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Nicotine Inhibits FcεRI-Induced Cysteinyl Leukotrienes and Cytokine Production without Affecting Mast Cell Degranulation Through α7/α9/α10-Nicotinic Receptors

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Smokers are less likely to develop some inflammatory and allergic diseases. In Brown-Norway rats, nicotine inhibits several parameters of allergic asthma, including the production of Th2 cytokines and the cysteinyl leukotriene LTC₄. Cysteinyl leukotrienes are primarily produced by mast cells, and these cells play a central role in allergic asthma. Mast cells express a high-affinity receptor for IgE (FcεRI). Following its cross-linking, cells degranulate and release preformed inflammatory mediators (early phase) and synthesize and secrete cytokines/chemokines and leukotrienes (late phase). The mechanism by which nicotine modulates mast cell activation is unclear. Using α-bungarotoxin binding and quantitative PCR and PCR product sequencing, we showed that the rat mast/basophil cell line RBL-2H3 expresses nicotinic acetylcholine receptors (nAChRs) α7, α9, and α10; exposure to exceedingly low concentrations of nicotine (nanomolar), but not the biologically inactive metabolite cotinine, for ≥8 h suppressed the late phase (leukotriene/cytokine production) but not degranulation (histamine and hexosaminidase release). These effects were unrelated to those of nicotine on intracellular free calcium concentration but were causally associated with the inhibition of cytosolic phospholipase A₂ activity and the PI3K/ERK/NF-κB pathway, including phosphorylation of Akt and ERK and nuclear translocation of NF-κB. The suppressive effect of nicotine on the late-phase response was blocked by the α7/α9-nAChR antagonists methyllycaconitine and α-bungarotoxin, as well as by small interfering RNA knockdown of α7-, α9-, or α10-nAChRs, suggesting a functional interaction between α7-, α9-, and α10-nAChRs that might explain the response of RBL cells to nanomolar concentrations of nicotine. This “hybrid” receptor might serve as a target for novel antiallergic/asthmatic therapies. *The Journal of Immunology*, 2010, 185: 588–596.

The prevalence and severity of atopic diseases, including allergic asthma, rhinitis, and eczema, have increased dramatically in recent years (1–5). Allergic diseases involve the allergen-induced Th2 response characterized by the production of Th2 cytokines, including IL-4, -5, and -13, which are critical in the development of the allergic response. Mast cells are critical tissue-based effector cells that mediate IgE-dependent allergic responses (6–8). Mast cells express IgE receptors (FcεRI), and binding of an allergen to IgE-FcεRI induces the release of three classes of proinflammatory mediators: 1) preformed granule-associated chemical mediators; 2) newly synthesized arachidonic acid metabolites, such as leukotrienes (LTs); and 3) proinflammatory cytokines, including TNF-α and Th2 cytokines (6–8). Among these mediators, the cysteinyl LTs (cysLTs) exert a number of patho-

physiological effects of allergic asthma, including proliferation and contraction of bronchial smooth muscle cells, mucus secretion, inflammatory cell migration, and increased vascular permeability (9–11). Indeed, cysLTs are important indicators of allergic asthma severity (12–14).

Several reports suggested an inverse correlation between cigarette smoking and the development of allergic diseases (15, 16). Smoking increases the risk for various diseases, including lung infections that may stem from the immunosuppressive effects of nicotine (NT) in cigarette smoke (17). Linneberg et al. (16) reported that smoking was negatively associated with the incidence of allergic sensitization, which is consistent with another population-based study that concluded that cigarette smokers were less likely to develop allergic sensitization during an 8-y follow-up period (15). Several cross-sectional studies also reported a lower incidence of aeroallergen sensitization among current smokers than among never-smokers; even past smokers were less likely to be sensitized than never-smokers (18–22).

NT, the major constituent of cigarette smoke, suppresses adaptive and inflammatory immune responses (23–25); recently, we demonstrated that NT pretreatment attenuated some parameters of ragweed- and house dust mite-induced allergic asthma in Brown Norway rats by primarily suppressing leukocytic infiltration and the production of LTs and Th2 cytokines/chemokines, without affecting the allergen-induced hexosaminidase/histamine release in the lung (26). Thus, in addition to its effects on T cells (27) and macrophages (24), NT affects the mast cell function in the lung (26), and the presence of nicotinic acetylcholine receptors (nAChRs) on murine bone marrow-derived mast cells (28) and

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Abbreviations used in this paper: α-BTX, α-bungarotoxin; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; CON, control; cPLA₂, cytosolic phospholipase A₂; CS, cromoglycate sodium; cysLT, cysteinyl leukotriene; LT, leukotriene; LTC₄, leukotriene C₄; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NT, nicotine; qPCR, quantitative PCR; RBL cells, RBL-2H3 cells; siRNA, small interfering RNA.

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human skin mast cells was suggested (29). To understand the mechanism by which NT modulates mast cell function, we used the rat mast cell/basophil cell line RBL-2H3 (RBL cells) to show that NT in nanomolar quantities blocked the delayed phase of mast cell activation through $\alpha 7/\alpha 9/\alpha 10$ -nAChRs and inhibited the cytosolic phospholipase A₂ (cPLA₂)/MAPK pathway.

Materials and Methods

Reagents

RBL cells were obtained from Dr. Janet M. Oliver, University of New Mexico Health Science Center (Albuquerque, NM). The following reagents were used: anti-ERK1/2, anti-phosphor-ERK1/2, anti-Akt, and anti-phospho-Akt (all from Cell Signaling Technology, Beverly, MA); HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (both from Santa Cruz Biotechnology, Santa Cruz, CA); and cell culture reagents (Life Technologies, Grand Island, NY). The vendors for the other reagents are included with the relevant assays.

Cell culture

RBL cells were cultured at $1\text{--}2 \times 10^6$ cells in a 75-ml flask at 37°C in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin in a humidified 5% CO₂ atmosphere. Cells were passaged every 3–4 d.

NT/cromoglycate sodium/cotinine treatment

RBL cells were treated with various concentrations of NT (MP Biomedicals, Solon, OH) or its biologically inactive metabolite cotinine (Sigma-Aldrich, St. Louis, MO) or 1 μ M cromoglycate sodium (CS; Sigma-Aldrich) for a predetermined optimal time (8 h), followed by stimulation with 10 ng anti-Fc ϵ RI Ab at 37°C. Cells and supernatants were collected and subjected to various assays.

Leukotriene C₄ assay

Leukotriene C₄ (LTC₄) was determined as described previously (30). After various treatments of RBL cells, cell supernatants were collected, and the LTC₄ content was determined using an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI), according to the manufacturer's protocol.

Histamine and β -hexosaminidase release assays

The amount of histamine released was measured by enzyme immunoassay, according to the manufacturer's instructions (Cayman Chemical). The β -hexosaminidase activity was assayed as described previously (31). Briefly, RBL cells were plated (4×10^5 cells/well) in 12-well microtiter plates; after overnight incubation, cells were washed with PIPES solution (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM CaCl₂, 0.4 mM MgCl₂, 25 mM PIPES, and 0.1% BSA) and stimulated with anti-Fc ϵ RI for 30 min at 37°C with gentle rotation. The supernatants were collected, and 0.5% Triton X-100 solution was added to the cells to quantify the unreleased β -hexosaminidase. Samples (20 μ l) were transferred to a 96-well microtiter plate, and 50 μ l the substrate solution (p-nitrophenyl-N-acetyl- β -D-glucosamide) was added to each well. The plates were incubated at 37°C for 60 min under gentle rotation. After the addition of 150 μ l the stop solution (0.2 M glycine [pH 10]) to each well, absorbance was recorded at 405 nm. The results of cell degranulation were expressed as the percentage of degranulation in control samples.

Western blot analysis for ERK and Akt

Whole-cell lysates were prepared as described (32). Briefly, cells were lysed on ice with ice-cold 1% Brij 96-containing lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1 mM orthovanadate, 1 mM PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) for 15 min. After clarification at 12,000 rpm, supernatants were assayed for protein concentration by the bicinchoninic acid method. Samples (40 μ g protein) were resolved on Criterion Gels (Bio-Rad, Hercules, CA), and the resolved proteins were transferred onto a nitrocellulose membrane, blocked with 2% BSA (Sigma-Aldrich), and probed with the phosphor-ERK1/2 and phosphor-Akt Abs. The membranes were washed three times, incubated with HRP-conjugated goat anti-rabbit secondary Abs, developed with ECL (Amersham Biosciences, Buckinghamshire, U.K.), and exposed to x-ray film. The same blots were stripped with Restore Western Blot Stripping Buffer (Pierce,

Rockford, IL) for 15 min at room temperature, washed, and reprobed with ERK1/2, Akt, and β -actin Abs to verify equal loading on the gel.

cPLA₂ activity assay

The cPLA₂ activity from homogenized cell extracts was assayed by enzyme immunoassay (Cayman Chemical), according to the manufacturer's instructions.

Nuclear translocation of NF- κ B

Nuclear extracts were prepared from RBL cells, essentially as previously described (33). Briefly, RBL cells were lysed on ice using hypotonic cell lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES [pH 7.8], 1 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, 1 mM DTT) at 4°C for 10 min. The nuclear pellet was extracted with high-salt nuclear extraction buffer (500 mM KCl, 10% glycerol, 0.5% Nonidet P-40, 25 mM HEPES [pH 7.8], 1 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml aprotinin, and 100 μ M DTT), and the suspension was centrifuged to yield the nuclear extract. The protein content of the extracts was determined by the bicinchoninic acid method. For the NF- κ B assay, an aliquot of the nuclear extract containing 8 μ g protein was incubated with ³²P-end-labeled 22-mer double-stranded NF- κ B oligonucleotide (Promega, Madison, WI) with the NF- κ B consensus sequence: 5'-AGTTGAGGGGACTTTCCAGGC-3' and 3'-TCAACTCCCCGTGAAAGGGTCCG-5' for 30 min at 37°C. The resulting DNA-protein complex was resolved by electrophoresis on a 5% native polyacrylamide gel; after drying the gel, the radiolabeled bands were visualized by Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Assay for intracellular free calcium concentration

The assay for intracellular free Ca²⁺ concentration [Ca²⁺]_i of RBL cells was determined as described (34). Briefly, 5×10^6 cells were loaded with indo-1 AM (Molecular Probes, Eugene, OR) for 20 min. After washing, the cells were suspended in Ca²⁺-containing medium (10 mM HEPES [pH 7.4], 126 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM dextrose, 2 mM Ca²⁺, 1 mM Mg²⁺, and 5% FBS, adjusted to an osmolarity of 285). [Ca²⁺]_i was determined by spectrofluorometry (PTI Deltascan 4000, South Brunswick, NJ). Indo-1-loaded cells were treated with the indicated reagents and [Ca²⁺]_i calculated as previously described (35).

Treatment of RBL cells with α -bungarotoxin and methyllycaconitine

Methyllycaconitine (MLA) (Calbiochem, La Jolla, CA) and α -bungarotoxin (α -BTX) (Molecular Probes) are established antagonists of $\alpha 7$ -nAChRs; however, more recent data suggest that they also bind $\alpha 9$ subunits of nAChRs (36, 37). To test the effects of these antagonists on NT-mediated effects on mast cells prior to NT and/or anti-Fc ϵ RI treatment, RBL cells were incubated with 100 nM MLA or 40 nM α -BTX for 30 min at 37°C.

Flow cytometry for α -BTX binding

To determine the presence of $\alpha 7$ -like-nAChRs on RBL cells, we used flow cytometry to determine the binding of Alexa Fluor 488-conjugated α -BTX, as described for T cells (27). Briefly, RBL cells (5×10^5 cells/ml) were incubated with 100 nM Alexa Fluor 488-conjugated α -BTX for 1 h. After washing with cold PBS, the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Real-time quantitative PCR analysis

Total RNA was isolated from RBL cells, as described (26). Briefly, cells were lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH) and after the addition of B cell precursors (Molecular Research Center), the lysate was centrifuged at $13,000 \times g$ for 10 min at 4°C. The aqueous layer was collected, mixed with isopropanol and, after 1 h at -20°C , the precipitate was collected by centrifugation. The pellets were resuspended in 75% ethanol, centrifuged, and air-dried. The RNA samples were dissolved in diethyl pyrocarbonate-treated water at 55°C for 10 min and quantified spectrophotometrically. Quantitative PCR (qPCR) analysis was performed on the ABI PRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). Primer/probes were purchased from Applied Biosystems.

Identification of nAChR expression by PCR and sequencing

RNA from RBL cells was reverse transcribed using the SuperScript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA) using oligonucleotide (dT20) and random hexamers for priming. PCR was performed with cDNAs

generated by both priming methods using Platinum Taq polymerase (Invitrogen), according to the manufacturer's instructions. Magnesium concentration was adjusted to produce the best product yields, and the primer-annealing temperatures were determined using Oligo 4.0 (National Biosciences, Plymouth, MN). Primers were designed using Oligo 4.0 and the National Center for Biotechnology Information Primer Blast primer designing tool. Primers were synthesized by Invitrogen, and PCR products were generated using both sources of cDNA with similar results.

The following three sets of primers were used for PCR analysis of $\alpha 10$ -nAChR: primer 1 between bases 799–1575 (upstream: 5'-CCCTGTGTG-TTCATCTCCCT-3'; downstream: 5'-GGTTGTGAGGTCAGAAGCACT-3'), primer 2 between bases 799–1324 (upstream: 5'-CCCTGTGTGTTC-ATCTCCCT-3'; downstream: 5'-TAGCCAGACGCTTCCAATCT-3'), and primer 3 between bases 1305–1696 (upstream: 5'-AGATTGGAAGCGTC-TGGCTA-3'; downstream: 5'-TGCTCATCTGGCATTGAGTCTTA-3'). PCR analysis of $\alpha 9$ used two sets of primers: primer 1 between bases 146–654 (upstream: 5'-TCAGAGCCGTAGAGACAGCA-3'; downstream: 5'-CAG-GGCATTGAATATGTCCA-3') and primer 2 between bases 1148–1752 (upstream: 5'-TGGGTGAGAGCTGCCTTAGT-3'; downstream: 5'-CTCTC-TTCTCCAGCCCTT-3'). To determine $\alpha 7$ -nAChR expression, a set of primers was designed to amplify a product between 1007–1509 bases containing transmembrane domains TM3 and TM4 and transmembrane domain 4 loop (upstream: 5'-TGAAGTGGTGTGCATGGTTT-3'; downstream: 5'-TTAAGCAAAGTCTTTGGACACA-3').

The PCR products were separated on a 1.0% agarose gel and purified using the QIAEX II gel extraction kit (Qiagen, Valencia, CA). The purified products were cloned into pCRII-TOPO vector (Invitrogen), and the clones were sequenced using a 3730 DNA Analyzer (Applied Biosystems) at The Ohio State University Plant-Microbe Genomics Facility. DNA sequences were used for a basic local alignment search tool search versus the GenBank database (38). Additional sequence analysis was done using GeneWorks (Intelligenetics, Hilton Head, SC).

Knockdown of $\alpha 7$, $\alpha 9$, and $\alpha 10$ expression by small interfering RNA

Small interfering RNAs (siRNAs) used in this study were purchased from Thermo Scientific (Lafayette, CO). The siRNA SMARTpool contained four pooled siRNA duplexes with "UU" overhangs and a 5' phosphate on the antisense strand. Each siRNA was complexed with the DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Rockford, IL) for a final concentration of 100 nM per well in a 6-well microtiter plate, and the cells were transfected according to the manufacturer's instructions. Briefly, the siRNA-DharmaFECT 1 complex was added to the cells in complete medium and incubated at 37°C in 5% CO₂ for 48 h. Cell viability was assessed 24 h after transfection using a trypan blue exclusion assay. The mRNA levels of $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs were assessed 48 h posttransfection using QuantiTect SYBR Green One-Step RT-PCR. Oligonucleotide primers and the SYBR Green One-Step RT-PCR Kit were purchased from Qiagen.

Statistical analysis

We used GraphPad Prism Software 3.0 (GraphPad, San Diego, CA) to determine statistical significance by the Student *t* test or two-way ANOVA. Values were considered significant at $p \leq 0.05$.

Results

NT inhibits LT and cytokine production but not histamine and β -hexosaminidase release

Inflammatory substances released by mast cells induce and maintain the allergic response. Upon stimulation of Fc ϵ RI, the mast cells degranulate rapidly (in seconds), releasing the preformed chemical mediators, including histamine and β -hexosaminidase that play a critical role in the immediate-type allergic response (39). In contrast, Fc ϵ RI activation also induces de novo synthesis of LTs, such as LTC₄, and cytokines, such as TNF- α and IL-1 β , which initiate the late-phase allergic response (40, 41). To determine the effects of NT on the Fc ϵ RI-mediated mast cell responses, we incubated RBL cells with different concentrations of NT for the predetermined optimal time (8 h) prior to stimulation with anti- ϵ Abs to ligate Fc ϵ RI. Fig. 1A shows that release of the LT peptide LTC₄ at 30 min after Fc ϵ RI ligation (predetermined

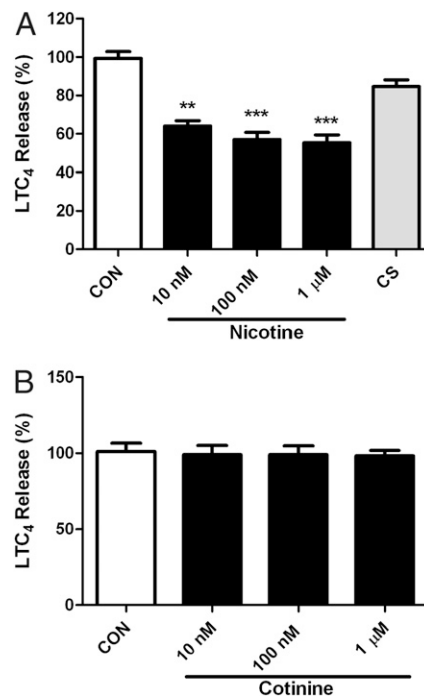


FIGURE 1. NT inhibits LT synthesis and secretion in RBL cells. RBL cells were treated with various concentrations of NT or 1 μ M CS (A) or cotinine (B) for 8 h, followed by stimulation with 10 ng of anti-Fc ϵ RI Ab. LTC₄ content in the supernatants was determined as described in *Materials and Methods*. Release of LTC₄ is expressed as the percentage of control RBL cells treated with anti-Fc ϵ RI alone. Data shown are the mean $\pm$ SE of four independent experiments. ** $p < 0.01$; *** $p < 0.001$.

optimal time for LTC₄ release) was significantly (40–60%) inhibited by an extremely low concentration of NT (10–100 nM). Under identical conditions, CS (Sigma-Aldrich), a mast cell stabilizer and an established antiasthma drug (42), inhibited the LTC₄ response by only 10–15% (Fig. 1A). Thus, NT is a relatively strong inhibitor of mast cell LT production. The effects of NT on the mast cell LT production are specific, and cotinine, the biologically inactive major metabolite of NT, did not affect LTC₄ production/release (Fig. 1B). Indeed, cotinine was ineffective, even at concentrations exceeding 1 μ M (0.1–1 mM), and the addition of cotinine to NT (1 μ M NT + 1 mM cotinine) did not significantly affect histamine release or the NT-induced inhibition of LTC₄ (data not shown). Moreover, the kinetics of the NT response indicated that LTC₄ production was not significantly affected until 8 h after NT treatment; it remained significantly depressed until 24 h after NT treatment (data not shown). Thus, the NT-induced suppression of LT production requires long-term exposure of mast cells to NT.

After Fc ϵ RI activation, in addition to LTs, proinflammatory cytokines, such as TNF- α and IL-1 β , are mostly synthesized de novo by mast cells (43). At 30 min postactivation, qPCR analysis indicated that pretreatment of RBL cells with NT (1 μ M) significantly inhibited the Fc ϵ RI-mediated mRNA expression of TNF- α and IL-1 β (Fig. 2A, 2B). Similarly, the concentration of released TNF- α in the culture medium at 2 h after anti-Fc ϵ RI Ab treatment was significantly reduced in NT-treated RBL cultures (Fig. 2C). These results suggest that, in addition to LTs, NT inhibits mRNA expression, as well as the production of the proinflammatory cytokine TNF- α by mast cells (i.e., both parameters of the late-phase response). However, under these conditions, the concentration of IL-1 β in the culture fluids was too low to clearly evaluate the effects of NT on the release of this cytokine.

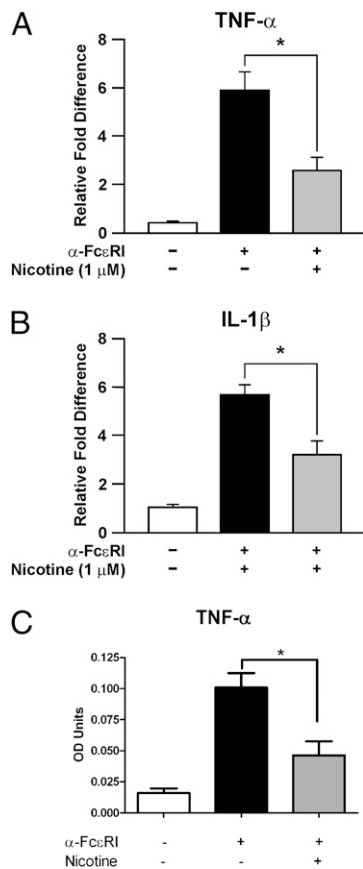


FIGURE 2. NT inhibits the expression of proinflammatory cytokines in RBL cells. Where indicated, RBL cells were pretreated with 1 μ M NT for 8 h, followed by stimulation with 10 ng of anti-Fc ϵ RI Ab. After 30 min, total RNA was isolated and analyzed for TNF- α (A) and IL-1 β (B) expression by qPCR. C, Two hours after anti-Fc ϵ RI stimulation, release of TNF- α was measured using ELISA. The results are the means $\pm$ SE of three independent experiments expressed as the fold change in the expression and release of these cytokines. * p < 0.05.

To ascertain the effects of NT on mast cell degranulation (characterized by the release of presynthesized compounds histamine and β -hexosaminidase), RBL cells were pretreated with various NT concentrations. β -hexosaminidase enzymatic activity and histamine release were measured in cell supernatants after activation by anti-Fc ϵ RI Ab. Results presented in Fig. 3 show that NT did not detectably affect the release of β -hexosaminidase or histamine, suggesting that NT does not significantly affect the acute-phase response of the mast cells.

Nicotinic receptors on RBL cells

Leukocytes, such as T cells and macrophages, have functional α 7-nAChRs (24, 27). There are reports that bone marrow-derived and skin mast cells express α 7-nAChRs (28, 29). To determine whether RBL cells expressed α 7-nAChRs, the cells were incubated with Alexa Fluor 488-conjugated α -BTX and analyzed by flow cytometry. Fig. 4A shows that essentially all RBL cells bind α -BTX, indicating the presence of α 7-nAChR-like receptors on RBL cells. However, in addition to α 7, α -BTX binds α 9- and α 10-nAChRs (36, 37); therefore, we examined the expression of α 7-, α 9-, and α 10-nAChRs on RBL cells by qPCR and sequence analysis. Data presented in Fig. 4B show that RBL cells express α 7-, α 9-, and α 10-nAChRs.

For α 7-nAChRs, the primer set yielded a clone covering 1007–1509, and the sequence of the amplified product matched exactly

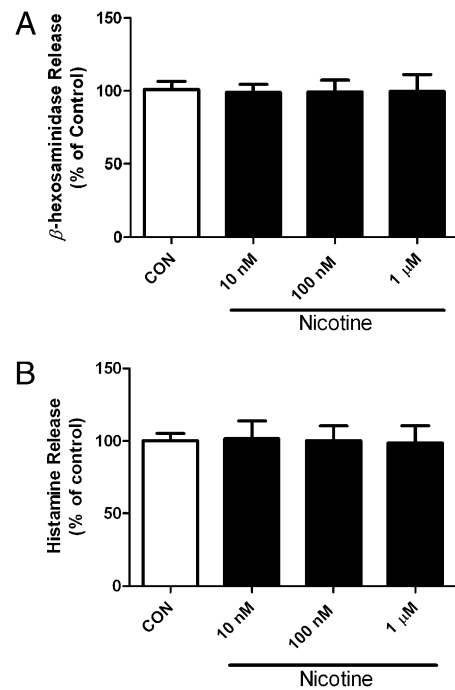


FIGURE 3. NT treatment does not affect degranulation in RBL cells. Cells were pretreated with various concentrations of NT for 8 h followed by stimulation with 10 ng of anti-Fc ϵ RI Ab for 20 min. Supernatants were analyzed for enzymatic activity of β -hexosaminidase (A) and histamine (B), as described in *Materials and Methods*. Data represent means $\pm$ SE for four independent experiments, each performed in triplicate.

the appropriate rat α 7-nAChR region. Unlike humans, the rat α 7 gene does not appear to be partially duplicated, which eliminated the need to ascertain the expression of a duplicated product encoding exons 5–10. For α 9-nAChR, six PCR clones, three each from the two primer sets covering sequences 146–654 and 1148–1752, were sequenced. The PCR products were identical to the corresponding regions of the rat α 9-nAChR cDNA. Of the six PCR clones sequenced for α 10-nAChR, one clone was derived from primer set 1, two clones were derived from primer set 2, and three clones were derived from primer set 3. The clones covered sequences from 799–1696, and the coding sequence ended at 1407. The sequences obtained from the PCR products were identical to the corresponding regions of the rat α 10-nAChR cDNA. Together, these results suggest that RBL cells express α 7-, α 9-, and α 10-nAChRs.

NT suppresses activation of NF- κ B, Akt, and ERK1/2

Activation of mast cells through Fc ϵ RI leads to activation of PI3K-dependent activation (phosphorylation) of Akt, activation of ERK1/2, and nuclear translocation of NF- κ B (44, 45); NF- κ B controls the expression of proinflammatory cytokine gene products in mast cells, including RBL cells (46). To determine whether NT affected the components of this pathway, we tested its effects (1 μ M) on the Fc ϵ RI-mediated activation of Akt, ERK1/2, and NF- κ B in RBL cells. Western blot analysis indicated that NT suppressed phosphorylation but not the total content of ERK1/2 (Fig. 5A) and Akt (Fig. 5B). These effects of NT were significantly detectable at 8 h but not at 2, 4, or 6 h after NT pretreatment (data not shown). Similarly, NT inhibited the activation (nuclear translocation) of NF- κ B (Fig. 5C) at 8 h but not at 2 h after incubation with NT. Thus, NT suppresses the PI3K/Akt/MAPK/NF- κ B signaling pathway, and these effects were discernable at $\sim$ 8 h after NT treatment. The inhibitory effects of NT on ERK

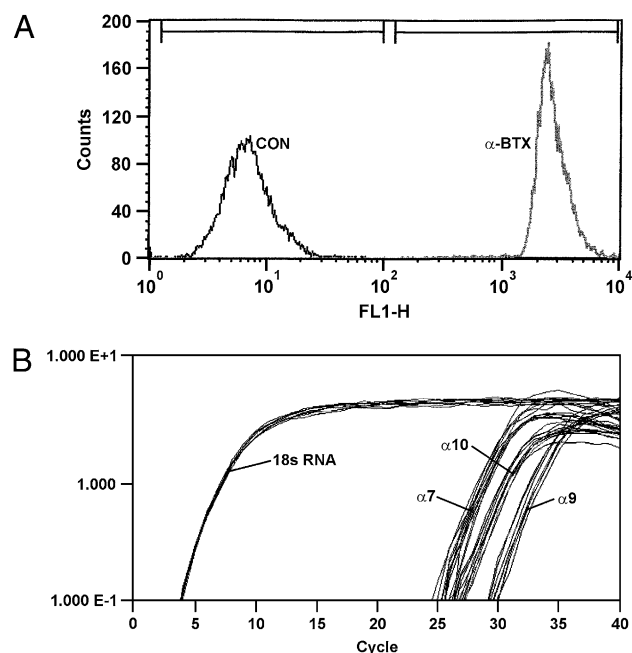


FIGURE 4. RBL cells bind α -BTX and express α 7-, α 9-, and α 10-nAChRs. **A**, RBL cells were incubated with Alexa Fluor 488-conjugated α -BTX and analyzed by flow cytometry, as described in *Materials and Methods*. After α -BTX treatment, there were no detectable α -BTX unbound cells, as seen by the lack of cells within the CON region of the graph. **B**, qPCR analysis for the expression of α 7-, α 9-, and α 10-nAChRs on RBL cells. Note that the expression of α 7-, α 9-, and α 10-nAChRs is approximately comparable. CON, control.

and Akt phosphorylation were also evident at 16 and 24 h after NT treatment (data not shown).

NT inhibits cPLA₂ activity

Mast cells and RBL cells contain phospholipase A₂ of low and high m.w. (cPLA₂) types (47); however, only cPLA₂ accounts for the anti-FcεRI-stimulated arachidonic acid production and is dependent on MAPK activation (48). Because the inhibitory effects of NT were correlated with suppression of ERK1/2 activity, we determined whether NT affected cPLA₂ activity in RBL cells. Results presented in Fig. 6 clearly show that NT-induced inhibition of ERK1/2 is also associated with the inhibition of cPLA₂ activity. Thus, it is likely that NT affects LT production by regulating ERK1/2-cPLA₂ activities.

Antagonists of α 7/ α 9 (α -BTX and MLA) suppress the effects of NT on FcεRI-induced ERK and Akt phosphorylation

In the absence of α 9, α 10-nAChR does not form a functional receptor (49). To ascertain whether the suppressive effects of NT on mast cells were mediated through the nAChRs expressed by RBL cells (i.e., α 7 and α 9/ α 10), prior to incubation with NT the cells were preincubated with the established antagonists of these receptors MLA or α -BTX (36). Western blots analysis suggested that α 7/ α 9-nAChR antagonists blocked the inhibitory effects of NT on Akt and ERK1/2 phosphorylation (Fig. 7). Thus, nAChRs α 7 and/or α 9/ α 10 are involved in mediating the effects of NT in the delayed phase of the mast cell response.

nAChRs α 7, α 9, and α 10 interact to mediate the effects of NT on mast cell function

Because antagonists that can specifically inhibit α 7- or α 9/ α 10-nAChRs are not readily available, we used siRNA to selectively knockdown α 7-, α 9-, and α 10-nAChRs on RBL cells. Depending

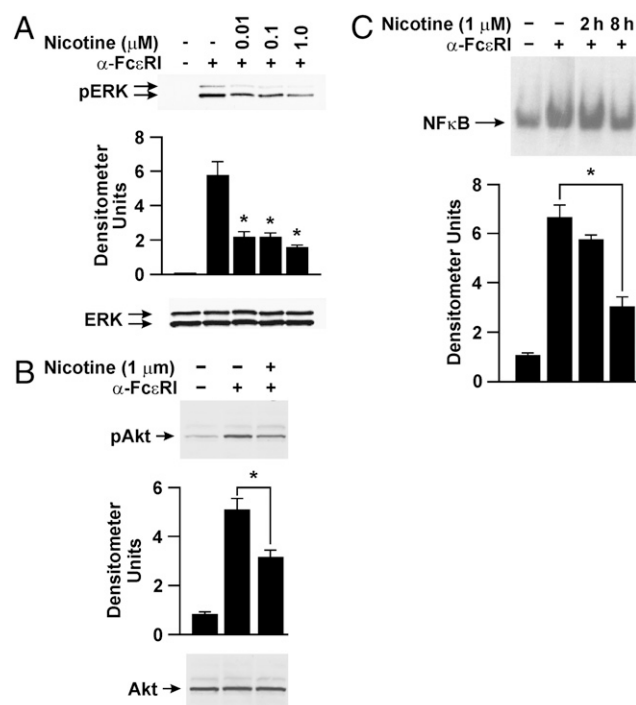


FIGURE 5. NT treatment suppresses the activation of ERK1/2, Akt, and NF- κ B in RBL cells. Cells were treated with the indicated concentrations of NT for 8 h, followed by stimulation with 10 ng of anti-FcεRI Ab for 20 min. Whole-cell lysates (40 μ g protein) were analyzed by Western blots, as described in *Materials and Methods*. Blots were probed by anti-phospho ERK1/2 (**A**) and anti-ERK1/2 anti-phospho Akt and anti-Akt (**B**). ERK1/2 and Akt were used as loading controls. Western blots shown are representative of six experiments. **C**, Nuclear extracts (8 μ g protein) from control and NT-treated RBL cells were incubated with ³²P-end-labeled NF- κ B oligonucleotide for 30 min at 37°C. The resulting DNA-protein complexes were analyzed by electrophoretic mobility gel shift assay, as described in *Materials and Methods*. Densitometry readings are representative of four to six independent experiments. * p < 0.05.

on the siRNA used, the treatment resulted in a nearly 70% reduction in α 7-, α 9- (Fig. 8A), or α 10-nAChRs mRNA (Fig. 9A), without significantly affecting the expression of the other mRNAs. Surprisingly, however, knocking down α 7-, α 9-, or α 10-nAChRs resulted in blocking the response of RBL cells to NT, as evidenced by the loss of the NT effect on anti-FcεRI-induced ERK1/2 phosphorylation (Figs. 8B, 9B). Thus, the lack of α 7, α 9, or α 10 expression abrogates the effects of NT on mast cells, sug-

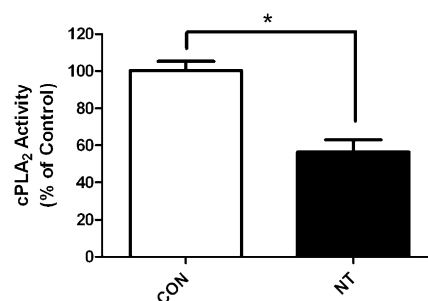


FIGURE 6. NT treatment inhibits cPLA₂ activation in RBL cells. Cells were treated with NT for 8 h, followed by stimulation with 10 ng of anti-FcεRI Ab for 20 min. cPLA₂ activity was measured by enzyme immunoassay, following the manufacturer's instructions. Data represent means $\pm$ SE of three independent experiments. * p < 0.05.

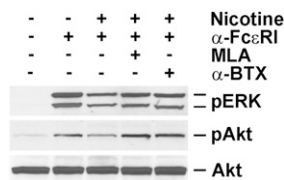


FIGURE 7. Antagonists of $\alpha 7$ - and $\alpha 9$ -nAChRs (α -BTX and MLA) attenuate the NT-induced inhibition of ERK and Akt. Prior to NT treatment, the cells were preincubated with 100 nM MLA or 40 nM α -BTX for 30 min. Cells were then stimulated with 10 ng of anti-Fc ϵ RI Ab for 20 min. Whole-cell extracts were fractionated on SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-phospho ERK1/2 and anti-phospho Akt Abs. Total ERK1/2 and Akt were used as loading controls. Western blots shown are representative of three experiments.

gesting that a function of nAChR on these cells might involve an interaction between $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs.

Role of Ca^{2+} in modulating NT response

A sustained increase in $[Ca^{2+}]_i$ is critical for degranulation and transcriptional regulation in mast cells and, as in TCR-mediated T cell activation, cross-linking of Fc ϵ RI increases the intracellular concentration of inositol 1,4,5-trisphosphate that releases Ca^{2+} from the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. The increased $[Ca^{2+}]_i$ triggers Ca^{2+} influx that stimulates mast cell degranulation (50). We observed that chronic exposure of animals to NT significantly reduced the TCR-mediated increase in $[Ca^{2+}]_i$ (34); therefore, it was possible that NT suppressed mast cell activation by inhibiting the Fc ϵ RI-mediated increase in $[Ca^{2+}]_i$. Results presented in Fig. 10 clearly show that increasing NT concentrations (10 nM–10 μ M) progressively decreased $[Ca^{2+}]_i$; however, the magnitude of LTC₄ inhibition by these NT concentrations was essentially identical (Fig. 1A). Moreover, none of these NT

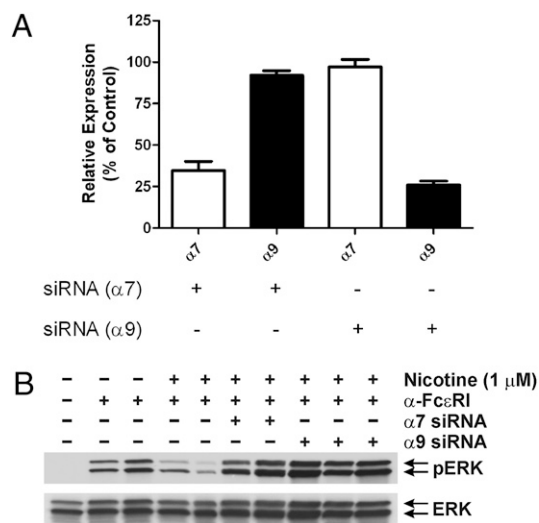


FIGURE 8. nAChRs $\alpha 7$ and $\alpha 9$ interact to mediate the effects of NT on mast cell function. **A**, Specific siRNAs selectively knockdown $\alpha 7$ - or $\alpha 9$ -nAChRs. RBL cells were treated with $\alpha 7$ - or $\alpha 9$ -specific siRNA, as described in *Materials and Methods*. At 48 h after siRNA treatment, $\alpha 7$ - and $\alpha 9$ -nAChR mRNA expression was assessed by qPCR. **B**, Knockdown of $\alpha 7$ - or $\alpha 9$ -nAChRs attenuates NT effects on RBL cell function. At 48 h after siRNA treatment, cells were incubated with 1 μ M NT and then stimulated with anti-Fc ϵ RI Ab. Whole-cell extracts were run on SDS-PAGE and probed with anti-phospho ERK1/2. Total ERK1/2 was used as loading control. The results are representative of three independent experiments.

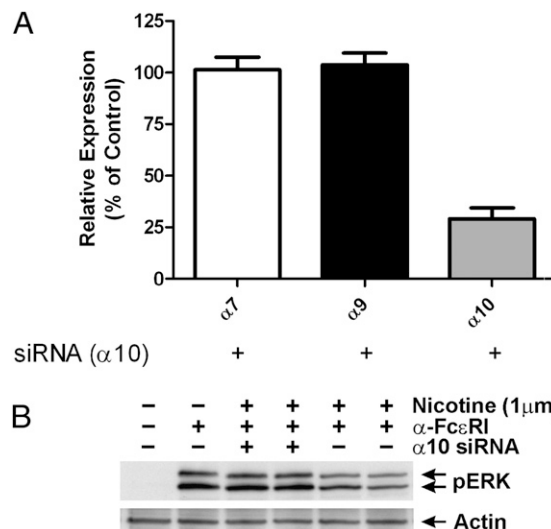


FIGURE 9. nAChRs $\alpha 7$, $\alpha 9$, and $\alpha 10$ interact to mediate the effects of NT in RBL cells. **A**, Specific siRNAs selectively knockdown $\alpha 10$ -nAChRs. RBL cells were treated with $\alpha 10$ -specific siRNA; 48 h after siRNA treatment, mRNA expression of $\alpha 7$ -, $\alpha 9$ -, and $\alpha 10$ -nAChRs was assessed by qPCR. **B**, Knockdown of $\alpha 10$ -nAChRs attenuates NT effect on RBL cells. At 48 h after siRNA treatment, cells were incubated with 1 μ M NT and then stimulated with anti-Fc ϵ RI Ab. Whole-cell extracts were run on SDS-PAGE and probed with anti-phospho ERK1/2. β -actin was used as loading control. Western blot shown is representative of three independent experiments.

concentrations affected the release of β -hexosaminidase or histamine (Fig. 3), which is believed to be very sensitive to $[Ca^{2+}]_i$. However, none of these NT concentrations totally blocked the α -Fc ϵ RI-induced increase in $[Ca^{2+}]_i$. Thus, ligation of Fc ϵ RI increases $[Ca^{2+}]_i$ in RBL cells that, even after partial inhibition by NT, is sufficient to promote degranulation and late-phase response. Therefore, it is unlikely that NT-induced suppression of the late-phase response is related to the reduction in the magnitude of increase in α -Fc ϵ RI-induced $[Ca^{2+}]_i$ in NT-treated mast cells.

Discussion

Mast cells play an important role in host defense, but they also mediate allergic and inflammatory diseases (51). Allergic diseases are less common in smokers than in non- or ex-smokers, and we previously reported that in animal models of allergic asthma, NT

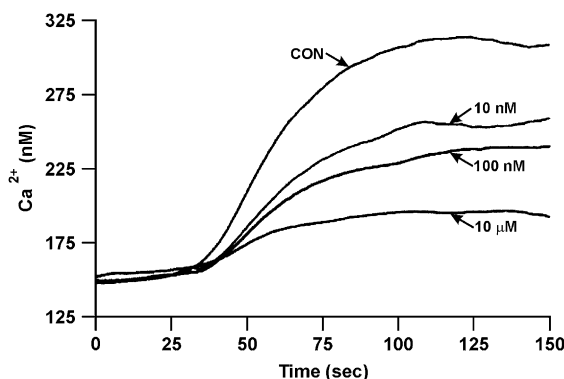


FIGURE 10. NT inhibits the increase in anti-Fc ϵ RI-induced $[Ca^{2+}]_i$. RBL cells were preincubated with the indicated concentrations of NT; after 8 h, cells were loaded with Indo-1, treated with anti-Fc ϵ RI Ab, and analyzed for changes in $[Ca^{2+}]_i$ by spectrofluorometry, as described in *Materials and Methods*. Results shown are representative of two independent experiments.

suppresses the allergen-induced production of LTC₄ and inflammatory cytokines without affecting the release of β -hexosaminidase/histamine in the lung (26). The experiments presented herein with RBL cells also showed that in response to cross-linking of the high-affinity Fc ϵ R1R, NT treatment does not affect the release of preformed compounds, such as β -hexosaminidase and histamine (degranulation). In contrast, the late-phase response, including the synthesis of LTC₄ and TNF- α , as well as the mRNA expression of TNF- α and IL-1 β , was significantly inhibited by NT treatment. This supports the recent reports that following Fc ϵ R1 activation in mast cells, the late-phase response (NF- κ B activation and cytokine production) is independent of degranulation (52, 53). These results suggest that NT affects the late, but not the early, degranulation phase of mast cell activation. Moreover, the response to NT was specific, and cotinine, the major metabolic product of NT, was essentially ineffective, even at concentrations that were 10–100-fold higher than NT used to inhibit the late-phase response. Cotinine was also ineffective in modulating the effects of NT on anti-Fc ϵ R1-induced ERK1/2 activation and LT production by RBL cells. Although the NT-induced inhibition of LTC₄ production varied between 40% and 60%, NT was far more effective than the established antiasthmatic drug CS (42) that optimally suppressed LTC₄ by only 10–15%. Thus, NT is a relatively strong suppressor of the late-phase response. A recent report (28) indicated that NT inhibited degranulation in bone marrow-derived mast cells. Although the reasons for this disparity in the NT response between RBL cells and bone marrow-derived mast cells are not clear at present, in addition to the difference in the type, the concentrations of NT required to detect the inhibition of degranulation exceeded 3.0 mM (28), a concentration that would be very difficult to achieve in live animals. In the present experiments, we used NT concentrations from 1 nM to 1 μ M that yielded effects on β -hexosaminidase and histamine release and the production of LTs akin to the *in vivo* effects observed after allergic sensitization of Brown Norway rats (26). Moreover, under these conditions, the plasma NT level of NT-treated rats is ~30–50 nM, which is comparable to the plasma NT levels of 1–2 pack per day human smokers.

The effects of NT on mast cell activation produced two surprising findings. First, the amount of NT required for optimal inhibition was extremely low, and significant inhibition was observed even at 1 nM of NT (data not shown). In contrast, the amount of NT required to elicit detectable action potential or whole-cell currents in α 7-nAChR-containing interneurons was ≥ 5 μ M (54), which is nearly 5000-fold greater than the amount of NT needed to inhibit the late-phase response of mast cells. As will be discussed later, the increased affinity of nAChRs for NT might reflect a complex interaction among three nicotinic receptor subunits on mast cells. Second, it took 8–16 h of preincubation with NT to detect inhibition of LTC₄ production in RBL cells, suggesting that the NT-induced suppression might represent the chronic effects of NT. Indeed, *in vivo*, the immunosuppressive effects of NT on allergic asthma require ≥ 2 wk of NT exposure (26, 55).

Because Fc ϵ R1-mediated mast cell activation is totally dependent on the increase in $[Ca^{2+}]_i$, it is conceivable that NT suppressed the Ca^{2+} response, thereby inhibiting the late-phase activation. Indeed, we observed that with increasing NT concentrations, the Fc ϵ R1-induced increase in $[Ca^{2+}]_i$ decreased progressively and did not affect degranulation. Moreover, there was no correlation among the concentration of NT, inhibition of $[Ca^{2+}]_i$, and the suppression of the late-phase response. It should be noted that because the concentrations of NT used in these experiments did not totally block the α -Fc ϵ R1-induced increase in $[Ca^{2+}]_i$, it is likely that the smaller increases in $[Ca^{2+}]_i$ in NT-treated cells were sufficient to activate

these cells. In fact, only modest increases in $[Ca^{2+}]_i$ are required for translocation of cPLA₂ into the membrane to initiate mast cell activation (56), and proper intracellular compartmentalization of Ca^{2+} is more critical than the overall increase in $[Ca^{2+}]_i$ (57).

The late-phase response of mast cells (i.e., production of cysLTs and proinflammatory cytokines/chemokines) requires PI3K/Akt/ERK/cPLA₂/NF- κ B activation (44–46, 48). We found that NT pretreatment inhibited cPLA₂ activity and phosphorylation of Akt and ERK1/2, but it did not affect the total content of Akt or ERK; in mast cells, ERK activation precedes cPLA₂ activation, NF- κ B activation, and production of LTC₄ and cytokines (48, 57, 58). Thus it is likely that NT affects the activity of kinases, such as Lyn and Fyn (59), or phosphatases, such as protein tyrosine phosphatase ϵ (60), which are upstream of PI3K/ERK. Upon activation of Fc ϵ R1, Lyn is quickly phosphorylated and activates Syk to stimulate degranulation in RBL cells (61, 62). The role of Lyn in mast cell degranulation is debatable (63, 64); however, there is evidence suggesting that it promotes degranulation but negatively regulates ERK activation and LTC₄ secretion in mast cells (65). Although Fyn is critical for cPLA₂ and MAPK activation (65), it might initiate complementary signals for mast cell degranulation in Lyn-deficient cells (32, 66). Thus, it is possible, but not proven, that NT suppresses Fyn, but not Lyn, activation to allow α -Fc ϵ R1-induced mast cell degranulation while suppressing the late-phase response. Interestingly, chronic NT treatment stimulates Fyn activity in rat T cells (S.P. Singh, N.C. Mishra, and M.L. Sopori, unpublished observations) and might reflect functional differences in the two cell types.

Neuronal nAChRs mediate synaptic transmission; however, nicotinic receptors are also present in peripheral cells, including the cells of the immune system (67, 68). Neuronal nAChRs are pentameric structures that function as ligand-gated ion channels (69). Nine neuronal nAChR α subunit genes (α 2– α 10) and three β subunits (β 2– β 4) have been identified; however, α 8 subunits are not found in mammals. Among the nicotinic receptors, α 7 is the most commonly found nAChR subunit on immune cells, and α 7 and α 9 can form functional homomeric nAChRs (69). The α 10-nAChR subunit is functional only in combination with the α 9 subunit (49); however, α 7 and α 10 colocalize in rat sympathetic neurons (70). We cloned α 7-nAChR from human and rat T cells and found it to interact with the T cell Ag receptor (27). Therefore, nAChRs may interact with other nAChRs or non-nAChRs to modulate cell function. Essentially all RBL cells bind α -BTX, and we identified the expression of α 7-, α 9-, and α 10-nAChR subunits by qPCR and sequence analysis of the PCR products. Moreover, the effects of NT on the late-phase response were inhibited by preincubation with α -BTX or MLA, and these agents were shown to inhibit the function of α 7- and α 9-nAChRs (36, 37). Thus, it is possible that α 7- or α 9/ α 10-nAChRs are involved in NT-induced changes in mast cell function. To differentiate between these possibilities, we used α 7-, α 9-, or α 10-specific siRNA to selectively knockdown α 7-, α 9-, or α 10-nAChRs from RBL cells. Indeed, the mRNA expression of siRNA-treated cells established the specificity of the knockdown method; however, to our surprise, ablation of α 7-, α 9-, or α 10-nAChRs by siRNA abrogated the response of RBL cells to NT. Therefore, it seems that on the mast cells, α 7, α 9, and α 10 subunits might interact to form a hybrid nAChR that strongly increases the affinity of this novel nAChR to NT by several orders of magnitude and may explain the nearly 5000-fold difference in the amount of NT required to activate α 7-nAChRs in neurons and the suppression of α -Fc ϵ R1-induced late-phase response in mast cells. This property of nAChRs on mast cells might be accessible to novel therapeutic approaches to control allergic diseases and allergic asthma.

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Disclosures

The authors have no financial conflicts of interest.

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