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LABORATORY INVESTIGATION

Porins and lipopolysaccharide stimulate platelet activating factor synthesis by human mesangial cells

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Porins and lipopolysaccharide stimulate platelet activating factor synthesis by human mesangial cells. Porins, a family of hydrophobic proteins located in the outer membrane of the cell wall of gram-negative bacteria and lipopolysaccharide (LPS), were shown to stimulate the synthesis of platelet activating factor (PAF), a phospholipid mediator of inflammation and endotoxic shock, by cultured human glomerular mesangial cells (MC). The synthesis of PAF induced by porins was rapid (peak at 20 min) and independent either from contamination by LPS or from generation of an endotoxin-induced cytokine such as tumor necrosis factor (TNF) since it was not prevented by cycloheximide, an inhibitor of protein synthesis or anti-TNF blocking antibodies. LPS also stimulated PAF synthesis by MC. However, the kinetic of PAF synthesis induced by LPS was biphasic with an early and transient peak at 10 minutes and a second and sustained peak at three to six hours. This second peak required an intact protein synthesis and was prevented by anti-TNF antibodies, suggesting the dependency on LPS-induced synthesis of TNF. Experiments with labeled precursors demonstrated that in MC, either after stimulation with porins or LPS, PAF was synthetized via the remodeling pathway that involves acetylation of 1-O-alkyl-sn-glyceryl-3-phosphorylcholine (2-lyso-PAF) generated from 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine by phospholipase A2 (PLA2) activity. Porins and LPS, indeed, induced PLA2-dependent mobilization of [^{3}H]-arachidonic acid that was inhibited by p-bromodiphenacylhemisuccinate (PBHDB). PBHDB, an inhibitor of PLA2, also blocked PAF synthesis by preventing the mobilization of 2-lyso-PAF, the substrate for PAF-specific acetyltransferase. The addition of 2-lyso-PAF restored PAF synthesis. The activity of acetyl CoA 2-lyso-PAF acetyltransferase was increased in porin- as well as in LPS-stimulated MC and, after cell preincubation with [^{3}H]-acetyl CoA, [^{3}H]-acetyl was incorporated in the newly synthetised PAF. The activation of PAF synthesis by porins was dependent on extracellular Ca^{2+}. Porins by forming trans-membrane channels determined a sustained influx of $^{45}Ca^{2+}$ into the cytosol. The inhibitory effect of trifluoperazine, an inhibitor of Ca^{2+}-calmodulin complexes, on PAF synthesis by porin-stimulated MC suggested that calmodulin mediated the Ca^{2+}-dependent activation of enzymes involved in PAF synthesis.

Platelet activating factor (PAF), a 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, is a mediator of endotoxin-induced inflammation and shock [1–4]. PAF is synthesized by polymorphonuclear neutrophils (PMN), monocytes/macrophages, basophils, platelets and vascular endothelial cells [5] after appropriate stimulation. Recently, rat and human mesangial cells (MC) have been found to be able to produce PAF and to be sensitive to its action [6–10]. It was demonstrated that cultured rat MC exhibit specific receptors for IgG and the occupancy of Fc receptors results in endocytosis associated with the generation of PAF [11]. Therefore, MC seem to behave as macrophages that synthesized PAF during phagocytosis [12]. PAF may be synthesized, either via the remodeling pathway, through acetylation of 2-lyso-PAF generated from 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine by phospholipase A2 (PLA2) activity, or via the *de novo* biosynthetic pathway that involves the synthesis of 1-O-alkyl-2-acetyl-sn-glycerol, which is then converted to PAF by a unique CDP-choline:1-alkyl-2-acetyl-sn-glycerol cholinephosphotransferase [13]. In human MC phagocytosis induces PAF synthesis mainly via the remodeling pathway [14].

The synthesis of PAF in endotoxin-induced inflammation and shock may be triggered either by endotoxin itself or by endotoxin-induced cytokines. Indeed, TNF, a cytokine primarily involved in endotoxin-induced inflammation and shock [15], stimulates PAF synthesis by activating the remodeling pathway in PMN, macrophages, endothelial and MC [14, 16]. In addition, endotoxin was found to be able to stimulate PAF synthesis by cultured rat MC [9]. In human MC, it remains to be determined whether the synthesis of PAF is directly stimulated by endotoxin or whether it is related to the autocrine effect of endotoxin-induced cytokines. Moreover, it is at present unknown whether other bacterial components can stimulate the synthesis of PAF by MC.

In the present investigation, we compared the effect of porins on the biosynthesis of PAF by cultured human MC with that of bacterial lipopolysaccharide (LPS). Porins are a family of hydrophobic proteins located in the outer membranes of the gram-negative bacteria cell-wall that form transmembranic channels (pores) for passive diffusion of small solutes [17]. Porins may also play a role in bacterial virulence due to their ability to bind the membrane of nucleated cells and to interfere with their functions [18].

The results obtained show that porins stimulate the synthesis of PAF from human MC in a manner distinct from that of LPS. Porins directly stimulate the MC by a mechanism that involves

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the influx of extracellular Ca^{2+} and the activation of the enzymes involved in the remodeling pathway. LPS activates a dual mechanism resulting in an early but transient direct activation of PAF synthesis, followed by a delayed and sustained synthesis dependent mainly on an autocrine action of TNF.

The effect of bacterial components on PAF synthesis by MC may be of significance for the pathogenesis of acute renal injury occurring during gram-negative sepsis.

Methods

Materials

Human recombinant tumor necrosis factor (TNF) and anti-human TNF antibodies were a gift of Dr. Baglioni (State University of New York at Albany, USA). 2 μg/ml of anti-human TNF antibodies were shown to prevent the cytotoxic activity of 10 ng/ml TNF on SK-MEL 109 melanoma cells tested using the method described by Ruggiero et al [19]. SDZ 63675 (Sandoz Research Institute, East Hanover, New Jersey, USA), WEB 2170 (Boehringer Ingelheim KG, Mannheim, Germany), and CV 3988 (Takeda Chemical Industries, Osaka, Japan) were used as specific PAF receptor antagonists. Polymyxin B, phospholipase A2, lipase A1, bovine serum albumin (BSA) fraction V (tested for not more than 1 ng endotoxin per mg), acetyl-coenzyme A (acetyl-CoA), p-bromodiphenacyl bromide (PBDB), sphingomyelin and lyso-2-phosphatidylcholine (lyso PC), and fluorescein-labeled phalloidin (F-PHD) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). PAF and 1-O-octadecyl-2-lyso-glycero-3-phosphocholine (lyso-PAF), phosphorylcholine (PC) and phosphatidylethanolamine (PE) were from Bachem Feinchemikalien (Bubendorf, Switzerland). [^{3}H]acetyl CoA (2 Ci/mmol), [methyl-^{3}H]-choline (75 Ci/mmol) and [^{14}C]arachidonic acid (55.5 mCi/mmol) were purchased from Amity, Milano, Italy. ^{45}Ca (30 mCi/mg calcium) was obtained from Amersham (Bucks, United Kingdom). Collagenase from *C. histolyticum* was from Boehringer; human factor VIII antiserum was from Nordic Immunology (Tilburg, The Netherlands); mouse monoclonal anti-HLA-DR and anti-leukocyte common antigen (CD45) antibodies were purchased from Becton-Dickinson (Milano, Italy); anti-smooth muscle cell myosin antibodies were from Immunotech (Marseille, France); mouse monoclonal anti-cytokeratin antibodies, anti-collagen type IV antibodies and anti-fibronectin were from Labometrics (Milano, Italy).

Preparation of porins

A strain of *Salmonella typhimurium* SH5014, provided by Nurminen (Central Public Health Laboratory, Helsinki, Finland), was used as the source of porins. Porins extracted by the method of Nurminen [20] were further purified by means of gel filtration in the presence of detergent (SDS) [21] to remove LPS contamination. As previously described [18], the SDS-polyacrylamide 12% gel electrophoresis of purified porins showed two bands with molecular weights of 34 kD and 36 kD in samples heated for five minutes at 100°C in SDS. LPS contamination in the final preparations of porins was only 10 pg of LPS, detected by Limulus assay, per 100 μg of porins. In selected experiments, porins were incubated with 5 μg/ml polymyxin B at room temperature for one hour to neutralize the biological activity of the traces of contaminating LPS as described by Blanchard et al [22].

LPS from *E. coli* 0111:B4 was purchased by Sigma Chemical Co., and LPS from *Salmonella typhimurium* was purchased from Difco Laboratories (Detroit, Michigan, USA). Stock solutions of LPS were prepared by suspending 10 mg of LPS in 2 ml 20 mM EDTA and sonicating until clarified (3 to 5 20-second bursts at maximum intensity using a W375 sonicator with a No. 419 microtip, Heat Systems-Ultrasonics, Farmingdale, New York). Aliquots of LPS stocks (200 μl) were stored at −20°C, and when thawed for use were sonicated for 15 seconds using a microsonicator (Microson, Heat Systems Ultrasonics). LPS working dilutions were prepared in 10 mM Hepes saline formulated using 1 M Hepes stock (Gibco Laboratories, Grand Island, New York) and sterile, non-pyrogenic saline.

Culture of human mesangial cells

Human glomeruli were isolated from surgical specimens of kidneys by the method described by Striker et al [23]. The separated cortex was sliced and forced through a graded series of stainless steel meshes, and isolated encapsulated glomeruli were recovered. MC were obtained from collagenase treated isolated glomeruli in order to remove the epithelial cell component [23]. Washed glomerular remnants were plated at a density of about 300 glomeruli/cm^2 in Dulbecco's modified Eagle's Medium and 20% fetal calf serum tested for endotoxin level less than 0.1 ng/ml (Sigma Chimica, Milano, Italy), 50 U/ml of penicillin and 50 μg/ml of streptomycin; culture flasks were kept in a 95% air–5% CO_2 environment at 37°C. After three weeks of primary culture, MC were harvested with 0.05% trypsin, 0.02% ethylene-diamine-tetraacetate (EDTA). Subcultures were grown in the same medium. The MC used were characterized by the following criteria [24]: (a) morphologic appearance of stellate cells growing in interwoven bundles; (b) uniform fluorescence with F-PHD specific for f-actin; (c) immunofluorescence staining for smooth muscle-type myosin; (d) immunofluorescence staining of extracellular matrix for type IV collagen and fibronectin using monospecific antisera; and (e) negative immunofluorescence staining for HLA-DR and leukocyte common antigen (CD-45) and human factor VIII antigens. In parallel experiments, cell viability was monitored by Trypan blue and ranged between 88% and 95%.

Experimental protocols

For PAF generation, MC were grown to confluence in six-well plates (Costar, Cambridge, Massachusetts, USA), washed three times with Tris-buffered Tyrode (2.6 mM KCl, 1 mM $MgCl_2$, 137 mM NaCl, 6 mM $CaCl_2$, 0.1% glucose, 1 mM Tris, pH 7.4) and equilibrated for 15 minutes in Tris-buffered Tyrode containing 0.25% delipidized BSA (fraction V) as previously described [14].

10^6 MC were incubated at 37°C for the indicated time with different stimuli: 0.1 to 20 μg/ml of porins or polymyxin B-treated porins or 10 μg/ml LPS. In selected experiments, MC (10^6) were preincubated with 1 μM PBDB in the absence or presence of 100 μM lyso-PAF, or with 100 μM lyso-PAF alone, for 30 minutes at 37°C and then treated with porins or with LPS.

After incubation of MC with porins or LPS, the supernatants were removed and cells adherent to plastic dishes were washed

in 1% BSA and scraped. Each individual experiment was performed in triplicate. In some experiments, MC were preincubated for 30 minutes with 30 μCi [^{3}H]-acetyl CoA or with 2.5 μCi [methyl-^{3}H]-choline. The supernatants and the cell pellets were extracted according to Bligh and Dyer [25]. PAF was quantitated after extraction and purification by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) by aggregation of washed rabbit platelets as previously reported [24]. In studies with labeled precursors of PAF, 0.5 cm sections of TLC plates were scraped and counted. The biologically-active material extracted from cells and supernatants in different experiments was characterized by comparison of physico chemical and biological characteristics with those of synthetic PAF as previously described [14]. In addition, the chemical identity with the synthetic PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) evaluated by a newly developed technique based on high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [26]. An API III (Perkin Elmer SCIEX) mass spectrometer with an ionspray articulated source, interfaced to a syringe pump HPLC (Applied Biosystems 140A) was used. Briefly each sample purified by TLC was resuspended in 60 μl of acetonitrile/water (90:10 vol/vol) with 0.1% trifluoroacetic acid (TFA). Fifty microliters of sample were injected in HPLC on a reverse phase butyl wide pore column (Hypersil WP-Butyl 100 × 1 mm Shandon) using a mobile phase gradient composed by (A) methanol with 0.1% TFA and (B) water with 0.1% TFA. The concentration of A was increased from 30% to 80% in 20 minutes, with a linear gradient, followed by an isocratic elution at 80% of A for 15 minutes at a flow rate of 50 μl/min.

Mass spectrometry analysis were performed under MS/MS conditions. Daughter ions, in the range of 40 to 500 m/z, were acquired in positive mode from parents of 524 m/z, that correspond to the protonated molecular ion of C16-PAF. Fragmentation was obtained by collision with argon at a collision gas thickness of 2.5×10^{15} atoms/cm and at an impact energy of 70 eV. Standard C16-PAF was purified and analyzed with the same technique.

The release of cytosolic lactate dehydrogenase activity (LDH) was determined by a spectrometric method. LDH release was expressed as the percentage of the total cellular content which was determined by treating MC with 0.01% Triton X-100 for one hour at room temperature.

The role of extracellular Ca^{2+} in porin-induced PAF synthesis was evaluated on MC incubated in a balanced salt solution (SSS: 0.159 M NaCl, 6.0 mM KCl, 0.8 mM $MgSO_4$, 11.1 mM glucose, 0.25% BSA, pH 7.4) in the absence or in the presence of various concentrations of $CaCl_2$ as described by Ludwig et al [27]. In some experiments EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol bis(beta-aminoethylether)-N,N'-tetraacetic acid) was added (final concentration, 10 mM) to MC incubated in Ca^{2+} containing Tyrode's buffer 10 minutes prior to porin addition. The extracellular Ca^{2+} uptake by porin-stimulated MC was evaluated using ^{45}Ca [28]. A total of 10^6 cells incubated in the balanced salt solution without $CaCl_2$ and containing 0.2 mM EGTA were challenged with porins. The reaction was started by adding 2 mM $CaCl_2$, 0.5 μCi ^{45}Ca, and porins. Termination was achieved by adding 2.5 mM EGTA and 5 μM ruthenium red (final concentration) on ice to remove cell surface-bound calcium. After washing, the cell layer was solubilized with 0.5 ml formic acid, and the radioactivity was counted in the presence of 4 ml Instagel. Correction for entrapment was made by using [^{3}H]inulin (1 μCi/40^6 MC); 0.014 ± 0.002 nl [^{3}H]inulin remained associated with stimulated and unstimulated MC.

Enzymatic assays

For the measurement of acetyltransferase activity, cell extract preparation and the enzyme assay were previously described [24]. Acetyltransferase activity was expressed as nmoles of acetyl-CoA incorporated into lyso-PAF molecule per minute of incubation and mg of protein in the lysate. The release of [^{3}H]arachidonic acid from cellular lipids, mainly phospholipids, was measured by a modification [14] of the method described by Hirata et al [29]. The release was evaluated after challenge with different stimuli at 37°C as described for the release of PAF.

Statistical analysis

Data within different experimental groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's or Newman-Keuls multiple comparison test where appropriate. Values are given as mean ± 1 SD. Values of $P < 0.05$ were considered statistically significant.

Results

The experiments shown in Figure 1 established that human MC synthesize PAF in response to porins. Porin-stimulated MC produced PAF in amounts comparable to that obtained after stimulation with TNF or LPS from *S. typhimurium* and ~ 50% of the amount produced after stimulation with LPS from *E. coli* 0111: B4) for three hours. PAF synthesized after stimulation with porins as well as TNF remained mainly associated with cells. Stimulation with LPS from *E. coli* also induced the release in the supernatant of about 20% of the total amount of PAF synthesized (Fig. 1). As shown in Figure 2, maximal PAF synthesis occurred 20 minutes after addition of porins and rapidly decreased thereafter being completely exhausted within 60 minutes. The synthesis of PAF induced by porins was more rapid and less sustained than that induced by TNF which peaked at 60 minutes. LPS induced a biphasic synthesis of PAF with an early transient peak at 30 minutes and a delayed and sustained peak between three to six hours. Pretreatment of porins or TNF with polymyxin B did not impair the synthesis of PAF (Table 1). In contrast, pretreatment of LPS with polymyxin B abrogated both the early transient and the delayed sustained synthesis of PAF. Treatment of MC with cycloheximide, an inhibitor of protein synthesis, prevented the delayed and sustained synthesis of PAF induced by LPS, but not the early transient peak of PAF induced by LPS. Cycloheximide did not affected PAF synthesis induced by porins and TNF. Anti-TNF antibodies abrogated the sustained synthesis of PAF induced by LPS, but not the early and transient peak. In contrast, anti-TNF antibodies were completely ineffective on PAF synthesis induced by porins. The synthesis of PAF by MC in response to different doses of porins was also measured (Fig. 3). PAF was extracted from cells and supernatant 20 minutes after porin addition. PAF synthesis was detectable with 5 μg/ml of porins and was maximal with 10 μg/ml. The viability of MC after treatment with the concentrations of agents used was

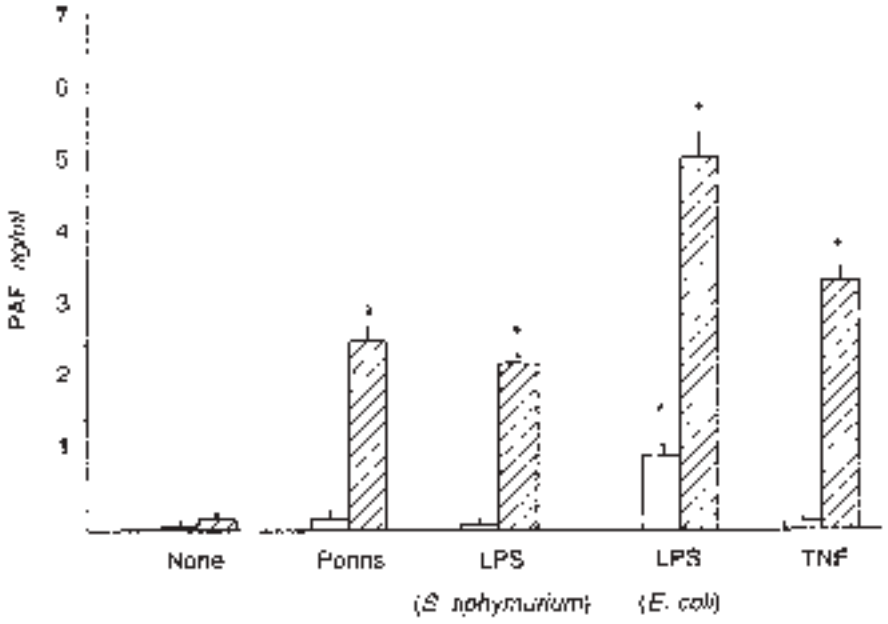

Fig. 1. *Synthesis and release of PAF by 10⁶ human MC treated with 10 μg/ml of porin (20 min) or with 10 μg/ml of LPS from S. typhimurium or E. coli 0111:B4. Then or with 10 ng/ml TNF (1 min) or by control MC incubated at 37°C for the same period of time without any addition (None). Symbols are: blank columns = supernatant PAF; shaded columns = cell-associated PAF. In this figure, PAF concentration is referred to 1 ml of supernatant and to the corresponding cell aliquot to allow comparison of the amount of PAF released with that remaining cell-associated. Vertical bars indicate the standard deviation of the mean of three different experiments performed in duplicate. ANOVA with Dunnett's multiple comparison test was performed between unstimulated cells (None) and cells treated with different stimuli. * $P < 0.05$.*

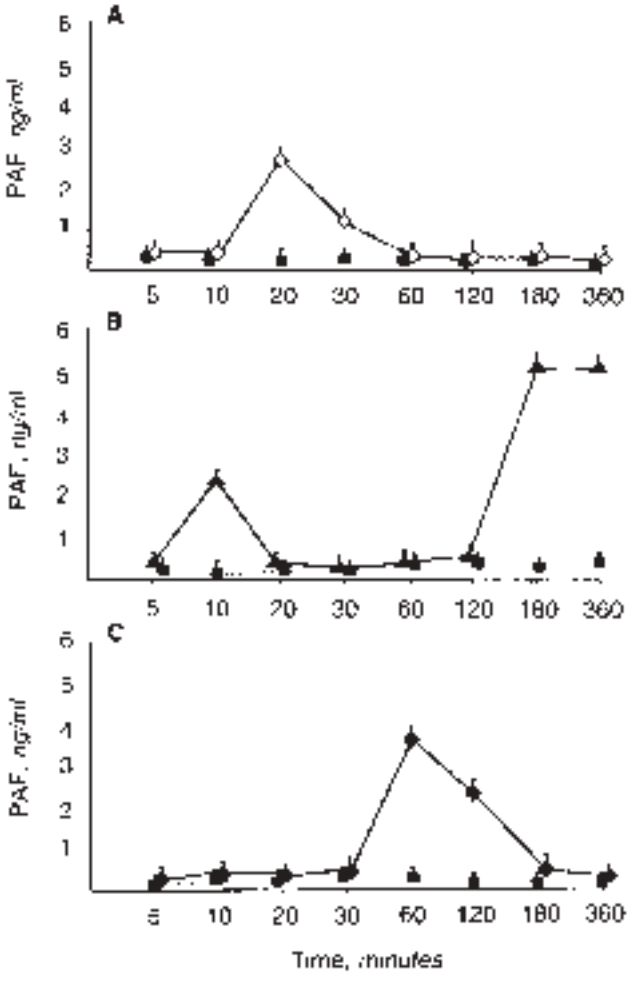

Fig. 2. *Time course of total PAF synthesis by 10⁶ human MC stimulated: A with 10 μg/ml of porins; B with 10 μg/ml of LPS from E. coli (▲—▲); or C with 10 ng/ml of TNF (◆—◆). Unstimulated cells were used as control (■—■). Data are mean ± 1 SD of three experiments.*

always greater than 90% and the release of LDH was <1%. A significant reduction of cell viability was observed for concentrations greater than 50 μg/ml and after 1 hour of treatment.

Characterization of PAF synthesized by MC

PAF was assayed after purification from cell and medium as described in the **Methods** section, and it had biological and physico-chemical characteristics identical to those of synthetic PAF. It induced platelet aggregation in an ADP- and arachidonic acid-independent way, which was specifically inhibited by the PAF receptor antagonists SDZ 64675, WEB 2170 and CV3988. PAF activity was destroyed after base-catalyzed methanolysis (6 to 2% residual activity) or treatment with phospholipase A2 (0 to 5% residual activity), indicating the presence of an ester linkage at sn-2 [30–32]. Treatment with phospholipase A1 did not inhibit PAF activity, suggesting the presence of an ether bond at sn-1 [31]. PAF activity was resistant to treatment with acids or weak bases [30]. After base-catalyzed methanolysis or digestion with phospholipase A2, treatments with acetic anhydride restored 80 to 90% of the biologic activity. The PAF obtained from MC had the same Rf (0.21) on TLC as synthetic PAF and migrated between lyso-PC (Rf = 0.11) and sphingomyelin (Rf = 0.29) using as solvent chloroform/methanol/water (65:35:6), and the same Rt (20 min) of synthetic PAF (μPorasil column) developed with chloroform/methanol/water (60:55:5). No PAF activity was detected in any other TLC or HPLC fraction. The MS/MS spectra obtained from porin- or LPS-stimulated MC samples at the retention time characteristic for C-16 PAF (29.9 to 30.2 min) exhibited a fragmentation pattern characterized by molecular ion (m/z 524) and a fragment corresponding to phosphocholine (m/z 183). Figure 4 shows typical spectra of porin (A) or LPS (B) stimulated MC sample containing 2.5 ng/ml and 3.4 ng/ml, respectively of PAF like bioactivity. An identical spectrum was obtained with synthetic C-16 PAF submitted to the same extraction and purification procedures of the samples (Fig. 4C).

Table 1. Synthesis of cell-associated PAF by human MC stimulated with porins, LPS or TNF in presence or absence of different additions

Additions	Stimulation with porins	Stimulation with LPS		Stimulation with TNF
		10 min	5 h	
None	2.65 ± 0.3	4.80 ± 0.2	5.06 ± 0.1	5.40 ± 0.2
Polymyxin B	2.54 ± 0.4	0.20 ± 0.1*	0.20 ± 0.1*	5.17 ± 0.3
Cycloheximide	2.68 ± 0.2	3.85 ± 0.3	0.20 ± 0.2*	3.21 ± 0.2
Anti-TNF antibodies	2.48 ± 0.3	4.02 ± 0.2	0.30 ± 0.2*	0.7 ± 0.2*

A total of 10^6 human MC were incubated with 10 μg/ml porins for 20 minutes or 10 μg/ml LPS from *E. coli* 0111:B4 for 10 minutes or 5 hours or with 10 ng/ml TNF for 1 hour or with the same stimuli pretreated with 5 μg/ml polymyxin B as described in the Methods section. When indicated, MC were stimulated in the presence of 0.1 mg/ml cycloheximide or 2 μg/ml of anti-TNF antibodies. Values are expressed as mean ± 1 SD of four different experiments. ANOVA with Dunnett's multiple comparison test was performed between control MC stimulated with porins, LPS or TNF in the absence of any addition (None) and experimental groups.

* $P < 0.05$

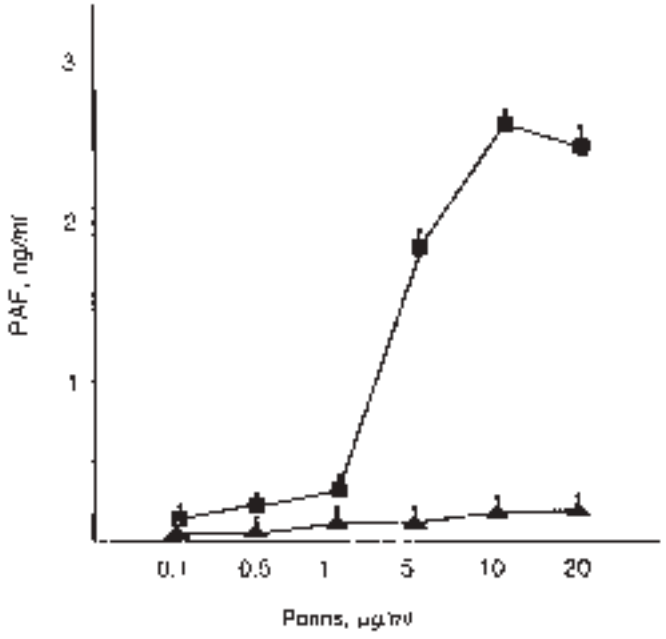

Fig. 3. *Dose response of PAF synthesis and release by human MC stimulated for 20 minutes with porins.* The cell-associated PAF (■) and that released into the supernatant (▲) by 1×10^6 cells treated with different concentrations of porins are shown. Data are mean ± 1 SD of three experiments.

Precursors and enzymes involved in PAF synthesis

Evidence that PAF was newly synthesized by MC treated with porins or LPS was obtained by following the incorporation of radioactive precursors. TLC analysis of lipid fractions extracted 20 minutes after addition of porins from MC preincubated with [^{3}H]-acetyl-CoA showed one main peak of radioactivity that comigrated with synthetic PAF (Fig. 5). This peak was absent in the lipid fractions extracted from untreated MC. Another set of experiments showed that exogenous 2-lyso-PAF may serve as substrate for porin-induced PAF synthesis. TLC analysis of the lipid fraction of MC preincubated with [^{3}H]2-lyso-PAF plus 0.1 mM unlabeled acetyl-CoA and then treated with porins for 20 minutes showed three peaks of radioactivity (Fig. 5). The first peak comigrated with 2-lyso-PAF, the second peak with PAF, and the third peak with L-2-phosphatidylcholine (PC). In contrast, preincubation of MC with [methyl-^{3}H]-choline did not result in its incorporation in PAF molecules synthesized either after stimulation with porins or with LPS (data not shown). These results indicate that in MC the synthesis of PAF induced by porins and LPS occurs via the remodeling pathway rather than the *de novo* pathway, as previously observed for TNF and phagocytosis. The activation of PLA2 is the first step involved in PAF biosynthesis via the remodeling pathway. As shown in Table 2, MC released [^{3}C]-arachidonic acid after stimulation with porins in amounts comparable to those observed after stimulation with LPS from *E. coli* 0111:B4. The basal level of [^{3}C]-arachidonic acid released by control cells was 0.48% of the label incorporated during preincubation. After stimulation, MC released 2.5 to 4.6% of the total label incorporated. PBDB, an inhibitor of PLA2 [33], markedly reduced the release of [^{3}C] arachidonic acid as well as the synthesis of PAF by porin- or LPS-stimulated MC (Table 2). The inhibition of PLA2 by PBDB possibly prevented the mobilization of 2-lyso-PAF, the substrate for the acetyl CoA: 2 lyso PAF acetyltransferase. The second step involved in PAF synthesis by the remodeling pathway is, in fact, the acetylation of 2-lyso-PAF generated from membrane 1-*O*-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine by PLA2. Indeed, the addition of 2-lyso-PAF to MC pretreated with PBDB restored the synthesis of PAF induced by porins or LPS (Table 2). The activity of acetyl CoA:2 lyso-PAF acetyltransferase was studied in parallel with the synthesis of PAF. In unstimulated MC, basal activity was 0.4 ± 0.1 nmol/min/mg protein. This enzymatic activity increased about seven- to ninefold during the synthesis of PAF after stimulation with porins or LPS. The activation of this enzyme paralleled the synthesis of PAF (Table 2) for both porin and LPS MC stimulation.

Role of extracellular calcium in porin-induced PAF synthesis

In experiments designed to evaluate the role of extracellular divalent cations on the synthesis of PAF, 10 mM EDTA or EGTA was added to MC 10 minutes before stimulation with porins (10 μg/ml) at 37°C for 20 minutes. The chelation of extracellular divalent cations inhibited the synthesis of PAF (control porin-stimulated cells: 2.65 ± 0.3 ng/ml; EDTA-treated cells: 0.10 ± 0.1 ng/ml; EGTA-treated cells: 0.7 ± 0.1 ng/ml). The addition of trifluoroperazine (10 μM), a drug that binds to Ca^{2+}-calmodulin complex blocking its action on target enzymes [34], five minutes before stimulation with porins also inhibited the production of PAF (0.30 ± 0.2 ng/ml).

Figure 6 shows the dose-response effect of extracellular Ca^{++} on PAF synthesis by MC incubated in calcium-free balanced

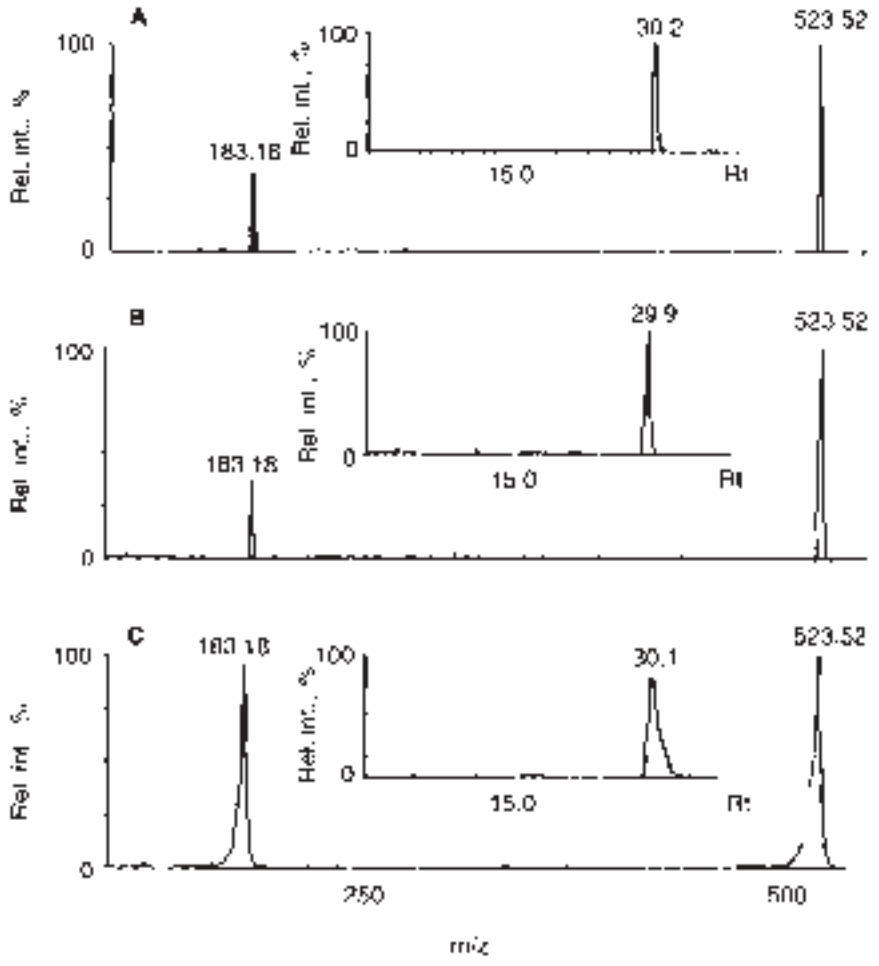

Fig. 4. *Spectra of the daughter ions from parents with m/z 524 of a representative sample of 10^6 MC treated with 30 μg/ml of porins for 20 minutes (A) or with 30 ng/ml of LPS from E. coli for three hours (B) and of synthetic C16-PAF used as standard (C).* In the insets, the reconstructed chromatograms of the daughter ions with m/z 183 from parents of m/z 524 of the corresponding samples are shown. The fragmentation spectra are identical in A, B and C. Only one peak can be observed in each chromatogram with superimposable retention time

salt solution (Methods). The different concentrations of Ca^{2+} were added to MC 10 minutes before stimulation with porins. PAF synthesis, detected as cell-associated PAF only, started at concentrations of 0.01 mM Ca^{2+}. As shown in the experiments with $^{45}Ca^{2+}$, porins rapidly increased the passage of Ca^{2+} from the extracellular to the intracellular compartment. This effect was sustained up to 30 minutes after porin addition (Fig. 7).

Discussion

The glomerular MC, a type of modified smooth muscle cell that contracts in response to a number of mediators, may be implicated in the endotoxin-induced acute hemodynamic renal insufficiency characterized by a greater reduction in glomerular filtration than in renal plasma flow [35]. MC react not only to vasoactive agents, but also to a variety of inflammation mediators, including cytokines, eicosanoids, and PAF [36]. Gram-negative sepsis causes exaggerated synthesis and release of a number of mediators which may contribute to the pathogenesis of acute renal failure and shock. The relative pathogenetic importance of the biological action of components of the cell wall of gram-negative bacteria and of mediators produced by the host, such as IL-1 and TNF, remains unknown.

TNF, in particular, is considered the main mediator of endotoxin-induced inflammation and shock [37]. This cytokine stimulates macrophages, PMN and vascular endothelial cells to produce and release PAF [16]. In addition, it was recently shown that cultured rat MC stimulated with LPS [9] and human MC stimulated with TNF synthesized PAF [34]. Since PAF, which contracts MC [7], may function as an autacoid that acts near its site of production, one may postulate that PAF, synthesized within the glomerulus produces renal functional alterations in endotoxin/septic shock. Indeed, PAF is produced during endotoxic shock and experimental sepsis by gram-negative bacteria [2–4, 38]. PAF infusion in experimental animals produces hypotension, decrease in cardiac output, hypovolemic shock [39], and mimics the renal hemodynamic effects of endotoxin [35]. PAF receptor antagonists inhibit and reverse endotoxin-induced hypotension in rats and significantly reduce mortality [2, 3]. In addition, PAF-receptor blockade prevents the endotoxin-induced acute hemodynamic renal insufficiency [35].

In endotoxic/septic shock, the synthesis of PAF may be triggered either directly by biologically-active bacterial components or toxins or by cytokines produced by the host. The LPS, indeed, directly stimulates monocytes, macrophages and probably PMN to synthesize PAF [40]. Beside LPS, the outer membrane layer of gram-negative bacteria cell wall particularly contains hydrophobic proteins [41] that are called porins because they form trans membrane channels for passive diffusion of solutes across the outer membrane [17]. Porins that in the membrane

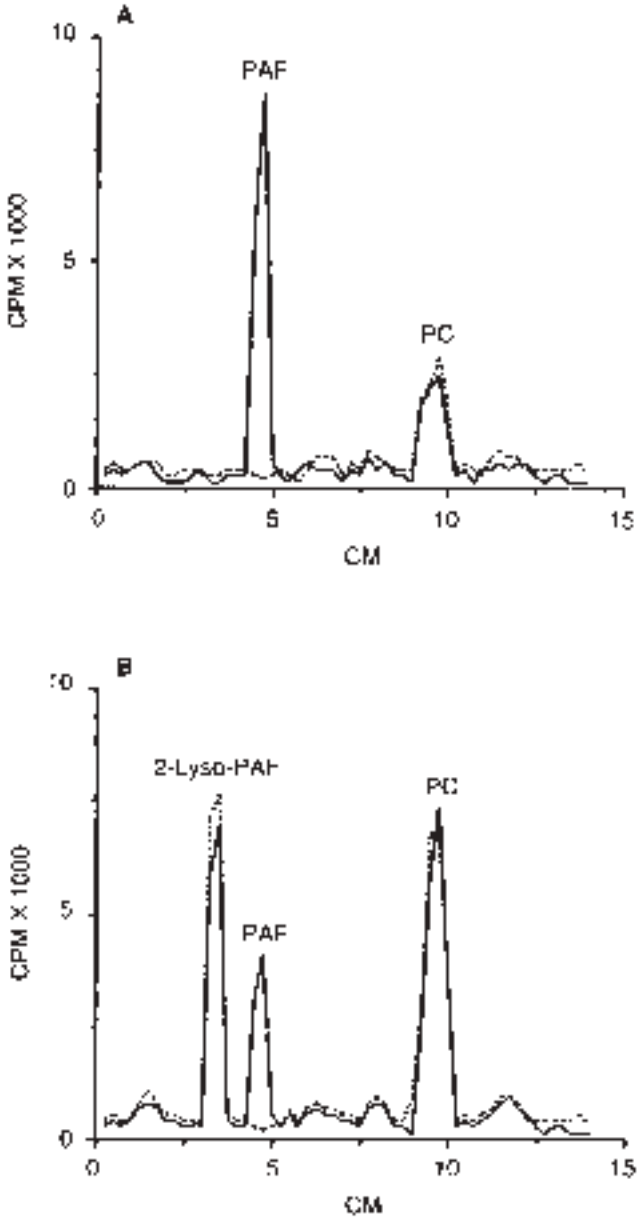

Fig. 5. *Representative TLC analysis of PAF synthesized by human MC stimulated for 20 minutes with 10 ng/ml of porin after 16 minutes preincubation with 15 μCi/ml [³H]-acetyl-CoA (A) or with 0.1 mM unlabeled acetyl-CoA and 2 μCi/ml [³H]-lyso-PAF (B).* The lipids extracted from cells and supernatants were analyzed by TLC using as solvent chloroform/methanol/acetic acid/water (50:25:8:4, vol/vol). The plates were divided in 0.5 cm sections and counted as described in the Methods section. The pattern obtained with porin-stimulated MC (line) and control unstimulated MC (dotted line) is shown. Three experiments were performed with similar results on cell associated and supernatant lipids.

are in trimeric form [42] may be released either during cell growth or during bacteriolysis [43]. Given their resistance to proteolysis [44], they are not degraded and may insert in the plasma-membrane of the cells of the host organism with marked changes in lipidic and protein phase relations [18]. Depending on the dose, porins may be cytotoxic for target cells or may interfere with cell functions [45]. Porins binding to human PMN were shown to reduce phagocytosis and intracellular killing of gram-negative bacteria [45] and to decrease the oxidative burst and the cell hydrophobicity causing alterations in cell morphology [18]. Moreover, subtoxic concentrations of porins act both as chemotaxins and chemotaxinogens on PMN [18]. Porins, once injected in the rat paw, were shown to induce an inflammatory reaction which was independent from the activation of complement [46], despite their ability to activate the complement system in vitro [47].

In the present study, we demonstrate that porins at non-toxic concentrations stimulate a transient rapid synthesis of PAF from human MC. This effect of porins does not depend on LPS contamination or TNF production. In fact, the treatment of porins with polymyxin B that abrogates the biological activity of LPS and incubation of MC with anti-TNF antibodies did not affect the synthesis of PAF induced by porins. In addition, the kinetic action of PAF synthesis induced by LPS differs from that of porins. After LPS stimulation a biphasic response was observed with a first transient peak of PAF synthesis at 10 minutes and a second delayed and sustained peak between three and 6 hours. Protein synthesis blockade by cycloheximide did not abrogate either PAF synthesis induced by porins or by TNF, or the early transient synthesis of PAF induced by LPS. In contrast, the delayed and sustained synthesis of PAF observed after incubation with LPS at three to six hours was dependent on protein synthesis and was inhibited by anti-TNF antibodies, suggesting the dependency of PAF synthesis on the production of TNF.

Therefore, human MC may synthesize PAF with a dual mechanism, a direct mechanism that triggers a rapid but transient synthesis of PAF also described in rat MC [9], and a cytokine-dependent mechanism that seems related to the LPS stimulated synthesis of TNF. The experiments with labeled precursors indicate that MC synthesized PAF both in response to porins and LPS via the remodeling pathway, as previously observed for stimulation with IL-1, TNF and phagocytosis [14]. The remodeling pathway involves the activation of two enzymatic steps, including the hydrolysis of 2-lyso-PAF by PLA2 and its acetylation at position-2, by a specific acetyltransferase. The first step requires Ca^{2+}, is inhibited by PBDB and is associated with the mobilization of arachidonic acid. The second step is detected by the incorporation of labeled acetate into PAF synthesized by cells preincubated with labeled acetyl-CoA.

The PLA2 activity in intact cells was estimated by measuring the release of label from MC preincubated with [^{14}C]arachidonic acid [14, 29]. Both porins and LPS induced the release of [^{14}C]-arachidonic acid. The release of arachidonic acid from cellular phospholipids may also depend on the phospholipase-C/diacylglycerol lipase pathways. Since 1,2 [^{3}H]-arachidonoyl diacylglycerol formation was not measured in the present study, a concomitant activation of this pathway cannot be excluded. However, the inhibitory effect of PBDB that prevents PLA2 activity [33] suggests the prevalence of a direct deacylation of phospholipids by PLA2. The inhibition of PLA2 by PBDB also blocks the synthesis of PAF by preventing the mobilization of 2-lyso-PAF, the substrate for PAF-specific acetyl-CoA:2-lyso-PAF acetyltransferase. Indeed, the addition of exogenous 2-lyso-PAF restores the synthesis of PAF in porin- as well as LPS-stimulated MC. The activation of the

Table 2. Release of ^{14}C-arachidonic acid, acetyl-CoA:2-lyso-PAF acetyltransferase activity and PAF synthesis by human MC stimulated by porins or LPS

Additions	^{14}C-arachidonic acid released c.p.m.	Acetyl-transferase activity nmol/min/mg of protein	PAF synthesis ng/ml
None	532 ± 99	0.48 ± 0.1*	0.10 ± 0.1
Porins	2890 ± 44*	3.17 ± 0.4*	2.65 ± 0.3*
LPS	4958 ± 76*	4.93 ± 0.2*	5.06 ± 0.4*
Porins plus PBDB	920 ± 68†	1.41 ± 0.3	0.30 ± 0.2†
LPS plus PBDB	1240 ± 52†	1.52 ± 0.4	0.90 ± 0.1†
Porins plus PBDB plus Lyso-PAF	914 ± 142‡	ND	2.46 ± 0.3‡
LPS plus PBDB plus Lyso-PAF	1142 ± 93‡	ND	4.90 ± 0.1‡

A total of 10^6 MC were incubated at 37°C with 10 μg/ml porins for 20 minutes or 10 μg/ml LPS for 3 hours with or without addition of PBDB (1 μM) or PBDB (1 μM) plus 2-lyso-PAF (10 μM). The release of [^{14}C]-arachidonic acid was performed as described by Bonsib et al [29]. The acetyl-transferase activity and the synthesis of PAF was measured as described in the **Methods** section. ANOVA with Newman-Keuls multiple comparison test was performed between unstimulated cells and cells stimulated with porins or LPS in the absence of additions (* $P < 0.05$); between porin- or LPS-treated cells in the absence of additions and after addition of PBDB († $P < 0.05$) or PBDB plus 2-lyso-PAF (‡ $P < 0.05$) and between porin- or LPS-treated cells in presence of PBDB and after addition of PBDB plus 2-lyso-PAF (‡ $P < 0.05$).

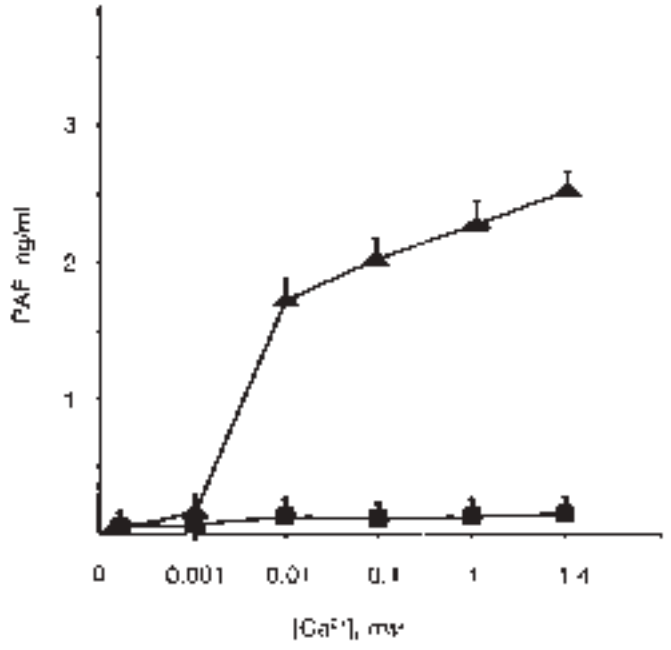

Fig. 6. *Effect of extracellular Ca^{++} concentrations on PAF synthesis by MC. Human MC incubated in calcium free balanced salt solution (Methods) and stimulated for 20 minutes with 10 μg/ml porins (■) or unstimulated (●). Calcium in the indicated concentrations was added 10 minutes before stimulation with porins. Data are mean ± SD of three experiments.*

remodeling pathway is also indicated by the incorporation of the label in PAF synthesized after preincubation with [^{3}H]-acetyl-CoA but not with [methyl-^{3}H]-choline. Porins and LPS, in addition, increase the activity of acetyl CoA:2-lyso-PAF acetyltransferase by about seven- to nineteenfold. We focused on the study of the mechanism of porin-induced PAF synthesis by evaluating the role of influx of extracellular Ca^{++}. Previous studies established that mobilization of intracellular Ca^{++} plays only a minor role in the induction of PAF synthesis, whereas the activation of enzymes involved in the remodeling pathway mainly requires an influx of extracellular Ca^{++} [27]. The studies on $^{45}Ca^{++}$ influx in porin-stimulated MC indicate that porins determine a sustained increase in membrane permeability to Ca^{++}. This observation may be explained by the binding of porins to MC membranes with formation of trans-membrane channels for passive diffusion of small solutes [18]. Since the concentration of free Ca^{++} in the cytosol is about 10^4 times lower than that of extracellular fluid, a large gradient tends to drive Ca^{++} into cytosol across porin-formed trans-membrane channels. The experiments with trifluoperazine, an inhibitor of Ca^{++}-calmodulin complexes, suggest that calmodulin regulates Ca^{++} dependent activation of enzymes involved in the synthesis of PAF by porin-stimulated MC, as observed for PMN stimulated with calcium ionophore [34]. Both the PLA2 and the acetyltransferase involved in PAF synthesis are calcium-dependent enzymes [48]. The PLA2 involved in the synthesis of PAF seems to cleave selectively arachidonic acid and translocate to the membranes in a Ca^{++}-dependent fashion [49]. Recently described was a cytosolic PLA2 that contains a 45 amino acid region with homology to protein kinase C, synaptic vesicle protein p65, GTPase activating protein, and phospholipase C, suggesting a Ca^{++}-dependent phospholipid-binding motif as mechanism for the second messenger Ca^{++} to translocate and activate cytosolic proteins [50]. The activation of PAF-specific acetyltransferase besides Ca^{++} seems to require phosphorylation [51].

The present demonstration, that besides LPS and cytokines other bacterial components directly promote the synthesis of PAF by MC, suggests that a number of stimuli may contribute, either independently or synergistically, to the stimulation of MC in gram-negative bacterial infections. The synthesis of PAF by MC may be relevant for the pathogenesis of acute renal injury related to gram-negative bacterial infections. However, the exact relationship between MC-derived PAF and other mediators, such as eicosanoids and cytokines, appears worthy of further study.

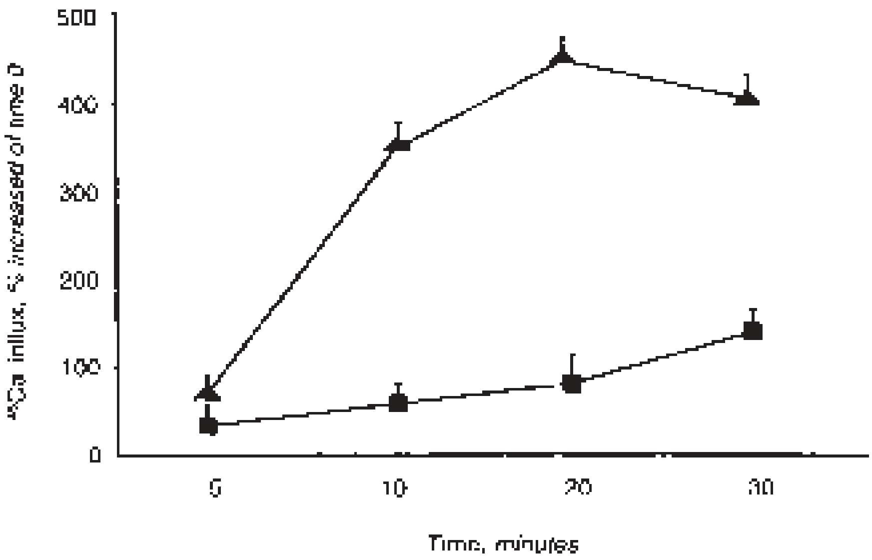

Fig. 7. *Time course of ^{45}Ca uptake by 10^{6} human MC unstimulated (■) or stimulated with 10 µg/ml porins (▲) at 37°C in balanced salt solution supplemented with 2 mM $CaCl_2$, 0.5 µCi ^{45}Ca.* Results are expressed as mean ± 1 SD of the percent increase of cpm of time 0 from a single typical experiment performed in triplicate. Three experiments were done with similar results.

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