Differences in Platelet-Activating Factor Receptor Mediated Ca\(^{++}\) Response Between Hamster and Guinea Pig Alveolar Macrophages\(^1\)

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ABSTRACT
The different platelet-activating factor (PAF) receptor subtypes were identified in alveolar macrophages of hamster and guinea pig, based on the distinct characteristics of PAF-induced Ca\(^{++}\) responses and PAF antagonist potencies to these responses. PAF, but not lyso-PAF (inactive PAF), induced Ca\(^{++}\) release from intracellular Ca\(^{++}\) stores and the influx of extracellular Ca\(^{++}\) in a dose-dependent manner in both hamster and guinea pig alveolar macrophages. The potency for PAF-stimulated Ca\(^{++}\) release, however, was significantly different between the two species with EC\(_{50}\) values being 30- and 50-fold higher in Ca\(^{++}\) release and Ca\(^{++}\) influx responses in guinea pig than hamster, respectively. In addition, there were distinct differences in Ca\(^{++}\) influx characteristics between the two species; guinea pig macrophages exhibiting a rapid Ca\(^{++}\) extrusion and high sensitivity to thapsigargin (depletion of intracellular Ca\(^{++}\) store). The PAF-induced Ca\(^{++}\) response was sensitive to G-protein inhibitor pertussis toxin in hamster but not in guinea pig, suggesting the coupling of different types of G-proteins to PAF receptors. Pretreatment of macrophages with tyrosine kinase inhibitor, herbimycin A, caused a dose-dependent decrease in PAF-induced Ca\(^{++}\) response in guinea pig but surprisingly an increased response in hamster. These observations suggest the possibility of a dual mechanism, for G-protein and tyrosine kinase signaling in response to PAF-mediated receptor signal transduction cascade. The PAF-induced Ca\(^{++}\) response was desensitized by repetitive stimulation with PAF or pretreatment with protein kinase C activator, mitogen-activated protein kinase, which had a slightly greater potency in guinea pig than hamster. Importantly, three structurally distinct PAF antagonists, WEB2086, L659,989 and CL184005, blocked PAF-induced Ca\(^{++}\) responses in a dose-dependent manner with a markedly different potencies between the two species. The IC\(_{50}\) values for inhibiting PAF-induced Ca\(^{++}\) release were 2.5- (WEB2086), 650- (L659,989) and 120- (CL184005) fold less in hamster than in guinea pig. The relative potencies of these PAF antagonists in hamster macrophages were L659,989 > CL184005 > WEB2086. However, in guinea pig these three antagonists showed roughly the same potency. Interestingly, the opposite inhibitory effects of these antagonists on PAF-induced Ca\(^{++}\) influx were found in the two species, in which the IC\(_{50}\) were 15- (WEB2086) and 5- (CL184005) fold greater in hamster than in guinea pig but no difference in the IC\(_{50}\) value of L659,989 between the two species. Pretreatment of macrophages from both species with these antagonists had no effect on ATP-induced Ca\(^{++}\) release. These observations suggest that the antagonism is specific to PAF receptors. Based on our data, it was concluded that the alveolar macrophages isolated from the bronchoalveolar lavage of hamsters contain a distinct subtype PAF receptor that differs from that of guinea pigs in modulating a different signal transduction pathway.

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ABBREVIATIONS: PAF, platelet activating factor; EC\(_{50}\), concentration that produces 50% of maximal response; IC\(_{50}\), concentration which inhibits 50% of response; PKC, protein kinase C; PT, pertussis toxin; TK, tyrosine kinase; IP\(_3\), inositol 1, 4, 5 triphosphate; PLC, phospholipase C; HBSS, Hanks’ balanced salt solution; PMA, phorbol 12-myristate 13-acetate; MAP, mitogen activated protein kinase; BSA, bovine serum albumin.
pathways that generate second messengers by coupling with the G-proteins (Chao and Olson, 1993). These include stimulation of phospholipid turnover via PLC, PL A2 and PL D pathways and activation of various protein kinases such as protein kinase C, tyrosine kinase, MAP kinase and PKC kinase (Honda et al., 1994; Franklin et al., 1995; Franklin et al., 1993). Furthermore, PAF stimulates the [Ca$$^{++}$$]i release via intracellular signaling process in almost all PAF responsive cells. The Ca$$^{++}$$ signaling can occur via both Ca$$^{++}$$ release from intracellular Ca$$^{++}$$ stores, mainly from endoplasmic reticulum, and extracellular Ca$$^{++}$$ influx. The Ca$$^{++}$$ release from endoplasmic reticulum is mediated by the IP3 that is one of the major products of PAF-stimulated phosphatidylinositol turnover through PLC activation (Chao and Olson, 1993). In addition to G-protein coupling system, PLC can also be stimulated by tyrosine kinase either by G-protein or directly by the receptor via a mechanism not yet known (Thurston et al., 1993). The mechanism of PAF-induced extracellular Ca$$^{++}$$ influx is not known. It might be mediated by PAF receptors or directly caused by PAF or PAF-generated other intracellular substances (Shukla et al., 1993).

The PAF-induced Ca$$^{++}$$ response is considered to be an ideal index to monitor the existence of a functional PAF receptor for investigating the mechanism of PAF receptor modulated signal transduction process. In this study we have used the PAF-induced Ca$$^{++}$$ signaling characteristics in alveolar macrophages to distinguish the PAF receptor in hamster from that in guinea pig by manipulating the upstream components of Ca$$^{++}$$ response such as: 1) inhibition of G-protein with PTX to test if there are different types of G-proteins linked to receptors; 2) inhibition of TK with herbimycin A to test PLC activation pathway and 3), blocking the PAF receptors with three structurally different PAF receptor antagonists (WEB2086, L659,989 and CL184005) to determine their relative potencies for inhibiting the PAF-induced Ca$$^{++}$$ response in the hamster and guinea pig alveolar macrophages. Our data suggest that the mechanism of PAF-induced signal transduction in the alveolar macrophages of hamster differs from that of the guinea pig.

Methods

Animals. Golden Syrian male hamsters weighing 100 to 120 g and Duncan Hartley male guinea pigs weighing 450 to 500 g (chronic respiratory disease free) were purchased from Simonsens, Inc. (Gillroy, CA). Hamsters were housed in groups of four in facilities with filtered air and constant temperature and humidity. All care was in accordance with the guidelines of the National Institute of Health for Animal Welfare. The hamsters were allowed to acclimate in animal facilities for 1 wk before starting the study. A 12 hr/12 hr light/dark cycle was maintained. Hamsters had access to Rodent Laboratory Chow 5001 and guinea pigs to Guinea Pig Laboratory Chow (Purina Mills, Inc. St. Louis, MO) and water ad libitum.

Alveolar macrophage preparation. The hamsters and guinea pigs were anesthetized with sodium pentobarbital (75–95 mg/kg i.p.) and subjected to bronchoalveolar lavage with Ca$$^{++}$$-free HBSS containing 15 mM HEPES. After centrifugation (200 x g for 10 min at 4°C) of the lavage fluid and washing twice, the resulting cell pellets were suspended in Ca$$^{++}$$-containing HBSS and kept on ice. Cell viability in all experiments was >95% as determined by trypan blue exclusion assay. The cell numbers were counted by hemocytometer. For measurement of cytoplasmic Ca$$^{++}$$ concentration in lavage cells, the cell suspensions were immediately loaded with fluorescence calcium indicator Fura-2-AM, as described below. Otherwise, the cells were resuspended in Dulbecco’s modified Eagle medium containing 5% fetal bovine serum, 15 mM HEPES, penicillin 100 U/ml and streptomycin 50 μg/ml, and plated on coverslips followed by incubation at 37°C for 1 hr to produce a monolayer of alveolar macrophages (Mosier, 1984).

Macrophages in the lavage cells and the monolayer cells were identified by nonspecific esterase staining. Briefly, an aliquot of the lavage cell suspension was taken to prepare a slide with cytopsin, and another aliquot was plated in Chamber slide to allow macrophages to attach on the slide and form monolayer cells. Both lavage cell film on slide and monolayer cells were then fixed and stained with AS-Naphth, and a-Naphth plus fluoride inhibition assay for nonspecific esterase for qualitative evaluation of macrophages, neutrophils and lymphocytes using a kit from Sigma Chemical Company (St. Louis, MO) following manufacturer’s protocol. Macrophages were about 95% in lavage cell suspension and more than 97% in monolayer cells.

The data is expressed as the mean ± S.D. The data were analyzed using analysis of variance and the level of significance was taken as P < 0.05.

Results

Characteristics of PAF-induced [Ca$$^{++}$$]i mobilization. We first tested whether PAF induced Ca$$^{++}$$ response characteristics in hamster alveolar macrophages are different from that of guinea pig alveolar macrophages. To accurately quantify PAF-induced Ca$$^{++}$$ signaling pathways, we dissociated Ca$$^{++}$$ release from intracellular stores from extracellular Ca$$^{++}$$ influx for most experiments using the Ca$$^{++}$$-free/Ca$$^{++}$$-reintroduction protocol (Clementi et al.,
1992; Zacchetti et al., 1991): the macrophages were placed in Ca++ free assay buffer (Ca++-free HBSS containing 0.5 mM EGTA, 0.1% BSA and 15 mM HEPES) immediately before [Ca++]i measurement, and, Ca++ (2 mM) was reintroduced into the buffer after PAF-stimulated Ca++ release response was over. Figure 1 demonstrates a representative PAF (10 nM)-induced Ca++ response tracing with the initial Ca++ release in Ca++-free medium, followed by Ca++ influx after exogeneous Ca++ reintroduction (fig. 1A and D). Depletion of intracellular Ca++ stores by pretreating the cells with 1 μM thapsigargin (Thastrup et al., 1990) completely inhibited PAF-induced Ca++ release (fig. 1B and E), which suggests that the PAF-induced [Ca++]i increase was contributed by both intracellular and extracellular Ca++ sources. Lyso-PAF, a biologically inactive form of PAF, had no effect on [Ca++]i mobilization in the macrophages from both species (fig. 1C and F). The slight increase of [Ca++]i after Ca++ reintroduction may be due to slow Ca++ influx through an unknown mechanism, probably a nonspecific effect of lyso-PAF.

However, there were marked differences in the Ca++ signaling characteristics between hamster and guinea pig alveolar macrophages. The PAF-induced Ca++ release and influx peaks were shallow in hamster and markedly sharp in guinea pig (fig. 1A and D). In addition, a rapid Ca++ extrusion after Ca++ influx was found in guinea pig relative to hamster (fig. 1D). It was reported that PAF-activated Ca++ extrusion was mediated by a product of arachidonic acid cascade generated in PAF-stimulated macrophages (Randriamampita and Trautmann, 1990). Therefore, this difference in the Ca++ extrusion between the two species could be attributed to a difference in the signal transduction mechanism triggered by the activation of the PAF receptor. Furthermore, depletion of intracellular Ca++ stores by thapsigargin equally prevented PAF-stimulated Ca++ release from intracellular stores in both species but had different effect on extracellular Ca++ influx (fig. 2A and B). For instance, the Ca++ influx in hamster was little affected whereas it was significantly increased in guinea pig when Ca++ stores were depleted by thapsigargin (fig. 2A and B). In contrast, depletion of intracellular Ca++ stores by thapsigargin increased ATP-induced Ca++ influx in hamster macrophage but not as much in guinea pig (fig. 2C and D). In addition, ATP-induced Ca++ extrusion was found in hamster alveolar macrophage but not in guinea pig macrophages. These data tend to support the hypothesis that there are distinct mechanisms for PAF and ATP receptor-mediated signaling pathways and also demonstrate that the macrophages from both species have similar Ca++ influx systems that are activated by different ligands suggesting different mechanisms of PAF receptor mediated Ca++ influx in hamster and guinea pig macrophages.

**Dose-response of [Ca++]i mobilization.** The dose-response curves of PAF-induced Ca++ signaling, both Ca++

![Fig. 1. PAF induced Ca++ release from intracellular stores and extracellular Ca++ influx in the alveolar macrophages of hamster and guinea pig. Alveolar macrophages were collected by pulmonary lavage and loaded with 1.5 μM Fura-2AM. The intracellular free Ca++ ([Ca++]i) of the cells in suspension was measured in Ca++-free HBSS followed by reintroduction with 2 mM Ca++ (Ca++-free/Ca++ reintroduction procedure as described in “Results”). The arrows indicate the times of addition. In A and D, the cells were stimulated by 10 nM PAF to indicate Ca++ release from intracellular Ca++ stores, followed by reintroduction of 2 mM Ca++ to show extracellular Ca++ influx. In B and E, the cells were pretreated with 1 μM thapsigargin (Tg) to deplete intracellular Ca++ stores and then stimulated by 10 nM PAF and Ca++ reintroduction. In C and F, the cells were stimulated by 10 nM lyso-PAF (inactive PAF). For comparison, the [Ca++]i measurements in hamster and guinea pig alveolar macrophages were performed in parallel. This is a representative tracing of three to five experiments.](image1)

![Fig. 2. Comparison of alveolar macrophage [Ca++]i mobilization induced by PAF and ATP in hamster and guinea pig. The Fura-2-loaded macrophages were pretreated with 1 μM thapsigargin (Tg) and then stimulated by 10 nM PAF (A and B) or 50 μM ATP (C and D). The arrows indicate the times of addition. Ca++ extrusion (efflux) after ATP-induced Ca++ influx was shown in hamster macrophages (C). PAF stimulated a slight Ca++ extrusion in hamster but marked Ca++ extrusion in guinea pig (A and B). This is a representative tracing of three to five experiments.](image2)
release and Ca\(^{2+}\) influx, were established by plotting the changes in peak [Ca\(^{2+}\)]\(_i\) levels against corresponding concentrations of PAF. As shown in figure 3, both transient Ca\(^{2+}\) release (fig. 3A) and sustained extracellular Ca\(^{2+}\) influx (fig. 3B) were increased in a concentration-dependent manner both in the alveolar macrophages of hamster as well as in the guinea pig. The responses peaked at 100 nM PAF for Ca\(^{2+}\) influx (fig. 3B) were increased in a concentration-dependent manner both in the alveolar macrophages of hamster as well as in the guinea pig. The responses peaked at 100 nM PAF for Ca\(^{2+}\) release (fig. 3A) but it required only 1 nM for Ca\(^{2+}\) influx (fig. 3B) in both species. However, significant differences in sensitivity and potency of the PAF-induced Ca\(^{2+}\) response were noted between hamster and guinea pig. The Ca\(^{2+}\) release response in guinea pig macrophages was stimulated by PAF at a concentration as low as 0.001 nM but it required a 100-fold more PAF (0.1 nM) for the hamster macrophages. The corresponding EC\(_{50}\) values (the concentration of PAF inducing 50% maximal response) were 0.3 and 10 nM in guinea pig and hamster macrophages, respectively, with approximately a 30-fold higher EC\(_{50}\) value in hamster than in guinea pig. Similar results were found in the case of PAF-induced extracellular Ca\(^{2+}\) influx with a 50-fold higher EC\(_{50}\) value in hamster (0.1 nM) than that in guinea pig (0.02 nM).

**Inhibition of Ca\(^{2+}\) signaling by PAF Antagonists.**

Pretreatment of the alveolar macrophages with three structurally different PAF antagonists WEB2086, L659,989 and CL184005 inhibited both PAF-stimulated Ca\(^{2+}\) release and Ca\(^{2+}\) influx in a dose-dependent manner. The Fura-2-loaded macrophages were pretreated with increasing concentration of PAF antagonists or their vehicle for 1 min at 37°C in an assay buffer followed by stimulation with 10 nM PAF and reintroduction with 2 mM Ca\(^{2+}\). Representative Ca\(^{2+}\) response tracings from the macrophages pretreated with or without each antagonist are shown in figure 4 for WEB2086, figure 5 for L659,989 and figure 6 for CL184005. For analysis of the dose-response relationship, the change in [Ca\(^{2+}\)]\(_i\) peak (above basal level) was measured and expressed as percentage of control (pretreated with vehicle) and plotted against the concentration of PAF. In both hamster and guinea pig, pretreatment of macrophages with these antagonists resulted in a concentration-dependent inhibition of PAF-induced intracellular Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) influx, although all antagonists were less sensitive and less potent to inhibit Ca\(^{2+}\) influx response. The inhibitory potencies, IC\(_{50}\) (concentration for 50% inhibition), of these antagonists for inhibiting both Ca\(^{2+}\) release and Ca\(^{2+}\) influx are summarized in table 1. With respect to inhibition of Ca\(^{2+}\) release, the hamster macrophages were more sensitive to all tested antagonists, specifically to L659,989, than the guinea pig macrophages. The IC\(_{50}\) values of WEB2086, L659,989 and CL184005 for inhibiting 10 nM PAF-induced intracellular Ca\(^{2+}\) release were 2.5-, 650- and 125-fold less in hamsters than in guinea pig. There was a wide variation in the inhibitory potencies between WEB2086 and L659,989 or CL184005 in hamster but not that much in guinea pig (fig. 7). Their relative potencies in hamster macrophages (fig. 7A) are L659,989 > CL184005 > WEB2086. However, in guinea pig, the potencies were not significantly different from each other (fig. 7B).

PAF-induced Ca\(^{2+}\) influx was blocked by all antagonists with much lower potencies in both species than Ca\(^{2+}\) release except CL184005 in guinea pig that showed roughly the same potency as inhibiting PAF-induced Ca\(^{2+}\) release. Moreover, the inhibitory effects of these antagonists on PAF-induced Ca\(^{2+}\) influx in hamster and guinea pig macrophages were markedly different from their Ca\(^{2+}\) release response. Both WEB2086 and CL184005 inhibited PAF-induced Ca\(^{2+}\) influx with lower potencies in hamster (IC\(_{50}\) = 7.3 and 1.1 μM, respectively) than in guinea pig (IC\(_{50}\) = 0.5 and 0.2 μM, respectively) (fig. 8A and C), and this was in contrast to their effects on Ca\(^{2+}\) release (fig. 4B and 6B). Interestingly, L659,989 had roughly the same potency for inhibiting the PAF-induced Ca\(^{2+}\) influx in both species with IC\(_{50}\) values of 1.3 μM in hamster and 1.6 μM in guinea pig (fig. 8B), whereas the potency of this antagonist in inhibiting the Ca\(^{2+}\) release response turned out to be 650-fold higher in the former than in the latter (fig. 5B).

The alveolar macrophages’ viability was not affected at any concentration, including the highest, of the antagonists as determined by trypan blue dye exclusion (data not shown).

**A. PAF-induced Internal Ca\(^{2+}\) Release**

**B. PAF-induced Extracellular Ca\(^{2+}\) Influx**

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Dose-dependent increase in PAF-induced [Ca\(^{2+}\)]\(_i\) mobilization in hamster and guinea pig alveolar macrophages. The Fura-2-loaded cells in suspension were stimulated with the different concentrations (0.001–100 nM) of PAF. The changes in Ca\(^{2+}\) release peaks (A) measured in Ca\(^{2+}\)-free condition and Ca\(^{2+}\) influx peak (B) measured after Ca\(^{2+}\) reintroduction were plotted against the logarithmic PAF concentrations. Each point represents the mean ± S.D. of three to five experiments.
Also, the use of antagonists perse did not alter the basal [Ca\(^{2+}\)]i level. To rule out the possibility that these antagonists may interfere with general cell Ca\(^{2+}\) signaling mechanism unrelated to PAF receptor, we compared PAF response with ATP-induced Ca\(^{2+}\) signaling in the antagonist-preincubated macrophages under identical conditions (performing side-by-side with PAF). The mechanisms for both ATP-induced [Ca\(^{2+}\)]i mobilization and extracellular Ca\(^{2+}\) influx are mediated by coupling with G-protein through stimulating PLC-IP3 pathway (thus intracellular Ca\(^{2+}\) release) (Coniglione and Jiang, 1995). We found that pretreatment of alveolar macrophages with or without PAF antagonists, WEB2086 (1 \(\mu\)M) or L659,989 (0.5 \(\mu\)M) did not change ATP-induced Ca\(^{2+}\) signaling, as shown in figure 9 for hamster and figure 10 for guinea pig macrophages. Interestingly the PAF- and ATP-stimulated Ca\(^{2+}\) influx effects were different between the two species. ATP stimulated a rapid Ca\(^{2+}\) influx with clear Ca\(^{2+}\) extrusion phase in hamster macrophages (fig. 9) whereas a sustained Ca\(^{2+}\) influx without Ca\(^{2+}\) extrusion phase in guinea pig macrophages (fig. 10). These observations suggest that the intrinsic intracellular Ca\(^{2+}\) homeostasis was not influenced by PAF antagonist pretreat-
A. Inhibition of PAF-induced Ca\(^{2+}\) Response by CL184005

![Figure 6](image1)

**Fig. 6.** Inhibition of PAF-induced [Ca\(^{2+}\)]\(i\) increase by PAF antagonist CL184005. A. The hamster and guinea pig alveolar macrophages were preincubated with 100 nM CL184005 (right panels) or its vehicle ethanol (left panels) at 37°C for 1 min followed by stimulation with PAF (10 nM) and reintroduction with Ca\(^{2+}\) (2 mM). The arrows indicate the times of addition. This is a representative tracing of three to five experiments. B. The dose-response of CL184005 for inhibiting 10 nM PAF-induced Ca\(^{2+}\) release. The changes in Ca\(^{2+}\) release peaks were measured and plotted against concentrations of CL184005, as described in figure 4. Each point represents the mean ± S.D. of three to five experiments.

B. Dose-Response

![Figure 7](image2)

**Fig. 7.** Inhibition of PAF-induced Ca\(^{2+}\) release in alveolar macrophages of hamster (A) and guinea pig (B) by PAF antagonists WEB2086, L659,989 and CL184005. The Fura2-loaded alveolar macrophages from both species were pretreated with different concentrations of each of three antagonists at 37°C for 1 min followed by stimulation with 10 nM PAF and reintroduction with 2 mM Ca\(^{2+}\). The changes in Ca\(^{2+}\) release peaks were calculated and expressed as percentages of controls (pretreated with vehicle only). The graphs are drawn from the data shown in figures 4 to 6 to compare the inhibitory effects of all three antagonists.

### TABLE 1

**Potencies of PAF antagonists for inhibiting PAF-induced [Ca\(^{2+}\)]\(i\) mobilization in alveolar macrophages from hamster and guinea pig**

<table>
<thead>
<tr>
<th>PAF Antagonist</th>
<th>Hamster Ca(^{2+}) release</th>
<th>Hamster Ca(^{2+}) influx</th>
<th>Guinea Pig Ca(^{2+}) release</th>
<th>Guinea Pig Ca(^{2+}) influx</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEB2086</td>
<td>51.5</td>
<td>7300</td>
<td>138</td>
<td>500</td>
</tr>
<tr>
<td>L659,989</td>
<td>1.2</td>
<td>1300</td>
<td>784</td>
<td>1600</td>
</tr>
<tr>
<td>CL184005</td>
<td>1.6</td>
<td>1100</td>
<td>200</td>
<td>230</td>
</tr>
</tbody>
</table>

The inhibitory potency expressed as the concentration (nM) of PAF antagonist that inhibits 50% of the response (IC\(_{50}\)). The IC\(_{50}\) values were derived from the data shown in figures 4, 5 and 6.
inhibited PAF-induced $[Ca^{++}]_i$ mobilization (both intracellular $Ca^{++}$ release and extracellular $Ca^{++}$ influx) in hamster but no significant change occurred in guinea pig macrophages under the same conditions (fig. 11). To establish an optimal time and concentration for inhibition, the time-course and dose-response of PT for inhibiting PAF-induced $Ca^{++}$ response (expressed as percentage of control) were carried out as shown in figure 12. PT inhibited the PAF-induced $Ca^{++}$ response in a time (up to 4 hr) and dose-dependent manner in hamster macrophage but not in guinea pig, although higher concentration (>500 ng/ml) led to slight inhibition in the latter. The 4-hr pretreatment of the hamster macrophages with PT reduced PAF-induced $Ca^{++}$ response by 60%. Interestingly, in hamster pretreatment with PT for more than 4 hr or at concentrations more than 50 ng/ml did not increase the inhibitory effect of PT on PAF-induced intracellular $Ca^{++}$ release, suggesting the involvement of additional mechanisms such as activation of TK.

**Effect of TK inhibitor on $Ca^{++}$ signaling.** PAF-induced stimulation of tyrosine phosphorylation could be an additional pathway for PAF-induced $[Ca^{++}]_i$ mobilization in hamster and guinea pig macrophages. This is based on the findings that TK has been found to be involved in PLC activation that stimulates IP3 production and subsequently $Ca^{++}$ release from intracellular $Ca^{++}$ stores (Rhee, 1991). Several investigators have demonstrated that TK inhibitors prevent PAF-induced stimulation of PLC (Dhar et al., 1990; Salari et al., 1990). In this study, we compared the effect of tyrosine kinase inhibitor, herbimycin A, on PAF-induced $[Ca^{++}]_i$ response between the two species. Figure 13A demonstrates that pretreatment of alveolar macrophages with 50 $\mu$M herbimycin A for 18 hr decreased both PAF-induced $Ca^{++}$ release and influx in guinea pig but it surprisingly potentiated the $Ca^{++}$ response in hamster. This contrasting effect of TK inhibitor on hamster and guinea was further confirmed by dose-response analysis as shown in figure 13B. About 200% increase in hamster and 50% decrease in guinea pig were found in PAF-induced $Ca^{++}$ response with herbimycin A pretreatment as compared to control cells (pretreated with DMSO). There was no change in cell viability and basal $[Ca^{++}]_i$ level under all concentrations of herbimycin A.

**Effects of PKC on $Ca^{++}$ signaling.** Phorbol ester-induced activation of PKC is known to suppress the PAF-mediated signal transduction through modification of G protein (Homma and Hanahan, 1988) and it also down-regulates the surface PAF receptors (O’Flaherty et al., 1989; Chao et al., 1990). PMA, a PKC activator, has been widely used to establish the role of PKC in signal transduction. In this study, pretreatment of the alveolar macrophages from both hamster and guinea pig with 10 nM PMA for 5 min at 37°C reduced both $Ca^{++}$ release and $Ca^{++}$ entry in response to 10 nM PAF (fig. 14A). The dose-response analysis of PMA for inhibiting PAF-induced $Ca^{++}$ release revealed that it had lower potency in hamster, with IC50 values of 6.3 nM than in guinea pig with IC50 value of 0.8 nM (fig. 14B).

**Discussion**

PAF receptor-mediated signal transduction has been well studied in several cell types such as human epidermoid carcinoma cell line A431 (Thurston et al., 1993), neurohybrid NCB-20 cell line (Yue et al., 1992), neurosecretory PC12 cells (Clementi et al., 1992), human erythroleukemia cell line (HEL) (Schwaner et al., 1992), human monocytes (Katnik and Nelson, 1993), and platelet (Dhar et al., 1990; Dhar and Shukla, 1991). $[Ca^{++}]_i$ mobilization stimulated by PAF is found in almost all PAF receptor-responsive cells (Chao and...
Olson, 1993) including platelets, neutrophils, macrophages (Gardner et al., 1993; Katnik and Nelson, 1993), vascular smooth muscle cells, endothelial cells (Gardner et al., 1993; Korth et al., 1995), tracheal epithelial cells (Kondo et al., 1994; Stoll et al., 1994) and neuronal cells. The PAF-induced \([\text{Ca}^{2+}]_i\) increase consists of: 1) extracellular \([\text{Ca}^{2+}]\) influx that occurs by an unclear mechanism, probably via a plasma membrane-associated \([\text{Ca}^{2+}]\) channel regulated by PAF receptors or by intracellular signaling molecules such as an unidentified \([\text{Ca}^{2+}]\) influx factor (Randriamampita and Tsien, 1993) and IP4 (Luckhoff and Clapham, 1992); and 2) \([\text{Ca}^{2+}]\) release from intracellular stores in response to IP3 produced during PAF receptor stimulation. PAF-induced IP3 production results from turnover of polyphosphoinositide mediated by PLC (Berridge, 1993). In this study PAF, but not lyso-PAF (inactive PAF) stimulated both \([\text{Ca}^{2+}]\) release and \([\text{Ca}^{2+}]\) influx responses in hamster and guinea pig alveolar macrophages in a dose-dependent manner, and the \([\text{Ca}^{2+}]\) response was selectively blocked by PAF antagonists. This suggests the existence of functional PAF receptors in both species. However, the \([\text{Ca}^{2+}]\) signaling characteristic features in hamster were significantly different from those found in guinea pig. First, PAF antagonists inhibited the \([\text{Ca}^{2+}]\) response with different potencies in these two species; second, PAF receptors trigger different signal transduction pathways as evidenced by PT-sensitive G-protein in hamster but PT-insensitive G-protein in guinea pig; third, opposite effects of tyrosine kinase inhibitor on \([\text{Ca}^{2+}]\) signaling with increasing \([\text{Ca}^{2+}]\) response in hamster but decreasing in guinea pig and fourth, a difference in PAF-induced \([\text{Ca}^{2+}]\) influx profile with
a sharp Ca\(^{2+}\) influx peak and marked Ca\(^{2+}\) extrusion in guinea pig as compared to hamster and this profile was in marked contrast to ATP-induced Ca\(^{2+}\) influx in the hamster and guinea pig alveolar macrophages. These findings indicate that different PAF receptor subtypes exist in hamster and guinea pig alveolar macrophages that mediate intracellular Ca\(^{2+}\) signaling by different mechanisms.

The PAF-induced Ca\(^{2+}\) signaling responses exhibit clear differences between hamster and guinea pig, as indicated by 1) higher potencies of PAF-induced stimulation [Ca\(^{2+}\)]\(_i\) mobilization and Ca\(^{2+}\) influx in guinea pig with 30-fold (Ca\(^{2+}\) release) and 50-fold (Ca\(^{2+}\) influx) higher EC\(_{50}\) value than in hamster (fig. 3); 2) a faster Ca\(^{2+}\) extrusion phase after Ca\(^{2+}\) influx in guinea pig than in hamster (fig. 1D); 3) increased PAF-induced Ca\(^{2+}\) influx in case of depletion of intracellular Ca\(^{2+}\) stores in guinea pig, but not in hamster (fig. 1E). These differences between hamster and guinea pig cannot be accounted for by differences in the intrinsic Ca\(^{2+}\) signaling system that exists between the two species because the ATP-induced Ca\(^{2+}\) extrusion phase was shown in hamster alveolar macrophage but not in guinea pig macrophages (fig. 2C and D). In addition, depletion of intracellular Ca\(^{2+}\) stores by thapsigargin increased ATP-induced Ca\(^{2+}\) influx in hamster but not in guinea pig. This suggests the existence of different subtypes of PAF receptors in hamster and guinea pig macrophages that are coupled with different signal transduction mechanisms in the same cell type.

The measurement of antagonist potency in functional assays has been one of the major criteria for defining receptor type and subtype (Kenakin et al., 1992). Antagonist data from our studies indicate that PAF acts through a receptor-dependent pathway to mobilize [Ca\(^{2+}\)]\(_i\) in hamster and guinea pig alveolar macrophages and provide evidence for the existence of PAF receptor subtypes in these two species. In both species, PAF-induced intracellular Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) influx were blocked by three structurally distinct PAF antagonists in a dose-dependent manner (fig. 4–6), especially Ca\(^{2+}\) release in the guinea pig. These antagonists caused inhibition of PAF-induced Ca\(^{2+}\) release in a concentration-dependent manner with the IC\(_{50}\) values of 2.5- (for WEB2086, fig. 4B), 650- (for L659,989, fig. 5B) and 120- (for CL184005, fig. 6B) fold less in hamster than in guinea pig, respectively. The relative potencies of the PAF antagonist in hamster macrophages ranked as follows: L659,989 > CL184005 > WEB2086 (fig. 7A), although in the guinea pig these three antagonists showed roughly the same potency with CL184005 slightly higher.
than the other two (fig. 7B). With any pretreatment, the antagonists did not change cell viability and had no effect on ATP-induced $[Ca^{2+}]_{i}$ mobilization (figs. 9 and 10) in the alveolar macrophages from both species. This suggests that PAF antagonists had no effect on intrinsic intracellular $Ca^{2+}$ homeostasis but they specifically blocked the PAF receptor-mediated signal transduction pathways responsible for $Ca^{2+}$ mobilization.

Interestingly, although pretreatment with these antagonists also led to a concentration-dependent inhibition of PAF-induced extracellular $Ca^{2+}$ influx with less potency and contrasting effects on macrophages from these two species after pretreatment with WEB2086 and CL184005. The inhibitory potencies were 15- (WEB2086) and 5-fold (CL184005) lower in hamster ($IC_{50} = 7.3$ and 1.1 $\mu$M, respectively) than in guinea pig ($IC_{50} = 0.5$ and 0.2 $\mu$M, respectively) (fig. 8A and C). The corresponding inhibitory potencies for inhibiting PAF-induced $Ca^{2+}$ release were 2.5- and 130-fold higher in hamster than in guinea pig (figs. 4B and 6B). In addition, L659,989 pretreatment, which showed most significant difference in inhibiting $Ca^{2+}$ release with 650-fold higher potency in hamster (fig. 5B), was equally effective on PAF-induced $Ca^{2+}$ influx in both species (fig. 8B). These variations in their inhibitory effects on the PAF-induced $Ca^{2+}$ release and $Ca^{2+}$ influx strongly suggest that there may be different binding sites for these antagonists in different subtype PAF receptors. Once these binding sites are occupied by the antagonists, the PAF receptor will be inhibited competitively (block the same sites as PAF binding) or noncompetitively (block near PAF binding sites). Further studies will distinguish the competitive or noncompetitive nature of PAF antagonists on alveolar macrophages from...
hamster and guinea pig with respect to blockade of PAF-induced Ca\(^{++}\) release and Ca\(^{++}\) influx.

G-protein inhibitor (PT), tyrosine kinase inhibitor (herbimycin A) and PKC activator (PMA) all had effects on both PAF-induced Ca\(^{++}\) release and Ca\(^{++}\) influx in hamster and guinea pig alveolar macrophages. Pretreatment of the alveolar macrophages from hamster with PT led to significant time- and dose-dependent inhibition of PAF-induced Ca\(^{++}\) response (fig. 12), both Ca\(^{++}\) release and Ca\(^{++}\) influx (fig. 11). In contrast, PT was ineffective on PAF-induced Ca\(^{++}\) response in guinea pig (fig. 12). This indicates that PAF receptor in the hamster macrophage is coupled with PT-sensitive G-protein and it is linked to the PT-insensitive G-protein in guinea pig. Interestingly, the maximal inhibition by PT pretreatment was about 60% in both time-course or dose-response studies (fig. 12). Obviously, additional mechanisms may be involved in PAF-induced PLC activation and/or other Ca\(^{++}\) signaling pathways in hamster as well as guinea pig. These results indicate that the PAF receptors in hamster and guinea pig macrophages are linked to different types of G-protein-coupled signaling systems.

Tyrosine phosphorylation by TK has been suggested to be additional mechanisms for PAF-induced stimulation of PLC, particularly PLC that turns over PIP2 to IP3 (Rhee, 1991). The involvement of TK in the PAF signaling mechanism has been investigated in rabbit platelet using TK inhibitor, genestein and antiphosphotyrosine antibody (Dhar et al., 1990). In that study, genestein inhibited PAF-induced platelet aggregation and PLC activation in a dose-dependent manner, suggesting that TK activity is an important early step in PAF receptor-stimulated production of IP3. Another study demonstrated that pretreatment of neutrophils with PAF but not lyso-PAF caused increases in tyrosine phosphorylation of 41-, 54-, 66-, 104- and 116-KDa proteins in a dose-dependent manner (Gomez-Cambronero et al., 1991). The PAF-induced tyrosine phosphorylation in neutrophils can be blocked by PAF antagonist BN-52021 and the phosphorylation of the proteins 66, 116 and 104 KDa were selectively inhibited by PT treatment. It has also been reported that PAF increased pp60c-src phosphorylation in platelet (Dhar and Shukla, 1991, 1994) and this phosphorylation can be blocked by the PAF antagonist CV-6209, whereas lyso-PAF had no effect on the phosphorylation (Dhar and Shukla, 1991). All these findings suggest that tyrosine phosphorylation may play an important role in the PAF receptor-mediated signaling mechanisms. Therefore, in our study TK was considered as the first candidate for alternative pathway involved in PAF-induced [Ca\(^{++}\)]i mobilization in hamster and guinea pig alveolar macrophages. Pretreatment of guinea pig macrophages with herbinycin A, a selective inhibitor for cytosolic TK (Uehara and Fukazawa, 1991), reduced PAF-induced Ca\(^{++}\) release in a dose-dependent manner with maximal inhibition of 50% (fig. 13B). Interestingly, in hamster, herbinycin A caused dose-dependent increase in PAF response with maximal increase to 200% (fig. 13B). Although we do not know the level of protein tyrosine phosphorylation, PLC activity and IP3 production after PAF stimulation and TK inhibition, these contrasting effects of tyrosine kinase inhibitor on PAF-induced Ca\(^{++}\) signaling indicate that there is a difference in PAF receptor signaling system between the hamster and guinea pig alveolar macrophages.

In several cell types or systems, the PAF-stimulated biochemical and physiological responses, including [Ca\(^{++}\)]i mobilization (O’Flaherty et al., 1989), protein phosphorylation (Shukla et al., 1989) and IP3 production (Shukla et al., 1993), are prone to the process of desensitization (homologous or heterologous) (Shukla et al., 1993). However, its mechanism is not fully understood. Recent study has suggested that Ser/Thr phosphorylation sites in PAF receptor cytoplasmic tail play an essential role in the PAF-induced desensitization (Takano et al., 1994), suggesting the phosphorylation of the receptor is important in the desensitization process. It has been found that PKC activation down-regulated high affinity PAF receptors and inhibited PAF-induced [Ca\(^{++}\)]i mobilization in human neutrophils (O’Flaherty et al., 1989). This may be due to PKC-induced receptor inactivation by protein phosphorylation of PAF receptor (Takano et al., 1994) or receptor internalization (Gerard and Gerard, 1994). PKC defines a family of Ser/Thr kinase involved in cell surface signal transduction for the control of rapid and delayed cellular responses (Nishizuka, 1986, 1988). In this study we found that PAF-induced [Ca\(^{++}\)]i mobilization in alveolar macrophages from both hamster and guinea pig showed desensitization to repetitive stimulation with PAF (data not shown). However, PMA, a PKC activator, inhibited PAF-induced Ca\(^{++}\) release and Ca\(^{++}\) influx in macrophages from both species (fig. 14A). The dose-response of PMA for inhibiting Ca\(^{++}\) release showed slightly less potency in hamster than guinea pig (fig. 14B). The inhibitory effects of PMA on the PAF-induced Ca\(^{++}\) response were partially reversed by pre- or posttreatment of the cells with the PKC inhibitor, staurosporine (data not shown).

Although the mechanisms of PAF receptor-coupled PLC desensitization remains unclear, it is possible that PKC- or TK-induced protein phosphorylation of PAF receptor or related signal transduction components such as PLC or G-protein play a critical role. This is supported by the evidence from our study in which PKC activator reduced PAF-stimulated Ca\(^{++}\) release in hamster and guinea pig macrophages and TK inhibitor, herbinycin A, increased Ca\(^{++}\) response in hamster and decreased in guinea pig. Other studies also demonstrated that PMA down-regulated [\(^{3}H\)]PAF binding and inhibited PAF-induced [Ca\(^{++}\)]i mobilization in human neutrophils; this could be reversed by PKC inhibitor, staurosporine (O’Flaherty et al., 1992). In contrast, pretreatment of rabbit platelets with the PKC inhibitors had no effect on the PAF-induced desensitization in both [\(^{3}H\)]PAF binding (Chau, 1991) and IP3 production (Morrison et al., 1989). These findings suggest that there are species and cell type-dependent differences in PKC-mediated desensitization.

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References

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