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Original Article

Involvement of both protein kinase C and G proteins in superoxide production after IgE triggering in guinea pig eosinophils

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ABSTRACT

To study the function and mechanism of eosinophils via the low affinity IgE receptor (FcεRII), we examined the production of O_2 metabolites by measuring the luminol-dependent chemiluminescence (LDCL) response and the generation of cysteinyl leukotrienes. Eosinophils obtained from guinea pig peritoneal fluid sensitized with horse serum were purified. Luminol-dependent chemiluminescence was induced by stimulation with monoclonal anti-CD23 antibody, but not by mouse serum (controls). The mean (±SEM) value of LDCL was $20.6 \pm 1.3 \times 10^3$ c.p.m. This reaction consisted of an initial rapid phase and a propagation phase and ended within 10 min. Guinea pig eosinophils were histochemically stained with monoclonal anti-CD23 antibody. The major product generated in the LDCL response was superoxide, as determined by the measurement of superoxide by cytochrome c reduction and the complete inhibitory effect of superoxide dismutase on the LDCL response. Pretreatment with either pertussis toxin or cholera toxin inhibited the LDCL reaction. Depletion of bivalent ions by EDTA inhibited this response and the protein kinase C inhibitor D-sphingosin inhibited both 1-oleoyl-2-acetyl-glycerol-induced and FcεRII-mediated LDCL. These findings suggest that the NADPH-protein kinase C pathway may be involved in the FcεRII-mediated LDCL response in guinea pig eosinophils.

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INTRODUCTION

Eosinophils have attracted a great deal of attention in bronchial asthma (BA) because they produce potent spasmogens, such as cysteinyl leukotrienes (LT)[1,2] and platelet activating factor (PAF),[3] and contain granule proteins, such as eosinophil peroxidase (EPO), eosinophil cationic protein and major basic protein, which have been reported to damage epithelia in the airways.[4] It has also been demonstrated that a number of activated eosinophils migrate into airways during the allergen-induced late asthmatic response.[5,6] In addition, the number of eosinophils in sputa and peripheral venous blood from asthmatics with attacks suggests the presence of a close relationship between eosinophils and BA.[5]

Recent investigations have demonstrated the presence of low affinity IgE Fc receptors (FcεRII) on certain cells other than histamine-containing cells,[7–9] although the reaction via high affinity IgE Fc receptors on the surface of histamine-containing cells has mainly been investigated in allergic asthma.[10] Activated eosinophils have been reported to be able to release greater amounts of spasmogens and basic granule proteins and have greater numbers of FcεRII on their surfaces than do non-activated eosinophils[11–13] and they have been reported to play an important role in the damage caused by helminth-infected patients with a high IgE titer. In addition, interleukin (IL)-4, which augments IgE production, regulates the expression of FcεRII on EoL-3 cells.[14] Thus, FcεRII on

the surface of activated eosinophils plays an important role in IgE-mediated allergic inflammation in BA and in helminth infection. Therefore, we examined the role of FcεRII on the eosinophil membrane by using monoclonal anti-CD23 antibody (CD23Ab).

In the present study, to evaluate the function of eosinophils via FcεRII, we examined luminol-dependent chemiluminescence (LDCL) responses and the production of cysteinyl LT. In addition, we investigated the signal transduction of the chemiluminescence response via FcεRII in eosinophils.

METHODS

Reagents

PIPES, 5-amino-2,3-dihydrol,4-phthalzinedione (luminol), cytochrome c from horse heart type IV, catalase from bovine liver, Hank's buffered saline solution (HBSS), pertussis toxin, cholera toxin, 3-amino-1,2,4-triazole (AMT), superoxide dismutase (SOD) from bovine blood, D-sphingosin from bovine brain and methyl green were all purchased from Sigma Chemical Co. (St Louis, MO, USA). 1-Oleoyl-2-acetyl-glycerol (OAG) and synthetic diacylglycerol were obtained from Calbiochem Co. Ltd (Los Angeles, CA, USA). Horse serum was from Gibco Laboratories Life Technologies Inc. (Rochester, NY, USA), while Giemsa solution and Wright eosine methylene blue were from Merck Co. Ltd (Tokyo, Japan).

Isolation of guinea pig eosinophils

As previously reported,[13] we obtained purified eosinophils by peritoneal lavage from horse serum-sensitized guinea pig eosinophils. Briefly, after male Hartley strain guinea pigs were immunized by intraperitoneal injection of 2 mL horse serum once a week for 2 months, peritoneal lavage fluid was obtained 48 h after the last injection and was centrifuged at 200 g for 8 min; the cell pellets were then resuspended in 3 mL of 1.070 g/mL Percoll solution. For the Percoll discontinuous gradients method,[13] the stock Percoll solution (density: 1.112 g/mL) was serially diluted with PIPES physiologic solution (pH 7.3) containing 24 mmol/L PIPES, 125 mmol/L NaCl, 5 mmol/L KCl and 0.03% bovine albumin (PA solution), providing a series of different densities of isotonic Percoll solutions. The samples suspended in 1.070 g/mL Percoll solution were carefully layered on top of four different densities of isotonic Percoll solution (from top to bottom: 3 mL of 1.080 g/mL, 5 mL of 1.085 g/mL, 10 mL of 1.095 g/mL and 3 mL of 1.112 g/mL) in a 25 mL conical polycarbonate tube (Beckman Instruments Inc., Fullerton, CA, USA). The tube was then centrifuged at 900 g for 15 min at 18°C. To obtain highly purified eosinophils, cells were obtained from the lowest of three distinct bands and were then washed twice with PA solution. Cells were examined in a Wright–Giemsa air-dried smear and the purity of the eosinophils was within the range of 99–100% (mean ±SEM: 99.8±0.3%). These purified eosinophils had over 1.095 g/mL density and were invariably contaminated with mononuclear cells. Alcian blue-safranin staining excluded the possibility of contamination by histamine-containing cells.

Measurement of LDCL reaction

Purified eosinophils were suspended in complete HBSS containing 1.7 mmol/L $CaCl_2$, 0.81 mmol/L $MgSO_4$, 0.50 mmol/L $KHPO_4$, 5.38 mmol/L KCl, 137 mmol/L NaCl, 0.48 mmol/L NaH_2PO_4, 4.2 mmol/L $NaHCO_3$ and 0.1% glucose adjusted to pH 7.3 by titration with 1 mol/L HCl. A final concentration of 3×10^6 cells/mL was suspended in HBSS solution containing 100 μmol/L luminol in a 10×30 mm plastic tube and was preincubated for 15 min at 37°C. Then, monoclonal mouse anti-CD23 antibody (2 μg) was added to the cell suspension. In controls, eosinophils were stimulated with normal mouse serum. In a preliminary study, 15 min was selected as the preincubation time.

Chemiluminescence was recorded every 8 s with a Biolumat LB 9500C (Laboratorium, ProF., Berthold, Wildbad, Germany) operated in the rate mode. The signals were fed into a PC-8801 mK II mR NEC Co. Ltd (Tokyo, Japan) with a PC-KD854 display. Chemiluminescence (CL) responses are expressed as peak values because the area obtained from CL responses was significantly correlated with the peak value ($y=1.203x-0.17$; $r=0.933$, $P<0.001$).

Immunohistochemical staining with monoclonal anti-CD23 antibody

After cytocentrifugation, specimens were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 10 min at room temperature and were stained immediately with the DAKO APAAP (alkaline phosphatase–anti-alkaline phosphatase) Kit™ System 40 K 670 according to the manufacturer's protocol (DAKO Corp., Carpinteria, CA, USA). In brief, after an incuba-

tion with normal rabbit serum to quench non-specific protein binding to certain tissue elements, samples were washed twice with Tris-buffered saline (TBS) and monoclonal anti-CD23Ab (1:25), or normal mouse serum (1:200) was then incubated with the samples for 48 h at 4°C. Samples were washed with TBS and were treated with rabbit antiserum to mouse IgG (link antibody) at room temperature for 30 min and were then washed and stained with APAAP at room temperature for 30 min. After washing with TBS, naphthol AS–MX phosphate and Fast Red TR agents were added as substrates and were reacted at room temperature for 20 min. Eosinophils were counterstained with Veronal-acetate buffered 1% methyl green solution (pH 4.0) for 10 s and were photographed to evaluate staining.

Effects of agents on the CL reaction

In order to determine the relative importance of O_2^- and H_2O_2 in the CL response through IgE receptors on the eosinophils, superoxide dismutase (SOD), catalase, sodium azide or AMT were added to the samples for 15 min as a preincubation period. Eosinophils treated with these agents were then stimulated with anti-CD23Ab and CL was recorded as described earlier. Controls were stimulated with normal mouse serum (2 μg).

Superoxide anion production

We also measured superoxide production in the LDCL reaction by testing cytochrome c reduction. Purified guinea pig eosinophils were resuspended in HBSS containing 120 nmol cytochrome c at a final concentration of 3×10^6 cells/mL and were stimulated with monoclonal anti-CD23Ab at 37°C. Superoxide production was quantified by measuring the difference in cytochrome c reduction between 550 and 540 nm using a spectrophotometer (model 557; Hitachi Co. Ltd, Tokyo, Japan). The concentration of cytrochrome c reduced was calculated from the equation $E_{550nm}=2.1\times10^4$ mol/L per cm.[15]

Intracellular mechanism of the LDCL reaction

In order to investigate the intracellular mechanism of the LDCL reaction we examined the effects of pertussis toxin, cholera toxin and bivalent ions on LDCL responses and on the production of cyclic adenosine monophosphate (cAMP) during the responses. Purified eosinophils were preincubated with pertussis toxin or cholera toxin for 60 min at 37°C. Cells were then stimulated with anti-CD23Ab and LDCL was recorded as described earlier.

To examine the role of bivalent cationic ions in the LDCL responses, purified eosinophils were resuspended with complete HBSS containing both Ca^{2+} and Mg^{2+}, HBSS free of either Ca^{2+} or Mg^{2+} or HBSS containing 2 μmol EDTA. Cells were stimulated and CL was then recorded as described earlier.

Measurement of cAMP

Aliquots of guinea pig eosinophils were removed at 0, 5, 15, 30 and 60 s and 5 and 15 mins after the addition of anti-CD23Ab. They were then treated for 60 s by ultrasonication (Isolator, model 200M; Kubota Co. Ltd, Tokyo, Japan) at 100 J in acidified ethanol. The supernatant was removed from the pellets of the denatured protein. cAMP was measured using a competitive protein-binding assay as described previously.[16]

Inhibition of OAG-induced LDCL by D-sphingosin

Chemiluminescence was measured in eosinophils stimulated with synthetic diacylglycerol. After preincubation at 37°C for 15 min, purified guinea pig eosinophils were stimulated with diacylglycerol and OAG (10^{-5} to 10^{-9} mol/L) and LDCL was recorded. In addition, we examined the effects of the protein kinase C (PKC) inhibitor D-sphingosin[18] on the OAG- and FcεRII-induced LDCL reactions. Purified eosinophils were preincubated with D-shingosin (10^{-5} mol/L) for 15 min at 37°C and the cells were then stimulated with OAG and CL was recorded.

Measurement of LT

Purified eosinophils (3×10^6 cells/mL) were suspended in complete PA solution containing 0.1% glucose 1 mmol/L Ca^{2+} and Mg^{2+} and were then stimulated with anti-CD23Ab at 37°C. After incubation, the supernatant was removed at 0, 15, 30 and 60 min by centrifugation at 400 g for 10 min and was stored in the dark under Argon gas at −20°C until measured.

Cysteinyl LT were assayed in the supernatant as previously reported.[13] Briefly, 2 mL sample were added to ethanol (8 mL), mixed gently and centrifuged at 2000 g for 15 min at 4°C. After the upper layer was partially purified on an octadecylsilyl silica column (Sep-pak C18 cartridge; Waters Chromatography Division, Millipore Co. Ltd, Boston, MA, USA), the extract was analyzed by means of reverse-phase, high-performance liquid chro-

matography (Waters Model 510). Fractions with the same retention time as LTC_4, LTD_4 and LTE_4 standards were collected, dried and resuspended in PBS. Concentrations of LTC_4, LTD_4 and LTE_4 were assayed in duplicate by a specific cysteinyl LT radioimmunoassay with antisera (Amersham Co. Ltd, IL, Chicago, USA). Based on the cross-reactivity ratio of the antisera, the measured values of LTC_4, LTD_4 and LTE_4 were corrected. In this assay, LTC_4, LTD_4 and LTE_4 could be measured with high reproducibility in quantities ranging from 10 to 200 pg. When the measured values were over 200 pg, the samples were diluted. The mean recovery rates of LTC_4, LTD_4 and LTE_4 were 60, 50 and 50%, respectively.

Cell viability was evaluated by trypan blue dye exclusion and was always greater than 95% throughout all experiments. All determinations were performed in duplicate.

Colorimetric assay of EPO

Guinea pig eosinophils (1×10^7 cells/mL) were suspended in HBSS. They were then stimulated with monoclonal anti-CD23 Ab and were incubated for 60 min at 37°C. They were centrifuged at 400 g for 2 min at 4°C and the supernatant was collected. Tris-buffered (pH 8.0) 0.1 mmol/L o-phenylene diamine dihydrochloride solution with 1 mmol/L H_2O_2 was added to the supernatant as a substrate and was incubated for 30 min at 37°C. After the addition of 4 mol/L sulfuric acid to stop the reaction, the absorbance was determined at 492 nm using a thermostated spectrophotometer. Eosinophil peroxidase activity was expressed as units referring to a standard curve using horse-radish peroxidase, type I.

Statistical analysis

Results are expressed as % of control in all experiments. Data are expressed as the mean ±SEM. Individual experiments were compared with Student's *t*-test. Significance was accepted at $P<0.05$.

RESULTS

Luminol-dependent chemiluminescence

Figure 1 shows an actual LDCL curve in the rate mode induced in guinea pig eosinophils stimulated by monoclonal anti-CD23Ab. The reaction consisted of an initial rapid phase and a propagation phase and ended within 10 min. The mean value of the rapid peak was $20.6\pm1.3\times10^3$ c.p.m. The LDCL response could be induced only by monoclonal anti-CD23Ab, but not by normal mouse serum. We showed that guinea pig eosinophils were histochemically selectively stained with anti-CD23Ab, in comparison with those stained with normal mouse serum (Fig. 2). Thus, these findings suggest that the LDCL reaction can be induced through FcεRII on the surfaces of eosinophils.

Release of mediators from eosinophils by stimulation of FcεRII

To evaluate the granular release and *de novo* synthesis of mediators by specifically stimulated FcεRII on

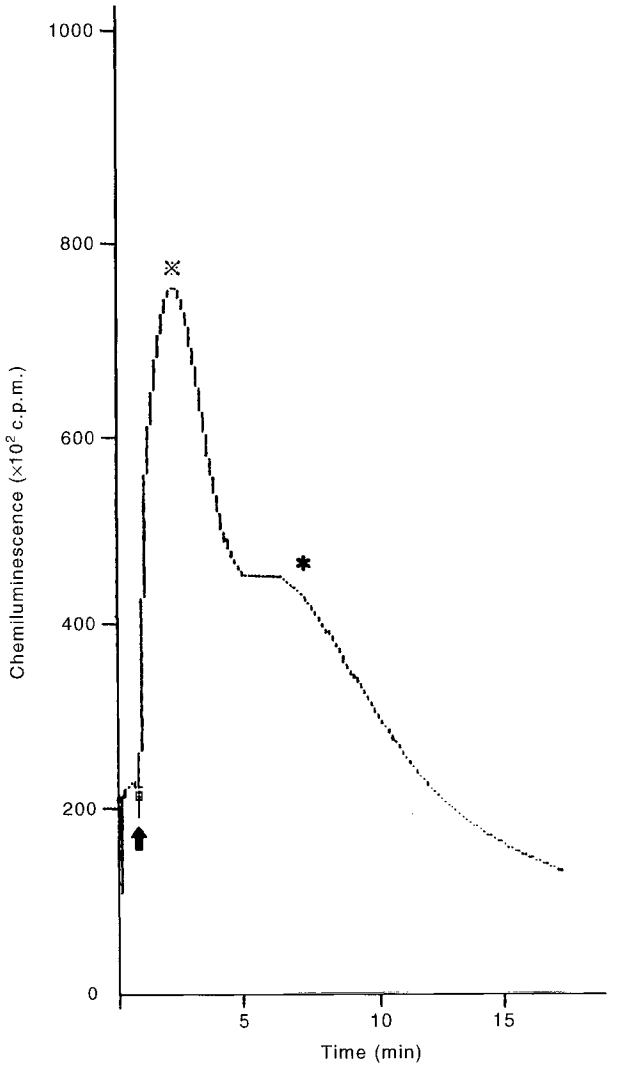

Fig. 1 Actual chemiluminescence curve measured in the rate mode in guinea pig eosinophils stimulated by monoclonal anti-CD23 antibody. The reaction consisted of an initial rapid phase (※) and a propagation phase (*). The arrow shows the time of addition of the anti-CD23 antibody.

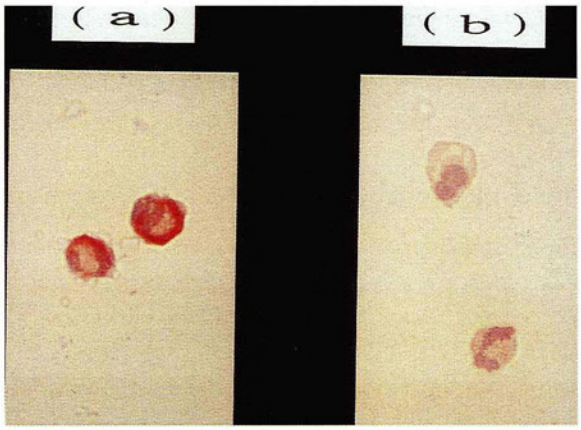

Fig. 2 Immunohistochemical staining with monoclonal anti-CD23 antibody using an alkaline phosphatase-anti-alkaline phosphatase kit system. Guinea pig eosinophils were stained with (a) monoclonal anti-CD23 antibody and (b) normal mouse serum. Original magnification ×1000.

eosinophils, we examined cysteinyl LT in the supernatant from guinea pig eosinophils. Stimulated with monoclonal anti-CD23Ab, the maximum amounts of LTC_4, LTD_4 and LTE_4 at 30 min after stimulation were 24.1±3.6 ($P<0.05$), 12.9±9.2 ($P>0.1$) and 24.9±3.8 pg/10^6 cells ($P<0.05$), respectively, but no LT were detected with normal mouse serum. Thus, we demonstrated that cysteinyl LT could be released by specific FcεRII stimulation, but the amount of LT was very small. However, EPO could be detected at 1 h after stimulation with anti-CD23Ab using a colorimetric assay (data not shown). Thus, we evaluated the mechanism of LDCL through FcεRII.

Major product of LDCL

Figure 3 shows the effects of SOD, catalase and NaN_3 on the CL responses in a dose-dependent manner. Although NaN_3 partially inhibited the reaction, both SOD and catalase completely inhibited the reaction. The IC_{50} of SOD was 7.1 U/mL and that of catalase was 53 U/mL. The specific EPO blocker AMT did not inhibit the responses (data not shown). Superoxide anion production in FcεRII was measured by testing cytochrome c reduction. The amount of superoxide anion was calculated as 2.4±0.2 nmol/min per 3×10^6 eosinophils by stimulation with anti-CD23Ab, but not detected with control normal mouse serum. In addition, as shown in Fig. 4, superoxide production was confirmed by testing cytochrome c reduction under SOD, which could scavenge superoxide anion. Figure 4 shows that SOD dose-

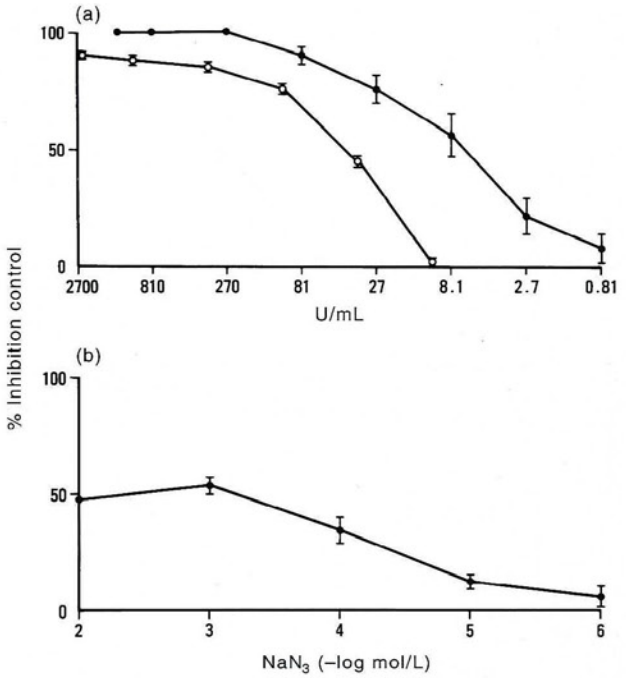

Fig. 3 Dose-dependent effects of (a) superoxide dismutase (●), catalase (○) and (b) NaN_3 on LDCL responses. Results are the mean±SEM of four experiments. The ordinate shows percent inhibition of controls and the abscissa is a logarithmic scale.

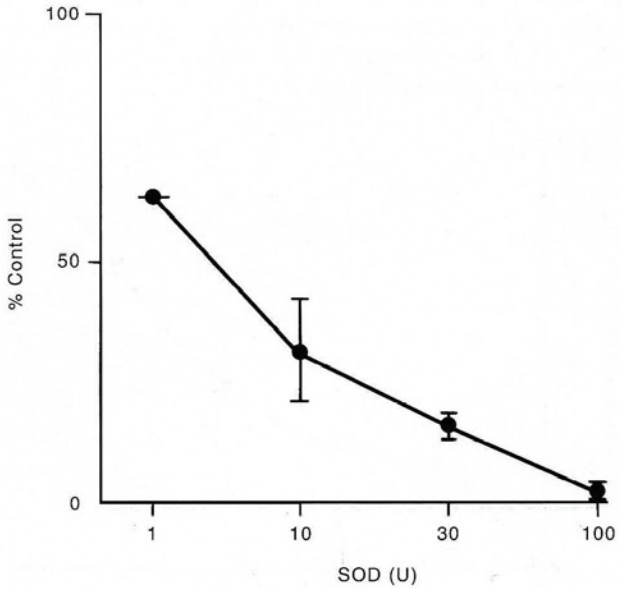

Fig. 4 Effect of superoxide dismutase (SOD) on superoxide anion production via FcεRII in guinea pig eosinophils by the reduction of cytochrome c. Results are expressed relative to no addition of SOD as 100%.

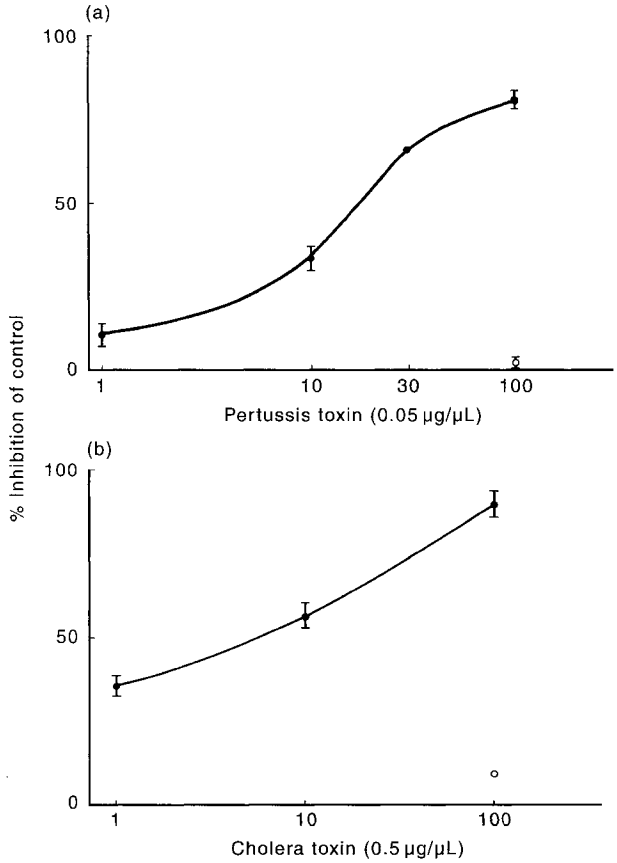

Fig. 5 Dose-dependent inhibition of luminol-dependent chemiluminescence (LDCL) responses in guinea pig eosinophils by either pertussis (a) or cholera (b) toxin. Results are the mean±SEM of five experiments. (●), experiments performed at 4°C; (○), experiments performed at 37°C. Both ordinates express doses of toxins (U/mL).

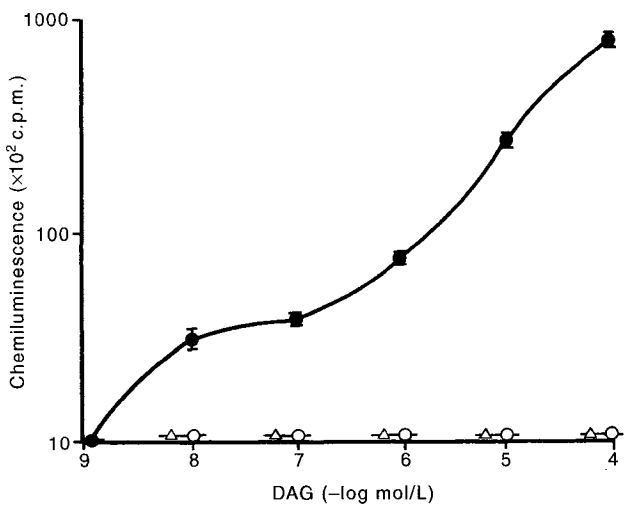

Fig. 6 Luminol-dependent chemiluminescence (LDCL) reaction induced by OAG, a synthetic diacylglycerol (●) and inhibitory effects of D-sphingosin (10^{-5} mol/L) on OAG-induced LDCL (○) and FcεRII-mediated LDCL (△) in guinea pig eosinophils. Results are expressed as the mean±SEM of three experiments.

dependently inhibited cytochrome c reduction. Thus, these findings suggest that superoxide was the major product in the LDCL responses via FcεRII of the eosinophil membrane.

Intracellular mechanism of the LDCL reaction

To investigate the participation of GTP-binding protein in the CL reaction induced in guinea pig eosinophils by the low affinity IgE receptor, we examined the effect of pertussis toxin and cholera toxin on the LDCL response. As shown in Fig. 5, both toxins inhibited the respiratory burst in a dose-dependent fashion at 37°C, but not at 0°C. The maximum per cent inhibition was 83.8±2.3% with 5 μg pertussis toxin and 90.6±3.8% with 50 μg cholera toxin. In addition, we confirmed that OAG, a synthetic diacylglycerol, could induce an LDCL reaction in purified eosinophils (Fig. 6) and found that D-sphingosin, an inhibitor of PKC,[18] inhibited not only OAG-induced CL, but also FcεRII-mediated CL (Fig. 6). Thus, these results

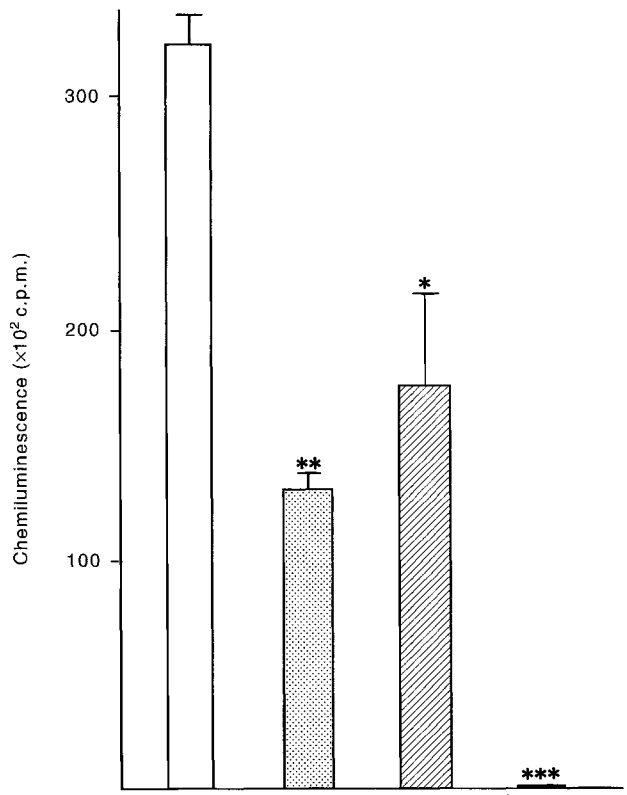

Fig. 7 Effects of Ca^{2+}, Mg^{2+} and EDTA (2 μmol/L) on FcεRII-mediated luminol-dependent chemiluminescence in eosinophils. Results are expressed as the mean±SEM of three experiments. Significant differences between results are indicated: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (□), Hanks' balanced salt solution (HBSS) containing both Mg^{2+} and Ca^{2+}, (⊡), HBSS containing Mg^{2+} only; (▨), HBSS containing Ca^{2+} only; (■) HBSS + EDTA.

demonstrate that PKC is implicated in FcεRII-mediated LDCL responses. The roles of calcium and magnesium ions in the LDCL responses were also evaluated. Figure 7 shows that LDCL responses were completely inhibited by the presence of 2 μmol EDTA in HBSS and partially inhibited by HBSS free of either Ca^{2+} or Mg^{2+}. Thus, both ions were necessary for complete CL responses. In addition, we found no increases in cAMP in cells during the LDCL reaction (data not shown).

Discussion

Although it has been reported that eosinophils emit CL with various stimulants, such as *N*-formyl-metionyl-leucyl-phenyl-alanine, phorbol myristate, calcium ionophore A23187, opsonized zymosan particles and PAF,[17-20] there are few reports concerning CL mediated via FcεRII on the cell surface.[21] In a preliminary study, we found that human eosinophils obtained from peripheral blood in asthmatic patients did not emit CL on pretreatment with human IgE or with serum from allergic asthmatics having a high titer. This may be partly because FcεRII is not always occupied by the IgE antibody, even in hypodense eosinophils[22] and because IgE binding inhibitory factors (blocking IgG antibody) were present in sera from atopic patients[23] and partly because the affinity of FcεRII binding of IgE has been reported to be lower than that of the high-affinity IgE receptor of histamine-containing cells.[24,25] Therefore, in the present study, we stimulated guinea pig eosinophils obtained from peritoneal lavage by using monoclonal anti-CD23Ab. Consequently, as shown in the Results, the eosinophils emitted LDCL. In addition, morphologically histochemical staining with anti-CD23Ab revealed the presence of IgE receptors on eosinophil cell surfaces. Therefore, these results suggest that the LDCL response is mediated via FcεRII on the cell surface of eosinophils.

As shown in Figure 1, the LDCL responses consisted of an initial phase and a propagation phase, which has been reported in normodense eosinophils from peripheral blood only after priming with PAF.[26] In the present study we used normodense eosinophils obtained from peritoneal fluid of guinea pigs sensitized with horse serum for 9 weeks. In a preliminary study we found that the LDCL reaction began to occur 5 weeks after the beginning of sensitization and that the CL induction was confirmed 9 weeks after that. Therefore, we examined the CL only 48 h after the last injection of horse serum, at which time responses could be induced in the most reproducible manner. Thus, we inferred that guinea pig eosinophils used in the present study were already activated and expressed FcεRII on their cell surfaces by at least 9 weeks after sensitization.

In the present study, we further evaluated the signal transduction of the LDCL responses. First, we demonstrated that the responses are mainly dependent on superoxide rather than H_2O_2, as indicated not only by the complete inhibition of the responses by SOD and catalase, but also by the cytochrome c reduction and the absence of effect of AMT on the responses. Thus, this FcεRII-related LDCL reaction could be mediated by the NADPH oxidase present on plasma membrane in eosinophils. Activation of NADPH oxidase in granulocytes could be initiated by stimulation binding to specific membrane receptors or by substrates bypassing receptor interraction, such as phorbol esters or calcium ionophores[27,28] and two distinct pathways implying either phospholipase A_2 or PKC.[29,30] In the present study the amounts of LTC_4 and LTE_4 that seemed to be released by phospholipase A_2 were small. Thus, we evaluated whether a sequence of biochemical reactions of PKC was involved in the IgE-mediated LDCL reaction by using OAG as a PKC activator and sphingosin as a PKC inhibitor. We showed that LDCL responses are mediated by PKC, because the specific PKC inhibitor D-shingosin[31] could significantly inhibit not only OAG[32]-induced LDCL responses but also FcεRII-induced LDCL responses.

Second, we found that the LDCL responses are significantly inhibited by treatment with either pertussis toxin or cholera toxin. This indicates that at least two types of G proteins are involved in the regulation of the LDCL reaction, as was recently reported.[33] Inhibitory doses of these toxins were greater than those reported previously in lymphocytes,[34] but this is because we used a short incubation time by increasing the doses of toxins in order to maintain cell activity.

Last, we demonstrated that the LDCL responses are regulated by calcium and magnesium ions, because depletion of either Ca^{2+} or Mg^{2+} attenuated the responses to approximately one-half and EDTA completely inhibited LDCL responses. These findings also suggest that FcεRII-mediated CL was via PKC and membrane-bound NADPH oxidase because the catalytic activity of the oxidase was enhanced by Ca^{2+} and Mg^{2+} and was inhibited by EDTA[35] and because eosinophils produce toxic metabolites by means of a membrane-bound NADPH oxidase[31,32] and because PKC seems to be involved in the activation of NADPH oxidase in intact leukocytes.[36]

In the present study we suggest that FcεRII on the surface of eosinophils may play physiologically important

roles in allergic reactions because stimulation of FcεRII could produce toxic O_2 metabolites by the NADPH-PKC pathway. These findings suggest the physiological significance of the induction of FcεRII on activated eosinophils attracted to allergic inflammatory tissues.

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