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Gα12 and Gα13 Subunits Modulate Ca^{2+}-Induced Histamine Release in Human Umbilical Cord Blood-Derived Mast Cells

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Abstract The role of Gα12 and Gα13 in modulating the IgE receptor-mediated histamine secretion in the streptolysin-O-permeabilized human cultured mast cell was investigated. The expression of Gα12 and Gα13 proteins were regulated during human cultured mast cell differentiation, and a significant correlation was observed between the levels of expression of Gα12 and Gα13 proteins and IgE receptor-mediated histamine secretion capability in human cultured mast cells. Antibodies against Gα12 and Gα13 effectively inhibited the IgE receptor-induced histamine release, and the concentration of anti-Gα12 antibody used to inhibit histamine secretion was shown to also inhibit the IgE receptor-mediated elevation of intracellular Ca^{2+}. Therefore, the results suggest that Gα12 and Gα13 play roles in modulating IgE receptor-activated Ca^{2+} influx, thereby regulating histamine release in cultured human mast cells. This is the first report to show that Gα12 and Gα13 are involved in the regulation of Ca^{2+}-mediated exocytosis in human cultured mast cells.

Key words: Human cultured mast cell, Gα12 and Gα13 subunits, histamine release, Ca^{2+} influx, anti-Gα12 and anti-Gα13 antibodies

Heterotrimeric G proteins, molecular switches in receptor-mediated transmembrane signaling systems, have been implicated in several secretory responses. Heterotrimers of the Gi family of pertussis toxin-sensitive Gα subunits, Gαi and Gαo, have been found localized in the membrane of secretory vesicles in diverse neuroendocrine cells, indicating that they are involved in the process of exocytosis [1, 8, 22]. The Gαi3 located in the plasma membrane appears to be essential in the activation of exocytosis in rat peritoneal mast cells [2].

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Several G proteins are involved in the regulation of cell proliferation signaling [reviewed in Ref. 4]. Gα12 and Gα13 have been shown to stimulate cell growth and transformation [23, 26]. In particular, it has been reported that Gα12 protein is involved in the regulation of cell proliferation signaling and its expression is tightly regulated during human myeloid differentiation [21], suggesting that Gα12 plays a certain role in modulating cellular function at each differentiation stage.

A strong positive correlation was observed between the levels of Gα12 and Gα13 expression and various culture stages of human mast cells, which also implicates a functional significance of these proteins in mast cells. Interestingly, Gα12 and Gα13 proteins have already been shown to inhibit Ca^{2+}-dependent exocytosis in mature PC12 cells [28]. This led to the hypothesis that Gα12 and Gα13 probably play a certain role in modulating histamine release at each differentiation stage. In order to confirm and extend this idea, we investigated whether there is any correlation in the expression of Gα12 and Gα13 with their functional significance in human cultured mast cells at various differentiation stages, by introducing the antibodies raised against several Gα subunits into the streptolysin-O-permeabilized human cultured mast cells and then measuring changes in histamine release together with those in Ca^{2+} influx pattern. The results showed that the antibodies against Gα12 and Gα13 inhibit histamine release by lowering the influx of Ca^{2+} in human cultured mast cells.

MATERIALS AND METHODS

Reagents

Heparinized human umbilical cord blood was kindly provided by Samsung Hospital (Seoul, Korea). Histopaque 1077, Iscove's modified Dulbecco's medium (IMDM), FCS,

Streptolysin-O, PGE_2, Hepes, L-glutamine, gentamycin, Fluo-3 AM, 1×MEM vitamin solution, 1×MEM amino acid solution (without L-glutamine), sodium pyruvate, and 2-ME were purchased from Sigma (St. Louis, MO, U.S.A.). Human rSCF (rhSCF), human rIL-6 (rhIL-6), myeloma IgE, and anti-human IgE antibody were purchased from Chemicon (Temecula, CA, U.S.A.). Penicillin-streptomycin was obtained from Gibco-BRL (Grand Island, NY, U.S.A.). Anti-Gαq antibody, anti-Gα11 antibody, and anti-Gαq/11 polyclonal antibody were kindly provided by M. I. Simon (California Institute of Technology, Pasadena, CA, U.S.A.). The anti-Gα12 and anti-Gα13 polyclonal antibodies were kindly provided by N. Dhanasekaran (Fels Institute, Temple University, PA, U.S.A.). West-Zol Western blot detection kit was purchased from Intron (Seoul, Korea).

Human Umbilical Cord Blood-Derived Mast Cell Culture

Human umbilical cord blood-derived mast cells (i.e., human cultured mast cell) were prepared as previously described with slight modifications [6, 17, 27]. Mononuclear cells clustered at the interface between plasma and Histopaque 1077 were collected by centrifugation at room temperature for 30 min at 400 ×g, washed, and maintained in culture medium [IMDM, 10% FCS, 80 ng/ml rhSCF, 50 ng/ml rhIL-6, 1 M PGE_2, 10 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamycin, 1×MEM vitamine solution, 1×MEM amino acid (without L-glutamine), 1 mM sodium pyruvate, 50 µM 2-ME]. The purity and viability of mast cells at each experiment time was shown to be more than 99%, as determined with Kimura's stain [7] and/or tryptase immunostaining [17].

Stimulation of Human Cultured Mast Cell and Measurement of Histamine Release

In each experiment, mast cells (2×10^5 cells) were sensitized with myeloma IgE (1.0 µg/ml) at 37°C for 1 h, washed, and then stimulated with anti-human IgE antibody (1.0 µg/ml) for 30 min at 37°C. To test the effects of various anti-Gα subunit antibodies on histamine release, cells were permeabilized with streptolysin-O (1.5 IU/ml) for 5 min at 30°C, washed once, and sensitized. Then, the cells were stimulated by preincubation with various anti-Gα subunit antibodies (1:500, 1:1000 dilution) for 5 min, and the amount of histamine released in the supernatant was measured with an automated fluorometric analyzer as described by Siraganian [19]. The detection limit of the histamine assay was approximately 5 ng/ml, and the amount of histamine released was expressed as percentage of the total histamine contents in nonstimulated mast cells.

Quantification of Intracellular Ca^{2+} Level During Stimulation of Human Cultured Mast Cells

The sensitized and streptolysin-O-permeabilized human mast cells (4×10^4 cells) were prewarmed for 5 min at 37°C, followed by incubation for another 30 min in the presence of Fluo-3 AM (5.0 µM), and stimulation with anti-human IgE antibody in the presence or absence of anti-Gα 12 antibody. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were monitored every 2 sec for 100 sec with a confocal laser scanning microscope (Leica TCS NT Confocal Microscopy, Germany) [10, 24]. The $[Ca^{2+}]_i$ was estimated by analyzing the optimal density (O.D.) with the computer software program, Image Gauge Ver 3.12 (Fuji Photo Film Co., Ltd.).

Expression of Recombinant Gαq Class and Gα12 Class Subunit G Proteins in Cos-7 Cells

Cos-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were seeded at a density of 1×10^5/well in a 12-well plate one day before transfection. A total of 1.0 µg of each cDNAs encoding pCMV, Gαq class subunits (Gαq, Gα11, Gα14, Gα15, and Gα16), and Gα12 class subunits (Gα12, Gα13) was mixed with 4 µl of lipofectamin in 0.5 ml of opti-MEM and the mixture was then added to the cells. Fetal bovine serum (20%) in 0.5 ml of DMEM was added after 5 h. Two days later, the cells were collected and then processed as described below.

Characterization of the Reaction Specificity of Various Anti-Gα Subunit Antibodies and the Expression of Gα12 and Gα13 in Human Cultured Mast Cells

Cos-7 cells expressing recombinant Gαq class and Gα12 class subunit G proteins and the umbilical cord blood-derived human mast cell (1×10^5 cells) at 9-, 10-, 11-, 12-, and 13-week old cultures were collected. After washing twice with phosphate-buffered saline (PBS), the cells (50 µg protein) were lysed in 45 µl of RIPA buffer (1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 4 µg/ml aprotinin, 2 µg/ml leupeptin, 200 µM vanadate, and 0.1% β-mercaptoethanol in PBS). Fifteen µl of 4×Laemmli's buffer was added, followed by boiling, and the samples were then subjected to SDS-gel electrophoresis and Western blot analysis. Recombinant Gαq class and Gα12 class subunit G proteins were used as authentic references to test the detection specificities of various antisera used in the experiments, and detection specificity was visualized with a West-Zol chemiluminescence Western blot detection kit.

Statistical Analysis

Experimental data are shown as mean±S.E.M. (standard error of the mean). Analysis of variance was performed by F-test to assess the difference between multiple groups. In the case where the F-statistic was significant, the mean values of each group were compared by Fisher's least significant difference method. P-values less than 0.05 were considered significant.

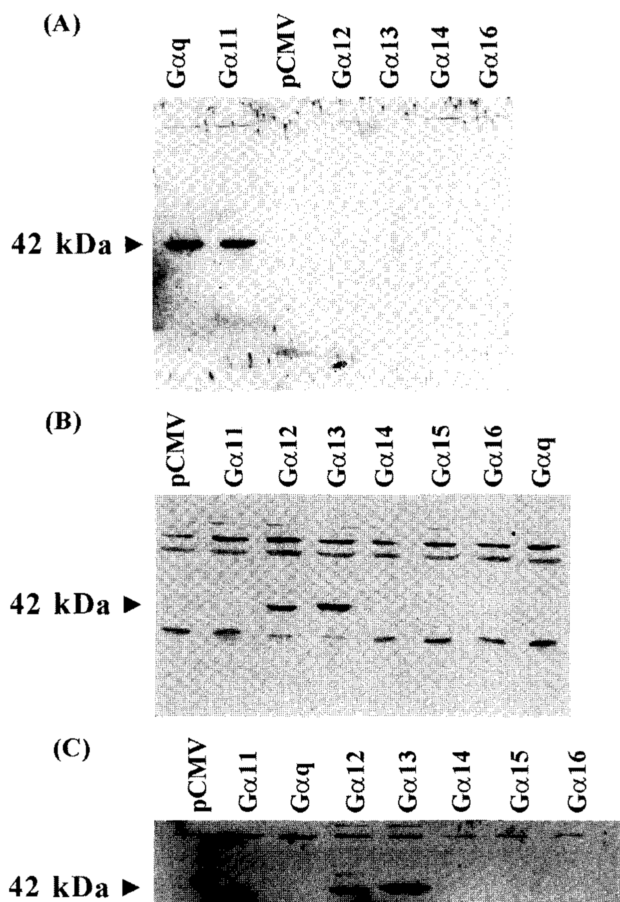

Fig. 1. Characterization of the reaction specificity of various anti-Gα subunits antibodies.

Cos-7 cells expressing each member of the recombinant Gαq class and Gα12 class subunits were collected in 25 μl of RIPA buffer, subjected to SDS-gel electrophoresis, and then electrotransferred onto PVDF membranes, as described in Materials and Methods. (A) Gαq and Gα11 proteins were detected with anti-Gαq/11 antibody (1:10,000) raised against the common carboxyl-terminal sequence (QLNLKEYNLV) of Gαq and Gα11. (B) Gα12 and Gα13 proteins were recognized by antibody (1:10,000) against the specific sequence (QENLKDIMLQ) of Gα12. (C) Gα12 and Gα13 proteins were recognized by antibody (1:10,000) against the specific sequence (HDNLKQLMLE) of Gα13. The level of expression was visualized with West-Zol Western blot detection kit.

RESULTS

Characterization of the Reaction Specificity of Various Anti-Gα Subunit Antibodies

As shown in Fig. 1(A), anti-Gαq/11 antibody specifically detected the recombinant Gαq and Gα11 proteins, but it did not recognize Gα12, Gα13, Gα14, and Gα16 subunit proteins. Figures 1(B) and 1(C) show that the anti-Gα12 and anti-Gα13 antibodies cross-reacted against both Gα12 and Gα13 proteins to a greater degree. Nevertheless, these antisera did not recognize Gαq, Gα11, Gα14, Gα15, and Gα16 subunit proteins, as shown in Figs. 1(B, C).

Developmental Stage-Dependent Histamine Release in Human Umbilical Cord Blood-Derived Mast Cells

As summarized in Table 1, 12-week-old culture cells showed more histamine release response than other cultures at each test dose of anti-IgE antibody, and the highest level of histamine release was observed in the very same culture, when treated with 1.0 μg anti-IgE antibody (36.3±1.53%, 12-week-old culture and 1.0 μg/ml anti-IgE). This was not due to stage-dependent cell viability variation, since purity and viability of mast cells at each experimented time were shown to be more than 99%, as determined with Kimura's stain [7] and/or tryptase immunostaining [17]. Additional control experiments were performed to determine the optimum conditions for sensitizing and activating mast cells for histamine secretion. As summarized in Table 2, the cells sensitized with 1.0 μg/ml IgE for 1 h followed by stimulation with 1 μg/ml anti-IgE antibody for 30 min was the most adequate for an efficient histamine release response (36.3±1.53%). Long duration (4 days) of sensitization with a large amount of IgE (5.0 μg/ml) and a longer period (2 h) of stimulation did not seem to be better than that mentioned above in eliciting histamine secretion (26.2±2.79% vs 36.3±1.53%). The spontaneous release rate was 7.8±2.43%, which reflects the basal level of histamine in the media.

Expression Level of Gα12 and Gα13 Proteins in Human Umbilical Cord Blood-Derived Mast Cells

Since anti-Gα12 antibody could recognize both Gα12 and Gα13 proteins, it was used to detect changes in the level of Gα12 and Gα13 proteins during the developmental stages of human cultured mast cells. Figure 2 clearly demonstrates a time-dependent expression profile of Gα12 and Gα13 proteins, showing the highest level in the 12-week-old culture cells, as compared with other cultures (9, 10, 11, and 13 week-cultures).

Effects of Various Anti-Gα Subunits Antibodies on the Histamine Release in Streptolysin-O-Permeabilized Human Umbilical Cord Blood-Derived Mast Cells

As shown in Fig. 3, anti-IgE-induced activation of mast cells released 37.5±0.84% of total histamine. Anti-Gα12 and anti-Gα13 antibodies added to anti-IgE activated mast cells (1:500 dilution) reduced the histamine release to 21.9±2.26% and 21.4±0.61%, which represent 41.6% and 42.9% inhibition of the anti-IgE-induced histamine release (37.5±0.84% release of total histamine content), respectively. In addition, the histamine release was further inhibited by applying anti-Gα12 and anti-Gα13 antibodies at 1:1,000 dilution to anti-IgE stimulated mast cells, releasing 13.6±2.18% and 11.3±2.92% of the total histamine that corresponded to 63.7% and 69.9% inhibition of anti-IgE stimulated histamine release, respectively. When pre-immune serum was applied to anti-IgE-stimulated mast cells, slight, but not significant enhancement of histamine release (15.5%

Table 1. Developmental stage-dependent histamine release in human cord blood-derived mast cells activated with IgE/anti-IgE antibody.[a]

IgE	Anti-IgE	Histamine release (%)					
		10 week	11 week	12 week	13 week	14 week	15 week
1 μg	0.1 μg	5.3±0.6	8.2±1.0	12.5±1.8	3.8±0.5	2.6±0.4	0.8±0.1
1 μg	1.0 μg	12.9±1.5	24.5±2.1	36.3±1.5	30.9±3.2	18.9±2.0	5.1±0.9
1 μg	10.0 μg	7.8±0.7	12.3±0.8	18.9±1.0	14.7±0.7	10.6±1.0	2.8±0.2

[a]Human umbilical cord blood-derived mast cells were cultured as described in Materials and Methods. The cells (2×10^5 cells) at each indicated time period (week) were washed and then suspended again with medium. The cells were incubated with 1.0 μg myeloma IgE, challenged with anti-human IgE antibody (N=6). The values of histamine released from nonstimulated mast cells were 5–10% of total histamine contents.

of anti-IgE stimulated release at 1:500 dilution) was observed. In addition, anti-Gαq and anti-Gα11 antibodies did not have any significant effects on histamine release.

Effects of Anti-Gα12 Antibody on the IgE Receptor-Mediated Ca^{2+} Influx in Streptolysin-O-Permeabilized Human Umbilical Cord Blood-Derived Mast Cells

As demonstrated in Fig. 4, $[Ca^{2+}]_i$ in the permeabilized human cultured mast cell increased in a dose- and time-dependent manner. Optical density that reflects the level of $[Ca^{2+}]_i$ reached peak value (275,700–289,300 AU) at approximately 25–50 s after stimulation with 1.0 μg/ml anti-IgE antibody and it lasted longer than that of the cell treated with 0.1 μg/ml anti-IgE antibody (Figs. 4A and 4B). The basal value in the nonstimulated cell was shown to be approximately 182,000 AU. In order to test the effects of anti-Gα12 antibody on the changes of cellular calcium level, the antiserum was included in the medium, however, no changes were observed in $[Ca^{2+}]_i$ at time zero (approximately 196,600 AU), indicating that anti-Gα12 antibody by itself did not have any effects on intracellular calcium concentration in nonstimulated cells (refer to $[Ca^{2+}]_i$ profiles at time zero of Figs. 4B and 4C). Moreover, in the presence of the anti-Gα12 antibody (1:1,000 dilution), no increase was observed in $[Ca^{2+}]_i$ at each time point, even in the anti-IgE-antibody-stimulated cells, except for a slight increase at 2 s after stimulation (approximately 203,000 AU that corresponded to only 3.3% increment of basal level) (Fig. 4C).

Table 2. Concentration- and time-dependent IgE/anti-IgE-induced histamine release in human cord blood-derived mast cells.[a]

	Incubation with IgE	Stimulation with anti-IgE (1.0 μg)	Histamine release (%)
1 h	1.0 μg	30 min	36.3±1.5
2 h	1.0 μg	1 h	21.2±1.8
overnight	10.0 μg	1 h	–[b]
4 days	0.05 μg	2 h	15.7±1.0
4 days	5.0 μg	2 h	26.2±2.8

[a]Human umbilical cord blood-derived mast cells were cultured for 12 weeks as described in Materials and Methods. The cells (2×10^5 cells) were sensitized with each indicated concentration of myeloma IgE, and followed by stimulation with 1.0 μg anti-IgE for the indicated time period (N=6).
[b]Histamine release was not detected in this experimental condition.

Discussion

It was shown that from the pharmacological view point, human cultured mast cells resemble lung mast cells [18], possessing a functional signaling pathway via the IgE receptor [20]. Thus, human cultured mast cells are considered to be suitable for studying developmental stage-dependent changes in IgE receptor-mediated histamine secretion.

In the human mast cells with streptolysin-O-induced pores on cell membrane, anti-Gα12 and anti-Gα13 antibodies effectively inhibited the IgE receptor-induced histamine release. With regards to this, all the data were obtained by using anti-Gα12 and anti-Gα13 antiserum.

As for the intracellular signaling molecules involved in the exocytosis, it is well known that Ca^{2+} ion plays an essential role in triggering histamine secretion in mast cells [15, 25]. Thus, it is highly possible that the effect of anti-

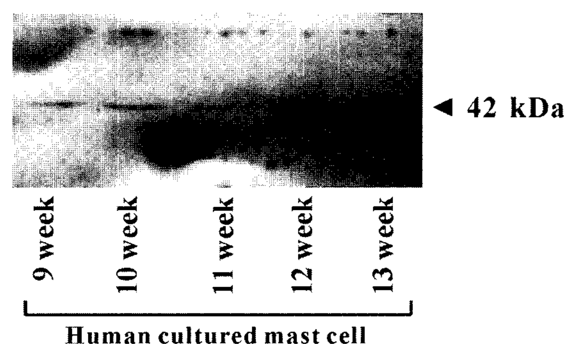

Fig. 2. Western blot analysis of the Gα12 and Gα13 protein expressions in human umbilical cord blood-derived mast cells.
Human umbilical cord blood-derived mast cells (1×10^5 cells) at 9, 10, 11, 12, and 13 week-old cultures were collected and washed twice with phosphate-buffered saline (PBS). Then, cell membranes were suspended in 25 μl of RIPA buffer, subjected to SDS-gel electrophoresis, and then electrotransferred onto PVDF membranes, as described in Materials and Methods. Gα12 and Gα13 proteins were detected with polyclonal antibody raised against Gα12 (1:1,000) that can recognize both Gα12 and Gα13 proteins. The level of expression was visualized with a West-Zol Western blot detection kit.

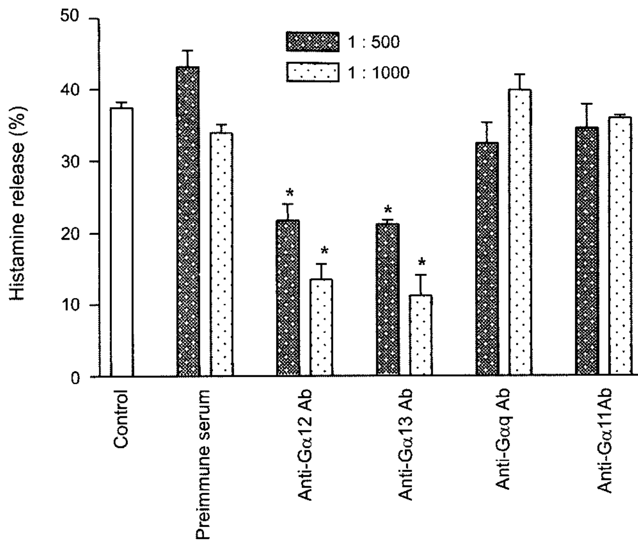

Fig. 3. Effects of various anti-Gα subunit antibodies on the histamine release in the activated human umbilical cord blood-derived mast cells.

Streptolysin-O-permeabilized mast cells (purity>99%, 12 weeks of culture) were sensitized with human myeloma IgE (1.0 μg/2×10^5 cells) for 1 h, followed by challenge with anti-IgE antibody (1.0 μg/ml for 30 min at 37°C) in the presence of anti-Gα12, anti-Gα13, anti-Gαq, or anti-Gα11 antibody, or pre-immune serum that was added 5 min before challenge, respectively. Amount of histamine released in the supernatant was measured and expressed as percentage of the total histamine contents in nonstimulated mast cells. Detection limit of the histamine assay was approximately 5 ng/ml. * denotes $P<0.05$ as compared with control.

Gα12 antibody on histamine release is due to modulation of Ca^{2+} response in mast cells. In the present study, therefore, anti-Gα12 antibody was investigated as a potential inhibitor of the Ca^{2+} influx pathway. Control experiments demonstrating the IgE receptor-mediated changes of $[Ca^{2+}]_i$ in a human cultured mast cell in the presence of external Ca^{2+} revealed the confocal microscopic $[Ca^{2+}]_i$ image profile to be similar to that obtained in a guinea pig lung mast cell [16]. Anti-Gα12 antibody at concentration similar to that required for inhibiting histamine secretion also inhibited the antigen-induced increase in $[Ca^{2+}]_i$, measured at the single cell level. This action of the anti-Gα12 antibody seemed to be mediated by both Gα12 and Gα13 proteins in cultured human mast cells, since anti-Gα12 and anti-Gα13 antibodies cross-reacted against both Gα12 and Gv13 proteins, but did not recognize the members of Gαq class subunits. In addition, as shown in Fig. 3, the anti-Gαq and anti-Gα11 antibodies did not have any significant effects on histamine release nor the pre-immune serum. Therefore, the results suggest that Gα12 and Gα13 may modulate IgE receptor-activated Ca^{2+} influx, thereby regulating histamine release in cultured human mast cells. However, this observation is contrary to the results obtained in PC12 cells, which may be due to differences in the exocytosis machinery of each cell type.

In human mast cells, the possibility that Gα12 and Gα13 are involved in the regulation of exocytosis by acting downstream of the Ca^{2+} signal was not excluded. Disruption of the peripheral actin barrier is required for efficient exocytosis in mast cells [12, 14]. Considering the facts that Gα12 and Gα13 induce cellular cytoskeleton changes via small G protein during the cell proliferation and differentiation, and that anti-Gα12 and anti- Gα13 antibodies effectively inhibited IgE receptor-mediated exocytosis, Gα12 and Gα13 may affect histamine release through alteration in actin peripheral network rearrangement in cultured human mast cells, which remains to be established.

It has been shown that Gαo and Gαi regulate the peripheral actin network and catecholamine secretion through mechanism involving the monomeric G protein Rho in the chromaffin cells [5]. In mast cells, it has been suggested that the plasma membrane-associated Gαi3 might transduce signals to monomeric GTPases, Rho and Rac, thereby leading to mediator secretion [9, 13, 14]. Rho together with Ca^{2+} is an important modulator of actin cytoskeleton that is implicated in the mechanism of exocytosis. Thus, in order to understand in detail the role of Gα12 and Gα13 in the exocytotic process in human cultured mast cells, it is worthwhile to study the possibility of whether this protein interacts with those monomeric G proteins in the mast cell.

Experiments were performed with streptolysin-O-permeabilized human cultured mast cells. It was highly possible that cell membrane disruption and subsequent leakage of soluble proteins were inherent and the progressive

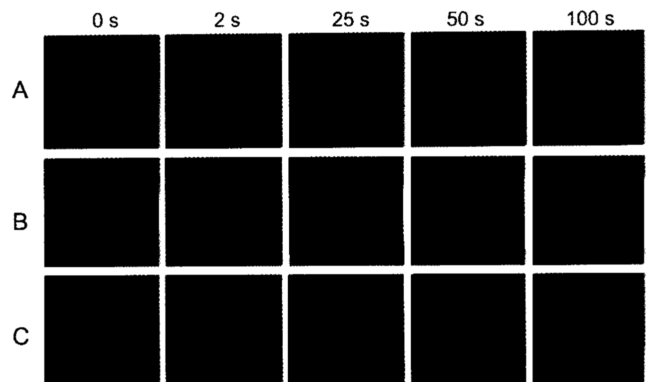

Fig. 4. Effect of anti-Gα12 antibody on the Ca^{2+} influx in a single human umbilical cord blood-derived mast cells activated with human myeloma IgE/anti-IgE antibody.

Fluo-3 AM (5.0 μM) was added to the streptolysin-O-permeabilized mast cells (4×10^5 cells), followed by incubating for 30 min. Cells were then fixed on a glass slide and activated with IgE/anti-IgE antibody in the absence or presence of anti-Gα12 antibody. Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were monitored at every 2 sec for 100 sec with a confocal laser scanning microscope as described in Materials and Methods. A, 0.1 μg anti-IgE antibody challenge; B, 1.0 μg anti-IgE antibody challenge; C, 1.0 μg anti-IgE antibody challenge in the presence of anti-Gα12 antibody (1:1,000 dilution). Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) at 0, 2, 25, 50, and 100 s are shown in each picture. S indicates the time in seconds after activation with anti-IgE antibody.

decrease in secretion efficiency was unavoidable. Preliminary experiments were performed in order to find the minimal concentration of streptolysin-O and the shortest exposure period to it, and it was found that it did not have any harmful effects on human cultured mast cells at 1.5 IU/ml and for 5 min treatment. Thus, permeabilized human cultured mast cells still maintained the IgE-induced secretory response that was similar to that of intact cells (37.5± 0.84% vs 36.3±1.53%). This indicated that functions of the cell membrane signaling components might not have been severely affected by the short-term (5 min) with low concentration treatment of streptolysin-O. In fact, for permeabilized adrenal chromaffin cells and for eosinophils, ultrastructural examination indicates that secretion occurs by an authentic exocytotic mechanism involving fusion of the secretory granule membranes with the plasma membrane [3, 11].

Considering that heterotrimeric Gα12 and Gα13 are membrane-bound proteins, and antibodies against these proteins induce the changes in the histamine-releasing capability via modulating the cellular Ca^{2+} profile, the role of Gα12 and Gα13 proteins as authentic regulators in exocytosis in cultured human mast cells is suggested. This is the first report showing that Gα12 and Gα13 are involved in the regulation of Ca^{2+}-mediated exocytosis in cultured human mast cells, although detailed mechanisms remain to be elucidated.

Acknowledgments

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