Differential Effects of β2-Adrenoceptor Desensitization on the 
IgE-Dependent Release of Chemical Mediators from 
Cultured Human Mast Cells

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Received May 13, 2004; accepted June 24, 2004

In the present study, we examined the inhibitory effects of the β2-adrenoceptor agonists isoproterenol, salbutamol, fenoterol, and clenbuterol, on the release of chemical mediators from cultured human mast cells after prolonged treatment with the agonists. Although preincubation of sensitized mast cells for 10 min with β2-adrenoceptor agonists profoundly inhibited mediator release, prolongation of the preincubation period up to 240 min attenuated the inhibition. The attenuation of histamine release inhibition was potent when compared with that of prostaglandin D2 (PGD2) and cysteiny1 leukotriene (LT) release inhibition. In contrast, forskolin inhibited mediator release and the inhibition increased gradually in proportion to the preincubation period. The reduced inhibition by the β2-adrenoceptor agonists was compensated for by simultaneous treatment with cholera toxin. The β2-adrenoceptor agonists elevated intracellular cAMP levels after 10-min incubation and the elevated levels were almost comparable to those after 240-min incubation. Forskolin elevated the intracellular cAMP levels more potently after incubation for 240 min than after 10 min. When mast cells were incubated for 3 d with the β2-adrenoceptor agonists, similar attenuation of mediator release inhibition was observed. Elevation of intracellular cAMP levels was also attenuated, although β2-adrenoceptor mRNA expression was potentiated. The present results collectively indicate that the attenuation of mediator release inhibition by β2-adrenoceptor agonists under the present experimental conditions involves uncoupling between β2-adrenoceptors and Gs proteins. Furthermore, the β2-adrenoceptor desensitization causes differential attenuating effects on the inhibition of histamine, PGD2, and LT release, suggesting that downstream events involved in each inhibitory pathway have different sensitivity to receptor desensitization.

Key words β2-adrenoceptor agonist; desensitization; human cultured mast cell; IgE; chemical mediator

Agonist binding to β2-adrenoceptors causes activation of adenylyl cyclase through coupling to a stimulatory component of GTP-binding protein (Gs protein) and results in an elevation of intracellular cAMP levels. cAMP plays an important role in inducing cellular responses as a second messenger. It is well known, however, that the responsiveness of β2-adrenoceptors wanes after continuous or repeated exposure to the agonists, a phenomenon referred to as desensitization. The initial step of the desensitization is caused by phosphorylation of intracellular domains of β2-adrenoceptors. The receptor phosphorylation results in an uncoupling between receptor proteins and Gs proteins. Two types of protein kinase are considered to be responsible for the phosphorylation. One is a cAMP-dependent protein kinase A (PKA) and the other is a cAMP-independent G protein-coupled receptor kinase such as β-adrenergic receptor kinase (β-ARK). Phosphorylation by β-ARK facilitates the binding of β-arrestin to the receptors, which interferes with the receptor coupling to Gs protein. Prolonged exposure of the receptors to agonists may facilitate the receptor internalization, followed by their degradation. Furthermore, β2-adrenoceptor gene expression may be downregulated when cells are exposed continuously to the agonists for a long period. Although receptor desensitization is an important mechanism to maintain homeostasis, the same mechanism may interfere with the therapeutic effects of β2-adrenoceptor agonists.

It has been reported that β2-adrenoceptor agonists inhibit IgE-mediated release of mediators such as histamine, prostaglandin D2 (PGD2), and cysteiny1 leukotrienes (LT) from human lung fragments and dispersed human lung and skin mast cells. On the other hand, desensitization has also been observed in the inhibition of human mast cell histamine release by β2-adrenoceptor agonists. Furthermore, β2-adrenoceptor agonists inhibit PGD2 and LT release more potently than disodium cromoglycate. Chong and Peachell reported that isoproterenol inhibition of histamine release from human lung mast cells is considerably more susceptible to desensitizing treatments than the isoproterenol relaxation of bronchial smooth muscle. In contrast to histamine release, however, desensitization of PGD2 and LT release inhibition by β2-adrenoceptor agonists has rarely been investigated.

In the present study therefore, we examined and compared the inhibitory effects of β2-adrenoceptor agonists on the release of histamine, PGD2, and LT from cultured human mast cells after prolonged treatment with the agonists.

MATERIALS AND METHODS

Reagents and Antibodies Isoproterenol, salbutamol, fenoterol, clenbuterol, and forskolin (Sigma Chemical Co.,
St. Louis, MO, U.S.A.) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. The stock solutions were diluted with a culture medium or Tyrode’s solution (NaCl 126 mM, KCl 4 mM, NaH₂PO₄ 0.64 mM, CaCl₂ 1 mM, MgCl₂ 0.6 mM, 0.1% glucose, HEPES 10 mM, 0.03% bovine serum albumin, pH 7.4) when used. Cholera toxin (A subunit, List Biological Laboratory) was dissolved in Tyrode’s solution and used at a final concentration of 100 ng/ml. In our preliminary experiments, we confirmed that the concentrations of DMSO in working solutions used in the present experiment had no effect on mediator release from cultured human mast cells. Human myeloma IgE (Chemicon Int. Inc., Temecula, CA, U.S.A.) and anti-human IgE (anti-IgE, Dako, Glostrup, Denmark) were used for sensitization and stimulation of mast cells, respectively.

**Culture of Human Mast Cells** Human mast cells were obtained according to the method described by Saito et al. with a slight modification. Briefly, mononuclear cells separated from heparinized umbilical cord blood with a Ficoll (Organon Teknika Co., Durham, NC, U.S.A.) gradient were cultured in Media I (IBL, Gunma, Japan) supplemented with 15% fetal bovine serum (Filtron Pty Ltd., Brooklyn, Australia), human recombinant stem cell factor 80 ng/ml (Kirin Brewery, Maebashi, Japan), and recombinant interleukin-6 50 ng/ml (Kirin Brewery). Cells were harvested and resuspended in a fresh medium weekly. Cultured cells recovered after 12 weeks or later were employed in the present study. Immunoperoxidase staining for tryptase revealed that the cells were almost 100% positive for tryptase.

**Activation of Mast Cells** Cultured mast cells were sensitized with 1 μg/ml of myeloma IgE under overnight incubation at 37°C. Sensitized cells were washed and resuspended in Tyrode’s solution at a concentration of 1×10⁵ cells/ml. Mediator release was induced by stimulating cells with 4 μg/ml of anti-IgE for 30 min and mediators released in the supernatant were quantified.

**Treatment of Mast Cells** In a series of experiments, sensitized mast cells at a concentration of 1×10⁵ cells/ml in Tyrode’s solution were incubated in the presence of β₂-adrenoceptor agonists for 10—240 min at 37°C before anti-IgE stimulation. Concentrations of the agonists employed were 10⁻⁷ M for isoproterenol, fenoterol, and clenbuterol, and 10⁻⁶ M for salbutamol, as determined based on our previous results and preliminary results. When cells were treated with cholera toxin simultaneously, it was added to the cell suspension 60 min before the treatment with β₂-adrenoceptor agonists.

In another series of experiments, sensitized mast cells suspended in a culture medium were treated with β₂-adrenoceptor agonists for 3 d. The agonists at a final concentrations of 2.5×10⁻⁸ M for isoproterenol, fenoterol, and clenbuterol, and 2.5×10⁻⁷ M for salbutamol were added 3 times to the cell suspension at a cumulative interval of 24 h. After the 3-d incubation period, mast cells were washed with and resuspended in Tyrode’s solution at a concentration of 1×10⁵ cells/ml. Mast cells were again treated with the same agonists (final concentration of 2.5×10⁻⁸ M or 2.5×10⁻⁷ M) for 10 min before stimulation with anti-IgE. When cells were treated with cholera toxin simultaneously, it was added to the cell suspension 60 min before the final treatment with β₂-adrenoceptor agonists.

**Measurement of Mediators Released** Histamine released in the supernatants was quantified by a postcolumn derivatization method, as reported previously. The minimal detectable concentration of histamine was 0.2 ng/ml. Cell-associated histamine was extracted in the presence of perchloric acid. Total cell-associated histamine was about 10 μg/10⁶ mast cells, and control mast cells released about 40% of total histamine upon IgE-dependent stimulation under the present experimental conditions. Spontaneous release was about 3%.

PGD₂ in the supernatants was measured with a PGD₂ MOX enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.). PGD₂ was converted to a stable methoxime derivative using methoxamine hydrochloride and then detected. The minimal detectable concentration of PGD₂ was 7.8 pg/ml. Under the present experimental conditions, control mast cells generated 150—200 ng/10⁶ cells of PGD₂ upon IgE-dependent stimulation.

For the analysis of cysteinyl LT, we employed a commercial ELISA kit (Buhmann Laboratory, Allschwile, Switzerland). The monoclonal antibodies involved in the kit recognize LTC₄, D₁, and E₂ equally. The minimal detectable concentration of LT was 12.5 pg/ml. Under the present experimental conditions, control mast cells released 150—200 ng/10⁶ cells of LT.

**Measurement of cAMP** Intracellular cAMP levels were evaluated using a cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech, UK). In brief, mast cell suspensions at a concentration of 5×10⁵ cells/ml in the presence or absence of a drug treatment were frozen in liquid nitrogen and then sonicated. Then cAMP was acetylated and measured. The minimal detectable concentration of cAMP was 2.0 fmol.

**mRNA Expression** Expression of mRNA for β₂-adrenoceptor and β-actin was evaluated using the reverse-transcriptase polymerase chain reaction (RT-PCR). In brief, mRNA was extracted from pelleted mast cells using Isogen (Nippon Gene Co., Ltd., Japan), and the total RNA was reverse-transcribed using a first-strand cDNA synthesis kit (Takara, Japan). PCR was performed under standard conditions, and PCR products were electrophoresed and visualized. Primers used were as follows: for β₂-adrenoceptor: 5’-CCCTTATCATCGCCGAGGCC-3’ and 5’-TCTCTCCTTGGAATGATGATCCTTATC-3’, and for β-actin: 5’-CAAGAGATGGCCACGGCCTTATC-3’ and 5’-TCCTTCTGCATCTCCGTGCAGCA-3’.

**Statistical Analysis** The data are expressed as mean values and standard error. Inhibition of mediator release is expressed as a percentage compared with controls. The statistical significance of differences was evaluated using Student’s or Welch’s t-test, or Tukey-Kramer’s multiple comparisons test using the InStat Program (GraphPad Software, San Diego, CA, U.S.A.). When the p value was less than 0.05, the difference was considered to be significant.

**RESULTS**

**Effects of Pretreatment for 10—240 min** Mast cells were incubated in the presence of isoproterenol, salbutamol, fenoterol, clenbuterol, and forskolin for 10—240 min and then stimulated with anti-IgE to cause mediator release. The results of histamine release are shown in Fig. 1A. Although...
Fig. 1. Effects of β₂-Adrenoceptor Agonists and Forskolin on IgE-Dependent Chemical Mediator Release from Human Mast Cells

Sensitized mast cells in the presence or absence of cholera toxin were incubated with isoproterenol, salbutamol, fenoterol, clenbuterol, and forskolin for 10 to 240 min before stimulation with anti-IgE. Experiments were repeated 4 or 5 times and the results are expressed as the mean ± S.E.M. for percent inhibition of control mediator release. A, histamine release; B, prostaglandin D₂ release; C, leukotriene release. * p<0.05, ** p<0.01, *** p<0.001 against 10-min incubation, # p<0.05, ## p<0.01 against corresponding untreated.
all β₂-adrenoceptor agonists employed inhibited histamine release by 70—80% after incubation for 10 min, prolongation of the preincubation period up to 240 min attenuated the inhibition. The most profound attenuation was observed in the case of clenbuterol. The attenuation of histamine release inhibition was recovered partially by simultaneous treatment with cholera toxin. In contrast, forskolin at a concentration of 10⁻⁴ M inhibited histamine release, and the inhibition increased gradually dependent upon the preincubation period. The inhibition by forskolin was also potentiated by cholera toxin.

The results of PGD₂ and LT release are shown in Figs. 1B and C. The β₂-adrenoceptor agonists inhibited PGD₂ and LT release by 80% or more after incubation for 10 min. Although an attenuation of the inhibition based on the prolongation of the preincubation period was observed similar to the case of histamine release, the degree of attenuation was very weak. Simultaneous treatment with cholera toxin showed a tendency to recover the attenuated inhibition. In contrast, forskolin inhibited PGD₂ and LT release by about 80%, and the inhibition increased gradually dependent upon the preincubation period. The inhibition by forskolin was potentiated slightly by cholera toxin.

Intracellular cAMP levels were examined after incubation of mast cells with the β₂-adrenoceptor agonists and forskolin for 10 and 240 min. As shown in Fig. 2, the β₂-adrenoceptor agonists elevated the intracellular cAMP levels to 2-4-fold the basal level after 10-min incubation and similarly elevated intracellular cAMP levels were also observed after 240-min incubation. In contrast, after incubation for 10 min, forskolin elevated the intracellular cAMP levels to 7-fold the basal level, and the levels were further elevated up to 15-fold after 240 min.

**Effects of Pretreatment for 3 d** Mast cells were incubated for a longer period of 3 d in the presence of the β₂-adrenoceptor agonists and then effects of the agonists on IgE-dependent mediator release were examined. The results of histamine release are shown in Fig. 3A. Although β₂-adrenoceptor agonists inhibited histamine release by 40—60%, the inhibition was significantly reduced by 3-d pretreatment with the agonists. Simultaneous treatment with cholera toxin attenuated the reduction, except for the case of fenoterol. The results of PGD₂ release are shown in Fig. 3B. The β₂-adrenoceptor agonists inhibited PGD₂ release by 50—70% and the 3-d pretreatment showed a tendency to attenuate the inhibition. The attenuation observed in the case of isoproterenol was significant. Simultaneous treatment with cholera toxin slightly recovered the attenuated inhibition. The results of LT release are shown in Fig. 3C. LT release was inhibited by 50—70% by the β₂-adrenoceptor agonists, and the inhibition was significantly attenuated by 3-d pretreatment. Cholera toxin treatment potentiated the attenuated inhibition partially, and the potentiation was significant in the case of clenbuterol.

Elevation of intracellular cAMP levels were examined in mast cells treated with β₂-adrenoceptor agonists for 3 d. The results of a time-course study using isoproterenol are shown in Fig. 4A, and those of the four β₂-adrenoceptor agonists are shown in Fig. 4B. Pretreatment with β₂-adrenoceptor agonists for 3 d depressed the elevation of intracellular cAMP levels.

**Fig. 2. Effects of β₂-Adrenoceptor Agonists and Forskolin on Intracellular cAMP Levels in Human Mast Cells**

Mast cells were incubated with isoproterenol, salbutamol, fenoterol, clenbuterol, and forskolin for 10 and 240 min and intracellular cAMP levels were examined. Results are expressed as the mean±S.E.M. of 7 experiments. Spontaneous cAMP levels at 10 and 240 min were 12.78±0.70 and 12.85±0.65 fmol, respectively. *p<0.05, **p<0.01, ***p<0.001 against spontaneous.

**Fig. 3. Effects of Prolonged Pretreatment with β₂-Adrenoceptor Agonists on the Inhibition of IgE-Dependent Histamine Release from Human Mast Cells by the Agonists**

Sensitized mast cells in the presence or absence of 3-d pretreatment with isoproterenol, salbutamol, fenoterol, and clenbuterol were incubated with the agonist for 10 min before stimulation with anti-IgE. Shaded columns indicate the results of mast cells treated simultaneously with cholera toxin. Experiments were repeated 5 times and the results are expressed as the mean±S.E.M. for percent inhibition of control mediator release. A, histamine release; B, prostaglandin D₂ release; C, leukotriene release. *p<0.05, **p<0.01, ***p<0.001 against corresponding control, #p<0.05, ##p<0.01 against without cholera toxin treatment.
Mast cells were treated with isoproterenol and salbutamol for 3-d and β₂-adrenoceptor mRNA expression was analyzed. As shown in Fig. 5, the 3-d treatment with β₂-adrenoceptor agonists induced the expression of β₂-adrenoceptor mRNA. mRNA expression could not be detected in control cells.

**DISCUSSION**

Cellular responses mediated through β₂-adrenoceptors wane after continuous or repeated exposure of cells to the agonists. Desensitization is also observed in the inhibition of human mast cell histamine release by β₂-adrenoceptor agonists. In the present study, we found that IgE-dependent histamine release from cultured human mast cells was inhibited significantly by isoproterenol, salbutamol, fenoterol, and clenbuterol and that the inhibition was apparently attenuated by prolonged treatment with the agonists. On the other hand, although forskolin, an activator of adenylate cyclase, apparently inhibited histamine release from cultured mast cells, it did not cause an attenuation of the inhibition even after prolonged treatment for up to 240 min. Simultaneous treatment with cholera toxin, a direct activator of Gs protein, recovered the attenuation of histamine release inhibition by prolonged treatment with the β₂-adrenoceptor agonists. Furthermore, even after the 3-d pretreatment with isoproterenol and salbutamol, β₂-adrenoceptor mRNA expression was not downregulated. Rather, the agonists apparently potentiated the expression. These results suggest that the attenuation of histamine release inhibition is caused by an inactivation of an immediate downstream event following receptor engagement, coupling between receptors and Gs proteins.

The β₂-adrenoceptor agonists induced an elevation of intracellular cAMP levels in human mast cells. The elevation after 10-min incubation with the agonists was 2—4-fold higher than the basal level and was comparable to that after 240-min incubation. In our previous study, isoproterenol and salbutamol induced a biphasic elevation of intracellular cAMP levels in cultured human mast cells. The first elevation was transient and peaked at around 10 min. Then the cAMP levels declined but again increased gradually for up to 360 min. Therefore elevated levels of intracellular cAMP appear not to be maintained when cells are incubated with β₂-adrenoceptor agonists for a long period. In contrast, forskolin elevated intracellular cAMP levels to 7-fold the basal level after 10-min incubation and to 15-fold after 240-min incubation. Peachell et al. indicated that elevated cAMP levels induced by forskolin persisted for at least 20 min, suggesting that forskolin may continuously elevate intracellular cAMP levels in cultured human mast cells for up to 240 min, which may contribute to the gradual increase in histamine release inhibition.

The β₂-adrenoceptor agonists inhibited the release of PGD₂ and LT from cultured human mast cells. The inhibition was slightly more potent than that for histamine release, as reported previously. The potent inhibitory effects of β₂-adrenoceptor agonists on PGD₂ and LT release were also reported by other investigators. Furthermore, forskolin inhibited the release of PGD₂ and LT far more potently than that of histamine, as reported by Marone et al. These results suggest that the inhibitory mechanisms of PGD₂ and LT release involve some steps sensitive to β₂-adrenoceptor-triggered signals or elevated intracellular cAMP levels. However, the inhibition of PGD₂ and LT release was resistant to prolonged treatment with the agonists. When mast cells were preincubated with the agonists for up to 240 min, significant attenuation of PGD₂ and LT release inhibition was not observed. Furthermore, even after 3-d preincubation, although attenuation of PGD₂ and LT release inhibition was observed, the degree of attenuation was less than that for histamine release inhibition.
release inhibition. After 3-d preincubation, elevation of intracellular cAMP levels was also attenuated, which may contribute to the attenuation of PGD₂ and LT release inhibition.

In the present study, we examined the β₂-adrenoceptor desensitization using four agonists. Isoproterenol is a first-generation, very short-acting β₂-adrenoceptor agonist, and salbutamol is a β₂-selective, orally active compound belonging to the second generation. Fenoterol and clenbuterol are third-generation drugs with relatively long-lasting actions¹⁵,³³⁰ and fenoterol has frequently been discussed in relation to asthma death.³⁵,³⁶⁰ As salbutamol exhibited less potent inhibitory effects on the mediator release than the other three agonists, higher concentrations were employed in the present experiments. These four agonists showed similar desensitizing effects on mediator release from cultured human mast cells in spite of their distinct characteristics, suggesting that the desensitizing phenomenon may not be affected significantly by the characteristics of each agonist under the present experimental conditions.

In summary, we found that prolonged treatment of cultured human mast cells with β₂-adrenoceptor agonists attenuated the inhibition of IgE-dependent mediator release by the agonists. The attenuation of mediator release inhibition appears to involve uncoupling between β₂-adrenoceptors and Gs proteins. Under the same receptor desensitization conditions, however, the attenuation of histamine release inhibition was more potent than that of PGD₂ and LT release inhibition. Histamine release inhibition appears to be more sensitive to receptor desensitization than PGD₂ and LT release inhibition. Therefore key events involved in the inhibitory pathways for PGD₂ and LT release by β₂-adrenoceptor agonists may have higher sensitivity to β₂-adrenoceptor-derived signals and their inhibitory functions may be longer lasting than those for histamine release.

Acknowledgments We are grateful to Dr. Shin-ichi Iwasa, Iwasa Hospital at Gifu, for providing cord blood. This study was supported in part by a Grant-in-Aid for Research on Eye and Ear Science, Immunology, Allergy and Organ Transplantation from the Ministry of Health, Labor and Welfare of Japan.

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