Binding of the Nicotinic Acetylcholine Receptor to SH2 Domains of Fyn and Fyk Protein Tyrosine Kinases*  

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The nicotinic acetylcholine receptor (AChR) is phosphorylated on tyrosine both in vitro and in vivo. To identify the protein tyrosine kinase that phosphorylates the receptor, we have previously cloned and characterized two protein tyrosine kinases, Fyn and Fyk, that are highly expressed in Torpedo electric organ, a tissue enriched in the AChR. Both kinases were shown by coimmunoprecipitation to be specifically associated with the AChR. In this study, we examined the molecular basis for the interaction of Fyn and Fyk with the AChR using fusion proteins containing the SH2 domains of the two kinases as affinity reagents. The AChR bound specifically and in a protein concentration-dependent manner to the SH2 domain fusion proteins of Fyn and Fyk. Quantitation of the association revealed that the binding of the AChR to Fyn and Fyk SH2 domain fusion proteins was to a single class of saturable high affinity sites. In addition, the association of the AChR with the SH2 domain fusion proteins was dependent on tyrosine phosphorylation of the AChR and was mediated by the δ subunit of the receptor. Furthermore, upon dissociation of the AChR into subunits, the δ subunit coimmunoprecipitated with both Fyn and Fyk. These data suggest that the association of the AChR with Fyn and Fyk is mediated by an interaction of the tyrosine-phosphorylated δ subunit of the receptor with the SH2 domains of the protein tyrosine kinases.  

Protein phosphorylation is a primary post-translational mechanism for the regulation of essentially all cellular processes (Edelman et al., 1987; Cohen, 1989). Based on their amino acid specificities, there are two major types of protein kinases: protein serine/threonine kinases and protein tyrosine kinases. Originally identified as mitogenic retroviral oncogene products, protein tyrosine kinases were subsequently shown to have normal cellular homologs (Hunter and Cooper, 1985) and to mediate differentiation in some cell types. For example, activation of the nerve growth factor receptor or expression of v-src in PC12 chromaffin cells induces a sympathetic neuron-like phenotype (Dichter et al., 1977; Allemà et al., 1985). Furthermore, protein tyrosine kinases are highly expressed in brain (Cotton and Brugge, 1983; Sudol and Hanafusa, 1986; Cooke and Perlmuter, 1989) where they are associated with synaptic structures (for review see Wagner et al., 1991) suggesting an involvement of protein tyrosine kinases in neuronal differentiation as well as in synaptic transmission.  

Ligand-gated ion channels mediate rapid synaptic transmission in the central and peripheral nervous system. The nicotinic acetylcholine receptor (AChR), which mediates postsynaptic depolarization at the neuromuscular junction, has served as a model for the study of the structure, function, and regulation of ligand-gated ion channels. The AChR is a 250-kDa pentameric complex of four homologous transmembrane subunits in a stoichiometry of α2βγδ (Galzi et al., 1991). Phosphorylation of the AChR both in vitro and in vivo by protein tyrosine kinases(s) has been reported (Hopfield et al., 1988; Qu et al., 1990). Phosphorylation of the AChR by protein tyrosine kinases is correlated with a modulation of the rapid rate of receptor desensitization and may play a role in the nerve-induced clustering of the AChR at the synapse (Hopfield et al., 1988; Qu et al., 1990; Wallace et al., 1991).  

We have identified two Src-like protein tyrosine kinases, Fyn and Fyk, that appear to be involved in the regulation of synaptic transmission at the neuromuscular junction by phosphorylating the AChR (Swope and Huganir, 1993). The two kinases are highly expressed in Torpedo electric organ, a tissue enriched in synaptic components including the AChR. As demonstrated by coimmunoprecipitation, Fyn and Fyk associate with the AChR. Furthermore, the AChR is phosphorylated in Fyn and Fyk immunoprecipitates. These results indicate that Fyn and Fyk are involved in the regulation of postsynaptic membrane function and suggest that these protein tyrosine kinases may phosphorylate the AChR in vivo. In the present study, we have investigated the molecular basis for the association of Fyn and Fyk with the AChR. Like other members of the Src class of protein tyrosine kinases (Sudol, 1993), Fyn and Fyk each contain a unique region, a Src homology two (SH2) domain, a Src homology three (SH3) domain, and a catalytic domain. SH2 domains mediate the association of Src-like tyrosine kinases and other SH2 domain-containing proteins with tyrosine-phosphorylated proteins such as autophosphorylated growth factor receptors and protein tyrosine kinase substrates (for review, see Koch et al., 1991). Therefore, using fusion proteins as affinity reagents we investigated the involvement of the SH2 domains of Fyn and Fyk in the association of the AChR with these two protein tyrosine kinases.  

EXPERIMENTAL PROCEDURES  

Preparation of Fusion Proteins—Fusion proteins derived from the sequence of fyn and fyk were prepared as previously described (Swope and Huganir, 1993). Polymerase chain reaction was used to generate the appropriate DNA fragments using 1 ng of full-length fyn or fyk in pBluescript KS- as a template. For fusion proteins derived from the  

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‡ The abbreviations used are: AChR, nicotinic acetylcholine receptor; SH2, Src homology two; SH3, Src homology three; α-Bgtx, α-bungarotoxin; BFGF, basic fibroblast growth factor; pp125Fps, focal adhesion-associated protein tyrosine kinase.
unique domains between the myristylation consensus motif and the SH3 domain of Fyn and Fyk, polymerase chain reaction products have been previously generated and subcloned as described (Swope and Huganir, 1993). For the Fyn unique domain, the 5'- and 3'-oligonucleotides were derived from the DREKKTIVY and TGGGOSLVY sequences for the SH3 domain, respectively. The Fyn unique domain 5'- and 3'-oligonucleotides were derived from the EKGTKQSAKV and TSRVGGVGTQV sequences, respectively. For Fyn SH2 domain, the 5'- and 3'-oligonucleotides were derived from the 142DSTQAAEVM and 142VPCFQGSM sequences, respectively; whereas for Fyk SH2 domain, the 5'- and 3'-oligonucleotides were derived from the 142VTQIAEVQ and 142VPCFQGSM sequences, respectively. For Fyn SH2 domain, the 5'- and 3'-oligonucleotides were derived from the GVLYFLVALY and 142PVDTSQAEE sequences, respectively; whereas for Fyk SH2 domain, the 5'- and 3'-oligonucleotides were derived from the GVMTIALIAY and 142PVDTSQAEE sequences, respectively.

Each 5'-oligonucleotide contained a RanH restriction site, whereas each 3'-oligonucleotide contained an EcoRI restriction site. The polymerase chain reaction products were subcloned into pGEX-2T.

The glutathione S-transferase fusion proteins were generated and purified as previously described (Swope and Huganir, 1993). However, after binding of the fusion proteins to the glutathione-agarose, the resin was subsequently washed five times with HEPES buffer containing 20 mM HEPES pH 7.6, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, and 25% glycerol, three times with phosphate-buffered saline, two times with 5X phosphate-buffered saline, two times with phosphate-buffered saline, and three times with TissueExtraction buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM EGTA and then resuspended as a 2X slurry in TissueExtraction buffer. Aliquots of the purified preparations were resolved by SDS-PAGE and visualized with Coomasie Brilliant Blue, and the protein concentration of each fusion protein was determined by comparison to a standard curve using bovine serum albumin.

Western blot with polyclonal rabbit antiserum generated against the 89KETYL?m16 and 79TRGGGSLTV87 sequences, respectively; whereas for the Fyk unique domain, the SH3 domain of Fyn and Fyk, polymerase chain reaction products have been previously generated and subcloned as described (Swope and Huganir, 1993). Thus, the dissociation constants were calculated using the equation

\[ \frac{B_o}{B_s} = k_d (t) \]

where

\[ B_o \] = the maximal binding,

\[ B_s \] = the ligand concentration. The association constants were calculated as described (Bylund, 1980).

For Fyn SH2 domain, the dissociation constants was then calculated using the equation

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where

\[ B_o \] = the maximal binding,

\[ B_s \] = the ligand concentration. The association constants were calculated as described (Bylund, 1980).

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Binding of the AChR to Fyn and Fyk Fusion Proteins—To electrode organ postsynaptic membranes enriched in AChR were prepared by methods previously described (Huganir et al., 1984). In 12 individual postsynaptic membrane preparations, the concentration of AChR receptor was 1430 ± 77 pmol/mg of membrane protein as assayed by [125I]-a-Bgt~ binding (Schmidt and Raftery, 1973). To examine the binding of the AChR to Fyn and Fyk fusion proteins, postsynaptic membranes were incubated with phosphorylation buffer (20 mM Tris, pH 7.4, 20 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM ouabain, 1 mM Na3VO4, 0.02 mg/ml NaN3, 0.1 mM dithiothreitol, 2 mM MgCl2, 0.1 mM of the Walsh peptide inhibitor of the CAMP-dependent protein kinase (TYYADFIASFGTGRNAIHAD), and 500 μM ATP) at 30°C for 30 min, after which EDTA was added to a final concentration of 12 mM and the membranes were put on ice. The postsynaptic membranes were diluted to 0.5 mg of protein/ml with fusion protein binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, and 1% Triton X-100, incubated for 15 min on ice, and centrifuged at 100,000 x g for 15 min. Supernatant representing the desired micrograms of membrane protein was incubated in 3°C with Fyn or Fyk glutathione S-transferase fusion protein bound to glutathione-agarose and then reacted with the Kemptide-agarose to a final volume of 100 μl of a 2X slurry. After 2 h, the agarose was washed three times with fusion protein binding buffer and two times with Tris/EDTA/EGTA buffer. The bound proteins were eluted with SDS sample buffer, resolved by SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose (Towbin et al., 1979). The postsynaptic membrane proteins were analyzed for phosphorylation content by Western blot using a commercially available monoclonal antibody (Upstate Biochemical Inc., Lake Placid, NY), enhanced chemiluminescence as commercially available peroxidase (Sigma) and Kodak X-Omat AR film. The dephosphorylated, control, and phosphorylated membrane preparations were used to examine AChR binding to Fyn and Fyk fusion proteins as described above.

Preparation of AChR with Various Stoichiometries of Tyrosine Phosphorylation—Phosphorylation of the AChR to three different stoichiometries was accomplished as previously described (Hopfield et al., 1988). Torpedo electric organ postsynaptic membranes were incubated under phosphorylating conditions as described above in the absence or presence of 500 μM ATP. After 30 min at 30°C, the membranes were pelleted on ice and diluted to 0.5 mg of protein/ml in fusion protein binding buffer, centrifuged as described above, resolved by SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose (Towbin et al., 1979). The postsynaptic membrane proteins were analyzed for phosphorylation content by Western blot using a commercially available monoclonal antibody (Upstate Biochemical Inc., Lake Placid, NY), enhanced chemiluminescence as commercially available peroxidase (Sigma) and Kodak X-Omat AR film. The dephosphorylated, control, and phosphorylated membrane preparations were used to examine AChR binding to Fyn and Fyk fusion proteins as described above.

Denaturation of Postsynaptic Membrane Proteins—AChR binding to Fyn and Fyk SH2 domain fusion proteins was incubated in 3°C with denaturant buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4 without (native protein) or with (denatured protein) 2% SDS. After 10 min at room temperature, the membrane proteins were diluted in fusion protein binding buffer to a final concentration of 0.5 mg of protein/ml, 2% Triton X-100, plus or minus 0.4% SDS, incubated on ice for 15 min, and centrifuged as described above. The native and denatured membrane preparations were then used to examine AChR binding to Fyn and Fyk fusion proteins as described above.

Preparation of AChR Subunit Phosphopeptides—Phosphopeptides derived from the known tyrosine phosphorylation sites of the AChR (Wagner et al., 1991b) were synthesized using Fmoc-Tyr(PO2Me)2-OH by the procedure of Kitas et al. (1989). For the β, γ, and δ subunits the peptides synthesized were SraneyDIYPORFIRPKA, MIKAEAEYPO2R, ILKPKPL, and ISKSAEYPO2RFNJKSR, respectively. Over 90% of each phosphopeptide was recovered and mass spectrometric data were shown demonstrated by matrix-assisted laser desorption (Coller, 1995). To examine the effect of the phosphopeptides on AChR binding to Fyn and Fyk SH2 domain fusion proteins, the affinity resin was preincubated overnight in the absence or presence of phosphoserine, phosphothreonine, phosphotyrosine, or phenylphosphorylation (Sigma) at a final concentration of 20 μM prior to incubation with the solubilized postsynaptic membrane proteins. The specifically bound proteins were analyzed for the presence of AChR as described above.

Coimmunoprecipitation of the AChR and AChR Subunits with Fyn and Fyk—After incubation under phosphorylating conditions as described above, the tyrosine phosphorylated membrane proteins were incubated in denaturant buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4 without (native protein) or with (denatured protein) 2% SDS. After 10 min at room temperature, the membrane proteins were diluted in fusion protein binding buffer to a final concentration of 0.5 mg of protein/ml, 2% Triton X-100, plus or minus 0.4% SDS, incubated on ice for 15 min, and centrifuged as described above. The native and denatured membrane preparations were then used to examine AChR binding to Fyn and Fyk fusion proteins as described above.

2 R. L. Huganir, unpublished observation.
Binding of AChR to the SH2 Domains of Fyn and Fyk

Using glutathione S-transferase fusion proteins as affinity reagents, we investigated whether the previously reported immunoprecipitation of the AChR with Fyn and Fyk was mediated by binding of the receptor to the SH2 domains of these two protein tyrosine kinases. Upon incubation of solubilized Torpedo electric organ postsynaptic membrane proteins representing between 1 and 30 μg of protein with the affinity reagents, we found that the AChR bound to the Fyn and Fyk SH2 domain fusion proteins in a manner that was dependent on the concentration of postsynaptic membrane protein (Fig. 1). By comparing the relative intensity of the AChR detected in the flow-through to that bound, we estimated that 4–20% (30–1 μg) of the receptor bound to the Fyn and Fyk SH2 affinity resins with a higher percentage bound with lower concentrations of membrane proteins. Additional experiments, as described below, demonstrated that up to 60% of the AChR bound to the Fyn SH2 domain while up to 28% bound to the Fyk SH2 domain when a high concentration of fusion protein (50 μg) was used. The data presented in Fig. 1 demonstrated that the binding of the AChR to the fusion proteins was specific for the SH2 domain since little if any AChR bound to the glutathione S-transferase backbone upon incubation with the highest concentration of protein (30 μg).

Since the AChR is phosphorylated on tyrosine residues, we had postulated that the immunoprecipitation of the receptor with Fyn and Fyk may be mediated by the SH2 domain of the two kinases. However, protein tyrosine kinases are known to associate with various membrane and cytoplasmic proteins via the SH3 and unique domains (Cicchetti et al., 1992; Bar-Sagi et al., 1993; Weng et al., 1993; Shaw et al., 1989). Therefore, the subdomain specificity for binding of the AChR to Fyn- and Fyk-derived glutathione S-transferase fusion proteins was examined. As shown in Fig. 1, the AChR bound to the SH2 domain fusion proteins, and more receptor bound to the Fyn-derived fusion protein than the Fyk-derived protein under these conditions (Fig. 2). In contrast, even at 200 μg of postsynaptic membrane proteins, AChR binding to the unique domain fusion proteins of Fyn and Fyk was not detected. In addition, the AChR did bind in a protein-dependent manner to the SH3 domain fusion protein of Fyk (Fig. 2) as well as the SH3 domain fusion protein of Fyn as detected by longer film exposures (data not shown). However, as discussed below, the binding of the AChR to the SH3 domain of Fyk may not be a direct interaction between the receptor and the fusion protein.

Quantitation of AChR Binding to the SH2 Domains of Fyn and Fyk—To quantitate the levels of AChR binding to the SH2 domains of Fyn and Fyk, we established a radioligand binding assay in which the AChR was tagged with [125I]a-Bgtx and then bound to the SH2 domain affinity resins. The time courses for association were very slow, reaching equilibrium after 5 h with 20 nM of AChR-[125I]a-Bgtx complex (Fig. 3). Qualitatively, the results obtained using the radioligand binding assay were similar to those obtained by analyzing the AChR binding using Western blotting. Thus, the AChR binding to the Fyn affinity resin was higher than for the Fyk affinity resin, and very little binding to the backbone fusion protein was observed. In addition, maximal binding to the Fyn and Fyk SH2 domain fusion proteins represented 8.0 ± 0.7% and 3.2 ± 0.3% of the total AChR-[125I]a-Bgtx complex in the binding reaction, in agreement with the data presented in Fig. 1. Additional experiments demonstrated that, using high concentrations of SH2 domain fusion protein (50 μg), up to 60% of the AChR-[125I]a-Bgtx complex bound to the Fyn SH2 domain while up to 28% bound to the Fyk SH2 domain (data not shown).

Dissociation experiments demonstrated that dissociation of the AChR-[125I]a-Bgtx complex from the SH2 domain affinity resins was also very slow; only 10% dissociated by 24 h (data not shown). Assuming a t½ for dissociation greater than 24 h the dissociation constant values were calculated to be 4.8 × 10⁻¹ min⁻¹. The k₆ values for Fyn and Fyk obtained from the

![Fig. 1](image-url)
FIG. 2. Subdomain specificity for binding of the AChR to Fyn and Fyk fusion proteins. The indicated micrograms of Torpedo electric organ post synaptic membrane proteins were incubated under phosphorylating conditions, solubilized, and incubated with glutathione-agarose containing 5 μg of either Fyn or Fyk SH2 (SH2) or SH3 (SH3) domain or the unique region (UNIQUE) fusion protein as described under "Experimental Procedures." The bound proteins were resolved by SDS-PAGE and analyzed by Western blot using an antiserum to the AChR as described under "Experimental Procedures." The positions of the α, β, γ, and δ subunits are as indicated.

FIG. 3. Time courses for AChR binding to Fyn and Fyk SH2 domain fusion proteins. Torpedo electric organ post synaptic membrane proteins, representing 5 pmol of AChR, were incubated under phosphorylating conditions and solubilized. The AChR was labeled with [125I]a-Bgtx, and the membrane proteins were incubated with glutathione-agarose containing 1 pg of either Fyn (FY) or Fyk (FYK) SH2 domain fusion protein or glutathione S-transferase backbone protein (PG) as described under "Experimental Procedures." The final concentration of AChR-[125I]a-Bgtx complex was 20 nM. After the indicated length of time, the resins were washed and the bound AChR-[125I]a-Bgtx complex measured as described under "Experimental Procedures." The data represent the mean of triplicate samples. Maximal binding to the Fyn and Fyk SH2 domain fusion proteins was 0.382 ± 0.004 and 0.14 ± 0.01 pmol of AChR-[125I]a-Bgtx complex, respectively. Binding to the backbone fusion protein was 0.0017 ± 0.0008 pmol.

time courses for binding (see Fig. 3) were 5.0 ± 1.0 x 10⁻³ min⁻¹ and 8.4 ± 0.6 x 10⁻³ min⁻¹ (mean ± range, n = 2), respectively. Thus, the calculated association constants, k⁺, for Fyn and Fyk were 2.5 ± 0.6 x 10⁻¹ and 3.7 ± 0.3 x 10⁻¹ min⁻¹ nM⁻¹, respectively. Based on these values, the calculated Kd apparent values were 1.9 ± 0.4 nM for Fyn and 1.3 ± 0.1 nM for Fyk. It should be noted that these values were minimum representations of the affinity since the dissociation constants were based on a minimum tₙ₀ for dissociation of 24 h.

To further quantitate the binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins, the ability of unlabeled AChR to inhibit the binding of the AChR-[125I]a-Bgtx complex was examined. For both Fyn and Fyk, over 95% of the AChR-[125I]a-Bgtx complex binding was inhibited by 300 nM AChR, indicating that the binding was to saturable sites (Fig. 4). The unlabeled AChR competed for the binding of the AChR-[125I]a-Bgtx complex in a dose-dependent manner with I₅₀ values for Fyn and Fyk of 12 ± 2 and 16 ± 5 nM, respectively (mean ± range, n = 2). Scatchard analysis of these data (Fig. 4, inset) indicated a single class of sites with Kd apparent values of 12 ± 2 nM for Fyn and 17.7 ± 0.2 nM for Fyk.

Dependence on Phosphorylation of the AChR Binding to SH2 Domains of Fyn and Fyk—Protein-protein interaction mediated by SH2 domains is known to be dependent on tyrosine phosphorylation of the associated proteins (Koch et al., 1991). Therefore, using two independent strategies we examined whether the binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins was dependent on tyrosine phosphorylation of the receptor. First, the effect of several phosphoamino acids on AChR binding was determined. Both phosphotyrosine and phenylphosphate blocked the binding of the receptor to the Fyn and Fyk SH2 domain fusion proteins (Fig. 5A). This inhibition was specific since phosphoserine and phosphothreonine were ineffective. Next, we examined whether the extent of AChR phosphorylation on tyrosine residues correlated with re-
Binding of AChR to SH2 Domains of Fyn and Fyk

FIG. 5. Dependence on tyrosine phosphorylation of AChR binding to Fyn and Fyk SH2 domain fusion proteins. A, Torpedo electric organ postsynaptic membranes (30 µg) were incubated under phosphorylating conditions, solubilized, and incubated with glutathione-agarose containing 5 µg of Fyn or Fyk SH2 domain fusion protein that had been preincubated in the absence (CON) or presence of the indicated phosphoamino acid, phosphoserine (PS), phosphothreonine (PT), phosphotyrosine (PY), or phenylphosphate (PP) at a final concentration of 20 mM for each. The bound proteins were resolved by SDS-PAGE and analyzed by Western blot using an antibody to the AChR as described under "Experimental Procedures." B, Torpedo electric organ postsynaptic membranes (30 µg) were incubated under either dephosphorylating (NP), control (CON), or phosphorylating (AP) conditions as described under "Experimental Procedures." The positions of the α, β, γ, and δ subunits are as indicated.

Receptor binding. Postsynaptic membranes were treated to produce AChR preparations that contained three different stoichiometries of phosphorytrosine. Thus, membranes were incubated under phosphorylating conditions to increase phosphorytrosine content, control conditions to produce basal phosphorytrosine content, and dephosphorylating conditions to decrease phosphorytrosine content. These three treatment conditions were effective at producing AChR preparations that contained a range of phosphorytrosine (Fig. 5B). The three receptor preparations were examined for the ability to bind to the Fyn and Fyk SH2 domain affinity resins. As shown in Fig. 5C, AChR binding increased with increasing phosphorytrosine content. The specific inhibition of AChR binding by phosphotyrosine and phenylphosphate as well as the correlation between phosphorytrosine content and receptor binding provided strong evidence that the AChR binding to Fyn and Fyk SH2 domain fusion proteins was dependent on tyrosine phosphorylation of the receptor.

Subunit Specificity for Binding of the AChR to Fyn and Fyk SH2 Domain Fusion Proteins—The binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins could be mediated by an ancillary protein or may be caused by a direct interaction of one or more receptor subunits with the kinase fragment. To address this question, postsynaptic membrane proteins were treated under denaturing conditions, 2% SDS, to dissociate the AChR from any bound proteins as well as to dissociate the receptor into individual subunits. After dilution of the membrane proteins into Triton X-100, we examined the ability of the individual receptor subunits to bind to the SH2 fusion proteins. In the control, incubation of native postsynaptic membrane proteins with the SH2 domain affinity resins resulted in AChR binding, and all four subunits were detected (Fig. 6) as demonstrated above. However, after denaturation only the AChR δ subunit was observed to bind to the Fyn and Fyk SH2 domain fusion protein (Fig. 6). In addition, after denaturation the individual AChR subunits did not bind to the Fyk SH3 domain fusion protein, suggesting that the association of the receptor with SH3 affinity resin may be mediated by an ancillary protein (data not shown; see "Discussion"). These data support a direct interaction between the AChR δ subunit and the Fyn and Fyk SH2 domain fusion proteins. In addition, these data indicate that not all tyrosine-phosphorylated postsynaptic membrane proteins, including the AChR β and γ subunits, bound to Fyn and Fyk SH2 domain fusion proteins.

To further investigate the subunit specificity of AChR binding to the Fyn and Fyk SH2 domain fusion protein, phosphopeptides based on the sequence surrounding the tyrosine phosphorylation site of the receptor β, γ, and δ subunits were synthesized. Fyn and Fyk SH2 affinity resin was preincubated overnight at 4 °C without or with 0.001–1.0 mM of each phosphopeptide. The ability of the native AChR to bind in the presence or absence of the phosphopeptides was then examined. The AChR δ subunit phosphopeptide completely blocked the binding of the receptor to the Fyn SH2 fusion protein at 0.1 mM (Fig. 7). The AChR γ and δ subunit phosphopeptides were less effective, 1.0 mM of each inhibited receptor binding to the Fyn affinity resin by approximately 50%. Thus, the δ subunit phos-
FIG. 7. Inhibition of AChR binding to Fyn and Fyk SH2 domain fusion proteins by AChR subunit phosphopeptides. Torpedo electric organ postsynaptic membranes (90 μg) were incubated under phosphorylating conditions, solubilized, and incubated with glutathione-agarose containing 5 μg of Fyn or Fyk SH2 domain fusion protein that had been preincubated in the absence (CON) or presence of the indicated concentration of AChR δ (δ), γ (γ), or β (β) subunit phosphopeptide. The bound proteins were resolved by SDS-PAGE and analyzed by Western blot using an antisera to the AChR as described under "Experimental Procedures." The positions of the α, β, γ, and δ subunits are as indicated.

FIG. 8. Subunit specificity for coimmunoprecipitation of the AChR with Fyn and Fyk. Torpedo electric organ postsynaptic membranes (250 μg) were incubated under phosphorylating conditions, treated without (NATIVE) or with (DENATURED) 2% SDS, and diluted to a final concentration of 0.4% SDS, 2% TritonX-100. The solubilized proteins were immunoprecipitated with preimmune (prei), anti-Fyn (fyn), or anti-Fyk (fyk) serum, resolved by SDS-PAGE, and analyzed by Western blot using a pool of monoclonal antibodies to the AChR as described under "Experimental Procedures." The positions of the α, β, γ, and δ subunits are as indicated.

The N-terminal half of Src-like protein tyrosine kinases contains four discrete functional domains (for review see Sadol, 1993). The most N-terminal region is necessary for myristylation. Just downstream in each kinase is a unique region, which for p56{lck} protein tyrosine kinase mediates its association with the plasma membrane binding protein CD4 (Shaw et al., 1989). The remaining portion of the N-terminal half is comprised of the SH3 followed by the SH2 domain. These two modulatory regions are conserved in a variety of signaling proteins including enzymes and adaptor proteins (Koch et al., 1991; Pawson and Gish, 1992). SH2 domains are now believed to mediate association with GTPase-activating proteins (Cicchetti et al., 1992) and cytoskeletal elements (Weng et al., 1993; Bar-Sagi et al., 1993). The SH2 domain was originally identified as a noncatalytic region that is conserved among cytoplasmic protein tyrosine kinases and modifies kinase function (Sadowski et al., 1986). SH2 domains, which are now known to be contained in a variety of signaling proteins, bind tyrosine-phosphorylated proteins such as autophosphorylated protein tyrosine kinases as well as substrates (Koch et al., 1991; Pawson and Gish, 1992) via the recognition of specific phosphopeptide sequences (Fantl et al., 1992; Songyang et al., 1993). Since the AChR is a substrate for protein tyrosine kinase(s), we postulated that the previously reported coimmunoprecipitation of the receptor with Fyn and Fyk may be mediated by an association between the SH2 domain of each kinase with the tyrosine-phosphorylated receptor. To address this possibility, we employed affinity chromatography using fusion proteins derived from each discrete N-terminal subdomain of Fyn and Fyk.

The AChR bound selectively to fusion proteins derived from the N-terminal region of Fyn and Fyk. Binding of the AChR to the SH2 domain fusion proteins was specific and dependent on protein concentration as well as receptor phosphorylation. In contrast, fusion proteins derived from the unique domain of Fyn and Fyk did not bind the AChR. Although the native receptor bound to the SH3 domain fusion proteins of Fyn and Fyk, we believe that this binding was mediated by an ancillary...
protein. Consistent with this interpretation, after treatment with SDS to denature all postsynaptic membrane proteins and thus dissociate the receptor into individual subunits as well as strip the receptor of any associated proteins, an AChR subunit bound to the SH2 but not the SH3 domain fusion protein. Although possible, inactivation of the SH3 domain binding site by denaturation is not consistent with previously published reports. For example, SH3 domains can bind to proteins resolved by SDS-PAGE and transferred to nitrocellulose (Cicchetti et al., 1992; Weng et al., 1993). Recently, the binding sites of two SH3 binding proteins were identified (Ren et al., 1993). Both binding sites are stretches of 9–10 amino acids comprised primarily of proline residues. Upon sequence analysis, no such motifs were evident in the AChR. In addition, we found that the binding of several Torpedo electric organ postsynaptic membrane proteins to the Fyn and Fyk SH3 domain fusion proteins was unaffected by SDS denaturation as analyzed by SDS-PAGE and silver staining.5 Taken together, these data indicated that the binding of the AChR to the SH2 domain fusion protein was mediated by a direct association whereas the binding to the SH3 domain was not. Although the binding of the AChR to the SH3 domain fusion proteins of Fyn and Fyk appears to not be a direct association, it may be physiologically important. For example, the AChR that binds to the SH3 domain fusion proteins may not be phosphorylated on tyrosine residues. Thus, the receptor in the unphosphorylated form may bind to the kinases via the SH3 domain, become phosphorylated, and then associate with Fyn and Fyk via a binding to the SH2 domain. This model would explain how some fraction of the AChR that immunoprecipitates with Fyn and Fyk can be phosphorylated by the two protein tyrosine kinases in the immunoprecipitates (Swope and Huganir, 1993).

Using a ligand binding assay, the association of the AChR to the SH2 domain fusion proteins was examined quantitatively. Both the association and dissociation rates were very slow. These kinetic experiments indicated apparent $K_d$ values of $\approx1–2$ nm for the binding of the AChR-[125]I-a-Bgtx complex to the SH2 domain fusion proteins. In contrast, competition experiments indicated IC$_{50}$ values of 10–20 nM for inhibition of AChR-[125]I-a-Bgtx complex binding by unlabeled AChR. The discrepancy in these values may have resulted from differing affinities of the AChR-[125]I-a-Bgtx complex and unlabeled AChR for the SH2 domain fusion proteins. However, both the $K_d$ and IC$_{50}$ values obtained were artificially high since the dissociation rate of the AChR-[125]I-a-Bgtx complex from the SH2 domain fusion proteins was so slow. Examination of the dissociation kinetics demonstrated that only 10% of the AChR-[125]I-a-Bgtx complex dissociated from the SH2 domain fusion proteins in 24 h. Because of our inability to accurately determine the dissociation constant, we used a $k_{-1}$ of 24 h as a minimum value to determine $k_{-1}$, thus resulting in artificially high $K_d$ values. In addition, the slow dissociation kinetics also resulted in high IC$_{50}$ values since at the lower concentrations of unlabeled AChR used in the competition experiments, binding may not have reached equilibrium. Although the $K_d$ and IC$_{50}$ values determined were probably higher than the actual values, they still indicated high affinity binding between the AChR and the SH2 domain fusion proteins. In addition, the competition experiments indicated that the binding was saturable. Furthermore, both Scatchard analysis of the data obtained by competition experiments as well as linear transformation of the association kinetics indicated a single class of sites. Taken together, these data indicated that the binding of the AChR to Fyn and Fyk SH2 domains was mediated by a single class of saturable high affinity sites.

The AChR is a 250-kDa pentameric complex comprised of four homologous transmembrane subunits in a stoichiometry of α$_1$β$_{2}$δ$_2$ (Galzi et al., 1991). The β, γ, and δ subunits are each phosphorylated on a single tyrosine residue, which is contained on the major intracellular loop between transmembrane domains three and four (Wagner et al., 1991b). By two independent approaches, we investigated the subunit specificity for the binding of the AChR to the Fyn and Fyk SH2 domain fusion proteins. First, subsequent to dissociation of the AChR by SDS, a dramatically specific binding of the δ subunit to the Fyn and Fyk SH2 fusion protein was observed. In addition, a δ subunit phosphopeptide derived from the known phosphorylation site was at least 10-fold more potent than the β or γ subunit phosphopeptide in blocking the binding of the native AChR to the Fyn and Fyk SH2 domain fusion proteins. Examination of the peptide sequences for the β, γ, and δ subunits revealed that the subunit specificity observed here was in agreement with previous reports. Phosphopeptides containing asparagine at position +2 to the phosphotyrosine and especially isoleucine at position +3 are preferred for binding by Src and Fyn SH2 domains (Songyang et al., 1993). The sequences of the tyrosine phosphorylation sites of the β, γ, and δ subunits are consistent with these data; the δ subunit sequence is YFNI, whereas the β and γ subunit sequences are YFIR and YILK, respectively (Wagner et al., 1991b). Thus, the association of the AChR with the SH2 domain of Fyn and Fyk appeared to be mediated by the tyrosine phosphorylation site of the receptor δ subunit. In addition, in agreement with the results using fusion protein affinity resin, the coimmunoprecipitation of the AChR with Fyn and Fyk appeared to be mediated by the δ subunit. Thus, the data presented here support a binding of the tyrosine-phosphorylated intracellular loop of the AChR δ subunit with the SH2 domain of Fyn and Fyk as mediating the association of the receptor with these two protein tyrosine kinases.

We found that the AChR bound to the SH2 domain fusion proteins in a protein-dependent manner. Although only 3–20% of the receptor bound, these data agreed with the low level of AChR (Swope and Huganir, 1993) and pp125Fak (Cobb et al., 1994) that associates with Fyn in vivo. These low levels of association are apparently not caused by low levels of AChR tyrosine phosphorylation, since under the conditions used, up to 2.7 mol of phosphotyrosine distributed between the β, γ, and δ subunits are detected per mol of receptor (Hopfield et al., 1988). In addition, up to 60% of the AChR-[125]I-a-Bgtx complex bound to the Fyn SH2 domain while up to 28% bound to the Fyk SH2 domain when high concentrations of fusion proteins (50 μg) were used. These results suggested that a major fraction of the AChR present in the postsynaptic membrane is capable of binding to the SH2 domains of Fyn and Fyk.

The functional consequence of association between the AChR and Fyn and Fyk remains to be determined. It has been postulated that binding of tyrosine-phosphorylated proteins to the SH2 domain of Src-like kinases and adapter proteins stabilizes the bound substrates by preventing rapid dephosphorylation (Koch et al., 1991). Alternatively, association between tyrosine-phosphorylated substrates and SH2 domain-containing proteins may provide a means for forming complexes of proteins containing a variety of functions. Consistent with this model, the slow dissociation of the AChR from the SH2 domains suggested that the association may promote the stabilization of enzymatic and structural elements involved in synaptic transmission at the neuromuscular junction.

Synaptogenesis is a complex developmental process for which the neuromuscular junction has served as a model. The clustering of postsynaptic components at the nerve-muscle contact appears to be regulated by factors contained in the extra-
cellular matrix (Burden et al., 1979) such as agrin (Magill-Soc and McMah, 1990; Wallace, 1989) and basic fibroblast growth factor (bFGF) (Rapraeger et al., 1991; Peng et al., 1991). The action of these components may be mediated by tyrosine phosphorylation. In fact, protein tyrosine kinase activation has been implicated in the effect of protein kinase (Qu et al., 1990) as well as a variety of other neuronal stimuli including polymeric agents (Baker and Peng, 1992; Baker et al., 1992), electric field (Peng et al., 1993), expression of exogenous 43-kDa protein, and in vitro treatment with bFGF (Peng et al., 1991) or agrin (Wallace et al., 1991) in the clustering of synaptic components. Formation of the postsynaptic specialization is associated with a rearrangement of cytoskeletal elements (Bloch and Pumplin, 1988) and an alteration in gene expression (Martinoiu et al., 1991). Therefore, structurally, biochemically, and developmentally, the neuromuscular junction shares many characteristics, including the involvement of tyrosine phosphorylation, with focal adhesions (Burrage et al., 1992). In fact, many of the membrane and cytoskeletal elements found in focal adhesions (Burridge et al., 1988) are concentrated at the postsynaptic membrane including Src-like protein tyrosine kinases (Swope and Huganir, 1993), integrins, and paxillin (for review see Hall, 1993). Paxillin, a tyrosine-phosphorylated cytoskeletal element, binds to the SH3 domain of Src (Weng et al., 1991) and/or Fyn and/or Fyk may associate with paxillin and a p125^A^-like tyrosine kinase, perhaps Fyn and/or Fyk. In addition, Fyn and/or Fyk may associate with paxillin and a pp125^A^-like kinase at the neuromuscular junction to mediate the formation of the postsynaptic specializations. These interesting possibilities remain to be investigated.

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