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IgE alone-induced actin assembly modifies calcium signaling and degranulation in RBL-2H3 mast cells

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Oka, Tatsuya, Masatoshi Hori, Akane Tanaka, Hiroshi Matsuda, Hideaki Karaki, and Hiroshi Ozaki. IgE alone-induced actin assembly modifies calcium signaling and degranulation in RBL-2H3 mast cells. *Am J Physiol Cell Physiol* 286: C256–C263, 2004. First published September 24, 2003; 10.1152/ajpcell.00197.2003.—In the mast cell signaling pathways, the binding of immunoglobulin E (IgE) to FcεRI, its high-affinity receptor, is generally thought to be a passive step. In this study, we examined the effect of IgE alone, that is, without antigen stimulation, on the degranulation in mast cells. Monomeric IgE (500–5,000 ng/ml) alone increased cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and induced degranulation in rat basophilic leukemia (RBL)-2H3 mast cells. Monomeric IgE (5,000 ng/ml) alone also increased $[Ca^{2+}]_i$ and induced degranulation in bone marrow-derived mast cells. Interestingly, monomeric IgE (5–50 ng/ml) alone, in concentrations too low to induce degranulation, increased filamentous actin content in RBL-2H3 mast cells. We next examined whether actin dynamics affect the IgE alone-induced RBL-2H3 mast cell activation pathways. Cytochalasin D inhibited the ability of IgE alone (50 ng/ml) to induce de novo actin assembly. In cytochalasin D-treated cells, IgE (50 ng/ml) alone increased $[Ca^{2+}]_i$ and induced degranulation. We have summarized the current findings into two points. First, IgE alone increases $[Ca^{2+}]_i$ and induces degranulation in mast cells. Second, IgE, at concentrations too low to increase either $[Ca^{2+}]_i$ or degranulation, significantly induces actin assembly, which serves as a negative feedback control in the mast cell Ca^{2+} signaling and degranulation.

mast cell; immunoglobulin E; cytochalasin D; Y-27632; wortmannin

THE HIGH-AFFINITY RECEPTOR for immunoglobulin E (IgE), FcεRI, that is expressed on mast cells is a crucial component in allergic responses. It is well known that FcεRI must be aggregated to initiate signal transduction (13). Cross-linking of IgE-bound FcεRI (IgE/FcεRI) by a multivalent antigen (allergen) initiates a biochemical cascade (6, 13). In addition, Segal et al. (18) demonstrated that covalently cross-linked aggregated IgE (dimers plus higher molecular weight aggregates) also initiates phosphorylation of tyrosines and induces degranulation. This suggests that aggregation of FcεRI is important for mast cell activation, regardless of antigen stimulation.

However, several recent studies have revealed the ability of monomeric IgE to enhance mast cell functions, such as FcεRI expression (22), cell survival (1), cytokine production (9), and cell adhesion (11), in the absence of antigen stimulation. It was also reported that, in bone marrow-derived mast cells (BMMC), monomeric IgE alone, that is, without antigen stimulation, increases the cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) (8, 11, 21). The increment in $[Ca^{2+}]_i$ is considered to play a pivotal role in the mast cell signaling pathways for triggering degranulation because the increase in $[Ca^{2+}]_i$ through capacitative Ca^{2+} entry by thapsigargin, a potent inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum membrane, or ionomycin, a Ca^{2+} ionophore, provokes degranulation without FcεRI activation (13, 15). These reports suggest that the increase in $[Ca^{2+}]_i$ can trigger mast cell degranulation. Although monomeric IgE alone can increase $[Ca^{2+}]_i$, it is not known whether monomeric IgE alone can induce degranulation.

We previously reported that IgE/FcεRI cross-linking by antigen stimulates filamentous actin (F-actin) assembly and that this reaction serves as a negative control in Ca^{2+} signaling and degranulation in rat basophilic leukemia (RBL)-2H3 mast cells (14). Very recently it was reported that monomeric IgE alone stimulates mast cell adhesion to fibronectin in the absence of antigen stimulation (11). Because F-actin is important in the regulation of cell adhesion in RBL-2H3 mast cells (16, 20), these findings led us to hypothesize that F-actin assembly, mediated by IgE in the absence of antigen stimulation, may play a role in mast cell activation.

In this study, we examined the effect of IgE on F-actin assembly and degranulation in the absence of antigen stimulation and found for the first time that, in RBL-2H3 mast cells, monomeric IgE and aggregated IgE, when added at relatively high concentrations, each induced not only $[Ca^{2+}]_i$ increase but degranulation as well. Furthermore, IgE at lower concentrations (5–50 ng/ml) stimulated actin assembly, and an inhibitor of actin polymerization enhanced the increase in $[Ca^{2+}]_i$ and degranulation in these cells. These results suggest that de novo synthesized F-actin represents a negative feedback control in mast cell activation.

MATERIALS AND METHODS

Cells. RBL-2H3 mast cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (BSA), 100 U/ml penicillin, and 100 μg/ml streptomycin. BMMC were obtained according to the method described previously (12). Bone marrow cells of $WBB6F_1^{+/+}$ mice were cultured in α-minimum essential medium supplemented with 10^{-4} M 2-mercaptoethanol, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml recombinant mouse IL-3. Culture flasks were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. One-half of the medium was replaced every 7 days. More than 90% of the cells were identified as immature mast cells 4 wk after the initiation of the culture. The cells were used at 5 to 6 wk of culture.

Preparation of monomeric IgE. Monomeric IgE was prepared by a method described previously (9). Anti-DNP IgE (Sigma; SPE-7) was fractionated using a high-performance liquid chromatography (HPLC)

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system (Waters, Milford, MA) with a BioSep SEC S3000 gel filtration column (300 × 7.8 mm; Phenomenex, Torrance, CA) equilibrated and run with PBS (50 mM Na-phosphate, 0.3 M NaCl, pH 6.8). Fractions were collected separately and, on the basis of optical density (OD) readings at 280 nm, each fraction was rechromatographed immediately. The prepared IgE content in each fraction was measured using the Bio-Rad Protein Assay kit (Bio-Rad, Tokyo, Japan).

Measurement of $[Ca^{2+}]_i$ and quantification of the Ca^{2+} oscillations. $[Ca^{2+}]_i$ was measured, using fura-PE3, by a method described previously (15). In brief, RBL-2H3 cells grown on glass coverslips were washed with HEPES-buffered solution (in mM: 125.4 NaCl, 11.5 glucose, 5.9 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, and 10 HEPES, pH 7.4). BMMC were attached to glass coverslips in HEPES-buffered solution. The cells were loaded with 5 μM fura-PE3 for 40 min in a dark room at 37°C. Every 3 s, the images at 340 nm were divided by the images at 380 nm to provide resultant ratio images that are indicators of $[Ca^{2+}]_i$.

Because the changes in $[Ca^{2+}]_i$ were oscillatory, asynchronous, irregular, and varied tremendously among cells, we used the area under the curve (AUC) analysis described elsewhere (15). In brief, data are expressed as relative values that are derived by taking the resting level of $[Ca^{2+}]_i$ as 0% and the level in the presence of 3 μM ionomycin with 3 mM Ca^{2+} as 100%. The area under the curve representing the change in $[Ca^{2+}]_i$ over time was measured to quantify each response.

β-Hexosaminidase degranulation. We measured the release of β-hexosaminidase as an index of degranulation, using a method described elsewhere (15). In brief, RBL-2H3 cells (2×10^5 cells/well) in 24-well plates or BMMC (2×10^5 cells/tube) were washed with PIPES-buffered solution (in mM: 140 NaCl, 5 KCl, 5.5 glucose, 0.6 $MgCl_2$, 1.5 $CaCl_2$, 10 PIPES, and 0.1% BSA, pH 7.4). The cells were stimulated at 37°C under gentle rotation. The supernatants were collected and transferred to a 96-well plate. Subsequently, Triton X-100 solution (0.5%) was added to the cells to quantify the remaining enzyme activity. The extracts were transferred to another 96-well plate. The absorbance at 405 nm (OD) of each well was measured. The percentage of degranulation was calculated using the following formula: $\%\text{degranulation} = OD_{supernatant}/(OD_{supernatant} + OD_{Triton\ X\text{-}100}) \times 100$.

F-actin assay. Total filamentous actin (F-actin) content in RBL-2H3 cells was measured as described previously (5, 14). In brief, cells were stained by 6 U/ml FITC-labeled phalloidin, and FITC-phalloidin bound to F-actin was extracted by incubating the cells with 75% methanol for 2 h at 4°C. The relative fluorescence of the supernatant, which included the extracts, was measured with an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The percentage of F-actin was given by taking the resting value to be 100%.

Chemicals. IL-3 was kindly provided by Kirin Brewery (Tokyo, Japan). Dinitrophenyl human serum albumin (DNP-HSA), monoclonal anti-DNP (mouse IgE isotype: SPE-7), thapsigargin, Triton X-100, cytochalasin D, *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide, DMEM powder, BSA, and 2-mercaptoethanol were from Sigma (St. Louis, MO). α-Minimum essential medium was from GIBCO (Grand Island, NY). Wortmannin and Y-27632 were from Calbiochem (Darmstadt, Germany). Fura-PE3/AM was from TEF Labs (Austin TX); cremophor EL was from Nacalai Tesque (Tokyo, Japan), EGTA was from Dojindo Laboratories (Kumamoto, Japan), and fetal bovine serum and fetal calf serum were from Filtron (Brooklyn, Australia).

Statistical analysis. Results of the experiments are expressed as means ± SE. Statistical evaluation of the data was performed using the unpaired Student's *t*-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA), followed by either Dunnett's test or the Tukey test for comparisons among more than two groups. A value of $P < 0.05$ was taken as significant.

RESULTS

IgE alone increased $[Ca^{2+}]_i$ and induced degranulation. We first investigated the effects of various concentrations of unfractionated IgE (5–5,000 ng/ml) on $[Ca^{2+}]_i$ in RBL-2H3 cells without DNP-HSA stimulation. We found that 5–50 ng/ml anti-DNP IgE did not change $[Ca^{2+}]_i$, whereas 500–5,000 ng/ml IgE increased $[Ca^{2+}]_i$ (Figs. 1, *A–E*) in a concentration-dependent manner (Fig. 1*F*). As shown in Fig. 1*F*, in cells sensitized to 50–5,000 ng/ml IgE, the addition of 10 ng/ml DNP-HSA induced a greater increase in $[Ca^{2+}]_i$. In the 50 ng/ml IgE-

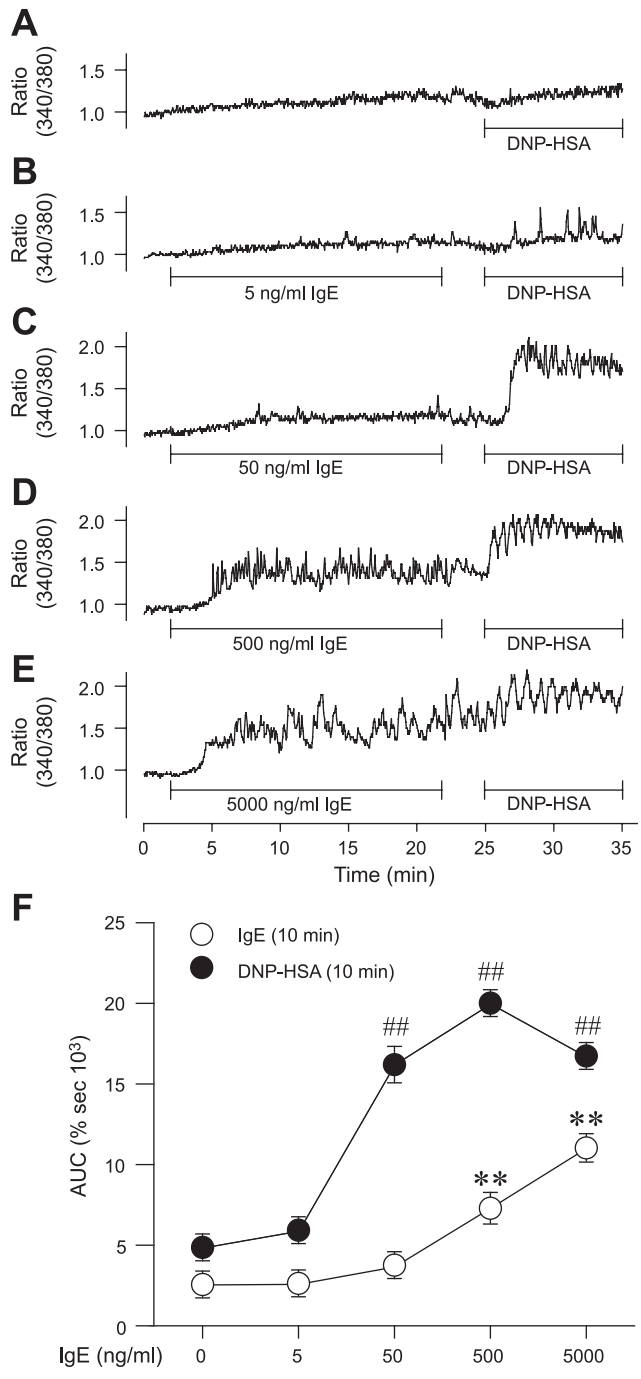


Fig. 1. Effect of IgE and the subsequent addition of dinitrophenyl human serum albumin (DNP-HSA) on cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in RBL-2H3 cells. Typical recordings show 0 (*A*), 5 (*B*), 50 (*C*), 500 (*D*), and 5,000 ng/ml IgE (*E*) (unfractionated)-induced increases in $[Ca^{2+}]_i$ in fura-PE3-loaded RBL-2H3 cells. Cells were subsequently stimulated with 10 ng/ml DNP-HSA. *F*: area under the curve (AUC) for 10 min after IgE and DNP-HSA stimulation shown in *A–E*. Results are expressed as means ± SE of 29–50 cells from 4 separate experiments. $^{**}P < 0.01$, $^{\#\#}P < 0.01$ compared with 0 ng/ml IgE.

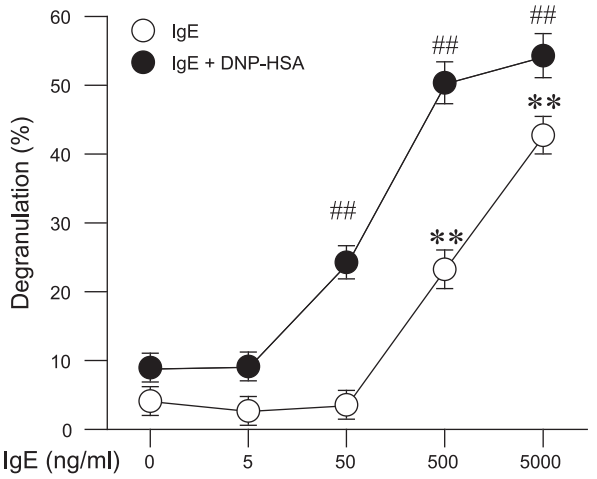


Fig. 2. Effect of IgE and the subsequent addition of DNP-HSA on degranulation in RBL-2H3 cells. Cells were stimulated with different concentrations of IgE (unfractionated) for 20 min. Subsequently, cells were stimulated with 10 ng/ml DNP-HSA for 10 min. The amount of β-hexosaminidase released by IgE or IgE + DNP-HSA was calculated as a percentage of the total amount of β-hexosaminidase. Results are expressed as means ± SE of 12–23 experiments. $^{**}P < 0.01$, $^{\#\#}P < 0.01$ compared with 0 ng/ml IgE.

sensitized cells, the increase in $[Ca^{2+}]_i$ was initiated only after the addition of DNP-HSA.

As shown in Fig. 2, treatment of RBL-2H3 cells with unfractionated IgE (500–5,000 ng/ml) alone for 20 min induced degranulation in a concentration-dependent manner. In the cells sensitized to 50–5,000 ng/ml anti-DNP IgE for 20 min, the addition of DNP-HSA (10 ng/ml) for 10 min induced greater degranulation. In the 50 ng/ml IgE-sensitized cells, degranulation was initiated only after DNP-HSA was added. Sensitization to 50 ng/ml IgE for 3 h did not change the ability of DNP-HSA to induce degranulation (28.7 ± 4.7%; data not shown) compared with the DNP-HSA-induced degranulation in the cells sensitized to 50 ng/ml IgE for 20 min (24.1 ± 3.3%; Fig. 2). This finding suggested that merely 20 min of IgE sensitization was sufficient to induce the subsequent response to antigen.

Monomeric IgE alone increased $[Ca^{2+}]_i$ and induced degranulation. It was reported (4) that anti-DNP IgE (Sigma; SPE-7) retains a small amount of aggregates after freezing and thawing. As shown in Fig. 3*A*, the unfractionated IgE contained a small amount of aggregated IgE in addition to monomeric IgE. To separate the two, we pooled gel-filtrated fractions every 30 s, from 4.5 min to 9 min during HPLC, and numbered each fraction from 1 to 9. The freshly prepared fractions were rechromatographed immediately (Fig. 3*B*). To calculate the percentage of aggregated IgE in unfractionated IgE, we measured the protein content (i.e., the IgE content) in each fraction. For convenience, we assumed that *fractions 1–3* and one-half of *fraction 4* contained aggregated IgE only and

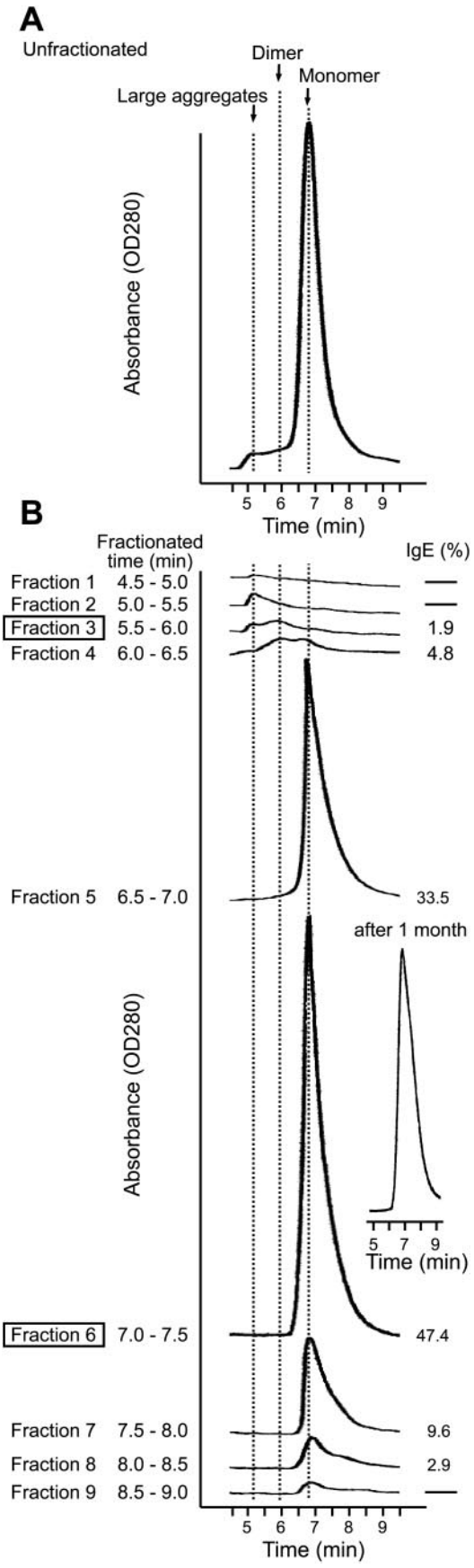


Fig. 3. Separation of monomeric IgE and aggregated IgE. Chromatogram shows typical recordings of unfractionated anti-DNP IgE (Sigma; SPE-7) (*A*), freshly fractionated IgE (*B, fractions 1–9*), and IgE after storage for 1 mo (*B, inset, fraction 6*). *B*: IgE content in each fraction was calculated as a percentage of the unfractionated IgE. An IgE% value represented by a dash indicates that IgE contained in that fraction was below our detection threshold. Results are representative of 3–4 separate experiments. OD, optical density.

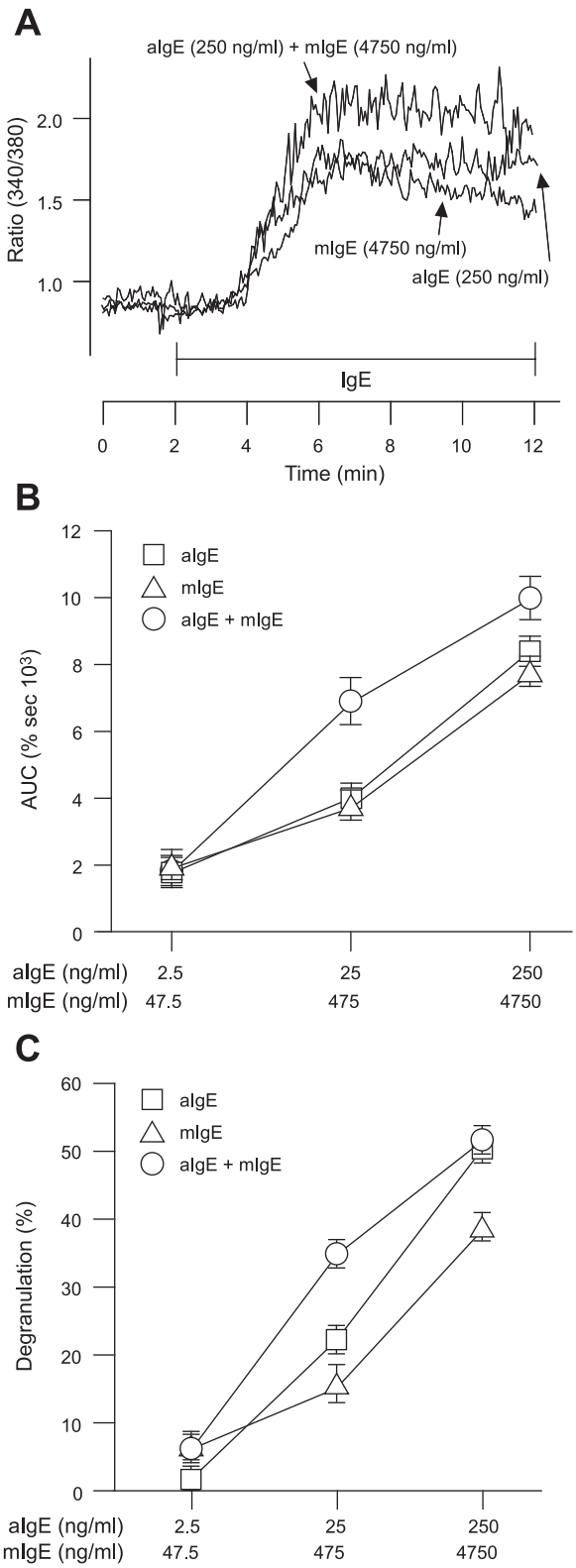


Fig. 4. Effect of IgE on $[Ca^{2+}]_i$ and degranulation in RBL-2H3 cells. *A*: typical recordings of aggregated IgE (aIgE; 250 ng/ml)-, monomeric IgE (mIgE; 4,750 ng/ml)-, and aIgE (250 ng/ml) + mIgE (4,750 ng/ml)-induced increase in $[Ca^{2+}]_i$ in the fura-PE3-loaded RBL-2H3 cells. *B*: AUC for 10 min after IgE stimulation in *A*. Results are expressed as means ± SE of 22–64 cells from 4 separate experiments. *C*: RBL-2H3 cells were stimulated with aIgE, mIgE, and aIgE + mIgE for 20 min. The released β-hexosaminidase was calculated as a percentage of the total amount of β-hexosaminidase. Results are expressed as means ± SE of 4 experiments.

that the rest of the fractions contained monomeric IgE only. Under that assumption, this lot of anti-DNP IgE contained 4–5% aggregated IgE. As shown in Fig. 3*B*, *inset*, the purified monomeric IgE remained in the monomeric state for at least 1 mo. Therefore, in the current study we used freshly prepared *fraction 3* as aggregated IgE (equilibrated to 5% of the unfrac-

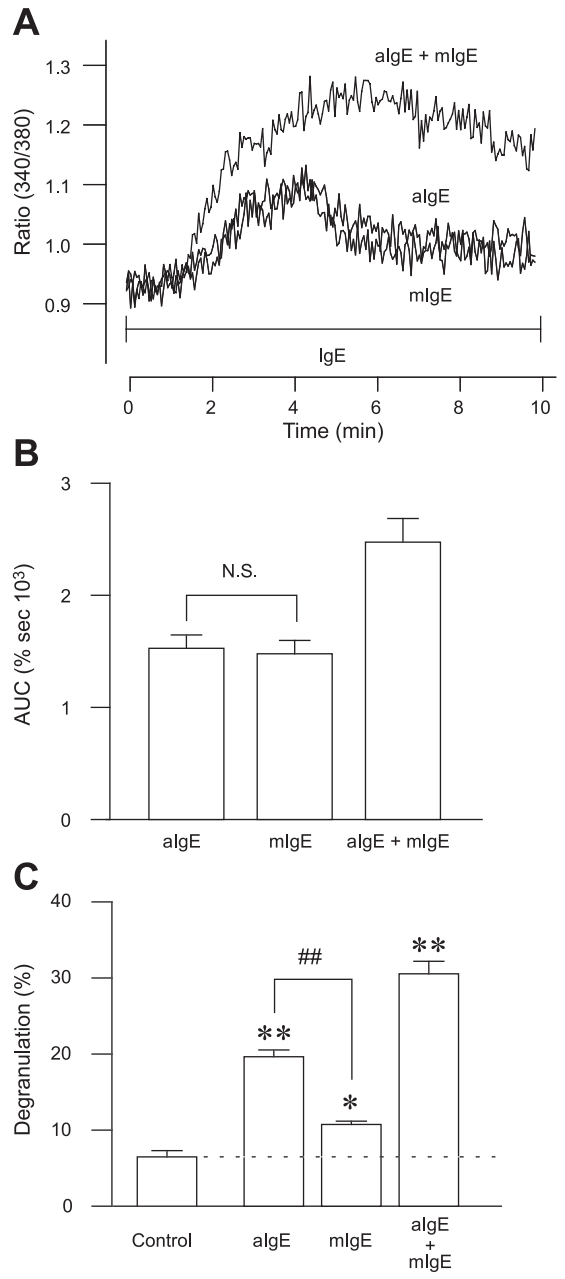


Fig. 5. Effect of IgE on $[Ca^{2+}]_i$ and degranulation in bone marrow-derived mast cells (BMMC). *A*: typical recordings of aIgE (250 ng/ml)-, mIgE (4,750 ng/ml)-, and aIgE (250 ng/ml) + mIgE (4,750 ng/ml)-induced increases in $[Ca^{2+}]_i$ in the fura-PE3-loaded BMMC. *B*: AUC for 10 min after IgE stimulation in *A*. Results are expressed as means ± SE of 32–39 cells from 3 separate experiments. *C*: BMMC were stimulated without (control) or with aIgE (250 ng/ml), mIgE (4,750 ng/ml), and aIgE (250 ng/ml) + mIgE (4,750 ng/ml) for 20 min. The released β-hexosaminidase was calculated as a percentage of the total amount of β-hexosaminidase. Results are expressed as means ± SE of 8 experiments. $*P < 0.05$, $**P < 0.01$, compared with control. $\#\#P < 0.01$, aIgE vs. mIgE. N.S., not significantly different.

tionated IgE concentration) and *fraction 6* as monomeric IgE (equilibrated to 95% of the unfractionated IgE concentration).

As shown in Fig. 4, *A* and *B*, in RBL-2H3 cells, freshly prepared monomeric IgE (*fraction 6*: 47.5–4,750 ng/ml) increased $[Ca^{2+}]_i$ in the absence of DNP-HSA stimulation in a concentration-dependent manner. Aggregated IgE (*fraction 3*: 2.5–250 ng/ml) induced similar increases in $[Ca^{2+}]_i$. A mixture of aggregated and monomeric IgE increased $[Ca^{2+}]_i$ to a greater efficacy than either aggregated or monomeric IgE alone.

As shown in Fig. 4*C*, in RBL-2H3 cells, aggregated IgE (*fraction 3*: 2.5–250 ng/ml) induced degranulation in the absence of DNP-HSA stimulation. Monomeric IgE (*fraction 6*: 47.5–4,750 ng/ml) induced degranulation to a slightly lesser extent than aggregated IgE. The mixture of aggregated IgE and monomeric IgE induced degranulation with greater efficacy than aggregated IgE alone.

To determine whether the effect of monomeric IgE was limited to RBL-2H3 mast cells, we examined the effect of monomeric IgE on BMMC. As was the case in RBL-2H3 cells, $[Ca^{2+}]_i$ in BMMC was increased to the same level by freshly prepared aggregated IgE (*fraction 3*: 250 ng/ml) as by monomeric IgE (*fraction 6*: 4,750 ng/ml) (Fig. 5, *A* and *B*). The mixture of aggregated IgE and monomeric IgE also increased $[Ca^{2+}]_i$ with greater efficacy in BMMC than either aggregated IgE or monomeric IgE alone.

As shown in Fig. 5*C*, aggregated IgE (*fraction 3*: 250 ng/ml) induced degranulation in BMMC. Likewise, monomeric IgE (*fraction 6*: 4,750 ng/ml) induced slight but still significant degranulation. The mixture of aggregated IgE and monomeric IgE also induced more extensive degranulation.

IgE alone increased F-actin content. As shown in Fig. 6*A*, in RBL-2H3 cells, unfractionated IgE (5–5,000 ng/ml) increased F-actin content in a concentration-dependent manner in the absence of DNP-HSA stimulation. At 5–50 ng/ml concentration, IgE significantly increased the F-actin content, whereas it did not induce an increase in $[Ca^{2+}]_i$ or degranulation (see Figs. 1 and 2). IgE (50 ng/ml) began to increase F-actin content no later than 5 min after stimulation, and the F-actin content reached a steady level within 5 min after stimulation (Fig. 6*B*).

It is well known that actin assembly in mast cells is regulated by multiple factors. We investigated the possible involvement of two of those factors, the Rho/ROCK (RhoA-dependent coiled coil serine/threonine kinase) and Ras/phosphatidylinositol 3-kinase (PI3K) pathways (19) in IgE-induced actin assembly, using selective inhibitors. As shown in Fig. 6*C*, a PI3K inhibitor, wortmannin (50 nM), and a ROCK inhibitor, Y-27632 (10 μM), each eliminated IgE-induced F-actin assembly.

As shown in Fig. 6*D*, the freshly prepared aggregated IgE (*fraction 3*: 0.25–25 ng/ml) and the monomeric IgE (*fraction 6*: 4.75–475 ng/ml) each increased F-actin content. The mixture of aggregated IgE and monomeric IgE induced more extensive actin assembly.

Inhibition of IgE-induced actin assembly augmented the increase in $[Ca^{2+}]_i$ and degranulation. Cytochalasin D, an inhibitor of de novo actin polymerization by binding and capping the barbed end of F-actin (2, 3), prevented the de novo polymerized F-actin by 50 ng/ml IgE in a concentration-dependent manner (Fig. 7). Because 300 nM cytochalasin D almost completely inhibited the 50 ng/ml IgE-induced F-actin

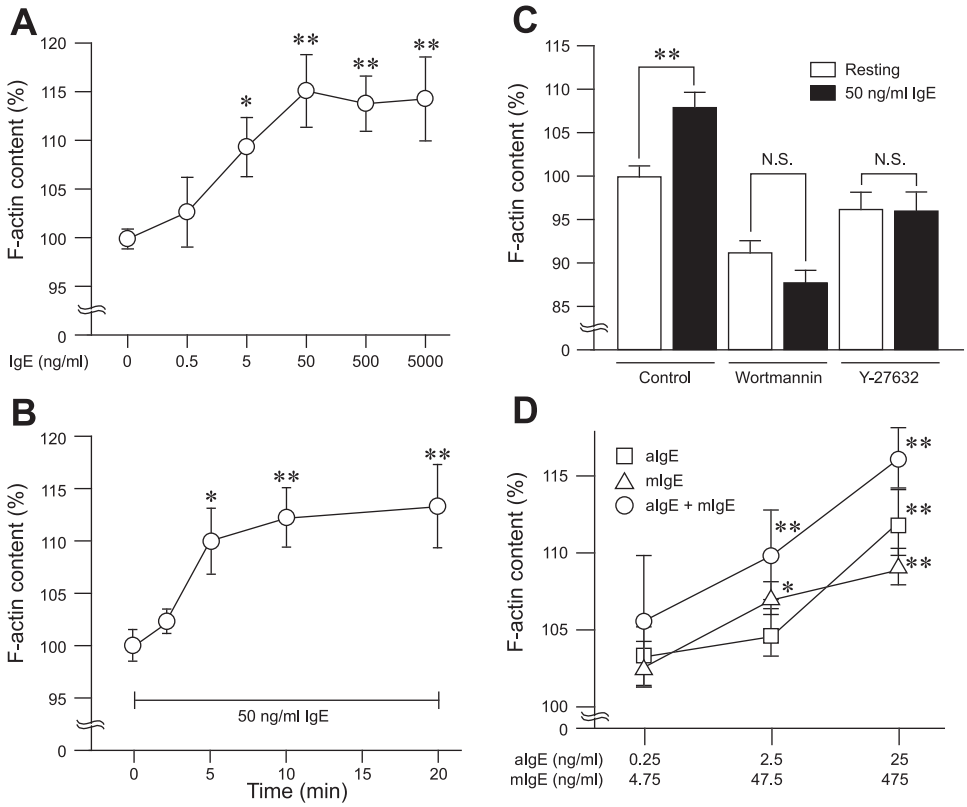


Fig. 6. Effect of IgE on F-actin content. *A*: RBL-2H3 cells were stimulated with the indicated concentrations of IgE (unfractionated) for 10 min. $*P < 0.05$, $**P < 0.01$ compared with 0 ng/ml IgE. *B*: RBL-2H3 cells were stimulated with 50 ng/ml IgE for the indicated times. $*P < 0.05$, $**P < 0.01$ compared with 0 min. *C*: RBL-2H3 cells were pretreated without or with 50 nM wortmannin or 10 μM Y-27632 for 30 min and subsequently stimulated with 50 ng/ml IgE for 10 min. $**P < 0.01$ compared with resting cells. *D*: RBL-2H3 cells were stimulated with freshly prepared aIgE, mIgE, and aIgE + mIgE for 10 min. F-actin content is shown as a percentage of the F-actin content in resting cells. $*P < 0.05$, $**P < 0.01$ compared with resting cells. Results are expressed as means ± SE of 9–26 experiments.

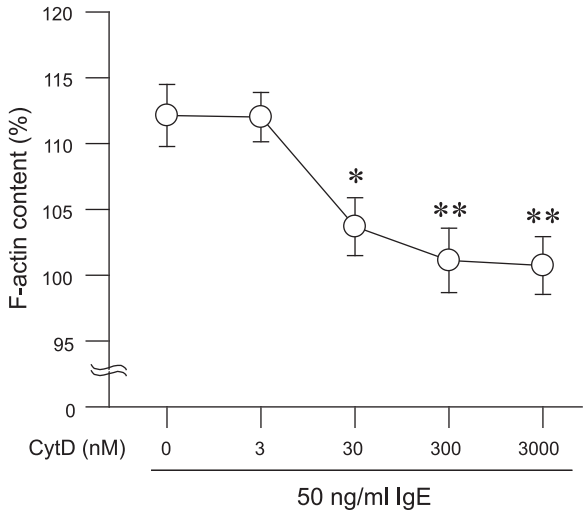


Fig. 7. Effect of cytochalasin D on IgE-induced F-actin assembly. RBL-2H3 cells were pretreated with the indicated concentrations of cytochalasin D (CytD) for 30 min and subsequently stimulated with 50 ng/ml IgE for 10 min. F-actin content is shown as a percentage of the F-actin content in resting cells. Results are expressed as means ± SE of 12 experiments. *$P < 0.05$, **$P < 0.01$ compared with 0 nM CytD.

assembly, this concentration of cytochalasin D was employed in the following experiments.

As shown in Fig. 8, *A* and *B*, treatment of the cells with 300 nM cytochalasin D for 30 min did not influence the resting $[Ca^{2+}]_i$. However, in the 300 nM cytochalasin D-pretreated cells, 50 ng/ml IgE induced the oscillatory increase in $[Ca^{2+}]_i$. AUC analysis demonstrated that 50 ng/ml IgE significantly increased $[Ca^{2+}]_i$ in the 300 nM cytochalasin D-treated cells (Fig. 8*C*).

As shown in Fig. 9*A*, 300 nM cytochalasin D did not influence the resting level of granule release. However, in the 300 nM cytochalasin D-pretreated cells, 50 ng/ml IgE significantly increased the level of degranulation (Fig. 9*B*).

DISCUSSION

As described in the Introduction, although Segal et al. (18) suggested that covalently cross-linked aggregated IgE initiates mast cell activation, several recent studies have demonstrated that monomeric rather than aggregated IgE induces cytokine production (9) or cell adhesion (11). In this study, we investigated the effect of IgE alone on Ca^{2+} signaling and degranulation, which are mediated by de novo actin assembly in RBL-2H3 mast cells, by using anti-DNP IgE (Sigma; SPE-7) and freshly fractionated IgE in both aggregated and monomeric forms (Fig. 3).

The present results (Fig. 5, *A* and *B*) and several studies (8, 11, 21) indicated that monomeric IgE alone, that is, without antigen-mediated FcεRI cross-linking, increase $[Ca^{2+}]_i$ in BMMC. Likewise, in our current study using RBL-2H3 mast cells, IgE at concentrations higher than 500 ng/ml increased $[Ca^{2+}]_i$ without antigen stimulation (Fig. 1, *A–F*). As demonstrated in Fig. 4, *A* and *B*, monomeric IgE in RBL-2H3 cells increased $[Ca^{2+}]_i$ to the same level that aggregated IgE did. These results suggest that monomeric IgE can increase $[Ca^{2+}]_i$ not only in BMMC but also in RBL-2H3 cells.

The increase in $[Ca^{2+}]_i$ is considered to play a pivotal role in the mast cell signaling pathways for degranulation, because the

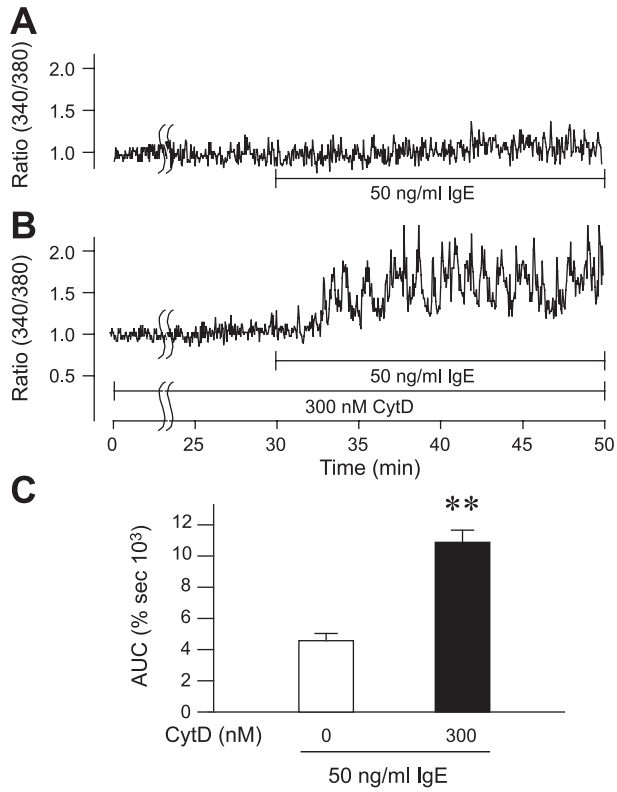


Fig. 8. Effect of CytD on IgE-induced increase in $[Ca^{2+}]_i$. Typical recordings are shown of the effect of 50 ng/ml IgE on $[Ca^{2+}]_i$ in the fura-PE3-loaded RBL-2H3 cells without (*A*) or with 300 nM CytD pretreatment for 30 min (*B*). Results are representative examples of 52–71 cells. *C*: AUC for 20 min after the 50 ng/ml IgE stimulation. Results are expressed as means ± SE of 52–71 cells. **$P < 0.01$ compared with control.

increase in $[Ca^{2+}]_i$ (induced by such substances as thapsigargin or ionomycin) provokes degranulation without FcεRI activation (see Introduction) (13, 15). As demonstrated in Fig. 2, IgE at the concentrations higher than 500 ng/ml in RBL-2H3 cells not only increased $[Ca^{2+}]_i$ but also induced degranulation. Furthermore, the freshly prepared monomeric IgE initiated

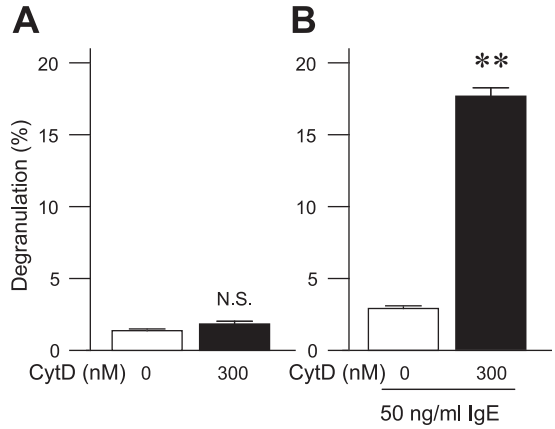


Fig. 9. Effect of CytD on degranulation. RBL-2H3 cells were pretreated with or without 300 nM CytD for 30 min. Subsequently, cells were stimulated with 50 ng/ml IgE for 20 min. The released β-hexosaminidase in the pretreatment step (*A*) or 50 ng/ml IgE treatment step (*B*) was calculated as a percentage of the total amount of β-hexosaminidase. Results are expressed as means ± SE of 12 experiments. **$P < 0.01$ compared with control.

degranulation (Fig. 4*C*). These results suggest that the increase in $[Ca^{2+}]_i$ is essential for RBL-2H3 mast cell degranulation, regardless of the antigen-mediated FcεRI cross-linking. In BMMC, however, several reports have demonstrated that monomeric IgE alone can induce a sustained increase in $[Ca^{2+}]_i$ (8, 11, 21), although in other studies it failed to initiate significant degranulation (8, 9). The present study also confirmed that monomeric IgE induced a sustained increase in $[Ca^{2+}]_i$ in BMMC (Fig. 5, *A* and *B*). In addition, monomeric IgE induced slight but significant degranulation in BMMC (Fig. 5*C*). However, considering the ability of monomeric IgE to increase $[Ca^{2+}]_i$, the efficacy of monomeric IgE-induced degranulation in BMMC was low. With regard to the dissociation between $[Ca^{2+}]_i$ and degranulation in BMMC, Huber et al. (8) speculated that the negative feedback activity of SHIP (*src* homology 2-containing inositol phosphatase) was sufficient to inhibit degranulation but not to inhibit Ca^{2+} entry. In RBL-2H3 mast cells, on the other hand, monomeric IgE-induced degranulation was almost equally effective as either aggregated IgE or a mixture of the two (Fig. 4*C*). The reason for this discrepancy between BMMC and RBL-2H3 cells may be that these two cell types require different $[Ca^{2+}]_i$ sensitivities for the degranulation to occur. In addition, it is reported that FcεRI expression in BMMC is originally very low and that IgE binding to FcεRI upregulates the receptor expression (10, 22). Taken together, these results suggest that RBL-2H3 mast cells may be more sensitive than BMMC to the $[Ca^{2+}]_i$ increment, thus more easily releasing granules by stimulation with an antigen or IgE alone. Further investigation is needed to clarify this possibility.

The major question is how monomeric IgE stimulates mast cells in the absence of antigen stimulation. Kalesnikoff et al. (9) proposed a model in which monomeric IgE binds to FcεRI and reduces an inherent repulsion between neighboring receptors, allowing them to slowly form small clusters in detergent-resistant membrane domains known as lipid rafts (6). Concerning antigen stimulation, it was reported that cross-linking of IgE/FcεRI complexes on the RBL-2H3 mast cell membrane leads to their association with lipid rafts, a step that precedes initiation of signaling events by these receptors (7). In addition, it was reported that these membrane lipids anchor the cytoskeleton, which is made up of actin microfilament (6, 17). Therefore, we investigated the effect of IgE alone on F-actin in RBL-2H3 cells. The present study demonstrated that 50 ng/ml IgE alone increased intracellular F-actin (Fig. 6) without increasing $[Ca^{2+}]_i$ (Fig. 1) or degranulation (Fig. 2). As demonstrated in Fig. 6*D*, monomeric IgE (47.5 ng/ml) increased F-actin content. In addition, cytochalasin D, which inhibits actin polymerization by capping the barbed end of F-actin, suppressed the 50 ng/ml IgE-induced de novo actin assembly to the resting level (Fig. 7). Interestingly, 50 ng/ml IgE alone increased $[Ca^{2+}]_i$ (Fig. 8) and induced degranulation (Fig. 9) in cytochalasin D-pretreated RBL-2H3 cells. These results led us to consider that de novo IgE-polymerized F-actin prevents the activation of mast cells. In our previous report, we showed that the IgE/FcεRI cross-linking-induced (IgE plus DNP-HSA) actin assembly serves as a negative feedback control in the mast cell signaling pathways (14). Taking all of these findings together, we hypothesize that the actin polymerization caused by stimulation with IgE alone might be a negative regulatory mechanism to reduce the repulsion between receptors. Sullivan et al. (19) demonstrated that actin assembly in mast cells is dynamically regulated by multiple factors, such as the Rho/ROCK and Ras/PI3K pathways. Indeed, our study demonstrated that in IgE-stimulated RBL-2H3 mast cells, actin assembly was inhibited by the PI3K inhibitor wortmannin and the ROCK inhibitor Y-27632 (Fig. 6*C*). It was also reported (11) that PI3K is required for IgE alone to activate cell adhesion. As just described, because PI3K could potentially be involved in the regulation of mast cell sensitivity to IgE via actin assembly, how the dynamics of actin assembly at steady state are controlled in mast cells, for instance in allergic disorders, should be clarified in future research.

We summarize the current findings into two points. First, monomeric IgE at higher concentrations increases $[Ca^{2+}]_i$ and induces degranulation in RBL-2H3 mast cells. Monomeric IgE also increases $[Ca^{2+}]_i$ and induces degranulation in BMMC, though at much lower efficacy. Second, monomeric IgE, at concentrations too low to increase either $[Ca^{2+}]_i$ or degranulation, significantly induces actin assembly in RBL-2H3 cells. The inhibition of this IgE-induced actin assembly by cytochalasin D initiates Ca^{2+} signaling and degranulation, suggesting that the de novo polymerized F-actin serves as a negative feedback control in the IgE-induced RBL-2H3 mast cell signaling pathways.

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