Platelet Activating Factor Raises Intracellular Calcium Ion Concentration in Macrophages

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Abstract. Peritoneal cells from thioglycollate-stimulated mice were allowed to adhere to coverglasses for 2 h to give a dense monolayer of adherent cells >95% of which were macrophages. After incubation with the tetra-acetoxymethyl ester of quin2, coverglasses were rinsed with Ca^{2+}-free saline, oriented at a 45° angle in square cuvettes containing a magnetically driven stir bar, and analyzed for changes in quin2 fluorescence in a spectrofluorimeter. Such fluorescence, taken as an indication of intracellular calcium ion concentration ($[Ca^{2+}]_i$), increased as exogenous calcium ion concentration ($[Ca^{2+}]_o$) was raised to 1 mM. At $[Ca^{2+}]_o \cong 10$ μM, $[Ca^{2+}]_i = 72 \pm 14$ nM ($n = 26$); at $[Ca^{2+}]_o = 1$ mM, $[Ca^{2+}]_i = 140$–220 nM, levels not increased by *N, N, N′, N′*-tetrakis (2-pyridylmethyl) ethylenediamine, a membrane-permeant chelator of heavy metals that can quench quin2. Addition of mouse α + β fibroblast interferon, lipopolysaccharide, thrombin, collagen, vasopressin, ADP, compound 48/80, or U46619 did not change $[Ca^{2+}]_i$. However, addition of platelet activating factor (PAF) (2–20 ng/ml) raised $[Ca^{2+}]_i$ by 480 nM within 1 min if $[Ca^{2+}]_o = 1$ mM. In the presence of 5 mM EGTA, PAF raised $[Ca^{2+}]_i$ by 25 nM. This suggests that PAF causes influx of exogenous Ca^{2+}, as well as releasing some Ca^{2+} from intracellular stores. Consistent with these results, when PAF was added to 1 mM Ca^{2+} in the presence of 100 μM Cd^{2+} or Mn^{2+} to block Ca^{2+} influx, $[Ca^{2+}]_i$ increased by only intermediate amounts; at the times of such dampened peak response, $[Ca^{2+}]_i$ could be raised within 1 min to normal PAF-stimulated levels by chelation of the exogenous heavy metals with diethylenetriaminepentaacetic acid. Normal PAF responses were observed in the presence of indomethacin. The lowest dose of PAF observed to raise $[Ca^{2+}]_i$ was 0.1 ng/ml. Response of $[Ca^{2+}]_i$ to 2–20 ng/ml PAF was transient, and second applications had no effect. The PAF response also was seen in cell suspensions. These results suggest that an increase in $[Ca^{2+}]_i$ may be an early event in PAF activation of macrophages.

ACTIVATION of macrophages appears to occur as a series of steps after the interaction of soluble substances, macroscopic particles, or tumor cells with corresponding receptors on the macrophage cell surface. Receptors mediating phagocytic functions include those for F_c immunoglobulin fragments and for complement; receptors mediating nonphagocytic functions include those for Ia antigen, transferrin, and for tumor cells; receptors involved in regulation include those for N-formylated peptides and for lymphokines (e.g., interferons) (1, 48, 54). In response to the binding of some of these substances to their receptors, macrophages are activated to secrete a large variety of products and display increased capacities for destroying microbes and tumor cells (1, 32, 34, 56). At least two steps appear to be involved in macrophage activation: (*a*) response to an initial or priming signal (e.g., lymphokines, lipopolysaccharide, or microbes), and (*b*) response to a subsequent or triggering signal (e.g., immune complexes, lipopolysaccharide, opsonized particles, or tumor cells) (1). The mechanisms by which these priming and secondary signals are transduced into intracellular messages may involve changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$).[1] Changes in $[Ca^{2+}]_i$ have been seen in guinea pig alveolar macrophages in response to formyl peptides (20, 21, 49) and in the macrophagelike cell line, J774, in response to antigen–antibody complexes and antibody-coated erythrocytes (60).

Platelet activating factor (PAF), 1-*O*-alkyl(C_{16} + C_{18})-2-acetyl-*sn*-glyceryl-3-phosphorylcholine, is a phospholipid synthesized and secreted by a variety of cell types, including macrophages, that are involved in response to nonimmunological and immunological inflammatory stimuli (reviews in references 33, 47, and 55). In response to PAF, platelets display rapid turnover of phosphoinositides (5), formation

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, intracellular calcium ion concentration; $[Ca^{2+}]_o$, exogenous calcium ion concentration; DTPA, diethylenetriaminepentaacetic acid; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IFN-α,β, mouse α and β fibroblast interferon; LPS, lipopolysaccharide; PAF, platelet activating factor; quin2/AM, tetra-acetoxymethyl ester of quin2; TPEN, *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine.

of phosphatidic acid, protein phosphorylation, and shape change (27, 38). When applied to macrophages, PAF stimulates the oxidative burst, prostaglandin E synthesis, thromboxane B_2 synthesis, glucose consumption, and spreading (14–18). When PAF is applied to human platelets, $[Ca^{2+}]_i$ increases 8–10-fold in a few seconds if exogenous calcium ion concentration ($[Ca^{2+}]_o$) is 1 mM; $[Ca^{2+}]_i$ undergoes an increase, albeit smaller, if PAF is applied in the absence of $[Ca^{2+}]_o$ (10, 11, 45). These data suggest that PAF causes influx of Ca^{2+} as well as some release of Ca^{2+} from internal stores.

Here we report that the same concentrations of PAF that cause Ca^{2+} influx and internal release in platelets have analogous effects on macrophages, raising $[Ca^{2+}]_i$ severalfold in a matter of seconds in a manner similarly dependent on $[Ca^{2+}]_o$. The facts that the effect of PAF on $[Ca^{2}]_i$ is transient and that second applications have no effect suggest that receptors for PAF exist on the surfaces of macrophages, as they appear on platelets and on at least some smooth muscles (22, 46), and that they may be rapidly desensitized, as hypothesized for platelets (10, 11) and smooth muscle (8).

Materials and Methods

Cells

Elicited macrophages were produced in MF1 female mice by intraperitoneal injection of 1.5 ml of aged, sterile thioglycollate broth 4–6 d before they were killed by decapitation. Peritoneal lavage cells were flushed from mice, using cold serum-free nutrient medium containing 10 U/ml heparin, and stored on ice. The cells were counted with a hemocytometer (>95% excluded trypan blue) and plated directly into rectangular trays at 0.95×10^6 cells/cm^2. Tray bottoms were covered with No. 1 coverglasses cut to 13 × 22-mm size and with upper left corners broken off to indicate clearly which side of the coverglass cells were on. Cells were allowed to adhere to coverglasses for 2 h at 37°C. Coverglasses then were transferred to individual 35-mm petri dishes where they were rinsed vigorously three times with Ca-free saline at 37°C and fed with nutrient medium containing 10% fetal calf serum (vol/vol). By this technique, coverglasses were covered evenly with a confluent monolayer of firmly adherent cells, approximately half of which were round and half of which were spread. More than 95% of both round and spread cells contained nonspecific esterase activity which could be abolished with 30 mM NaF, a characteristic of macrophages, but not of neutrophils (24). Cells on coverglasses were used immediately or after as long as 24 h in vitro at 37°C. The entire plating and incubation procedure also could be performed in serum-free nutrient medium, with equivalent results.

Loading with quin2

Coverglasses in their dishes were rinsed twice with Ca-free saline at 37°C and transferred to individual tubes (15-mm inner diam) containing loading medium at 37°C. Loading medium consisted of nutrient medium containing 10% fetal calf serum, 2 mM freshly added glutamine, 50 μM tetraacetoxymethyl ester of quin2 (quin2/AM), and 0.1% dimethylsulfoxide. Tubes were immersed in a 37°C bath at a 30° angle and rotated at 10 rpm so that the coverslips would be flopped gently back and forth through the loading medium, but without touching an air–water interface (the cells do not touch the tube walls). After incubating for 30 min, coverglasses were transferred back to individual 35-mm petri dishes for rinsing (rinsing in the loading tubes seemed to remove many cells, presumably because of interfaces sweeping past). Coverglasses were rinsed three times with Ca-free saline at 37°C and then stored in the third rinse solution in the dark at room temperature. As judged by trypan blue exclusion (>95%), such cells remained viable for at least 3 h.

Measurement of $[Ca^{2+}]_i$

Just before use, each coverglass stored in the last rinse solution was placed in a diagonal position in a standard 1-cm square cuvette containing 3 ml Ca-free saline. In the bottom of the cuvette was a small magnetic stirring bar and a " ∩ " shaped glass rod which spanned from corner to corner and kept the edge of the coverslip just above the stirring bar. A 5-mm long piece of glass tubing (9–10-mm outer diam) placed on top of the coverslip prevented its movement during stirring. The cuvette was then placed in a Perkin-Elmer fluorescence spectrophotometer (model MPF-44A, Perkin-Elmer Corp., Instrument Div., Norwalk, CT) and maintained at 37°C while being stirred continuously. After equilibration for 5 min and tapping to dislodge air bubbles, fluorescence was recorded (excitation, 339 nm; emission, 500 nm). To optimize coverglass position in the excitation beam, the cuvette was rotated through the four possible positions until maximum emission was detected. This always was found to be a position in which the coverglass would not have simply reflected light into the photometer. This position then was maintained as additions were made to the cuvette. Cells began in Ca-free saline, which contained ~10 μM Ca^{2+}. In this solution, or after additional $CaCl_2$ was added, the amount of extracellular quin2 dye (leakage) was estimated by adding 0.1 mM $MnCl_2$ and recording the extent to which the fluorescence was immediately depressed (leak) (intracellular quin2 is not exposed immediately to Mn^{2+}, whereas extracellular quin2 is accessible and so its fluorescence is abolished when binding to Mn^{2+} occurs [10, 39, 40]). After ~1 min, the extracellular Mn^{2+} was chelated by addition of 0.2 mM Ca diethylenetriaminepentaacetic acid (DTPA) (membrane-impermeant) to terminate the leak test, and experimental agents were then added sequentially. Experiments were terminated in the presence of 1 mM Ca^{2+} by rapid addition of 50 μM digitonin to the sector of the cuvette facing the cells. Fluorescence then rose rapidly to a value that we have designated as F_{max} and then fell as intracellular quin2 diffused from the permeabilized cells. Finally, the value for F_{min} was calculated from the fluorescence level recorded after 1 mM $MnCl_2$ was added ($F_{Mn^{2+}}$) (equals essentially autofluorescence) (11, 40):

$$F_{min} = F_{Mn^{2+}} + \frac{(F_{max} - leak) - F_{Mn^{2+}}}{6}$$

Values for $[Ca^{2+}]$ then were calculated from the observed fluorescence (F) (11, 52):

$$[Ca^{2+}]_i = \frac{(F - leak) - F_{min}}{(F_{max} - leak) - (F - leak)} \cdot K_d$$

K_d for quin2 is 115 nM. In the two equations above, the value for the leak was that determined at the beginning of each experiment, as described above.

F_{max} was determined by permeabilizing cells with digitonin because levels of fluorescence reached after addition of even high concentrations of ionomycin or A23187 (the alternative method for recording F_{max}) were always somewhat below those attained when cells were permeabilized with digitonin (also see reference 37). This effect also was observed with thioglycollate-elicited peritoneal lavage cells that were loaded with quin2, rinsed, and examined in the cuvette in suspension (according to methods in reference 39), as well as with peritoneal cells attached to coverslips (macrophages). Unlike the ~25% enhancement of autofluorescence seen upon addition of digitonin to lymphocyte suspensions (52), addition of 50 μM digitonin to macrophages on coverglasses did not increase autofluorescence detectably.

Materials

Nutrient medium consisted of RPMI 1640 with 2 mM glutamine and 25 mM Hepes included, without bicarbonate (Gibco Ltd., Paisley, Scotland), to which was added 50 μg/ml gentamycin (Gibco Ltd.) and NaOH to pH 7.4, stored frozen; for daily use an additional 2 mM glutamine was freshly added. Fetal calf serum was myo-clone fetal calf serum (Gibco Ltd.) containing 0.22–0.36 ng/ml endotoxin; it was heat-inactivated at 56°C for 30 min and stored frozen. Ca-free saline contained (mM): 145 NaCl, 5 KCl, 1 $MgSO_4$, 10 glucose, 10 Hepes, titrated to pH 7.4 with NaOH. Stock solutions of CaDTPA contained (mM): 100 DTPA, 100 $CaCl_2$, 1 Hepes, and sufficient NaOH to yield pH 7.4. Stock solutions of EGTA contained (mM): 500 EGTA, 1 Hepes, and NaOH to pH 7.4.

ADP, compound 48/80, DTPA, EGTA, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), Hepes, indomethacin, mouse α and β fibroblast interferon (IFN-α, β), and lipopolysaccharide (LPS) from *E. coli* serotype 026:B6 (phenol extract) were purchased from Sigma Chemical Co. (Poole, Dorset, UK); PAF, ionophore A23187, thrombin, and vasopressin were from Calbiochem (Cambridge BioScience, Harwick, Cambridge, UK); quin2/AM was from Lancaster Synthesis (Morecambe, Lancs, UK); collagen was from Hormon-Chemie (Munich, West Germany); thioglycollate medium (with dextrose, without indicator) was from Gibco Ltd.; ionomycin was from Squibb (Hounslow, Middlesex, UK); U46619 was from Upjohn Co.

(Kalamazoo, MI); *N,N,N',N'*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was a gift from Dr. R. Y. Tsien.

Results

Effects of PAF in 1 mM $[Ca^{2+}]_o$

When PAF was applied to macrophages in the presence of 1 mM Ca^{2+}, $[Ca^{2+}]_i$ increased, reaching maximal values after 1.5 min: Beginning in the presence of Ca-free saline, $[Ca^{2+}]_i$ was 72 ± 14 nM (n = 26) (Fig. 1). Fig. 1 then illustrates the rise in $[Ca^{2+}]_i$ as $[Ca^{2+}]_o$ was raised to 1 mM. Addition of 0.1 mM Mn^{2+} suppressed fluorescence slightly, indicating the presence of only small amounts of extracellular quin2 (leak). Chelation of the extracellular Mn^{2+} with an excess of CaDTPA restored the fluorescence level of 1 mM Ca^{2+}. Addition of PAF, at a standard concentration of 20 ng/ml, a maximally effective concentration on human platelets (11; Simpson, and T. J. Rink, manuscript in preparation), then caused a rapid rise in $[Ca^{2+}]_i$ to a peak. Addition of A23187 at the time of the PAF-induced peak caused no further rise in $[Ca^{2+}]_i$, but addition of 50 μM digitonin sent $[Ca^{2+}]_i$ up to a transitory F_{max}. After the subsequent fall in fluorescence, presumably as quin2 diffused from the permeabilized cells, a plateau was reached. Addition of 1 mM Mn^{2+} caused a further rapid fall to a plateau level ($F_{Mn^{2+}}$) (Fig. 1). These results indicate that $[Ca^{2+}]_i$ in macrophages rapidly rises in response to PAF and that A23187, when applied shortly thereafter, causes no further detectable increase in $[Ca^{2+}]_i$.

In contrast, if A23187 was added before PAF, fluorescence rose to a level that was not increased when PAF was added subsequently (Fig. 2). In the absence of cells or quin2, A23187 could be used at concentrations even as high as 600 nM without causing a detectable increase in fluorescence (data not shown). Even concentrations of 1,000 nM (1 μM) A23187 contributed only a very small increase in fluorescence. The results in Fig. 2 therefore indicate that A23187 caused a significant increase in $[Ca^{2+}]_i$, as expected for this ionophore, and that PAF, when applied shortly after the A23187-induced peak was reached, did not raise $[Ca^{2+}]_i$ further.

When 1 mM $[Ca^{2+}]_o$ was attained by a single addition of $CaCl_2$, $[Ca^{2+}]_i$ rose in a single step to a plateau level of 140 ± 14 nM (n = 4) (e.g., Figs. 1, 2, and 7), whereas if the $CaCl_2$ was added in two unequal portions, $[Ca^{2+}]_i$ rose to 192 ± 17 nM (n = 6) in response to 400 μM $[Ca^{2+}]_o$ and then rose in a second step to 220 ± 17 nM (n = 6) when the remaining $CaCl_2$ was added to bring $[Ca^{2+}]_o$ up to 1 mM (e.g., Figs. 5, 6, 8, and 9). By whatever protocol it was accomplished, once 1 mM $[Ca^{2+}]_o$ had been attained, followed by the leak test, addition of 20 ng/ml PAF caused $[Ca^{2+}]_i$ to rise by 483 ± 86 nM (n = 6).

The resting level of $[Ca^{2+}]_i$ before the addition of PAF was not raised by addition of 20 μM TPEN, a membrane permeant chelator of intracellular heavy metals (e.g., Zn^{2+}) which are known to suppress quin2 fluorescence in some cell types (3) (see Fig. 6). The resting level of $[Ca^{2+}]_i$ also was not raised by addition of 1 or 2 μg/ml compound 48/80, suggesting the absence of significant numbers of mast cells on

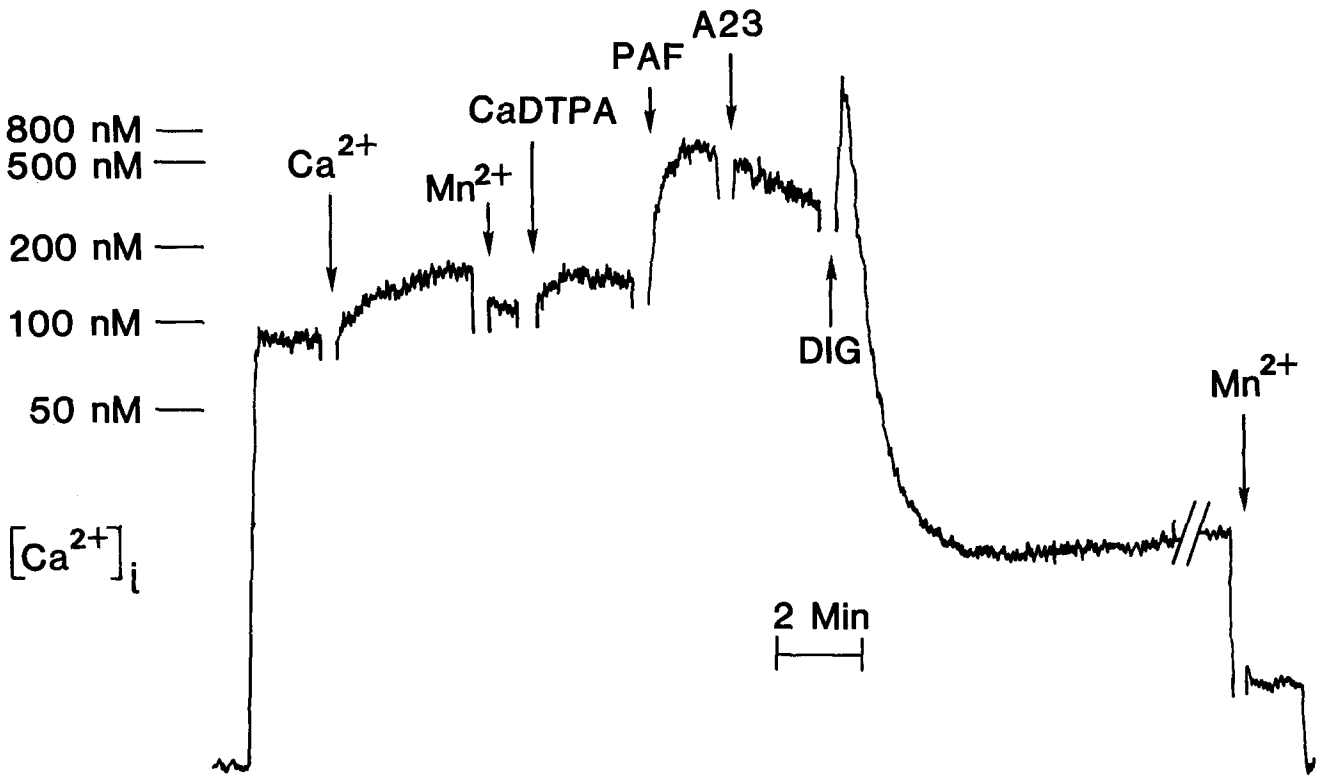


Figure 1. Response of macrophage $[Ca^{2+}]_i$ to $[Ca^{2+}]_o$ and PAF. Fluorescence was recorded at 500 nm from macrophages on coverglasses loaded with quin2. Beginning with cells in Ca-free saline additions were made as indicated: 1 mM Ca^{2+}, 0.1 mM Mn^{2+} (leak test), 0.2 mM CaDTPA to chelate the Mn^{2+}, 20 ng/ml PAF, 500 nM A23187, 50 μM digitonin (slash in the record represents a 7-min gap), and 1 mM Mn^{2+}.

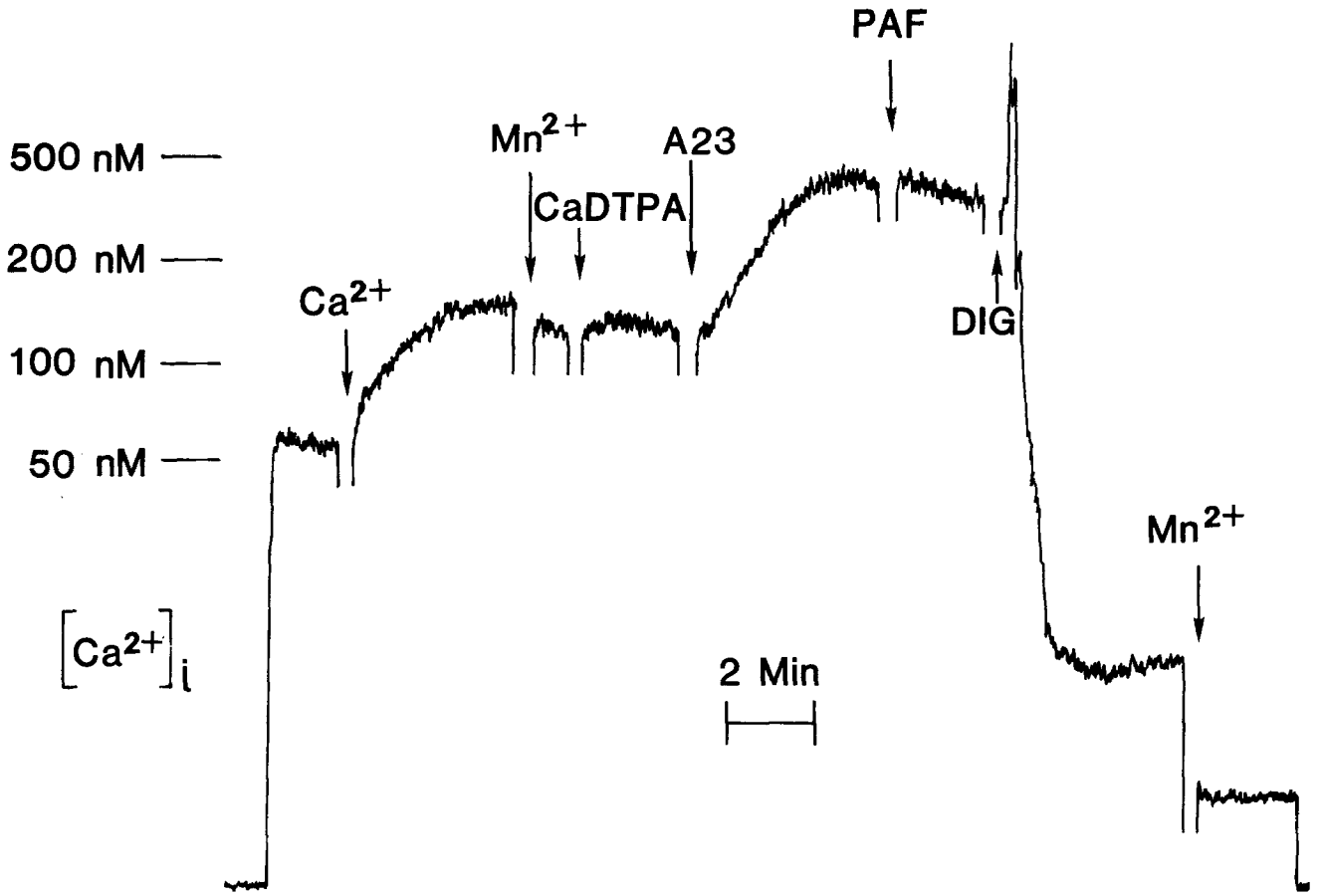


Figure 2. Response of macrophage $[Ca^{2+}]_i$ to $[Ca^{2+}]_o$ and A23187. Cells prepared as in Fig. 1. Additions made: 1 mM Ca^{2+}, 0.1 mM Mn^{2+}, 0.2 mM CaDTPA, 500 nM A23187, 20 ng/ml PAF, 50 μM digitonin, and 1 mM Mn^{2+}.

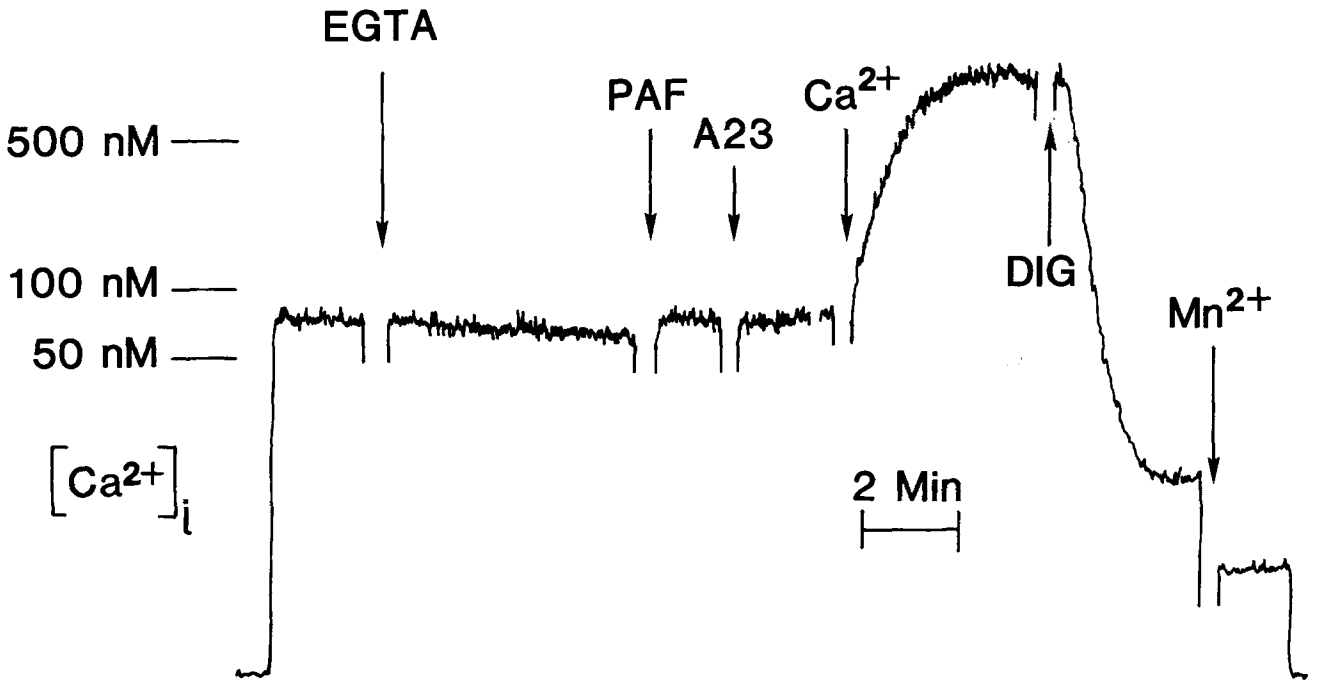


Figure 3. Effect of PAF in submicromolar $[Ca^{2+}]_o$. Cells prepared as in Fig. 1. Additions made: 5 mM EGTA, 20 ng/ml PAF, 500 nM A23187, 1 mM Ca^{2+} (free), 50 μM digitonin, and 1 mM Mn^{2+}.

the coverglasses (58). The presence of compound 48/80 did not interfere with the normal macrophage response to PAF described in Fig. 1 (data not shown).

As a control for the possible re-uptake of extracellular (leaked) quin2 by fluid-phase pinocytosis, a process that occurs very rapidly in macrophages (50), cells were incubated for the usual 30-min loading period in 50 μM quin2 (i.e., in the free acid, rather than in the usual 50 μM quin2/AM ester), followed by the normal rinses in Ca-free saline. The very low, background level fluorescence in such cells was not changed detectably by the standard additions of 1 mM Ca^{2+}, 0.1 mM Mn^{2+}, 0.2 mM CaDTPA, 20 ng/ml PAF, 50 μM digitonin, and 1 mM Mn^{2+} (data not shown), suggesting little uptake of leaked quin2 and no detection of general luminescence at 500 nm in response to adding PAF (chemiluminescence burst in response to PAF; reference 16).

Effect of PAF in Submicromolar $[Ca^{2+}]_o$

When PAF was applied to macrophages in the absence of exogenous Ca^{2+}, $[Ca^{2+}]_i$ still displayed a slight rise (Fig. 3), but it was greatly diminished compared with the rise seen in the presence of 1 mM Ca^{2+} (Fig. 1). Alternatively, if A23187 was added first, $[Ca^{2+}]_i$ rose no higher than it did in response to PAF (Fig. 4). In both cases, subsequent addition of A23187 (Fig. 3) or of PAF (Fig. 4) did not raise $[Ca^{2+}]_i$ beyond the level induced by the first agent. Regardless of the order in which the two agents were added, addition of 1 mM Ca^{2+} caused $[Ca^{2+}]_i$ to rise sharply, essentially to F_{max} (Figs. 3 and 4). The small, but consistent response to PAF in the virtual absence of exogenous Ca^{2+} was seen when the quin2-loaded cells were subjected to a variety of pretreatments: (*a*) simply left in Ca-free saline (thus left in ~10 μM $[Ca^{2+}]_o$, or (*b*) immediately incubated for 5 min in 5 mM EGTA (as in Fig. 3), or (*c*) incubated for a few minutes in 100 μM $[Ca^{2+}]_o$, to preload intracellular stores to some extent, followed by 1–5-min incubation in 1–5 mM EGTA. Regardless of which of these three protocols was followed, upon subsequent addition of 20 ng/ml PAF, $[Ca^{2+}]_i$ increased by 25 ± 7 nM (n = 6). Alternatively if cells were preincubated in 100 μM $[Ca^{2+}]_o$ (but not treated with EGTA), addition of 20 ng/ml PAF increased $[Ca^{2+}]_i$ by 138 nM (one experiment), a response intermediate between that seen in the presence of 10 μM or lower $[Ca^{2+}]_o$ (e.g., Fig. 3) and that seen in the presence of 1 mM $[Ca^{2+}]_o$ (e.g., Fig. 1). These data suggest that the increase in $[Ca^{2+}]_i$ induced by PAF arose from an influx of exogenous Ca^{2+}, together with some release of Ca^{2+} from intracellular stores.

Effect of Mn^{2+} and Cd^{2+} on the Response to PAF

If, indeed, PAF stimulates Ca^{2+} influx, then the presence of exogenous Mn^{2+} or Cd^{2+} might interfere with the response to PAF by blocking Ca^{2+} influx (2, 9, 26, 31, 57). Moreover, subsequent addition of DTPA, a membrane-impermeant chelator of heavy metals (52), with very high affinity for Mn^{2+} and Cd^{2+} but low affinity for Ca^{2+}, ought to relieve the inhibition quickly and allow Ca^{2+} influx. All of this assumes that Mn^{2+} and Cd^{2+} do not enter cells quickly. If such entry occurred, these ions would bind cytosolic quin2, suppress its fluorescence, and prevent the fluorescence increase in response to PAF. Such intracellular binding of quin2 would not be relieved quickly by addition of DTPA (such phenomena have been seen in platelets [10], but were not seen here).

Macrophages first were allowed to equilibrate to 1 mM Ca^{2+} and then were exposed to either 100 μM Mn^{2+} (Fig. 5) or 100 μM Cd^{2+} (Fig. 6) for 2 min. The instantaneous slight drop in fluorescence upon addition of Mn^{2+} or Cd^{2+} arose

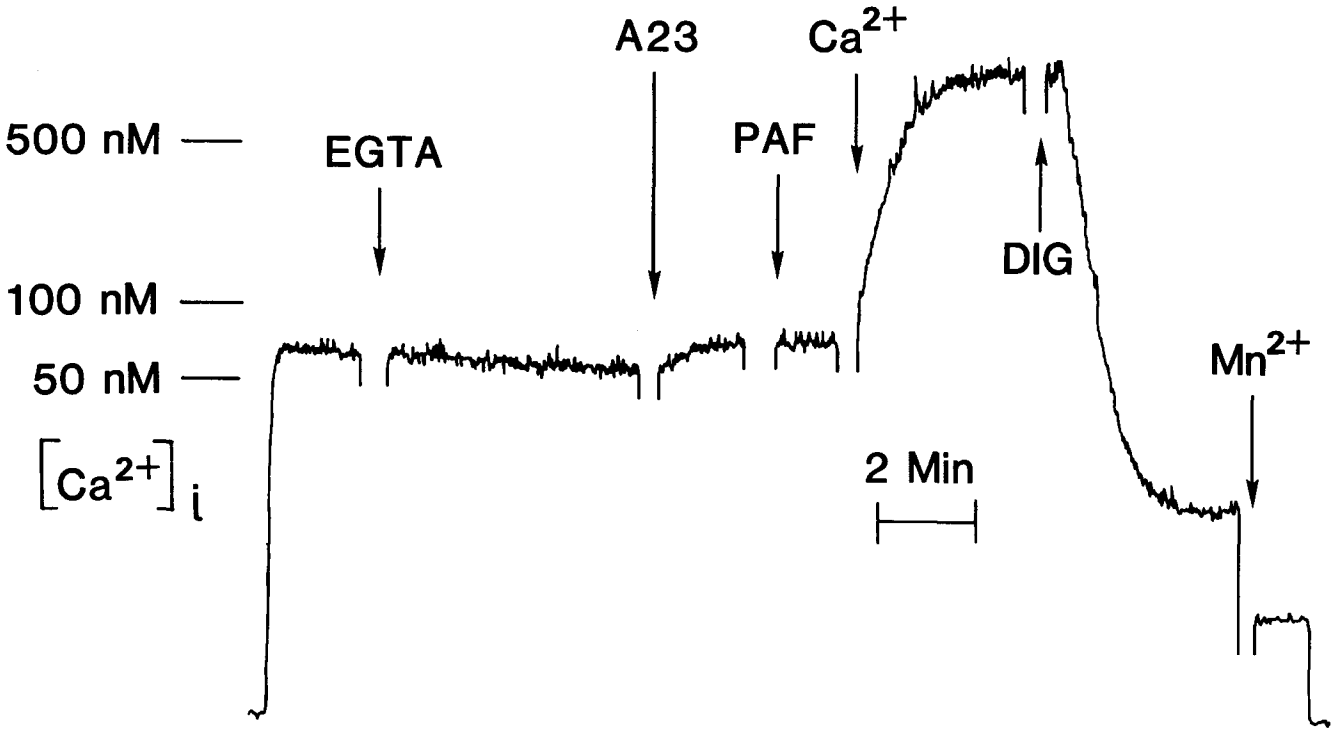


Figure 4. Effect of A23187 in submicromolar $[Ca^{2+}]_o$. Cells prepared as in Fig. 1. Additions made: 5 mM EGTA, 500 nM A23187, 20 ng/ml PAF, 1 mM Ca^{2+} (free), 50 μM digitonin, and 1 mM Mn^{2+}.

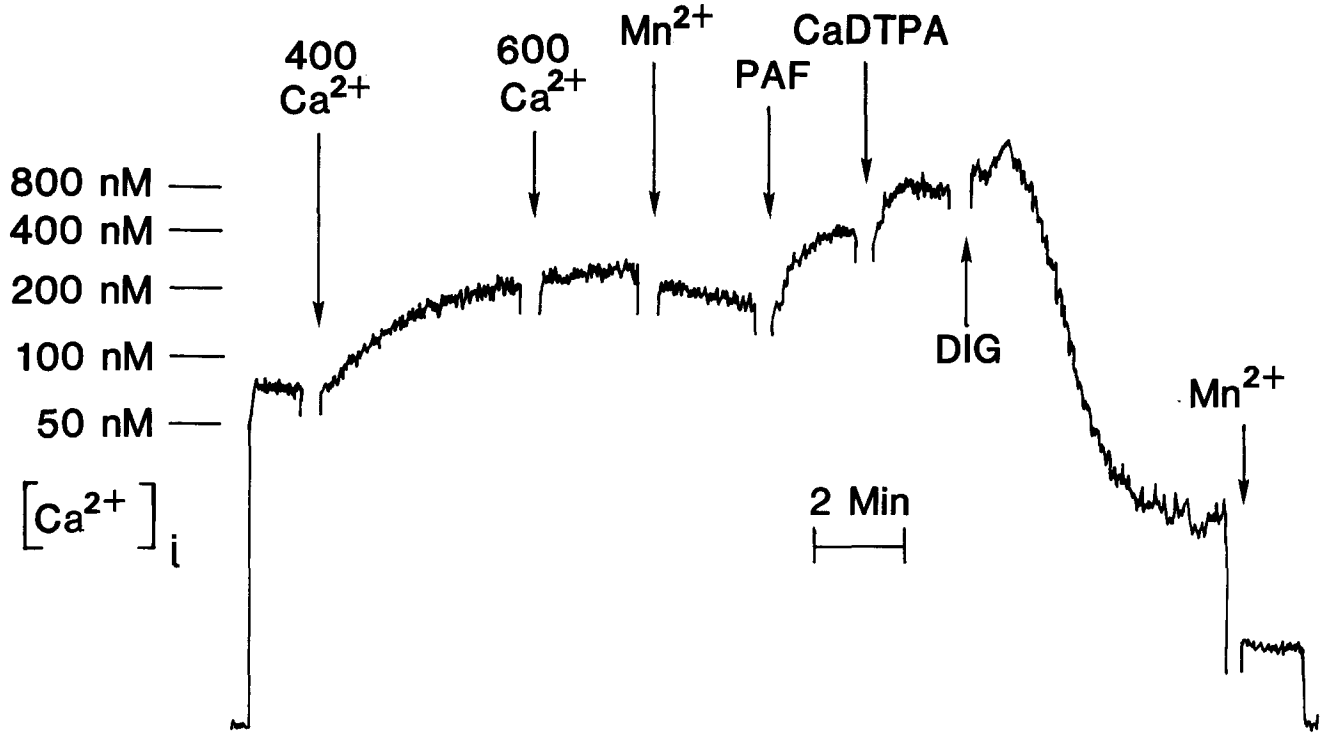


Figure 5. Effect of Mn^{2+} on the response to PAF. Cells prepared as in Fig. 1. Additions made: 400 μM Ca^{2+}, 600 μM Ca^{2+} (1 mM Ca^{2+}, total), 0.1 mM Mn^{2+}, 20 ng/ml PAF, 0.2 mM CaDTPA, 50 μM digitonin, and 1 mM Mn^{2+}.

from the quenching of extracellular quin2 (leak). The subsequent steady decline in fluorescence during the next 2 min presumably arose as the Mn^{2+} or Cd^{2+} slowly entered the cells and quenched some portion of the intracellular quin2 fluorescence (as seen in platelets, 10). Addition of PAF then caused a rise in $[Ca^{2+}]_i$ larger than that seen in the presence of submicromolar $[Ca^{2+}]_o$ (perhaps because intracellular stores had not been depleted by exogenous EGTA), but less than that seen in the presence of 1 mM Ca^{2+} alone: an increase of 295 nM in the case of Mn^{2+} (Fig. 5) and an in-

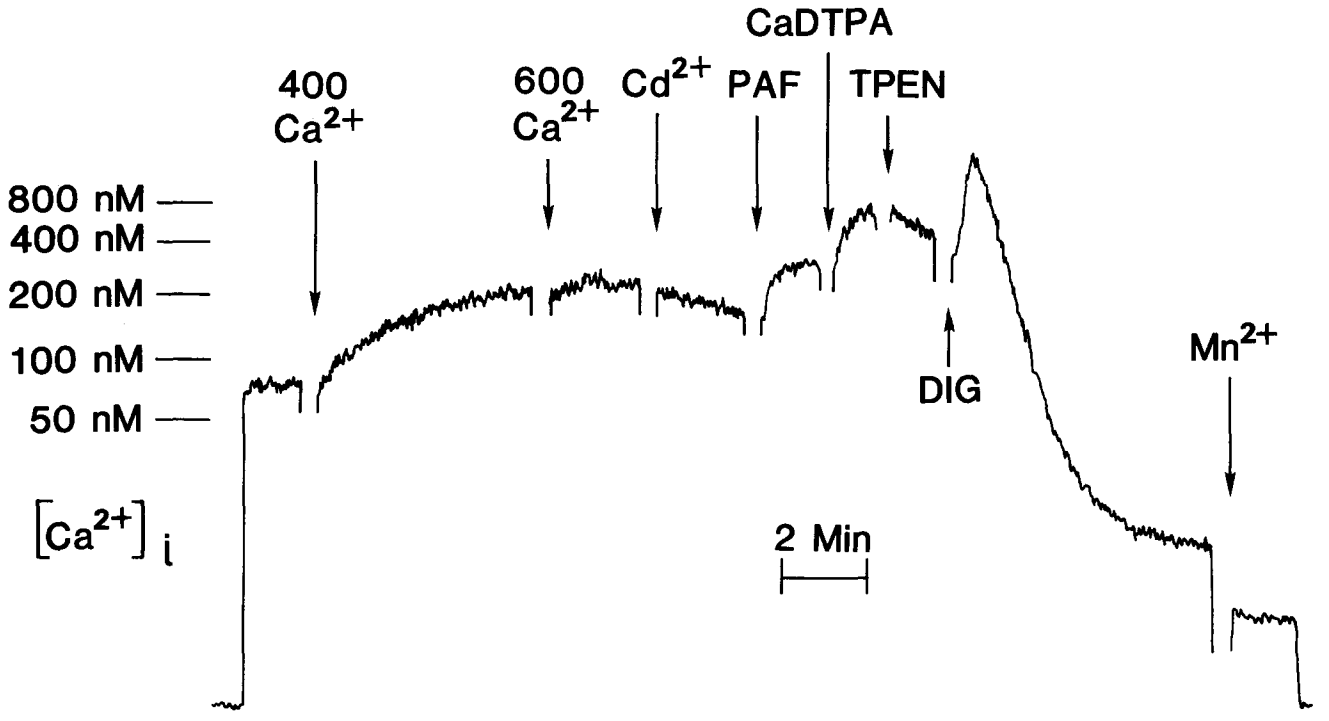
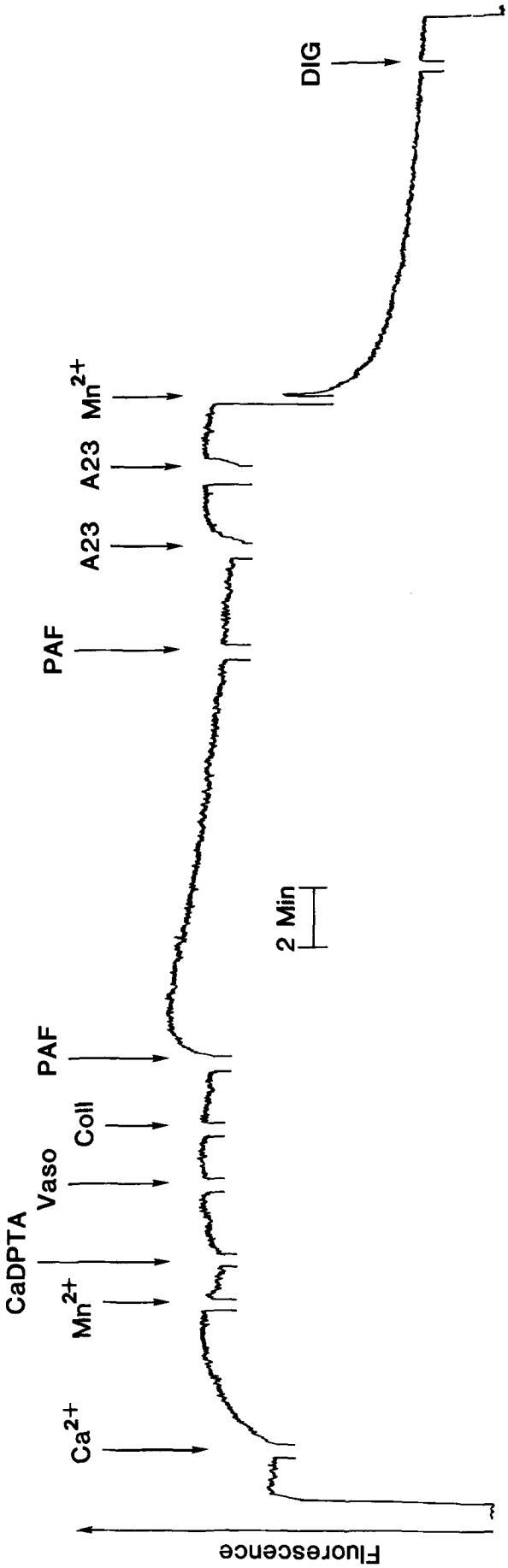


Figure 6. Effect of Cd^{2+} on the response to PAF. Cells prepared as in Fig. 1. Additions made: 400 μM Ca^{2+}, 600 μM Ca^{2+} (1 mM Ca^{2+}, total), 0.1 mM Cd^{2+}, 20 ng/ml PAF, 0.2 mM CaDTPA, 20 μM TPEN, 50 μM digitonin, and 1 mM Mn^{2+}.



crease of 142 nM in the case of Cd^{2+} (Fig. 6). Upon chelation of the exogenous heavy metals with 200 μM CaDTPA, $[Ca^{2+}]_i$ rose rapidly to levels normally attained when PAF was applied simply in the presence of 1 mM Ca^{2+}, presumably because exogenous Ca^{2+} was then free to enter the cells in response to the PAF that had been added previously.

Characteristics of the Response to PAF

The response was transitory: $[Ca^{2+}]_i$ rose to a peak in response to PAF and then gradually fell over the next several minutes (Fig. 7). Much of this fall must have occurred because of an actual fall in $[Ca^{2+}]_i$ (rather than leakage of dye) because even after a 15-min incubation period in PAF, application of A23187 caused a rise in $[Ca^{2+}]_i$ (Fig. 7). Second applications of PAF had no effect on $[Ca^{2+}]_i$ (Fig. 7). Primary doses of PAF of 2 ng/ml had the same effect as 20 ng/ml, as in human platelets (11; and Simpson, and T. J. Rink, manuscript in preparation). Primary doses as low as 0.1 and 0.2 ng/ml caused some elevation of $[Ca^{2+}]_i$; doses of 0.02 ng/ml were without effect. Unlike platelets, whose $[Ca^{2+}]_i$ can be increased by application of thrombin, even after responding to and recovering from a dose of PAF (11), the $[Ca^{2+}]_i$ of macrophages was not altered by thrombin, before or after PAF. Indomethacin at 10 μM did not suppress the response to PAF in either 1 mM $[Ca^{2+}]_o$ or in submicromolar $[Ca^{2+}]_o$ (data not shown).

In Fig. 7, the order of addition of digitonin and 1 mM Mn^{2+} was reversed from that used in previous figures to demonstrate that after cells were treated with A23187, they became permeable not only to Ca^{2+} but also to Mn^{2+}. The latter, no longer excluded from the cytosol, as it was during the leak test conducted earlier in Fig. 7, therefore could then quench intracellular as well as extracellular quin2 fluorescence (similar Mn^{2+} permeation into quin2-loaded, ionomycin-treated lymphocytes had been reported previously [19]). In the present study, the viability of such cells, as judged by trypan blue exclusion, remained above 95%; staining of cells occurred in all experiments only upon addition of digitonin. Thus, in Fig. 7 the cells in the monolayer retained intracellular quin2 until they were permeabilized with digitonin. The fluorescence level ($F_{Mn^{2+}}$) of quin2-loaded, Mn^{2+}-saturated viable cells remained unchanged upon addition of digitonin (Fig. 7), indicating that the auto-fluorescence level could be determined alternatively by adding digitonin first and 1 mM Mn^{2+} second. The latter method therefore was used routinely in this study (Figs. 1–6, 8, and 9) because it allowed determination of the transitory F_{max} value seen in the presence of digitonin, as well as $F_{Mn^{2+}}$.

An increase in $[Ca^{2+}]_i$ in response to PAF also was seen when the thioglycollate-elicited peritoneal lavage cells were loaded, rinsed, and maintained in the cuvette in suspension (data not shown) (39). Such cell populations would have contained mainly macrophages, together with other peritoneal cell types (e.g., lymphocytes and mast cells). In such preparations, the amount of extracellular quin2 (leak) was always

Figure 7. Effect of vasopressin, collagen, and PAF on $[Ca^{2+}]_i$. Cells prepared as in Fig. 1. Additions made: 1 mM Ca^{2+}, 0.1 mM Mn^{2+}, 0.2 mM CaDTPA, 1 μM vasopressin, 10 μg/ml collagen, 20 ng/ml PAF, another 20 ng/ml PAF, 200 nM A23187, another 200 nM A23187, 1 mM Mn^{2+}, and 50 μM digitonin. No quantitation was performed because Mn^{2+} was added before digitonin.

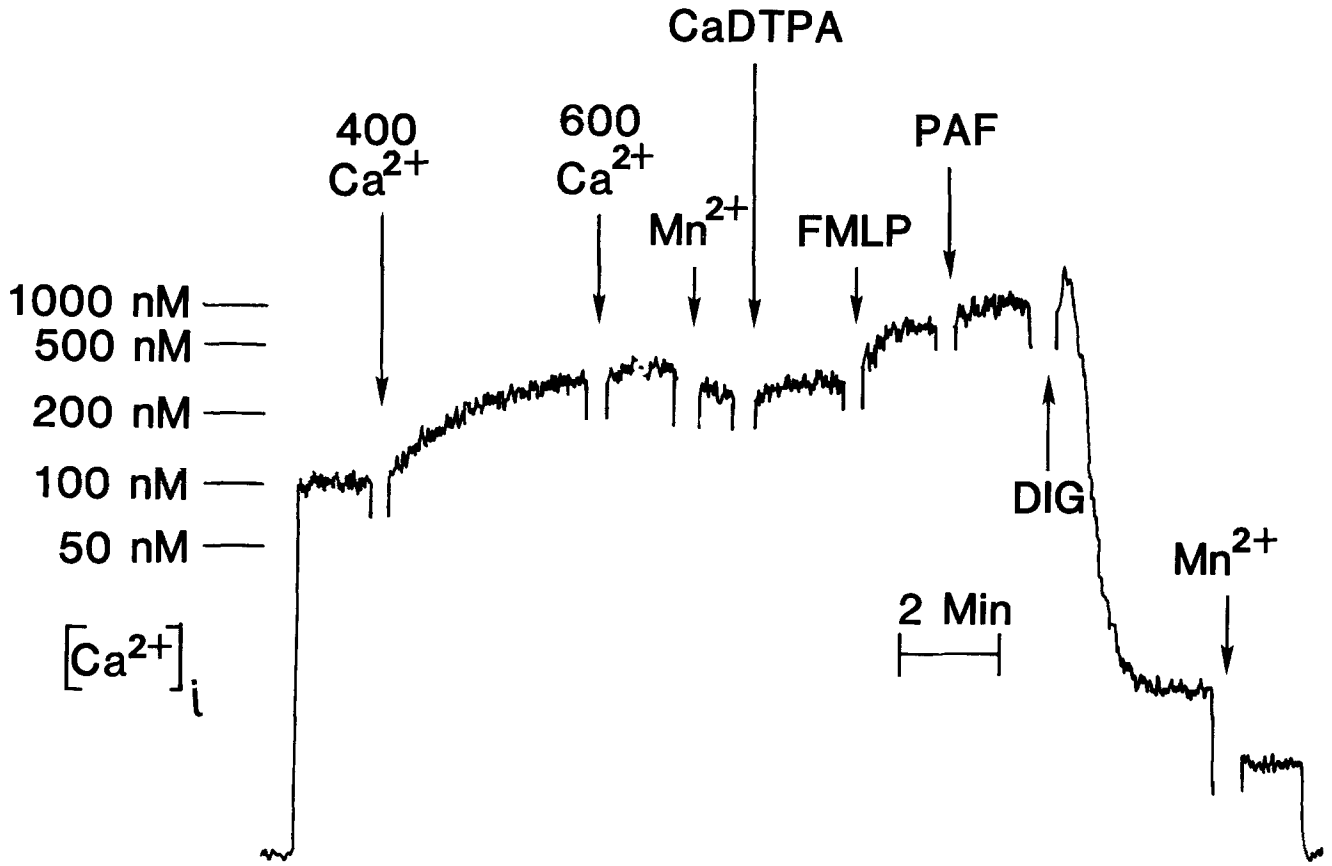


Figure 8. Effect of FMLP in presence of 1 mM $[Ca^{2+}]_i$. Cells prepared as Fig. 1. Additions made: 400 μM Ca^{2+}, 600 μM Ca^{2+} (1 mM Ca^{2+}, total), 0.1 mM Mn^{2+}, 0.2 mM CaDTPA, 1 μM FMLP, 20 ng/ml PAF, 50 μM digitonin, and 1 mM Mn^{2+}.

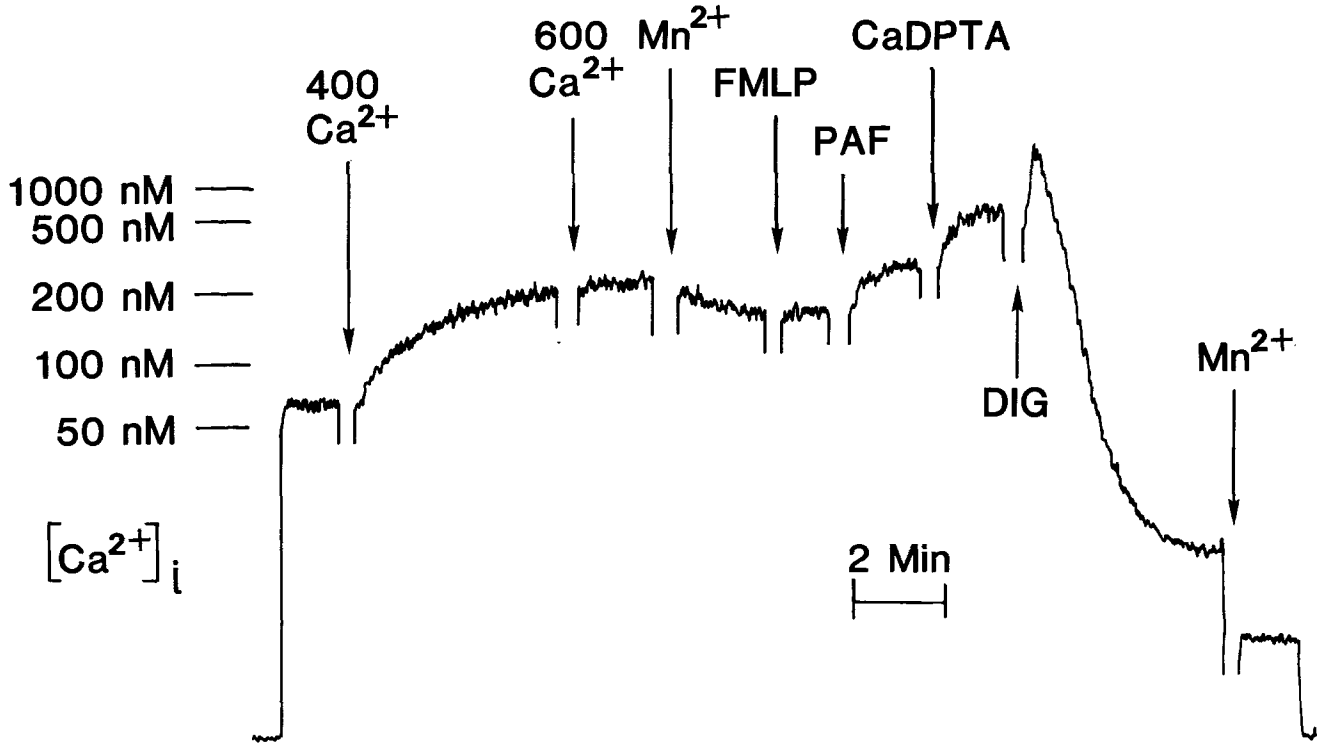


Figure 9. Effect of FMLP in presence of Mn^{2+}. Cells prepared as in Fig. 1. Additions made: 400 μM Ca^{2+}, 600 μM Ca^{2+} (1 mM Ca^{2+}, total), 0.1 mM Mn^{2+}, 1 μM FMLP, 20 ng/ml PAF, 0.2 mM CaDTPA, 50 μM digitonin, and 1 mM Mn^{2+}.

much larger than when using cells adhering to coverglasses (i.e., just macrophages); this substantial amount of leaked dye was present in suspended cells washed one to four times by centrifugation (250 *g*), as well as in cells collected and washed gently by gravity on a Millipore filter (8-μm pore size, Millipore Corp., Bedford, MA). The observations above suggested that both adherent and nonadherent macrophages could respond to PAF.

Other Agents Applied to Macrophages

In addition to PAF, the only other compound that was found to affect $[Ca^{2+}]_i$ in the macrophages was FMLP. In preliminary experiments, application of 1 μM FMLP in the presence of 1 mM Ca^{2+} caused $[Ca^{2+}]_i$ to rise by 363 nM (Fig. 8). Subsequent addition of PAF raised $[Ca^{2+}]_i$ by an additional 542 nM (Fig. 8). Alternatively, if 1 μM FMLP was applied in the presence of 1 mM Ca^{2+} and 100 μM Mn^{2+}, an increase of only 19 nM was seen (Fig. 9). That level was increased by another 123 nM after addition of PAF, and was increased by 233 nM by chelation of the exogenous Mn^{2+} with CaDTPA to allow Ca^{2+} influx (Fig. 9).

In survey experiments similar to those in Fig. 7, agents other than PAF and FMLP were tested for their ability to raise $[Ca^{2+}]_i$ in the macrophages. The following compounds did not raise $[Ca^{2+}]_i$ when applied before PAF: 1 U/ml thrombin, 10 μg/ml collagen, 10 μM ADP, 0.1 and 1 μM vasopressin, 1 and 2 μg/ml compound 48/80, 1 μM U46619 (a stable prostaglandin endoperoxide, a thromboxane A_2 mimic [42]), or 20 μM TPEN. The following compounds did not raise $[Ca^{2+}]_i$ when applied after PAF: 1 U/ml thrombin, 2 μg/ml concanavalin A, 1 μM transferrin, 1 mM histamine, or 20 μM TPEN. In addition, the $[Ca^{2+}]_i$ level of macrophages on coverglasses, as well as of total thioglycollate-elicited peritoneal lavage cells in suspension, remained unchanged upon addition of 5 μg/ml LPS and/or of 1,000 U/ml IFN-α,β, either when applied to previously untreated cells or when applied to cells preincubated for 4 h with either 5 μg/ml LPS or 1,000 U/ml IFN-α,β.

Discussion

The results above demonstrate that PAF induces a rapid rise in $[Ca^{2+}]_i$ in thioglycollate-elicited mouse macrophages. Most of the Ca^{2+} necessary for this rise comes from an influx of exogenous Ca^{2+}, but a small component appears to result from the release of some internally stored Ca^{2+}. The PAF-induced rise in $[Ca^{2+}]_i$ is transitory, and once the $[Ca^{2+}]_i$ returns to normal levels, a second application of PAF has no effect on $[Ca^{2+}]_i$. A variety of other compounds, some of which, like PAF, raise $[Ca^{2+}]_i$ in platelets, neither raise $[Ca^{2+}]_i$ in macrophages nor interfere with the PAF-induced rise in $[Ca^{2+}]_i$. Ionophore A23187 produces a rise in $[Ca^{2+}]_i$ which precludes a PAF-induced rise in $[Ca^{2+}]_i$, whereas FMLP elicits a rise in $[Ca^{2+}]_i$ which does not appear to interfere with a further PAF-induced rise in $[Ca^{2+}]_i$.

Measuring $[Ca^{2+}]_i$ in Cells on Coverglasses

To our knowledge, measurement of $[Ca^{2+}]_i$ in adherent, living macrophages has not been reported previously (in contrast to lysed cells; reference 28). There are several advantages to measuring quin2 fluorescence in macrophages on coverglasses rather than in suspension. (*a*) Cell purity is increased compared to suspensions of nonfractionated peritoneal lavage cells. (*b*) Less quin2 is leaked from macrophages on coverslips than from nonfractionated peritoneal lavage cells in suspension. (*c*) Rinsing of cells on coverslips can be performed faster than that of cells in suspension. (*d*) Perfusion is possible (29, 30) and effluents can be collected for assay of substances released in response to agonists, if desired. (*e*) Small populations of cells can be analyzed efficiently by inoculating them at high densities only along the central region of the coverglass, specifically in the area illuminated by the excitation beam and monitored by the emission photometer.

On the other hand, one possible problem encountered in calculating the $[Ca^{2+}]_i$ of macrophages on coverslips concerned the determination of F_{max}. Although F_{max} may be reached for some adherent cell types by addition of ionomycin (smooth muscle cells; reference 6) or A23187, we found for both of these ionophores that quin2 fluorescence in macrophages rose still higher when digitonin was applied but then fell as expected, presumably as the Ca^{2+} diffused from the permeabilized cells. Moreover, higher values for F_{max} were observed when digitonin was added to the sector of the cuvette in contact with the cells than when it was added to the sector to which cells were not immediately exposed. We do not think that this is an artifact of studying adherent cells or an artifact of digitonin. When platelets loaded with quin2 and maintained in suspension are first treated with ionomycin in the presence of 1 mM Ca^{2+}, the fluorescence rises sharply to stable level, but then rises to a somewhat higher level (designated as F_{max}) upon addition of Triton X-100 (37). The same phenomenon was observed in the present study when peritoneal lavage cells in suspension were treated with ionomycin or A23187 and then with digitonin (data not shown).

The possibility in the present experiments that absolute F_{max} was not reached may explain why the resting levels of $[Ca^{2+}]_i$ in 1 mM $[Ca^{2+}]_o$ determined here for adherent mouse macrophages (140 ± 14 nM, $n = 4$) are somewhat higher than those reported previously for rabbit alveolar macrophages (25), guinea pig alveolar macrophages (49), or adherent mouse peritoneal macrophages (28). Nevertheless, our values for resting $[Ca^{2+}]_i$ after a one-step addition of 1 mM $[Ca^{2+}]_o$ may indeed be accurate, because they were the same for both adherent macrophages determined as above and for suspensions of peritoneal lavage cells in suspension, determined according to Rink and Pozzan (39), (140 ± 14 nM, $n = 4$, and 151 ± 20 nM, $n = 4$, respectively).

The present observation that the $[Ca^{2+}]_i$ of adherent mouse macrophages was greater when $[Ca^{2+}]_o$ was brought to 1 mM in two steps rather than in one step also has been noted for lymphocytes (52). The $[Ca^{2+}]_i$ of lymphocytes appears to be less affected by $[Ca^{2+}]_o$ (52) than is that of macrophages (25, 53). The reasons for these differences remain unresolved.

Effects of PAF

The mechanism by which PAF activates platelets and interacts with other cell types remains under study and has been reviewed extensively (33, 47, 55). Both in platelets (10, 11, 45) and as shown here in macrophages, 2–20 ng/ml of PAF rapidly raises $[Ca^{2+}]_i$ in a transitory manner and renders

both cell types nonresponsive to second applications of PAF. These data suggest the presence of receptors for PAF on platelets (10, 11, 22, 46) and on macrophages. Binding of PAF to specific receptor sites on the plasma membrane of rabbit platelets is rapid and reversible (22). Specific PAF receptor sites could be detected on the plasma membranes of human and bovine platelets, bovine and guinea pig polymorphonuclear leukocytes, rabbit ileum, guinea pig ileum and lung, and rat trachea, but not on rabbit erythrocytes or on rat alveolar macrophages or tracheal and lung tissue of some other animals (22). Nevertheless, application of PAF to elicited guinea pig peritoneal macrophages causes a variety of responses (14–16). The structural requirements of the PAF receptor on guinea pig peritoneal macrophages (18) are similar to those of the PAF receptor on platelets (review; reference 47), although quantitative differences have been detected.

The mechanism of desensitization of the putative PAF receptors in vitro in platelets (11) and guinea pig ileal smooth muscle (8) is unknown. Thioglycollate-elicited mouse peritoneal macrophages do not appear to actively degrade PAF (44), whereas guinea pig peritoneal macrophages do degrade PAF (17).

Effect of PAF on $[Ca^{2+}]_i$

To our knowledge, an effect of PAF on $[Ca^{2+}]_i$ in macrophages has not been reported previously. The effect of PAF observed here appears to involve a major influx of exogenous Ca^{2+} through receptor-mediated channels, as it does in platelets (10, 11). The effect may be related to the increase in membrane permeability to Ca^{2+} which occurs in rat renal juxtaglomerular cells in response to PAF (35) and in isolated cardiac muscle fibers (51). The uptake of exogenous Ca^{2+} was demonstrated in the present study by the greatly diminished changes in $[Ca^{2+}]_i$ in response to PAF both in the presence of submicromolar $[Ca^{2+}]_o$ and in the presence of 1 mM Ca^{2+} containing 100 μM Mn^{2+} or Cd^{2+} as blockers of Ca^{2+} influx (2, 9, 26, 31, 57). Although Mn^{2+} and Cd^{2+} are known to have intracellular effects, their effects here appeared to be exerted predominately on the plasma membrane because their inhibition of quin2 fluorescence could be immediately relieved by addition of DPTA, a chelator of heavy metals, which does not cross cell membranes (10, 52). In addition to the major influx of exogenous Ca^{2+}, the studies reported here also show a small but consistent response of $[Ca^{2+}]_i$ to PAF in the presence of submicromolar exogenous Ca^{2+}, suggesting that PAF can cause release of some Ca^{2+} from intracellular stores, as in platelets (11, 45). An apparent increase in membrane permeability to Mn^{2+}, seen in platelets in response to PAF (10), was not observed here in macrophages.

Effects of Other Compounds

The present study gives preliminary evidence that the chemotactic peptide, FMLP, raises $[Ca^{2+}]_i$ in mouse macrophages. A similar effect for FMLP has been observed in human and rabbit neutrophils (36, 59) and for the related N-formylated peptides, N-formyl-L-methionyl-L-phenylalanine and N-formyl-L-norleucyl-L-leucyl-L-phenylalanine, in guinea pig alveolar macrophages (20, 21, 49). The present data affirming the responsiveness of mouse macrophages to FMLP contradicts an earlier suggestion of the apparent nonresponsiveness of mouse macrophages to FMLP and other N-formylated peptides (1) and requires further experimental examination.

Collagen, vasopressin, thrombin, U46619, and ADP are compounds that have been demonstrated to raise $[Ca^{2+}]_i$ in human platelets (10–12, 38, 41, 42), but in the present work, they did not have detectable effects on the $[Ca^{2+}]_i$ of macrophages. IFN-α and IFN-β when applied individually to elicited mouse peritoneal macrophages at concentrations of 1,000 U/ml have been shown to be equivalent to IFN-γ at 1 U/ml in priming the cells for tumor cell killing (34). In contrast, a mixture of IFN-α and IFN-β, even at 1,000 total U/ml does not activate protein kinase C activity, whereas IFN-γ at 1 U/ml causes a fivefold stimulation of activity (13). In the present experiments, neither a mixture of IFN-α and IFN-β at 1,000 total U/ml nor LPS at 5 μg/ml changed $[Ca^{2+}]_i$ detectably, even when cells were pretreated for 4 h with LPS or with IFN-α,β.

Implications

In this paper we have demonstrated that PAF raises the $[Ca^{2+}]_i$ of thioglycollate-elicited mouse peritoneal macrophages adhering to a substratum. We did not determine whether this treatment, in addition, activated the cells (1) or would have had the same effect on resident macrophages or on macrophages from other sites (e.g., alveolar). Because it is known that PAF can prime or totally activate guinea pig peritoneal elicited macrophages (14–18), it will be interesting to determine whether the PAF-induced rise in $[Ca^{2+}]_i$ seen here is necessary for the other events of normal macrophage activation and for release of endogenous PAF (43, 44). Treatment of mouse bone marrow–derived macrophages for 4 h with 1–3 μM A23187 resulted in macrophage priming for tumor cell killing, whereas incubation for 4 h with 3 μg/ml PAF did not cause priming (23); our results would suggest that these high concentrations of A23187 and PAF would have caused rapid elevation of $[Ca^{2+}]_i$, but it is not possible to predict how long it would have been maintained or how rapidly the cells would have degraded these agents. The PAF released from other cell types, and from the macrophages themselves, may mediate macrophage participation in such conditions as endotoxin-induced hypotension (7), PAF-induced circulatory collapse (4), and other conditions of shock, asthma, allergic responses, and anaphyllaxis.

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