mol of subunit, respectively.

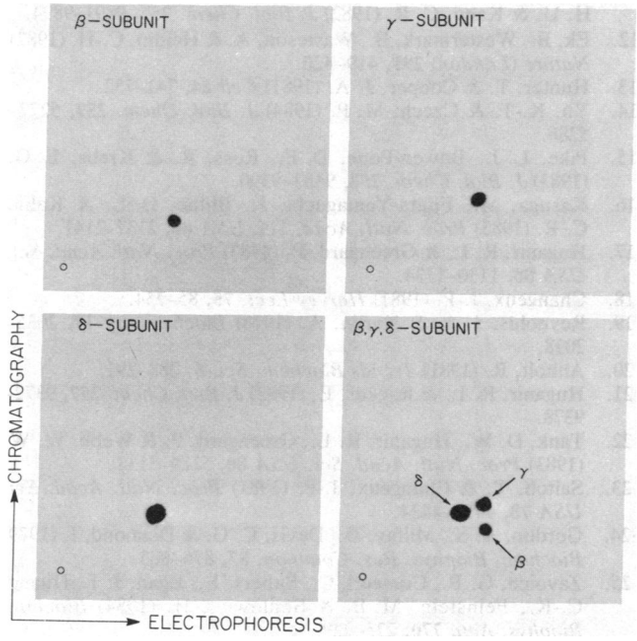

FIG. 5. Two-dimensional tryptic phosphopeptide maps of the β, γ, and δ subunits of the acetylcholine receptor phosphorylated by endogenous tyrosine protein kinase. Endogenous phosphorylation was carried out under standard conditions with 100 μM [γ-^{32}P]ATP. The final stoichiometry of the phosphorylation of the β, γ, and δ subunits was 0.3–0.5 mol of phosphate per mol of receptor.

Phosphopeptide Maps of the Phosphorylated β, γ, and δ Subunits of the Acetylcholine Receptor. When the phosphorylated β, γ, and δ subunits were subjected to limit digestion with trypsin and the resultant phosphopeptides analyzed by two-dimensional thin-layer chromatography, each subunit yielded a single phosphopeptide (Fig. 5). The phosphopeptides from the β, γ, and δ subunits had similar but not identical mobilities in the two dimensions. Thermolysin digestion of the phosphorylated subunits also produced a single distinct phosphopeptide for each subunit (data not shown).

DISCUSSION

The results presented here demonstrate that postsynaptic membranes rich in the nicotinic acetylcholine receptor contain an endogenous tyrosine protein kinase that specifically phosphorylates the β, γ, and δ subunits of the receptor. The phosphorylation of these subunits by the tyrosine kinase is stimulated by Mn^{2+}, a characteristic of other tyrosine kinases (5–7). The tyrosine kinase appears to be the most active protein kinase in the postsynaptic membrane preparation and phosphorylates each of the three subunits on a single site to a high stoichiometry. The phosphorylation of the receptor subunits by the tyrosine kinase is inhibited by low concentrations of the nonionic detergent NP-40 and all other detergents examined. This inhibition appears to be due to a specific effect on the ability of the receptor to serve as a substrate, since tyrosine phosphorylation by the postsynaptic membranes of exogenous substrates such as [5-valine]angiotensin II (36) is activated rather than inhibited by NP-40 (data not shown). In agreement with this interpretation, it has been reported that other membrane-associated tyrosine kinases are generally unaffected or activated by nonionic detergents (5–7).

δ-SUBUNIT
358 P P P 378
ARG-ARG-SER-SER-SER-VAL-GLY-TYR-ILE-SER-LYS-ALA-GLN-GLU-TYR-PHE-ASN-ILE-LYS-SER-ARG

γ-SUBUNIT
350 P P 370
ARG-ARG-ARG-SER-SER-PHE-GLY-ILE-MET-ILE-LYS-ALA-GLU-GLU-TYR-ILE-LEU-LYS-LYS-PRO-ARG

β-SUBUNIT
340 P 360
SER-PRO-ASP-SER-LYS-PRO-THR-ILE-ILE-SER-ARG-ALA-ASP-ASP-GLU-TYR-PHE-ILE-ARG-LYS-PRO

α-SUBUNIT
314 P 334
LYS-ILE-PHE-ILE-ASP-THR-ILE-PRO-ASN-VAL-MET-PHE-PHE-SER-THR-MET-LYS-ARG-ALA-SER-LYS

FIG. 6. Proposed locations of the phosphorylated amino acid residues on the α, β, γ, and δ subunits of the nicotinic acetylcholine receptor. The kinases and their proposed phosphorylation sites are tyrosine-specific protein kinase (β-subunit, Tyr-355; γ-subunit, Tyr-364; δ-subunit, Tyr-372), cAMP-dependent protein kinase (γ-subunit, Ser-354; δ-subunit, Ser-361), and protein kinase C (α-subunit, Ser-333; δ-subunit, Ser-377). The numbering of the amino acids is according to Noda *et al.* (37, 39, 40).

The identity of the endogenous tyrosine kinase responsible for phosphorylation of the β, γ, and δ subunits of the nicotinic acetylcholine receptor has not yet been determined. The phosphorylation does not appear to be regulated by insulin, EGF, or PDGF (data not shown) and, therefore, is probably not mediated through a receptor for one of these ligands. A431 cell membranes, which are extremely rich in the EGF receptor (5), do specifically phosphorylate purified acetylcholine receptor on the β, γ, and δ subunits in an EGF-stimulated manner (data not shown). Moreover, purified pp60^{src} of Rous sarcoma virus specifically phosphorylates the β, γ, and δ subunits of purified acetylcholine receptor (unpublished results in collaboration with D. Anthony, L. Rubin, and A. Goldberg). However, we have no evidence that the endogenous tyrosine kinase is the EGF receptor or a normal cellular pp60^{src} homologue. It remains to be determined whether the nicotonic acetylcholine receptor, by analogy with the insulin receptor, the EGF receptor, and the PDGF receptor, is itself a tyrosine kinase.

We have previously demonstrated that postsynaptic membrane preparations contain an endogenous cAMP-dependent protein kinase that specifically phosphorylates the γ and δ subunits of the receptor (17) and an endogenous calcium/phospholipid-dependent protein kinase (protein kinase C) that specifically phosphorylates the δ and α subunits of the receptor (ref. 9 and unpublished results). The complete amino acid sequence of all four subunits of the receptor has been deduced from the cDNA sequence (37–40). Therefore, we have examined the sequences of the various subunits for possible phosphorylation sites for the three protein kinases, taking the following into account: (*i*) the specificity of the three protein kinases for the subunits of the receptor; (*ii*) peptide maps of the subunits phosphorylated by the three kinases using proteases and CNBr; and (*iii*) the known primary amino acid sequence preferences of tyrosine-specific protein kinases (41) and cAMP-dependent protein kinase (42). The proposed locations of the various phosphorylation sites in the primary amino acid sequence of the α, β, γ, and δ subunits of the nicotinic receptor are shown in Fig. 6. All three of the tyrosine residues proposed as phosphorylation sites for the tyrosine-specific kinases are preceded by glutamic and/or aspartic acid residues; acidic amino acid residues preceding the phosphorylated tyrosine residue are characteristic of known tyrosine phosphorylation sites (41). The two serine residues proposed as phosphorylation sites for cAMP-dependent protein kinase are preceded by three (γ subunit) and two (δ subunit) arginine residues; basic amino acids preceding the phosphorylated serine residue are characteristic of the known phosphorylation sites for the cAMP-dependent protein kinase (42). These proposed phosphorylation sites, as well as the two phosphorylation sites proposed for protein kinase C (Fig. 6), will have to be confirmed by sequencing the isolated phosphopeptides.

Although it has been demonstrated that the nicotinic acetylcholine receptor is multiply phosphorylated *in vitro* by three protein kinases endogenous to the postsynaptic membrane (refs. 9 and 17; and this paper), it is not known what effect(s) these phosphorylation events have on the function of the receptor. Characteristics of the nicotinic receptor such as ion channel properties, isoelectric point, antigenic properties, metabolic stability, and receptor clustering at the neuromuscular junction are known to change during development (18). It has been suggested that these changes may be due to post-translational modifications such as phosphorylation (see ref. 18). It has been reported recently that chicken myotubes transformed with Rous sarcoma virus do not cluster acetylcholine receptors even in the presence of active clustering factors (43). This inhibition of receptor clustering appears to be mediated by the src gene product $pp60^{src}$ (43), which is a tyrosine protein kinase. It is possible that phosphorylation of the β, γ, and δ subunits of the nicotinic receptor by $pp60^{src}$ contributes to the observed inhibition of receptor clustering in the transformed cells.

The phosphorylation sites proposed in this paper are located within 20 amino acids on a conserved region of each of the subunits. If the proposed phosphorylation sites are correct, it would suggest that phosphorylation of the acetylcholine receptor by these three protein kinases may be involved in regulating a common property of the receptor. These proposed phosphorylation sites are located on the major intracellular loop in theoretical models of the structure of the receptor subunits (38, 40, 44, 45). Thus, phosphorylation of these areas of the subunits might regulate the interaction of the subunits with cytoskeletal elements and thereby affect receptor clustering. Alternatively, phosphorylation of these areas, which are adjacent to a membrane-spanning region thought to form the ion channel of the receptor (44, 45), might regulate the receptor ion channel properties.

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1. Nestler, E. & Greengard, P. (1983) *Nature (London)* **305,** 583–588.
2. Gordon, A. S., Davis, C. G., Milfay, D. & Diamond, I. (1977) *Nature (London)* **267,** 539–540.
3. Teichberg, V. I., Sobel, A. & Changeux, J.-P. (1977) *Nature (London)* **267,** 540–542.
4. Stadel, J. M., Nambi, P., Shorr, R. G. L., Sawyer, D. F., Caron, M. G. & Lefkowitz, R. J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3173–3177.
5. Cohen, S., Carpenter, G. & King, L. (1980) *J. Biol. Chem.* **255,** 4834–4842.
6. Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215,** 185–187.
7. Heldin, C. H., Ek, B. & Rönnstrand, L. (1983) *J. Biol. Chem.* **258,** 10054–10061.
8. Vandlen, R. L., Wu, W. C.-S., Eisenach, J. C. & Raftery, M. A. (1979) *Biochemistry* **18,** 1845–1853.
9. Huganir, R. L., Albert, K. A. & Greengard, P. (1983) *Soc. Neurosci. Abstr.* **9,** 578.
10. Ushiro, H. & Cohen, S. (1980) *J. Biol. Chem.* **255,** 8363–8365.
11. Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Häring, H. U. & Kahn, C. R. (1982) *J. Biol. Chem.* **257,** 9891–9894.
12. Ek, B., Westermark, B., Wasteson, A. & Heldin, C.-H. (1982) *Nature (London)* **295,** 419–420.
13. Hunter, T. & Cooper, J. A. (1981) *Cell* **24,** 741–752.
14. Yu, K.-T. & Czech, M. P. (1984) *J. Biol. Chem.* **259,** 5277–5286.
15. Pike, L. J., Bowen-Pope, D. F., Ross, R. & Krebs, E. G. (1983) *J. Biol. Chem.* **258,** 9383–9390.
16. Kasuga, M., Fujita-Yamaguchi, J., Blithe, D. L. & Kahn, C. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2137–2141.
17. Huganir, R. L. & Greengard, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1130–1134.
18. Changeux, J.-P. (1981) *Harvey Lect.* **75,** 85–254.
19. Reynolds, J. A. & Karlin, A. (1978) *Biochemistry* **17,** 2035–2038.
20. Anholt, R. (1981) *Trends Biochem. Sci.* **6,** 288–291.
21. Huganir, R. L. & Racker, E. (1982) *J. Biol. Chem.* **257,** 9372–9378.
22. Tank, D. W., Huganir, R. L., Greengard, P. & Webb, W. W. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5129–5133.
23. Saitoh, T. & Changeux, J.-P. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4430–4434.
24. Gordon, A. S., Milfay, D., Davis, C. G. & Diamond, I. (1979) *Biochem. Biophys. Res. Common.* **87,** 876–883.
25. Zavoico, G. B., Comerci, C., Subers, E., Egan, J. J., Huang, C.-K., Feinstein, M. B. & Smilowitz, H. (1984) *Biochim. Biophys. Acta* **770,** 225–229.
26. Smilowitz, H., Hadjian, R. A., Dwyer, J. & Feinstein, M. B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4708–4712.
27. Damle, V. N., McLaughlin, M. & Karlin, A. (1978) *Biochem. Biophys. Res. Commun.* **84,** 845–851.
28. McPherson, J. M., Whitehouse, S. & Walsh, D. A. (1979) *Biochemistry* **18,** 4835–4844.
29. Sobel, A., Weber, M. & Changeux, J.-P. (1977) *Eur. J. Biochem.* **80,** 215–224.
30. Davis, C. G., Gordon, A. S. & Diamond, I. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3666–3670.
31. Schmidt, J. & Raftery, M. A. (1973) *Anal. Biochem.* **52,** 349–354.
32. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
33. Bylund, D. B. & Huang, T.-S. (1976) *Anal. Biochem.* **73,** 477–485.
34. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
36. Wong, T. W. & Goldberg, A. R. (1983) *J. Biol. Chem.* **258,** 1022–1025.
37. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. & Numa, S. (1982) *Nature (London)* **299,** 793–797.
38. Claudio, T., Ballivet, M., Patrick, J. & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1111–1115.
39. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T. & Numa, S. (1983) *Nature (London)* **301,** 251–255.
40. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. & Numa, S. (1983) *Nature (London)* **302,** 528–532.
41. Patschinsky, T., Hunter, T., Esch, F. S., Cooper, J. A. & Sefton, B. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 973–977.
42. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G. (1977) *J. Biol. Chem.* **252,** 4888–4894.
43. Anthony, D. T., Schuetze, S. M. & Rubin, L. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2265–2269.
44. Devillers-Thiery, A., Giraudat, J., Bentaboulet, M. & Changeux, J.-P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2067–2071.
45. Finer-Moore, J. & Stroud, R. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 155–159.