

Protein kinase C δ functions downstream of Ca²⁺ mobilization in Fc ϵ RI signaling to degranulation in mast cells

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Background: Mobilization of Ca²⁺ plays an important role in the degranulation of mast cells. Although events upstream of Ca²⁺ mobilization in the regulation of degranulation are relatively well characterized, the downstream mediators of Ca²⁺ remain largely unknown.

Objective: We sought to characterize the downstream signaling mechanism by which Ca²⁺ mobilization mediates degranulation in antigen-stimulated mast cells.

Methods: The effect of various inhibitors was examined in the antigen-induced or Ca²⁺ ionophore A23187-induced degranulation process, and the effect of inhibitors on histamine release was tested in the mouse model of asthma.

Results: The δ isoform of protein kinase C (PKC) functions downstream of Ca²⁺ in the signaling pathway from Fc ϵ RI to degranulation in RBL-2H3 mast cells. Stimulation of cells with either antigen or the Ca²⁺ ionophore A23187 induced a rapid translocation of PKC- δ from the cytosol to the cellular membranes, and either treatment with the PKC- δ -specific inhibitor rottlerin or infection with an adenovirus encoding a dominant negative mutant of PKC- δ markedly inhibited degranulation induced with antigen or A23187. Furthermore, both the translocation of PKC- δ and degranulation induced by A23187 were inhibited by prevention of the accumulation of reactive oxygen species normally elicited by the ionophore. Finally, intraperitoneal injection of rottlerin prevented the increase in the concentration of histamine in bronchoalveolar lavage fluid induced by means of antigen challenge in a mouse model of allergic asthma.

Conclusion: PKC- δ plays an essential downstream mediatory role in the degranulation elicited by Ca²⁺ mobilization, and reactive oxygen species mediate the activation of PKC- δ by Ca²⁺ in the regulation of degranulation. (*J Allergy Clin Immunol* 2004;114:1085-92.)

Key words: Fc ϵ RI signaling, Ca²⁺, degranulation, PKC- δ , mast cells, asthma

Abbreviations used

BAL:	Bronchoalveolar lavage
[Ca ²⁺] _i :	Intracellular free Ca ²⁺ concentration
DAG:	Diacylglycerol
DMEM:	Dulbecco modified Eagle medium
DNP:	Dinitrophenol
DPI:	Diphenyleneiodonium chloride
EGTA:	Ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid
IP ₃ :	Inositol 1,4,5-trisphosphate
NAC:	N-acetyl cysteine
OVA:	Ovalbumin
PKC:	Protein kinase C
PLC- γ :	Phospholipase C γ
ROS:	Reactive oxygen species

Aggregation of Fc ϵ RI on mast cells or basophils triggers the release of inflammatory mediators, including histamine and serotonin, by means of degranulation and thereby underlies the allergic reaction.^{1,2} The initial signaling events elicited by cross-linking of multiple IgE-bearing Fc ϵ RI molecules by polyvalent antigen include the rapid activation of several nonreceptor-type protein tyrosine kinases and of other signaling proteins that mediate the regulation of degranulation.^{3,4} An important event in this signaling cascade is the activation of phospholipase C γ (PLC- γ), which catalyzes the generation both of diacylglycerol (DAG), an activator of protein kinase C (PKC), and of inositol 1,4,5-trisphosphate (IP₃), an inducer of intracellular Ca²⁺ mobilization.⁵

Members of the PKC family of serine-threonine kinases regulate mast cell functions, including leukotriene generation, cytokine synthesis, and degranulation.⁶⁻⁸ For example, PKC- δ and PKC- θ have been implicated in the regulation of antigen-induced mast cell degranulation.^{7,9-11} However, the precise mechanisms by which these novel PKC isoforms are activated in mast cells and the relationship between their activation and antigen-induced Ca²⁺ mobilization remain largely unknown.

The degranulation of mast cells and basophils triggered by antigen stimulation is dependent on the mobilization of intracellular Ca²⁺.¹²⁻¹⁵ The Rho family of small GTPases, including Rac and Cdc42, is also implicated in the regulation of Ca²⁺ mobilization and degranulation in mast cells and rat basophilic RBL-2H3 cells.¹⁶⁻¹⁸ Rac and Cdc42 have been suggested to promote degranulation by

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Supported by a Korea Research Foundation grant (KRF-2003-041-C20227), the SRC Program (Aging and Apoptosis Research Center, 2002) of the Korean Science and Engineering Foundation (KOSEF), the BioDiscovery Research Program, and Frontier 21 Programs (Proteomics, to J.-H.K.) of the Korean Ministry of Science and Technology.

Received for publication April 19, 2004; revised July 15, 2004; accepted for publication July 16, 2004.

Available online October 4, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.07.035

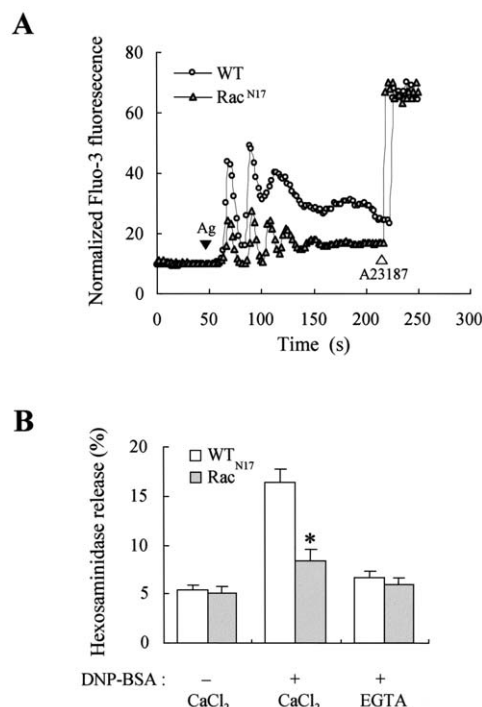


FIG 1. Role of Rac in antigen-induced Ca^{2+} mobilization and degranulation in RBL-2H3 cells. **A**, Data are means of normalized Fluo-3 fluorescence intensity obtained from at least 24 individual cells and are representative of at least 3 independent experiments. **B**, Cells were incubated for 1 hour in the absence or presence of DNP-BSA (0.5 $\mu\text{g}/\text{mL}$) in medium containing either 2 mmol/L CaCl_2 or 2 mmol/L EGTA, as indicated. The released enzyme activity was expressed as a percentage of the total activity. Data are presented as means $\pm$ SD for 3 experiments. * $P < .05$ versus the corresponding value for wild-type (WT) cells.

enhancing the production of IP_3 and Ca^{2+} mobilization in antigen-stimulated mast cells.¹⁹

Although the events upstream of Ca^{2+} mobilization in the signaling pathway from Fc ϵ RI to degranulation are relatively well characterized, the mechanism by which the increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) mediates degranulation is less clear. The principal aim of the present study was therefore to characterize the downstream signaling mechanism by which Ca^{2+} mediates degranulation in antigen-stimulated RBL-2H3 cells. Our results indicate that Ca^{2+} induces degranulation through a PKC- δ -dependent pathway and that reactive oxygen species (ROS) mediate Ca^{2+} -induced PKC- δ activation in these cells.

METHODS

Reagents

A mouse monoclonal IgE antibody (clone SPE-7) to dinitrophenol (DNP), dimethyl sulfoxide, *N*-acetyl cysteine (NAC), diphenyleneiodonium chloride (DPI), phorbol 12-myristate 13-acetate, 2',7'-dichlorofluorescein diacetate, Fluo-3 acetoxymethyl ester, *o*-phthalaldehyde, and ovalbumin (OVA) was obtained from Sigma (St Louis, Mo). DNP-conjugated BSA, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, A23187, U73122, GF109203X,

Gö6976, rottlerin, and *N*(G)-monomethyl-L-arginine, a nonselective nitric oxide synthase (NOS) inhibitor, were from Calbiochem (La Jolla, Calif). Mouse mAbs to PKC- δ and Rac1 were from BD Biosciences (Palo Alto, Calif). Dulbecco modified Eagle medium (DMEM), phenol red-free DMEM, heat-inactivated FBS, and nonessential amino acids were from Invitrogen (Carlsbad, Calif). Aluminum hydroperoxide gel was from Pierce (Rockford, Ill), and glutathione-Sepharose 4B beads and an ECL kit were from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell culture and construction of stable clones

RBL-2H3 cells were maintained, as described previously.²⁰ Cells were transfected with pcDNA-HA-Rac^{N17} encoding a dominant negative Rac1 mutant by using the lipofectamine transfection method. Stably transfected clones were selected with 0.4 mg/mL G418 (Invitrogen, Carlsbad, Calif), after which they were screened for HA-Rac1 expression by means of immunoblotting the cell lysates with anti-HA rabbit antibody.

Assay of β -hexosaminidase activity

RBL-2H3 cells were plated in 24-well plates at a density of 2×10^5 cells per well, sensitized with monoclonal DNP-specific IgE (1 $\mu\text{g}/\text{mL}$) for 12 hours, and incubated for 6 hours in DMEM supplemented with 0.5% FBS. They were then washed twice with Tyrode buffer (135 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 5.6 mmol/L glucose, 1 mg/mL BSA, and 20 mmol/L Hepes-NaOH [pH 7.4]) and stimulated at 37°C with DNP-BSA (0.5 $\mu\text{g}/\text{mL}$) or 1 $\mu\text{mol/L}$ A23187 in Tyrode buffer for 1 hour or 30 minutes, respectively. The incubation medium and cell lysates (prepared by addition to the cells of the same volume of Tyrode buffer containing 1% Triton X-100) were then recovered for quantitation of β -hexosaminidase release as a measure of degranulation activity. The samples (100 μL) were incubated for 1 hour at 37°C with 100 μL of *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (1.3 mg/mL) in 0.1 mol/L sodium citrate buffer (pH 4.5), after which the reaction was terminated by the addition of 200 μL of 0.2 mol/L glycine buffer (pH 10.7). The generation of *p*-nitrophenol was monitored by measurement of absorbance at 405 nm. The released β -hexosaminidase activity was expressed as a percentage of the total activity present in the cells and medium.

Measurement of $[\text{Ca}^{2+}]_i$

The $[\text{Ca}^{2+}]_i$ was measured with the use of the fluorescent indicator Fluo-3. Cells were plated on cover slips and incubated for 12 hours in DMEM supplemented with 15% FBS. They were then sensitized with anti-DNP IgE (1 $\mu\text{g}/\text{mL}$) for 12 hours, incubated in DMEM containing 0.5% FBS for 6 hours, washed twice with Tyrode buffer, incubated in the dark for 1 hour at 25°C in Tyrode buffer containing 5 $\mu\text{mol/L}$ Fluo-3 acetoxymethyl ester, and exposed to DNP-BSA (0.5 $\mu\text{g}/\text{mL}$). In experiments with various inhibitors, the cells were exposed to these agents for 20 minutes before treatment with DNP-BSA. Fluo-3 fluorescence was monitored in 24 randomly selected cells for each experimental condition with a TCS 4D laser-scanning microscope (Leica, Nussloch, Germany); it was excited at 488 nm with an argon laser, and the evoked emission was filtered with a 515-nm long-pass filter.

Subcellular fractionation

RBL-2H3 cells grown in 60-mm dishes and stimulated as indicated were washed twice with ice-cold PBS, scraped into the same solution, harvested by means of centrifugation, and resuspended in 0.2 mL of buffer A (137 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4 , 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4 , 2.5 mmol/L EDTA, 1 mmol/L dithiothreitol,

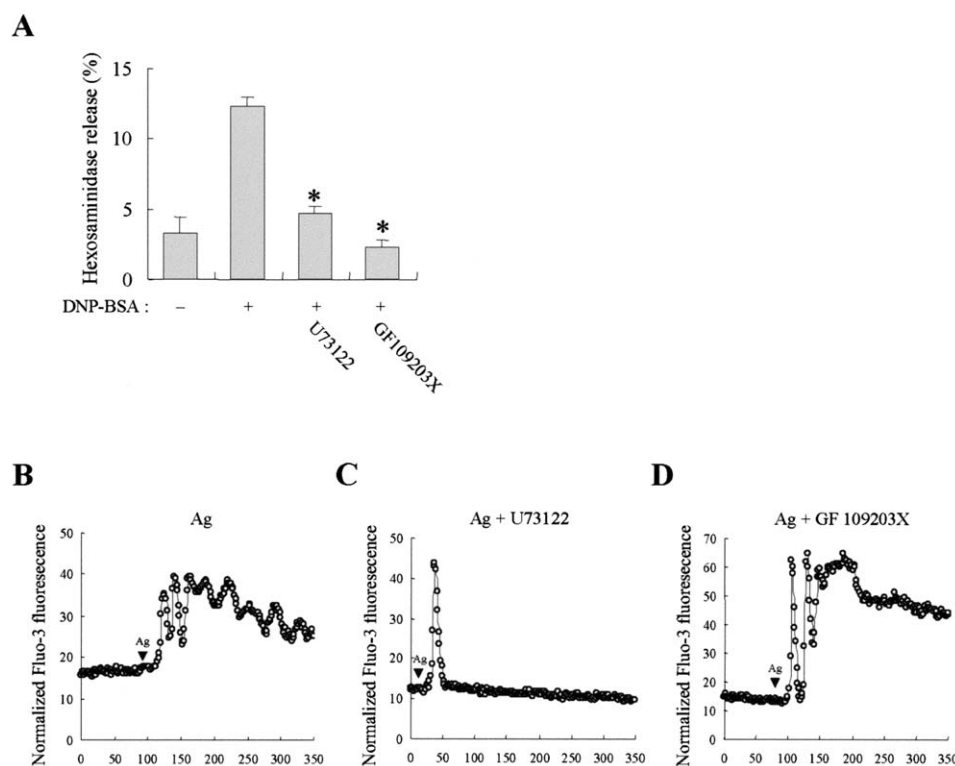


FIG 2. Participation of PLC- γ , but not PKC, in antigen-induced Ca^{2+} mobilization in RBL-2H3 cells. **A**, The extent of degranulation was determined. Data are presented as means $\pm$ SD for 3 experiments. * $P < .05$ versus the value for cells stimulated with antigen in the absence of inhibitor. **B-D**, The $[\text{Ca}^{2+}]_i$ was monitored on the basis of Fluo-3 fluorescence. Data are representative of 3 independent experiments. Ag, Antigen.

0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 $\mu\text{g}/\text{mL}$ leupeptin, adjusted to pH 7.5). The cells were lysed on ice either by means of sonication (two 15-second pulses) or by passing them 20 times through a 21.1-gauge needle. The lysates were centrifuged at 80,000g for 1 hour at 4°C to obtain cytosolic and total particulate fractions. The pellet was resuspended in 50 μL of buffer A, whereas the supernatant was mixed with 5 volumes of acetone, incubated for 5 minutes on ice, and centrifuged at 12,000g for 10 minutes at 4°C. The new pellet was resuspended in 200 μL of buffer A containing 1% Triton X-100.

Adenovirus infection

Adenovirus expression vectors for wild-type PKC- δ (Ax-PKC- δ -WT) or for a dominant negative mutant thereof (Ax-PKC- δ -DN) were kindly provided by J. S. Chun (Kwangju Institute of Science and Technology). Subconfluent RBL-2H3 cells grown in 24-well plates were incubated for 1 hour with either of these viruses or with the control virus (Ax-LacZ) at a multiplicity of infection of 100 in DMEM containing 0.1% FBS. The viruses were then removed, and the cells were cultured for an additional 30 hours in DMEM supplemented with 15% FBS.

Measurement of ROS

Intracellular H_2O_2 was measured by means of flow cytometric analysis of DCF fluorescence, as described previously.²¹ DCF fluorescence was excited at 488 nm, and the evoked emission was filtered with a 515-nm long-pass filter.

Mouse sensitization and challenge

Female C57BL/6J mice, 6 weeks of age and free of murine-specific pathogens, were obtained from the Korean Research Institute

of Chemistry Technology (Daejeon, Korea). All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of the Korea University. Antigen sensitization and challenge in mice were performed as previously described.²² GF109203X (20 μg), rottlerin (5 μg), or NAC (300 μg) was injected into the peritoneum of mice 1 and 24 hours before OVA challenge.

Measurement of histamine

Histamine in bronchoalveolar lavage (BAL) fluid obtained from OVA-challenged mice was assayed by using an automated fluorometric method.²³ Histamine in BAL fluid was purified and coupled with *o*-phthalaldehyde with the use of an Astoria Histamine Analyzer (Astoria-pacific, Clackamas, Ore). The fluorescence of the histamine-*o*-phthalaldehyde complex was then measured with a fluorometer.

Statistical analysis

Data are expressed as means $\pm$ SD, and comparisons between groups were performed with the Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

Essential role of Rac in antigen-induced Ca^{2+} mobilization and degranulation

Previous studies have implicated the role of Rac in regulation of the IP_3 - Ca^{2+} signaling pathway and exocytosis in mast cells.^{17,19,24} To examine the role of Rac in

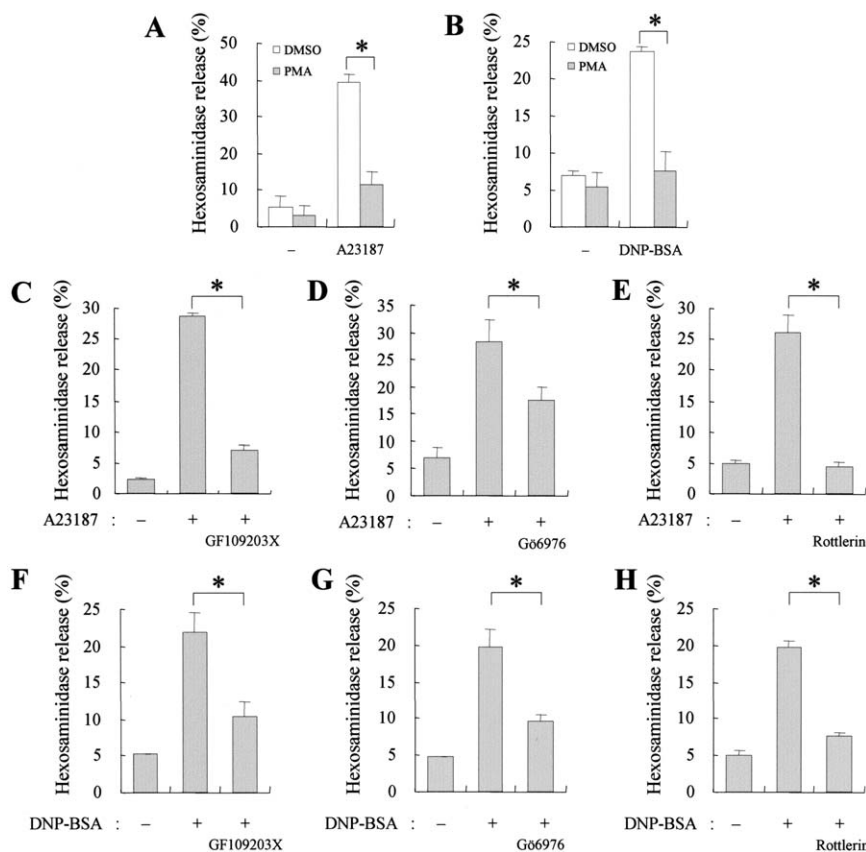


FIG 3. Participation of PKC in signaling to degranulation induced by A23187 or by antigen in RBL-2H3 cells. Data are presented as means $\pm$ SD of values from 3 independent experiments. * $P < .05$ for the indicated comparisons. DMSO, Dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate.

Fc ϵ RI signaling to Ca^{2+} mobilization and degranulation, we generated RBL-2H3 cell lines that stably express the dominant negative mutant of Rac1. Antigen (DNP-BSA, 0.5 μ g/mL) stimulation of wild-type RBL-2H3 cells induced a rapid increase in $[Ca^{2+}]_i$ (Fig 1, A). This effect of antigen was markedly inhibited in cells expressing Rac^{N17}, a dominant negative mutant of Rac1. In contrast, the Ca^{2+} ionophore A23187 (1 μ mol/L) elicited a rapid increase in $[Ca^{2+}]_i$ in all these cell lines.

Antigen-induced Ca^{2+} mobilization eventually leads to the release of preformed granules in RBL-2H3 cells. This degranulation effect was markedly inhibited in cells expressing Rac^{N17} (Fig 1, B), which is consistent with previous observations.²⁴ The degranulation elicited by antigen stimulation in these cell lines was strictly dependent on the presence of extracellular Ca^{2+} , given that it was not apparent in the presence of ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Together these results indicate that Ca^{2+} is essential for degranulation and that Rac plays a critical role in antigen-induced Ca^{2+} mobilization and subsequent degranulation.

PLC- γ , but not PKC, is important for antigen-induced Ca^{2+} mobilization

To clarify the mediators of antigen-induced Ca^{2+} mobilization and degranulation, we tested the effects of

inhibitors of PLC- γ (U73122, 10 μ mol/L) and PKC (GF109203X, 200 nmol/L). Each of these compounds markedly inhibited antigen-induced degranulation, suggesting that this process is dependent on PLC- γ and PKC in RBL-2H3 cells. To our interest, however, whereas U73122 greatly inhibited antigen-induced Ca^{2+} mobilization, GF109203X had no such effect (Fig 2, B-D). These results thus suggest that PLC- γ , but not PKC, mediates Ca^{2+} mobilization in response to antigen and that PKC mediates the following degranulation step in RBL-2H3 cells.

PKC mediates A23187- or antigen-induced degranulation

Although PKC does not appear to contribute to antigen-induced Ca^{2+} mobilization, previous studies have suggested that certain PKC isoforms, including PKC- δ and PKC- θ , are critically involved in Fc ϵ RI signaling.^{7,9-11} We therefore next investigated whether PKC acts downstream of Ca^{2+} mobilization in Fc ϵ RI signaling. Bypassing the IgE receptor with the Ca^{2+} ionophore A23187 resulted in degranulation to an extent similar to that observed in response to antigen (Fig 3, A and B), indicating that Ca^{2+} influx is sufficient to trigger degranulation in these cells. Prolonged (12 hours) treatment of cells with phorbol 12-myristate 13-acetate, which induces downregulation of

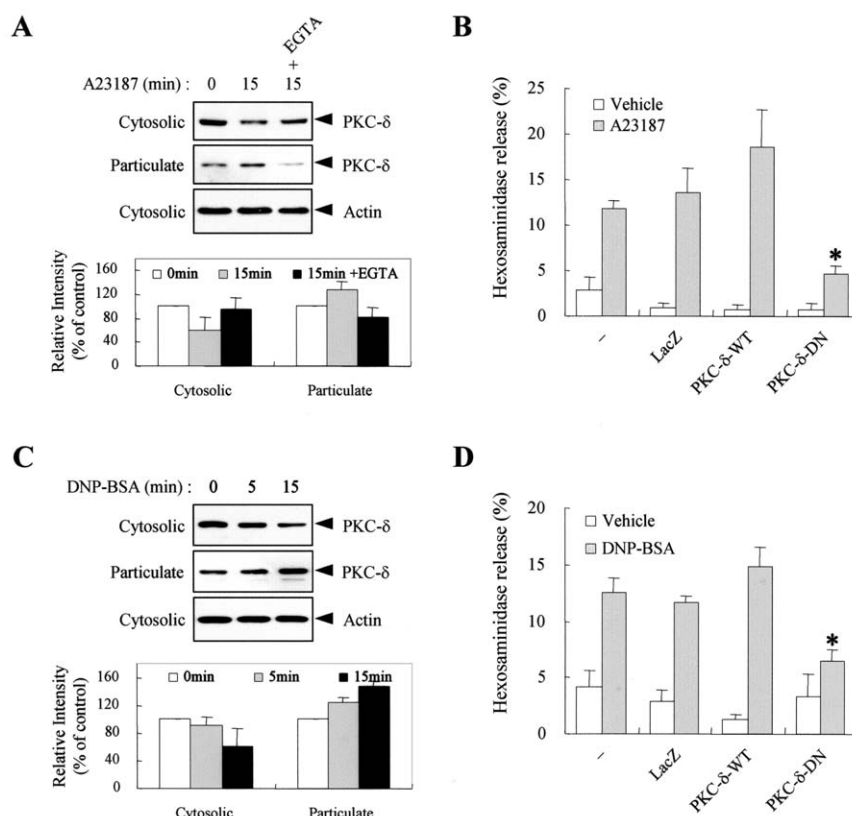


FIG 4. Specific role of PKC- δ in degranulation induced by A23187 or by antigen in RBL-2H3 cells. **A** and **C**, Cytosolic and particulate fractions were prepared and subjected to immunoblot analysis with antibodies to PKC- δ . Data are representative of at least 3 independent experiments. Additionally, scan densitometry was used to determine the relative intensity of the bands, and these data are presented as graphs. Data are presented as means $\pm$ SD of values from 3 independent experiments. * $P < .05$ for the indicated comparisons. **B** and **D**, The extent of degranulation was determined. Data are presented as means $\pm$ SD of values from 3 independent experiments. * $P < .05$ versus the corresponding value for noninfected control cells.

PKC activity,²⁵ resulted in remarkable inhibition of degranulation in response either to A23187 or antigen. To investigate which isoform of PKC might be responsible for A23187-induced degranulation, we examined the effects of various inhibitors, including GF109203X (a general PKC inhibitor), Gö6976 (an inhibitor of Ca^{2+} -dependent isoforms of PKC), and rottlerin (a PKC- δ -specific inhibitor). All 3 compounds inhibited A23187-induced degranulation, although the effect of rottlerin was most pronounced (Fig 3, C-E). Similar results were obtained for the effects of these PKC inhibitors on antigen-induced degranulation (Fig 3, F-H), suggesting that PKC- δ plays a critical role in antigen-induced degranulation, acting downstream of Ca^{2+} mobilization, in RBL-2H3 cells, although we cannot eliminate other PKC isoforms from having potential roles in the process.

Role of PKC- δ in Ca^{2+} -induced degranulation

PKC- δ is a DAG-dependent, Ca^{2+} -independent isoform of PKC. Given that our results implicated PKC- δ in A23187-induced degranulation, we next examined the effect of A23187 on PKC- δ activity by monitoring the translocation of the enzyme from the cytosol to the

membranes. A23187 (1 $\mu\text{mol/L}$) indeed induced the translocation of PKC- δ , and this effect was dependent on the presence of extracellular Ca^{2+} (Fig 4, A). To examine further the role of PKC- δ , we introduced wild-type PKC- δ or a dominant negative mutant PKC- δ into RBL-2H3 cells by means of adenoviral infection. Although infection of cells with a control virus (Ax-LacZ) or with the virus for wild-type PKC- δ (Ax-PKC- δ -WT) did not significantly affect A23187-induced degranulation, infection with the virus for the dominant negative mutant (Ax-PKC- δ -DN) significantly inhibited this action of A23187 (Fig 4, B). Similarly, antigen (DNP-BSA, 0.5 $\mu\text{g/mL}$) stimulation induced PKC- δ translocation to membranes (Fig 4, C), and the dominant negative mutant of PKC- δ significantly inhibited antigen-induced degranulation (Fig 4, D). Together, these results suggest that PKC- δ is required for antigen-induced degranulation in RBL-2H3 cells and that it acts downstream of Ca^{2+} mobilization.

Role of ROS in Ca^{2+} -induced activation of PKC- δ

A ROS-dependent pathway has been shown to mediate the activation of PKC in various cell types.²⁶⁻²⁸ In

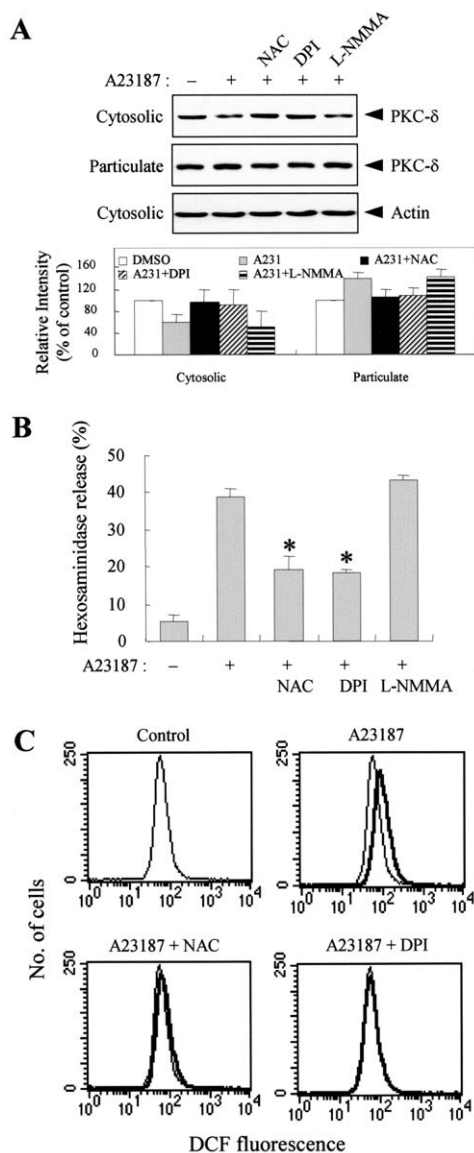


FIG 5. Role of ROS in Ca^{2+} -induced activation of PKC- δ in RBL-2H3 cells. **A**, The translocation of PKC- δ was analyzed by means of immunoblot analysis. Additionally, the scan densitometry was used to determine the relative intensity of the bands, and these data are presented as graphs. Data are presented as means $\pm$ SD of values from 3 independent experiments. $*P < .05$ for the indicated comparisons. **B**, Data are presented as means $\pm$ SD of values from 3 independent experiments. $*P < .05$ versus the corresponding value for cells stimulated with A23187 in the absence of inhibitor. **C**, DCF fluorescence was quantified by means of flow cytometry; the control profile is shown for comparison in all panels. Data are representative of 3 independent experiments. L-NMMA, N(G)-monomethyl-L-arginine; DMSO, dimethyl sulfoxide.

addition, ROS have been implicated in degranulation in mast cells and basophils.^{29,30} To determine whether ROS mediate the activation of PKC- δ and subsequent degranulation induced by A23187 in RBL-2H3 cells, we examined the effects of DPI, an inhibitor of flavoenzymes, such as reduced nicotinamide adenine dinucleotide phos-

phate oxidase, and NAC, an ROS scavenger, on these processes. Both PKC- δ translocation and degranulation induced by A23187 were markedly inhibited in cells pretreated with 10 $\mu\text{mol/L}$ DPI or 1 mmol/L NAC (Fig 5, A and B). To determine whether A23187 induces the production of ROS in RBL-2H3 cells, we measured the generation of H_2O_2 . A23187 indeed elicited an approximately 2-fold increase in the level of ROS, and this effect was abolished by pretreatment of cells with DPI or NAC (Fig 5, C). Together, these results suggest that ROS mediate Ca^{2+} signaling to PKC- δ in RBL-2H3 cells.

Effect of PKC- δ inhibition on mast cell degranulation *in vivo*

Finally, we investigated the physiologic relevance of PKC- δ in mast cell degranulation *in vivo* with the use of an OVA-induced mouse model of asthma. Challenge with OVA induced a marked increase in the concentration of histamine in BAL fluid compared with that apparent in animals challenged with saline (Fig 6). This effect of OVA was greatly inhibited by prior intraperitoneal administration of GF109203X (20 μg), rottlerin (5 μg), or NAC (300 μg), suggesting that both PKC- δ and ROS contribute to the release of histamine from mast cells *in vivo*.

DISCUSSION

Although signaling events upstream of the Ca^{2+} mobilization induced by antigen stimulation are relatively well characterized, those that link the increase in $[\text{Ca}^{2+}]_i$ to degranulation have remained unclear. We have now provided evidence for a critical role of PKC- δ in the process of degranulation evoked by Ca^{2+} mobilization in RBL-2H3 cells. In addition, our results suggest that ROS generated in response to the increase in $[\text{Ca}^{2+}]_i$ mediate the activation of PKC- δ .

Several mediators of Fc ϵ RI signaling to degranulation, including the tyrosine kinases Lyn and Syk, as well as PLC- γ , IP $_3$, and Ca^{2+} , have been identified.^{14,31} Recently, an alternative novel Fyn kinase-Grb2-PI $_3$ -kinase-PKC- δ -linked pathway was demonstrated to be also required for IgE-dependent mast cell degranulation.¹⁰ Therefore both Lyn/Syk-dependent Ca^{2+} increase and Fyn-linked PKC- δ activation might somehow collaborate with each other to efficiently mediate mast cell degranulation, as suggested previously.¹⁰ The precise mechanism by which intracellular Ca^{2+} regulates degranulation has been unknown, however, and the linkage between Ca^{2+} and PKC- δ has not previously been described. There is one report proposing that PKC- δ is the main isoform implicated in Ca^{2+} -dependent degranulation in mast cells, thus supporting the linkage for PKC- δ to calcium flux.³² The PKC isoforms α , β I, β II, δ , ϵ , and ξ are expressed in RBL-2H3 cells (unpublished data), and our results now indicate that PKC- δ , a Ca^{2+} -independent, DAG-dependent isoform, specifically contributes to the triggering of degranulation in response to Ca^{2+} increase induced by antigen or A23187 in these cells. However, other isoforms of PKCs might

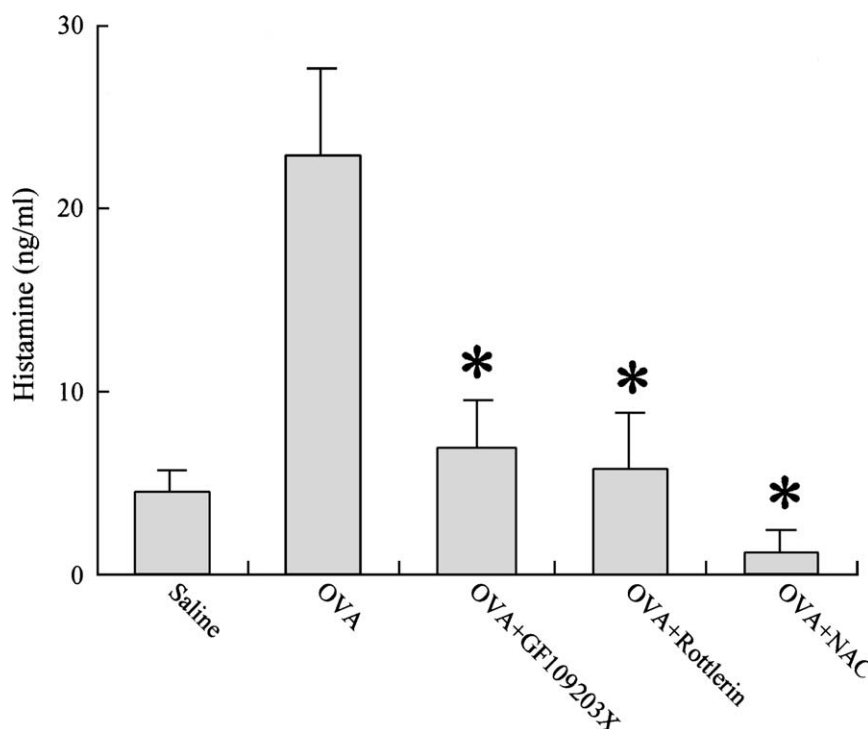


FIG 6. Roles of ROS and PKC- δ in mast cell degranulation in an OVA-induced mouse model of asthma. Two days after OVA challenge, BAL fluid was collected and assayed for its histamine content. Data are presented as means $\pm$ SD of values from 5 mice. * $P < .05$ versus the corresponding value for animals challenged with OVA in the absence of inhibitor.

also have potential roles in the degranulation process in general, and, for example, overexpression of PKC- β was reported to significantly enhance degranulation activity in mast cells.³³ Interestingly, Leitges et al³⁴ proposed that PKC- δ might act as a negative regulator of degranulation in response to antigen, which is inconsistent with other findings.^{10,31,32} The reason for the discrepancy is unclear and remains to be resolved. In any event our results suggest that PKC- δ lies at the crucial downstream center of a network of pathways from both Lyn/Syk-linked cascade and Fyn-linked cascade, acting as a key converging mediatory molecule in the IgE signaling to degranulation in mast cells.

Inhibitors of PLC- γ (U73122) or of phospholipase D (propranolol) did not affect the translocation of PKC- δ induced by A23187 in RBL-2H3 cells (data not shown), suggesting that Ca^{2+} ionophore, bypassing the IgE receptor, activates PKC- δ without involving PLC- γ or PLD. Oxidative stress has also been shown to activate PKC- δ through tyrosine phosphorylation,²⁶⁻²⁸ and inhibition of ROS generation by DPI prevented antigen-induced or A23187-induced tyrosine phosphorylation and degranulation in mast cells.^{29,30} Consistently, we also observed that A23187 induced ROS generation in RBL-2H3 cells, and prevention of ROS accumulation inhibited A23187-induced PKC- δ activation and degranulation (Fig 5). We thus propose that the increase in $[\text{Ca}^{2+}]_i$ induced by antigen stimulation triggers the generation of ROS, which

might in turn activate a tyrosine kinase (or kinases) that phosphorylates PKC- δ . The precise mechanism by which Ca^{2+} induces ROS generation and the source of the ROS remain to be determined. In addition, how PKC- δ activated by Ca^{2+} -ROS cascade triggers degranulation is unclear, although it is possible that PKC- δ regulates actin polymerization through phosphorylation of the myosin light chain. Indeed, A23187-induced secretion was shown to be correlated with myosin light chain phosphorylation by PKC in RBL-2H3 cells, although the specific PKC isoform was not characterized.^{35,36}

Finally, we demonstrated the physiologic relevance of PKC- δ in antigen-induced mast cell degranulation in a murine asthma model (Fig 6). Given that histamine contributes to asthmatic attacks in several ways, such as by inducing vasodilation, bronchoconstriction, and mucus secretion, inhibition of this isoform might be expected to alleviate the symptoms of such attacks. Indeed, we observed that inhibition of PKC- δ attenuated both eosinophil infiltration and airway obstruction in this animal model (data not shown). Together, our findings suggest that inhibition of PKC- δ might contribute to the therapeutic alleviation of allergic inflammation associated with mast cell degranulation. In conclusion, the present study demonstrates a previously unrecognized direct link between Ca^{2+} and PKC- δ in the degranulation pathway, thus proposing PKC- δ as a key signaling molecule responsible for the early events in the IgE signaling in mast cells.

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