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Mechanism of *N*-Formyl-methionyl-leucyl-phenylalanine- and Platelet-Activating Factor-Induced Arachidonic Acid Release in Guinea Pig Alveolar Macrophages: Involvement of a GTP-Binding Protein and Role of Protein Kinase A and Protein Kinase C

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SUMMARY

Various pharmacological effectors were used to investigate the mechanism of arachidonic acid release by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet-activating factor (PAF) in guinea pig alveolar macrophages. The fMLP- and PAF-stimulated arachidonic acid release (i) was mimicked by sodium fluoride and inhibited by *Bordetella* pertussis toxin, suggesting the participation of a guanine nucleotide-binding protein; ii) was mimicked by A23187 but was insensitive to the calmodulin inhibitor R24571, making the involvement of a calmodulin-dependent pathway unlikely; and (iii) was mimicked by 12-*O*-tetradecanoyl phorbol 13 acetate (TPA) and was, like the TPA-stimulated release, markedly decreased when protein kinase C (PKC) had been down-regulated by TPA (65% decrease) or inhibited by sphingosine, a diacylglycerol-competitive PKC inhibitor shown to completely abolish the enzyme activity from alveolar macrophages at 40 μM. Moreover, PAF and fMLP, under conditions where they stimulated arachidonic acid release, promoted an appreciable, albeit transient, translocation of PKC, suggesting a possible involvement of the enzyme in the agonist-stimulated process. However, staurosporine, another PKC inhibitor decreasing PKC activity from alveolar macrophages by 60% at 20 nM, failed to alter fMLP- and PAF-stimulated release. These data lead us to suggest that fMLP- and PAF-stimulated arachidonic acid release is mediated by mechanisms involving either a staurosporine-insensitive PKC isoform or a sphingosine-sensitive coupling between a pertussis toxin-sensitive guanine nucleotide-binding protein and phospholipase A_2. Finally, the fMLP- and PAF-stimulated arachidonic acid release was inhibited by cholera toxin and was, like A23187-stimulated release, potentiated by *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8), an exclusive protein kinase A inhibitor in alveolar macrophages, suggesting a negative regulation by protein kinase A.

The chemotactic peptide fMLP and PAF are involved in alveolar macrophage activation, one aspect of which consists of the release of arachidonic acid as well as of peroxidative products (1, 2). This effect has been related to PLA_2 stimulation (3), which might account also for the generation of PAF from rat alveolar macrophages. The initial event elicited in peritoneal macrophages by fMLP (4) or PAF (5) is the hydrolysis of phosphatidylinositol diphosphate by a specific PLC, leading to a rise in inositol triphosphate and DAG. Such a mechanism is also suggested for the activation of guinea pig alveolar macrophages by fMLP or PAF, because the stable DAG analogue TPA and the ionophore A23187 synergize in inducing arachidonic acid release (3). Of particular interest is the fact that, in various cell types, both agonists stimulate a phosphatidylinositol diphosphate-specific PLC through a pertussis toxin-sensitive G protein (6–8). They also promote the translocation of PKC from the cytosol to the membrane (9–11), a process thought to reflect the activation of the enzyme (12).

These findings prompted us to investigate whether fMLP- and PAF-induced arachidonic acid release in guinea pig alveolar macrophages might implicate G protein- and PKC-mediated processes. We made use of various pharmacological effectors. The involvement of a G protein was investigated with PT, CT (8), and fluoroaluminate (13) and that of calmodulin with R24571 (14). The involvement of PKC in fMLP- and PAF-stimulated arachidonic acid release was investigated using different approaches, including (i) testing the effect of TPA on

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ABBREVIATIONS: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; PT, *Bordetella* pertussis toxin; CT, cholera toxin; G protein, guanine nucleotide-binding protein; H8, *N*-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamidedihydrochloride; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; PLA_2, phospholipase A_2; LDH, lactate dehydrogenase; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*, *N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; IBMX, isobutyl-methylxanthine.

arachidonic acid release, (ii) examining the effect of PKC inhibitors or PKC down-regulation of fMLP, PAF, and TPA effects on this process, and (iii) evaluating the ability of the two agonists to promote PKC translocation under conditions where they increase arachidonic acid release. Finally, we investigated a possible role of PKA in the regulation of fMLP- and PAF-stimulated arachidonic acid release.

Experimental Procedures

Materials. Fetal calf serum and RPMI 1640 medium were from GIBCO (UK). Histone III-S, histone II-AS, phosphatidylserine, sodium fluoride, PT, CT, TPA, fMLP, sphingosine, fatty acid-free bovine serum albumin, R24571, sphingomyelin 1,2-diolein, ATP, cAMP, PMSF, β-mercaptoethanol, aprotinin, pepstatin, leupeptin, and antipain were purchased from Sigma (St. Louis, MO). Staurosporine, A23187, and the LDH kit were from Boehringer Mannheim (FGR). PAF was from Bachem (Switzerland) and H8 from Seikagaku America Inc. (St. Petersberg, FL). [1^{14}C]Arachidonic acid (58.4 mCi/mmol) was from the Commissariat à l'Energie Atomique (CEN Saclay France). [γ-^{32}P]ATP (30.7 Ci/mmol) was from NEN Research Products (Boston, MA).

Macrophage culture, labeling, and stimulation. Alveolar macrophages were harvested by bronchoalveolar lavage from Hartley guinea pigs (300–500 g; Saint-Antoine, 22 740 Pleudaniel France) and cells were allowed to adhere, as already described (1).

The adherent cells, which were washed twice with RPMI medium, were labeled using 0.1 μCi of [1^{14}C]arachidonic acid added with 0.2% fatty acid-free albumin in RPMI medium. After 1 hr, the cells were washed twice with isotonic saline containing 0.2% fatty acid-free albumin and twice with isotonic saline alone.

Cells were stimulated at 37° by 5 nM fMLP, by 1 μM PAF added with 0.5% fatty acid-free albumin in RPMI 1640 medium, by 1 μM TPA in dimethyl sulfoxide, by 1 μM A23187 in ethanol, or by various amounts of sodium fluoride. Controls were made with the pure vehicles (0.1% final) or, in the case of experiments with sodium fluoride, with the same concentration of sodium chloride.

After the stimulation, the media were collected and centrifuged at 600 × *g* for 10 min in order to remove nonadherent cells, and aliquots of the media were collected. The adherent cells were scraped with a rubber policeman. Cellular lipids were extracted as already described (3). Aliquots of the media and cell lipid extracts were used for radioactivity measurements.

When required, the cells were preincubated with PT (10 to 100 ng/ml) for 3 hr or with CT (1 μg/ml) for 3 hr. Stock solutions (50 μg/ml) were made in 2 M urea, 50 mM phosphate buffer, pH 7.4, and diluted into isotonic saline before use. Sphingosine (10 to 150 μM) and sphingomyelin (100 μM) complexed to fatty acid-free albumin in an equimolar ratio, R24571 (1 μM) in ethanol, or H8 (6.5 to 50 μM) in isotonic saline were added 10 min before cell stimulation. Staurosporine (0.1 to 1 μM) in isotonic saline was added 5 min before cell stimulation. The release of LDH activity was used as an index of cell viability.

Macrophage preparation for PKC assays. After three washings with ice-cold phosphate-buffered saline, pH 7.4, cells were scraped from the dish into the homogenization buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 5 mM EGTA, 2 mM PMSF, 50 mM β-mercaptoethanol, 0.25 M saccharose). They were disrupted by sonication and the homogenate was centrifuged at 105,000 × *g* for 1 hr. The supernatant was used as the source of cytosolic enzyme. The pellet, resuspended in the same volume of homogenization buffer containing 0.1% Triton X-100, was incubated for 1 hr at 4° and centrifuged at 105,000 × *g* for 1 hr. The resulting supernatant was used as the source of membrane-bound enzyme.

In assays for staurosporine and H8 inhibition, the cells were disrupted in the homogenization buffer containing 0.1% Triton X-100, incubated at 4°, and centrifuged at 105,000 × *g* for 1 hr, and the supernatant was used as total PKC source.

Macrophage preparation for PKA assays. Cells were washed three times, as indicated above, scraped into a homogenization buffer (20 mM KH_2PO_4/K_2HPO_4, pH 7, 3 mM EDTA, 4 mM $MgCl_2$, 6 mM β-mercaptoethanol, 1 mM PMSF, 10 units/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml antipain) and then disrupted by sonication. Homogenates were centrifuged at 30,000 × *g* for 20 min at 4°. Ammonium sulfate was added at 2.5 M to the supernatant. After stirring at 4° for 30 min, the supernatant was centrifuged at 30,000 × *g* for 40 min at 4°. The pellet was resuspended in the homogenization buffer and dialyzed against 5000 volumes of the same buffer overnight. The precipitate was removed by centrifugation and the supernatant was used as enzyme source.

Protein kinase assays. PKC was assayed in a reaction mixture containing, in a final volume of 250 μl, 20 mM Tris·HCl, pH 7.4, 5 mM magnesium chloride, 200 μg/ml histone III-S, 100 μM [γ^{32}P]ATP (5 × 10^6/dpm), and 2.5 μg of enzyme protein source, in the presence or absence of an excess of 125 μM calcium chloride, 20 μg/ml diolein, and 80 μg/ml phosphatidylserine. The incubation was carried out at 30° for 5 min. ATP (10 μM) was used in the experiments in which H8 and staurosporine were tested. To study the inhibitory effect of sphingosine, mixed micelles of substrates were made. Diolein, phosphatidylserine, and sphingosine solutions were dried under a stream of nitrogen. They were then solubilized in 0.8% Triton X-100 by vortexing and incubated at 30° for 5 min. The final proportion in the micelles was 2.5 mol % diolein, 80 mol % phosphatidylserine, and various amounts of sphingosine.

PKA was assayed in a reaction mixture containing in a final volume of 250 μl, 20 mM KH_2PO_4/K_2HPO_4, pH 7, 0.3 mM EGTA, 0.5 mM IBMX, 0.7% dithiothreitol (w/v), 7 mM NaF, 4.3 mM magnesium acetate, 420 μg/ml histone II-AS, and 10 μM [γ-^{32}P]ATP, in the presence or absence of 7 M cAMP. The incubation was carried out at 30° for 8 min.

The reactions were stopped by the addition of 1 ml of ice-cold 25% trichloroacetic acid, followed by 3 ml of 5% trichloroacetic acid and 60 μg of fatty acid-free serum albumin as a carrier protein. The samples were centrifuged at 3000 rpm for 15 min. The pellets were dissolved in ice-cold 5% trichloroacetic acid and the centrifugation/resuspension procedure was performed three times. The final pellet was dissolved in 250 μl of 0.2 N NaOH, and the radioactivity was measured in a liquid scintillation counter.

Down-regulation of PKC. Adherent macrophages were preincubated for various time periods at 37° with TPA at 1 μM or 0.1 μM. When required, cells were labeled during the last hour of the preincu-

TABLE 1

Stimulation of arachidonic acid release by fMLP, PAF, TPA, or A23187 in guinea pig alveolar macrophages

Alveolar macrophages were labeled with [1-^{14}C]arachidonic acid and stimulated as indicated in Experimental Procedures. Values are the mean ± standard error of arachidonic acid release observed in different macrophage preparations after subtraction of the spontaneous release.

Arachidonic acid release			
fMLP (5 nM, 15 min)	PAF (1 μM, 5 min)	TPA (1 μM, 15 min)	A 23187 (1 μM, 15 min)
% of total cell radioactivity			
17.3 ± 0.7 (n = 22)	6.4 ± 0.3 (n = 21)	8.5 ± 0.5 (n = 19)	9.0 ± 0.8 (n = 15)

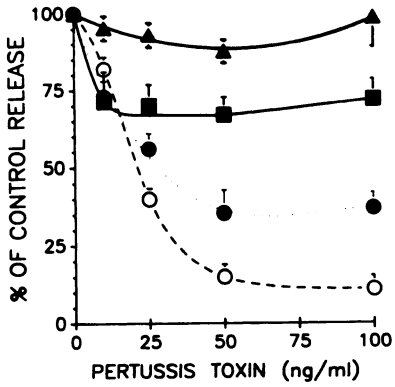

Fig. 1. Effect of PT pretreatment on arachidonic acid release stimulated by A23187 (▲), TPA (■), PAF (●), or fMLP (○). Values are the mean ± standard error of triplicate determinations. Values are expressed as percentage of the respective control. Total radioactivity incorporated in unstimulated cells was 65,381 ± 5,180 cpm. Spontaneous release after 5 and 15 min was, respectively, 1,272 ± 154 and 2,406 ± 810 cpm and was subtracted from the respective values of stimulated arachidonic acid release. Stimulated release without PT was, respectively, 19.0 ± 0.5%, 5.2 ± 0.5%, 8.3 ± 1.8%, and 6.2 ± 1.6% for fMLP, PAF, TPA, and A23187.

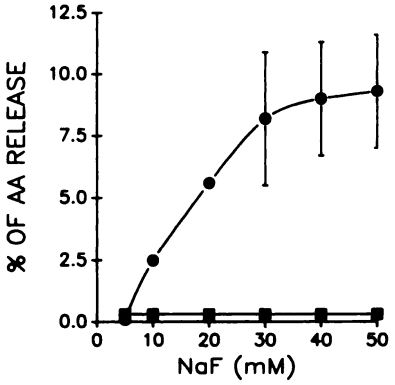

Fig. 2. Effect of sodium fluoride (●) or sodium chloride (■) on arachidonic acid (*AA*) release. Values are the mean ± standard error of triplicate determinations from a typical experiment (three independent experiments). Results are expressed as percentage of total cell labeling released as free arachidonic acid after 30 min minus spontaneous release. Total radioactivity incorporated in unstimulated cells was 44,375 ± 5,087 cpm. Spontaneous release after 30 min was 722 ± 65 cpm.

bation time. Then the cells were washed and stimulated by the various agonists. Total PKC activity and the release of arachidonic acid were measured as described above.

Statistical analysis. Results are given as means ± standard errors for the indicated numbers of independently performed experiments. Statistical differences between the means were evaluated by Student's *t* test for nonpaired data.

Results

Involvement of G protein in PAF- and fMLP-induced arachidonic acid release. Table 1 shows the arachidonic acid release by adherent alveolar macrophages stimulated with 5 nM fMLP, 1 μM TPA, or 1 μM A23187 for 15 min with 1 μM PAF for 5 min. In agreement with our previous results (3), fMLP was more efficient than A23187 or TPA, with PAF being a less effective agonist whose response plateaued after 5 min. Pretreatment of the alveolar macrophages for 3 hr with PT inhibited fMLP- and PAF-induced arachidonic acid release (Fig. 1). The maximal inhibition (85% for fMLP stimulation and 65% for PAF stimulation) was reached with 50 ng/ml PT. This concentration is close to that required for the maximal inhibition of fMLP-induced arachidonic acid release in guinea pig neutrophils (8, 15), higher than that required for the full inhibition of PAF-induced reactive oxygen intermediate production by bone marrow-derived macrophages (16), but lower than the concentration used to inhibit thrombin-induced arachidonic acid release in human platelets (17). No PT-inhibitory effect was seen on A23187-stimulated arachidonic acid release and a slight (25%) but significant effect was observed for TPA stimulation of this process at a PT concentration as low as 10 ng/ml (Fig. 1). Like fMLP and PAF, sodium fluoride (but not sodium chloride) induced the release of arachidonic acid by alveolar macrophages, the plateau being reached at 30 mM fluoride (Fig. 2). This is related to the formation of aluminium fluoride (AlF^{4-}) and provides additional evidence for a role of G protein in this process (18).

Translocation of PKC induced by TPA, fMLP, and PAF in alveolar macrophages. Our first approach to investigate the involvement of PKC in fMLP- and PAF-stimulated arachidonic acid release consisted of testing the ability of both agonists to promote the translocation of PKC, in comparison with that induced by TPA.

As shown in Fig. 3A, treatment of guinea pig alveolar macrophages with 1 μM TPA induces a rapid decrease in cytosolic PKC. Concomitantly, the membrane-bound PKC activity increases until 5 min, from which time it remains constant up to 15 min. In contrast to mouse peritoneal macrophages, in which 100% translocation was achieved after 10 min (19), we found that in alveolar macrophages the translocation amounted to 50% after 15 min.

Fig. 3B shows that TPA induces a down-regulation of PKC, which starts immediately after TPA addition and reaches 65%. No further decrease was observed up to 5 hr, which was the last time tested. Therefore, in alveolar macrophages, like in lymphocytes (20), the down-regulation is not complete. This is in contrast to other cell types, in which nearly all the PKC activities are destroyed after 2 hr (19, 21). The large differences in the down-regulation process between cell lines have been stressed by Adams and Gullick (22) and may reflect differences in the respective proportions of the multiple species of PKC as well as in the proteolytic equipment of the various cells.

Fig. 3C shows that macrophage stimulation by 5 nM fMLP induces a rapid decrease in the cytosolic PKC activity, which is already maximal after 2 min. This decrease is transient, because after a longer period of time the activity recovers in the cytosol. Concomitant with the initial decrease in cytosolic PKC activity, the membrane-bound activity increases and reaches, after 5 min, a maximal value of 164% of the control. As shown in Fig. 3D, PAF is also able to translocate PKC in alveolar macrophages. Again, the response is essentially transient and starts to reverse after 10 min. At that time, PKC activity is minimal in the cytosolic fraction and is maximal in the particulate fraction. The results presented in Fig. 3, C and D, show that both fMLP and PAF proved to be able to induce, presumably through DAG generation, a translocation of PKC that was only transient, in agreement with what has been already observed in neutrophils challenged with either agonist (9–11). In this regard, it is worth noting that 1,2 dioctanoylglycerol, a permeant DAG considered to be a physiological

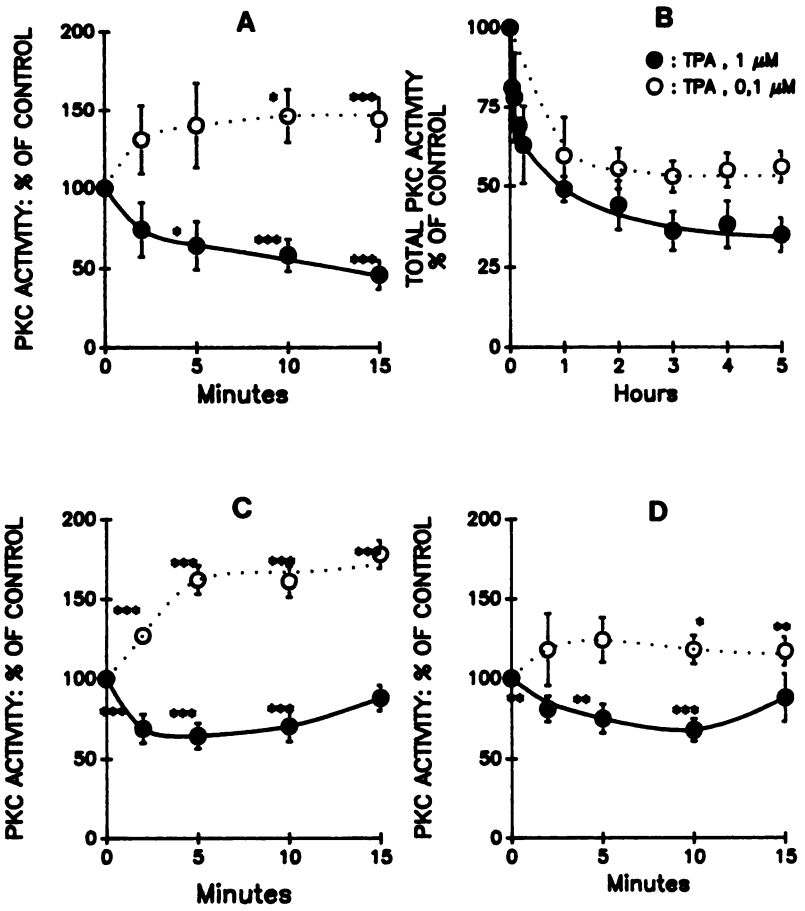

Fig. 3. Translocation and down-regulation of PKC in guinea pig alveolar macrophages. PKC activity variations induced by TPA (A), fMLP (C), or PAF (D) in cytosolic (○) or particulate (●) fractions are expressed as percentage of control values. Values are mean ± standard error of four different macrophage preparations performed in duplicate. Statistical analyses were performed for each time in comparison with control results. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$. B, Time course of TPA-induced down-regulation of total PKC activity in guinea pig alveolar macrophages. Results are expressed as percentage of control values and are the mean ± standard error of three different macrophage preparations, performed in duplicate.

activator of PKC, was also reported to induce a transient and discrete translocation of PKC in MCF-7 cells (23).

Sensitivity of PKC and PKA from alveolar macrophages to various inhibitors. Before testing sphingosine and staurosporine as potential inhibitors of fMLP- and PAF-stimulated arachidonic acid release, we evaluated their effectiveness on PKC and PKA activity from alveolar macrophages. Fig. 4A shows that sphingosine induces a dose-dependent inhibition of total homogenate PKC, which reaches a maximum at a 40 μM concentration of the agent. This observation indicates that sphingosine, when integrated in micelles of Triton X-100, exerts a competitive effect on the stimulation of PKC by diolein. Such a competitive effect was first demonstrated on PKC purified from brain by Hannun *et al.* (24). As shown in Fig. 4B, staurosporine is able to inhibit both PKC and PKA from alveolar macrophages with a similar efficiency, because a maximal inhibition of 60–70% was obtained for both kinases at a 15 nM concentration of the drug, suggesting that only certain isoforms of these enzymes are targets for staurosporine inhibition. The IC_{50} values were, respectively, 2 and 3 nM for PKC and PKA. These results confirm the observation of Tamaoki *et al.* (25), who first reported the ability of staurosporine, considered so far as a potent specific PKC inhibitor, to also inhibit PKA. This PKA inhibition proved to be as efficient as that induced by H8 (Fig. 4C), shown to be a potent inhibitor of PKA but a weak inhibitor of PKC (26). The maximal inhibition of PKA from alveolar macrophages by H8, which is obtained at a 10 μM concentration, reaches 70%, close to the same level as that induced by 15 nM staurosporine. Finally, it must be noted that, in alveolar macrophages, H8 is totally unable to inhibit PKC (result not shown).

Effect of PKC inhibition and of PKC down-regulation on the release of arachidonic acid induced by various agonists. Fig. 5A shows that sphingosine inhibited TPA-, fMLP-, PAF-, and A23187-stimulated arachidonic acid release in a dose-dependent manner, the maximal inhibition being obtained at 100 μM. The IC_{50} values were 20 μM for TPA and 50 μM for fMLP, PAF, and A23187. Even at 150 μM, sphingosine did not increase LDH release (result not shown).

The specificity of the sphingosine effect was checked three ways. Firstly, 100 μM sphingomyelin, a sphingosine analogue with no effect on PKC (27), did not significantly modify fMLP-, A23187-, TPA-, or PAF-stimulated arachidonic acid release (not shown). Secondly, R24571, a potent inhibitor of calmodulin-dependent enzymes (14), did not modify the effect of the various agonists (not shown), ruling out the possibility that sphingosine inhibition occurs through calmodulin-dependent enzymes, in contrast to what was reported in GH_3 cells (28). Thirdly, the inhibition of TPA-, fMLP-, and PAF-stimulated arachidonic acid release by sphingosine was gradually reversed by increasing amounts of TPA (Fig. 5B).

Considered together, these data suggest that PKC may be involved in the stimulation of arachidonic acid release elicited by A23187, fMLP, and PAF. For the two latter agonists, this hypothesis was reinforced by the fact that a 5-hr pretreatment of cells with 1 μM TPA, shown to induce a 65% down-regulation of PKC (Fig. 3B), markedly reduced fMLP- and PAF-stimulated arachidonic acid release (Table 2).

Staurosporine and H8 effects on agonist-induced ar-

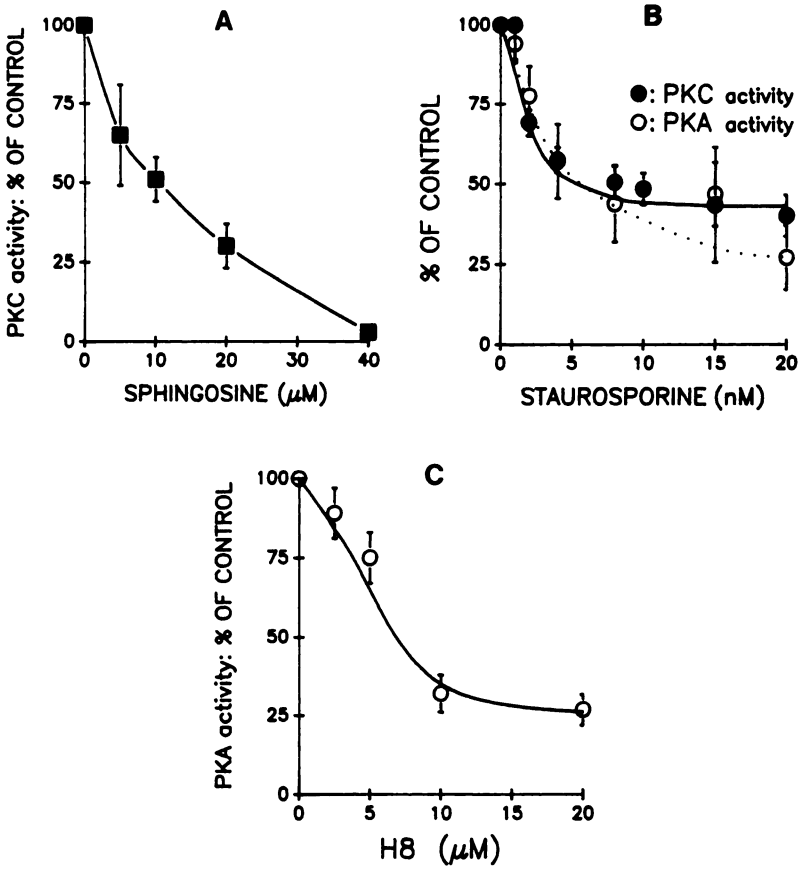

Fig. 4. A, Sphingosine inhibition of PKC activity from alveolar macrophages. The control value is 346 ± 38 pmol/min/mg of protein. B, Staurosporine inhibition of alveolar macrophage PKC and PKA activity in the presence of 10 μM ATP. Control values are 12.1 ± 1.8 and 17.8 ± 4.0 pmol/min/mg of protein for PKC (○) and PKA (●), respectively. C, H8 inhibition of alveolar macrophage PKA activity in the presence of 10 μM ATP. The control value is 17.8 ± 4.0 pmol/min/mg of protein. Values are the mean from two different macrophage preparations performed in duplicate. Bars represent standard error.

achidonic acid release. Fig. 5C shows that staurosporine inhibits TPA-mediated release, with the half-maximal effect and maximal effect being obtained at 0.1 and 0.5 μM, respectively. This TPA-induced release is not affected by H8 (Fig. 5D). Staurosporine fails to inhibit the effect of fMLP, PAF, or A23187. The same results were obtained using H8. On the contrary, a stimulatory effect of the two drugs was observed (Fig. 5, C and D). The concentrations of staurosporine giving half-maximal and maximal stimulation of fMLP-induced arachidonic acid release are the same as those giving half-maximal and maximal inhibition of TPA-induced arachidonic acid release (Fig. 5C). Such results suggest that fMLP- and PAF-stimulated arachidonic acid release may be negatively controlled by PKA. In support of this hypothesis, we observed that the stimulation of cAMP synthesis by CT decreased, respectively, by 31.5 ± 11.5% and by 36.3 ± 2.3% the fMLP- and PAF-stimulated arachidonic acid release in alveolar macrophages.

Discussion

PT interacts with alveolar macrophages and blocks practically all fMLP-induced arachidonic acid release (85%) but blocks only partially that induced by PAF (65%) (Fig. 1). This effect is likely due to ADP-ribosylation of a G protein, as proposed for the inhibition of fMLP-induced arachidonic acid release by guinea pig neutrophils (8, 15). There are several possibilities to explain the mechanism by which PT substrate is involved in fMLP- or PAF-mediated arachidonic acid release. One is a decrease in the cAMP content of the alveolar macrophages resulting from receptor occupancy, because in some cells the PT substrate is the inhibitory subunit coupled with adenyl cyclase (8); after ADP-ribosylation, this inhibitory subunit no longer exerts its inhibitory effect. This possibility is unlikely, at least for fMLP, because cellular cAMP was transiently increased by fMLP in neutrophils (29). The most likely possibility is that the PT substrate transduces the effect of receptor occupancy towards a specific PLC. Such a possibility is further supported by the fact that fluoroaluminate induces an arachidonic acid release in alveolar macrophages (Fig. 2).

Under those conditions, the fMLP- or PAF-stimulated increase in PLA_2 activity that we demonstrated in alveolar macrophages (3) must be related to inositol triphosphate-stimulated cytoplasmic Ca^{2+} rise, DAG appearance in membranes, or both (4–7) or to a direct coupling between a G protein and PLA_2, as suggested in endothelial cells (27). The effect of PAF is mediated only in part by a PT-sensitive G protein (Fig. 1). The up to 25% inhibition by PT of TPA-induced arachidonic acid release suggests that some positive feedback does exist. It might involve the release of thromboxane A_2, which has been shown in guinea pig alveolar macrophages (3).

The involvement of the calcium pathway was investigated using the calcium ionophore A23187, which induced arachidonic acid release only at high concentrations (Table 1 and Ref. 3). This effect does not involve a PT-sensitive G protein (Fig. 1). It has been suggested that arachidonic acid release in activated alveolar macrophages requires the action of a calmodulin-dependent enzyme, because its release is inhibited by trifluoroperazine, chlorpromazine, or W-7 (30). However, these agents inhibit also PKC activity (31) and the potent calmodulin antagonist R24571 has no significant effect, even at 1 μM.

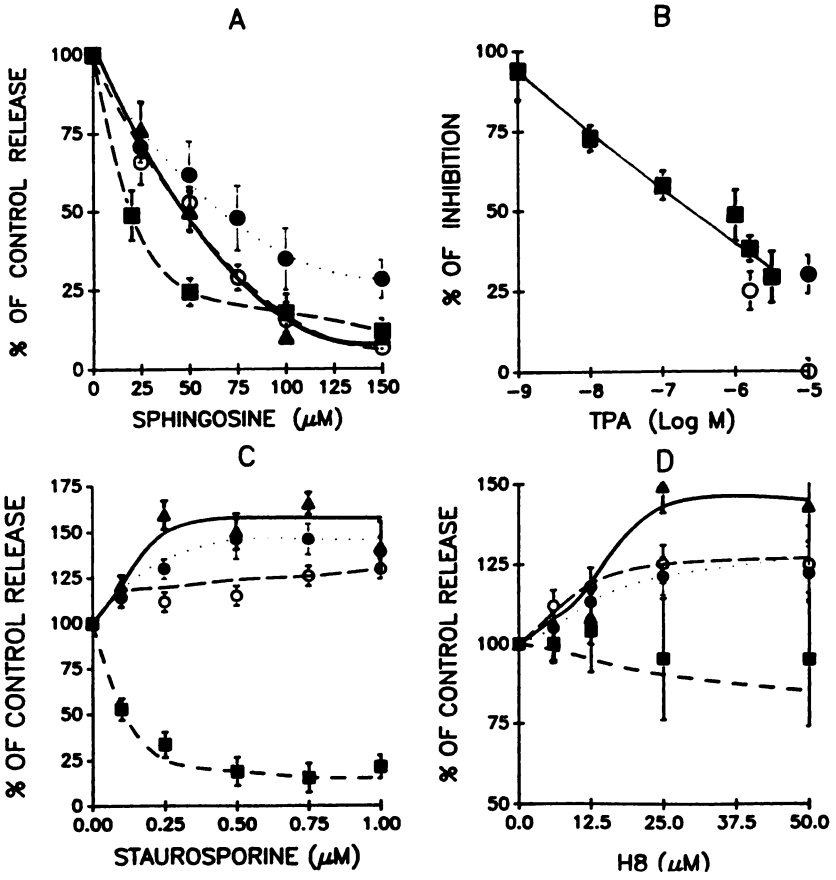

Fig. 5. Effect of various protein kinase inhibitors on agonist-stimulated arachidonic acid release. Each value is the mean ± standard error of triplicate determinations from a typical experiment (three independent experiments). A, Effect of sphingosine on arachidonic acid release stimulated by A23187 (▲), TPA (■), PAF (●), or fMLP (○). Total radioactivity incorporated in unstimulated cells was 54,298 ± 3,536 cpm. Spontaneous release after 5 and 15 min was, respectively, 907 ± 157 and 2,131 ± 114 cpm and was subtracted from the respective values of stimulated arachidonic acid release. Stimulated release without sphingosine was, respectively, 7 ± 3%, 7 ± 1%, 8 ± 2%, and 5.8 ± 0.7% for fMLP, PAF, TPA, and A23187. B, Reversal of the inhibitory effect of sphingosine on TPA- (■), PAF- (●), or fMLP- (○) stimulated arachidonic acid release by increasing concentrations of TPA [10 μM sphingosine (■, ○); 20 μM sphingosine (△, ●)]. Total radioactivity in unstimulated cells was 49,871 ± 5,968 cpm. Spontaneous release was 2,865 ± 285 cpm and was subtracted from the respective values of TPA-stimulated arachidonic acid release. Stimulated release without sphingosine was, respectively, 2.6 ± 1.5%, 7.7 ± 2.1%, 12.4 ± 3.0%, 17.1 ± 1.4%, 18.7 ± 2.5%, and 23.8 ± 0.6% for TPA, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 2×10^{-6} M and 10×10^{-6} M. C, Effect of staurosporine on arachidonic acid release stimulated by A23187 (▲), TPA (■), PAF (●), or fMLP (○). Total radioactivity in unstimulated cells was 40,063 ± 6,671 cpm. Spontaneous release after 5 and 15 min was, respectively, 927 ± 276 and 1,809 ± 333 cpm and was subtracted from the respective values of stimulated arachidonic acid release. Stimulated release without staurosporine was, respectively, 21.6 ± 0.4%, 6.7 ± 0.9%, 6.5 ± 1.5%, and 9.6 ± 0.5% for fMLP, PAF, TPA, and A23187. For all agonists, $p < 0.002$ at all concentrations of staurosporine tested. D, Effect of H8 on arachidonic acid release stimulated by A23187 (▲), TPA (■), PAF (●), or fMLP (○). Total radioactivity in unstimulated cells was 43,339 ± 5,862 cpm. Spontaneous release after 5 and 15 min was, respectively, 1,022 ± 61 and 2,506 ± 58 cpm and was subtracted from the respective values of stimulated release. Stimulated arachidonic acid release without H8 was, respectively 25.4 ± 2.2%, 6.5 ± 0.5%, 13.2 ± 0.9%, and 13.2 ± 2.1% for fMLP, PAF, TPA, and A23187. For A23187, fMLP, and PAF, $p < 0.002$ at concentrations higher than 12.5 μM. Each value is the mean ± standard error of triplicate determinations. Values are expressed as percentage of the respective control.

Therefore, a role for calmodulin in the arachidonic acid release induced in alveolar macrophages seems rather unlikely.

Because PKC has been suggested to be involved in the activation of PLA_2 in human neutrophils (32), we have investigated the role of this enzyme in arachidonic acid release. In guinea pig alveolar macrophages, TPA, a direct activator of PKC (Fig. 3A), stimulates arachidonic acid release (Table 1). This effect was completely abolished by pretreatment with 1 μM TPA (Table 2), which decreases by 65% the total PKC activity in alveolar macrophages (Fig. 3B). Furthermore, the TPA-induced arachidonic acid release is inhibited by sphingosine (Fig. 5A) in a competitive manner (Fig. 5B) and by staurosporine (Fig. 5C).

The involvement of PKC in fMLP- and PAF-stimulated arachidonic acid release is suggested by the inhibitory effect of sphingosine (Fig. 5A), which can be reversed by TPA (Fig. 5B), and by the fact that these agonists translocate PKC (Fig. 3, C and D), together with stimulated arachidonic acid release (3). Arachidonic acid release elicited by fMLP is inhibited by TPA pretreatment in the same range as the extent of PKC down-regulation (Table 2 and Fig. 3B). In contrast, the incomplete down-regulation of PKC completely blocks PAF-induced arachidonic acid release. The differences observed in the sensitivity of fMLP- and PAF-induced arachidonic acid release to TPA pretreatment could suggest that the two agonists act through PKC isoforms that may be differentially down-regulated by TPA (34). On the other hand, the inability of staurosporine to inhibit both fMLP- and PAF-induced arachidonic acid release casts doubt on the actual involvement of PKC in this process.

Staurosporine elicits the same stimulatory effect on fMLP- and PAF-stimulated arachidonic acid release as H8, which is without any effect on PKC (Fig. 5, C and D). A similar stimulatory effect of staurosporine has been described on fMLP- and PAF-stimulated peroxide liberation in neutrophils (35). The ability of H8 to inhibit PKA on the one hand and to potentiate fMLP- and PAF-stimulated arachidonic acid release on the other hand suggests that this stimulation is under the negative control of PKA. This hypothesis is supported by results ob-

TABLE 2

Effect of TPA-induced down-regulation of PKC on fMLP-, PAF-, and TPA-stimulated arachidonic acid release in guinea pig alveolar macrophages

Alveolar macrophages were incubated in RPMI medium with TPA or its vehicle for 5 hr and labeled for the last hour with [1-^{14}C]arachidonic acid. They were challenged as indicated in Experimental Procedures. Values are the mean ± standard error of a triplicate assay from a typical macrophage preparation (three independent preparations have been used). Values in parentheses represent the percentage of inhibition of respective controls. The incorporated radioactivity before stimulation was, respectively, 37,318 ± 8,388, 30,293 ± 6,122, and 24,643 ± 6,650 cpm for control cells and cells pretreated with 0.1 or 1 μM TPA. Unstimulated release was 1.6 ± 0.5% after 5 min and 3.4 ± 1.5% after 15 min and was subtracted. No difference in the spontaneous release was observed between control cells and TPA-pretreated cells.

Treatment	Arachidonic acid release		
	fMLP (5 nM, 15 min)	PAF (1 μM, 5 min)	TPA (1 μM, 15 min)
	% of total cell radioactivity		
Control	18.3 ± 1.9	5.3 ± 0.2	8.8 ± 0.5
TPA (0.1 μM, 5 hr)	11.3 ± 4.2 (39%)	ND[a]	5.2 ± 0.5 (41%)
TPA (1 μM, 5 hr)	5.1 ± 2.3 (73%)	0.3 ± 0.2 (95%)	0 (100%)

[a] ND, not done.

tained in cells treated with mediators that, in the same way, increase cellular cAMP content and decrease fMLP- and PAF-stimulated arachidonic acid release. This is actually the case for CT-treated guinea pig alveolar macrophages or IBMX- and prostaglandin E_2-treated guinea pig alveolar macrophages (3), for CT-treated guinea pig neutrophils (8), and for IBMX- or dibutyryl-cAMP-treated human monocytes (36).

In conclusion, our results strongly suggest that the stimulation of arachidonic acid release by fMLP and PAF involves several mechanisms, some of which might implicate G protein-mediated processes, and is negatively regulated by PKA. Concerning the role that PKC could play in these mechanisms, such a role is supported by several experimental arguments, including (i) the ability of these agonists to translocate PKC, (ii) the ability of TPA, a well known PKC activator, to stimulate arachidonic acid release and to reverse sphingosine inhibition, and (iii) the marked decrease in the effect of both agonists after PKC had been down-regulated by TPA or inhibited by sphingosine. However, it remains to be elucidated why staurosporine, which inhibits 60% of PKC activity in alveolar macrophages (Fig. 4B), not only fails to inhibit the fMLP- and PAF-stimulated arachidonic acid release but also potentiates this effect to the same extent as H8, which is an exclusive PKA inhibitor in alveolar macrophages. One possible explanation might be that some of the pathways involved in fMLP- and PAF-stimulated arachidonic acid release are mediated by PKC isoforms that are not inhibited by staurosporine. Another explanation would be that stimulated arachidonic acid release is mediated by a PKC-independent, but sphingosine-sensitive, mechanism through a direct coupling between a PT-sensitive G protein and PLA_2. Further studies are required to discriminate between these hypotheses.

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