

Monomeric IgE Stimulates NFAT Translocation Into the Nucleus, a Rise in Cytosol Ca^{2+} , Degranulation, and Membrane Ruffling in the Cultured Rat Basophilic Leukemia-2H3 Mast Cell Line¹

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Mast cells are key regulators in allergy and inflammation, and release histamine, cytokines, and other proinflammatory mediators. In the classical view, IgE acts merely to prime mast cells, attaching to FcεRs but not evoking any cell signaling response until cross-linked by the presence of a multivalent allergen. However, several recent studies have reported that IgE alone can promote cell survival and cytokine production in the absence of cross-linking by allergen. In this study we demonstrate that acute addition of monomeric IgE elicits a wide spectrum of responses in the rat basophilic leukemia-2H3 mast cell line, including activation of phospholipases Cγ and D, a rise in cytosol Ca^{2+} , NFAT translocation, degranulation, and membrane ruffling within minutes. Calcium transients persist for hours as long as IgE is present resulting in the maintained translocation of the transcription factor NFAT to the nucleus. Removal of IgE reverses the signaling processes. Our results indicate that, far from simply preparing the cells for a response to allergen, monomeric IgE can stimulate signaling pathways that lead to degranulation, membrane ruffling, and NFAT translocation. The mechanism of activation is likely to be via aggregation of the FcεR1 because activation by IgE can be inhibited with monovalent hapten. *The Journal of Immunology*, 2004, 172: 4048–4058.

Mast cells and basophils are the main effector cells in IgE-mediated pathological symptoms seen in allergic disorders, such as urticaria, hay fever, and asthma. In addition, they provide host resistance to parasites, and both functions are mediated by the secretion of proinflammatory mediators (1, 2). High-affinity receptors on their surface (FcεR1) bind circulating IgE (allergen-specific) sensitizing the cells to respond subsequently to the allergen (3). The FcεR1 is a tetramer composed of $\alpha\beta\gamma_2$ with the α -chain responsible for binding the Fc portion of the IgE, whereas the β - and γ -chains contain the tyrosine-based activation motifs responsible for transducing the activation signal within the cell. FcεR1 activation is thought to require the cross-linking of the α -chain, which is normally accomplished by multivalent allergen. The cross-linking of the receptor initiates a cascade of phosphorylation events including the activation of the Src family kinases, Lyn and Fyn, as well as the kinase Syk (4–6). This phosphorylation cascade leads to the promotion of signaling complexes organized by adaptor proteins, including linker for T cell activation, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa, and Grb-2-associated binder 2 (GAB2)⁴

(6–8). This initiates the downstream activation of signal transducing enzymes including phospholipase C (PLC)γ, phosphoinositide 3-kinase (PI3K) (4), and phospholipase D (PLD) (9, 10). These lipid-derived second messengers (phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (recruits and activates specific proteins), inositol 1,4,5-trisphosphate (IP₃) (mobilizes Ca^{2+}), diacylglycerol (activates protein kinase C (PKC)), and phosphatidic acid (regulates phosphatidylinositol 4,5-bisphosphate production)) act in concert to evoke numerous downstream functional responses. These include the release of secretory granules (degranulation), the de novo synthesis of proinflammatory lipid mediators, the synthesis and secretion of cytokines, and changes in the actin cytoskeleton.

The classical view of mast cell activation has been that IgE acts merely to prime the cells, attaching to FcεR1 but not evoking any response until allergen promotes the cross-linking of the FcεR1 (4, 11). However, recent results from bone marrow-derived mast cells (BMMCs) indicate that IgE alone could activate several signaling pathways that lead to cytokine production and to cell survival but not degranulation (12). Monomeric IgE was shown to stimulate multiple phosphorylation events including the activation of the mitogen-activated protein kinases (extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase), protein kinase B (PKB), and the β -subunit of the FcεR1 receptor. Phosphorylation was maintained for a longer period compared with IgE-primed cells subsequently challenged with allergen. Synthesis and secretion of cytokines such as IL-6, TNF- α , IL-4, and IL-13 was also enhanced and was suggested to cause increased cell survival (12). The BMMCs also showed increased adhesion to fibronectin (13) but did not degranulate (14). Degranulation could however be triggered in BMMC derived from mice in which the gene for the PIP₃ phosphatase, src homology 2-containing inositol phosphatase (SHIP)1,

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⁴ Abbreviations used in this paper: GAB2, Grb-2-associated binder 2; PLC, phospholipase C; PLD, phospholipase D; PI3K, phosphoinositide 3-kinase; SHIP, src homology 2-containing inositol phosphatase; IP₃, inositol (1,4,5)trisphosphate; HSA, human serum albumin; EGFP, enhanced green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate; BMMC, bone marrow-derived mast cell; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C.

had been deleted. From these studies it was suggested that SHIP is the "gatekeeper" of mast cell degranulation (13, 14). In a separate study, IgE was only shown to promote cell survival but no evidence was found that the binding of monomeric IgE could induce detectable signaling or production of mediators by BMMCs (15). These studies used two different sources of IgE, SPE-7 IgE and H1 DNP- ϵ -206 IgE, which may account for the differences in their experimental results.

In this paper we report that monomeric IgE (SPE-7 IgE and H1 DNP- ϵ -206 IgE) stimulates rat basophilic leukemia (RBL)-2H3 mast cells to raise cytosol Ca^{2+} , PLC and PLD activation, nuclear translocation of the transcription factor NFAT, membrane ruffling, and degranulation. Our studies indicate that monomeric IgE induces intracellular signaling cascades, which are similar to those observed in IgE-primed cells that are subsequently challenged with allergen.

Materials and Methods

Cell culture of RBL-2H3 mast cells

RBL-2H3 cells (London cells) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in a growth medium of DMEM supplemented with 10% (v/v) FCS, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 4 mM glutamine. RBL-2H3 cells also obtained from Dr. M. Wakelam (University of Birmingham, Birmingham U.K.) were cultured similarly, and referred to as Birmingham cells. We also obtained cells from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Animal Cell Culture 312; Braunschweig, Germany) and from Sheffield, U.K., and both cultures were gifts from Dr. J. Coleman (University of Liverpool, Liverpool, U.K.). These two cell lines were cultured at 37°C in a humidified atmosphere at 5% CO_2 in a growth medium of RPMI 1640 supplemented with 10% (v/v) FCS, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 4 mM glutamine.

Imaging of NFAT translocation

To image NFAT translocation, RBL-2H3 mast cells were scraped from the flask and resuspended in electroporation buffer (137 mM NaCl, 3 mM KCl, 1 mM MgCl_2 , 5.6 mM glucose, 20 mM HEPES, pH 7.2) to which was added 30 μg of cDNA for enhanced green fluorescent protein (EGFP)-NFAT1c. Cells were electroporated in a Bio-Rad GenePulser (Hercules, CA) with two 500-V pulses as previously described (10). The cells were immediately placed on ice for 5 min before being plated on glass coverslips and incubated at 37°C for 4–6 h, after which the growth medium was renewed and cells incubated for a further 18 h. IgE was added into the growth medium, and incubated at 37°C in the incubator (5% CO_2) for the indicated times. At the end of the incubation, the growth medium was removed and the cells washed twice with PBS and fixed with 4% paraformaldehyde and mounted with Mowiol mounting medium. This process was conducted at room temperature. The cells were imaged using a Zeiss LSM510 using $\times 40$ oil objective. Five random fields per cover glass were imaged per condition, and used for counting cells. Each condition was done in duplicate, and data were accumulated from two independent experiments.

Measurement of intracellular Ca^{2+}

To measure intracellular calcium responses during acute application of stimulants, cells were loaded with Ca^{2+} indicator, fura 2-AM ester form. Stock solutions (2 mM) of the AM ester form of the fluorescent Ca^{2+} indicator fura 2 (Molecular Probes, Eugene, OR) were made using a solution of 2.5% (w/v) Pluronic F-127 in DMSO. This stock was diluted 1000-fold in the growth medium in the culture dishes and the cells incubated for 30 min at 25°C and 5% CO_2 . At the end of this incubation, the cells were washed with HEPES buffered saline (120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 20 mM HEPES, pH 7.2) and mounted on the stage of the imaging system at room temperature. Ratiometric measurements of intracellular calcium were made every 2 s as stimulants were applied. Two imaging systems (Kinetic Imaging, Wirral, U.K. and TILL Photonics, Munich, Germany) were used and independently calibrated in vitro to determine their R_{min} , R_{max} , and S (16). K_d of fura 2 was taken to be 236 nM at 20°C and 285 nM at 37°C (17). Agonist-evoked calcium increases were calculated cell by cell as the maximum calcium value recorded after agonist application less the prestimulation value. To measure the maintained response of cells to IgE that had been present overnight, the cells were loaded with dye in the same medium bathing the cells. After loading, cells were transferred to fresh growth

medium with or without IgE and the dishes were maintained at 34°C in an atmosphere of 5% CO_2 on the stage of an imaging microscope. Ratiometric measurements of intracellular calcium were made at 2-s intervals. Cells were scored as generating calcium transients by observation of the images by an observer who was unaware of whether IgE was present.

The data in Fig. 2, *H* and *I*, are sample proportions. For example, Fig. 2*H* (first bar) had 1 of a total of 472 naive cells generating a calcium transient in 1 min. For each bar, the predicted population proportion is equal to the sample proportion (in this case, 0.2%), and the error bars represent the 5% confidence limits. For this example, a population proportion of 1% active would, in 5% of trials, yield a measured sample of 472 in which one or fewer cells were active, so the upper confidence limit is set at 1%. This form of confidence limit analysis is described in Ref. 18 (page 131) and the limit values used in this study are taken from Table F of that study.

Measurement of inositol phosphates

For measurement of inositol phosphates, the cells were labeled for 2 days with [^3H]inositol (2.5 $\mu\text{Ci}/\text{ml}$) in 6-well plates. For priming the cells, 0.5 $\mu\text{g}/\text{ml}$ IgE was added to the wells for 18 h. All the cells were washed in HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1 mg/ml glucose, pH 7.2), which was supplemented with 10 mM LiCl to inhibit the breakdown of inositol phosphates back to inositol. Cells were stimulated at 37°C for 30 min with IgE alone to naive cells or with allergen DNP-human serum albumin (DNP-HSA) for primed cells. At the end of the incubation, the medium was removed and retained for measurement of hexosaminidase, and replaced with 500 μl of ice-cold methanol. The cells were vigorously scraped on ice and transferred to a clean tube. The wells were rinsed with a further 500 μl of methanol and combined with the cellular extract. Chloroform (1 ml) was added followed by 1 ml of water to the methanol cell extracts. Following centrifugation to separate the phases, the [^3H]inositol phosphates were recovered in the top phase and were analyzed on Dowex columns as previously described (19).

Measurement of PLD activity

Adherent RBL-2H3 cells, which were seeded the day before at 1×10^5 per well in 24-well plates, were labeled with [^3H]myristic acid for 1 h at 37°C in HEPES buffer. In experiments in which DNP-HSA was used as the allergen for cross-linking IgE as a stimulus, cells were primed for 18 h with 50 ng/ml anti-DNP-IgE. After the labeling period, [^3H]myristic acid was removed and the cells incubated with the stimuli in HEPES buffer in the presence of 0.5% butanol for 30 min. Reactions were terminated by adding 0.5 ml of ice-cold methanol to HCl (98:2; v/v) ratio. Cells were then scraped and transferred to test tubes. The wells were rinsed with 500 μl methanol and the volume was transferred to the tubes. Two phases were generated by combining 1 ml of chloroform and 1 ml of H_2O . The lower organic phase was removed and the chloroform evaporated. The lipids were resuspended in 20 μl chloroform and spotted onto silica gel-60 TLC plates that were developed with chloroform to methanol to acetic acid to H_2O (75:45:3:0.4, v/v/v/v) ratio. The areas corresponding to [^3H]phosphatidylbutanol and [^3H]phosphatidylcholine were identified with authentic standards after iodine staining, scraped and counted for radioactivity. The results are expressed as percentage of [^3H]phosphatidylbutanol/[^3H]phosphatidylcholine.

Degranulation assay

RBL-2H3 cells were grown overnight on 19-mm glass coverslips for Cy3-Annexin V binding. Cells were subsequently primed for 1–4 h with 50 ng/ml IgE. Following washing in HEPES buffer, (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1 mg/ml glucose, 1 mg/ml BSA, pH 7.4), cells were stimulated in the presence of 1:100 Cy3 to Annexin V (MBL, Woburn, MA), and in the presence or absence of 0.5 $\mu\text{g}/\text{ml}$ IgE or 40 ng/ml DNP for 20 min. The reaction was stopped on ice. Cy3-Annexin V stained samples were washed in PBS and then fixed for 10 min in 4% formaldehyde. Following washing with PBS, cells were mounted with Mowiol. Images were obtained with the LSR Ultraview confocal microscope.

Degranulation was also measured by release of β -hexosaminidase. RBL-2H3 cells were seeded at 2×10^5 per well on a 24-well plate and left overnight. Where indicated, the cells were primed overnight or for 4 h with 50 ng/ml anti-DNP-IgE as indicated. Following washing with HEPES buffer, cells were preincubated for 10 min at 37°C in the presence of inhibitors, wortmannin and Ro-31-7549 (where indicated). Cells were incu-

bated for 20 min in the presence or absence of IgE or 40 ng/ml allergen for primed cells. At the end of the incubation, the plate was transferred to ice and the cells centrifuged at $2000 \times g$ for 5 min at 4°C. An aliquot of the supernatant (50 μ l) was analyzed for β -hexosaminidase as previously described (20).

Fluorescent staining of the actin cytoskeleton to monitor membrane ruffling

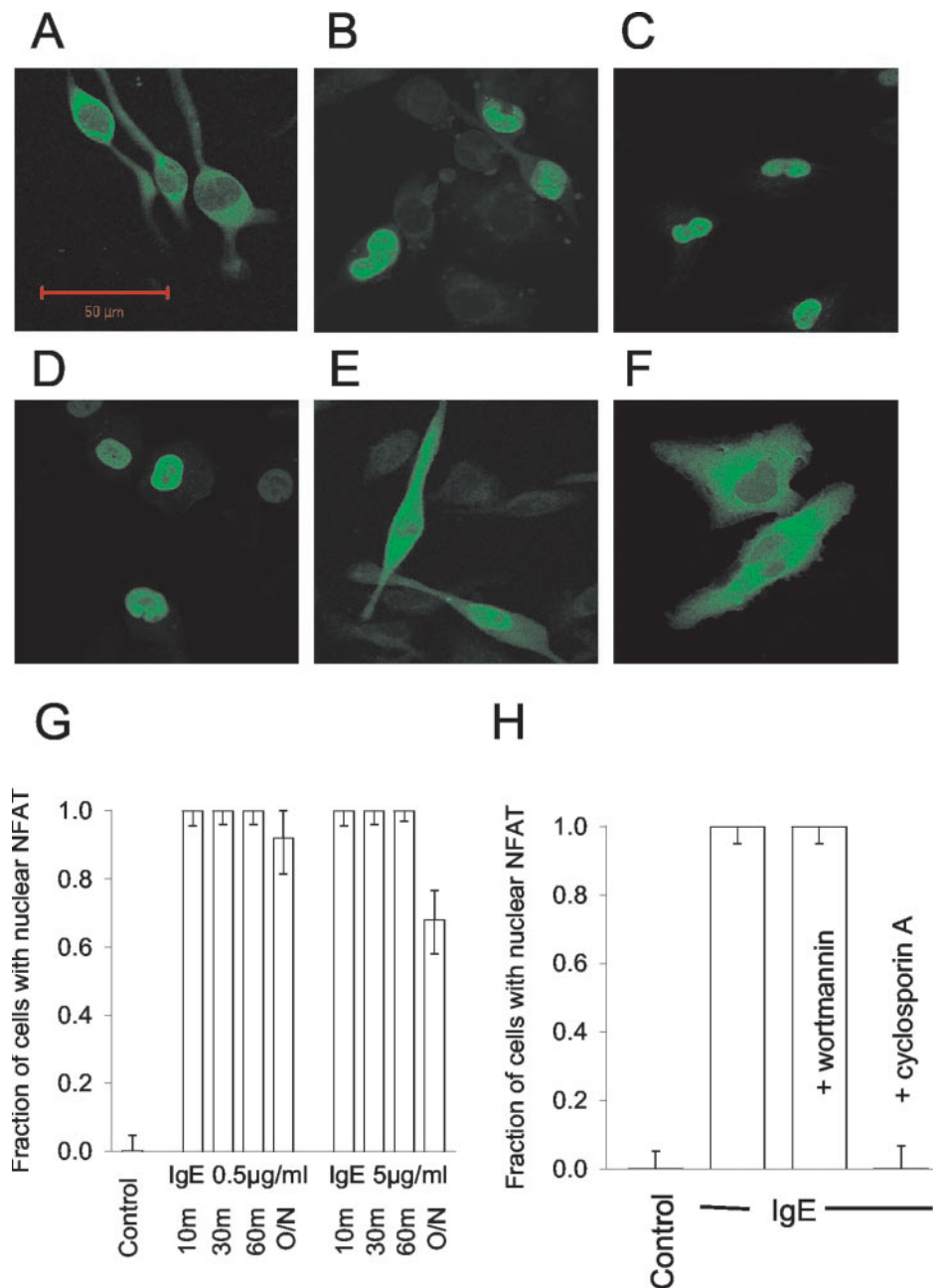
For the staining of the cytoskeleton, the cells were plated on glass coverslips and left overnight. The following day, the cells were washed with HEPES buffer and stimulated with IgE at 37°C for 10 min. For labeling of total cellular filamentous actin, cells were first fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 and incubated with 0.5 μ M tetramethylrhodamine isothiocyanate (TRITC) phalloidin for 20 min at room temperature. The cells were then washed three times with PBS and coverslips were mounted. Fluorescence was viewed with a PerkinElmer/Olympus confocal microscope using a $\times 100$ oil-immersion objective. Images were acquired using a PerkinElmer charge-coupled device camera cooled to -35°C . Localization of TRITC-phalloidin in single cells was captured by exciting the cells at 510 nm and taking sequential laser confocal slices (0.5 μ m apart) through the whole of the cells. Random

fields of control and stimulated cells were taken with a $\times 40$ oil immersion lens and sequential confocal slices were taken from the top of the cell to the adhesion plane. The total numbers of cells was counted from 10 random fields and were categorized according to whether the cell was or was not ruffled.

Preparation of monomeric IgE

To purify monomeric IgE, IgE (SPE-7 anti-DNP clone; Sigma-Aldrich, St. Louis, MO) was chromatographed on a size-exclusion column (Superose 12; Amersham Biosciences, Piscataway, NJ) with Gel Filtration on an FPLC. The column was equilibrated with 20 mM HEPES, 137 mM NaCl, and 3.7 mM KCl (pH 7.4). A total of 200 μ l (200 μ g of protein) was injected, 200- μ l fractions were collected, and monomer and aggregate peaks based on OD₂₈₀ readings were identified. The fractions containing the monomer peak were pooled and concentrated. Following concentration, the sample was reanalyzed by Gel Filtration and no aggregate peak was observed. Greater than 95% of the protein was found in the monomer peak. The purified IgE was used immediately and the remainder of the material was stored in 20 μ l aliquots at -20°C . Once thawed for an experiment, the IgE aliquot was discarded. Identical results were obtained with the freshly

FIGURE 1. Translocation of NFAT into the nucleus following addition of monomeric IgE. RBL-2H3 cells were transfected with EGFP-NFAT. Localization of NFAT in control RBL-2H3 cells (A), 2 μ M ionomycin-stimulated cells for 30 min (B); 0.5 μ g/ml IgE for 10 min (C); 0.5 μ g/ml IgE for 1 h (D); 0.5 μ g/ml IgE for 18 h (E); cyclosporin A (1 μ M) treated cells (20 min) followed by 0.5 μ g/ml IgE for 10 min (F); and a quantitative analysis of the fraction of cells showing localization of NFAT in the nucleus as a function of time (G). H, Effect of cyclosporin A (1 μ M) and wortmannin (100 nM) on the translocation of NFAT into the nucleus stimulated by 0.5 μ g/ml IgE for 10 min. Error bars in G and H are 5% confidence limits taken from Table F of Box et al. (18).



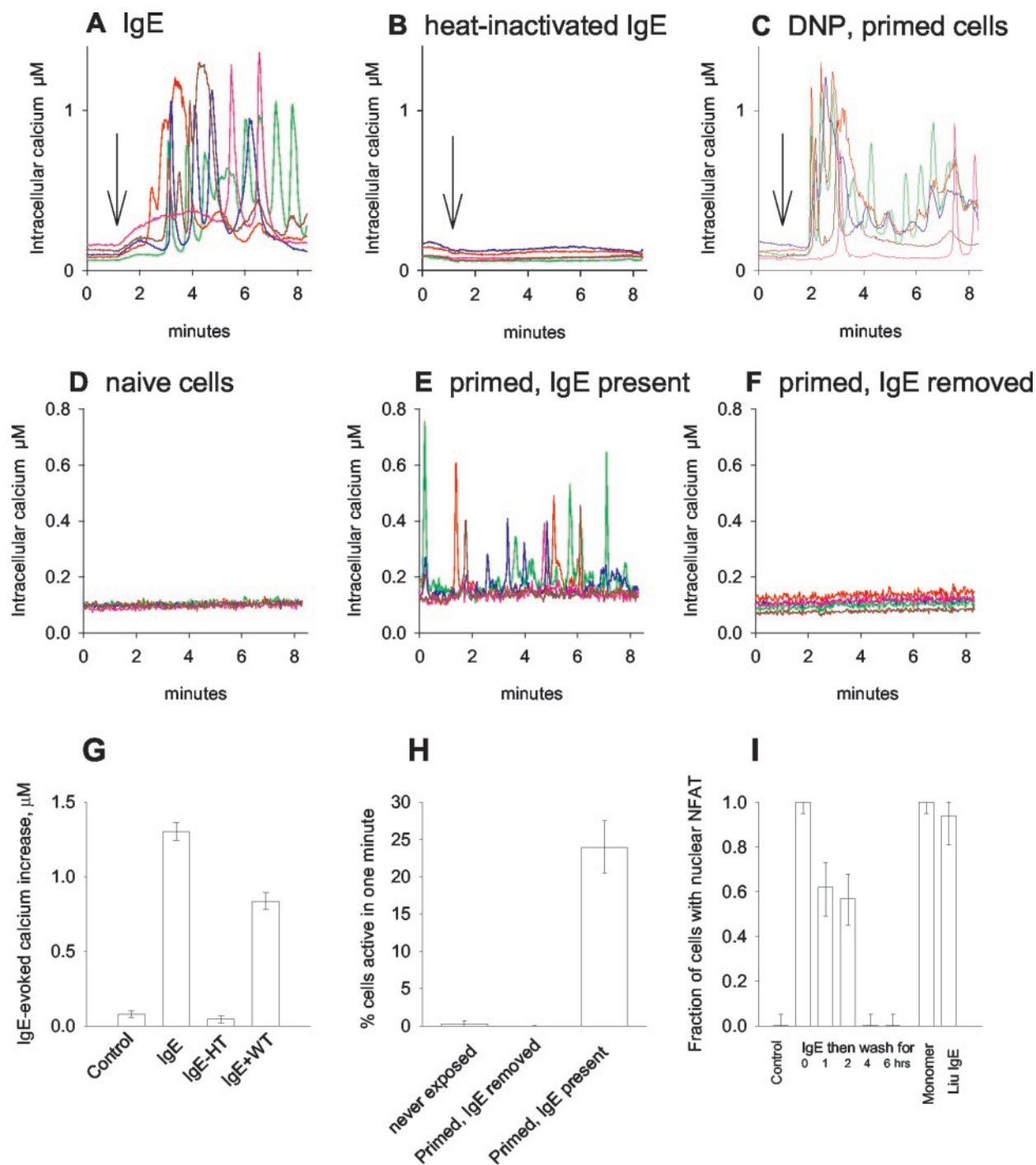


FIGURE 2. Monomeric IgE stimulates Ca^{2+} transients that are maintained for 18 h provided that IgE is present. RBL-2H3 cells were loaded with fura 2-AM for 30 min. Acute addition of IgE (0.5 $\mu\text{g}/\text{ml}$) (A) and heat treatment of IgE (30 min at 56°C) (B) abolishes calcium transients. C, Cells (0.5 $\mu\text{g}/\text{ml}$ IgE) were “primed” for 18 h, washed, and subsequently challenged with allergen, DNP-HSA (40 ng/ml). D, RBL-2H3 cells not exposed to IgE. E, RBL-2H3 cells were primed for 18 h and examined for Ca^{2+} activity in the presence of IgE (0.5 $\mu\text{g}/\text{ml}$). F, RBL-2H3 cells were primed for 18 h and examined for Ca^{2+} activity after removal of IgE. G, Histogram of mean ΔCa^{2+} ($\pm$ SEM) from experiments of types illustrated in A and B. IgE-HT, IgE inactivated by heat treatment; IgE+WT, cells pretreated with wortmannin (100 nM). H, Ca^{2+} transients are maintained over long periods and are reversed upon removal of IgE. The cells were not exposed to IgE; cells were primed with IgE (0.5 $\mu\text{g}/\text{ml}$) for 18 h and washed three times to remove the IgE; cells were primed with IgE (0.5 $\mu\text{g}/\text{ml}$) for 18 h and IgE was present throughout the experiment. I, RBL-2H3 cells were treated with IgE for 10 min ($t = 0$). The cells were washed three times and incubated for the indicated lengths of time, and assessed for the localization of NFAT. Removal of IgE causes the movement of NFAT back into the cytosol. Purified Sigma-Aldrich IgE as well as Liu IgE (obtained from a different clone) is still effective at stimulating translocation of NFAT into the nucleus. Error bars in H and I were calculated as described in the Fig. 1 legend.

purified material and the frozen material. To obtain sufficient material for the aggregates, several runs were combined, and the aggregates concentrated and used immediately for the degranulation assays.

An alternative way of removing aggregates was to centrifuge the IgE preparation before use. The IgE solution was centrifuged at $15,000 \times g$ for 1 h at 4°C or ultracentrifuged at $100,000 \times g$ for 1 h at 4°C to remove

aggregated IgE. Ultracentrifugation was more effective at removing aggregates than centrifugation at $15,000 \times g$ as analyzed by gel filtration.

Results

IgE alone causes translocation of NFAT into the nucleus

NFAT is a transcriptional activator, which has been previously shown to translocate to the nucleus upon allergen stimulation of IgE-primed cells (21). NFAT is located in the cytosol of unstimulated cells and translocates to the nucleus when dephosphorylated by the calcium-calmodulin-dependent phosphatase calcineurin. Thus a rise in cytosol Ca^{2+} is the triggering event for this process, and therefore Ca^{2+} ionophore is similarly effective at NFAT translocation (22). NFAT translocation is dependent on Ca^{2+} mobilization from both internal and external stores (22) and recent studies have implied a requirement for PIP_3 in Ca^{2+} mobilization (23, 24). Its mechanism of action is not clear, and in one study it was implied that PIP_3 directly regulated Ca^{2+} entry (24), whereas in another it augmented the increase in IP_3 levels (23). This study was initiated to examine the requirement for PI3K activation for the translocation of NFAT into the nucleus upon activation of IgE-primed cells with allergen.

RBL-mast cells were transfected with EGFP-NFAT and cells to be challenged with allergen were primed with IgE (0.5 $\mu\text{g}/\text{ml}$) overnight (16–18 h). In unprimed RBL-2H3 cells, EGFP-NFAT was cytosolic and following addition of ionomycin (30 min), NFAT translocated into the nucleus (Fig. 1, A and B). However, in the IgE-primed cells, NFAT was already nuclear (Fig. 1, C and D). To examine this phenomenon in further detail, we incubated EGFP-NFAT-transfected RBL-2H3 cells with IgE for 10 min, 30 min, 1 h, and overnight. Within 10 min of addition of IgE (0.5 $\mu\text{g}/\text{ml}$), all cells show NFAT redistribution into the nucleus, and this response is maintained in the cells incubated for 18 h with IgE although at this time some cytosolic localization is also observed (Fig. 1E). The histogram in Fig. 1G provides a quantitative analysis of the cytosol vs nuclear distribution of the number of cells counted in several random fields. Similar results were obtained with 5 $\mu\text{g}/\text{ml}$ IgE, concentrations used previously by Krystal and his colleagues (12).

NFAT interacts with calcineurin and is retained in the cytoplasm by a complex of other proteins in the resting cells. In the absence of Ca^{2+} and calmodulin, the active site of calcineurin is blocked, and calcineurin is inactive. The binding of calmodulin triggered by a rise in cytosol Ca^{2+} leads to a conformational change in calcineurin, and activated calcineurin can now dephosphorylate NFAT. Cyclosporin A is an inhibitor of calcineurin and it was effective at preventing the IgE-mediated translocation of NFAT (Fig. 1, F and H). It is noted that the cyclosporin A does not prevent activation of cell flattening by IgE (compare Fig. 1, A and F). These data indicate that NFAT translocation to the nucleus stimulated by acute addition of IgE alone is dependent on calcineurin, and therefore on Ca^{2+} . We also examined whether wortmannin, the PI3K inhibitor, disrupts NFAT translocation, and this drug was found to have no effect (Fig. 1H).

IgE alone can reversibly maintain cytosol Ca^{2+} transients over a long period

In T cells, if intracellular Ca^{2+} remains elevated, calcineurin activity remains high, and NFAT remains activated and nuclear for many hours (25). In B cells, NFAT remains nuclear as long as calcium signaling continues but returns to the cytosol as soon as calcium transients cease (26). We therefore examined the effects of IgE on the Ca^{2+} levels in individual RBL-2H3 cells. Acute application of 0.5 $\mu\text{g}/\text{ml}$ IgE to RBL-2H3 cells elicited a strong calcium signal (Fig. 2A) that was not significantly different from the re-

sponse of primed cells to allergen (IgE plus allergen) (Fig. 2C). Mild heat treatment (30 min at 56°C) disrupts the Fc domain of IgE, and therefore inhibits binding to $\text{Fc}\epsilon\text{R1}$, but does not damage the allergen binding site (27–29). Heat treatment of IgE markedly attenuated the calcium response (Fig. 2B) indicating that binding of IgE to the Fc receptor was the triggering event. Because the calcium responses of individual cells are asynchronous, the mean calcium at any one time (as would be measured in a cuvette system) underestimates the calcium levels experienced by the cells. We therefore calculated the agonist-evoked calcium increase for each cell as the difference between the maximum calcium value recorded after agonist application, whenever that occurred, and the prestimulus value. Values for individual cells were then averaged and plotted $\pm$ SEM as shown in Fig. 2G. In the presence of wortmannin, Ca^{2+} responses were only mildly reduced, thus permitting full translocation of NFAT into the nucleus (Fig. 1H).

Because NFAT remained nuclear even after 18 h in the presence of IgE (Fig. 1G), we examined calcium responses to IgE over a long period. RBL-2H3 cells were treated overnight with 0.5 $\mu\text{g}/\text{ml}$ IgE, then imaged the following day in the continued presence or absence of IgE. Calcium transients were observed in the cells, provided IgE was continually present (Fig. 2E). In contrast when cells were treated overnight with IgE but imaged in IgE-free medium, their intracellular calcium concentration was low and stable (Fig. 2F) as was calcium in cells that had never been exposed to IgE (Fig. 2D). Fig. 2H shows that the fraction of cells generating calcium transients in a 1-min test period was markedly higher in cells maintained overnight in the presence of IgE.

A similar pattern is seen with the NFAT translocation. Removal of IgE causes the movement of NFAT back to the cytosol, and in this case, it takes ~ 4 h for all the NFAT to return to the cytosol following the removal of IgE (Fig. 2I). This may be the time taken for the kinases to rephosphorylate NFAT and effect its export, because the Ca^{2+} signals return to baseline much sooner after IgE removal.

Because IgE is able to stimulate a rise in cytosol Ca^{2+} , we examined the possibility that IgE accomplishes this by activating PLC and generating IP_3 . RBL-2H3 cells were prelabeled with [^3H]inositol for 2–3 days to label the inositol lipid pool. The cells were stimulated with IgE for 30 min in the presence of lithium chloride to trap the inositol phosphates resulting from PLC activity. Fig. 3 illustrates that IgE is able to stimulate inositol phosphate accumulation upon acute addition of IgE and the response is very similar to that seen in primed cells subsequently challenged with allergen.

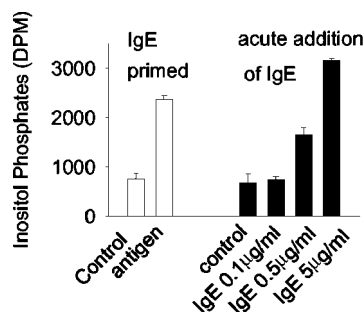


FIGURE 3. Monomeric IgE stimulates an increase in PLC activity. RBL-2H3 cells were labeled with [^3H]inositol for 2–3 days. Where indicated, a batch of cells was primed with 0.5 $\mu\text{g}/\text{ml}$ IgE for 18 h. The cells were stimulated with 40 ng/ml allergen (primed cells) or with IgE at the indicated concentrations for 30 min. Total inositol phosphates were monitored as an indicator of PLC activity.

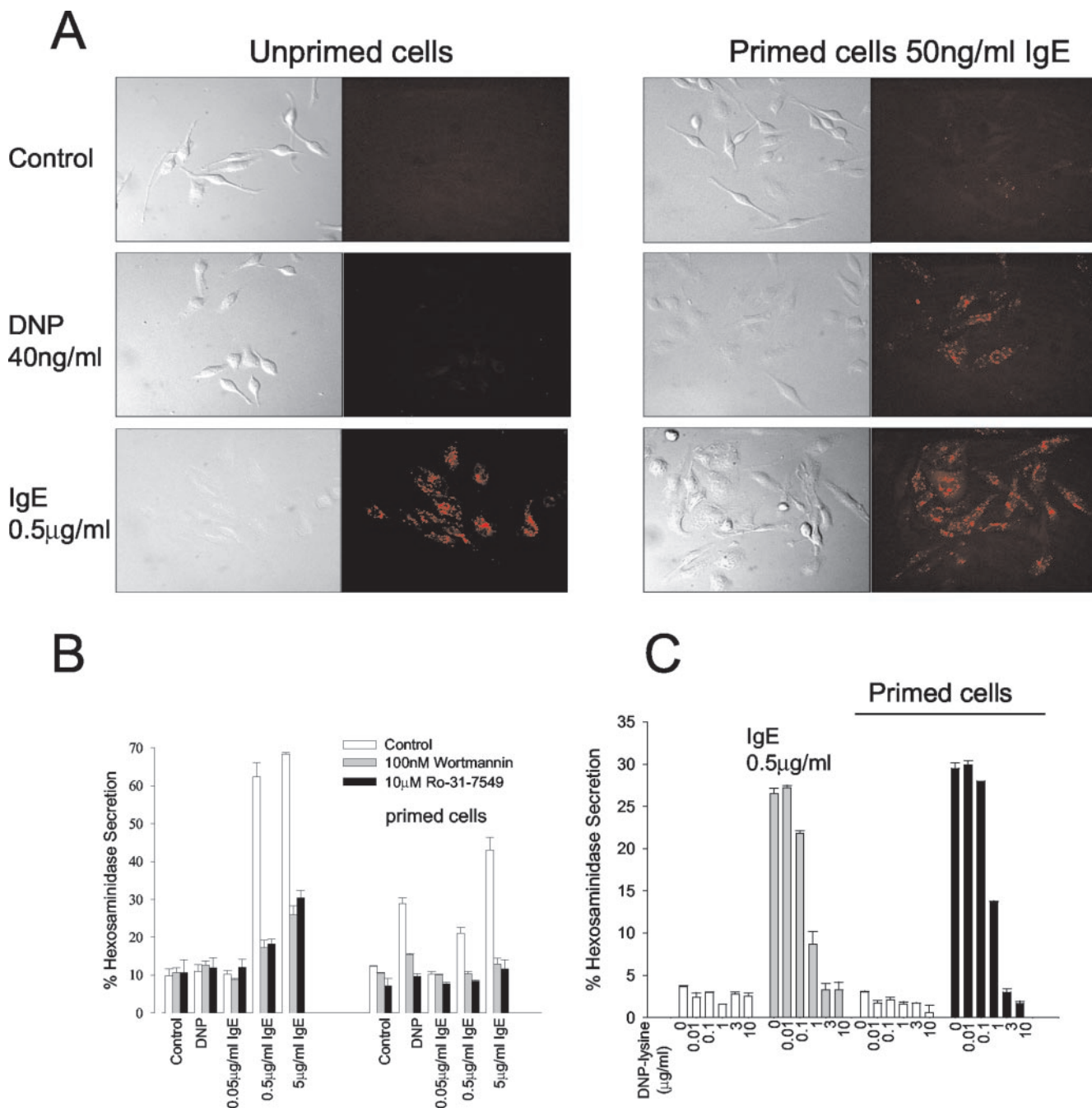


FIGURE 4. IgE stimulates degranulation. RBL-2H3 cells were primed with 50 ng/ml IgE for 4 h where indicated. **A**, Degranulation was assessed by monitoring the exposed phosphatidylserine using Cy3-labeled annexin V. The corresponding brightfield image is also shown, which reveals cell spreading. **B**, Degranulation was assessed by measuring the release of β -hexosaminidase. Both wortmannin (100 nM) and Ro-31-7549 (10 μ M) inhibits degranulation stimulated by IgE or in primed cells stimulated by allergen (40 ng/ml). **C**, DNP-lysine inhibits IgE-mediated degranulation. RBL-2H3 cells were stimulated with 0.5 μ g/ml IgE or multivalent allergen (40 ng/ml, DNP-BSA) for 20 min in the presence of indicated concentrations of monovalent allergen DNP-lysine. Because DNP-lysine can interfere with the hexosaminidase assay, we also checked the degree of inhibition, and at 3 and 10 μ g/ml, DNP-lysine inhibited the hexosaminidase assay by 5 and 10%, respectively.

IgE alone stimulates degranulation in RBL-2H3 mast cells

The observation that IgE can stimulate PLC activation, Ca^{2+} transients and NFAT translocation provides a complementary set of data to the studies that have recently been published identifying a signaling role for IgE from the laboratory study of Krystal and colleagues (12), but not that of Asai et al. (15). Krystal and colleagues (14) reported that IgE alone could stimulate Ca^{2+} influx, multiple phosphorylation events of extracellular signal-regulated kinases, p38, c-Jun N-terminal kinase, PKB, and the Fc ϵ R1 β -subunit, and release of cytokines (IL-6,

IL-13, IL-4, and TNF- α) but not degranulation in BMMCs. Degranulation could only be stimulated from BMMCs obtained from SHIP knockout mice, leading to the conclusion that SHIP, by hydrolyzing PIP_3 , was restraining degranulation. In many respects, this result was surprising because PKB activation could be observed in IgE-treated SHIP $^{+/+}$ BMMCs implying that an increase in PIP_3 must be taking place in these cells (12).

We have examined whether acute addition of IgE could stimulate degranulation from RBL-2H3 cells and compared the response

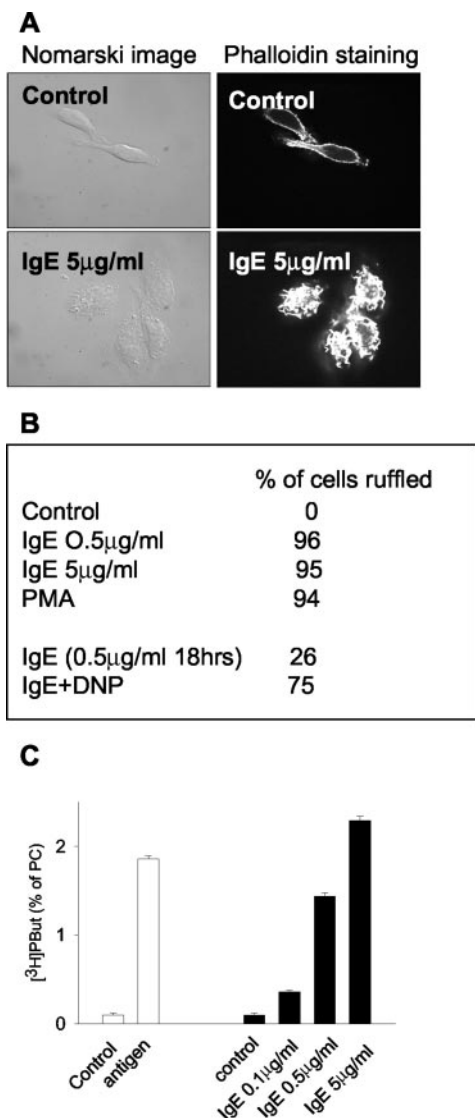


FIGURE 5. IgE stimulates membrane ruffling. **A**, RBL-2H3 cells (unprimed) were stimulated with IgE alone or with 100 nM PMA. For priming, RBL-2H3 cells were incubated with 0.5 μ g/ml IgE for 18 h and stimulated with 40 ng/ml allergen for 10 min. The cells were fixed and stained with TRITC-phalloidin. A representative field of cells is shown. Random fields of cells were also imaged and the number of ruffled cells counted. **B**, For each condition, over 100 cells were counted, and the proportion of ruffled cells calculated. **C**, RBL-2H3 cells were labeled with [^{3} H]myristic acid for 1 h to label phosphatidylcholine. Where indicated a batch of cells were primed with 0.05 μ g/ml IgE for 18 h. The cells were stimulated with 40 ng/ml allergen (primed cells) or with IgE at the indicated concentrations for 30 min in the presence of 0.5% butanol. Phosphatidylbutanol was separated by TLC and the data are expressed as a percentage of label incorporated into phosphatidylcholine.

to primed cells challenged with allergen. The priming concentration used for these experiments was reduced to 0.05 μ g/ml, which was established by titration as being the minimum concentration that still supports a response to subsequent challenge. This concentration is not stimulatory and is likely to lend to only partial occupation of the Fc receptors (30). (Previous studies from our laboratory and those of others have used between 0.5 and 1 μ g/ml for priming (10, 20, 31–33)). Degranulation was assessed by monitoring the exposure of phosphatidylserine that accompanies granule fusion with the plasma membrane (34, 35). Phosphatidylserine exposure was monitored by using Cy3-labeled annexin V. Fig. 4

illustrates that acute IgE is capable of causing degranulation in naive cells as well as in primed cells, but only primed cells can be stimulated with DNP. Allergen stimulation leads to a flattening of the cells due to spreading and this is evident from the brightfield image. IgE stimulation also causes flattening of the cells in a similar manner. To quantitate the amount of degranulation, the release of hexosaminidase was also measured and IgE plus allergen elicited similar responses (Fig. 4, B and C).

Wortmannin inhibits IgE-mediated secretion but not NFAT translocation or Ca^{2+} signaling

In primed RBL-2H3 cells, inhibition of PI3K reduces a subset of responses stimulated in primed cells by allergen, which include membrane ruffling and degranulation (33, 36, 37). In BMMCs, wortmannin also inhibits secretion but not IL-6 production (38). These data suggest that PI3K is at a branch point in the intracellular signaling process that controls different aspects of mast cell activation (degranulation vs cytokine production). We have examined the effect of wortmannin on IgE-evoked degranulation in RBL-2H3 cells and it inhibited degranulation similarly to that seen with allergen stimulation (Fig. 4B). In contrast, wortmannin did not affect EGFP-NFAT translocation (Fig. 1H), and only reduced Ca^{2+} signals (Fig. 2G). Another inhibitor of allergen-stimulated mast cell degranulation is the PKC inhibitor, Ro-31-7549 and this compound also inhibited IgE-mediated degranulation (Fig. 4B). These data support the conclusion that IgE stimulation uses the same signaling pathways as allergen, which includes PLC and PI3K to trigger degranulation.

IgE stimulates membrane ruffling and PLD activity

Treatment of RBL-2H3 cells with PMA or IgE-primed cells challenged with allergen evokes membrane ruffles and this response is dependent on the activation of PLD (10, 39). We first examined IgE-stimulated membrane ruffling that was monitored by labeling the actin with fluorescent phalloidin (Fig. 5A). IgE at 0.5 μ g/ml was as effective as 5 μ g/ml at triggering membrane ruffling, and the number of cells that scored positive for membrane ruffles was counted by examining random fields. In unprimed cells, in the presence of IgE (0.5 μ g/ml), the majority of the cells had a ruffled morphology. In cells primed with IgE (0.5 μ g/ml) overnight, for subsequent challenge the following day, we noted that a higher proportion of the cells exhibited a ruffled morphology. Addition of allergen increased the proportion of cells that exhibited membrane ruffles.

The formation of membrane ruffles stimulated by allergen is exquisitely dependent on the continual activation of PLD and therefore it was of interest to examine whether acute addition of IgE could activate PLD activity. PLD activity was monitored by measuring the increase in phosphatidylbutanol, the unique product produced by phospholipase activity in the presence of 0.5% butanol. IgE stimulated PLD activity in a concentration-dependent manner and the lowest concentration at 0.1 μ g/ml was able to register a response. The maximal response was observed at 5 μ g/ml IgE and this response was comparable to that observed with primed cells subsequently challenged with allergen (Fig. 5C).

These data so far demonstrate that IgE stimulation of RBL-2H3 cells evokes responses very similar to those observed with allergen stimulation. To ensure that the stimulation by IgE observed in these experiments was not unique to the RBL-2H3 cell clone used in our laboratory, we also examined three other RBL-2H3 clones obtained from different laboratories (Fig. 6). In all cases, all the cells were responsive to IgE, and the maximal stimulation achieved was very similar to that seen with IgE plus allergen.

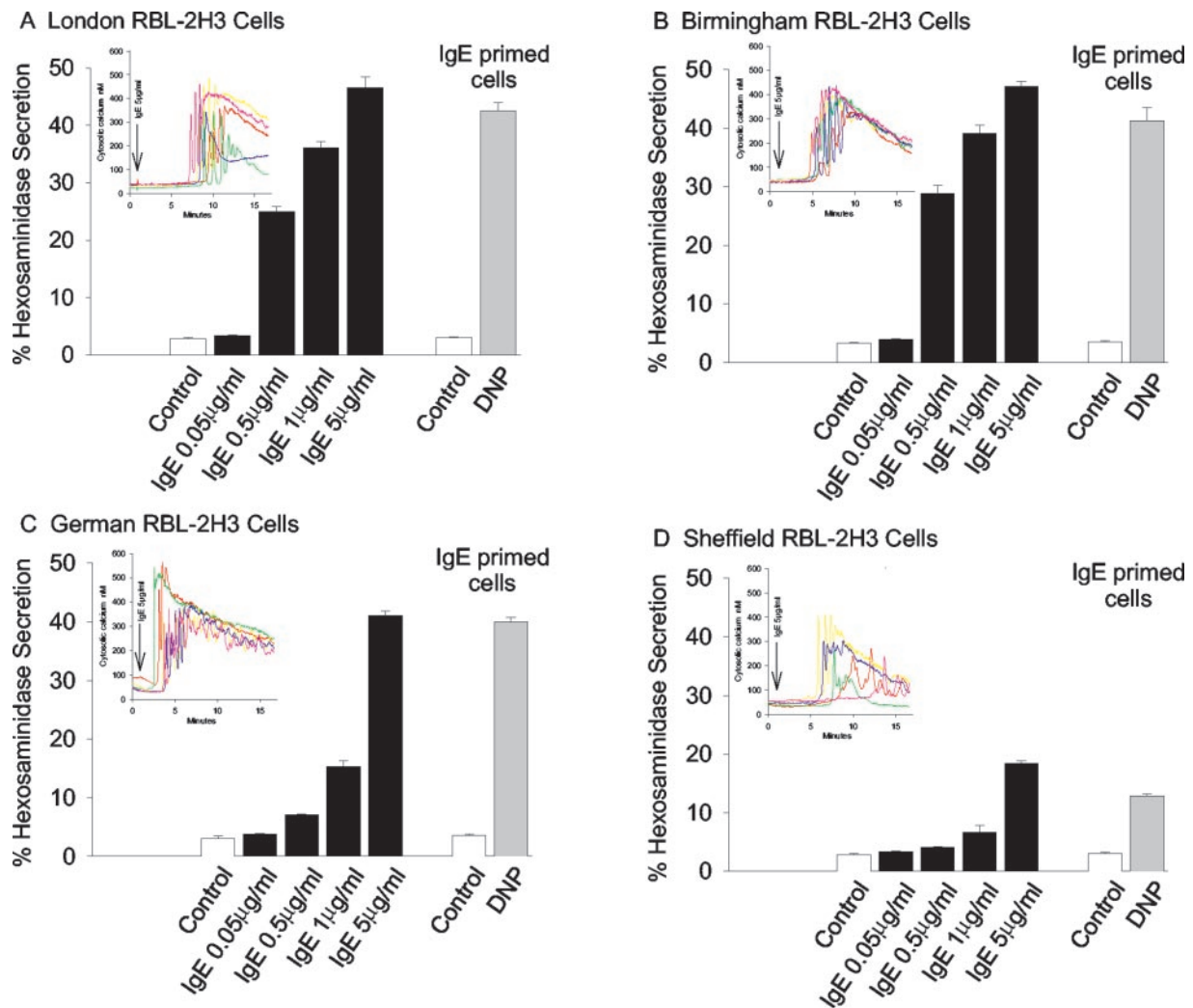


FIGURE 6. RBL-2H3 cells from different sources respond to IgE. Degranulation and a rise in cytosol Ca^{2+} were assessed. A batch of cells including London (A), Birmingham (B), German (C), and Sheffield (D) were primed for 4 h with 50 ng/ml IgE and were stimulated with allergen (40 ng/ml). *Insets* show the rise in cytosol Ca^{2+} in these cells.

Sensitivity at lower concentrations of IgE (0.5 $\mu\text{g/ml}$) varied depending on the source of the RBL-2H3 cells. In all cases, 5 $\mu\text{g/ml}$ was stimulatory. In addition to secretion we also measured Ca^{2+} signaling and all cell lines tested were responsive to IgE (all tested at 5 $\mu\text{g/ml}$ only) (Fig. 6, *insets*).

IgE purified to remove aggregates and IgE from a different source are equally effective at activating mast cells

In the classical view of mast cell activation, allergen cross-links FcεRs and thereby allows tyrosine phosphorylation and the activation of downstream targets including PLCγ and PI3K (4). Our results could be explained within the classical model if the IgE used contained multimolecular complexes. Such complexes could bind and therefore link two or more FcεRs. To examine this possibility, IgE (SPE-7 IgE; Sigma-Aldrich) was purified by size-exclusion column chromatography (Fig. 7A). The major peak, corresponding to monomeric IgE (Fig. 7A), was still effective at stimulating degranulation (Fig. 7B). This monomeric IgE also elicited calcium responses (data not shown) and caused nuclear translocation of EGFP-NFAT (Fig. 2J). The fractions containing the aggregates were accumulated from several runs, concentrated, and examined for their ability to stimulate degranulation. Approximately 10% hexosaminidase secretion was observed with 5 $\mu\text{g/ml}$

aggregated IgE compared with 50% secretion with 0.5 $\mu\text{g/ml}$ IgE or with IgE plus allergen (Fig. 8). A total of 0.5 $\mu\text{g/ml}$ aggregated IgE was unable to stimulate degranulation (Fig. 8C). Aggregates can be removed by centrifugation ($15,000 \times g$ for 1 h) and Fig. 8C illustrates that centrifugation of the aggregated IgE led to loss of degranulating activity.

Having established that centrifugation is effective in removing aggregates, we examined the ability of the IgE to stimulate degranulation and changes in cytosol Ca^{2+} before and after centrifugation. The IgE sample was centrifuged at $15,000 \times g$ and at $100,000 \times g$ for 1 h and was compared with noncentrifuged IgE. It was anticipated that ultracentrifugation would be more effective at removing aggregates compared with centrifugation at $15,000 \times g$. The dose response for degranulation remained unchanged whether the IgE was centrifuged or not (Fig. 8A). All three IgE types elicited comparable cytosolic Ca^{2+} responses (data not shown).

The study by Krystal and colleagues (14) used the same source of IgE (SPE-7 clone; Sigma-Aldrich) as used in this study, whereas the study of Asai et al. (15) used a different source of IgE (40). Both IgE types were directed against DNP. IgE types generated by different cell clones may differ in their ability to activate mast cells and may account for the different results obtained in these two

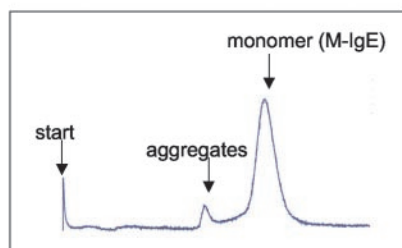
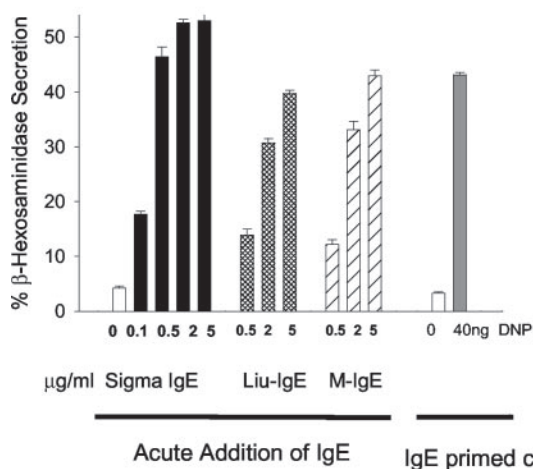
A**B**

FIGURE 7. IgE from two different sources is effective at stimulating degranulation as is monomeric IgE. **A**, Purification of commercial IgE to obtain the monomeric IgE; IgE was purified by size-exclusion chromatography and the monomeric peak was retained and concentrated. **B**, Degranulation of RBL-2H3 cells stimulated with Sigma IgE (SPE-7 clone; Sigma-Aldrich), purified IgE (M-IgE), and Liu IgE. Primed cells (Sigma IgE, 50 ng/ml) stimulated with 40 ng/ml allergen were also analyzed in parallel.

studies (12, 15). We therefore compared both types of IgE. Asai et al. (15) used IgE purified from a clone originally generated by Liu et al. (40). We have obtained this IgE (H1 DNP- ϵ -206 IgE) as a gift from Dr. J. Rivera (National Institutes of Health, Bethesda, MD) and refer to this IgE as Liu IgE. Liu IgE was effective in stimulating degranulation (Fig. 7B), calcium responses (data not shown), and nuclear translocation of EGFP-NFAT (Fig. 2I). The IgE used in our study was made against DNP, and therefore it was of interest to test whether the addition of monovalent DNP would be inhibitory. Monovalent DNP inhibits stimulation by multivalent DNP-directed allergen by competing for the binding site on the IgE molecule. DNP-lysine at 3 μ g/ml effectively blocked degranulation stimulated with multivalent DNP from IgE primed cells (Fig. 4C). Because DNP-lysine is fluorescent, we also checked its effect on the hexosaminidase assay that is also based on fluorescence. DNP-lysine does inhibit the measurement of β -hexosaminidase and was therefore monitored within each experiment. In the experiment shown in Fig. 4C, 3 and 10 μ g/ml, DNP-lysine inhibited the hexosaminidase assay minimally by 5 and 10%, respectively. We tested DNP-lysine between 0 and 10 μ g/ml and report that DNP-lysine dose dependently inhibits degranulation stimulated by IgE (from SPE-7 clone; Sigma-Aldrich) acutely applied at 0.5 μ g/ml. Maximal inhibition was observed at 3 μ g/ml DNP-

lysine, a concentration that had a very slight effect on the hexosaminidase assay.

Discussion

In this paper we report that IgE alone evokes a number of functional responses in RBL-2H3 mast cells, which are identical with those seen when IgE-primed cells are triggered with multivalent allergen. These results indicate that sensitization of mast cells with IgE is not a passive process and that IgE can trigger a remarkably similar array of intracellular signaling pathways that include the activation of a rise in cytosol Ca^{2+} , PLC, PLD, and NFAT translocation into the nucleus. It is the persistent Ca^{2+} transients that cause the transcription factor NFAT to translocate to the nucleus and remain there until the IgE is removed. IgE-evoked calcium signals, and the subsequent translocation of NFAT, appear to be the result of activation of Fc ϵ R and the subsequent activation of PLC because disruption of the Fc domain by mild heat treatment markedly reduced the calcium signal.

A simple explanation of our results would be that the commercial IgE product used contained IgE complexes that would bind to multiple Fc ϵ Rs, mimicking the effect of monomeric IgE plus allergen. IgE was analyzed by size exclusion chromatography and the majority of IgE was present as a monomer with a small peak of IgE aggregates. The monomer peak was still effective in evoking degranulation, calcium responses, and NFAT translocation. The responses are not unique to the commercial IgE product because an independent product evoked the same responses. IgE was obtained as a gift from Dr. J. Rivera, and as illustrated in Fig. 7B, it is able to stimulate degranulation, and calcium responses as effectively as IgE (Sigma-Aldrich). Our results lend support to the recent proposal, based on work on BMMCs, that IgE alone stimulates intracellular signaling. The classical view that IgE treatment represents merely a priming process, attaching to Fc ϵ R1 but not evoking any response until the appearance of allergen needs to be reconsidered. The concentrations of IgE that stimulate RBL-2H3 mast cells are 0.1–0.5 μ g/ml, which are present in humans during parasite infection and in patients suffering from various forms of allergy. Thus, high circulating levels of IgE may have a stimulatory effect that needs to be experimentally verified. Additionally, one-third of patients with chronic urticaria have circulating IgG autoantibodies against Fc ϵ R1 or IgE. The intradermal injection of autologous serum causes a weal and flare in many patients with chronic urticaria. However the autoantibodies were only detected in some, but not all of the patients with the positive skin tests. This might indicate that IgE stimulates their mast cells, although further examination would be needed (41, 42).

How does monomeric IgE activate mast cells? Firstly, it is not the presence of some impurity in the preparation that is responsible for this activation. Disrupting the Fc portion of the IgE by mild heat treatment inhibits the effects of IgE and secondly, monovalent allergen also inhibits, suggesting that the variable region in which the allergen binds is required for activation.

It is well established that IgE alone can up-regulate the surface expression of the Fc ϵ R1, and it is thought that this is due to stabilization of the receptor at the cell surface (43, 44). Thus occupation of the Fc ϵ R1 by IgE must provide some kind of internal signal to inhibit endocytosis. The nature of this signal is unknown, and our study suggests an alternative mechanism. Our results suggest that the initial binding of IgE to the receptor can initiate a transient signaling process, that is not dissimilar to that observed with cross-linking of the receptor with allergen. The receptor must undergo a change in conformation at this stage, and that transient change is the signaling step. Transient cross-linking may take place because monovalent allergen is inhibitory. This is supported

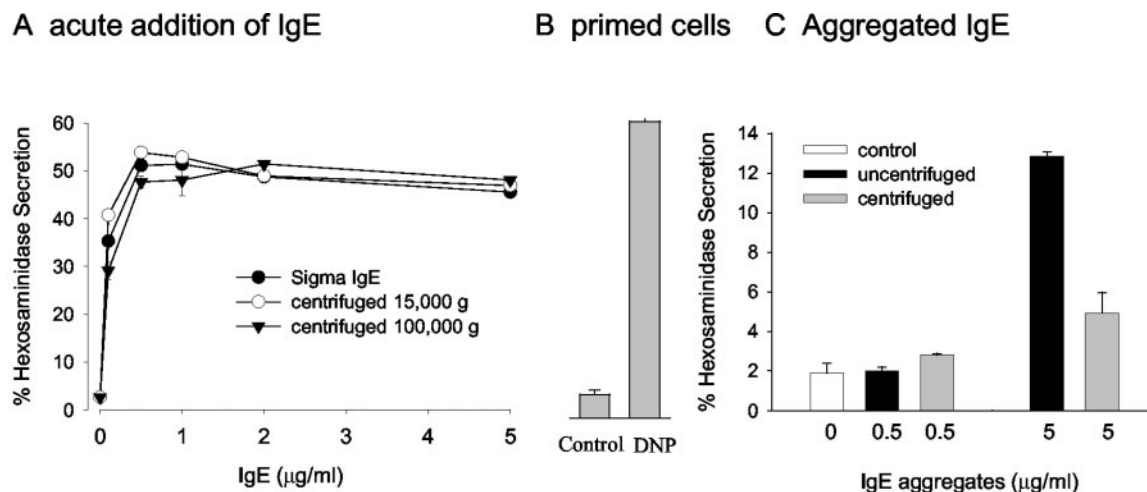


FIGURE 8. Centrifugation of IgE to remove aggregates does not affect the ability of IgE to cause degranulation. *A*, Degranulation stimulated with IgE before and after centrifugation at $15,000 \times g$ or at $100,000 \times g$ for 1 h. *B*, A batch of cells were primed with 50 ng/ml IgE overnight, and stimulated with allergen (DNP) in parallel. *C*, Aggregated IgE stimulates a low level of degranulation and this is lost upon centrifugation of the sample to remove the aggregates.

by the observation that the same signaling pathways are activated with monovalent IgE as that seen with allergen and this includes PLC, PLD, and Ca^{2+} leading to similar functional outcomes including spreading, membrane ruffling, and degranulation. Since submission of this paper, Kitaura et al. (45) have reported that IgE alone causes receptor aggregation as determined by phosphorescence anisotropy measurements.

Signaling by monomeric IgE is persistent over many hours (16 h at least) as long as free IgE is present. Removal of IgE reverses the cells to quiescence. Such IgE-treated cells remain sensitive to subsequent addition of allergen, suggesting that once IgE is bound to the receptor, it no longer triggers further activation. When IgE-primed cells are stimulated with a further addition of IgE, signaling is promptly initiated indicating that bound IgE does not prevent the free IgE from causing activation. From this we conclude that free IgE has to be present for intracellular signaling to be maintained. IgE binds to its receptor with high affinity and dissociates slowly, therefore removal of IgE would not cause much dissociation from the FcεR1 (30). This bound IgE is not responsible for signaling as removal of free IgE causes the Ca^{2+} transients to be inactivated (Fig. 2, *F* and *H*). A similar pattern is seen with the NFAT translocation. Removal of IgE causes the movement of NFAT back to the cytosol, and in this case, it takes ~4 h for all the NFAT to return to the cytosol following the removal of IgE (Fig. 2*J*). This may be the time taken for the kinases to rephosphorylate NFAT and affect its export, because the Ca^{2+} signals return to baseline much sooner after IgE removal.

Wortmannin inhibits degranulation but not NFAT translocation into the nucleus. Wortmannin inhibits PI3K activity and hence PIP_3 production. It is also a potent inhibitor of degranulation in mast cells. PIP_3 has several targets including PLCγ1, Btk, and PDK1, and it is not immediately obvious which of these enzymes are required for degranulation. Moreover, in SHIP-deficient BMMCs, degranulation was now observed with monomeric IgE, indicating that increased levels of PIP_3 promote degranulation. There is an accumulation of literature that supports the notion that stimulation of PLCγ1 requires the prior activation of PI3K (33, 46–48). PIP_3 by binding the pleckstrin domain of PLCγ1 can recruit the enzyme to the plasma membrane. However, in mast cells, the major PLCγ isoform is PLCγ2 and this isoform is not dependent on prior activation of PI3K (33, 49). In BMMCs from PLCγ2

knockout mice, degranulation is considerably reduced, whereas IL-4 production was comparable to wild-type cells (49). Thus it is unlikely that wortmannin inhibition of degranulation is accounted for by the inhibition of PLCγ isozymes. In mast cells, there are two signaling arms that are stimulated; one is via Fyn/GAB2/PI3K/ PIP_3 /PKCδ and the other is LYN/SYK/LAT/PLCγ/ Ca^{2+} and both arms intersect in several places and cooperate to stimulate degranulation (50). In Fyn $^{-/-}$, Vav1 $^{-/-}$, Syk $^{-/-}$, GAB2 $^{-/-}$, and PLCγ2 $^{-/-}$ BMMCs, degranulation is considerably reduced, but not in Lyn $^{-/-}$ knockout cells (7, 8, 51, 52). We speculate that PIP_3 is required for recruitment and activation of Rac and ADP-ribosylation exchange factors, and inhibition of Rac and ADP-ribosylation factor GTPases may be responsible for inhibition of degranulation. Previous studies have shown that both ADP-ribosylation factor and Rac participate in regulating degranulation from mast cells (9, 53). Because wortmannin has only a mild effect on the calcium signals and no effect on NFAT translocation but markedly inhibited IgE-evoked secretion, it appears that for optimal degranulation, signals from both arms of the pathway are required for degranulation.

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