

Characterization of platelet-activating factor-induced cytosolic calcium mobilization in human eosinophils

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Abstract

Background Activated eosinophils play an important role in the pathogenesis of bronchial asthma and other allergic diseases, and platelet-activating factor (PAF) is a potent activator of eosinophils.

Objective To characterize the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) mobilization in human eosinophils in response to PAF.

Methods $[\text{Ca}^{2+}]_i$ responses to PAF were examined in human eosinophils using a microscopic fura-2 fluorescence-ratio imaging system.

Results PAF caused a significant and dose-dependent increase in $(\text{Ca}^{2+})_i$, which consisted of an initial rapid rise followed by a sustained elevation. This PAF-induced $(\text{Ca}^{2+})_i$ rise was inhibited by WEB 2086, a specific PAF receptor antagonist. The addition of 5 mM EGTA or 1 mM Ni^{2+} to a nominally Ca^{2+} -free solution did not appreciably reduce the initial rise but significantly inhibited the sustained rise. The application of a protein kinase C inhibitor, Ro31-8220, augmented the sustained increase by PAF. Thapsigargin, a microsomal Ca^{2+} -ATPase inhibitor, induced no appreciable change in a nominally Ca^{2+} -free solution but induced a marked increase in $(\text{Ca}^{2+})_i$ when changed to a Ca^{2+} -containing solution.

Conclusions The initial rapid rise and the following sustained rise in $(\text{Ca}^{2+})_i$ by PAF depends on Ca^{2+} release from the intracellular Ca^{2+} stores and Ca^{2+} influx, respectively, which are regulated by protein kinase C in human eosinophils. Furthermore, the so called Ca^{2+} -capacitative entry is possibly involved in the Ca^{2+} influx from the extracellular solution in human eosinophils.

Keywords: Ca^{2+} -capacitative entry, cytosolic-free calcium, human eosinophils, platelet-activating factor, protein kinase C, thapsigargin

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Introduction

Eosinophils play an important role in the pathogenesis of bronchial asthma and other allergic diseases. Eosinophil infiltration is observed at sites of inflammation, and the granule proteins are released from activated eosinophils in response to different stimuli [1,2]. However, the signal transduction pathways through which human eosinophil responses to inflammatory mediators are effected are not completely understood. Platelet-activating factor (PAF) is

a potent eosinophil-activating substance, which is synthesized and released by several inflammatory cells [3] and has been reported to induce phosphoinositide metabolism mediated by GTP-binding proteins and to form inositol 1,4,5-trisphosphate (IP_3) [4,5]. In many other cells, IP_3 causes an increase in the intracellular-free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to Ca^{2+} release from intracellular Ca^{2+} stores, followed by a sustained Ca^{2+} influx [6]. In guinea pig peritoneal eosinophils, it has been reported that the PAF-induced elevation of $[\text{Ca}^{2+}]_i$ arises mainly from the influx of extracellular Ca^{2+} rather than from the Ca^{2+} release from intracellular Ca^{2+} stores [7], although a highly significant positive correlation was found between the peak increase in IP_3 and the peak rise in $[\text{Ca}^{2+}]_i$ [8]. Furthermore, in a variety

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of nonexcitable cells other than eosinophils, release or depletion of Ca^{2+} from IP_3 -sensitive stores activates Ca^{2+} influx or entry from the extracellular solution [6,9,10]. However, it is still unknown if a mechanism similar to the so-called Ca^{2+} -capacitative entry is also active for the Ca^{2+} influx in human eosinophils. Namely, there have no or few studies that have focused on the precise mechanism of Ca^{2+} mobilization in human eosinophils. In the present study we therefore examined the $[\text{Ca}^{2+}]_i$ responses to PAF in human eosinophils using a microscopic fura-2 fluorescence-ratio imaging system.

Materials and Methods

Materials

O, O-Bis(2-aminoethyl)ethyleneglycol-N, N, N, N-tetraacetic acid (EGTA), piperazine-N, N'-bis(2-ethanesulphonic acid) (PIPES), N-2-hydroxy ethyl-piperazine-N'-2-ethansulphonic acid (HEPES), and fura-2 acetoxymethyl ester (fura-2/AM) were obtained from Dojindo Chemical Co (Kumamoto, Japan). Ethylenediamine tetraacetic acid (EDTA), PAF, human albumin, thapsigargin and Hanks' balanced salt solution (HBSS) (ordinary and without calcium chloride and magnesium sulphate) were purchased from Sigma (St Louis, MO, USA). Dextran T500 and polyvinylpyrrolidone-coated silica gel (Percoll[®]) were purchased from Pharmacia (Uppsala, Sweden). Bovine serum albumin was obtained from Boehringer Mannheim (Mannheim, Germany). Cremphor EL was purchased from Nakarai Chemicals Ltd, (Kyoto, Japan). WEB 2086 and Ro31-8220 were kind gifts from Boehringer Ingelheim (Ingelheim, Germany) and Roche Products Ltd, (Welwyn Garden City, UK), respectively. All other reagents were of the highest quality.

Cell preparation

The purification of eosinophils was as described previously [11] with slight modifications. In brief, 30 mL of venous blood was obtained from nonatopic volunteers and mixed with 10 mL of 3% Dextran T500 in divalent cation-free or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline with 10 mM EDTA. After sedimentation of the cells for 30 min, the dextran-plasma was collected and centrifuged at 400g for 8 min. The pellets were washed three times with PIPES solution containing 120 mM NaCl, 5 mM KCl, 25 mM PIPES, and 0.3% bovine serum albumin (pH 7.3), and resuspended in Percoll solution (1.070 g/mL). Percoll solutions of 1.124 and 1.087 g/mL were then prepared and layered successively into a 50-mL polycarbonate tube using a peristaltic pump. The tube was centrifuged at 1300g for 20 min (20°C), resulting in the separation of the suspension into two distinct bands. The lower band was

collected, washed three times with a PIPES solution, resuspended and kept on ice until each fura-2/AM loading. The purity of specimens ranged from 90 to 98% (mean, 95%), and the viability of the specimens judged by trypan blue exclusion test was more than 95% during the course of the experiments, which were performed within 4 h.

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

$[\text{Ca}^{2+}]_i$ measurement was performed as previously described [12] with slight modifications. In brief, 20 mL of the cell suspension was transferred into a Petri dish with a cover glass bottom. Sedimentation of the cells occurred within a few minutes and the dish was then incubated with PIPES solution containing 5 mM fura-2/AM for 1 h at 37°C. In addition, we added Cremphor EL (final concentration, 0.02%) to the fura-2/AM; this permitted uniform loading without detectable compartmentalization of fura-2. After loading, the settled cells were washed three times with HBSS containing either 1 mM Ca^{2+} with Mg^{2+} , or containing 1.8 mM (ordinary) Ca^{2+} , and buffered with 10 mM HEPES (HBSS, pH 7.4). The dish was then transferred to the stage of a TMD-inverted microscope (Nikon, Tokyo, Japan) where the temperature of the solution was kept constant at 37°C. On the stage, cells were incubated in either 1 or 1.8 mM Ca^{2+} HBSS for 15 min before PAF application. We chose these concentrations of Ca^{2+} since the 1 mM Ca^{2+} solution was used in guinea pig eosinophils [7] and 1.8 mM Ca^{2+} was the Ca^{2+} concentration of ordinary HBSS. In some experiments the solution was exchanged with HBSS containing 5 mM EGTA or 1 mM NiCl with no additional Ca^{2+} 3 min before the PAF application. In some other experiments, either WEB 2086, a PAF receptor antagonist [13], or Ro31-8220, a protein kinase C inhibitor [14], was applied 5 min before PAF stimulation.

In order to measure $[\text{Ca}^{2+}]_i$, cells were illuminated alternately at 360 and 380 nm (slit width = 10 nm) using a microscopic dual-wavelength fluorometer (CAM-230, Jasco Corp, Tokyo, Japan). An electric shutter was used to restrict the illumination to the duration of the data sampling. The excitation light was deflected with a dichroic mirror through a $\times 40$ objective (NCF Fluor, NA = 0.85, Nikon). Fluorescence emission was collected through a 510-nm band pass filter (20 nm band width) with a SIT video camera (C2400, Hamamatsu Photonics Corp, Hamamatsu, Japan) connected to an image processor (Argus-50/Ca, Hamamatsu Photonics Corp) and a computer (MAXY-DT2, Mitsubishi Electric Corp, Tokyo, Japan). An average of 32 frames at each wavelength were collected every 10 s in the preliminary experiments, and the peak responses of $[\text{Ca}^{2+}]_i$ were observed 8–10 s after the application of PAF. The averaged images were background-subtracted and divided pixel-by-pixel to yield ratio images, which were stored on a

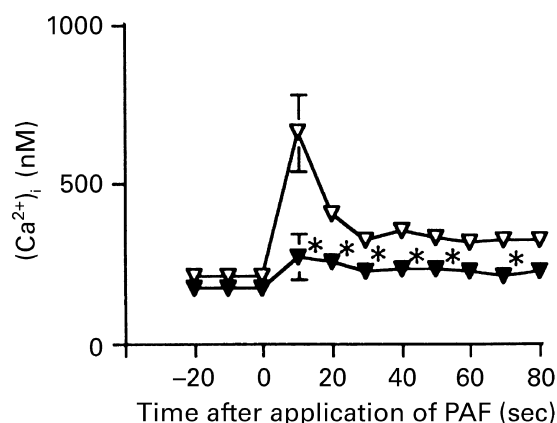


Fig. 1. Effect of EGTA on PAF-induced $[\text{Ca}^{2+}]_i$ changes in 1 mM Ca^{2+} . The eosinophils were incubated for 15 min in HBSS with 1 mM Ca^{2+} , further treated with either 1 mM Ca^{2+} -HBSS (∇ , control) or 5 mM EGTA-HBSS ($\blacktriangledown$), and then stimulated with 100 nM PAF. Mean $\pm$ SE of five experiments. * $P < 0.05$ as compared with control.

rewritable optical disk unit (ME-5U1, Mitsubishi Electric Corp, Tokyo, Japan) for off-line analysis. In addition, the regional unevenness of sensitivity of the video camera was corrected by a 'shading' procedure in which 200 mM fura-2 solution with K-aspartate at 0.1 mM Ca^{2+} (buffered with total of 4 mM EGTA) was used as a standard. $[\text{Ca}^{2+}]_i$ was estimated for the average ratios of rectangular regions of individual cells using the equation

$$[\text{Ca}^{2+}]_i = K_d \{ (R - R_{\min}) / (R_{\max} - R) \} \beta$$

Each value was determined according to the method by Scanlon *et al* [15]. $R_{\max}$ was measured as the R -value of intact cells perfused with 20 mM ionomycin and HBSS containing 1.8 mM CaCl_2 . $R_{\min}$ was determined as the R -value of the cells perfused with 20 mM ionomycin and HBSS in the presence of 5 mM EGTA without any additional Ca^{2+} . β was determined as the fluorescence intensity ratio at 380 nm in zero (5 mM EGTA) and saturating (1.8 mM) Ca^{2+} . K_d is the dissociation constant of fura-2 for Ca^{2+} , assumed to be 224 nM [16] at 37 °C. PAF was applied to the eosinophils within 300 ms by using the 'Y-tube' method according to Wakamori *et al* [17]. Since the eosinophils were firmly attached to the cover glass even during the application of rapid perfusion, the rectangular regions for $[\text{Ca}^{2+}]_i$ measurements of individual cells did not appreciably change during the course of the experiments. This was confirmed by the consistent ratio results, determined by different investigators at different times. The vehicle for PAF alone (methanol, maximum concentration to be 0.001%) did not induce any appreciable increase in $[\text{Ca}^{2+}]_i$.

Statistical analysis

In each experiment, $[\text{Ca}^{2+}]_i$ was obtained as the mean $[\text{Ca}^{2+}]_i$ of 5–6 cells and the data were expressed as the mean $\pm$ SE of 5–7 experiments. Comparisons were performed using the same samples when any comparison of the data was required. Statistical analysis of data was performed with Student's t -test, and the Cochran–Cox t -test was used when Bartlett's test for uniformity of variance showed it to be non-uniform. A value of $P < 0.05$ was considered to be significant.

Results

First, we examined the PAF-induced Ca^{2+} responses of human eosinophils in HBSS containing 1 mM Ca^{2+} . In this solution, 0.1–100 nM PAF induced a rapid increase in $[\text{Ca}^{2+}]_i$ followed by a steady plateau increase, and both increases were blocked by 2 mM WEB 2086, a specific PAF receptor antagonist [13]. The eosinophils were then pre-incubated with 1 mM Ca^{2+} HBSS for 15 min and the solution was changed to 5 mM EGTA HBSS. The baseline $[\text{Ca}^{2+}]_i$ was not altered but both the rapid-initial and steady-state $[\text{Ca}^{2+}]_i$ responses were significantly inhibited by 5 mM EGTA, although a small initial increase was transiently observed (Fig. 1). This finding was consistent with previous results observed in guinea pig eosinophils [7].

In the following routine experiments, we examined PAF-induced $[\text{Ca}^{2+}]_i$ responses when the cells were incubated with 1.8 mM Ca^{2+} . As illustrated in Fig. 2, PAF caused a rapid increase in $[\text{Ca}^{2+}]_i$, which reached a peak in 10 s

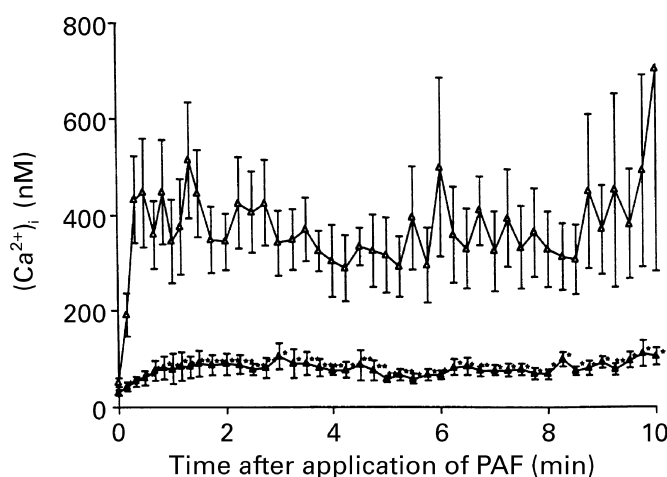


Fig. 2. Effect of WEB 2086 on PAF-induced $[\text{Ca}^{2+}]_i$ elevation in 1.8 mM Ca^{2+} . The eosinophils were incubated for 5 min in the presence of 20 mM WEB 2086 ($\blacktriangle$) or vehicle ($\triangle$, control), and then stimulated with 100 nM PAF. Mean $\pm$ SE of five experiments. * $P < 0.05$ and as compared ** $P < 0.01$ with control.

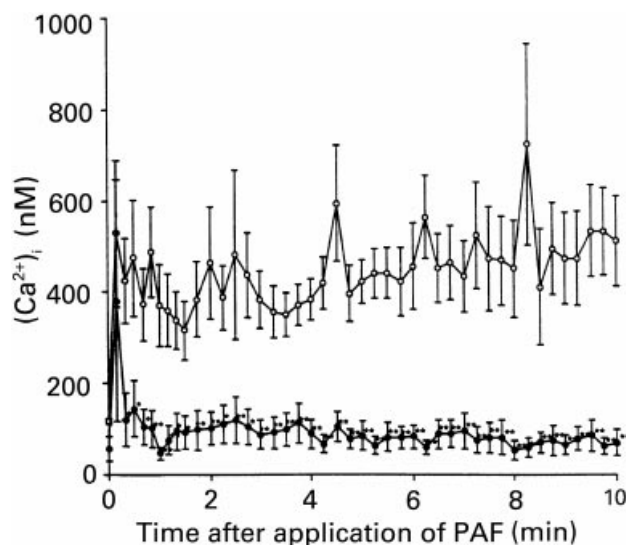


Fig. 3. Effect of EGTA on PAF-induced $[Ca^{2+}]_i$ in 1.8 mM Ca^{2+} . The eosinophils were perfused with 5 mM EGTA and stimulated with 100 nM PAF as shown (●). Mean $\pm$ SE of 6–7 experiments. * $P < 0.05$ and ** $P < 0.01$ as compared with control (○).

and was followed by a sustained rise. The PAF-induced (10–100 nM) $[Ca^{2+}]_i$ rises of both the initial and sustained phases were significantly inhibited by 2–20 mM WEB 2086, indicating that these also were caused via specific PAF receptors (Fig. 2).

We then examined the dose response and effect of EGTA on the PAF-induced $[Ca^{2+}]_i$ responses in HBSS containing 1.8 mM Ca^{2+} or 5 mM EGTA with no additional Ca^{2+} (Fig. 3). PAF (0.1–100 nM) dose-dependently increased $[Ca^{2+}]_i$ with a rapid peak followed by the steady-state responses. When the eosinophils were pre-incubated with

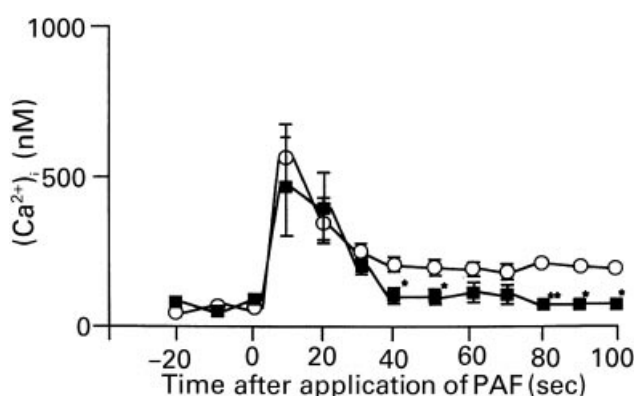


Fig. 4. Effect of Ni^{2+} on the $[Ca^{2+}]_i$ -rise by 100 nM PAF. The experimental protocol was the same as that of Fig. 3 except that 5 mM EGTA was replaced with Ni^{2+} (■). * $P < 0.05$ and ** $P < 0.01$ as compared with control (○). Mean $\pm$ SE of five experiments.

HBSS containing 1.8 mM Ca^{2+} and the solution was then changed to that containing 5 mM EGTA with no additional Ca^{2+} , the PAF-induced $[Ca^{2+}]_i$ initial rise was not significantly different from that in the presence of 1.8 mM Ca^{2+} , whereas the sustained rise was significantly inhibited (Fig. 3). Since Ni^{2+} has been reported to inhibit the PAF-induced Ca^{2+} influx in guinea pig eosinophils [7], we examined the effect of Ni^{2+} on the PAF-induced $[Ca^{2+}]_i$ response in human eosinophils pre-incubated with 1.8 mM Ca^{2+} HBSS. As illustrated in Fig. 4, the addition of 1 mM Ni^{2+} in place of Ca^{2+} did not significantly inhibit the initial phases of the PAF-induced Ca^{2+} response, whereas the sustained rise was significantly inhibited. These results indicate that the PAF-induced $[Ca^{2+}]_i$ initial and sustained elevations depend mainly on intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular solution, respectively, in human eosinophils when these cells are pre-incubated with 1.8 mM Ca^{2+} .

Although the role of protein kinase C has been studied mostly in other cells [18], there are few studies that examined the effect of protein kinase C on the Ca^{2+} mobilization in human eosinophils [19]. Therefore, we examined the effect of a specific protein kinase C inhibitor, Ro31-8220 [14], on the $[Ca^{2+}]_i$ response induced by PAF. Ro31-8220 at 10 mM was applied 5 min before the addition of 100 nM PAF. The baseline $[Ca^{2+}]_i$ level increased slightly and the application of PAF promptly evoked an elevation of $[Ca^{2+}]_i$ that was significantly prolonged for more than 1 min (Fig. 5). In order to determine whether the prolonged elevation of $[Ca^{2+}]_i$ by the addition of Ro31-8220 was due to the increase of Ca^{2+} influx by the inhibition of protein kinase C, we examined the effect of extracellular Ca^{2+} on this sustained $[Ca^{2+}]_i$ elevation. Eosinophils exposed to 10 mM Ro31-8220 for 5 min were stimulated with 100 nM in either HBSS containing 1.8 mM Ca^{2+} or Ca^{2+} -free HBSS with 5 mM EGTA. The prolonged elevation of $[Ca^{2+}]_i$ was significantly inhibited by the addition of EGTA, whereas there was no significant difference between the initial $[Ca^{2+}]_i$ rise in the presence of 1.8 mM Ca^{2+} and that in Ca^{2+} -free HBSS with 5 mM EGTA. These results indicate that inhibition of protein kinase C caused an augmentation in the sustained elevation of the PAF-induced $[Ca^{2+}]_i$. This conclusion is consistent with the fact that the Ca^{2+} influx is negatively regulated by protein kinase C in human neutrophils [16].

Thapsigargin, a Ca^{2+} ATPase inhibitor of the intracellular IP_3 -sensitive Ca^{2+} store, has been known to deplete the IP_3 -sensitive Ca^{2+} store and greatly increase Ca^{2+} influx in other cells, although the mechanism of activation of this so called capacitative entry is not well understood [6,9,10]. To determine whether a similar mechanism is active in human eosinophils, we examined the effects of thapsigargin and extracellular Ca^{2+} on $[Ca^{2+}]_i$. Eosinophils were treated with

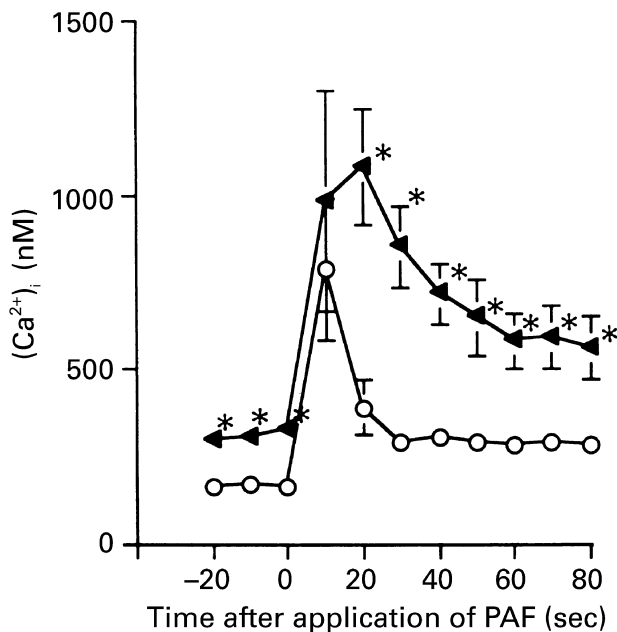


Fig. 5. The effect of Ro31-8220, a protein kinase C inhibitor, on PAF-induced $[\text{Ca}^{2+}]_i$ changes. The experimental protocol was the same as that of Fig. 3 except that the eosinophils were incubated for 5 min with 10 mM Ro31-8220 (▲) or vehicle (○) and stimulated with 100 nM PAF. Mean $\pm$ SE of five experiments. * $P < 0.05$ as compared with control.

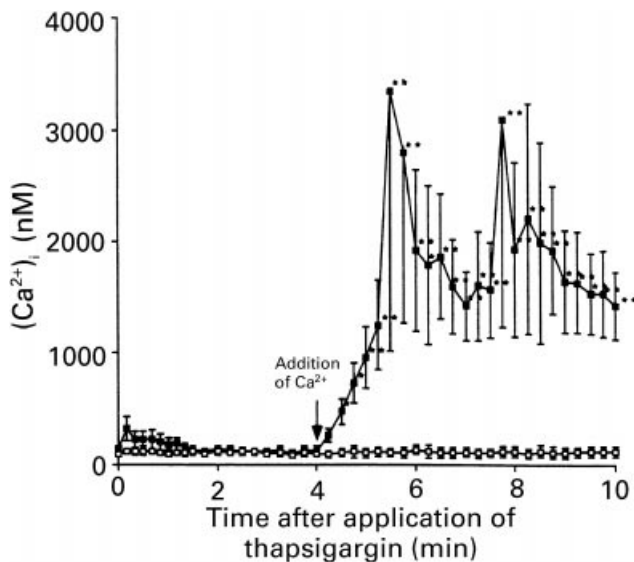


Fig. 6. Effect of thapsigargin and extracellular Ca^{2+} on $[\text{Ca}^{2+}]_i$. The eosinophils were treated with 1 μM thapsigargin in a nominally Ca^{2+} -free solution and perfused with a solution containing 1.8 mM Ca^{2+} 4 min after the application of thapsigargin (■). Control eosinophils (□) were perfused with the Ca^{2+} -free solution throughout the experiments. Mean $\pm$ SE of 6–7 experiments.

1 M thapsigargin in a Ca^{2+} -free HBSS that was changed to HBSS containing 1.8 mM Ca^{2+} 4 min after the application of the thapsigargin. As shown in Fig. 6, a marked increase in $[\text{Ca}^{2+}]_i$ of eosinophils was observed when the Ca^{2+} -free solution was changed to the Ca^{2+} -containing one.

Discussion

In the present experiments, PAF evoked an initial $[\text{Ca}^{2+}]_i$ elevation that was significantly inhibited by 5 mM EGTA when the eosinophils were incubated with 1 mM Ca^{2+} . This result was consistent with that observed in guinea pig peritoneal eosinophils using an extracellular solution with 1 mM Ca^{2+} [7]. In contrast, the initial increase was not inhibited with EGTA or Ni^{2+} when the eosinophils were exposed to 1.8 mM Ca^{2+} , indicating that the major source of the initial $[\text{Ca}^{2+}]_i$ elevation was the intracellular Ca^{2+} stores. These responses to PAF in both 1 mM Ca^{2+} - and 1.8 mM Ca^{2+} -containing solutions were significantly inhibited by WEB 2086, indicating that these responses were evoked through a PAF receptor-mediated mechanism. Although we have no direct evidence, we can speculate on the inhibition of the initial increase by EGTA in a 1-mM Ca^{2+} -containing solution. There have been some reports describing a mechanism for coupling between extracellular $[\text{Ca}^{2+}]_i$ and the release of Ca^{2+} from intracellular stores, and reduced extracellular $[\text{Ca}^{2+}]_i$ has been reported to induce the release of Ca^{2+} from intracellular stores in other cells [20,21]. Therefore, it seems likely that, during the incubation in 1 mM Ca^{2+} solution, the intracellular Ca^{2+} stores were decreased, resulting in the apparent inhibition by EGTA in human eosinophils.

PAF has been shown to induce the accumulation of IP_3 after stimulation of the PAF receptor [5]. Generally, receptor-activated Ca^{2+} mobilization via the inositol phosphate Ca^{2+} signalling system involves two phases: (1) Ca^{2+} release from intracellular Ca^{2+} stores and (2) a more prolonged phase of extracellular Ca^{2+} entry. The present experiments confirmed the presence of these two phases in the Ca^{2+} signalling of human eosinophils. The second phase is presumed to be coupled to the depletion of intracellular Ca^{2+} stores by IP_3 , and this process is termed 'capacitative Ca^{2+} entry' [6]. The present experiment using thapsigargin showed this capacitative Ca^{2+} entry in the Ca^{2+} mobilization of human eosinophils. Furthermore, the low concentration of extracellular $[\text{Ca}^{2+}]_i$ may have facilitated the Ca^{2+} entry by the stimulated release and resultant decrease in the intracellular Ca^{2+} stores as described above.

N-formyl-methionyl-leucyl-phenylalanine (FMLP) has been shown to induce a biphasic rise in $[\text{Ca}^{2+}]_i$ in human neutrophils [18]. The initial phase is not appreciably affected by a protein kinase C inhibitor, staurosporine, although the second phase is markedly increased by this

treatment [18]. In order to determine the role of protein kinase C in human eosinophils, we used a specific protein kinase C inhibitor to inhibit the activation of protein kinase C during PAF stimulation and examined the $[Ca^{2+}]_i$ responses. Although calphostin C has been shown to be a specific protein kinase C inhibitor [22], we did not use this compound because it raised the baseline $[Ca^{2+}]_i$ a mM to a level much higher than that of eosinophils in HBSS alone (our unpublished data). Furthermore, selectivity of the blocking effect of staurosporin on protein kinase C has recently been questioned [23]. Therefore, we used Ro31-8220 as a specific protein kinase C inhibitor. Although the addition of this compound also significantly increased baseline $[Ca^{2+}]_i$, the effect was slight compared with that of calphostin C, which caused an augmentation in the sustained elevation of the PAF-induced $[Ca^{2+}]_i$. This augmentation was significantly inhibited by the application of EGTA with no additional Ca^{2+} , indicating that it was mainly due to the augmentation in the extracellular Ca^{2+} entry. The effect on the increase in the initial transient response was not clear in the present experiments. Therefore, these findings suggest an inhibitory action of protein kinase C on Ca^{2+} influx in human eosinophils, as reported in human neutrophils [18]. Meanwhile, a protein kinase C inhibitor has been shown to enhance the antigen-induced hydrolysis of inositol phospholipid in a rat basophilic leukaemia cell line [24]. Also, Ro31-8220 has been reported to enhance leukotriene B₄-induced IP₃ accumulation and the duration of the Ca^{2+} transient in guinea pig peritoneal eosinophils [25]. Furthermore, a recent experiment by Dent *et al* [19] has shown that bisindolylmaleimide I and calphostin C both augmented the initial increase as well as the sustained increase in $[Ca^{2+}]_i$ in response to PAF in human eosinophils. Therefore, it remained to be determined whether the activation of protein kinase C increases the hydrolysis of inositol phospholipid and enhances Ca^{2+} release from intracellular Ca^{2+} stores in human eosinophils. In addition to the direct interaction of protein kinase C with inositol phosphate metabolism, we can speculate on other possibilities. Activation of protein C has been demonstrated to down-regulate leukotriene B₄ receptors in human neutrophils [26] and PAF receptors in guinea pig eosinophils [27]. Therefore, it is possible that protein kinase C inhibitors potentiate the $[Ca^{2+}]_i$ responses by preventing receptor down-regulation, rather than through an interaction with intracellular signalling pathways. Furthermore, it has also been suggested that protein kinase C may inactivate PAF receptor-operated Ca^{2+} channels in human neutrophils, in which PAF induces $[Ca^{2+}]_i$ elevation independently of IP₃ accumulation [28].

In conclusion, the initial rapid and the following sustained rises in $[Ca^{2+}]_i$ by PAF depend on the Ca^{2+} release from the intracellular Ca^{2+} stores and on the Ca^{2+} influx, respectively, and the latter is regulated by protein kinase C

in human eosinophils. Furthermore, the so-called Ca^{2+} capacitative entry is possibly involved in the Ca^{2+} influx from the extracellular solution in human eosinophils. These findings are important for understanding the pathophysiology of bronchial asthma and other allergic diseases.

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References

- 1 Gleich GJ, Adolphson CR. The eosinophil leukocyte: structure and function. *Adv Immunol* 1986; 39:177–253.
- 2 Venge P. What is the role of the eosinophil? *Thorax* 1990; 45:161–3.
- 3 Chung KF. Platelet-activating factor in inflammation and pulmonary disorders. *Clin Sci* 1992; 83:127–38.
- 4 Honda Z, Nakamura M, Miki I, Minami M, Watanabe T, Seyama Y, Okado H, Toh H, Ito K, Miyamoto T, Shimizu T. Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature* 1991; 349:342–6.
- 5 Honda Z, Takano T, Gotoh Y, Nishida E, Ito K, Shimizu T. Transfected platelet-activating factor receptor activates mitogen-activated protein (MAP) kinase in Chinese hamster ovary cells. *J Biol Chem* 1994; 269:2307–15.
- 6 Putney JW Jr, Bird GS. The inositol phosphate-calcium signalling system in nonexcitable cells. *Endocrine Rev* 1993; 14:610–31.
- 7 Kroegel C, Pleass R, Yukawa T, Chung KF, Westwick J, Barnes PJ. Characterization of platelet-activating factor-induced elevation of cytosolic free calcium concentration in eosinophils. *FEBS Lett* 1989; 243:41–6.
- 8 Kroegel C, Chilvers ER, Gienbycz MA, Challiss RA, Barnes PJ. Platelet-activating factor stimulates a rapid accumulation of inositol (1, 4, 5) trisphosphate in guinea pig eosinophils: Relationship to calcium mobilization and degranulation. *J Allergy Clin Immunol* 1991; 88: 114–24.
- 9 Parekh AB, Terlau H, Stuhmer W. Depletion of InsP₃ stores activates a Ca^{2+} and K^{+} current by means of a phosphatase and a diffusible messenger. *Nature* 1993; 364:814–8.
- 10 Petersen CCH, Berridge MJ. The regulation of capacitative calcium entry by calcium and protein kinase C in *Xenopus* oocytes. *J Biol Chem* 1994; 269:32246–53.
- 11 Aizawa T, Kakuta Y, Yamauchi K, Ohkawara Y, Maruyama N, Nitta Y, Tamura G, Sasaki H, Takishima T. Induction of granule release by intracellular application of calcium and guanosine-5'-O-(3-thiotriphosphate) in human eosinophils. *J Allergy Clin Immunol* 1992; 90:789–95.
- 12 Nagaki M, Shimura S, Irokawa T, Sasaki T, Oshiro T, Nara M, Kakuta Y, Shirato K. Bradykinin regulation of airway submucosal gland secretion: role of bradykinin receptor subtype. *Am J Physiol* 1996; 270:L907–L913.
- 13 Casals-Stenzel J, Muacevic G, Weber KH. Pharmacological actions of WEB 2086, a new specific antagonist of platelet-activating factor. *J Pharmacol Exp Ther* 1987; 241:974–81.

- 14 Davis PD, Hill CH, Keech E, Lawton G, Nixon JS, Sedgwick AD, Wadsworth J, Westmacott D, Wilkinson SE. Potent inhibitors of protein kinase C. *FEBS Lett* 1989; 259:61–3.
- 15 Scanlon M, Williams DA, Fay FS. A Ca^{2+} -insensitive form of fura-2 associated with polymorphonuclear leukocytes. *J Biol Chem* 1987; 262:6308–12.
- 16 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260:3440–50.
- 17 Wakamori M, Hidaka H, Akaike N. Hyperpolarizing muscarinic responses of freshly dissociated rat hippocampal CA1 neurones. *J Physiol* 1993; 463:585–604.
- 18 Wong K, Parente J, Prasad KVS, Ng D. Auranofin modulated cytoplasmic free calcium in neutrophils by mobilizing intracellular calcium and inhibiting protein kinase C. *J Biol Chem* 1990; 265:21454–61.
- 19 Dent G, Munoz NM, Ruhlmann E, Zhu X, Leff AR, Magnussen H, Rabe KF. Protein kinase C inhibition enhances platelet-activating factor-induced eicosanoid production in human eosinophils. *Am J Respir Cell Mol Biol* 1998; 18:136–44.
- 20 Zanotti S, Charles A. Extracellular calcium sensing by glial cells: low extracellular calcium induces intracellular release and intracellular signalling. *J Neurochem* 1997; 69:594–602.
- 21 Shiina Y, Kaneda M, Matsuyama K, Tanaka K, Hiroi M, Doi K. Role of the extracellular Ca^{2+} on the intracellular Ca^{2+} changes in fertilized and activated mouse oocytes. *J Reprod Fertility* 1993; 97:143–50.
- 22 Kobayashi E, Nakano H, Morimoto M, Tamaoki T. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Comm* 1989; 159:548–53.
- 23 Kageyama M, Mori T, Yanagisawa T, Taira N. Is staurosporine a specific inhibitor of protein kinase C in intact porcine coronary arteries? *J Pharmacol Exp Ther* 1991; 259:1019–26.
- 24 Ozawa K, Szallasi Z, Kazanietz MG, Blumberg PM, Mischak H, Mushinski JF, Beaven MA. Ca^{2+} -dependent and Ca^{2+} -independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. *J Biol Chem* 1993; 268:1749–56.
- 25 Perkins RS, Lindsay MA, Barnes PJ, Giembycz MA. Early signalling events implicated in leukotriene B₄-induced activation of the NADPH oxidase in eosinophils: role of Ca^{2+} , protein kinase C and phospholipase C α . *Biochem J* 1995; 310:795–806.
- 26 Yamazaki M, Gomez-Cambronero J, Durstin M, Molski TF, Becker EL, Shaafi RI. Phorbol 12-myristate 13-acetate inhibits binding of leukotriene B₄ and platelet-activating factor and the responses they induce in neutrophils: site of action. *Proc Natl Acad Sci USA* 1989; 86:5791–4.
- 27 Kroegel C, Giembycz MA, Matthys H, Westwick J, Barnes PJ. Modulatory role of protein kinase C on the signal transduction pathway utilized by platelet-activating factor in eosinophil activation. *Am J Respir Cell Mol Biol* 1994; 11:593–9.
- 28 Naccache PH, Molski MM, Volpi M, Becker EL, Shaafi RI. Unique inhibitory profile of platelet activating factor induced calcium mobilization, polyphosphoinositide turnover and granule enzyme secretion in rabbit neutrophils towards pertussis toxin and phorbol ester. *Biochem Biophys Res Commun* 1985; 130:677–84.