Biochemical and Functional Responses Stimulated by Platelet-activating Factor in Murine Peritoneal Macrophages

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Abstract. Platelet-activating factor (PAF) is a potent stimulant of leukocytes, including macrophages. To analyze the mechanisms of its effects upon macrophages, we determined whether macrophages bear specific surface receptors for PAF. By competitive radioactive binding assays, we determined two classes of specific receptors to be present on purified membranes derived from murine peritoneal macrophages (one having a K_d of $\sim 1 \times 10^{-10}$ M and one a K_d of $\sim 2 \times 10^{-9}$ M). When the macrophages were incubated with PAF, rapid formation of several inositol phosphates including inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate were observed. PAF also elevated intracellular levels of calcium to 290 ± 27% of basal levels which were 82.7 ± 12 nM. Increases in calcium were observed first in submembranous areas of the macrophages. PAF also led to increases of 1,2-diacylglycerol of $\sim$200 pmol/10^7 cells. A characteristic pattern of enhanced protein phosphorylation, similar to that initiated by both phorbol 12,13-myristate and lipopolysaccharide, was observed and involved enhanced phosphorylation of proteins of 28, 33, 67, and 103 kD. The half-maximal dose of PAF for initiating all the above effects was $\sim 5 \times 10^{-9}$ M. PAF also initiated significant chemotaxis of the cells; the half-maximal dose for this effect was $\sim 1 \times 10^{-11}$ M. Taken together, these observations suggest that murine mononuclear phagocytes bear specific membrane receptors for PAF and that addition of PAF leads to generation of breakdown products of polyphosphoinositides, subsequent changes in intracellular calcium and protein phosphorylation, and chemotaxis.

PLATELET-ACTIVATING factor (PAF),[1] a naturally occurring ether lipid which possesses potent biological activities, is released upon stimulation of a wide variety of cells, such as platelets, basophils, macrophages, and neutrophils (8, 18, 47). These and other cells, including endothelial and muscle cells, are responsive to PAF. PAF has been implicated as a mediator of a variety of inflammatory, respiratory, and cardiovascular disorders (for reviews see references 8, 18, 47). In view of the widespread occurrence of macrophages in inflammatory sites and of the emerging role of macrophage–platelet interactions in potentiation of atherogenesis, the interactions of PAF with macrophages may be of considerable consequence to the host.

The molecular mechanisms underlying macrophage regulation by extracellular signals have recently come under considerable scrutiny (3, 17). Excellent models now exist for studying the slow acquisition of function (i.e., development of competence over many hours). By contrast, one of the best models for studies on rapid execution of leukocyte function, the response to N-formylated peptides (35, 38, 44), has not been well characterized in mice (2). Definition of a receptor on murine macrophages leading to the rapid and transient breakdown of polyphosphoinositides would thus be useful (1).

The interactions of PAF with and its effects upon macrophages, however, remain to be explored in depth. PAF can stimulate increased glucose consumption, enhanced synthesis of prostaglandins and thromboxane B_2, and increased release of H_2O_2 in guinea pig macrophages (19, 20, 22). PAF has recently been reported to induce rises in intracellular calcium in murine peritoneal macrophages, although the mechanism of such fluxes was not established (10). A surface receptor for PAF on macrophages has been inferred from studies that compared the structure–function order of a series of PAF-like compounds between macrophages and platelets that bear a defined surface receptor for PAF (22). Taken together, these data raise the possibility that murine macrophages possess a surface receptor for PAF, which can lead to the breakdown of polyphosphoinositides and functional consequences.

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1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DAG, 1,2-diacylglycerol; InsP, myo-inositol phosphate; LPS, lipopolysaccharide; PAF, platelet-activating factor; $PInsP_2$, phosphatidylinositol-4,5-bisphosphate; PMA, phorbol 12,13-myristate acetate; pp, phosphoprotein; TG, thioglycollate.

We report here that murine peritoneal macrophages do have specific membrane binding sites for PAF; that addition of PAF initiates the generation of *myo*-inositol-1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG), rises in intracellular calcium, and a specific pattern of enhanced protein phosphorylation; and that PAF initiates chemotaxis of macrophages.

Materials and Methods

Reagents

PAF was purchased from Sigma Chemical Co., St. Louis, MO or Avanti Polar Lipids, Inc., Birmingham, AL. DAG kinase was from Lipidex Inc. and 1,2 Dioleoylglycerol from Avanti Polar Lipids, Inc. 1-0-[^{3}H]Octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine [^{3}H]PAF was from Amersham Corp., Arlington Heights, IL. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA). All other reagents were obtained as described previously (41).

Cell Culture

Specific pathogen-free, inbred C57Bl/6 mice (6 wk old) were purchased from the Trudeau Institute (Saranac Lake, NY) and from Charles River Breeding Laboratories, Inc., Wilmington, MA. Thioglycollate (TG)-elicited and proteose peptone-elicited macrophages were obtained by peritoneal lavage with 10 ml of Hank's balanced salt solution (HBSS) containing 10 U of heparin/ml as reported previously (41, 49). TG-elicited cell suspensions contained >90% macrophages as determined by differential cell counts. The cell monolayers were routinely found to contain >98% macrophages as determined by morphology with Giemsa stain or histochemical assay for nonspecific esterase (49). Suspension of leukocytes from peptone-treated mice contained fewer macrophages and a greater number of leukocytes.

Analysis of Protein Phosphorylation

For studies of protein phosphorylation, macrophages were plated in MEM containing 5% FBS, 2 mM glutamine, 125 U/ml penicillin, and 6.25 µg/ml streptomycin, at 3 × 10^6 cells/4.5-cm^2 plastic tissue culture wells, incubated for 2-3 h at 37°C in an atmosphere of 5% CO_2, and washed three times with HBSS to remove nonadherent cells (41, 49). Macrophages were then radiolabeled with 200 µCi/ml ^{32}Pi in a modified Thyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM $MgCl_2$, 0.05 mM NaH_2PO_4, 5 mM glucose, 5 mM Hepes, pH 7.4) for 3 h in 5% CO_2 at 37°C. Stimulants were then added for various times. Cultures were washed five times with ice cold Thyrode's buffer, drained, solubilized in electrophoretic sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol), boiled for 2 min, and analyzed on 10% polyacrylamide slab gels according to the method of Laemmli (31). Equal amounts of radioactivity were added to each gel line. Acid-precipitable radioactivity was quantitated by the addition of 50 µl of 5% BSA and 1 ml of 20% TCA to 20-µl aliquots of labeled samples. Precipitated protein was collected on 0.45 µm type HA filters (Millipore Corp., Bedford, MA), washed with 5% TCA, dried, and counted by liquid scintillation spectrometry. Gels were fixed, stained with Coomassie Blue, dried under vacuum, and exposed for autoradiography using Kodak X-Omat RP-5 x-ray film with Dupont Co. (Wilmington, DE) "lightning plus" intensifying screens for appropriate times at −70°C.

Quantification of Inositol Phosphates

For rapid time course of ^{3}H-inositol phosphates production after stimulation of macrophages with PAF, TG-elicited intraperitoneal macrophages from 10-20 mice were pooled and resuspended (50 × 10^6 cells/ml) in inositol-free DME, and labeled with 100 µCi [2-^{3}H]myo-inositol/ml for 3 h, at 37°C in 5% CO_2 with frequent agitation, according to previously described methods (41). Macrophages were washed three times and resuspended in HBSS medium with 10 mM Hepes, pH 7.4, at concentrations of 5-10 × 10^6 cells/ml, and kept at 37°C. 10 min before assay 10 mM LiCl was added. Control samples received saline, while others were stimulated with PAF for the indicated times. 0.2 ml of 60% TCA was added to 1 ml of cell suspension and the mixture was placed on ice. After centrifugation, TCA was removed from the supernatants by four washes with 3 ml of ether.

For other studies macrophages were plated at 12-15 × 10^6 cells/28.2-cm^2 plastic tissue culture wells in RPMI 1640 containing 5% FBS, 2 mM glutamine, 125 U/ml penicillin, and 6.25 µg/ml streptomycin, incubated for 2-3 h at 37°C, and washed three times in 5 ml of HBSS to remove nonadherent cells. Macrophages were then radiolabeled with 10 µCi/ml of [2-^{3}H]-myo-inositol in inositol-free DME for 12 h, at 37°C in 5% CO_2. Cultures were then washed five times with 5 ml of HBSS and 10 mM Hepes, pH 7.4, and bathed in the same buffer supplemented with 10 mM LiCl, for 10 min at 37°C. PAF was then added for the desired period of time, and the reaction stopped with 10% TCA (final concentration). Denatured cultures were then scraped and quantitatively transferred into tubes on ice. After centrifugation, TCA was removed from the supernatants as described above and samples were concentrated by lypholyzation.

The various inositol phosphates were separated using two methods. (*a*) Samples were applied to 1.5-ml columns of AG 1-X8 ion-exchange resin (formate form) (Bio-Rad Laboratories, Richmond, CA) and were sequentially eluted in batch fashion with H_2O, 5 mM tetraborate, and 0.06 M ammonium formate; and three buffers containing 0.1 M formic acid and 0.2, 0.4, and 1.0 M ammonium formate, respectively (7, 41, 46, 48). This method has been shown to separate inositol, glycerophosphoinositol, *myo*-inositol-phosphate (InsP), *myo*-inositol-bisphosphate ($InsP_2$), and $InsP_3$, in that order (7). (*b*) We used an HPLC method described by Irvine et al. (27), using a Partisil-10 SAX (Whatman, Inc., Clifton, NJ) column (The Anspec Company, Inc., Ann Arbor, MI) together with a radial compression separation system silica guard Precolumn (Millipore Corp., Bedford, MA) (41, 46). Samples were loaded onto the column and eluted with a flow rate of 1.4 ml/min and a gradient from H_2O (pump A) to 2 M ammonium formate adjusted to pH 3.7 with orthophosphonic acid (pump B) as follows: 0-5 min 0% B, 28 min 45% B, 38 min 50% B, 40 min 100% B, 60 min 100% B, 62 min 0% B. For either method, the fractions were collected; the ones obtained by the HPLC method were diluted 1:1 with H_2O, and the radioactivity was determined by liquid scintillation counting. The peaks that contained radioactivity were identified using the relative retention times of various inositol phosphates in comparison to the retention time of commercially available [^{3}H]$Ins_{1,4,5}P_3$ (27, 28, 46). In our shallower salt elution profile, *myo*-inositol-1,3,4-trisphosphate ($Ins_{1,3,4}P_3$) does not coelute with [^{32}P]ATP as in the originally described procedure (27). For this reason, we used as standards $Ins_{1,3,4}P_3$ and *myo*-inositol-1,3,4,5-tetrakisphosphate ($Ins_{1,3,4,5}P_4$) prepared from the T cell line, Jurkat (46), a generous gift from Dr. S. Dillon, Duke University Medical Center, Durham, NC.

Quantification of Intracellular Calcium Levels

Measurements of intracellular calcium were performed using the fluorescent indicator Fura 2 (15, 50). For these studies two conditions were used: macrophages in suspension and adherent macrophages. For measuring cytosolic free calcium concentration $[Ca^{2+}]_i$ levels in well-spread adherent macrophages, we used peptone and TG-elicited macrophages and obtained essentially similar data. Uniform labeling of the TG-elicited macrophages was more difficult but could be routinely achieved provided strict adherence to the protocol described below.

When macrophages in suspension (TG-elicited, 5 × 10^6/ml) were used, Fura 2/AM (5 µM) was added at 23°C, and suspensions were kept in the dark in RPMI 1640, 5% FBS, 10 mM Hepes, pH 7.4, medium. After 20 min, cells were washed and resuspended in HBSS containing 10 mM Hepes, pH 7.4, at a concentration of 2.0 × 10^6/ml. Intracellular Ca^{2+} was then measured using the following formula: $[Ca^{2+}]_i = K_d(F - F_{min})/F_{max} - F)$ (9, 15, 39), where F is the measured fluorescence; F_{max} is the maximal fluorescence when the cells were lysed with 0.02% digitonin (final concentration), thus exposing Fura 2 to 2.5 mM Ca^{2+}; and F_{min} is the minimal fluorescence determined after adding 10 mM EGTA, and correcting the pH to 8.5 with 20 mM Tris base. Autofluorescence was measured under the same conditions but using cells without Fura 2, and found to be negligible, and essentially similar to $F_{Mn}{}^{2+}$ (fluorescence level recorded after addition of 20 mM Mn^{2+} to Fura 2-loaded and lysed cells). The contribution of free Fura 2 in the extracellular medium was determined after addition of 3 mM EGTA or 20 µM $MnCl_2$ as a sudden drop in fluorescence, and this value was taken into account as described in references 5 and 10.

For measurement of intracellular calcium using single cells, 1 ml of cell suspension containing 1 × 10^6 TG- or peptone-elicited macrophages in RPMI 1640, 5% FBS was allowed to adhere onto glass coverslips (22 × 22 mm), and these were incubated for 2-3 h in 5% CO_2 at 37°C. Single dishes were then cooled to room temperature one at a time and 10 mM Hepes, pH 7.4, and 1 µM Fura 2/AM added for 20 min in the dark. The monolayers of macrophages were then washed in HBSS containing 10 mM Hepes, pH 7.4. Glass coverslips with the macrophage monolayers in the same medium were placed on the fluorescent microscope stage. Intracellular Ca^{2+} was measured by digital video imaging techniques (11, 33). The digitized video microscope uses a Carl Zeiss Inc. (Thornwood, NY) model IM35 microscope and a 100 × NA 1.4 Nikon Inc. (Garden City, NY) UVF objective. After collecting baseline data, PAF was added. Excitation light for fluorescence was provided by a 75-W xenon lamp. Temperature was maintained at 37°C using an air curtain incubator. Digitized video was obtained by averaging up to 256 frames with the following filter combination: Fura 2-excitation, 340 and 380 nm; emission, >450 nm. Video frames were collected using an ISIT-66 camera (DAGE-MTI, Inc., Michigan City, IN) and digitized with an IP-512 series board set (Imaging Technologies Inc., Woburn, MA). Routinely, excitation intensity was attenuated 100–1,000-fold before reaching the cells, and background images (areas on the coverslip devoid of cells) were obtained at the beginning and end of each experiment. Calcium concentration was measured by subtracting background from images taken at 340 and 380 nm and then dividing the resultant images on a pixel-by-pixel basis. To obtain $[Ca^{2+}]_i$ for an individual cell, the mean value of the pixel ratios for the cell was compared to values obtained with the same equipment using Fura 2–containing EGTA-Ca^{2+} buffers (15).

Basal values for calcium differ in our hands between suspended and adherent macrophages, which contained ~160 and ~80 nM calcium, respectively (see Figs. 5–7 for details). Several factors may be considered in regard to this difference. (*a*) Suspended macrophages can have substantial amounts of Fura 2 in the medium, as evidenced by a sharp drop in fluorescence after addition of 3 mM EGTA and/or 20 μM $MnCl_2$ (5, 9, 10). This has been attributed to "leaky" macrophages but could also be due to the presence of Fura 2 secreted by macrophages in the extracellular medium (13, 45). Even after correcting for this, our data on resting calcium levels in suspended macrophages (~160 nM) are higher than values in adherent cells. Such discrepancy might also be attributable to quenching of the fluorescent signal of the adherent cells by heavy metals. However, the addition of 20 μM *N,N,N′,N′*-tetrakis (2-pyridylmethyl)ethylenediamine (a membrane-permeant chelator of heavy metals that can quench Quin-2 fluorescence) (5) to adherent macrophages did not increase levels of $[Ca^{2+}]_i$ (reference 10, and data not shown). (*b*) Another possibility is that in suspension experiments there may exist a subpopulation of cells which has sequestered Fura 2 into noncytosolic compartments and as such have differing levels of Ca^{2+} than the cytoplasm. This situation is avoided using digitized video microscopy as individual cells are examined and can be prescreened for diffuse cytosolic labeling. Other workers have noted resting $[Ca^{2+}]_i$ levels in the range of 140 to 220 nM in macrophages (10, 30); in one of these reports investigators used adherent macrophages (10). When we measured $[Ca^{2+}]_i$ from macrophages in suspension using quantitative digitized video microscopy <2 min after adding cells to the microscope stage chamber before cells had become adherent, basal $[Ca^{2+}]_i$ was found to be 60 ± 10 nM, $n = 4$. These observations raise the possibility that the differences we see regarding $[Ca^{2+}]_i$ levels using digitized video microscopy and in the suspension studies reflect a difference in the calibration methods used (9, 23) and the potential presence of sequestered Fura 2 in a subpopulation of the cells in suspension. In any event our measured basal $[Ca^{2+}]_i$ levels are well within the range reported by other investigators for this same cell type (10, 30) and PAF addition elicits quantitatively the same changes in $[Ca^{2+}]_i$ in suspensions or adherent macrophages (see below).

Quantification of Binding of [^{3}H]PAF to Macrophage Membranes

Plasma membranes were isolated by sucrose density gradient sedimentation of macrophage lysates as described in reference 43. Binding of PAF was performed by incubating 50 μg of plasma membrane protein in HBSS, 10 mM Hepes, pH 7.4, and 1 mg/ml BSA with different concentrations of [^{3}H]PAF (sp act 44 Ci/mmol). After a 90-min incubation at 2°C, membranes were diluted with 4 ml of the above ice cold buffer and rapidly filtered through presoaked GF/C filters (Whatman, Inc.). Filters were washed three more times with 4 ml of buffer, dried, and counted. Nonspecific binding was determined in the presence of 10^{-5} M unlabeled PAF. Because of the specific activity of [^{3}H]PAF and the conditions used, accurate binding could not be determined below ~0.05 nM [^{3}H]PAF.

Quantification of Chemotaxis

Cells from peritoneal lavage were washed twice in HBSS containing 10 mM Hepes, pH 7.0, 4.2 mM $NaHCO_3$, and 0.5% BSA, and resuspended at 1 × 10^6/ml in similar buffer. Chemotaxis was assayed in a 48-well microchemotaxis assembly with a 5-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) separating the chambers (14, 34). The assay proceeded for 2 h at 37°C. Chemotactic activity was measured by counting the number of migrating cells in response to PAF or activated mouse serum as the source of C5a.

Quantification of Intracellular Levels of DAG

Proteose peptone–elicited macrophages were plated at 2 × 10^6 macrophages/4.5-cm^2 well in 2 ml of RPMI 1640 containing 10% FBS, 2 mM glutamine, 125 U/ml penicillin, and 6.25 μg/ml of streptomycin. After a 2-h incubation at 37°C in an atmosphere of 5% CO_2, the cells were washed three times with 2 ml of the above medium to remove nonadherent cells and incubated 18 h in 2 ml of the same. Cells were then washed three times with 2 ml of HBSS containing 10 mM Hepes, pH 7.4, and incubated for 1 h in 0.5 ml Hepes-buffered HBSS. Stimulants (0.1 ml) were added for the indicated times at 37°C. The reaction was terminated with 2 ml of methanol, the wells were scraped, and the contents transferred to tubes containing 1 ml of $CHCl_3$. Neutral lipid extraction and analysis of DAG content was performed basically as described by Preiss et al. (40).

Results

Binding of Radiolabeled PAF to Macrophage Membranes

To determine the presence of specific binding sites on macrophages for PAF, we initially used a radiolabeled probe of very high specific activity and methods that we have previously successfully used to demonstrate specific binding of a wide variety of proteins, such as maleylated BSA, mannose, fucoidin, transferrin, and IFN_γ to monolayers of mononuclear phagocytes held at 2°C (2). These experiments were, however, not successful. Specific binding could not be observed, since the radiolabeled PAF was very rapidly and extensively taken up by the macrophages, even at 2°C (data not shown). We next examined binding of labeled PAF at 2°C to purified preparations of membranes prepared from macrophages. Saturable reversible binding, over and above noncompetitive binding, was readily observed (Fig. 1 *A*). When these data were analyzed by the method of Scatchard (42), two classes of binding sites (one having a K_d of ~0.1 nM and one having a K_d of ~2 nM [2.4 ± 0.9, $n = 3$]) were observed (Fig. 1 *A*, *inset*). The concentration of unlabeled PAF required to displace 50% of specific binding at 1 × 10^{-10} M [^{3}H]PAF was ~2 nM (Fig. 1 *B*). Taken together, these data suggest PAF binds to a specific receptor(s) on macrophage membranes, which has two classes (i.e., high and low) of binding sites.

Stimulation of Inositol Phosphates Production by PAF

Since PAF leads to intracellular rises in Ca^{2+} in murine macrophages (10), we examined whether this was attributable to generation of breakdown products of polyphosphoinositides. To examine this question, suspensions of macrophages were prelabeled with [^{3}H]inositol and stimulated with PAF. The breakdown products of phosphatidylinositol-4,5-bisphosphate ($PInsP_2$) were analyzed by HPLC (Fig. 2). The formation of $Ins_{1,4,5}P_3$ was quite rapid and was observable at the earliest time point measured; i.e., 10 s (Fig. 3). At this time, intracellular levels of $InsP_3$ were ~50-fold of those in controls. Significant increases in $Ins_{1,3,4,5}P_4$ were also observed, although over a somewhat slower time course (Fig. 3). Formation of $Ins_{1,4,5}P_3$ was followed by $Ins_{1,3,4,5}P_4$

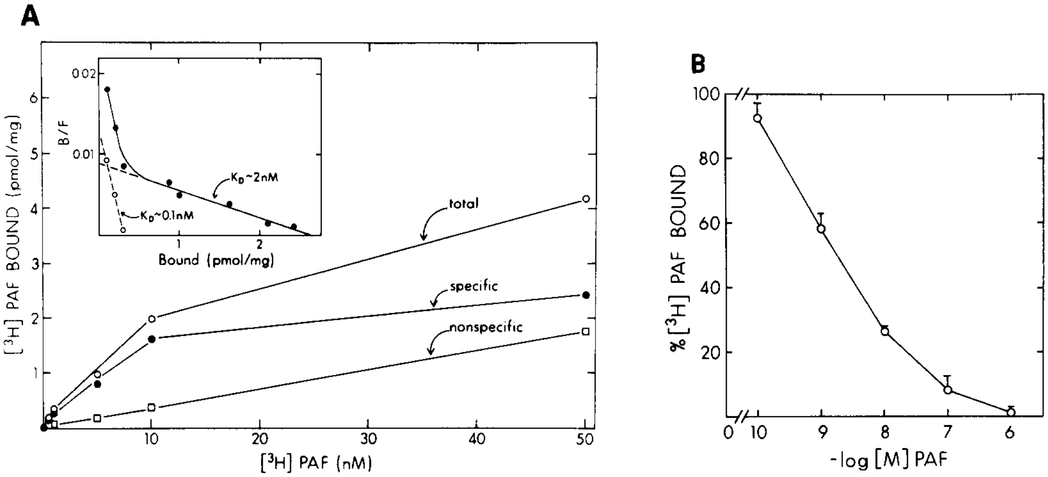

Figure 1. [^{3}H]PAF binding to macrophage membranes. (*A*) Binding of [^{3}H]PAF to macrophage membranes was performed as described in Materials and Methods, using [^{3}H]PAF concentrations from 0.05 to 50 nM. Nonspecific binding was determined in the presence of 10^{-5} M unlabeled PAF. (*Inset*) Scatchard analysis of specific [^{3}H]PAF binding to macrophage membranes, indicating estimated affinities for the two sites. Detailed analysis of high-affinity binding was precluded under the binding conditions used, because of the specific activity of [^{3}H]PAF. (*B*) Displacement of specific binding was determined in the presence of 1×10^{-10} M [^{3}H]PAF and the indicated concentrations of unlabeled PAF. Nonspecific binding was determined in the presence of 10 μM PAF. Based on the amount of [^{3}H]PAF maximally bound to the membrane preparation (2.4 pmol/mg protein) ~15-fold enrichment of plasma membrane markers (43), and 140 μg protein/10^6 macrophages, we can estimate the number of PAF receptors per macrophage to be ~13,000.

and subsequently $Ins_{1,3,4}P_3$, $InsP_2$, and InsP (Fig. 3). Overall, these data suggest a complex breakdown of $PInsP_2$ consistent with the initial formation of $Ins_{1,4,5}P_3$, phosphorylation of this to form $Ins_{1,3,4,5}P_4$, and then subsequent degradation via $Ins_{1,3,4}P_3$ and $InsP_2$ to InsP, a pattern observed in other cells (12, 27, 28, 46). Essentially, the same results were obtained using adherent macrophages prelabeled with [^{3}H]*myo*-inositol, although taking samples at such short times were less accurate. Maximum stimulation of $InsP_3$ formation in adherent macrophages stimulated with PAF for 30 s was observed at 10^{-7} M PAF and half-maximum stimulation at 5×10^{-9} M (Fig. 4). The lesser index of stimulation observed in these experiments can be attributed to the different methodology used to separate inositol phosphates (compare Fig. 4 with Figs. 2 and 3). Note the higher background level of an unidentified isomer of $InsP_3$ as well as of $Ins_{1,3,4}P_3$, since these isomers are included in the pool of $InsP_3$ shown in Fig. 4. When macrophages were cultured in medium containing no added calcium, 30–40% less $InsP_3$ was generated for a given dose of PAF than in medium containing Ca^{2+} (data not shown).

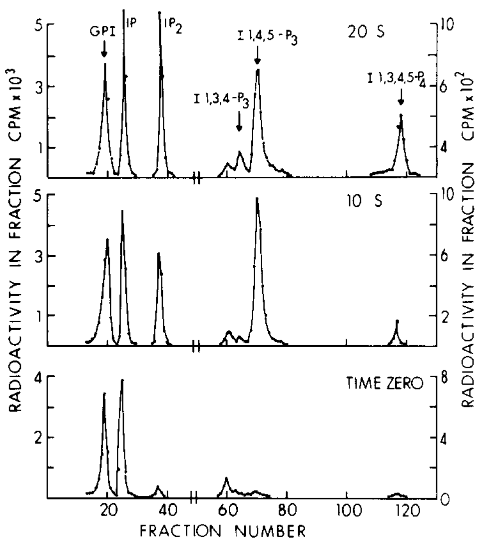

Figure 2. Chromatogram of ^{3}H-myoinositol phosphates generated in macrophages stimulated with PAF. Macrophages (5×10^7/ml) prelabeled with 100 μCi/ml of [^{3}H]*myo*-inositol for 3 h at 37°C in 5% CO_2 were washed and resuspended at 10×10^6/ml in HBSS, 10 mM LiCl, and 10 mM Hepes, pH 7.4, for 10 min before stimulation with 2×10^{-7} M PAF. Control samples (*TIME ZERO*) received saline, while others were stimulated with PAF. The reaction was stopped with 10% TCA (final concentration), and the mixture placed on ice. The neutral water-soluble inositol-containing compounds were analyzed by HPLC on a Partisil 10 SAX (Whatman, Inc.) column. One representative experiment of four separate experiments is shown. Note change in scale at fraction 50.

Effects of PAF on Intracellular Levels of Calcium

To monitor $[Ca^{2+}]_i$ levels, well-spread adherent macrophages were loaded with Fura 2/AM. Fluorescence was then quantified in single cells with a digitized, low-light video microscope system attached to a computer, which provided frame averaging, background subtraction, ratio imaging, and data storage (see Materials and Methods for details). In single macrophages, PAF at 10^{-8} M induced rapid and large rises in intracellular calcium. When fluorescent intensity ratios in the various pixels were plotted as different colors (each representing a different range of $[Ca^{2+}]_i$ levels) in order to analyze cytosolic distribution of calcium, increases in calcium were observed as early as 10 s in some areas of the macrophages (Fig. 5). Maximum intensity, over the entire macrophage was observed by 10–30 s (Fig. 5); this was waning by 1–2 min. Overall, the basal $[Ca^{2+}]_i$ of 40 nM in the macrophages were raised to ~180 nM by 10^{-8} PAF (Fig. 5). The increase in cytosolic calcium was proportional to the concentration of PAF added (Fig. 6). The maximum response

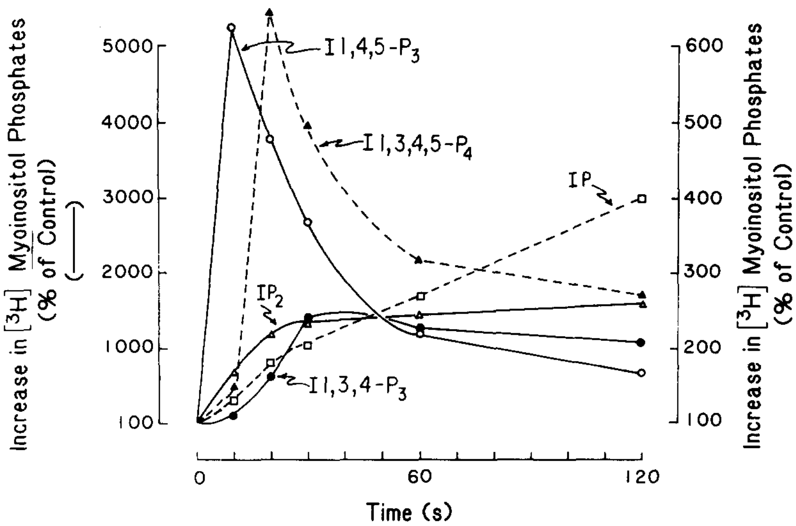

Figure 3. Time course of the various ^3H-myoinositol phosphates induced by 2×10^{-7} M PAF. Macrophages were prelabeled with $[^3H]$*myo*-inositol and treated as described in Fig. 2 for the designated periods of times. InsP (□), $InsP_2$ (△), $Ins_{1,4,5}P_3$ (○), $Ins_{1,3,4}P_3$ (●), and $Ins_{1,3,4,5}P_4$ (▲) were separated from each other by HPLC on a Partisil 10 SAX (Whatman, Inc.) column. Basal levels of the ^3H-inositol phosphates did not change over the time course (not shown). A representative experiment of three separate experiments is shown.

(i.e., 290 ± 27% of control) was observed with 10^{-8} M PAF; the half-maximum response was observed at ~5×10^{-9} M PAF (Fig. 6).

We next examined the role of extracellular calcium in the generation of these fluxes by culturing macrophages in calcium-free medium. When adherent macrophages in culture medium containing 2.5 mM Ca^{2+} were exposed to PAF at 10^{-8} M, maximal increases in the intracellular calcium concentration were significantly greater than when similar macrophages were exposed to PAF in medium not containing added calcium (in four separate experiments, the $[Ca^{2+}]_i$ was 461 ± 81% of basal levels in medium with added Ca^{2+}, and 209 ± 62% in medium without added Ca^{2+}). Macrophages cultured in suspension showed similar responses to PAF. After addition of PAF, levels of intracellular Ca^{2+} rose rapidly but to a lesser extent in Ca^{2+}-free medium; i.e., ~294 vs. 201% of control (Fig. 7, compare *A* and *B*).

The subsequent fall in levels of intracellular calcium was biphasic. An initial rapid fall from 30 to 60 s was followed by a slower decrease after 60 s, which persisted until ~10 min when cytosolic values had returned again to basal levels (Fig. 7; and data not shown). This biphasic decline was observed at all doses of PAF. Note that the slow phase of the decline in calcium levels was not readily seen in the macrophages in which calcium was omitted in the extracellular medium (Fig. 7).

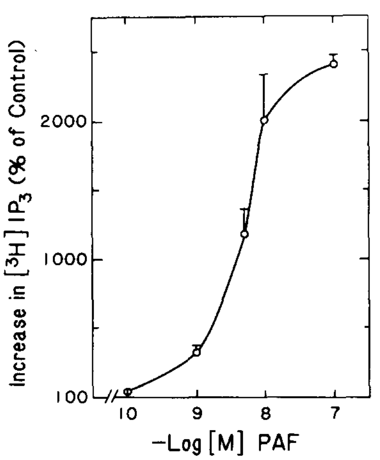

Figure 4. Dose response of PAF on generation of $[^3H]InsP_3$ at 30 s. Macrophages were plated at 12–15 × 10^6 cells/well, prelabeled with 10 μCi/ml $[^3H]$*myo*-inositol for 12 h at 37°C in 5% CO_2 and treated as described in Materials and Methods. Cultures were incubated in HBSS medium plus 10 mM Hepes, pH 7.4, at 37°C, and 10 mM LiCl for 10 min before the stimulation with PAF. The reaction was stopped with 10% TCA (final concentration) 30 s after the addition of PAF. After centrifugation, TCA was extracted and the neutral water-soluble products were chromatographed on AG 1-X8 resin (formate form). The data shown are calculated as percent change from control, and represent the mean ± SEM (n = 3) from one representative experiment of three separate experiments. Basal level of $InsP_3$ was 809 cpm ± 190.

Effects of PAF on Generation of DAG and Enhanced Phosphorylation of Macrophage Proteins

We next determined if PAF would lead to the intracellular accumulations of DAG. PAF, at an optimal concentration of 10^{-7} M, led to increases in intracellular levels of DAG of ~200 pmol/10^7 cells from a basal level of ~400 pmol/10^7 cells. The effect was clearly observable 5 min after pulsing the cells (Fig. 8 *A*) and persisted for ≥30 min. The increased generation of DAG was dependent upon the dose of PAF (Fig. 8 *B*). Maximum stimulation was observed at 10^{-7} M PAF and half-maximal stimulation at 5×10^{-9} M.

Because of the effects of PAF on raising intracellular levels of DAG, a well-known stimulant of protein kinase C (37), we next determined if PAF would induce altered protein phosphorylation in macrophages. The effects on protein phosphorylation in macrophages of two other stimulants of protein kinase C (i.e., lipopolysaccharide [LPS] and phorbol-12,13-myristate acetate [PMA]) have already been reported (41, 49). To this end, we took monolayers of macrophages prelabeled with ^{32}P to equilibrium and exposed them to PAF at 2×10^{-7} M (see Materials and Methods for experimental details; gels were loaded with equal amounts of radioactivity in these experiments). PAF led to a characteristic pattern of enhanced ^{32}P labeling of proteins with molecular masses of 28, 33, 67, and 103 kD (i.e., phos-

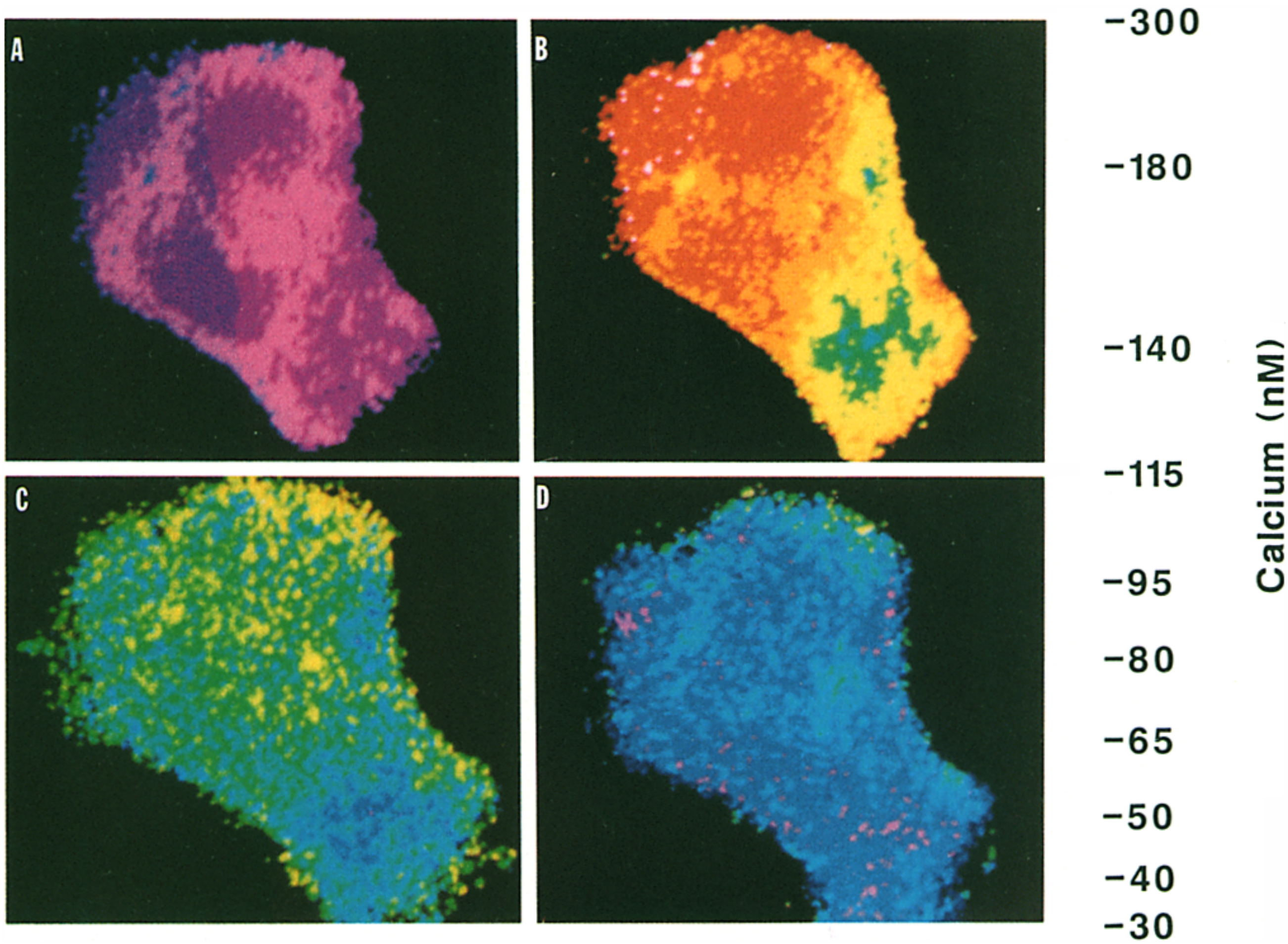

Figure 5. Color-enhanced images of 340:380 fluorescence ratio of a macrophage loaded with Fura 2/AM and stimulated with PAF. Macrophages were plated and loaded with 1 μM Fura 2/AM as described in Materials and Methods. Intracellular $[Ca^{2+}]_i$ in macrophages stimulated with 10^{-8} M PAF at (*A*) time zero, (*B*) 10 s, (*C*) 20 s, and (*D*) 30 s was then estimated by digital video imaging techniques. Data representative of five separate experiments is shown.

phoprotein (pp)28, pp33, pp67 and pp103) (Fig. 9 *A*). Phosphorylation of other bands (i.e., the pp at 80 kD) was not consistently observed in this study or in previous work (41, 49). Overall, these effects were visible at 5 min, maximum at 15 min, and were waning by 30 min. LPS, as previously reported (49), enhanced labeling of pp28, pp33, pp67, and pp103 (Fig. 9 *A*). PMA enhanced phosphorylation of pp45 in addition to pp28, pp33, and pp67 (Fig. 9 *B*). The time course of phosphorylation was quite distinct between PAF and LPS. Phosphorylation in response to LPS was barely visible at 15 min and maximum at 30 min (Fig. 9 *A*). The time course in response to PMA was quite similar to that induced by PAF (Fig. 9 *B*).

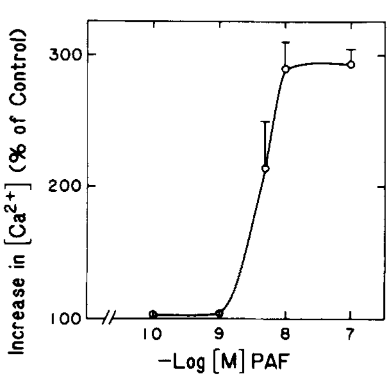

Figure 6. Dose response for PAF on intracellular Ca^{2+} increase in Fura 2-loaded macrophages using single cell measurements. Data points at 30 s after the addition of PAF were taken and are shown as the mean ± SEM of four separate experiments. The basal intracellular Ca^{2+} level was 82.7 ± 12.0 nM. See Materials and Methods for other details.

Effects of PAF on Chemotaxis

PAF induced significant chemotaxis of peritoneal macrophages in a dose-dependent fashion (Fig. 10). Maximum effects observed with PAF at 10^{-9} M, and half-maximal effects were observed with PAF at $\sim 10^{-11}$ M.

Discussion

These observations support the existence of a specific receptor for PAF on murine peritoneal macrophages. Highly specific and active, radiolabeled PAF bound to purified plasma membrane preparations from murine peritoneal macrophages (Fig. 1). This binding was saturable and could

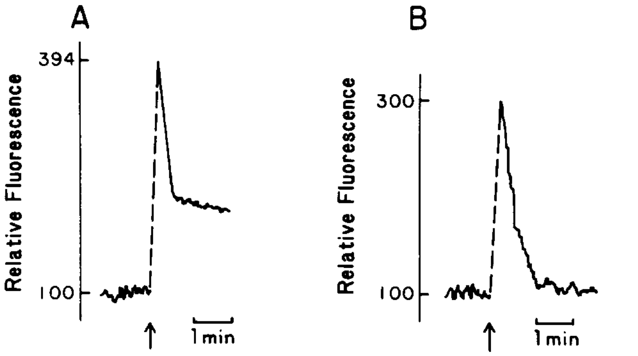

Figure 7. Effect of extracellular Ca^{2+} levels on calcium mobilization for macrophages in suspension. Macrophages were loaded with 5 μM Fura 2/AM and resuspended in Hepes-buffered HBSS medium, pH 7.4, at 37°C containing (*A*) 2.5 mM Ca^{2+} or (*B*) no added Ca^{2+}. Changes in fluorescence were monitored before and after addition of 10^{-8} M PAF, using an excitation wavelength set at 339 nm and the emission wavelength set at 500 nm. Resting levels of $[Ca^{2+}]_i$ in suspended macrophages, after correction for extracellular Fura 2 with 3 mM EGTA or 20 μM $MnCl_2$, was ∼165 ± 4 nM. A representative experiment of three is shown.

be readily displaced by addition of excess unlabeled ligand. Analysis of the binding by the method of Scatchard (42) yielded a curvilinear plot, indicating two classes of binding sites; the higher affinity site exhibited a K_d of ∼0.1 nM and the lower a K_d of ∼2 nM. We did not demonstrate specific binding of the radiolabeled ligand to monolayers of macrophages, even at 2°C, a finding observed in other cells and attributed to rapid uptake and metabolism of the lipophilic ligand (8). The number of binding sites per cell thus could not be estimated directly. By an indirect method of estimate (see Fig. 7), one can estimate ∼13,000 binding sites per macrophage. The actions of PAF on platelets have been well documented (8), although both binding affinities and number of receptors have been reported over a wide range (see reference 8), presumably due to the lipophilic nature of PAF. The similarities observed between binding affinity and physiological action in macrophages suggest that ligation of the physiologically relevant receptor is being measured (see below).

The presence of an additional high affinity (K_d ∼0.1 nM) component in membrane preparations has routinely been observed for receptors that couple to a guanine nucleotide regulatory protein (32). It has recently been reported that guanine nucleotides are capable of modulating binding of low concentrations of [^{3}H]PAF to membranes from polymorphonuclear leukocytes and platelets (24, 36).

PAF, at concentrations consistent with the K_d of the receptor on murine peritoneal macrophages (i.e., a half-maximum concentration of 5 nM) led to formation of breakdown products of polyphosphoinositides. Specifically, PAF initiated the rapid, extensive, and transient formation of $Ins_{1,4,5}P_3$ (Figs. 2 and 3). As observed in other cells (12, 26, 27, 46), the breakdown of $Ins_{1,4,5}P_3$ is complex and involves formation of $Ins_{1,3,4,5}P_4$, and subsequent breakdown of $Ins_{1,3,4,5}P_4$ and of $Ins_{1,4,5}P_3$, respectively, into $Ins_{1,3,4}P_3$ or $InsP_2$, and ultimately into InsP. $Ins_{1,3,4,5}P_4$ is probably formed by phosphorylation of $Ins_{1,4,5}P_3$ (12, 26, 46); the dephosphorylation product of this reaction is $Ins_{1,3,4}P_3$ (26, 27). $Ins_{1,4,5}P_3$ can also be dephosphorylated directly into $Ins_{1,4}P_2$, which in turn is dephosphorylated into Ins_1P or Ins_4P, and finally into free inositol which enters back into phosphatidylinositol (12). Of interest, the ultimate extent of formation of $InsP_3$ in macrophages stimulated with PAF is dependent, to some extent, upon the presence of calcium into the extracellular medium, a finding observed in other cell types. This has been attributed to a calcium requirement of the polyphosphoinositide phosphodiesterase (48). The breakdown of $PInsP_2$ after ligation of the receptor for PAF has been linked in other cells to a guanine nucleotide regulatory protein (e.g., reference 44), and the role of such a protein in macrophages is currently under investigation in our laboratory. In addition to the inositol phosphates described above, various other inositol phosphates were observed during the course of these studies and were relatively insensitive to PAF addition (Fig. 2, and V. Prpic, unpublished observations). One of these eluted before $Ins_{1,3,4}P_3$ but was clearly distinguishable from glycerophosphoinositol-4,5-bisphosphate and unlikely to represent a cyclic $InsP_3$ (41). Later-eluting, PAF-insensitive inositol phosphates were also observed and tentatively identified as $InsP_5$ and $InsP_6$ (Prpic, V., unpublished observations). the additional $InsP_3$ peak demonstrated might thus represent a novel dephosphorylation product.

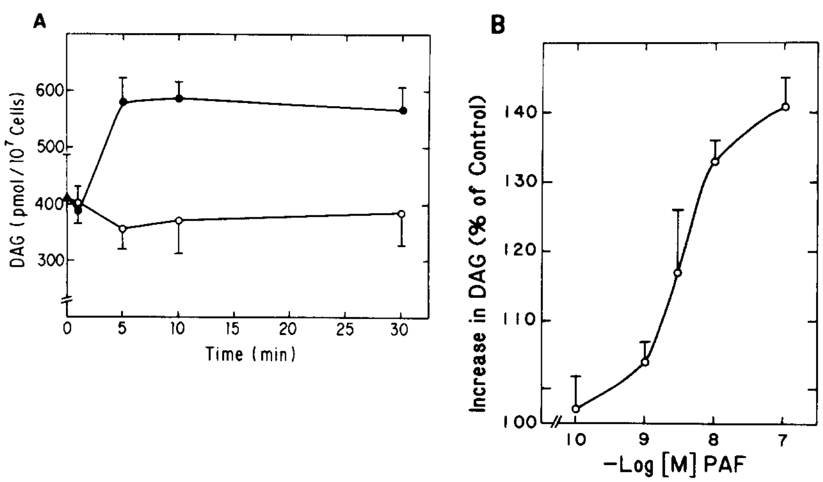

Figure 8. PAF stimulation of DAG production. (*A*) Peritoneal macrophages cultured in HBSS containing 10 mM Hepes, pH 7.4, were incubated in the presence of buffer (○) or 10^{-7} M PAF (●) at 37°C for the indicated times. Incubations were terminated and samples processed as described in Materials and Methods. Results are presented as mean ± SEM for triplicate determinations of a representative experiment. (*B*) Dose response of DAG accumulation in response to PAF. Peritoneal macrophages were incubated as indicated above for 10 min in the presence of buffer or the indicated concentrations of PAF. Results are presented as mean ± SEM for triplicate determinations of a representative experiment.

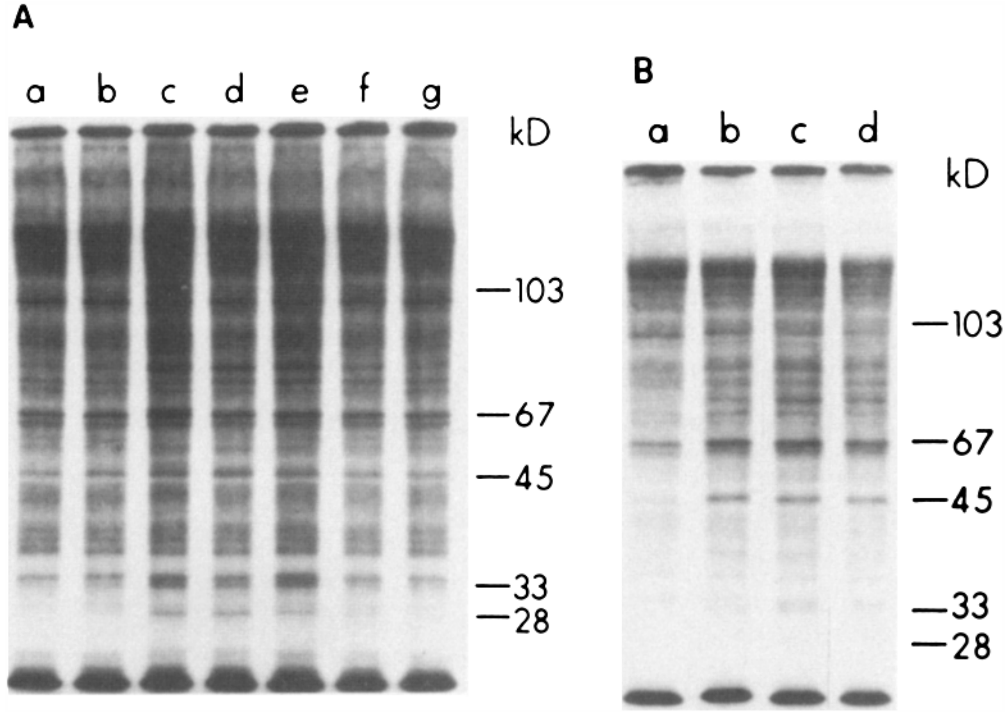

Figure 9. Time course of PAF and LPS on endogenous protein phosphorylation. (*A*) TG-elicited macrophages were prepared, labeled with $^{32}P_i$ for 3 h, and treated with either 50 ng/ml LPS (lanes *a–c*) or 2×10^{-7} M PAF (lanes *d–f*) for 5 (lanes *a* and *d*), 15 (lanes *b* and *e*), and 30 min (lanes *c* and *f*) before analysis of endogenous protein phosphorylation as described in Materials and Methods. Lane *g* is a control. (*B*) Time course of PMA (10 ng/ml) on endogenous phosphorylation. Control (lane *a*); 5, 15, and 30 min (lanes *b–d*, respectively).

A likely consequence of the generation of breakdown products of polyphosphoinositides in response to PAF was the generation of rapid intracellular fluxes of Ca^{2+} (Figs. 5 and 6). In other cells, the formation of $Ins_{1,4,5}P_3$ has been linked to the liberation of calcium from nonmitochondrial intracellular stores, while the formation of $Ins_{1,3,4,5}P_4$ has been linked to opening of membranous channels and influx of extracellular Ca^{2+} (26) or to inhibition of calcium reuptake into intracellular stores (29). The partial dependence of generation of intracellular fluxes of Ca^{2+} in macrophages exposed to PAF upon the availability of extracellular calcium (Fig. 7) supports the former interpretation. The calcium transients observed in adherent macrophages were maximal $\leqslant$30 s after the cells were exposed to PAF (Fig. 5). The cellular increases in calcium were ultimately observed diffusely over macrophages exposed to PAF, although they were initially observed mainly in submembranous areas (Fig. 5).

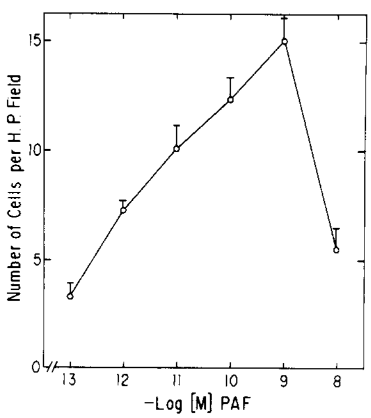

Figure 10. The effect of PAF on macrophage chemotaxis. The isolated cells were resuspended in HBSS–BSA buffer at 1×10^6/ml. Buffer or the attractants (PAF or activated mouse serum) were placed in the wells of a multiwell chemotaxis chamber. The cells were added to the top wells and incubated for 2 h, after which migrated cells were stained and counted. Chemotaxis was assessed by the number of cells crossing the millipore filter (5 μm). Results are expressed as the mean number of migrating cells per 10 high-power (*H.P.*) fields in triplicate samples. Buffer alone was 1.5 ± 0.6 and 3% activated mouse serum was 10.2 ± 1.2 cells/high-power field. Triplicates of each dilution were performed in four separate experiments.

An additional consequence of stimulating macrophages with PAF is the formation of DAG (Fig. 8). Maximal response of macrophages to PAF led to increases of ∼200 pmol of DAG/10^7 macrophages. This rise, which was observed in the absence of cytochalasin B, was clearly visible at 5 min after exposure of cells to PAF and remained elevated for $\geqslant$30 min, a time course similar to that observed in other cells (40).

A likely consequence of the generation of DAG was enhanced phosphorylation of a characteristic subset of macrophage proteins. We have previously observed a characteristic pattern of phosphorylation in macrophages in response to LPS and to PMA (41, 49). Both of these stimuli led to enhanced phosphorylation of 28-, 33-, and 67-kD proteins (i.e., pp28, pp33, and pp67, respectively). The phosphopeptide breakdown products of pp28, pp33, and pp67, when analyzed by Cleveland digests, were previously reported to be similar whether the enhanced phosphorylation was induced by PMA or by LPS (49). These two signals did differ, in that LPS caused enhanced phosphorylation of pp103 while PMA caused enhanced phosphorylation of pp45. In the present experiments, we observed enhanced phosphorylation of all five of these phosphoproteins in response to PAF (Fig. 9). We have also observed that LPS in macrophages causes the generation of breakdown products of polyphosphoinositides (41) and of DAG (Uhing, R. J. unpublished observations). Taken together, these observations imply that all three stimulants, acting via protein kinase C, lead to enhanced phosphorylation of four or more of the five pp's.

The reason(s) for and the significance of the fine differences in the three phosphorylation patterns remain to be established, although distinct differences also exist in the amount of DAG produced in response to each (Uhing, R. J., unpublished observations). Note that LPS and PMA, though sharing some common functional effects on macrophages, have distinct functional effects as well (2–4, 17). PMA, for example, initiates a respiratory burst in macrophages while LPS does not (2); phosphorylation of a component of the oxidase complex of ~45 kD has been recently suggested to be a participant in the oxidative burst (21). Differences in substrate phosphorylation via protein kinase C have also been suggested to bear upon the differential regulation of transcription and message stability for early competence genes such as *c-fos*, *JE*, and *KC* in fibroblasts and macrophages (16; Koerner, T. J., S. E. Yu, D. O. Adams, unpublished observations). In sum, these qualitative differences in enhanced phosphorylation may be important clues to the role of phosphorylation of various substrate proteins in different functional responses by macrophages.

The time course of phosphorylation initiated by PAF was rapid and comparable to that initiated by PMA (Fig. 9). By contrast, the course of phosphorylation initiated by LPS was much slower. This is consistent with the rapid and transient breakdown of polyphosphoinositides observed in response to PAF, in comparison with the slower developing and longer persisting course of such breakdown initiated by LPS (41). Current evidence from our laboratory indicates that such differences can be reflected in the speed and extent with which gene transcription is initiated in response to LPS in comparison to ligation of a defined receptor (T. J. Koerner, S. E. Yu, D. O. Adams, unpublished observations).

The present observations document that PAF, when applied to murine peritoneal macrophages, initiates an integrated, functional response; i.e., chemotaxis (Fig. 10). Considerable evidence, in a variety of systems, implicates the breakdown of polyphosphoinositides plus a subsequent increase in $[Ca^{2+}]_i$ and phosphorylation of substrate proteins as important to chemotaxis in macrophages and neutrophils (38, 44). For example, the well-known chemotactic receptor for N-formylated peptides is also coupled to the hydrolysis of polyphosphoinositides; inhibitors of both calcium and protein kinase C reduce chemotaxis (38, 44). In the present studies, the half-maximal dose of PAF for initiating chemotaxis was 10^{-11} M (Fig. 10), while the half-maximal dose for $InsP_3$ production, for generation of Ca^{2+} fluxes, and of DAG production was $\sim 5 \times 10^{-9}$ M (Figs. 4, 6, and 8). Such an apparent disparity has been observed in many systems and has been related to the concept of "spare receptors" (1). In other words, the number of receptors that must be engaged to initiate a functional response is far less than the total number of receptors (engaging all of which can lead to maximum biochemical responses, e.g., $PInsP_2$ breakdown). The present observations are also in accord with other known functional effects of PAF on macrophages. PAF is known to initiate a respiratory burst and release of metabolites of arachidonic acid, from various macrophages (6, 19, 20; and Prpic, V., R. Fry and D. O. Adams, manuscript in preparation). These responses too have been linked to the breakdown of polyphosphoinositides (25, 39, 47).

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