

Regulation of monomeric IgE responses by PKC β II

Critical role of PKC β II in activation of mast cells by monomeric IgE

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Accumulating evidence suggests that IgE-mediated activation of mast cells occurs even in the absence of antigen, which is referred to as 'monomeric IgE' responses. Although monomeric IgE was found to induce a wide variety of responses, such as up-regulation of the Fc ϵ RI, survival, cytokine production, histamine synthesis, and adhesion to fibronectin, it remains to be clarified how mast cells are activated in the absence of antigen. It has been controversial whether monomeric IgE responses are mediated by a similar signaling mechanism to antigen stimulation, although recent studies suggest that IgE can induce the Fc ϵ RI aggregation even in the absence of antigen. In this study, we focused on the role of conventional PKC (cPKC), since this response is suppressed by a specific inhibitor for cPKC. Monomeric IgE-induced Ca²⁺ influx was not observed in a mouse mastocytoma cell line, which lacks the expression of PKC β II, although Ca²⁺ influx induced by cross-linking of the Fc ϵ RI was intact. Transfection of PKC β II cDNA was found to restore the Ca²⁺ influx induced by monomeric IgE in this cell line. Furthermore, the dominant negative form of PKC β II (PKC β II/T500V) significantly suppressed the Ca²⁺ influx, histamine synthesis, and IL-6 production in another mouse mast cell line, which is highly sensitive to monomeric IgE. Expression of PKC β II/T500V was found not to affect the antigen-induced responses. These

results suggest that PKC β II plays a critical role in monomeric IgE responses, but not in antigen responses.

Activation of mast cells triggers allergic and inflammatory responses through the release of a wide variety of mediators, such as histamine, arachidonic acid metabolites and neutral proteases, and modulates immune responses through the production of cytokines and chemokines (1, 2). One of the prominent mechanisms for activation of mast cells is cross-linking of the Fc ϵ RI, the high-affinity receptor for IgE, by the multivalent antigen, and various signaling molecules have been identified in this pathway (3, 4). Furthermore, accumulating evidence has indicated that IgE-mediated activation of mast cells can occur even in the absence of the multivalent antigen (5). Sensitization of IL-3¹-dependent mouse bone marrow derived mast cells (BMMCs) with IgE induces an array of events, such as up-regulation of the Fc ϵ RI (6, 7), resistance to apoptosis under IL-3 deprivation (8-11), cytokine production (9, 10), histamine synthesis (12), and adhesion to fibronectin (13, 14). These results have clearly indicated that sensitization with IgE (monomeric IgE) is able to activate mast cells in the absence of antigen. However, it remains to be clarified as to how monomeric IgE activates mast cells and what kinds of signaling molecules are involved in this pathway.

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Some preceding studies revealed that signaling molecules are shared between monomeric IgE responses and responses induced by cross-linking with multivalent antigen; weak but sustained tyrosine phosphorylation of several signaling components, which are intensively phosphorylated upon antigen stimulation, were observed in BMMCs stimulated with monomeric IgE (9, 14). High concentrations of monomeric IgE were found to have a potential to induce degranulation (10, 15, 16). Furthermore, monovalent haptens, for which the IgE is specific, were found to abrogate these responses (9, 10, 17). These results have raised the possibility that monomeric IgE can induce aggregation of the Fc ϵ RI via its antigen binding portion. Indeed, Kitaura et al. demonstrated the increase in anisotropy of the surface Fc ϵ RI of a rat mast cell line stimulated with monomeric IgE (10). Very recently, Schweitzer-Stenner and Pecht performed a simulation analysis for the distance between the surface IgE molecules bound to the Fc ϵ RI and proposed a hypothesis that monomeric IgE induces the receptor aggregation via low affinity interaction between the IgEs or between the IgE and another cell surface component (18).

We recently demonstrated that activation of BMMCs by monomeric IgE requires Ca²⁺ influx, which is mediated by pharmacologically distinct channels from those activated upon antigen stimulation (17). Based upon this result, we hypothesized that signal transduction activated by monomeric IgE is not only quantitatively but also qualitatively different from that upon antigen stimulation. Since monomeric IgE-induced responses, such as histamine synthesis and IL-6 production were drastically suppressed by a conventional protein kinase C (cPKC) inhibitor, Gö6976, we particularly focused on the role of cPKC and found the critical role of PKC β II in monomeric IgE-induced activation of mast cells.

MATERIALS AND METHODS

Cell culture

A mouse mastocytoma cell line, P-815 and its transfectants were maintained in RPMI-1640 containing 10% heat-inactivated FCS.

P-815/Fc ϵ RI (P-815/Fc), which is a stable cell line that expresses rat Fc ϵ RI α , β , and γ chain cDNAs, is a generous gift from Dr. H. Metzger (NIH) (19). A mouse mast cell line, MC9, was maintained under the same conditions in the presence of 1 ng/ml IL-3 (R&D Systems, Minneapolis, MN).

Stable P-815 and MC9 clones

Mouse PKC β II cDNA was amplified by RT-PCR using a pair of primers (forward; 5'-CCG GAT CCG CGC GCG CAA GAT GGC TGA CCC G-3', reverse; 5'-GGC TCG AGA CGG ATC TAC TTA GCT CTT GAC TTC AGG TT-3', underlines indicates the restriction sites for *Bam*HI and *Xho*I) and cloned into the *Bam*HI and *Xho*I sites of pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA). P-815/Fc cells were transfected with pcDNA3.1/Hygro containing PKC β II cDNA (pcDNA3.1/Hygro/ β II) using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) and stable transfectants were selected in the presence of hygromycin (0.1 mg/ml). Dominant negative form of PKC β II (PKC β II/T500V) was constructed as described (20), and subcloned into a lentiviral vector, pLenti6/V5-D-TOPO (Invitrogen). MC9 cells were infected with the recombinant lentivirus prepared according to the manufacturer's instruction and stable transfectants were selected in the presence of blasticidin (6 μ g/ml).

PKC β II expression in COS-7 expression system

COS-7 cells were transfected with pcDNA3.1/Hygro/ β II and its mutant (pcDNA3.1/Hygro/ β II-T500V), respectively or simultaneously using GenePORTER transfection reagent. Expression of PKC β II was confirmed by immunoblot analysis as described below.

Preparation of IgEs

IgEs (an anti-DNP IgE, clone SPE-7, from Sigma, St. Louis, MO, an anti-TNP IgE, clones IgE-3 and c38-2, from BD Biosciences, San Diego, CA) were purified before use by centrifugation at 100,000 $\times$ g at 4°C for 1 hr for removal of the aggregated form. No significant differences were observed between the IgE purified by centrifugation and the IgE purified by gel-filtration in activation of mast cells as a monomeric IgE. We used the clone SPE-7 through this study otherwise stated.

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Immunoblot analysis

Cells were homogenized in 50 mM HEPES-NaOH, pH 7.3 containing 1 mM dithiothreitol, 0.5% Triton X-100, 0.5% deoxycholate, 0.05% SDS, and a protease inhibitor mixture (0.2 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 0.1 mM benzamidin, 1 μ g/ml pepstatin A, and 10 μ g/ml E-64) and were centrifuged at 15,000 $\times$ g for 20 min at 4°C. The resultant supernatant was subjected to SDS-PAGE (10% slab gel). The separated proteins were electrophoretically transferred onto a PVDF membrane (Millipore, Billerica, MO). Primary and secondary antibodies were commercially obtained and used; anti-PKC α antibody (1:250, BD Biosciences, San Jose, CA), anti-PKC β I antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-PKC β II antibody (1:200, Santa Cruz), anti-PKC γ antibody (1:200, BD Biosciences), anti-actin antibody (1:1000, Chemicon, Temecula, CA), horse radish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:3000, Dako, Glostrup, Denmark), and HRP-conjugated anti-rabbit IgG antibody (1:3000, Dako). The membrane was stained using the ECL kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction.

Flowcytometry

Cells were pretreated with 10 μ g/ml anti-CD16/32 (2.4G2, BD biosciences) at 4°C for 10 min, then with 12.5 μ g/ml each IgE at 4°C for 50 min. Labeling of the cells was performed by incubation with an FITC-conjugated anti-mouse IgE (BD Biosciences) at 4°C for 25 min. Flowcytometric analysis of the stained cells was performed with FACSCalibur (Becton Dickinson) equipped with the CELLQUEST software.

IgE-mediated activation of mast cells

Cells were treated with the indicated concentrations of an anti-DNP IgE. In case of antigen stimulation, cells were sensitized with 0.3 μ g/ml anti-DNP IgE for 24 hr, washed three times to remove unbound IgE, and then stimulated with the indicated concentrations of DNP-conjugated human serum albumin (Sigma).

Measurement of cytosolic Ca²⁺ concentrations

Cells were loaded with 2 μ M Fura-2/AM in the modified Tyrode's buffer (130 mM NaCl

containing 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, NaOH, pH 7.3 and 0.1% bovine serum albumin) for 45 min at room temperature and then washed in the modified Tyrode's buffer. Fluorescent intensities were measured, at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrometer (Jasco, CAF-100, Tokyo, Japan).

Membrane translocation of cPKCs

MC9 Cells were treated with 1 μ g/ml IgE for the indicated periods or with 100 nM TPA for 10 min. The cells were homogenized and lysed by sonication in 20 mM Tris-HCl, pH 7.5 containing 10 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF, and 20 μ g/ml leupeptin. The resultant homogenate was centrifuged at 100,000 $\times$ g for 15 min at 4°C to separate the cytosolic and membrane fractions. Each fraction was subjected to the immunoblot analysis using the specific PKC antibodies.

Measurement of HDC activity and IL-6 release

Cells were rinsed with PBS followed by centrifugation and the cell pellet was lysed (1 $\times$ 10⁷ cells/ml) with 10 mM HEPES-NaOH, pH 7.3, containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100 and the protease inhibitor mixture on ice for 10 min. The cells were centrifuged at 10,000 $\times$ g for 30 min at 4°C and the supernatant was used for the measurement of HDC activity as previously described (21). IL-6 release was measured using mouse IL-6 BD OptEIA ELISA system (BD Biosciences).

RESULTS

PKC β II is required for monomeric IgE-induced Ca²⁺ mobilization in a mastocytoma cell line, P-815

Since activation of mast cells by monomeric IgE was found to be suppressed by a specific inhibitor for cPKC, Gö6976 (22), we investigated the expression of cPKC subtypes in mast cell lines, MC9 and P-815 by immunoblot analysis. PKC α , β I, and β II, were expressed in MC9 cells, whereas PKC α , and β I, but not β II, in P-815 cells (Fig. 1A). Expression of PKC γ was not observed in P-815

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and MC9 cells (data not shown). Since P-815 cells lack Fc ϵ RI α and β chains and the expression level of γ chain is low, we used a stable clone, P-815/Fc, which expresses sufficient amount of all three chains. IgE (clone SPE-7)-mediated Ca^{2+} mobilization was not observed in the parental P-815 cells due to the lack of Fc ϵ RI expression (Fig. 1B and 1C). Although IgE-mediated antigen stimulation induced the intracellular Ca^{2+} mobilization in P-815/Fc cells, monomeric IgE failed to induce Ca^{2+} mobilization (Fig. 1C). We then prepared a stable clone, P-815/Fc/ β II, which expresses PKC β II (Fig. 1A). In this clone, intracellular Ca^{2+} mobilization was observed not only upon antigen stimulation but also by monomeric IgE (Fig. 1C). Optimal condition of antigen stimulation was not changed in this clone (data not shown). Equal levels of surface expression of Fc ϵ RI were verified by flowcytometry in P-815/Fc and P-815/Fc/ β II cells, indicating that PKC β II did not affect the surface expression of Fc ϵ RI (Fig. 1B). No or little changes of Ca^{2+} mobilization induced by thrombin, which evokes Ca^{2+} mobilization by acting on the heterotrimeric G protein-coupled receptor, were observed between P-815/Fc and P-815/Fc/ β II cells. We investigated the monomeric IgE-induced Ca^{2+} mobilization using the other IgE clones, which exhibit the similar levels of surface binding to the clone, SPE-7 (Fig. 2A). An IgE clone, c38-2, was found to induce Ca^{2+} mobilization as well as SPE-7, whereas another clone, IgE-3, was not. These differential effects of the IgE clones were also observed in BMMCs (17). Monomeric IgE-induced Ca^{2+} mobilization in P-815/Fc/ β II cells was suppressed by Gö6976, staurosporine, and a specific inhibitor of store-operated Ca^{2+} channels, SK&F96365, but not by La^{3+} , Gd^{3+} , which is consistent with our previous results using BMMCs (17) (Fig. 2B).

Lentiviral expression of the dominant negative form of PKC β II in MC9 cells

Since our preliminary study revealed that MC9 cells are highly sensitive to monomeric IgE, we first investigated the membrane translocation of cPKCs. A minor portion of cPKCs expressing in MC9 cells was found to rapidly translocate to the

membrane fractions by monomeric IgE. Relatively prolonged translocation of PKC β II was observed compared with PKC α and β I (Fig. 3). Previous study demonstrated that Thr⁵⁰⁰, which is located in the activation loop of PKC and phosphorylated, is one of the critical residues for kinase activity of PKC β II and that the mutation of this residue to Val (PKC β II/T500V) resulted in the loss-of-function (20). We, therefore, prepared the stable MC9 clones, which express this mutant form of PKC β II, to evaluate its dominant negative effects on monomeric IgE-induced activation. Several clones that express unphosphorylated PKC β II were obtained. We selected two clones with high expression levels of the mutant PKC β II, MC9/DN1 and MC9/DN2. In these clones, high expression levels of the unphosphorylated PKC β II were observed, whereas little or no expression of the phosphorylated form was detected (Fig. 4A). It remains unknown why the phosphorylated form disappears in the presence of PKC β II/T500V. We investigate the expression of PKC β II and PKC β II/T500V in a mammalian expression system using COS-7 cells, which lack endogenous PKC β II. The phosphorylated form was also undetectable in the cells co-transfected with the wild and mutant PKC β II cDNA, although each form was detectable in the cells transfected with its cDNA alone (Fig. 4B). The expression of PKC β II/T500V did not affect the expression levels of the other cPKCs, PKC α and β I (Fig. 4A). Expression of PKC β II/T500V did not affect the surface expression of Fc ϵ RI in MC9 cells (Fig. 4C).

Suppression of monomeric IgE-induced activation in MC9 cells expressing the dominant negative form of PKC β II

We then evaluated the dominant negative effects of PKC β II/T500V on monomeric IgE-induced activation. Ca^{2+} mobilization induced by monomeric IgE was partially suppressed in MC9/DN clones, whereas that upon antigen stimulation unchanged (Fig. 5). Since induction of histidine decarboxylase (HDC) activity was observed in MC9 cells both by monomeric IgE and upon antigen stimulation (Fig. 6A, B), we then

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investigated the effects of PKC β II/T500V on induction of HDC. PKC β II/T500V was found to significantly suppress the induction of HDC by monomeric IgE, but not upon antigen stimulation (Fig. 6C). No changes in the dose response curve were observed in between MC9 cells and each DN clone (data not shown). Similarly, monomeric IgE-induced IL-6 release was also significantly suppressed by PKC β II/T500V, whereas antigen-induced IL-6 release was not (Fig. 6D).

DISCUSSION

We focused on the role of cPKC in IgE-mediated activation of mast cells and found that PKC β II plays a critical role in monomeric IgE-induced responses, but not in antigen responses. Previous studies have cast some doubt on the differences in signal transduction between monomeric IgE responses and antigen responses; they speculated that monomeric IgE can induce the Fc ϵ RI-aggregation and that a similar signaling pathway is activated upon both responses with different intensities (10, 18). Accumulating evidence has indicated that the Fc ϵ RI-mediated activation of mast cells is finely tuned. Gonzalez-Espinosa et al. demonstrated that several chemokines, such as MCP-1, are released at low antigen concentrations or at low receptor occupancy with IgE compared with several cytokines such as IL-3 and IL-10 (23). This result indicated that antigen-induced IL-3 release requires a 'strong' stimulation, in which linker for activation of T cells (LAT) and ERK are extensively phosphorylated. On the other hand, Kohno et al. demonstrated that monomeric IgE-induced survival of BMMC is mediated by a large amount of IL-3 autocrine production (11). In this study, IL-3 production by monomeric IgE was found to be much larger than that upon antigen stimulation. Since monomeric IgE was found to elicit a 'weak and sustained' signal transduction including ERK phosphorylation (9), it is likely that IL-3 production by monomeric IgE is regulated by a different mechanism from that upon antigen stimulation. We also found that IL-6 production in MC9 cells stimulated with monomeric IgE was much larger than that upon antigen stimulation

under the optimized condition. Our current results clearly demonstrated that PKC β II is a prerequisite component for monomeric IgE-induced activation of mast cells, but not for antigen responses. We previously demonstrated that Ca²⁺ influx induced by monomeric IgE is mediated by channels different from Ca²⁺ release-activated Ca²⁺ channel (CRAC), which is utilized upon antigen responses, using several channel blockers (17). We propose that the signaling cascades activated by monomeric IgE are distinguishable from those upon cross-linking of the Fc ϵ RI by multivalent antigen.

A mouse mastocytoma cell line, P-815, is a suitable model for the analysis of Fc ϵ RI-mediated activation, since it lacks the surface expression of Fc ϵ RI (19). We can exhibit the direct evidence of involvement of the Fc ϵ RI in monomeric IgE responses using this cell line, which is consistent with the recent study using BMMCs derived from the Fc ϵ RI α deficient mice (24). Restored Ca²⁺ influx by PKC β II was observed only in the presence of certain IgE clones and exhibited a similar sensitivity to various kinase inhibitors and channel blockers to that observed in BMMCs (17), indicating that the responses in P-815 cells reproduce those observed in BMMCs. However, no or little induction of HDC and IL-6 was observed upon monomeric IgE treatment or antigen stimulation in P-815 and its transfectants. Since potent activators of mast cells, such as A23187 and thapsigargin, failed to induce histamine synthesis and IL-6 release in P-815 cells (maximum IL-6 release < 0.1 pg/ml, data not shown), it is quite difficult to evaluate the effects of IgE-mediated responses. Although detectable levels of IL-6 was found in the concentrated medium of unstimulated P-815 cells, no increase of IL-6 was observed in the presence of A23187. P-815 cells may lack signaling molecules downstream of Ca²⁺ influx, which are required for detectable levels of induction of HDC and IL-6.

The suppressive effects of the mutant PKC β II/T500V on monomeric IgE-induced activation of MC9 cells were consistent with the results obtained in P-815 cells transfected with PKC β II. These suppressive effects were found exclusively in monomeric IgE responses, not in antigen stimulation. Since PKC β I and β II are

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generated by alternative splicing of the carboxy-terminal exons and exhibit little differences in enzymatic characters (25), there have been few studies on the specific roles of each isoform of PKC β II in mast cells. Although previous study using PKC β deficient mice revealed that BMMC lacking both PKC β I and β II exhibited lower levels of degranulation and IL-6 release than the wild type cells in response to antigen (26), it remains to be clarified which isoform is responsible for degranulation and IL-6 release. Kawakami et al. demonstrated that PKC β II was only weakly activated upon the Fc ϵ RI cross-linking compared with PKC β I in BMMCs (27, 28). Weak activation of PKC β II upon antigen stimulation is consistent with our results that PKC β II did not affect the antigen responses. Very recently, Kawakami et al. demonstrated that PKC β II plays a dominant role in phosphorylation of Akt on Ser⁴⁷³, which contributes to cytokine production (29), in BMMC upon antigen stimulation (30). They compared the role of PKC β I and β II retrovirally transduced into BMMC derived from PKC β deficient mice. Although antigen-induced histamine release was restored by either of PKC β isoform, antigen-induced IL-2 release was found to be completely dependent on PKC β II. This result clearly indicated the specific role of PKC β II in the signal transduction downstream of Fc ϵ RI. PKC β II-Akt pathway may play an important role in monomeric IgE-induced activation of mast cells.

It remains unknown how PKC β II is activated by monomeric IgE. Kawakami et al. demonstrated that 20-70% of PKC β II was localized in the membrane fraction of resting BMMC and only a part of it was tyrosine-phosphorylated upon antigen stimulation (28). We found that less than 1% of PKC β II is localized in the membrane fraction of MC9 cells and that a minor population of PKC β II was translocated to the membrane fraction by monomeric IgE. Antigen stimulation failed to induce detectable membrane translocation of PKC β II in MC9 cells (data not shown). We prepared another stable MC9 clone, in which an

RNAi sequence for the unique region of PKC β II is retrovirally transduced. Although ~70% suppression of PKC β II expression on immunoblot analysis was achieved in this clone, no significant suppression was detected in Ca²⁺ mobilization, histamine synthesis, and IL-6 production, when the cells were stimulated with monomeric IgE (data not shown). These results suggest that a small portion of PKC β II may be able to mediate the monomeric IgE-induced signal transduction. The suppression of monomeric IgE-induced activation by the mutant PKC β II/T500V was found to be significant but partial. However, potency of suppression by each DN clone was correlated with the amount of the unphosphorylated form expressed in each clone. A very low and undetectable level of phosphorylated form of PKC β II may be responsible for the residual HDC and IL-6 expression induced by monomeric IgE in these clones.

The region specific for PKC β II contains 52 amino acids and is homologous to the corresponding region of PKC β I. PKC β II was found to bind to RACK1 (the first receptor of activated C kinase) via its C2 domain and carboxy terminal specific region (V5 region) with higher affinity than PKC β I (31). Although we could detect the binding of PKC β II to RACK1 in MC9 cells, the amount of bound PKC β II was not changed by monomeric IgE (data not shown).

Although specific physiological functions of PKC β II remain to be figured out, several studies have suggested the critical role of PKC β II *in vivo*. Liu et al. demonstrated that azoxymethane-induced colon carcinogenesis was abolished in PKC β KO mice and that transduction of PKC β II can restore its susceptibility (32). Todt et al. suggested that PKC β II is involved in phagocytosis of apoptic cells by murine peritoneal macrophages with pharmacological approaches (33). We revealed in this study that PKC β isoforms are specifically exploited in the Fc ϵ RI-mediated signal transduction in mast cells.

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FOOTNOTES

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LEGENDS TO FIGURES

Figure 1 Monomeric IgE-induced Ca²⁺ mobilization in P-815 is restored by reconstitution with Fc ϵ RI and PKC β II

(A) Expression of PKC α , β I, and β II in MC9, P-815, and its transfectants (P-815/Fc, and P-815/Fc/ β II) was investigated by immunoblot analysis as described in 'MATERIALS AND METHODS'. The amount of actin in each lane was also determined as a loading control. (B) Surface expression of Fc ϵ RI was measured by flowcytometry using an anti-DNP IgE and an FITC-conjugated anti-IgE antibody. (C) Cells were stimulated with monomeric IgE (IgE, 3 μ g/ml), antigen (IgE/Ag; cells were sensitized with 0.3 μ g/ml anti-DNP IgE for 24 hr, and then stimulated with 100 ng/ml DNP-conjugated HSA), and thrombin (Thrombin, 1 U/ml). Changes in the cytosolic Ca²⁺ concentrations were monitored using Fura2/AM and presented as a ratio. The time point of each stimulation is indicated by the arrow. Bar = 1 min. This is a representative figure of three independent experiments showing similar results.

Figure 2 Characterization of monomeric IgE-induced Ca²⁺ mobilization in P-815/Fc/ β II cells

(A) Surface binding of various IgE clones (SPE-7, IgE-3, and c38-2) was measured by flowcytometry using an FITC-conjugated anti-IgE antibody (left). Ca²⁺ mobilization induced by each IgE clone (3 μ g/ml) was investigated as described in the legend to Fig. 1C (right). These are representative figures of three independent experiments showing similar results. (B) P-815/Fc/ β II cells were pretreated with SK&F 96365 (100 μ M, 3 min), LaCl₃ (30 μ M, 3 min), GdCl₃ (30 μ M, 3 min), Gö6976 (1 μ M, 60 min), or staurosporine (10 nM and 100 nM, 30 min), before stimulation with IgE (3 μ g/ml). Changes in the cytosolic Ca²⁺ concentrations were monitored using Fura2/AM and presented as a ratio. The time point of each stimulation is indicated by the arrow. Bar = 1 min. This is a representative figure of three independent experiments showing similar results.

Figure 3 Translocation of cPKCs to the membrane fraction in MC9 cells stimulated with IgE

MC9 cells were stimulated with 1 μ g/ml IgE for the indicated periods (min) or 100 nM TPA for 10 min. The cells were then homogenized and separated into the cytosol (C) and membrane (M) fractions according to the procedures described in 'MATERIALS AND METHODS'. Each fraction was subjected

Regulation of monomeric IgE responses by PKC β II

to immunoblot analysis. This is a representative blot of five independent experiments showing similar results.

Figure 4 Expression of the dominant negative form of PKC β II in MC9 cells

(A) Expression of PKC α , β I, and β II in MC9, and its transfectants with PKC β II/T500V (two clones, DN1 and DN2) was investigated by immunoblot analysis as described in 'MATERIALS AND METHODS'. The amount of actin in each lane was also determined as a loading control. The arrows indicate the phosphorylated (upper band) and unphosphorylated (lower band) forms. (B) COS-7 cells were transfected with the wild type PKC β II cDNA (WT), T500V mutant cDNA (DN), and the mixture of both cDNAs (WT/DN). Expression of PKC β II was investigated by immunoblot analysis, respectively. The arrows indicate the phosphorylated (upper band) and unphosphorylated (lower band) forms. (C) Surface expression of Fc ϵ RI was measured by flowcytometry using an anti-DNP IgE and an FITC-conjugated anti-IgE antibody.

Figure 5 Attenuated Ca²⁺ mobilization by monomeric IgE in MC9 cells expressing PKC β II/T500V

Cells were stimulated by monomeric IgE (IgE, 3 μ g/ml), antigen (IgE/Ag; cells were sensitized with 0.3 μ g/ml anti-DNP IgE for 24 hr, and then stimulated with 10 ng/ml DNP-conjugated HSA), and thrombin (Thrombin, 1 U/ml). Changes in the cytosolic Ca²⁺ concentrations were monitored using Fura2/AM and presented as a ratio. The time point of each stimulation is indicated by the arrow. Bar = 1 min. This is a representative figure of three independent experiments showing similar results.

Figure 6 PKC β II/T500V suppresses histamine synthesis and IL-6 release induced by monomeric IgE, not upon antigen stimulation in MC9 cells

(A, B) Induction of HDC activity in MC9 cells was measured. MC9 cells were stimulated with the indicated concentrations of IgE for 6 hr (A) or were sensitized with 0.3 μ g/ml IgE for 24 hr followed by the antigen stimulation at the indicated concentrations for 6 hr (B). Values are represented by the means $\pm$ SEM (n=6). (C, D) HDC activity (C) and IL-6 release (D) induced by monomeric IgE (IgE, 1 μ g/ml, upper panel) or antigen stimulation (IgE/Ag, cells were sensitized with 0.3 μ g/ml anti-DNP IgE for 24 hr, and then stimulated with 10 ng/ml DNP-conjugated HSA, lower panel) was presented. Values are represented by the means $\pm$ SEM (n=3). * p <0.01 is regarded as significant by the Student's t test. Basal activity of HDC was <10 pmol/min/mg and no spontaneous release of IL-6 was detected in each MC9 clone.

Figure 1

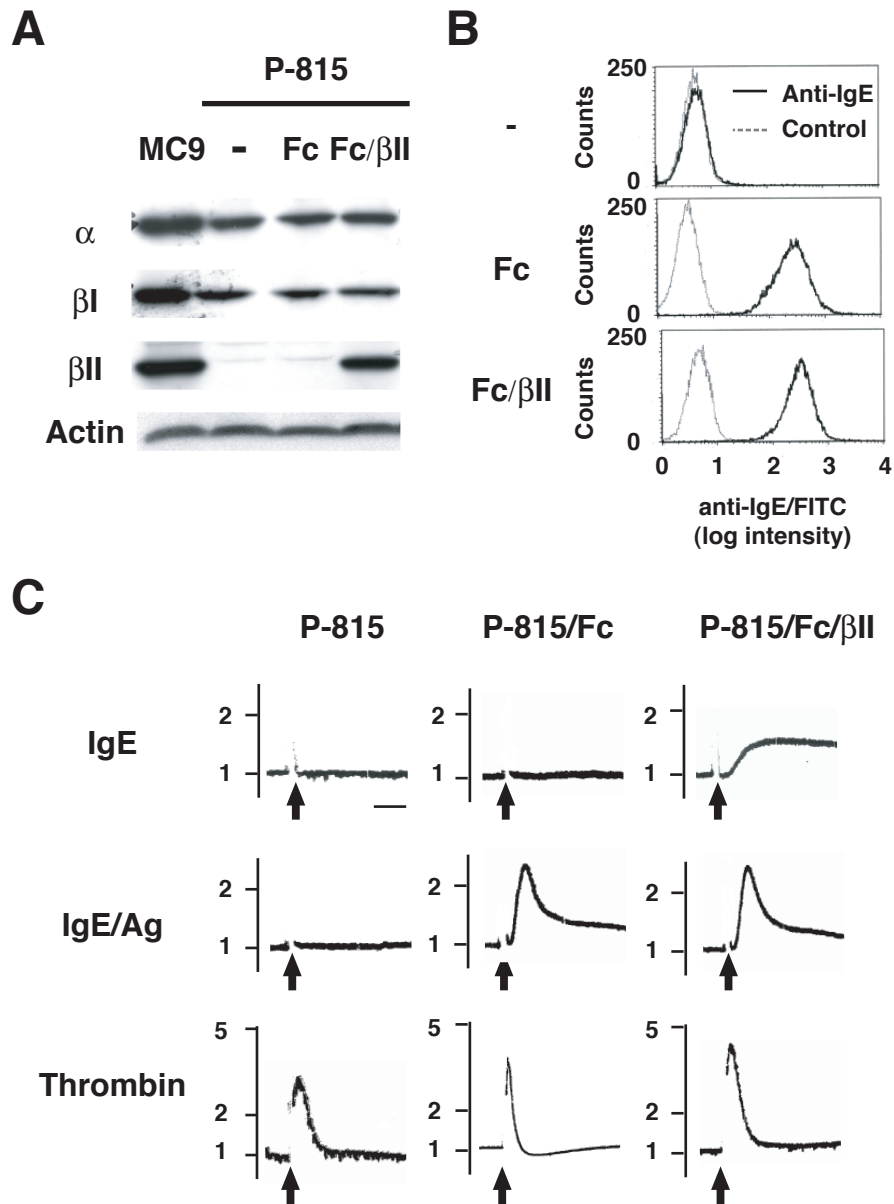
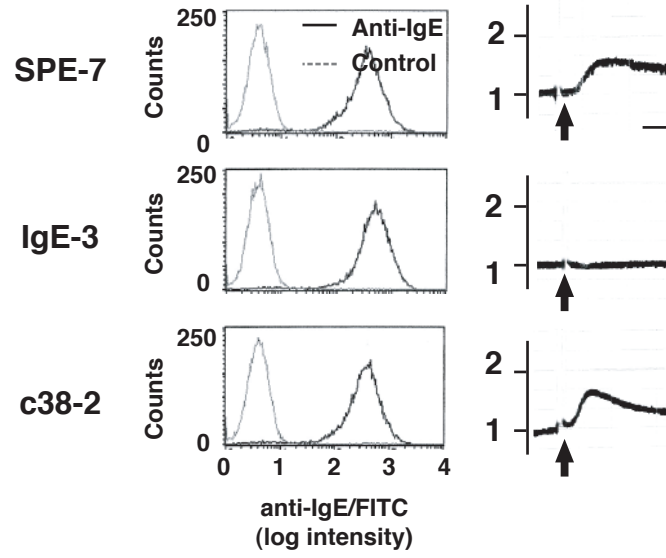


Figure 2

A



B

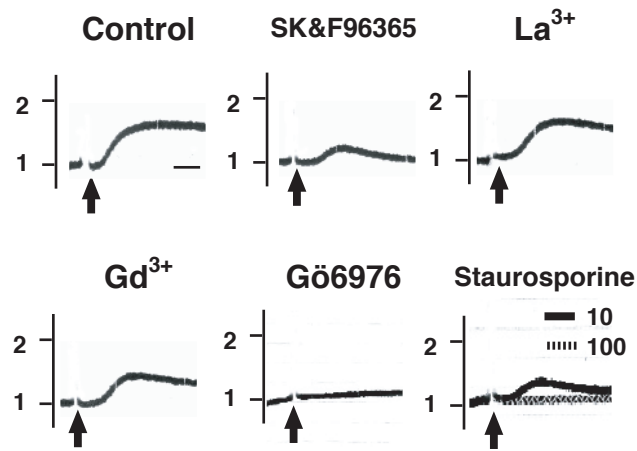


Figure 3

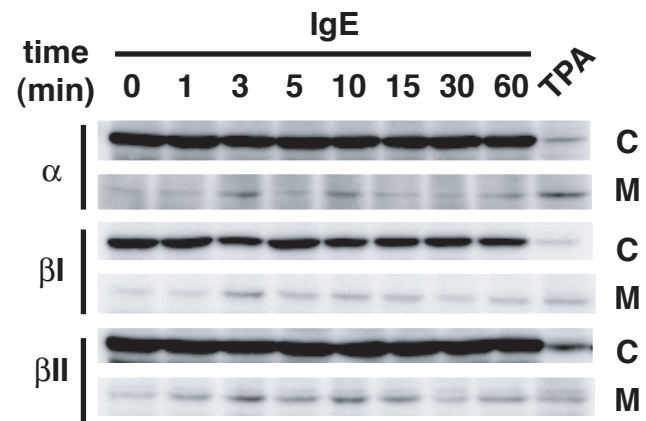
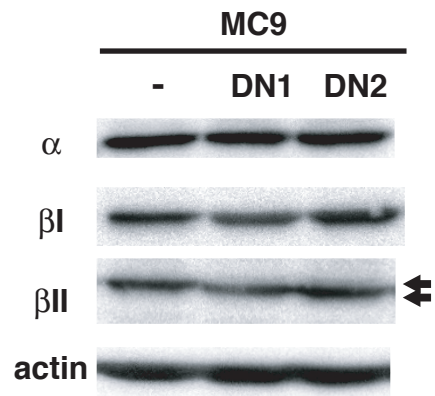
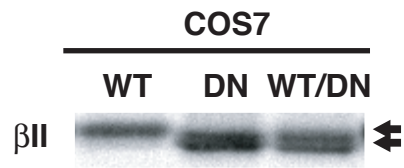


Figure 4

A



B



C

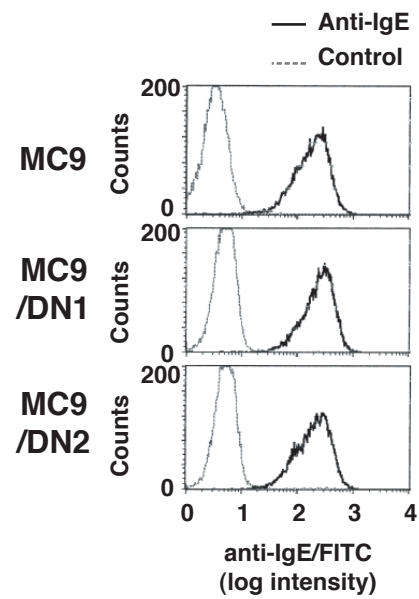


Figure 5

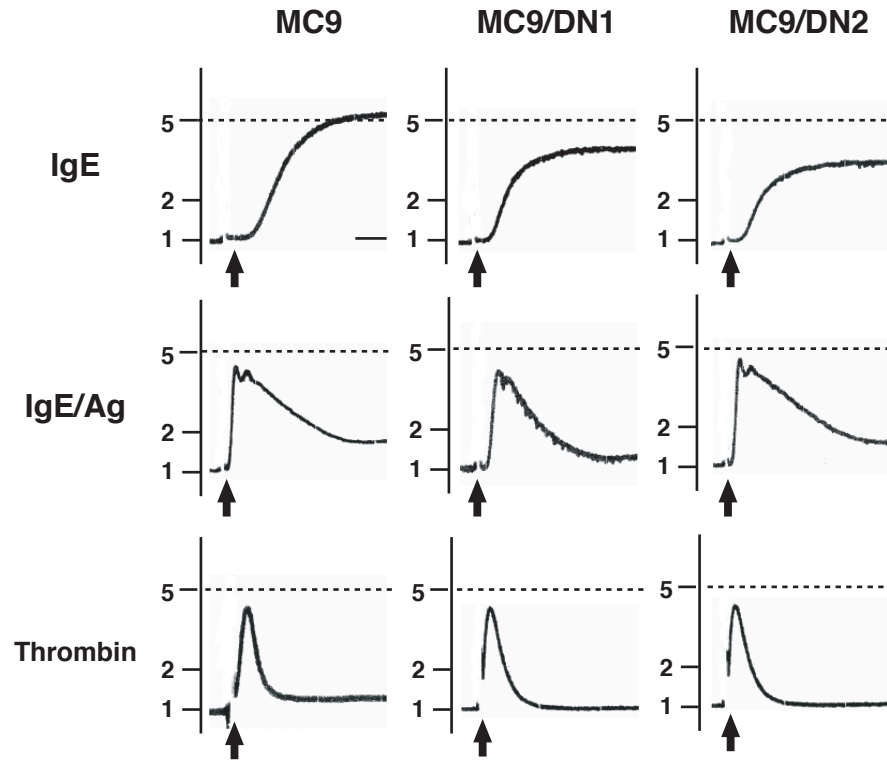


Figure 6

