Regulation of the Neuronal Nicotinic Acetylcholine Receptor by Src Family Tyrosine Kinases*

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Src family kinases (SFKs) are abundant in chromaffin cells that reside in the adrenal medulla and respond to cholinergic stimulation by secreting catecholamines. Our previous work indicated that SFKs regulate acetylcholine- or nicotine-induced secretion, but the site of modulatory action was unclear. Using whole cell recordings, we found that inhibition of SFK tyrosine kinase activity by PP2 (4-amino-5-(4-chlorophenyl)-7-(4-butylyl)pyrazolo(3,4-d)pyrimidine) treatment or expression of a kinase-defective c-Src reduced the peak amplitude of nicotine-induced currents in chromaffin cells or in human embryonic kidney cells ectopically expressing functional neuronal α3β4δ5 acetylcholine receptors (AChRs). Conversely, the phosphotyrosine phosphatase inhibitor, sodium vanadate, or expression of mutationally activated c-Src resulted in enhanced current amplitudes. These results suggest that SFKs and putative phosphotyrosine phosphatases regulate the activity of AChRs by opposing actions. This proposed model was supported further by the findings that SFKs physically associate with the receptor and that the AChR is tyrosine-phosphorylated.

The Src family kinases (SFKs),¹ Src, Fyn, and Yes, are expressed ubiquitously in many cell types but are especially abundant in neural tissue (1–4). Their catalytic and protein-protein interaction activities function in a large number of signaling pathways (5–7) that regulate cell growth, proliferation, differentiation, synaptic plasticity, and synaptic vesicle endocytosis and exocytosis (8–12). One striking feature of SFKs is their ability to regulate the activities of a variety of neuronal channels, including potassium channels (13, 14), the neuromuscular acetylcholine receptor (15), the NMDA receptor (16, 17), and the GABA receptor (18–20).

SFKs are also highly expressed in chromaffin cells (21–24), which are derived from the neural crest during embryogenesis and reside in the adrenal medulla of the adult. Chromaffin cells are innervated by the preganglionic cholinergic neurons of the sympathetic nervous system, which secrete acetylcholine, the ligand that induces secretion of catecholamines from chromaffin cells. Binding of either acetylcholine or nicotine to the AChR on the surface of chromaffin cells opens the receptor channel, permitting cations to enter the cell through an electrochemical gradient. The resultant depolarization of the membrane opens voltage-gated calcium channels (L-type) followed by a subsequent calcium influx that triggers release of catecholamines (25, 26).

AChRs of chromaffin cells are similar in structure to neuronal AChRs (27). These receptors are pentamers, composed of three β and two α subunits that mediate high affinity ligand binding (28, 29). To date, eight different α subunits and three β subunits of the neuronal AChR have been identified. Various combinations of these subunits form functional receptors that relay excitatory impulses throughout the nervous system and regulate secretion from chromaffin cells (27, 30). The neuronal AChR on chromaffin cells which regulates secretion is thought to be composed of two α3, one α5, and two β4 subunits, with α5 presumed to take the role of one β4 subunit (31–34). This structure is different from the muscle AChR in composition and function, and its regulation by phosphorylation has not been reported. In comparison, the muscle receptor contains α, β, δ, and γ, or ε subunits (35, 36) and transmits signals that regulate muscle contraction. All subunits of this receptor can be phosphorylated by serine/threonine and tyrosine kinases (15), an event that appears to increase the rate of receptor desensitization (37).

Previous studies have shown that serine/threonine and tyrosine kinases influence chromaffin cell secretion (38–40) and that phosphotyrosine phosphatase (PTPase) activity associates with both c-Src and AChRs in large multimeric complexes isolated from the adrenal medulla (41). SFKs localize to the cell membrane as well as to secretory vesicle membranes, spatially juxtaposing SFKs to the AChR (21, 22). These findings suggest that SFKs and PTPases may coordinate the activity of the neuronal AChR found on chromaffin cells.

The current study was undertaken to investigate the potential role of SFKs in regulating AChR on chromaffin cells. Using pharmacological inhibitors of SFKs and mutational variants of c-Src, we demonstrate that SFK activity modulates both AChR channel currents and catecholamine secretion. Evidence for involvement of an opposing PTPase in regulating channel currents is also described. Biochemical analysis demonstrates co-
immunoprecipitation of the nAChR with SFKs and tyrosine phosphorylation of the receptor, suggesting that the channel activity may be modulated by tyrosine phosphorylation. These are the first studies to reveal a functional relationship between SFKs and the neuronal AChR.

MATERIALS AND METHODS

Culture and Treatment of Chromaffin Cells and α3β4α5 AChR Human Embryonic Kidney Cells—Chromaffin cells were cultured from freshly harvested bovine adrenal glands as described previously (42) and modified (43). Cells were maintained in serum-free N2 medium (Invitrogen) at 37 °C in a humidified, 5% CO2 environment. All stimulations and treatments were carried out at 37 °C. For secretion assays, 3–6-day cultures were stimulated with 20 μM nicotine, 55 mM KCl, or 50 μM PP2. Cells were then washed twice with BSSG (15 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5 mM glucose, 2 mM CaCl2, 14.4 mM NaHCO3, 1 mg/ml bovine serum albumin, pH 7.2) for 15 min before stimulation with replacement with BSSG ± secretagogue for the indicated times. Nicotinic was purchased from Sigma and stored as 100 mM stock in water. A23187 was purchased from Sigma and stored at room temperature as a 10 mM stock in dimethyl sulfoxide. When appropriate, BSSG was modified to accommodate the increase in KCN concentration to 55 mM by reducing the NaCl concentration to 90 mM. The indicated doses are maximum effective amounts, determined in previous dose-response analyses. The Src family tyrosine kinase inhibitor PP2 and the negative control analogue PP3 were purchased from Calbiochem and stored at −80 °C. Basal efflux of [3H]NA was extracted in 95% ethanol, 0.5 M HCl and counted. Basal efflux of [3H]NA was measured by liquid scintillation counting. Basal efflux of [3H]NA was measured by liquid scintillation counting.

Release from stimulated cultures was measured by modification of the procedure described previously (40). As a primary immunoblotting antibody, Q0 mouse, anti-rat, or anti-rabbit antiserum (Amersham Biosciences), as described below. 2 mg of protein G-purified mouse mAb 35 was incubated with a 1:ml slurry of protein G-Sepharose beads at room temperature for 1 h, then washed twice with 10 volumes of 0.2 m sodium borate, pH 9.0. The bead-bound antibodies were resuspended in 10 volumes of the same sodium borate buffer and cross-linked by incubation in 20 mM dimethyl pIM (Pierce) for 2 h at room temperature rock. The reaction was stopped by the addition of 0.2 ml amylanol, 80 °C, and incubation for 2 h at room temperature. Beads were then washed twice with phosphate-buffered saline (PBS) and resuspended in PBS made 0.10% sodium azide and stored at 4 °C.

Immunoprecipitation and Immunoblotting—Fresh bovine adrenal glands were cleared of blood by perfusion with cell buffer A (15 mM HEPES, pH 7.4, 11.2 mM glucose, 145 mM NaCl, 5.4 mM KCl, 1.0 mM NaHCO3) at room temperature and trimmed of external fat. The glands were then perfused with digestion buffer (0.25% collagenase (Roche Applied Science) and 0.005% DNase (United States Biochemical) in cell buffer A), incubated with gentle agitation for 5 min at a 37 °C water bath, and passed with 95% O2 and 5% CO2. The perfusion and incubation with digestion buffer were repeated twice. Medullae were dissected from cortices, frozen immediately in liquid nitrogen, and stored at −80 °C. Frozen tissues were ground with mortar and pestle and homogenized in radioimmune precipitation assay protein lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris, pH 8.0) supplemented with 1 μg/ml leupeptin and 2 μg/ml aprotinin, α3β4α5 AChR cells at −70 °C confuence were washed three times with PBS and lysed in radioimmune precipitation assay buffer. All lysates were clarified by centrifugation at 10,000 × g for 10 min at 4 °C, and protein concentrations were determined by the Bradford method (Bio-Rad). Clarified cell lysate (1 mg) was immunoprecipitated with 5 μg of the indicated antibody and 1% sodium borate slurry. Upstate (Lake Placid, NY), as described previously (38). Precipitated proteins were detected on 8% or 12% SDS-polyacrylamide gels, immunoblotted with specific primary antibodies, and visualized by enhanced chemiluminescence (ECL) using horseradish peroxidase-conjugated anti-mouse, anti-rat, or anti-rabbit antisera (Amersham Biosciences), as described previously (40). As a primary immunoblotting antibody, Q0 antisera was diluted 1:1,000. Other antibodies were used at a concentration of 1 μg/ml in rinsing buffer (50 mM Tris-HCl, pH 7.2, 0.15 mM NaCl, 0.05% Tween 20) made with 5% milk with the conventional blotting procedure was modified for detection of phosphoryrosine by blocking the filters and diluting the antibodies in 5% bovine serum albumin instead of milk.

Whole Cell Voltage Clamp Recording—For these experiments, chromaffin cells, α3β4α5 AChR, or α3β4α5 AChR cells ectopically expressing GFP alone or GFP + e-Variants were seeded on laminin-coated coverslips at densities ranging from 103 to 105 cells/coverglass and incubated in the appropriate medium in 35-mm plastic dishes until use. Coverslips were coated by incubation in a 100 μg/ml solution of 0.5 mg/ml laminin in PBS for 2 h at 37 °C and washed twice with PBS before use. Seeded coverslips were transferred to a specially designed chamber where they were held by a Sygord insert and viewed at ×800 magnification with Hoffman Modulation Contrast optics. Recordings were made with glass pipettes using standard patch clamp techniques. To obtain G1 seals whole cell recordings were started by adding 2 M KCl to the bath, and the electrodes were as follows. The control bath solution contained 150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 10 mM HEPES, pH 7.4, 11 mM glucose, 10 μM nicotine, or 10 μM PP2, or both as indicated. The patch electrode solution contained 150 mM CsCl, 0.1 KCl.
With the previous studies (38, 40). The addition of PP2 significantly inhibited secretion elicited by nicotine but failed to reduce secretion induced by either 55 mM KCl or the Ca\(^{2+}\)-ionophore (Fig. 1B). These results replicated those of Ely et al. (38), who utilized kinase-defective c-Src as a dominant negative inhibitor of endogenous c-Src to demonstrate that the action of SFKs in the secretory pathway occurred at the level of or immediately downstream of the AChR. The identical results obtained by the two methods of inhibiting SFKs substantiated the use of PP2 as a SFK inhibitor in this system. In further support of its use, PP3, an analogue of PP2 which has no effect on the kinase activity of SFKs, was approximately 1 order of magnitude less potent as an inhibitor of catecholamine release than PP2 (Fig. 1C). Overall, these results indicate that SFKs play a role in regulating catecholamine secretion at the level of the AChR.

**Src Family Kinases Positively Affect Nicotine-evoked Currents through the Neuronal AChR**—To determine whether SFKs affect properties of the AChR channels themselves in the secretion process, whole cell recordings of chromaffin cells stimulated with nicotine in the presence or absence of PP2 were obtained. 10 \(\mu M\) nicotine was added to the bath, and inward currents were evoked in the absence of PP2 (Fig. 2A, top tracing), while the voltage was kept constant to avoid opening of downstream voltage-gated Ca\(^{2+}\) channels. After the addition of nicotine, we next sought to examine the mechanism by which c-Src and/or its family members (SFKs) regulate the secretion process more specifically, whether this mechanism involved regulation of the channel current of AChR. To gain insights into the site of action of SFKs in the signaling cascade that regulates secretion, we utilized a specific pharmacological inhibitor of SFKs (PP2) plus different secretagogues, which induce catecholamine secretion at different levels in the signal transduction stream, to assess the inhibitory effect of PP2 on secretion. Nicotine, as an acetylcholine agonist, activates the system at the level of the AChR, whereas 55 mM extracellular KCl intersects the cascade by depolarizing the membrane and activating voltage-gated calcium channels. Calcium ionophore bypasses both of these upstream stages by introducing a bolus of Ca\(^{2+}\) into the cell from extracellular stores (Fig. 1A). Fig. 1B shows that of the three secretagogues used, nicotine induced the greatest amount of catecholamine release, consistent with our previous studies (38, 40). The addition of PP2 significantly inhibited secretion elicited by nicotine but failed to reduce secretion induced by either 55 mM KCl or the Ca\(^{2+}\)-ionophore (Fig. 1B). These results replicated those of Ely et al. (38), who utilized kinase-defective c-Src as a dominant negative inhibitor of endogenous c-Src to demonstrate that the action of SFKs in the secretory pathway occurred at the level of or immediately downstream of the AChR. The identical results obtained by the two methods of inhibiting SFKs substantiated the use of PP2 as a SFK inhibitor in this system. In further support of its use, PP3, an analogue of PP2 which has no effect on the kinase activity of SFKs, was approximately 1 order of magnitude less potent as an inhibitor of catecholamine release than PP2 (Fig. 1C). Overall, these results indicate that SFKs play a role in regulating catecholamine secretion at the level of the AChR.
of 10 μM PP2 to the bath, the middle tracing in Fig. 2A shows that the peak amplitude decreased substantially compared with the amplitude recorded from an untreated cell. The PP2-induced decrease in current recovered to a mean of –86% of the initial amplitude, as shown in the bottom tracing of Fig. 2A and in 2C. The nicotine-evoked current of cells treated with 10 μM PP3 was not significantly different from control (mean of 1.06 ± 0.06 versus control, set at 1.00). The reversibility of PP2 inhibition and the lack of inhibition by PP3 indicated that inhibition was not the result of a persistent toxic effect of PP2 on the cells. Fig. 2B is a time course analysis of the effect of PP2 on a series of measurements made from a single chromaffin cell. PP2 caused a decrease in the nicotine-evoked current amplitude as long as it was present in the bath, but its inhibitory effects were again shown to be partially reversible upon its withdrawal. Fig. 2C shows the mean ± S.E. peak current amplitude of 10 different chromaffin cells that were treated with nicotine alone (control), with nicotine + PP2, or with nicotine after PP2 withdrawal. Complete recovery was seen in only 2 cells of 10 total. Part of the reason for incomplete recovery was deterioration of the nicotinic responses after multiple stimulations.

The same experiment was repeated in the a3β4α5 AChR cell line, which stably expresses the a3, α5, β4 subunits of human AChRs. Results from whole cell recordings of a3β4α5 AChR cells were similar to those from chromaffin cells (Fig. 3, A–C), showing that PP2 inhibited by –50% the nicotine-induced peak current amplitude in a reversible fashion. In contrast, the same concentration of PP3 showed no effects relative to controls (1.16 ± 0.24 versus 1.00). Taken together (Figs. 1–3), our results suggest that one mechanism by which SFKs can affect secretion is through a kinase-dependent up-regulation of nicotinic-induced channel currents.

Because internalization of other neuronal receptors has been reported to occur within the same time frame as the PP2 pretreatment in our studies (54, 55), we used a radiolabeled ligand binding assay to determine whether PP2 pretreatment caused internalization of the AChR. Fig. 4 shows that the level of [3H]nicotine binding to intact AChR on chromaffin cells was not significantly different between PP2-pretreated and non-pretreated cells (p > 0.05). The specificity of [3H]nicotine binding was demonstrated by competition with cold, excess nicotine. Together, these results indicate that the reduction of current amplitude in the presence of PP2 was not caused by premature AChR internalization.

To examine further the ability of SFKs to influence AChR activity, we generated a3β4α5 AChR clonal cell lines that stably express either dominant negative (kinase-defective), wt, or constitutively active forms of chicken c-Src and tested them for nicotine responses, using whole cell recordings. Fig. 5A shows that the presence of kinase-defective or constitutively active c-Src resulted in a 75–85% reduction or a 2-fold increase, respectively, in the mean peak amplitude compared with that of a3β4α5 AChR cell lines that express either wt c-Src or GFP alone. Because we used whole cell recordings to examine channel activity, the possibility existed that the stable expression of c-Src variants might result in altered levels of AChRs. Fig. 5B shows that the levels of α5 and β4 subunit expression were not changed appreciably by ectopic expression of GFP or any variant of c-Src. Fig. 5C demonstrates that similar levels of kinase-defective and wt c-Src but a lesser level of activated c-Src were expressed in the cell lines. Thus, results depicted in Fig. 5 strongly support the involvement of c-Src and potentially other SFKs in the regulation of nicotine-evoked channel currents.

Vanadate Treatment Enhances Nicotine-induced Current Amplitudes—Previous studies from our laboratory showed that both AChR and c-Src immune complexes from chromaffin cells contain an associated PTPase activity of unknown identity (41). This finding suggested that the channel activity of AChRs may be dually regulated by tyrosine kinases and opposing PTPases. To test this hypothesis, we treated a3β4α5 AChR cells with sodium vanadate, a broad spectrum PTPase inhibitor, and examined the effects of this treatment on nicotine-evoked channel currents. In these experiments, vanadate was added intracellularly through the micropipette, and nicotine was added to the bath. Fig. 6A shows that the peak current amplitude of vanadate-treated cells was significantly greater than that of untreated cells. This enhancement was maintained with continued exposure to vanadate and increased over a time course of multiple nicotine stimulations (Fig. 6B), reaching a maximum of >2.5-fold over untreated after a 15-min vanadate treatment (Fig. 6C). These results support the hypothesis that the channel activity of the neuronal form of the AChR can be regulated negatively by PTPases(s), thereby counterbalancing the effects of the SFKs.

Coinmunoprecipitation of AChR with SFKs—To determine whether SFKs physically associate with AChRs, as do PTPases (41), a series of coinmunoprecipitation experiments was performed, wherein AChRs from adrenal medullary lysates were immunoprecipitated with mAb 35, and precipitated proteins were immunoblotted with mAb 337 (specific for the β4 subunit of the AChR) or with antibodies specific for various SFKs. Fig. 7A depicts the coinmunoprecipitation of SFKs with AChRs, wherein the pan-SFK antibody, Q0, was used to immunoblot an
AChR immunoprecipitate. Immunoblotting of AChR immunoprecipitates with antibodies specific for various SFKs identified c-Src as the predominant SFK that coassociates with the receptor (10.5% of total c-Src associates with the AChR) compared with Fyn (3.9% total Fyn associates with the AChR) and c-Yes (undetectable association). Anti-β4 blotting of the immunoprecipitated complexes confirmed the ability of mAb 35 to immunoprecipitate AChRs. To verify further the specificity of the common immunoprecipitation of c-Src with the AChR, anti-transferrin receptor was immunoprecipitated from chromaffin cell lysates. In contrast to the AChR, the transferrin receptor immunoprecipitate contained no detectable c-Src (Fig. 7B) while blotting positively for the transferrin receptor. These findings, depicting the physical association of SFKs (particularly c-Src) with AChRs, provide at least one biochemical basis for the biological interactions between the two molecules.

AChRs from Chromaffin Cells and a3β4α5 AChR Cells Are Tyrosine-phosphorylated—The intracellular domains of each of the subunits of AChRs, α3, β4, and α5 contain multiple tyrosine residues. To investigate the tyrosine phosphorylation status of the AChR, we immunoprecipitated AChRs from either chromaffin or α3β4α5 AChR cells and blotted precipitated proteins with phosphotyrosine-specific antibody. Fig. 8 shows that AChRs are tyrosine-phosphorylated in both primary cultures of chromaffin cells and in α3β4α5 AChR cells. The electrophoretic migration of the tyrosine-phosphorylated bands in each cell type is most consistent with that of either the α3 and/or the β4 subunit. (The molecular weight of human and bovine α3 and β4 subunits is ~54–55, whereas that of human and bovine α5 subunits is ~52.)

DISCUSSION

This study demonstrates that chromaffin cells of the adrenal medulla require catalytically active c-Src (or other Src family members) to respond optimally to nicotine stimulation, as measured either by the receptor channel activity or the release of catecholamines. Along with pharmacological inhibitors of SFKs and ectopic expression of wt, dominant negative, and constitutively active forms of c-Src, whole cell recordings were applied to two different model cell systems (namely, chromaffin cells and α3β4α5 AChR cells that ectopically express functional neuronal AChRs) to elucidate these effects of SFKs. Treatment of cells with sodium vanadate also revealed a role for a putative PTPase in negatively regulating channel currents. Supportive evidence for a functional interaction among the AChR, SFKs, and a PTPase are provided by experiments that demonstrate tyrosine phosphorylation of the receptor (Fig. 8 of this paper) and the presence of stable associations between c-Src and the AChR (Fig. 7 of this paper), between an unidentified PTPase and the neuronal-type AChR (41), and between a PTPase and c-Src (41). Molecular sieving chromatography further revealed a comigration among c-Src, the PTPase activity, and the AChR (41). Together, these findings support a model in which a trimeric complex composed of c-Src, a PTPase, and the AChR serves as the structural basis for regulation of the AChR. The activity of this receptor in turn directly influences the secretory responses of chromaffin cells, thereby pinpointing one step in the exocytotic pathway which is regulated by SFKs.

Although our studies are the first to reveal a role for SFKs and PTPases in regulating the current amplitude of neuronal AChR, kinases and phosphatases have previously been shown to influence critical properties of other neuronal channels. Such properties include desensitization, peak current amplitude, and run-down. For example, tyrosine phosphorylation or phosphorylation by protein kinase A has been found to increase the desensitization rate of neuromuscular AChRs (56, 57), and protein kinase C has been shown to phosphorylate serines 1303 and 1323 directly in the C terminus of the NR2B subunit, leading to an increase in peak current amplitude of the NMDA receptor (58). In addition, the phosphotyrosine state of the GABA receptor is controlled by a PTPase, which regulates the run-down of GABA-activated chloride currents (59).

Of the many candidate tyrosine kinase families that could mediate these effects on channel properties, members of the...
Src family of tyrosine kinases have been most frequently implicated. Phosphorylation of the Torpedo AChR by the SFKs, Fyk, and Fyn was found to modulate synaptic transmission at the neuromuscular junction (15), and microinjection of isolated c-Src into vascular smooth muscle cells resulted in increased voltage-operated calcium channel currents that were dependent on c-Src kinase activity (60). c-Src was also found to associate with the tyrosine-phosphorylated NMDA receptor which may regulate the function of this receptor (17), and SFKs bind heteromultimeric Shaker potassium (61) channels by interactions between the Src homology 3 (SH3) domain of SFKs and the proline-rich SH3 binding domains of the channels (62). In our studies, we demonstrated coimmunoprecipitation between c-Src and AChR. At this point in time, we do not know whether this interaction is direct or indirect, but the physical association provides a structural basis for the possible functional interaction between the AChR and Src kinases, which could involve tyrosine phosphorylation. We also tested coimmunoprecipitation of AChR with c-Yes or Fyn (these SFKs are also richly expressed in chromaffin cells), but no c-Yes was observed to associate with the receptor, and less Fyn than Src was stably complexed with the receptor. Thus, c-Src appears to be the predominant SFK that stably associates with the neuronal AChR.

In post-translation modulation mechanisms, the phosphorylation of proteins is reversible, which means that kinases are usually counterbalanced by corresponding phosphatases. For instance, synchronized activities of tyrosine kinases and alkaline phosphatase coordinately regulate the activity of Kv1.3 potassium channels (13). Protein kinase A and type 1 protein phosphatase bind the NMDA receptor via a scaffold protein, and the activity of protein kinase A confers the rapid enhancement of NMDA receptor currents, whereas type 1 protein phosphatase limits current amplitudes (63). Sodium channels are also associated with a receptor-type PTPase, PTPase-β, and dephosphorylation of the channel by this phosphatase in-
creases whole cell sodium current (64). Studies described in this report reveal a requirement for SFKs for neuronal AChRs to achieve optimal current amplitude after nicotine stimulation, whereas an unidentified PTPase is postulated to regulate this event negatively.

What could be the mechanism of this dual regulation? Our published data have demonstrated coprecipitation of a PTPase activity with both the AChR and c-Src from adrenal medulla and provided evidence for multimeric complexes containing c-Src and PTPase activity (41). One of these complexes is greater than 669 kDa in mass, and the other measures around 450 kDa. The smaller complex comigrates with the AChR during molecular sieving chromatography, suggesting the existence of a trimeric interaction among c-Src, the AChR, and a PTPase. The larger complex contains no detectable AChR. This observation raises the possibilities that the two complexes are in equilibrium with one another and that the activity of the AChR could be influenced either by the stoichiometry of the various components or by the molecular composition (or both) of the two complexes.

Identification of the PTPase(s) associated with c-Src has been attempted by immunoblotting c-Src immunoprecipitates with a panel of antibodies directed against various PTPases, such as Syp (SH-PTP2) (65), R-PTPα (66), R-PTPβ (67), PTP1B (68), and ERP (extracellular-regulated PTPase) or MKP-1 (MAP kinase PTPase) (69). None of these PTPases could be detected in association with c-Src from chromaffin cells, suggesting either that the level of PTPase is below the sensitivity of detection, or it is a PTPase not yet tested or shown to associate with c-Src. Alternatively, it could be a novel PTPase. Efforts are currently under way to identify this PTPase activity.

Based on the results of our studies, we propose that c-Src, a putative PTPase, and AChR form a multimeric complex that regulates the channel currents as well as secretion in accordance with the appropriate biological environment. One model of regulation might be that in a resting chromaffin cell, the equilibrium between the activities of SFKs and PTPases favors the PTPases, thereby maintaining the AChR in an inactive state. Upon stimulation by acetylcholine or nicotine, the activities of the SFKs become predominant, whereas the phosphatase activities are suppressed. This state favors opening of the channel, increasing the inward current, and eventual triggering of the release of catecholamines. Meanwhile, the c-Src/AChR-associated PTPase gradually recovers its activity, an event that potentiates the closing of the channel. This model is supported by our previous findings in chromaffin cells, which showed that the PTPase activity associated with c-Src decreases in the first few seconds after stimulation with nicotine, then recovers at the later stages of secretion (41). An alternative model suggests that the agonist determines activation of the receptor, but the ratio of SFK to PTPase activity determines the proportion of competent receptors, probability of receptor opening, or, perhaps most likely, the basal level of desensitization within the

**FIG. 7.** Physical association between c-Src and the neuronal AChR found on chromaffin cells. A, lysates were prepared from adrenal medullary chromaffin cells, and 1 mg of lysate protein was immunoprecipitated (IP) with anti-c-Src 2–17 mAb, anti-Fyn polyclonal, anti-c-Yes mAb, anti-AChR mAb 35, or negative control antibody, as described under "Materials and Methods." Precipitated proteins were resolved on a 12% SDS-polyacrylamide gel and immunoblotted with the anti-pan SFK antibody, Q0, anti-c-Src 2–17, anti-Fyn, anti-Yes, or anti-AChR 337. (The c-Yes mAb used and others tested were found to immunoprecipitate poorly, thus no signal in the first lane of the c-Yes panel was observed.) For direct immunoblotting, 200 μg of cell lysate protein was applied to the gel. B, 1 mg of lysate protein from chromaffin cells was immunoprecipitated with anti-transferrin receptor mAb, anti-c-Src 2–17 mAb, or negative control antibody. Precipitated proteins were resolved on a 12% SDS-polyacrylamide gel and immunoblotted with anti-transferrin receptor mAb or anti-c-Src 2–17 mAb.

**FIG. 8.** AChR receptors from chromaffin cells and α3β4α5 AChR cells are tyrosine-phosphorylated. 1 mg of lysate protein from chromaffin cells, α3β4α5 AChR, or tsA201 cells was immunoprecipitated (IP) with anti-AChR mAb 35 or mouse IgG, each conjugated to Sepharose beads. Precipitated proteins were resolved on a 12% SDS-polyacrylamide gel and immunoblotted with anti-AChR mAb 337 or PY99 anti-phosphotyrosine (pTyr) mAb.
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receptor population at rest. Our data also support a bifunctional role for SFKs, one to target PTases to the AChR via their SH2 domains, and another to mediate phosphorylation via their kinase domains.

The presence of phosphotyrosine on the AChR and the effects of SFK inhibitors on receptor channel activity suggest that SFKs could phosphorylate the receptor directly. We tested this hypothesis by examining the levels of receptor tyrosine phosphorylation in resting and nicotine-stimulated cells expressing the various c-Src mutants or in cells treated with PP2 or vanadate. Surprisingly, we found no significant differences among any of the treatment groups or cell lines, suggesting that tyrosine phosphorylation of the receptor may not be the critical regulator of channel activity or that tyrosine phosphorylation of the receptor is important but not the sole (sufficient) regulator of the channel. Additional studies are needed to understand the physiological significance and effects of tyrosine phosphorylation on receptor function. A third explanation for our findings is that because the a3 subunit has six and the β4 subunit has three potential tyrosine phosphorylation sites, if modulation of one site is critical (against a background of phosphorylation of the other sites), our assay would not have been sensitive enough to detect such a small change in overall tyrosine phosphorylation. Finally, it could be postulated that the receptor is not the direct target of SFK kinase activity. Perhaps another kinase or a phosphatase mediates the effects of SFKs, and regulation of this protein may require multiple inputs, that of the SFKs being only one. Clearly, identification of the direct target of SFKs in this system is needed to address these issues.

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