Full Paper

Carbachol-Induced Secretion and Homologous Desensitization in Rat Basophilic Leukemia (RBL-2H3) Cells Transfected With Human m2 Muscarinic Acetylcholine ReceptorsKazuhiko Oishi^{1,*}, Akihiro Sakurai¹, Noriyasu Seki¹, Tomokazu Yoshizumi¹, and Masaatsu K. Uchida¹¹Department of Pharmacology, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo 204-8588, Japan

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Abstract. Carbachol (CCh) caused a dose-dependent release of β -hexosaminidase and an increase in the production of inositol 1,4,5-trisphosphate (IP₃) in RBL-2H3 cells transfected with m2 mAChR cDNA (RBL-m2 cells). The secretion was completely inhibited by LaCl₃ and pertussis toxin. The secretion was dependent on extracellular Ca²⁺ and mediated through the pertussis toxin-sensitive G protein. Exposing RBL-m2 cells to 100 μ M CCh for 30 min in Ca²⁺-free medium (desensitizing treatment) inhibited the secretion induced by the subsequent addition of 10 μ M CCh plus Ca²⁺, but not by stimulating the high affinity IgE receptor (Fc ϵ RI). Desensitizing treatment of RBL-m2 cells reduced the affinity of the lipophilic ligand [³H]quinuclidinyl benzilate to m2 mAChR without a reduction of the total m2 mAChR number. The treatment also decreased the cell surface mAChR number to 14% with a slight reduction in its affinity. Desensitizing treatment of RBL-m2 cells inhibited the CCh-induced transient increase in levels of IP₃ and intracellular Ca²⁺ concentration. The results suggested that the CCh-induced desensitization of m2 mAChR-mediated secretion is due to the receptor sequestration followed by blocking the increase in [Ca²⁺]_i and that this desensitizing mechanism is receptor-subtype-specific.

Keywords: m2 muscarinic receptor, desensitization, secretion, RBL-2H3 cell, transfection

Introduction

Most cells contain a set of receptor proteins that allows them to respond to each of the chemical signals that initiate or modulate each cell function. In responding to almost any type of stimulus, a cell reversibly adjusts its sensitivity to that stimulus. Desensitization, the phenomenon of the decay of cellular responses during persistent or repeated stimulation by agonists, is a fundamental regulatory mechanism for this mode of adaptation.

Molecular studies have revealed that the desensitization of G protein-coupled receptors might involve at least two distinct processes. One may be at the level of the receptor and/or receptor/G protein interaction that involves receptor phosphorylation by G protein-coupled receptor kinases or second messenger-dependent protein

kinases, which may regulate the coupling of the receptor to G proteins and receptor sequestration and down-regulation. The second process involves negative regulation at steps which are considerably downstream of G protein and mostly common to multiple types of receptor-mediated signaling. Although there has been much progress in the study of receptor desensitization and its associated intracellular signaling, little is known about the essential contribution of these desensitizing features to diminishing cellular responsiveness.

Rat basophilic leukemia (RBL-2H3) cells, which are analogues of mast cells, secrete inflammatory mediators such as histamine and β -hexosaminidase by means of the high affinity IgE receptor (Fc ϵ RI) aggregating in the plasma membrane. A Ca²⁺ requirement for regulated secretion has been identified in RBL-2H3 and many other secretory cells (1, 2). The aggregation of Fc ϵ RI results in a number of biochemical events through tyrosine phosphorylation. These include the activation of phospholipase C γ (1, 3), phospholipase D (4), and

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phospholipase A₂ (5), and phosphatidylinositol 3-kinase (6), processes which may lead to exocytosis. The muscarinic acetylcholine receptor (mAChR) family consists of five receptor subtypes; m1, m3, and m5, which are coupled to the activation of phospholipases C_β, and m2 and m4, which are coupled to the inhibition of adenylyl cyclase (7) and the activation of inward-rectifier potassium channels (8). We established RBL-2H3 cells stably transfected with cDNA encoding the human m3 (RBL-m3) mAChR. Therefore, they contain entirely homologous populations of m3 mAChR (9). Since transfected cells caused secretion in response to the mAChR agonist carbachol, a m3 mAChR-mediated short-term desensitization of secretory response was investigated to assess the essential contribution to diminishing cellular responsiveness. Using RBL-m3 cells, we identified a homologous desensitization of the secretory response (9). We found that RBL-m3 cells were desensitized without any modification of the m3 receptors and functional uncoupling between the receptor and phospholipase C. The desensitization of the m3 mAChR-mediated secretory response was supposed to occur considerably downstream of phospholipase C and that steps independent of the increased intracellular calcium participated in this phenomenon (9). These results indicated that the essential desensitizing features to diminishing cellular responsiveness could occur without any changes at the level of receptor and/or receptor/G protein interaction.

We transfected RBL-2H3 cells with the cDNA encoding human m2 mAChR, to test whether this desensitizing mechanism is receptor subtype-specific. Since, similar to other pertussis toxin-sensitive G protein-coupled receptors such as *N*-formylmethionyl-leucyl-phenylalanine (fMLP) and C5a chemoattractant receptors (10), the stimulation of expressed m2 mAChR caused many functional responses such as polyphosphoinositide hydrolysis, Ca²⁺ mobilization, and exocytosis, we examined the desensitization of m2 mAChR-mediated secretion. We demonstrated that desensitization of secretion occurs as a result of the receptor sequestration.

Materials and Methods

Transfection

The transfection proceeded as described (9). Either plasmid pEFm2 or pEFm3 was co-transfected into RBL-2H3 cells with pEF-neo, using the calcium phosphate method (11). Clones were selected in the presence of 600 μg/ml G418 for 2 weeks. Single colonies were isolated using cloning rings and propagated to generate continuous cell lines. Positive colonies were

identified by the binding of [³H]quinuclidinyl benzilate ([³H]QNB).

Cell culture

RBL-2H3 cells, provided by JCRB (Tokyo), and transfected colonies were maintained as monolayers in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum, 4 mM L-glutamate, 100 units/ml penicillin, and 100 mg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂. The cells were harvested with 0.125% trypsin plus 0.01% EDTA and recovered by centrifugation (250 × *g* for 5 min at 4°C). Cultures were replated overnight in 24-well culture plates to measure β-hexosaminidase release (10⁵ cells/well) and on glass coverslips of glass bottomed culture dishes (Meridian Instruments Corp., Okemos, MI, USA) for measuring the intracellular concentration of Ca²⁺ ([Ca²⁺]_i) and fluorescent images. Where appropriate, adherent RBL-2H3 cells were incubated for 2 h with dinitrophenol (DNP)-specific mouse monoclonal IgE (100 ng/ml) in the culture medium prior to experiments.

Desensitizing treatment and measurement of β-hexosaminidase

RBL-m2 cells grown in 24-well culture plates were washed three times and incubated in Ca²⁺-free Hepes-buffered Tyrode solution (HT) containing 100 μM carbachol for 30 min at 37°C (desensitization) as described (9). Thereafter, the cultures were washed and allowed to stand for 5 min. Ca²⁺-free HT was then replaced with HT containing 10 μM carbachol plus various concentrations of CaCl₂. The cells were incubated for 10 min at 37°C to induce secretion, which was assessed by measuring the release of β-hexosaminidase into the medium. HT consisted of 137 mM NaCl, 2.68 mM KCl, 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) (pH 7.4 at 37°C), 1 mM MgCl₂, 5.6 mM glucose, 0.03% bovine serum albumin (BSA), and the indicated concentrations of CaCl₂. Ca²⁺-free HT had the same composition except for the omission of CaCl₂. All solutions were equilibrated to 37°C before addition to the cultures. The release of β-hexosaminidase was assayed as described (12). In brief, aliquots (250 μl) of the incubation medium were incubated with 250 μl of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 30 min. Thereafter, 500 μl of 1 M Tris was added to quench the reactions and the absorbance was measured at 410 nm. The data are presented as a percentage of the total radioactivity, determined by lysing an aliquot of unstimulated cells with 0.2 % Triton X-100. Background release (in the absence of secretagogues)

was less than 4% in all experiments and was not subtracted from the values shown. All determinations were carried out in duplicate, and experiments were repeated on at least three occasions, with similar results.

Radioligand binding experiments

The binding activity of [^3H]-radiolabeled ligand was measured as described by Tobin et al. (13). After incubation with carbachol, cultures were washed with 1 ml of ice-cold Ca^{2+} - and BSA-free HT (binding medium) per 1 well for three times and incubated with various concentration (0.05 – 1.0 nM) of [^3H]QNB or [^3H]N-methylscopolamine ([^3H]NMS) in binding medium (1 ml/well) at 4°C for 16 h. Cells were then washed with 1 ml of ice-cold binding medium per 1 well for three times. Cells were dissolved in 0.1 N NaOH, and the radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 5 μM atropine. Ca^{2+} - and BSA-free HT (binding medium) had the same composition except for the omission of CaCl_2 and BSA. Protein was determined by the method of Lowry et al. (14).

Fluorescence microscopy

The cells on glass coverslips of glass bottomed culture dishes were washed three times and incubated with Ca^{2+} -free HT. The medium was then replaced with Ca^{2+} -free HT containing 10 μM carbachol. The cells were incubated for various periods at 37°C and then fixed with 2% paraformaldehyde containing 0.02% saponin in PBS for 30 min, rinsed in PBS, and incubated for 30 min in 0.05% BSA with 20 U/ml rhodamine-phalloidin. After washing, the coverslips were mounted in 90% (v/v) glycerol in 50 mM sodium carbonate buffer (pH 9.5). Cells were examined using a confocal laser scanning microscope (LSM GB-200; Olympus Corp., Tokyo) with an argon ion laser capable of excitation at 510 nm.

Measurement of inositol 1,4,5-trisphosphate

RBL-m2 cells were harvested and washed three times with Ca^{2+} -free HT. Cell suspensions (10^6 cells/0.2 ml) were then incubated in Ca^{2+} -free HT containing 100 μM carbachol for 30 min at 37°C. After desensitization, the cells were washed and the Ca^{2+} -free HT was replaced with HT containing 10 μM carbachol plus 1 mM CaCl_2 . After an incubation at 37°C for the indicated periods, the reaction was terminated by adding 100 μl of 10% perchloric acid. Samples were titrated to pH 7.4 with 1.54 M KOH/75 mM Hepes and then centrifuged to remove precipitated KClO_4 . The inositol 1,4,5-trisphosphate (IP_3) content in 100 μl of the resultant supernatant was determined using the IP_3 assay kit.

[Ca^{2+}] $_i$ measurements of single cells

RBL-m2 cells were trypsinized and replated overnight on glass coverslips in glass bottomed culture dishes (Meridian Instruments). The cells were loaded for 1 h at 37°C in Eagle's essential medium with 5 μM fura-2 AM in a humidified atmosphere containing 5% CO_2 . The cells were washed and incubated in Ca^{2+} -free HT containing 100 μM carbachol for 30 min and 5% CO_2 . The dishes were placed in a heating chamber on an Olympus microscope and maintained at 37°C for the duration of the experiment. The 340 nm/380 nm ratio with emission at 510 nm was recorded every 0.2 s as an index of [Ca^{2+}] $_i$ using a calcium photometry system (OSP-10CA, Olympus) and processed using OSP-SFCA software (Olympus). Calibrated calcium values were determined using the ratiometric analysis as described (15).

Data analysis

Values were means $\pm$ S.E.M. for the number of separate experiments performed. Statistical significance was evaluated by Student's paired *t*-test.

Drugs

Plasmid pEFm2 was a generous gift from Dr. T. Haga (Tokyo University, Tokyo). The IP_3 assay kit was purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). [^3H]QNB and [^3H]NMS were from New England Nuclear (Boston, MA, USA). Geneticin (G418) was from Gibco BRL (Gaithersburg, MD, USA). *p*-Nitrophenyl-*N*-acetyl- β -D-glucosaminide and dinitrophenol (30 – 40 mol DNP/mol human serum albumin)-conjugated human serum albumin (DNPs-HSA) were from Sigma (St. Louis, MO, USA). Hepes was from Dojindo Laboratories (Kumamoto). Rhodamine-phalloidin was from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were of reagent grade.

Results

Transfection and secretory responses to agonist

RBL-2H3 cells were stably transfected with cDNA encoding the human m2 mAChR (RBL-m2 cells). Ligand binding characteristics of expressed m2 mAChR were studied in intact cells using [^3H]QNB. A Scatchard plot of the specific binding of [^3H]QNB to the RBL-m2 subclones gave a straight line. The RBL-m2 subclone used here expressed mAChR levels with a B_{max} (the mean values for the dissociation constant) of 1.19 ± 0.06 pmol/mg protein and a K_d (the mean binding sites) of 0.20 ± 0.01 nM (Table 1). Prior to transfection, RBL-2H3 cells displayed no muscarinic receptor binding to carbachol.

Table 1. The characteristics of [3 H]quinuclidinyl benzilate ([3 H]QNB) and [3 H]*N*-methylscopolamine ([3 H]NMS) binding to control and desensitized RBL-m2 cells

Cells	[3 H]QNB binding			[3 H]NMS binding		
	K_d (nM)	B_{max} (pmol/mg protein)	r	K_d (nM)	B_{max} (pmol/mg protein)	r
Control	0.20 ± 0.01	1.19 ± 0.06	0.98 ± 0.01	0.13 ± 0.01	0.43 ± 0.03	0.99 ± 0.01
Desensitized	$0.48 \pm 0.06^*$	1.14 ± 0.07	0.94 ± 0.03	$0.17 \pm 0.01^*$	$0.06 \pm 0.01^{***}$	0.97 ± 0.01

Each value represents the means $\pm$ S.E.M. of the results from three independent experiments. *Significantly different from the values for control cells ($P < 0.05$) by Student's *t*-test for paired data; *** $P < 0.005$. K_d , dissociation constant; B_{max} , maximum number of binding sites; r , relative coefficient

Carbachol produced a dose-dependent release of β -hexosaminidase from RBL-m2 cells (Fig. 1a). The EC_{50} value for secretion was 2.3 and 100 μ M carbachol gave maximal secretion from spontaneous levels to 23% (Fig. 1B). The secretion was completely inhibited by 10 nM atropine, 30 ng/ml islet-activating protein (IAP) as a pertussis toxin, and 1 mM $LaCl_3$ (Fig. 2), indicating that m2 mAChR-mediated secretion was dependent on extracellular Ca^{2+} and mediated through a pertussis toxin-sensitive G protein pathway. Carbachol induced an increase in the production of IP_3 (Table 2), indicating the m2-mAChR-mediated activation of phospholipase C. In contrast to these observations, RBL-2H3 and RBL-neo (RBL-2H3 cells transfected with plasmid pEF-neo alone) cells, which do not express mAChRs on their cell surface, responded to DNPs-HSA as an antigen but not to carbachol (Fig. 1A).

We studied the reorganization of actin filaments during desensitization by confocal fluorescence microscopy. Carbachol, a potential secretagogue for RBL-m3 cells (9), caused a remarkable transformation in the dorsal cell surface topography. Incubation of RBL-m3 cells with carbachol in Ca^{2+} -free medium caused a membrane ruffling that formed a highly folded or plicated cell surface (Fig. 3), whereas incubation of RBL-m2 cells with 100 μ M carbachol did not cause membrane ruffling and the cells maintained a finely microvillous surface (Fig. 3). These data suggested that m2 mAChR was not linked to the signal pathway leading to membrane ruffling that was involved in RBL-m3 cells.

Homologous desensitization of secretion

RBL-m2 cells were desensitized by an incubation with 100 μ M carbachol for 30 min in Ca^{2+} -free medium. The cells did not secrete during this period. After the cells were washed with the same medium and allowed to stand for 5 min, they were incubated with 10 μ M carbachol plus various concentrations of $CaCl_2$ for 10 min to induce secretion. As shown in Fig. 4A, 10 μ M

carbachol induced β -hexosaminidase release in a $CaCl_2$ concentration-dependent manner. The release was maximal at 0.1 mM $CaCl_2$. Exposing RBL-m2 cells to 100 μ M carbachol completely inhibited the secretion induced by the subsequent addition of 10 μ M carbachol plus $CaCl_2$. At 0.1 mM $CaCl_2$, $17.1 \pm 0.5\%$ of β -hexosaminidase released in the control was decreased to $3.5 \pm 0.9\%$ by desensitization and at 1 mM $CaCl_2$, from $16.7 \pm 1.4\%$ to $4.5 \pm 0.1\%$. However, the release induced by 20 ng/ml DNPs-HSA was not influenced by desensitization (Fig. 4B), indicating a homologous desensitization of the secretory response.

A Scatchard plot of the specific binding of [3 H]QNB to RBL-m2 cells gave a straight line, and K_d and B_{max} were 0.20 ± 0.01 nM and 1.19 ± 0.06 pmol/mg protein, respectively (Table 1). On the other hand, [3 H]QNB bound to the desensitized cells with a K_d and B_{max} of 0.48 ± 0.06 nM and 1.14 ± 0.07 pmol/mg protein, respectively (Table 1). These results indicated that the K_d value increased, whereas the B_{max} value was not significantly different from those estimated in the control cells, and suggested that the affinity of [3 H]QNB to m2 mAChR was reduced upon a 30 min incubation with carbachol without a reduction of the total m2 mAChR number.

A hydrophilic ligand [3 H]NMS which is known to bind exclusively to mAChRs were used to determine changes in cell surface mAChRs. A Scatchard plot of the specific binding of [3 H]NMS gave a straight line, and K_d and B_{max} were 0.13 ± 0.01 nM and 0.43 ± 0.03 pmol/mg protein respectively (Table 1). On the other hand, [3 H]NMS bound to the desensitized cells with a K_d and B_{max} of 0.17 ± 0.01 nM and 0.06 ± 0.01 pmol/mg protein, respectively (Table 1). These results suggested that approximately 86% of the cell surface m2 mAChR was lost upon a 30-min exposure of RBL-m2 cells to carbachol with a slight reduction in affinity.

Fig. 1. The release of β -hexosaminidase from RBL-m2 cells in response to carbachol. A: RBL-2H3, RBL-neo, and RBL-m2 cells were sensitized by incubating for 2 h at 37°C in Eagle's essential medium with dinitrophenol (DNP)-specific mouse monoclonal IgE (100 ng/ml) in a humidified atmosphere containing 5% CO₂. The cells were washed and incubated in HEPES-buffered Tyrode solution (HT) containing 1 mM CaCl₂ plus 100 μ M carbachol (solid columns), 20 ng/ml DNPs-HSA (hatched columns), or vehicle (open columns) for 20 min at 37°C to induce secretion. The data are expressed as percentage of total cellular content. Columns and bars represent means $\pm$ S.E.M. from three independent experiments. B: RBL-m2 cells were incubated in HT containing 1 mM CaCl₂ plus various concentrations of carbachol for 20 min at 37°C to induce β -hexosaminidase release. The data are expressed as a percentage of the total cellular content. Points and vertical bars represent means $\pm$ S.E.M. from three independent experiments.

Desensitization of both phosphoinositide and Ca²⁺ signaling

Measurements using fura-2 revealed that carbachol increased [Ca²⁺]_i in RBL-m2 cells in a biphasic manner. A transient [Ca²⁺]_i peak was followed by a rather sustained phase (Fig. 5). Desensitizing these cells

completely blocked the elevation of [Ca²⁺]_i induced by carbachol (Fig. 5). This suggested that a change in the elevation of [Ca²⁺]_i is involved in the desensitization of secretion in RBL-m2 cells. Carbachol caused a transient increase in the levels of IP₃ in RBL-m2 cells. Adding 10 μ M carbachol to RBL-m2 cells resulted in a rapid elevation of mass IP₃, from basal levels of 0.43 ± 0.51 to 5.56 ± 1.12 pmol/10⁶ cells within 10 s, followed by a decrease in the basal levels within 30 s. A 30 min exposure of RBL-m2 cells to carbachol followed by a wash period inhibited the carbachol-induced transient increase in the levels of IP₃ (Table 2).

Discussion

The present study demonstrated that the ligation of expressed m2 mAChR stimulates many functional responses such as polyphosphoinositide hydrolysis, Ca²⁺ mobilization, and exocytosis. We also found that m2 mAChR-mediated secretion is sensitive to pertussis toxin. These findings suggested that the secretion was mediated through the pertussis toxin-sensitive G protein. m2 mAChR activates phospholipase C β through a pertussis toxin-sensitive G protein pathway similar to α_2 -adrenergic, metabotropic glutamate, fMLP, C5a, and other G protein-linked receptors (10, 16, 17). Moreover, Ali et al. (10) have shown that ligation of fMLP and C5a receptors stably transfected in RBL-2H3 cells can induce exocytosis and require the pertussis toxin-sensitive G protein activation of phospholipase C as well as Ca²⁺ mobilization. These results suggested that m2 mAChR-mediated secretion is mediated through the pertussis toxin-sensitive G protein activation of phospholipase C as well as Ca²⁺ mobilization.

The maximal secretion at 100 μ M carbachol was 23% of the total, which was over twofold lower than m3 mAChR-mediated secretion. The possibility that the lower level of secretion is due to the expression of fewer receptors could be excluded because all of nine subclones with various numbers of expressed receptors obtained in this study caused secretion to a similar extent (data not shown). It is therefore conceivable that the lower level of secretion in RBL-m2 cells is due to a different signaling pathway between m2 and m3 mAChRs. Cytochalasin B, an actin-binding fungal metabolite, augments many chemoattractant-induced responses of polymorphonuclear leukocytes, including calcium influx, aggregation, superoxide generation, and exocytosis (18). Ali et al. (10) have shown that cytochalasin B also augments fMLP and C5a receptor-mediated secretion from RBL-2H3 cells transfected with the fMLP and C5a receptors. Therefore, the lower secretion level may be due to the lack of activation of

Table 2. Peak agonist changes in the IP₃ mass 10 s after carbachol addition in control and desensitized RBL-m2 cells

Cells	IP ₃ (pmol/10 ⁶ cells)
Unstimulated	0.43 ± 0.51
Control	5.56 ± 1.12
Desensitized	2.04 ± 0.69*

RBL-m2 cell suspensions (10⁶ cells/0.2 ml) were incubated in Ca²⁺-free HEPES-buffered Tyrode solution (HT) containing 100 μM carbachol (desensitized) or vehicle (control) for 30 min at 37°C. After desensitization, the cells were washed and allowed to stand for 5 min. Ca²⁺-free HT was replaced with HT containing 10 μM carbachol plus 1 mM CaCl₂ and incubated at 37°C for 10 s. IP₃ mass of unstimulated RBL-m2 cells was also measured. Each value represents the means ± S.E.M. of the results from three independent experiments. *Significantly different from the values for control cells ($P < 0.05$) by Student's *t*-test for paired data.

the cytochalasin-sensitive component of signaling.

Cytoskeletal actin reorganization is an early response to many extracellular signals. In secretory cells, such a change in the organization of the actin network has been suggested to play a role in granule migration and in regulating the access of secretory granules to the plasma membrane (19, 20). The disassembly of F-actin in the cortical region and the appearance of actin filaments in the interior of cells is a necessary preliminary step for exocytosis in mast cells (21, 22). Moreover, a heterotrimeric G protein has been suggested to release actin filaments from the cortex, which are then trapped within the cell by a mechanism dependent on the small G protein Rac. Another small G protein, Rho, mobilizes

Fig. 2. Inhibition of the carbachol-induced β -hexosaminidase release by atropine, LaCl₃, and islet-activating protein (IAP) in RBL-m2 cells. RBL-m2 cells were incubated with various concentrations of atropine (A) or LaCl₃ (B) for 10 min at 37°C in HEPES-buffered Tyrode solution (HT) containing various concentrations of CaCl₂ and challenged with carbachol (final concentration of 10 μM) to induce β -hexosaminidase release. The data are expressed as a percentage of the total cellular content. In the case of IAP (C), the cells were incubated for 2 h at 37°C in Eagle's essential medium with various concentrations of IAP and dinitrophenol (DNP)-specific mouse monoclonal IgE (100 ng/ml), if necessary, in a humidified atmosphere containing 5% CO₂. The cells were washed and incubated in HT containing various concentrations of CaCl₂ plus 100 μM carbachol (filled circles) or 20 ng/ml DNPs-HSA (unfilled circles) for 20 min at 37°C to induce β -hexosaminidase release. The data are expressed as a percentage of the control release. The percentages of β -hexosaminidase release induced by 10 μM carbachol and 20 ng/ml DNPs-HSA were 15.1 ± 1.9% and 44.5 ± 3.1%, respectively. Points and vertical bars represent means ± S.E.M. from three independent experiments. *Significantly different from the values for control cells ($P < 0.05$) by Student's *t*-test for paired data; ** $P < 0.01$, *** $P < 0.005$.

Fig. 3. Dorsal cell surface topography in carbachol stimulation of RBL-m2 (a, b, c) and RBL-m3 (d, e, f) cells. RBL-m2 cells in glass bottomed culture dishes were incubated with 100 μ M carbachol for 0 min (a, d), 10 min (b, e), and 30 min (c, f) in Ca^{2+} -free HEPES-buffered Tyrode solution. Cell surface topography was observed by means of confocal fluorescence microscopy of F-actin, visualized by rhodamine-phalloidin. Fluorescent images were obtained by focusing at the dorsal cell surface with a confocal laser scanning microscope. Scale bar represents 50 μ m.

Fig. 4. β -Hexosaminidase release from RBL-m2 cells in response to carbachol or 20 DNPs-HSA with (unfilled circles) or without (filled circles) desensitization. A: RBL-m2 cells were incubated in Ca^{2+} -free Hepes-buffered Tyrode solution (HT) containing 100 μM carbachol for 30 min at 37°C. After desensitization, the cells were washed and allowed to stand for 5 min. Ca^{2+} -free HT was then replaced with HT containing 10 μM carbachol plus various concentrations of CaCl_2 . The cells were incubated for 10 min at 37°C to induce secretion. B: RBL-m2 cells were sensitized by incubating for 2 h at 37°C in Eagle's essential medium with dinitrophenol (DNP)-specific mouse monoclonal IgE (100 ng/ml) in a humidified atmosphere containing 5% CO_2 . The cells were washed and incubated in Ca^{2+} -free HT containing 100 μM carbachol for 30 min at 37°C. After desensitization, the cells were washed, and allowed to stand for 5 min. The cells were incubated in HT containing 20 ng/ml DNPs-HSA plus various concentrations of CaCl_2 for 20 min at 37°C to induce β -hexosaminidase release. The data are expressed as a percentage of the total cellular content. Points and vertical bars represent means $\pm$ S.E.M. from three independent experiments. *Significantly different from the values for control cells ($P < 0.05$) by Student's *t*-test for paired data; ** $P < 0.01$, *** $P < 0.005$.

a membrane associated G-actin pool and mediates polymerization of actin in the cell interior. We demonstrated that RBL-2H3 cells possess a mechanism for preventing exocytosis until an appropriate signal is received by the cell, and that the significance of the cytoskeleton, particularly the actin network, lies in its preventing exocytosis (12). We also demonstrated in the preparation of RBL-m3 cells that Rho plays an essential role in the exocytosis mediated by a multiple type of receptor including $\text{Fc}\epsilon\text{RI}$ and m3 mAChR and suggested that Rho functions as an integration point for exocytosis by regulating actin reorganization (23). Such a receptor-mediated reorganization of the actin network, a process independent of Ca^{2+} , may reveal the capacity to induce exocytosis. We found that ligation of m3 mAChR by carbachol developed membrane ruffling in RBL-m3 cells, but not in the case of RBL-m2 cells (Fig. 3). Thus, m2 mAChR-mediated secretion is unlikely to involve the regulation of actin reorganization leading to membrane ruffling.

Cytochalasin B enhances the activation of phospholipase D in response to pertussis toxin-sensitive G protein-coupled peptide chemoattractants such as fMLP and C5a (24). On the other hand, the activation of m3 mAChR, which is coupled to pertussis toxin-

insensitive G protein, increases phospholipase D activity in the absence of cytochalasins (25). The involvement of G protein in agonist-induced activation of phospholipase D has been demonstrated in RBL-2H3 cells (26), chromaffin cells (27), neutrophils (28, 29), and fibroblasts (30); and two low molecular weight G proteins, ARF and a Rho-like protein, may be involved in phospholipase D regulation. Together with our finding that there is a lack of carbachol-induced membrane ruffling in RBL-m2 cells, the lower secretion level in RBL-m2 cells may be due to lack of activation of phospholipase D which may be regulated through the Rho-like protein activation of actin reorganization.

Desensitization is of two types: homologous and heterologous. Homologous desensitization occurs in response to a specific agonist and affects only the signaling mediated by agonist-occupied receptors. In contrast, heterologous desensitization is characterized by a diminished responsiveness to multiple types of agonists in response to a desensitizing agent. In our study, desensitizing RBL-m2 cells completely inhibited the secretion induced by stimulating the cells with carbachol (acting on pertussis toxin-sensitive G protein-coupled m2 mAChR) but not by stimulating $\text{Fc}\epsilon\text{RI}$ (acting on receptor tyrosine kinase) (Fig. 4). These

Fig. 5. Representative traces of the time course of $[Ca^{2+}]_i$ elevation in adherent single RBL-m2 cells in response to $10 \mu M$ carbachol with or without desensitization. RBL-m2 cells in glass bottomed culture dishes were loaded for 1 h at $37^\circ C$ in Eagle's essential medium with $5 \mu M$ fura-2 AM in a humidified atmosphere containing 5% CO_2 . The dish was placed in a heating chamber on an Olympus microscope and maintained at $37^\circ C$ for the duration of the experiment. The cells were washed and incubated in Ca^{2+} -free Hepes-buffered Tyrode solution (HT) containing $100 \mu M$ carbachol (+) or vehicle (-) for 30 min. After desensitization, the cells were again washed and allowed to stand for 5 min. The solution was then replaced with HT containing $1 mM$ $CaCl_2$. To initiate the increase in $[Ca^{2+}]_i$, carbachol was added to a final concentration of $10 \mu M$. The increase in fluorescence intensity due to $[Ca^{2+}]_i$ increases in single cells was monitored using the calcium photometry system OSP-10CA (Olympus). Traces are representative of at least five repeated experiments.

findings indicated that the desensitization of the secretory response was homologous. This notion was supported by Didsbury et al. (31), who showed that stimulation of fMLP receptors coupled to the pertussis toxin-sensitive G protein activation of phospholipase C_β , does not desensitize the α_1 -adrenergic receptor coupled to the pertussis toxin-insensitive G protein activation of phospholipase C_β . It is notable that m3 mAChR-mediated secretion was heterologously desensitized (unpublished data). The different mode of desensitization between m2 and m3 mAChRs may be due to different signaling pathways leading to secretion.

Unlike m2 mAChRs, $Fc\epsilon RI$ does not couple to a pertussis toxin-sensitive G protein and activates phospholipase C_γ by receptor tyrosine kinase. Therefore, the homologous desensitization revealed by this study must occur upstream of phospholipase C_β activation. Measuring $[Ca^{2+}]_i$ using fura-2 revealed that carbachol induced a transient elevation of $[Ca^{2+}]_i$ in RBL-m2 cells. Desensitizing these cells completely blocked the increase in $[Ca^{2+}]_i$ induced by subsequent carbachol

addition (Fig. 5). This suggested that a change in the elevation of $[Ca^{2+}]_i$ is involved in the desensitization of secretion in RBL-m2 cells. Dell'Acqua et al. (32) have shown that desensitization of m2 mAChR signaling, including $[Ca^{2+}]_i$ elevation and the stimulation of polyphosphoinositide hydrolysis, selectively occurs following the activation of phospholipase C rather than inhibition of adenylyl cyclase, and it involves negative regulation at the level of the receptor and G protein. We have observed that the desensitizing treatment also blocked carbachol-induced transient increase in the levels of IP_3 . Therefore, it seems likely that the carbachol-induced desensitization of m2 mAChR-mediated secretion is a consequence of changes at the level of the receptor and/or receptor/G protein interaction followed by blocking the increase in $[Ca^{2+}]_i$. This notion was supported by the finding that there was no heterologous desensitization of $Fc\epsilon RI$ -mediated secretion, which did not involve the G protein-coupled activation of phospholipase C. Desensitizing treatment led to an 86% reduction in the cell surface m2 mAChR number and complete reduction of m2 mAChR-mediated $[Ca^{2+}]_i$ increases. In contrast, the treatment reduced m2 mAChR-mediated IP_3 production to 30% of control values. The different degrees of desensitization of m2 mAChR-mediated responses may be due to different receptor reserves among m2 mAChRs signaling to $[Ca^{2+}]_i$ increases and IP_3 production. Alternatively, it cannot be excluded that the different degrees of m2 mAChR-mediated responses are simply due to the exclusion of Ca^{2+} from the medium during desensitization.

Receptor phosphorylation is thought to be involved in the desensitization of receptors via the regulation of receptor functions and distribution. Such phosphorylation inactivates the receptor by uncoupling it from its associated G protein, sequestration, and finally by down-regulation. Several groups have shown that mAChR m2 subtypes in the third cytoplasmic loop are phosphorylated in an agonist-dependent manner by G protein-coupled receptor kinases (33) and that it is correlated with desensitization of the mAChR (34, 35). Pals-Rylaarsdam and Hosey (36) have shown that substitution of serine and threonine residues in amino acids 307–311 by alanine residues attenuates the sequestration of m2 mAChRs. Tsuga et al. (37) have found that about 40% of m2 mAChR on COS-7 cells was sequestered after an incubation with $10 \mu M$ or higher concentrations of carbachol for 60 min. Our data from the $[^3H]QNB$ binding study demonstrated that the total m2 mAChR number did not significantly decrease upon a 30-min incubation with $100 \mu M$ carbachol. In contrast to the $[^3H]QNB$ binding, the $[^3H]NMS$ binding markedly

decreased upon the incubation with carbachol. These results indicate that the decrease in [^3H]NMS binding is not due to down-regulation of m2 mAChR, but is due to sequestration of m2 mAChR. Taken together, our present study suggests that the homologous desensitization of secretory response in RBL-m2 cells is due to the sequestration of m2 mAChR. Sequestration of m2 mAChR has been reported to be facilitated by their phosphorylation by G protein-coupled receptor kinase 2 (GRK2) (37). Further investigations are required to clarify whether receptor phosphorylation is involved in the desensitization of m2 mAChR-mediated secretion. Bmax value of [^3H]NMS binding was significantly smaller than that of [^3H]QNB binding. It is conceivable, at present, that the overexpression of recombinant m2 mAChRs in RBL-2H3 cells, which originally lack m2 mAChRs, caused a saturation of the trafficking mechanism.

In conclusion, this study demonstrated that ligation of m2 mAChR by carbachol causes secretion from RBL-2H3 cells transfected with m2 mAChR and that the secretion is mediated through the pertussis toxin-sensitive G protein. Furthermore, m2 mAChR-mediated secretion was homologously desensitized, which completely blocked the carbachol-induced transient increase in levels of inositol IP₃ and the elevation of the intracellular Ca²⁺ concentration. Our findings suggest that the carbachol-induced desensitization of m2 mAChR-mediated secretion is due to the sequestration of m2 mAChR followed by blocking the increase in [Ca²⁺], and that this desensitizing mechanism is receptor subtype-specific. Desensitization would be one mechanism by which a cell reversibly adjusts its sensitivity to an agonist and terminates the signal for cellular functions. This study revealed that m2 mAChRs are homologously desensitized and functionally regulates cell responses.

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