Nicotinic acetylcholine receptor $\alpha_7$ regulates cAMP signal within lipid rafts

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Oshikawa, Jin, Yoshiyuki Toya, Takayuki Fujita, Masato Egawa, Junichi Kawabe, Satoshi Umemura, and Yoshihiro Ishikawa. Nicotinic acetylcholine receptor $\alpha_7$ regulates cAMP signal within lipid rafts. Am J Physiol Cell Physiol 285: C567–C574, 2003. First published May 14, 2003. 10.1152/ajpcell.00422.2002.—Neuronal nicotinic acetylcholine receptors (nACHRs) are made of multiple subunits with diversified functions. The nACHR $\alpha_7$-subunit has a property of high Ca$^{2+}$ permeability and may have specific functions and localization within the plasma membrane as a signal transduction molecule. In PC-12 cells, fractionation by sucrose gradient centrifugation revealed that nACHR$\alpha_7$ existed in low-density, cholesterol-enriched plasma membrane microdomains known as lipid rafts where flotillin also exists. In contrast, nACHR $\alpha_5$- and $\beta_2$-subunits were located in high-density fractions, out of the lipid rafts. Type 6 adenylyl cyclase (AC6), a calcium-inhibitable isoform, was also found in lipid rafts and was coimmunoprecipitated with nACHR$\alpha_7$. Cholesterol depletion from plasma membranes with methyl- $\beta$-cyclodextrin redistributed nACHR$\alpha_7$ and AC6 diffusely within plasma membranes. Nicotine stimulation reduced forskolin-stimulated AC activity by 35%, and this inhibition was negated by either treatment with $\alpha$-bungarotoxin, a specific antagonist of nACHR$\alpha_7$, or cholesterol depletion from plasma membranes. The effect of cholesterol depletion was negated by the addition of cholesterol. These data suggest that nACHR$\alpha_7$ has a specific membrane localization relative to other nACHR subunits and that lipid rafts are necessary to localize nACHR$\alpha_7$ with AC within plasma membranes. In addition, nACHR$\alpha_7$ may regulate the AC activity via Ca$^{2+}$ within lipid rafts.

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MATERIALS AND METHODS

Cell culture. PC-12, a rat pheochromocytoma cell line, was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum and 10% horse serum in a humidified 95% air-5% CO₂ incubator. In our experiments, the cells were used in the undifferentiated state.

Cell fractionation by sucrose gradient centrifugation. Lipid rafts were obtained by the sodium carbonate-based detergent-free method (29). Briefly, four 10-cm plates of PC-12 cells with 80% confluence were homogenized in a solution containing 0.5 M sodium carbonate (pH 11) and protease inhibitors with three 10-s bursts of a Polytron tissue grinder and four 20-s bursts of a sonicator. The homogenates were adjusted to 45% sucrose by adding 90% sucrose in 25 mM MES (pH 6.5)-0.15 M NaCl (MBS) and placed at the bottom of an ultracentrifugation tube. A 5–35% discontinuous sucrose gradient was formed above and centrifuged at 39,000 rpm at 4°C for 35 min. The supernatant was discarded, and the pellet was resuspended in the same buffer, followed by immunoblotting.

Immunoblotting. Each fraction obtained from sucrose gradient centrifugation or the particulate fraction from PC-12 cells was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h with 5% nonfat dry milk and 5% bovine serum albumin in Tris-buffered saline and 0.1% Tween 20 (TBS-T) at room temperature. Polyclonal antibodies against nAChRα5 (no. SC-9345)-α7 (no. SC-1447), and -β2 (no. SC-11372) and AC type 3 (AC3) (no. SC-588) and AC5/6 (no. SC-590) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal or monoclonal antibodies against caveolin-1 [nos. C37120 and C13630 from Transduction Laboratories (Lexington, KY)] and no. SC-894 from Santa Cruz Biotechnology and no. F65020 from Transduction Laboratories were used (13, 23). Monoclonal antibody against flotillin-1 (no. F65020) was purchased from Transduction Laboratories. The membranes were incubated with specific primary antibodies for 2 h at room temperature, followed by visualization with horseradish peroxidase-conjugated specific secondary antibodies. Immunoreactive bands were detected with a SuperSignal substrate kit (Pierce, Rockford, IL).

Immunoprecipitation. PC-12 cells were lysed in a lysis buffer (10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA, 60 mM octylglucoside, and 1% Triton X-100, and protease inhibitors) at 4°C for 30 min. The lysates were incubated with primary antibodies for 12 h. Immune complexes were formed by incubation with protein G-Sepharose for 2 h. The immune complexes were washed with washing buffer (10 mM Tris-HCl pH 8.0, 0.15 M NaCl, 5 mM EDTA, 0.1% Triton X-100) three times, solubilized, and subjected to immunoblotting.

Intracellular Ca²⁺ concentration measurements. The intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) of individual cells was examined as previously described (14) with some modifications. PC-12 cells were incubated for 1 h at 37°C in a basal salt solution (in mM: 130 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 10 HEPES pH 7.3, and 5.5 glucose) containing 4 μM fura 2-AM (Dojin, Kumamoto, Japan) and 0.025% Pluronic F-127 (Molecular Probes, Eugene, OR). After cells were washed, changes in [Ca²⁺]ᵢ were measured by the fluorescence emitted at 510 nm resulting from alternate excitation at 340 and 360 nm with an ARGUS-50/CA apparatus (Hamamatsu Photonics, Hamamatsu, Japan). Conversion of fluorescence signals into absolute [Ca²⁺]ᵢ values was performed with ARGUS-50/CA analysis software.

Fig. 1. Protein distribution of PC-12 cells after sucrose gradient centrifugation and subcellular localization of nicotinic acetylcholine receptor (nAChR) α5-, β2-, and α7-subunits. A: PC-12 cells were fractionated to 13 fractions, and the protein concentration of each fraction was determined. A representative result is shown. Fraction 1 had the lowest sucrose density, and fraction 13 was the pellet. B: PC-12 cell membranes and mouse lung tissues were subjected to immunoblotting with polyclonal anti-caveolin (cav)-1, monoclonal anti-caveolin-2, and anti-flotillin-1 antibodies. C: an aliquot from each of the 13 fractions was subjected to SDS-PAGE, followed by immunoblotting for flotillin-1 and nAChRα5-, β2-, and -α7. Molecular masses are shown on right.
cAMP accumulation assay. cAMP accumulation in intact cells was measured according to the method of Kawabe et al. (20) with a modification. The PC-12 cells were incubated in normal growing medium containing 3H-labeled adenine for 24 h. The cells were washed three times with HEPES-buffered serum-free DMEM (pH 7.4). Nicotine and H9251-Bungarotoxin (H9251-BTX) were added and incubated for 30 min with 20 μM N-(2-[p-bromocinnamylamino]ethyl)-5-isouquinolinesulfonamide (H89). The cells were incubated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min before reactions. Reactions were started by adding 1 μM forskolin at 37°C and terminated after 10 min by the addition of cold 12% trichloroacetic acid, 0.25 mM ATP and 0.25 mM cAMP. 3H-labeled ATP and 3H-labeled cAMP were separated according to the method of Alvarez and Daniels (1). cAMP production was calculated by [3H]cAMP/([3H]ATP) x 10³. All assays were performed four to six times, and the values are shown as percentage of control in RESULTS. Student’s t-test was used for statistical analysis.

Cholesterol depletion. To deplete cholesterol from plasma membrane in PC-12 cells, cells were washed twice with PBS and incubated with DMEM containing 10 mM methyl-β-cyclodextrin (MβCD) at 37°C for 1 h (11). In some experiments, cholesterol (16 μg/ml) was applied in addition to MβCD (12). The cells were then harvested and used for immunoblotting, immunoprecipitation, and cAMP accumulation assays.

RESULTS

Subcellular distribution of nAChRs. PC-12 is a rat pheochromocytoma cell line that expresses various nAChR subunits endogenously. A previous study reported that PC-12 express nAChR α3-, α5-, α7-, β2-, and β4-subunits (16). In our preliminary experiments, nAChR α5-, α7-, and β2-subunits were readily detected by immunoblotting in our PC-12 cells. At first, to determine the subcellular distribution of these subunits, homogenates of PC-12 cells were fractionated by sucrose gradient centrifugation. Figure 1A shows the distribution of the protein concentration of 13 fractions after fractionation. The amount of protein in fractions 4–6 was no more than 5% of the total cellular protein, and a major portion of protein was in fractions 9–13. Fractions 4–6 may be similar to caveolae fractions in nonneuronal tissues. However, no caveolin any subtype were detected in the particulate fraction in our PC-12 cells by immunoblotting, whereas they were readily detected in mouse lung tissues (Fig. 1B); the results were similar when various caveolin antibodies that have been used in previous studies were used (13, 23).

Because caveolin was not detectable in our PC-12 cells, we examined the expression and distribution of
flotillin, a marker of cholesterol-enriched lipid rafts in tissues that do not express caveolin (3). Figure 1B demonstrates that flotillin was present in PC-12 cells whereas caveolin was not. Figure 1C demonstrates the subcellular localization of flotillin in our PC-12 cells. Flotillin was found mainly in fractions 4–6, indicating that these fractions represented lipid rafts in our PC-12 cells.

To determine whether nAChRs exist within or outside of lipid rafts, 13 fractions obtained from sucrose gradient centrifugation were subjected to immunoblotting. Polyclonal antibodies against specific nAChRs were used for immunoblotting, which found that nAChRoβ7 was localized to fractions 4–6 but that nAChRoα7 and β2 were found mainly in fractions 9–13 (Fig. 1C). Doublet bands of nAChRoα7 were detected as shown in previous studies (5, 10). This distinct subcellular localization of α7 relative to that of the other subunits might reflect functional significance of this subunit. We thus investigated whether α7-subunit may interact with the other molecules located in the same lipid rafts.

Interaction of nAChRo7 with AC. nAChRo7 is α-BTX sensitive and has a higher Ca2+ permeability than the other subunits. A previous study demonstrated that nicotine induced the elevation of [Ca2+]i in PC-12 cells (14). Accordingly, the high Ca2+ permeability of nAChRo7 may regulate Ca2+-sensitive molecules within lipid rafts. AC is the enzyme that produces cAMP from ATP. At least nine isoforms of AC (AC1–AC9) have been cloned and characterized in mammals (18). AC5 and AC6 are directly inhibited by low concentrations of Ca2+ (15, 22). It is possible that nicotinic receptor agonists may regulate the activity of AC5 or -6 through increasing the entry of Ca2+. In this regard, we previously demonstrated (25) that molecules involved in cAMP signaling such as AC and G proteins are located in caveolar microdomains.

We first examined the subcellular distributions of AC in PC-12 by sucrose gradient centrifugation. The fractionated PC-12 cellular proteins were subjected to immunoblotting with polyclonal antibodies for AC3 and AC5/6 (17). It should be noted that the polyclonal antibody against AC5/6 potentially detects both AC5 and AC6. However, this antibody detected a single AC species in PC-12, which is most likely AC6 because the mRNA expression of AC6, but not AC5, was readily detected (data not shown). We also examined the protein expression of AC1, -2, and -4, but they were not readily detected by immunoblotting (data not shown).

Figure 2A shows that both AC3 and AC6 were in fractions 4–6, indicating that AC3 had little association with nAChRα7 and AC6. Similarly, reciprocal immunoblotting assays of the supernatants with antibodies against AC6 or nAChRα7 after immunoprecipitation with nAChRα7 or AC6 antibodies, respectively, showed that most, but not all, AC6 and nAChRα7 were associated with each other. In contrast, AC3 had little association with nAChRα7 (Fig. 2B, right). Our results suggest that nAChRα7 and AC6 are colocalized and associated in lipid rafts.

Nicotinic stimulation and cAMP accumulation. To examine whether the activation of nAChRs leads to changes in [Ca2+]i, and thus altered cAMP signal within the cell (19), cAMP accumulation assays were conducted in the presence of nicotinic stimulation.

First, we confirmed the effect of nicotine to elevate [Ca2+]i in PC-12 cells (Fig. 3A). The treatment of PC-12 cells with 100 μM nicotine elicited a rise in [Ca2+]i at two peaks of the elevation as reported previously (14). This rise of [Ca2+]i was blocked in the presence of α-BTX, a specific nAChRα7 antagonist, indicating that the effect of nicotine was due to the stimulation of nAChRα7.

Stimulation of PC-12 with forskolin (1 μM), a direct AC stimulator, for 10 min increased cAMP accumulation by 10-fold over the basal level. In the presence of nicotine, this accumulation was inhibited by ~35% (Fig. 3B). The subcellular distribution of nAChRα7 and AC was not shifted by nicotine stimulation (data not shown). In the presence of α-BTX, the effect of nicotine

Fig. 3. Effect of nicotine on intracellular Ca2+ concentration ([Ca2+]i) and cAMP accumulation. A: changes in [Ca2+]i of PC-12 cells were analyzed in the presence of nicotine (1–100 μM; left). α-Bungarotoxin (α-BTX; 10 nM) was added 10 min before nicotine treatment (100 μM; right). Values shown are averages of 6–8 cells in each condition. B: [3H]adenine-labeled PC-12 cells were incubated with nicotine in the presence (open bars) or absence (filled bars) of α-BTX (10 nM) for 30 min. cAMP accumulation assays were conducted in the presence of forskolin (1 μM) for 10 min. Values are shown as mean ± SE % of control (n = 4–6). *P < 0.05; **P < 0.01.
on cAMP accumulation was negated (Fig. 3B), suggesting that nAChRα7 contributed to the inhibition of AC6.

**Effect of cholesterol depletion from plasma membrane.** It is known that MβCD depletes plasma membrane cholesterol, leading to the attenuation of the function of several molecules within lipid rafts (11). PC-12 cells were incubated with 10 mM MβCD, followed by fractionation and immunoblotting. Figure 4A shows the subcellular distribution of nAChRα7, AC6, and flotillin in MβCD-treated cells. In contrast to the finding in the absence of MβCD pretreatment (Fig. 1C), these molecules were found diffusely in fractions 4–10, indicating that the localization of nAChRα7, AC6, and flotillin was altered by cholesterol depletion. When cholesterol was added to MβCD to negate its cholesterol-depleting effect (12), nAChRα7, AC6, and flotillin were relocalized to lipid rafts, suggesting that the localization of these molecules to lipid rafts requires the presence of cholesterol in the plasma membrane. Furthermore, the association between nAChRα7 and AC6 was mostly lost by MβCD treatment, as demonstrated by reciprocal immunoprecipitation assays with nAChRα7 and AC6 antibodies (Fig. 4B, center). This ablation of the association between nAChRα7 and AC6 was also restored by adding cholesterol to MβCD (Fig. 4B, right).

Figure 5A demonstrates that MβCD or cholesterol treatment did not alter nicotine-induced rise in [Ca^{2+}].

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**Fig. 4. Effect of cholesterol depletion on subcellular distribution and association of nAChRα7 and AC6.**

A: PC-12 cells were treated with methyl-β-cyclodextrin (MβCD; 10 mM) or MβCD (10 mM) + cholesterol (16 μg/ml), followed by fractionation and immunoblotting for nAChRα7, AC6, and flotillin. B: cells untreated (control) or treated with MβCD or with MβCD + cholesterol were homogenized and subjected to immunoprecipitation assays with anti-AC6 (AC) and nAChRα7 (a7) antibodies, followed by immunoblotting for AC6 and nAChRα7. Note that only the lower band (arrow) of the nAChRα7 doublets was visible after immunoprecipitation because the upper band of the nAChRα7 doublets (Fig. 1C) overlapped with IgG.
relative to that in nontreated cells shown in Fig. 3A. The peaks of \([\text{Ca}^{2+}]_i\) showed no significant differences among the control, MβCD, and MβCD + cholesterol groups (282.3 ± 4.1, 273.5 ± 3.9, and 280.8 ± 5.1 nM, respectively). The second elevation of \([\text{Ca}^{2+}]_i\) also showed no differences among these conditions (127.3 ± 2.9, 124.5 ± 3.4, and 126.3 ± 2.8 nM, respectively). Similarly, MβCD treatment did not alter forskolin-stimulated cAMP accumulation but negated the nicotine-induced inhibition of cAMP accumulation in PC-12 cells (Fig. 5B). We also examined the specificity of the effect of MβCD. When cholesterol was added to MβCD in PC-12 cells, the nicotine-induced inhibition of cAMP production was restored (Fig. 5B). These data suggest that the presence of intact lipid rafts is necessary to localize nAChRβ7 to the lipid rafts as well as to regulate cAMP signal through the nicotinic activation of nAChRβ7.

**DISCUSSION**

In neuronal cells, where the rapid transmission of signal and thus prompt regulation of enzymatic activity are required, the close association of nAChR with AC may play an important role in regulating intracellular cAMP signals. Unlike the receptor-mediated regulation of cAMP signals, which requires the activation of G protein by the receptor and the association and activation of AC by the G protein, the direct regulation of AC activity by Ca2+ may occur promptly. The influence of Ca2+ may be restricted to the microdomain, where the concentration of Ca2+ rises high enough to inhibit AC activity. Chiono et al. (7) demonstrated that diffuse elevation of \([\text{Ca}^{2+}]_i\) by releasing Ca2+ from intracellular stores in modulating cAMP synthesis. The local elevation of intracellular Ca2+ concentration by Ca2+ entry, especially in cholesterol-rich domains, is necessary for inhibition of AC6 activity (11). In this study we have shown that both nAChRα7 and AC6 exist within lipid rafts and interact both physically (immunoprecipitation) and functionally (cAMP accumulation assay). The elevation of Ca2+ concentration by stimulation of nAChRα7 (14) might be restricted to lipid rafts and influence AC6 activity. In this regard, the close association between nAChRα7 with a high Ca2+-permeable and AC6 with a Ca2+-inhibitable property within lipid rafts may play an important role in regulating neuronal cAMP signals. It is of interest that the other nAChR subunits such as β2 or α5 were not found in lipid rafts.

We found that AC3 was also in lipid rafts but was not readily coimmunoprecipitated with nAChRα7. This finding suggests that AC3 may not be bound to nAChRα7 as tightly as AC6, and thereby the regulation of AC3 by nAChRs might not be so potent. Unfortunately, the effect of Ca2+ on this isoform is controversial (8, 31), and we cannot predict the effect of nAChRs specifically on AC3. Alternatively, the sensitivity and/or affinity of the AC3 antibody may not be sufficiently high, although we have successfully used this antibody in past studies (17). Nevertheless, nAChRα7 may play a distinct role from the other nAChR subunits in regulating intracellular signals because of its unique subcellular localization in lipid rafts. In support of this hypothesis, it was recently demonstrated that nAChRα7 associates with phosphatidylinositol 3-kinase (PI3K) and a tyrosine kinase (Fyn) but nAChRα4 did not (21). PI3K and the Src family of ty-
Rosine kinases are reported to exist within caveolae (32, 33). These findings suggested that nAChRox7, PI3K, and Fyn are closely located and interact with each other. We do not deny, however, that the other nAChR subunits also play important roles in regulating other signaling pathways.

We have not examined the mechanism that localizes nAChRox7 and AC to the same lipid rafts. In caveolae of peripheral cells, caveolin, a structural component of caveolae, directly binds to and anchors the molecules within caveolae (24). However, we did not find caveolin expression in lipid rafts of our PC-12 cells. A few studies in which the same antibodies were used as in our study reported that caveolin was readily detected in PC-12 cells (13, 23). This difference may be due to the difference in PC-12 cell type or experimental conditions, such as the state of cellular differentiation. We instead demonstrated that flotillin, a marker for lipid rafts (3), was in the same fractions as nAChRox7 and AC6. Nevertheless, our results suggest that the cholesterol depletion that is known to disrupt both caveolae and lipid rafts (11) destroyed the interaction between nAChRox7 and AC. The robust finding of our study is the suggestion that subcellular microdomains such as lipid rafts provide a site of regulation within neuronal cells between specific Ca2+-handling molecule sub-type and a Ca2+-regulated enzyme subspecies.

DISCLOSURES

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