Determination of the Tyrosine Phosphorylation Sites of the Nicotinic Acetylcholine Receptor*

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The peripheral nicotinic acetylcholine receptor (nAChR) is phosphorylated on tyrosine residues in vitro and in vivo at a high stoichiometry. We have previously reported that this tyrosine phosphorylation occurs on the β, γ, and δ subunits of the receptor and is implicated in both the modulation of the function of the receptor and localization of the receptor at the synapse. The specific tyrosine residue of each subunit which is phosphorylated is now identified. The endogenously phosphorylated nAChR from the electric organ of Torpedo californica was phosphorylated to maximal stoichiometry in vitro exclusively on tyrosine residues as indicated by phosphoamino acid analysis. Two-dimensional phosphopeptide maps of thermolysin limit digests of the isolated phosphorylated subunits indicated that each subunit is phosphorylated at a single site. To determine the site of tyrosine phosphorylation of the β, γ, and δ subunits, phosphorylated subunits were isolated and digested with trypsin. A single phospho-tyrosine containing peptide from each subunit was purified by antiphosphotyrosine antibody affinity chromatography and reverse phase high performance liquid chromatography. The purified phosphorylated peptides were subjected to sequential Edman degradation and sequence analysis. Comparison of the phosphopeptide sequence data with the deduced amino acid sequence of each subunit indicated that Tyr-355 of β, Tyr-364 of γ, and Tyr-372 of δ are the sites of in vitro and in vivo tyrosine phosphorylation of the nAChR. Identification of these sites should facilitate further studies of the role of tyrosine phosphorylation in the regulation of receptor function.

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel that mediates signal transduction at the postsynaptic membrane of cholinergic synapses such as the neuromuscular junction and the electroplaque of electric fish. It is a pentameric complex composed of four homologous transmembrane subunits in the stoichiometry α2β2δ (Galzi et al., 1991; Changeux et al., 1984). Recent studies have demonstrated that the nAChR is regulated in vitro and in vivo by serine and tyrosine phosphorylation (Huganir and Miles, 1989). Postsynaptic membranes of the Torpedo electric organ, rich in nAChR, contain endogenous protein kinases which phosphorylate the receptor as well as protein phosphatases which dephosphorylate the receptor (Gordon et al., 1977; Teichberg et al., 1977; Huganir and Greengard, 1983; Huganir, 1987; Huganir et al., 1984; Mei and Huganir, 1991). Endogenous cAMP-dependent protein kinase phosphorylates the γ and δ subunits, while protein kinase C phosphorylates the δ subunit (Huganir and Greengard, 1983; Huganir, 1987; Safran et al., 1987). In addition, an unidentified protein tyrosine kinase has been demonstrated to specifically phosphorylate the β, γ, and δ subunits at a high stoichiometry (Huganir et al., 1984; Hopfield et al., 1988).

The phosphorylation of the receptor by all three of these protein kinases appears to modulate the function of the receptor channel by increasing its rate of rapid desensitization (Huganir et al., 1986; Hopfield et al., 1988; Eusebi et al., 1985). In addition, recent results have suggested that tyrosine phosphorylation of the receptor may be important for synapse formation. Tyrosine phosphorylation of the nAChR in the postsynaptic membrane has been shown to be dependent on neuronal innervation (Qu et al., 1990). The effect of innervation on phosphorylation appears to be mediated by agrin, a neuronally derived extracellular matrix protein, which has been shown to induce receptor aggregation underneath the nerve terminal (Wallace et al., 1991). These results suggest that tyrosine phosphorylation of the nAChR may play an integral role in the formation of the neuromuscular junction.

The location of the phosphorylation sites for each of the various protein kinases within the protein sequence of the nAChR subunits were originally proposed based on subunit specificity and known consensus sequence preferences of the different protein kinases (Huganir et al., 1984). All of the proposed phosphorylation sites are within the major intracellular loop of each subunit in close proximity to each other. The location of the cAMP-dependent protein kinase phosphorylation sites on the γ and δ subunits have been confirmed by protein sequencing techniques (Yee and Huganir, 1987). Recent studies using synthetic peptide substrates and site-specific antibodies have strongly suggested that the protein
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kinase C sites are also contained within this region (Safran et al., 1987). In this paper, we have determined the tyrosine phosphorylation sites within the \( \beta \), \( \gamma \), and \( \delta \) subunits of the \( n \)AChR which are phosphorylated \textit{in vitro} and \textit{in vivo} by the endogenous protein tyrosine kinase. The primary structural requirements of protein tyrosine kinase substrates have begun to be defined by the use of synthetic peptide substrates and the analysis of protein tyrosine kinase autophosphorylation sites (Kemp and Pearson, 1987; Gehlen and Harrison, 1990). However, relatively few sites of physiological substrates have been determined. The identification of the sites of tyrosine phosphorylation of the \( n \)AChR may contribute to a better understanding of the structural determinants required for substrate recognition by protein tyrosine kinases. In addition, this may provide a better understanding of the molecular mechanisms involved in the regulation of receptor function by tyrosine phosphorylation.

\section*{EXPERIMENTAL PROCEDURES}

\textbf{Materials—} \textit{Live} \textit{Torpedo californica} were obtained from Winkler Enzyme (Sacramento, CA). Leupeptin and antipain were purchased from Chemicon (Los Angeles, CA). Aprotinin was from Miles, Inc. (West Haven, CT). \( \gamma ^{32} \text{P} \text{ATP} \) and \( \text{[35S]Protein A} \) were obtained from Du Pont-New England Nuclear. Acetylcholine affinity resin was synthesized (Reynolds and Karlin, 1978) by reacting bromoacetylcholine (Damle et al., 1978) with reduced Affi-Gel 401 (Bio-Rad). Agarose-conjugated antiphosphotyrosine monoclonal IgG was obtained from UBI. Oubain, vanadate, trypsin, and all other chemicals were purchased from Sigma.

\textbf{Tyrosine Phosphorylation of the nAChR—} Postsynaptic membranes, rich in the \( n \)AChR and in the endogenous protein tyrosine kinase which phosphorylates the receptors were prepared from the electric organ of \textit{Torpedo californica} as previously described by Sobel et al. (1977), and modified by Qu et al. (1990). The \( n \)AChR was tyrosine phosphorylated \textit{in vitro} by incubating 50 mg of postsynaptic membrane protein at 0.5 mg/ml in 20 mM Tris-HCl, pH 8.0, 20 mM MgCl\(_2\), 2 mM NaN\(_3\), 1 mM EDTA, 1 mM ouabain, 1 mM Na\(_2\)V\(_3\)O\(_4\), 100 mM dithiothreitol, 1 mM Walsh peptide, and 20 \( \mu \)g of each of antipain, leupeptin, and aprotinin. Phosphorylation was initiated with the addition of ATP: 40 \( \mu \)g of membrane protein were phosphorylated with 200 \( \mu \)M ATP and 10 \( \mu \)g were phosphorylated with 200 \( \mu \)M \( \gamma ^{32} \text{P} \text{ATP} \) at 800 cpm/\( \mu \)g in order to quantitate the in vitro phosphorylation and to follow the phosphopeptide residues by Cerenkov counting throughout the experiment. The reaction was stopped after 45 min at \( 30^\circ \text{C} \) by placing the samples on ice.

\textbf{Purification of Phosphorylated nAChR Subunits—} Phosphorylated membranes were centrifuged at 114,000 \( \times \text{g} \) at 4 \( ^\circ \text{C} \) for 20 min. The pellet was resuspended in one-half volume of 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and then solubilized for 30 min at 4 \( ^\circ \text{C} \) with 1% Triton X-100. Following a 20-min centrifugation at 114,000 \( \times \text{g} \), at 4 \( ^\circ \text{C} \), the receptor was purified from the supernatant by acetylcholine affinity chromatography as described previously (Huganir and Raiker, 1982). One milligram of pooled \( ^{32} \text{P} \)-labeled and unlabeled phosphorylated receptor, at a ratio of approximately 1:4, was electrophoresed on two preparative 8\% acrylamide gels (Laemmli, 1970), and transferred to nitrocellulose (Towbin et al., 1979). Regions of nitrocellulose containing the phosphorylated \( \beta \), \( \gamma \), and \( \delta \) subunits were detected by autoradiography and cut out of strips.

\textbf{Enzymatic Cleavage of Electroblotted Subunits—} Nitrocellulose strips were denatured with 2\% urea and 100 mM NH\(_4\)HCO\(_3\), reduced with 1.125 \( \text{mM} \) dithiothreitol and carboxyamidomethylated with 2.5 \text{mM iodoacetamide} as described by Tone et al. (1989). The subunits were then separately digested in situ as described by Aebersold et al. (1987), using a trypsin/subunit ratio of 1:20 \( \text{w/w} \) in 100 mM NH\(_4\)HCO\(_3\), pH 8.2. At the end of the digestion any remaining peptide was eluted from the strips with 5\% acetonitrile.

\textbf{Purification of Tyrosine Phosphopeptides—} Approximately 15 nmol of each digested subunit in 0.5 ml was diluted 5\( \times \)fold in 100 mM Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride. After removing reducing agent with 0.45\( \mu \)mol \( \text{NaBH}_4\), the peptide samples were boiled for 10 min to completely inactivate the trypsin. The samples were then cooled to room temperature and separately loaded onto 1.0-mLagarose-conjugated antiphosphotyrosine antibody affinity col-
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FIG. 1. Tyrosine phosphorylation of the β, γ, and δ subunits of the nAChR in vitro and in vivo. nAChR was purified from postsynaptic membranes or phosphorylated in vitro with [γ-32P]ATP or nonradioactive ATP prior to purification and the subunits were then resolved on 8% SDS-PAGE. Lane 1, protein stain of purified receptor; lane 2, autoradiography of 32P-labeled tyrosine-phosphorylated receptor; lane 3, immunoblot with antiphototyrosine antibody of endogenously phosphorylated receptor; lane 4, immunoblot with antiphototyrosine antibody of receptor phosphorylated in vitro with nonradioactive ATP. The stoichiometry of tyrosine phosphorylation of the subunits was determined by comparison of the specific activity of the 32P incorporated into the nAChR subunits with the 32P-protein A labeling of the nAChR subunits before and after phosphorylation with nonradioactive ATP as described under "Experimental Procedures."

FIG. 2. Phosphoamino acid analysis of the β, γ, and δ subunits phosphorylated by the endogenous protein tyrosine kinase. Following in vitro phosphorylation with [γ-32P]ATP the subunits were acid hydrolyzed and electrophoresed as described. The 32P-labeled phosphoamino acids were detected by autoradiography. Circles indicate position of phosphothreonine (THR), phosphoserine (SER), and phototyrosine (TYR) standards detected by ninhydrin staining.

FIG. 3. Two-dimensional phosphopeptide maps of the in vitro tyrosine-phosphorylated nAChR subunits. Thermolysin digests of the 32P-labeled β, γ, and δ subunits were separated by electrophoresis and then by ascending chromatography. 32P-Labeled phosphopeptides were detected by autoradiography. Open circles indicate the origin.

FIG. 4. HPLC elution profile of the δ subunit tryptic phosphopeptide. The δ phosphopeptide was purified from other tryptic peptides on an agarose-conjugated antiphototyrosine antibody affinity column, eluted with phenyl phosphate, and isolated by HPLC using a C18 column. The single major peak of UV absorbance coeluted with >90% of the radioactivity (eluting at 17% acetonitrile). The fraction corresponding to this peak was collected and subjected to sequence analysis.

The column and the final fractions containing the phosphopeptide were pooled.

The phosphopeptides were then separated from the phenyl phosphate and trace levels of other tryptic peptides by reverse phase chromatography. There was one major peak of UV absorbance for each subunit which corresponded to the peak of radioactivity as shown for the δ subunit in Fig. 4. Similar column profiles were seen with the β and γ phosphopeptides. The presence of a single phosphorytosine containing peptide in each subunit suggests that the in vitro and in vivo tyrosine phosphorylation sites are the same. For the δ subunit, 67% of the radioactivity from the antiphototyrosine antibody column eluted in a major peak at 17% acetonitrile on HPLC. Seventy-four percent of the radioactivity from the γ subunit were recovered at 14% acetonitrile and 71% of the radioactivity from the δ subunit were recovered at 17% acetonitrile.
The peak from the reverse phase HPLC purification for each tryptic phosphopeptide was judged to be pure by peptide sequencing. Sequence analysis of the peptides identified the residues shown in Table I. The sequence obtained for the β subunit tryptic phosphopeptide corresponds uniquely to residues 351-358 of the deduced amino acid sequence (Noda et al., 1983a). The γ subunit sequence corresponds to residues 361-367 and the δ subunit sequence corresponds to residues 369-376 (Fig. 5) (Claudio et al., 1983; Noda et al., 1983a, 1983b). All of these regions lie within the major intracellular loop of each subunit and adjacent to sites for serine phosphorylation. Phosphoryl groups bind tightly to the treated glass-fiber filter used in the sequence cartridge and therefore the PTH-derivative of phosphotyrosine does not appear in any of the sequenator cycles. From comparison of the peptide sequences with the sequences deduced from cDNA, it is clear that the blank cycles in each subunit correspond to the position of the phosphorylated tyrosine residue. We conclude then that the tyrosine phosphorylation site in the β subunit is Y-355, the γ subunit is Y-364, and the δ subunit is Y-372.

The yield of PTH-derivatives obtained from each cycle of the sequential degradation was quantified. The initial yield of each peptide was larger than the yield predicted from the calculation based on the in vitro phosphorylation stoichiometry. This suggests that the endogenously phosphorylated peptides co-purified with the in vitro phosphorylated peptides and is additional evidence that the in vivo and the in vitro phosphorylation sites are identical.

**Table I**

Amino acid sequence of tryptic phosphopeptides of the β, γ, and δ subunits

Trptic phosphopeptides purified by affinity chromatography and HPLC were subjected to automated Edman degradation. PTH-derivatives were identified and quantitated by reverse phase HPLC. The PTH-derivative of phosphotyrosine did not elute from the treated glass-fiber filter used in the sequence cartridge and therefore the PTH-derivative of phosphotyrosine does not appear in any of the sequenator cycles. From comparison of the peptide sequences with the sequences deduced from cDNA, it is clear that the blank cycles in each subunit correspond to the position of the phosphorylated tyrosine residue. We conclude then that the tyrosine phosphorylation site in the β subunit is Y-355, the γ subunit is Y-364, and the δ subunit is Y-372.

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| Cycle | PTH-derivative | Residue | Yield (pmol) |
|-------|----------------|---------|-------------|
| β Subunit | 1 | A | 784 |
|        | 2 | N | 475 |
|        | 3 | D | 390 |
|        | 4 | E | 387 |
|        | 5 |  |  |
|        | 6 | F | 375 |
|        | 7 | I | 295 |
|        | 8 | R | 208 |
| γ Subunit | 1 | A | 137 |
|        | 2 | E | 55 |
|        | 3 | E | 72 |
|        | 4 |  |  |
|        | 5 | I | 37 |
|        | 6 | L | 65 |
|        | 7 | K | 57 |
| δ Subunit | 1 | A | 582 |
|        | 2 | Q | 386 |
|        | 3 | E | 403 |
|        | 4 |  |  |
|        | 5 | F | 128 |
|        | 6 | N | 187 |
|        | 7 | I | 151 |
|        | 8 | K | 33 |

**FIG. 5** Identified and proposed sites of tyrosine phosphorylation in the major intracellular loops of ligand-gated ion channels. The sequences surrounding the sites of tyrosine phosphorylation on the β, γ, and δ subunits of the nAChR which are located in each of the subunits’ major intracellular loops are similar to potential tyrosine phosphorylation sites in homologous domains of the GABAB and glycine receptor subunits.

**DISCUSSION**

This paper has described the determination of the sites of tyrosine phosphorylation on the β, γ, and δ subunits of the nAChR. The receptor was phosphorylated selectively on tyrosine residues, purified over an acetylcholine affinity column, and its subunits isolated by SDS-PAGE. The phosphorylated β, γ, and δ subunits were individually digested and shown to each contain only one phosphopeptide after trypsin or thermolysin cleavage. The tryptic phosphopeptides were purified over an agarose-conjugated antiphosphotyrosine affinity column and then by reverse phase HPLC. The sequences obtained from the purified phosphopeptides were: Ala-Asp-Glu-Tyr(P)-Phe-Ile-Arg for the β subunit, Ala-Glu-Glu-Tyr(P)-Ile-Leu-Lys for the γ subunit, and Ala-Glu-Tyr(P)-Phe-Asn-Ile-Lys for the δ subunit. These sequences correspond uniquely to residues 351-358 of the β subunit, 361-367 of the γ subunit, and 369-376 of the δ subunit, which are all homologous regions within the major intracellular loops of the integral membrane subunits (Noda et al., 1983a, 1983b).

The sites for in vivo and in vitro tyrosine phosphorylation of the nAChR are identical. The receptor used in this study was phosphorylated on tyrosine residues in vivo at approximately 1 mol of phosphate/mol of receptor as determined by quantitative immunoblotting. In addition to this endogenous level of phosphorylation, the receptor was phosphorylated in vitro to a final level of 2 mol of phosphate/mol of receptor. However, a single tryptic phosphopeptide was obtained from the affinity column for each subunit as judged by the coelution of single peaks of absorption and radioactivity observed during reverse phase chromatography. Based on initial yields from the sequencing runs, the amount of phosphopeptide recovery was greater than that estimated from values obtained using the in vitro phosphorylation stoichiometry. These observations indicate that tryptic phosphopeptides from receptor phosphorylated in vivo co-purified with those derived from in vitro phosphorylation and were identical by sequence analysis.

The sequence surrounding the tyrosine phosphorylation sites of the other nAChR subunits are similar to those of most of the other known substrates of protein tyrosine kinases, with acidic amino acids immediately preceding the tyrosine residue (Kemp and Pearson, 1990; Geahlen and Harrison, 1990). Acidic amino acids adjacent to the phosphorylated tyrosine residues are found at the autophosphorylation sites of most protein tyrosine kinases, at the sites of tyrosine phosphorylation of in vivo substrates such as phospholipase C-γ and erythrocyte Band 3, and in effective synthetic peptide substrates for protein tyrosine kinases such as poly(Glu,Tyr)α4. (Hanks et al., 1988; Kim et al., 1990; Wahl et al., 1990;
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Dekowski et al., 1983; Braun et al., 1984). The phosphorylation sites of the three subunits of the nAChR also share additional features, including hydrophobic residues followed by basic residues on the COOH-terminal side of the tyrosine residue. However, these similarities may represent shared features of very homologous proteins rather than primary structural determinants that are important for site recognition by the protein tyrosine kinase. Secondary or tertiary structural determinants may play an important role in substrate recognition by the endogenous protein tyrosine kinase which phosphorylates the nAChR subunits. Short synthetic peptides which lack higher order structural determinants have been reported to be poor substrates for protein tyrosine kinases (Geahlen and Harrison, 1990). Similarly, synthetic peptides corresponding to the region phosphorylated on the nAChR are inefficiently phosphorylated by the endogenous protein tyrosine kinase with $K_m$ values in the millimolar range (data not shown). Thus, secondary and tertiary structural determinants are likely to be important for the recognition of substrates by protein tyrosine kinases.

The sites of tyrosine phosphorylation of the nAChR are conserved in the nAChR from all species examined except for the $\gamma_2$ subunit, whose tyrosine phosphorylation site is present only in Torpedo and chicken (Huganir and Miles, 1989). This suggests that tyrosine phosphorylation plays an integral role in the regulation of the function of the receptor. Recent studies indicate that tyrosine phosphorylation of the receptor may be important during development of the neuromuscular junction. Innervation with chick myotubes in culture by ciliary ganglion neurons and innervation of rat muscle during development induce tyrosine phosphorylation of the nAChR (Qu et al., 1990). This nerve-induced phosphorylation appears to be mediated by agrin, a factor released from the neuron, which is known to cause aggregation of the receptor in myotubes (Wallace, 1986; Nitkin et al., 1987). Agrin has recently been shown to specifically induce tyrosine phosphorylation of the $\beta$ subunit of the nAChR in chick myotubes, suggesting that tyrosine phosphorylation of the nAChR is directly involved in aggregation of the nAChR under the nerve terminal (Wallace et al., 1991).

Phosphorylation may be a common mechanism of modulating ligand-gated ion channels. Similar to the nAChR, other ligand-gated ion channels are multimeric structures composed of homologous subunits with several membrane-spanning domains (Barnard et al., 1987). The GABA$\alpha$ receptor has been shown to be phosphorylated by cAMP-dependent protein kinase and protein kinase C in vitro and the glycine receptor is phosphorylated by protein kinase C in vitro (Kirkness et al., 1989; Browning et al., 1990; Ruiz-Gomez et al., 1991). In addition, the $\gamma_1$ and $\gamma_2$ subunits of the GABA$\alpha$ receptors and the $\beta$ subunit of the glycine receptor have a tyrosine residue surrounded by acidic amino acids in their major intracellular loops (Fig. 5) (Ymer et al., 1990; Pritchett et al., 1989; Genningslo et al., 1990). The similarity of these sites to the tyrosine phosphorylation sites within the nAChR subunits suggests that these ligand-gated ion channels may also be substrates of protein tyrosine kinases and tyrosine phosphorylation may be a general mechanism in the regulation of neurotransmitter receptor function.

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