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IgE-induced degranulation of mucosal mast cells is negatively regulated via nicotinic acetylcholine receptors

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ABSTRACT

The autonomic nervous system is known to mediate mast cell activation. We investigated expression of nicotinic acetylcholine receptors (nAChRs) in mucosal-type mast cells and their contribution to the regulation of mast cell activation. Expression of mRNA of nAChR α4, α7, and β2 subunits were detected in specially differentiated mucosal-type murine bone marrow-derived mast cells (mBMMCs). Pretreatment with non-specific nAChRs agonists, acetylcholine, nicotine and epibatidine and a specific α7 subunit agonist GTS-21 significantly inhibited antigen-induced degranulation of mBMMCs in a dose-dependent manner and GTS-21-induced inhibition was significantly blocked by α7 subunit antagonist, α-bungarotoxin. Furthermore, confocal microscopy also demonstrated surface binding of α-bungarotoxin on mBMMCs. Our findings indicate that mucosal mast cell activation may be negatively regulated mainly through nAChR α7 subunit, suggesting that nAChRs are involved in neuronal-mucosal mast cell interactions.

Ulcerative colitis (UC), a form of inflammatory bowel disease, is characterized by contiguous inflammation of the colonic lamina propria. It has been suggested that T helper cell type 2 (Th2)-derived cytokines play a key role in UC pathogenesis. The mast cell count in UC patients is increased when compared with that in control subjects, and the mast cell count in inflamed tissues is greater than that in normal tissues, suggesting that mast cells play an important role in the development of this condition [1]. However, the exact mechanism of UC is still not clear, and specific and useful medicines for UC have not yet been established. It has been reported that up to 35% of patients with UC exhibit autonomic imbalance [2], which suggests that autonomic nervous system are considered to be implicated in the pathogenesis of the disease. Recently, we established a mouse model of UC by using a modification of a previously described method [3] and found that both chemical stimulation of vagus nerve or subcutaneous nicotine injection improved the colitis [4]. Previous reports also demonstrate that the relative risk of UC among cigarette smokers is lower when compared with that among non-smokers [5] and that the condition of patients with active UC improved when they were treated with transdermal nicotine [6].

Interestingly, we found that nicotine also had a therapeutic effect on a murine model of food allergy in which gastrointestinal symptoms and Th2-dominant responses were present [7]. In cases of both UC and food allergy, mucosal mast cells are believed to play a central role in gut hypersensitivity and inflammation. Because nicotine is an agonist of nicotinic acetylcholine receptors (nAChR), we hypothesized nAChR is a negative regulator of mucosal mast cell activation.

There are two distinct populations of mast cells, namely, mucosal mast cells and connective tissue mast cells. Recently, there has been considerable evidence demonstrating that mucosal mast cells are morphologically, biochemically, and functionally distinct from connective tissue mast cells. Previous reports have shown that these two types of cells have the different abilities upon stimulation with various secretagogues [8]. Mucosal mast cells are known to play a pivotal role in gastrointestinal hypersensitivity and protective responses against parasitic infections [9]. These results indicate that it is important to use mast cells of the mucosal type for examining the role of nAChRs on intestinal mast cells; such studies will contribute to elucidating the pathogenesis of gastrointestinal diseases.

Miller et al. reported that mBMMCs cultured with a combination of 4 cytokines, namely, stem cell factor (SCF), interleukin (IL)-3, IL-9, and transforming growth factor β1 (TGF-β1), were homologous to mouse mucosal mast cells based on the morphology and expression of mouse mast cell protease (mMCP)-1 and mMCP-2 [10]. In the present study, we investigated the effect of nAChRs agonists on mBMMCs grown with Miller's tetrad cytokine combination. We show that nAChRs are expressed in the mBMMCs and that nAChRs agonists have inhibitory effects on mBMMC degranulation. Our results suggest that nAChRs are involved in the negative regulation of mucosal mast cell activation.

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Materials and methods

Cell culture: We prepared mBMMCs from a 6-week-old male mouse (BALB/c) according to the method described previously [10]. Briefly, bone marrow cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS), 10 μM 2-mercaptoethanol (Wako, Osaka, Japan), 20 mM Hepes buffer (Sigma), 1 mM sodium pyruvate (Sigma), 100 μM MEM non-essential amino acids (Sigma), 2 μg/ml gentamicin solution (Sigma), 20 μl/ml penicillin–streptomycin solution stabilized (Sigma), 20 ng/ml recombinant murine interleukin-3 (IL-3; Peprotech, Rocky Hill, NJ), 40 ng/ml recombinant murine SCF (Peprotech), 5 ng/ml recombinant murine IL-9 (R&D, Minneapolis, MN) and 1 ng/ml TGF-β1 (Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. Mast cell purity was examined by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ), and more than 98% of the non-adherent cells were FcεRI- and c-kit-positive (data not shown).

Reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was isolated from 1×10^7 mBMMCs using Sepasol®-RNA I Super (Nacalai Tesque, Kyoto, Japan). After incubation with 2.5 U DNase I (Roche, Basel, Switzerland) and 50 U RNase inhibitor (Takara Bio, Shiga, Japan), 1.5 μg total RNA was subjected to cDNA synthesis in a 20-μl reaction volume using PrimeScript Reverse Transcriptase (Takara Bio) with oligo$(dT)_{15}$ primers. Reactions were performed in the presence or absence of reverse transcriptase. We used 2-μl cDNA aliquots as templates and amplified them using a PCR kit (AccuPower™ PCR Premix; BioNeer, Daejeon, Korea) with the following thermal cycling conditions: denaturation at 94 °C for 5 min, followed by 37 cycles of 30 s at 94 °C, 45 s at 60 °C, and 45 s at 68 °C, and a final extension at 72 °C for 10 min. A 422-bp fragment of the nAChR α3 subunit was amplified with primers 5′-CCTCATGACCTCCCAAAC AGCAT-3′ and 5′-TGAAATATGAGCCACAGTGAATTGC-3′. A 457-bp fragment of the α4 subunit was amplified with primers 5′-GTG GCTCCAACCACAAGAAATGC-3′ and 5′-TGATGGCTAGCCGAACTG GTCTC-3′. A 478-bp fragment of the α7 subunit was amplified with primers 5′-CCCAAACTTTGTGGAGGCTGTGT-3′ and 5′-AAGG GGCAGCTGTTGAAATGGAT-3′. A 466-bp fragment of the β2 subunit was amplified with primers 5′-CTGTGCCCCAAAACATGTCACTG-3′ and 5′-CCTCAGCACTTTCCCTGCTTCAA-3′. A 412-bp fragment of the β4 subunit was amplified with primers 5′-GCTCTCTGGGCTCCAGGTGTTTT-3′ and 5′-TCCAGGGGATCTAGTGCCCTTTC-3′. A 454-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified with primers 5′-GAAGGGCTCATGACCACAGTCCATG-3′ and 5′-T GTTGCTGTAGCCGTATTCATTGTC-3′. The PCR products were separated on 1.2% agarose gels stained with ethidium bromide. Total mouse brain RNA was used as a positive control for nAChR expression.

Degranulation assay: The degree of degranulation was assessed by measuring β-hexosaminidase release. mBMMCs were suspended at a density of 1.5×10^5 cells/ml in the medium and sensitized with 1.5 μg/ml mouse monoclonal anti-dinitrophenyl (DNP) IgE (Yamasa Corporation, Tokyo, Japan) for 6 h at 37 °C. The cells were washed and resuspended at a density of 6×10^5 cells/ml in 50 μl Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, and 0.1% BSA, pH 7.5) containing various concentrations of nAChR agonists. After 15 min, the cells were stimulated with 100 ng/ml DNP-bovine serum albumin (BSA) at 37 °C for 1 h. nAChR antagonists were allowed to remain in contact with the cells for 15 min before adding agonists. Samples were centrifuged and supernatants were collected. Cell pellets were solubilized with 0.5% Triton X-100 in Tyrode's buffer. The enzymatic activities of β-hexosaminidase in supernatants and cell lysates were measured using *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M sodium citrate (pH 4.5) at 37 °C for 1 h. The reaction was stopped by the addition of 0.2 M NaOH and 0.2 M glycine. Production of *p*-nitrophenol was detected by absorbance at 405 nm. The extent of degranulation was calculated by dividing *p*-nitrophenol absorbance in the supernatant by the sum of absorbance in the supernatant and cell lysate. Data are expressed as the means ± S.E. Statistical comparisons were made using a one-way ANOVA followed by a post hoc Dunnett's *t*-test for multiple comparisons. Probability values (P) of <0.05 were considered as statistically significant.

α-Bungarotoxin staining and confocal microscopy: Cells (10^6) mBmM Cs were washed with PBS containing 1% BSA and 0.2% NaN_3, stained with 5 μg fluorescein isothiocyanate labeled α-Bungarotoxin (FITC-α-Btx; Sigma) for 30 min on ice, washed three times, and mounted on slides. Confocal images were acquired using a DMI6000 confocal system (Leica Microsystems, Mannheim, Germany) controlled and analyzed by TCS-SP5 software (Leica Microsystems).

Reagents: Nicotine, ACh, epibatidine, mecamylamine and α-Btx were purchased from Sigma. GTS-21 was obtained from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Results

mBMMCs express nAChRs

To determine whether nAChRs were expressed in mBMMCs, mRNA was extracted from the mBMMCs, reverse-transcribed and amplified using mouse nAChR-specific primers (Fig. 1). Bands of the sizes predicted for the amplified DNA were obtained at around 500 bp for α4, α7, and β2. This result suggests that mBMMCs express the mRNAs of these three nAChR subunits constitutively.

nAChR agonists inhibit mBMMC degranulation

To examine the direct effects of nAChRs agonists on mucosal mast cells, mBMMCs were treated with serial dilutions of the agonists before being activated by the antigen DNP-BSA, and the extent of degranulation was assessed by β-hexosaminidase release (Fig. 2). The percentage of degranulation was significantly decreased by the treatment of $\geqslant 3.2$ mM nicotine. The toxicity of nicotine was tested by the dye exclusion test using propidium iodide (PI) performed by FACS analysis and 32 mM nicotine for 2 h was not cytotoxic for BMMC (data not shown). Epibatidine ($\geqslant 3.2$ mM), a potent nAChR agonist, and ACh ($\geqslant 10$ mM) also significantly inhibited mBMMC degranulation. We found that GTS-21, a specific agonist of the nAChR α7 subunit, markedly inhibited the DNP-BSA-induced degranulation ($\geqslant 100$ μM) and that GTS-21 was more effective than the other agonists.

Previous studies have demonstrated that long-term exposure to low concentration of nicotine upregulates its own receptors in some cell lines or in *Xenopus* oocytes injected with the mRNAs of nAChR subunits by a process that requires hours to weeks of chronic exposure depending on the nicotine concentration [11]. However, when mBMMCs were cultured in a medium containing 1 nM–10 μM nicotine for 72 h, no significant differences in nicotine sensitivity were detected between the nicotine-treated and untreated mBMMCs, suggesting that the expression level of nAChRs in mBMMCs was not altered by long-term nicotine exposure (data not shown).

Inhibitory effect of GTS-21 is partially blocked by -Btx

To investigate the effect of nAChR antagonists on the inhibitory effect of nicotine, mecamylamine (a non-specific nAChR antagonist but a weak antagonist of nAChR α7 subunit), and α-Btx (a potent and highly specific antagonist of nAChR α7 subunit) were added

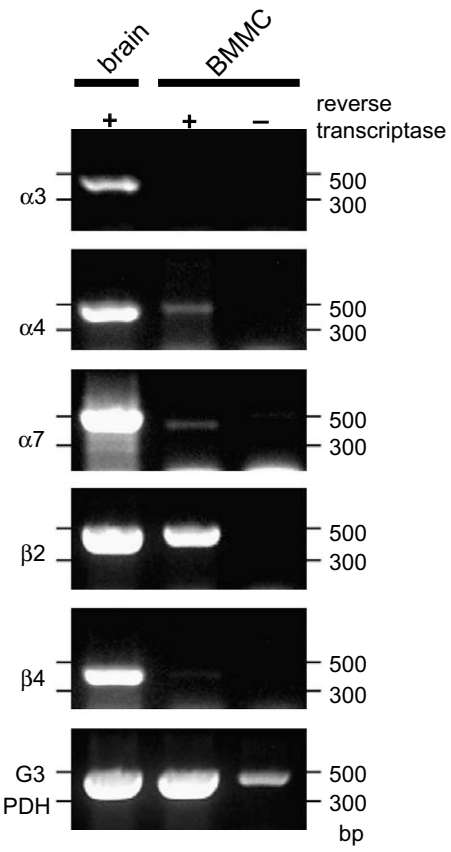

Fig. 1. Expression of nAChRs in mBMMCs. Total RNA from mBMMCs was extracted, subjected to cDNA synthesis with (+) or without (−) reverse transcriptase, and the expression of the α3, α4, α7, β2, and β4 subunits was detected by PCR using nAChR-specific primers. Primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and mouse brain RNA were used as positive controls.

to the cells before treatment with 100 μM GTS-21. 100 μM GTS-21 again significantly reduced the DNP-BSA-induced degranulation. α-Btx (100 μM) significantly diminished the GTS-21-evoked inhibitory effects on degranulation (Fig. 3, lane 4), whereas mecamylamine did not abrogate the inhibitory effects of GTS-21 (data not shown).

FITC-α-Btx binds on the surface of mBMMCs

To examine whether nAChR α7 subunits were indeed expressed on surface of mBMMCs, mBMMCs were labeled with FITC-α-Btx and viewed by fluorescent confocal microscopy. Binding of FITC-α-Btx was observed on the mBMMCs surface (Fig. 4). As previously reported in human mastocytoma cell line [12], bounded FITC-α-Btx was clustered on the surface of mast cells.

Discussion

The existence and role of nAChRs in mucosal mast cells have not yet been investigated. In this study, we found that mRNAs of α4, α7 and β2 nAChR subunits are expressed in the mBMMC. Furthermore, we found that nicotine (⩾3.2 mM) has an inhibitory effect on mBMMC degranulation. The response was mimicked by epibatidine, ACh, and GTS-21. Among these agonists, the agonist rank order potency was GTS-21 > epibatidine > nicotine > Ach. GTS-21, a nAChR α7 subunit agonist [13], has the strongest effect on mBMMCs, and ⩾100 μM GTS-21 significantly inhibits the degranulation. In addition, epibatidine, an agonist of nAChR α3, α4, and α7 subunits, is more potent than either nicotine or ACh [14]. These results indicate that nAChR agonists may affect mBMMC degranulation mainly through the nAChR α7 subunit. We also found that α-Btx, a nAChR α7 antagonist, significantly diminished the inhibitory effect of GTS-21, whereas mecamylamine did not. Confocal microscopy further demonstrated surface binding of α-Btx on mBMMCs. These results are reinforcing the possibility that

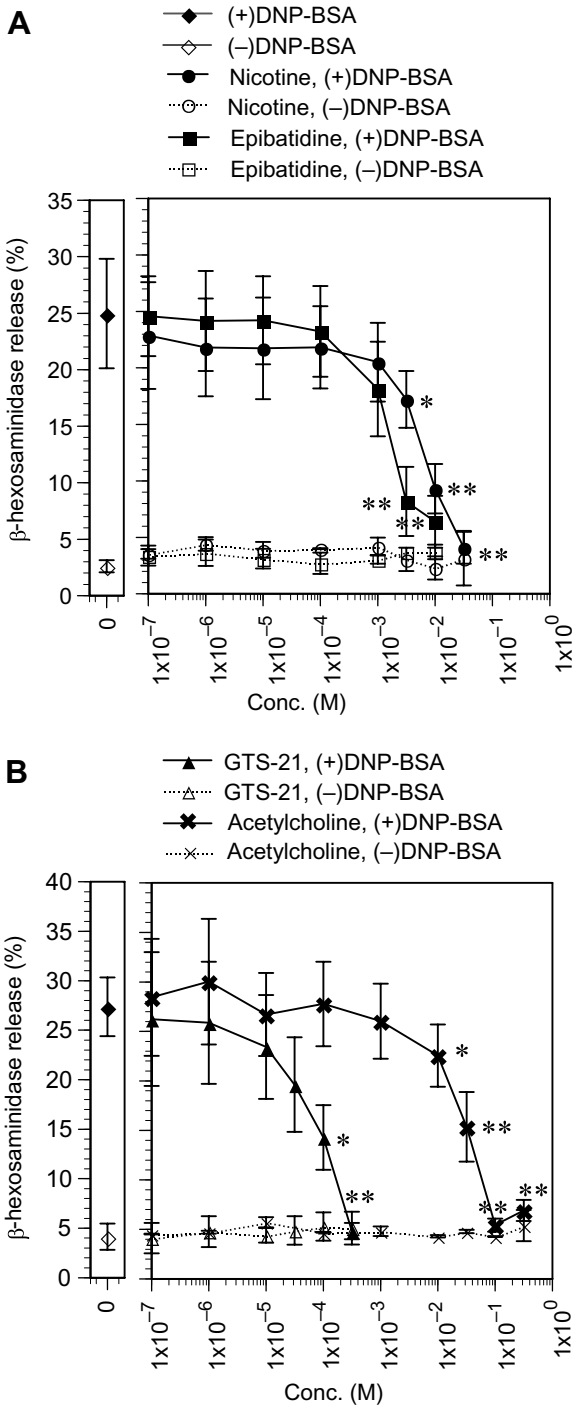

Fig. 2. Effect of nAChR agonists on mBMMC degranulation. mBMMCs were sensitized for 6 h with IgE anti-DNP, and nicotine (circles), epibatidine (squares) (A), GTS-21 (triangles), and ACh (crosses) (B) at concentrations noted were added 15 min before stimulation. Tyrode's buffer with (filled) or without (open) DNP-BSA was added to the treated cells, and β-hexosaminidase release was determined after 1 h. Each point represents the means ± S.D. values of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to cells treated with Tyrode's buffer instead of nicotine antagonists (filled diamond).

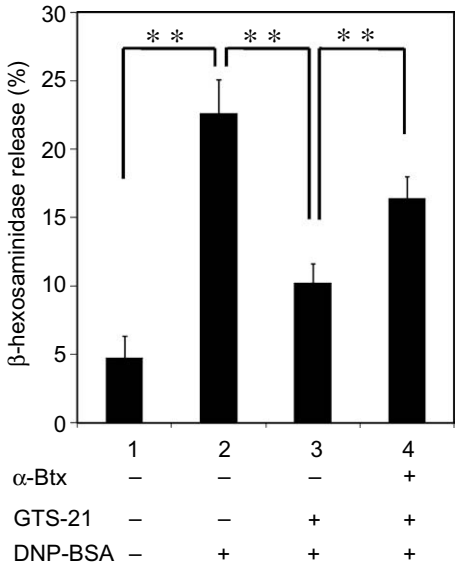

Fig. 3. Effect of α-Btx on the inhibitory effect of GTS-21. Sensitized mBMMCs were treated with (lane 4) or without (lane 1–3) 100 μM α-Btx for 15 min, then 100 μM GTS-21 (lane 3 and 4) or tyrode buffer (lane 1 and 2) was added for 15 min. The cells were activated by DNP-BSA (lane 2–4) for 1 h, and β-hexosaminidase release was examined. $^{**}P<0.01$

mucosal mast cells could be regulated by the cholinergic pathway mainly through nAChR α7 subunit.

Recent studies have indicated that nicotine has anti-inflammatory properties. The cholinergic anti-inflammatory pathway is a now well-established mechanism to control macrophage activation [15–17]. This so-called 'cholinergic anti-inflammatory pathway' is characterized by a nicotine dose-dependent decrease in the production of proinflammatory mediators, including tumor necrosis factor (TNF), IL-1β, IL-6 and IL-18 [18]. Here, we have shown that a nAChR agonist has anti-inflammatory effects on mast cells. Our results in mBMMCs were consistent with our *in vivo* data, as we found that an agonist of nAChR has a therapeutic effect on a murine model of UC and food allergy [4,7]

This study has demonstrated that nAChR agonists inhibit antigens-IgE-induced degranulation of mucosal mast cells. However, significance was only achieved at relatively high concentrations ($\geqslant$3.2 mM nicotine) compared to the nicotine concentration which is found in plasma of smokers (100 nM) [19]. There have been reports showing that high nicotine concentration (in mM) is required to observe the conspicuous effects on some type of cells. Thompson–Cree and coworkers reported that an IgE-induced histamine release of basophils is inhibited by high concentration (1×10^{-3} and 1×10^{-5} M) of nicotine [20]. In neutrophils, high concentration of nicotine (1.89 mM at EC_{50}) induces production of reactive oxygen intermediates and of IL-8 [21]. There are some possible explanations for the requirement of relatively high concentrations for nicotine effect. First, local concentration of nicotine is differed from systemic concentration. It has been reported that the concentrations in the tissues of the respiratory tract of smokers are as high as those in their saliva, in which nicotine concentrations reached mM levels [22,23]. Second, the sensitivity for nicotine is different between the cell types or their activation/differentiation status. Differentiated human macrophages or monocytic cell line, U937 cells, are significantly more sensitive to cholinergic agonists than peripheral blood mononuclear cells or neutrophils: nM–μM for the former and μM–mM for the latter [18,24,25]. Third, the effective concentrations of nicotine differ with the nAChR subtypes. The nicotine concentrations equivalent to those in smokers' blood are adequate to regulate the function of the α 4 β 2 subtype in the central nervous system (200–300 nM at EC_{50}). In contrast, the muscle-type AChR or α 3 AChR requires much higher doses (1 mM or higher) [26–28]. Such differences in the local and systemic concentrations of nicotine or in the sensitivity of the cell types and that of nAChR subtypes to nicotine may account for the high concentrations needed to inhibit the degranulation of mast cells *in vitro*.

Our results highlight the interactions between nerves and mucosal mast cells. Accumulating evidence has so far indicated that there is functional cross-talk between nerves and mast cells. Morphologically, mast cells are often located in close proximity to nerves. Functionally, both cell types communicate with each other in a bidirectional manner. Substance P released from nerves and proteases and cytokines from mast cells have proved to be

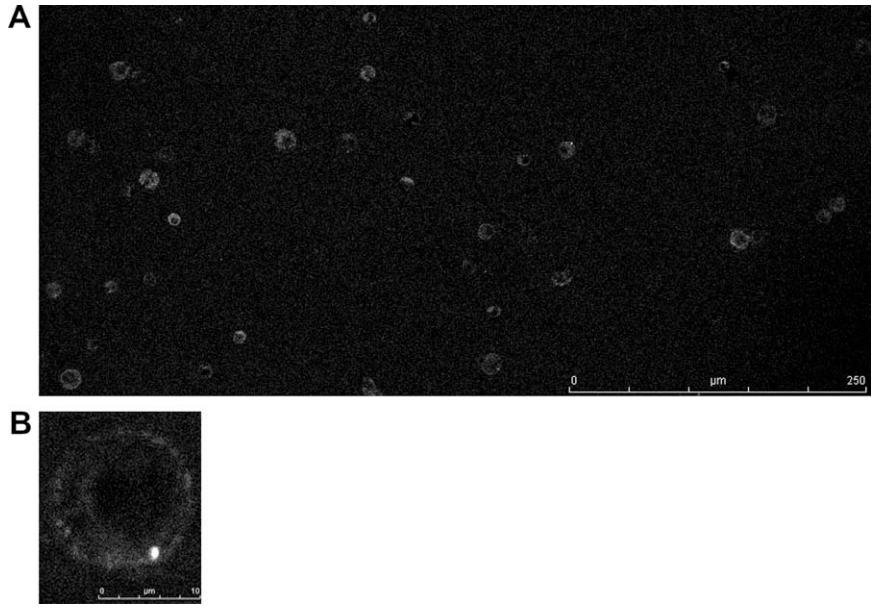

Fig. 4. Confocal microscope images of FITC-α-Btx on mBMMCs. mBMMCs were stained with FITC-α-Btx and viewed by fluorescent confocal microscopy. bars: (A), 250 μM; (B), 10 μm.

important mediators in such communications [29]. In this study, we, for the first time, have demonstrated that nAChR agonists inhibit mBMMC degranulation and that some nAChR subunits are expressed in these cells. The mutual interactions between nerves and mucosal mast cells through nAChRs may play an important role in the pathogenesis of several Th2-dominated cholinergic diseases such as UC and food allergy, and the presence of nicotine may have an influence on the response of mucosal mast cells in the gastrointestinal tract.

In conclusion, we have demonstrated that nAChR α 4, α 7, and β2 subunits are expressed in mBMMCs. Furthermore, we suggest a possible role of nicotine and ACh in the negative regulation of mucosal mast cell activation through nAChRs, particularly those containing the α7 subunit. The present results may provide a new insight into the mechanism of regulation of mucosal mast cell activation. This finding also could have therapeutic implications. Although the health risks associated with smoking are immense, specific α7 nAChR agonists might be a candidate for a new therapeutic drug for Th2-dominated allergic disorders in the gut.

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