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INHIBITION BY MANOALIDE OF fMLP-STIMULATED ELASTASE RELEASE FROM HUMAN NEUTROPHILS

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Abstract—Incubation of human polymorphonuclear leukocytes (PMNLs) with the chemotactic factor *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) resulted in a concentration-dependent release of the neutral protease elastase. This response was inhibited by pretreatment of the PMNLs with manoalide (IC_{50} approximately 0.08 μM). To understand the mechanism of this inhibition, we examined the effect of manoalide on the signal-transduction pathway believed to mediate fMLP stimulation. We observed in fura-2 loaded cells that pretreatment with manoalide blocked fMLP-induced increases in cytosolic free-calcium (IC_{50} approximately 0.15 μM). However, manoalide had no effect on inositol 1,4,5-trisphosphate (IP_3) production at concentrations which completely inhibited the Ca^{2+} signal. Furthermore, manoalide was approximately 50-fold less potent as an inhibitor of phospholipase C activity in membrane preparations of PMNLs than as an inhibitor of calcium mobilization in whole cells. These data indicate that manoalide can block stimulation of human PMNLs through inhibition of Ca^{2+} mobilization, but that this occurs at a site beyond phospholipase C activation and inositol phosphate turnover.

Manoalide, a marine natural product, has been shown to have activity in *in vivo* models of inflammation [1]. In light of studies demonstrating manoalide to be a potent inhibitor of phospholipase A_2 (PLA_2)† from both bee and cobra venoms and of β-bungarotoxin neurotoxicity [2–4], it was suggested that its anti-inflammatory effects may be due, in part, to inhibition of phospholipases *in vivo*. Recent studies have shown manoalide to inhibit PLA_2 activity in cytosolic fractions from several mammalian tissues [5], and to block arachidonic acid release from calcium ionophore A23187-stimulated rabbit polymorphonuclear leukocytes (PMNLs) [6]. However, the drug also has been shown to decrease significantly Ca^{2+} mobilization in a number of cell types [7], as well as to inhibit phospholipase C and 5-lipoxygenase enzymes [8, 9]. Thus, further study of manoalide was undertaken to clarify its mechanism of action as an anti-inflammatory drug.

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† Abbreviations: PLA_2, phospholipase A_2; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TRIS, tris (hydroxymethyl) aminomethane; PMNL, polymorphonuclear leukocyte; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LDH, lactate dehydrogenase; IP, inositol 1-phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; TMB-8, 8-(*N*,*N*-diethylamino)-octyl:3,4,5-trimethoxybenzoate hydrochloride; fura-2 AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5-methylphenoxy)-ethane-*N*, *N*, *N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester; FDA, fluorescein diacetate; MAPTAM, 1,2-bis-5-methyl-aminophenoxylethane-*N*,*N*,*N*′-tetraacetoxymethylacetate; and NAD, nicotinamide adenine dinucleotide.

Polymorphonuclear leukocytes have shown to be a major component of the cellular infiltrate accompanying the early stages of inflammation and allergic skin reactions. It is the local accumulation of neutrophils, in fact, which is believed to contribute in large measure to the associated tissue damage. In the presence of a variety of pro-inflammatory stimuli, these cells exhibit a series of responses including aggregation, chemotaxis, generation of superoxide and other reactive oxygen metabolites, and degranulation [10]. Since release of lysosomal enzymes contributes to the inflammatory response seen *in vivo*, we examined the effect of manoalide on the release of elastase from human neutrophils stimulated with the chemotactic peptide fMLP.

Mobilization of intracellular calcium appears to be a necessary early step in this process, with the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by phospholipase C being responsible for the initial increase in cytosolic free Ca^{2+} [11]. The products formed from the hydrolysis of PIP_2 are inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which have been shown to raise intracellular Ca^{2+} levels and to activate protein kinase C, respectively [12, 13]. Since manoalide has been reported to inhibit phosphatidylinositol specific phospholipase C [8] and to block Ca^{2+} channels [7], we examined the possibility that the effect of the drug on elastase release may be due, at least in part, to inhibition of this signal transduction pathway.

MATERIALS AND METHODS

Materials. Histopaque, HEPES, TRIS, digitonin, cytochalasin B, fMLP, TMB-8, 7-amino-4-methylcoumarin and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methyl-coumarin were obtained from Sigma. Fura-2-AM was purchased from Molecular

Probes, Inc. [2-^{3}H]Inositol (16.3 Ci/mmol) and L-3-phosphatidyl[2-^{3}H]inositol (10–15 Ci/mmol) were purchased from Amersham. All inorganic reagents were obtained from Mallinckrodt or Sigma.

PMNL isolation. Polymorphonuclear leukocytes were isolated as previously described [9]. Briefly, human whole blood was layered on discontinuous Histopaque gradients and the PMNLs were separated from monocytes and red blood cells (RBCs) by centrifugation at 700 *g* for 30 min at room temperature. Contaminating RBCs were lysed by treatment with 144 mM NH_4Cl, 17 mM TRIS (pH 7.2) at 37°. The final mixed granulocyte preparation contained 95 ± 0.4% neutrophils, as determined by Giemsa–Jenner staining.

Loading of fura-2 into PMNLs and measurement of calcium mobilization. After isolation, PMNLs were washed with medium containing 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 1 mg/mL glucose, and 1 mg/mL sodium pyruvate, pH 7.4, and resuspended in the same at 10^6 cells/mL. The calcium indicator dye fura-2-AM was added (2 μM) and incubated with the suspension for 15 min at 37°. After centrifugation for 10 min at 300 *g*, PMNLs were washed with medium containing 1 mM $CaCl_2$ before being suspended at 10^6 cells/mL. Intracellular calcium determinations were made on a Perkin–Elmer LS-5 fluorescence spectrophotometer. Cells were kept in suspension with a mechanical stirring device, and the cuvette temperature was maintained at 37° via a water-filled jacket connected to a circulating water bath. A constant fluorescence signal was recorded with the excitation wavelength set at 340 nm, and the emission wavelength set at 500 nm. Then 1.5 mL of PMNL suspension was placed in a cuvette along with 1.5 mL of medium. The cells were allowed to equilibrate for 5 min before drug or vehicle was added. After an additional 5 min, fMLP was added, and the change in fluorescence was determined. Twenty microliters of 100 mg/mL digitonin in DMSO was added in order to lyse the cells and determine the maximum fluorescence. Then 20 μL of 500 mM $MnCl_2$ was added to quench the activity of the fura-2 dye and deliver the basal fluorescence level (fMn). The concentration of free cytosolic (intracellular) Ca^{2+} was determined [14] by applying the equation:

$$[Ca^{2+}]_i = 220\ \text{nM} \cdot (f - f_{min})/(f_{max} - f)$$

where f_{max} = maximum fluorescence and f_{min} = fMn + 1/6 (f_{max} − fMn). Results are expressed as:

$$\%\ \text{of resting level} = \frac{[Ca^{2+}]\ \text{at maximum of agonist-induced peak} \cdot 100\%}{[Ca^{2+}]\ \text{at resting (pre-agonist) level}}.$$

Measurement of elastase release. Release of the neutral protease elastase from PMNLs was measured by a spectrofluorometric method described by Sklar *et al.* [15]. Cells (2×10^6 were suspended in a cuvette with 3 mL of medium containing 1 mM $CaCl_2$, 20 μM MeO-Suc-Ala-Ala-Pro-Val-MCA (substrate), 1 μg/mL of cytochalasin B, and either vehicle or drug. Fluorescence was recorded using an SLM 8000 fluorescence spectrophotometer with the excitation and emission wavelengths set at 370 and 440 nm respectively. After a 5-min incubation period, either vehicle or fMLP was added. The initial (maximum) rate of signal increase was determined, and the enzyme rate was calculated using methylcoumarin amide (MCA) as a standard. The enzyme rate was a reflection of the amount of elastase released from the cells.

To measure levels of lactate dehydrogenase (LDH), cell suspensions were centrifuged, and the resultant cell-free supernatants analyzed on a Beckman AS-8-ASTRA system. LDH activity was determined by a spectrophotometric assay which measured the reduction of NAD. Release of LDH from PMNLs was used as an indication of cytotoxicity.

[^{3}H]*Inositol labeling of PMNLs and measurements of inositol phosphates.* After isolation, PMNLs were washed with medium containing 0.5 mM $CaCl_2$, and resuspended in the same at 5×10^6 cells/mL. Then cells were pulsed with [^{3}H]inositol at 100 μCi/mL for 4 hr at 37°. LiCl was added (5 mM), and the suspension was centrifuged for 10 min at 300 *g*. Then PMNLs were washed twice with equal volumes of medium containing 0.5 mM $CaCl_2$ and 5 mM LiCl before a final suspension was prepared at 1×10^6 cells/mL. Aliquots (1.0 mL) were incubated for 5 min at 37° with manoalide or vehicle before fMLP (10^{-8} M) was added. Cells were incubated for the specified amount of time before 1.8 mL of chloroform : methanol : 4 N HCl (100 : 200 : 2) was added to stop the reaction. The inositol phosphates were extracted and separated on AG-1 X8 resin columns according to the method described by Beaven *et al.* [16]. IP, IP_2, and IP_3 were eluted from the columns with 200 mM, 400 mM, and 1 M concentrations, respectively, of ammonium formate in 0.1 M formic acid. Disintegrations per minute (dpm) were measured for each of the column fractions, and the time course for IP_3 was calculated according to the following equation:

$$\%\ \text{of basal dpm} = (\text{dpm at time point})/(\text{dpm at time zero}) \cdot 100\%.$$

Measurement of phospholipase C activity. Isolated PMNLs were resuspended at 40×10^6 cells/mL in homogenization buffer (300 mM sucrose, 5 mM HEPES, 2 mM EGTA, pH 7.0). Cells were disrupted first by mechanical homogenization, and then by sonication (2 × 15 sec) on ice. After centrifugation at 100,000 *g* for 60 min, the pellet was resuspended in homogenization buffer. The pellet was assayed subsequently for phospholipase C activity by measurement of the release of [^{3}H]inositol phosphates from [^{3}H]phosphatidylinositol-4,5-bisphosphate (PIP_2), as described by Bennett *et al.* [8]. Briefly, the reaction mixture consisted of the 100,000 *g* pellet from approximately 800,000 cells, assay buffer (50 mM HEPES, 50 mM NaCl, pH 7.0) and manoalide or vehicle. This mixture was preincubated for 20 min at 37°. The reaction was initiated by the addition of LiCl (5 mM), $CaCl_2$ (10 μM in excess of EGTA) and 0.2 mM PIP_2 (approximately 50,000 dpm) in 1.5 mM deoxycholate. The final volume was 200 μL. After incubation for 10 min, the reaction was stopped by the addition of 750 μL of

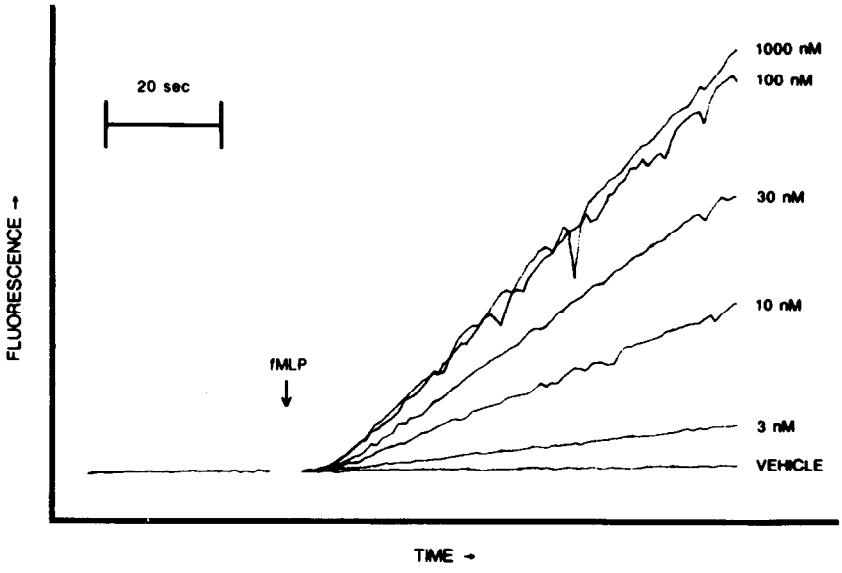

Fig. 1. Elastase release from human neutrophils in response to fMLP. The data are presented as an increase in fluorescence resulting from cleavage of the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA and release of aminomethylcoumarin. Cell suspensions (0.7×10^6 cells/mL) were equilibrated for 5 min at 37° prior to addition of the stimulus. At the indicated time, vehicle or various concentrations of fMLP were added and fluorescence measurements were made with an SLM 8000 spectrofluorometer with continuous data acquisition capabilities.

chloroform:methanol:4 N HCl (100:200:2). This was followed by adding 0.25 mL each of $CHCl_3$ and 0.5 N HCl. Samples were vortexed, and the organic and aqueous phases were separated by centrifugation at 1000 *g* for 10 min. After centrifugation, 0.6 mL from the aqueous layer was removed and radioactivity was measured in a Beckman LS3801 scintillation counter. Under conditions in which the substrate conversion was <7%, the assay was linear with protein concentration.

RESULTS

Elastase release. Incubation of human PMNLs in the presence of fMLP led to a linear increase in fluorescence after an initial lag period of approximately 10 sec (Fig. 1). The response remained linear for at least 70 sec, with the level of fluorescence directly proportional to the amount of the fluorophore MCA released. The measured elastase activity represented secretion of the enzyme because removal of the PMNLs by centrifugation led to a loss of <5% of elastase activity in the supernatant. Furthermore, this was not a generalized release of enzymes since LDH activity in the supernatant showed no increase over background even at the highest concentration of fMLP tested (Table 1). The amount of elastase released was concentration-dependent with an EC_{50} for fMLP of approximately 2×10^{-8} M (Fig. 2). We found that 75–85% of the total cellular elastase activity could be released by maximum concentrations of fMLP. Preincubation of the PMNLs with manoalide led to a time- and concentration-dependent inhibition of this response, with a maximum effect seen after 2 min (Fig. 3) and an IC_{50} of approximately 8×10^{-8} M (Fig. 4). To determine if manoalide was inhibiting elastase release or having a direct effect on enzyme activity, samples were stimulated with fMLP, and the cells removed by centrifugation; then manoalide (5×10^{-7} M) was added to the supernatants. Under these conditions, manoalide did not inhibit elastase activity (data not shown). Manoalide alone had no measurable effect on LDH release until concentrations > 10^{-6} M (Table 1).

Ca^{2+} mobilization. The addition of fMLP to suspensions of fura-2-loaded PMNLs led to a rapid, transient increase in fluorescence (Fig. 5). It has been shown previously that this measured increase in the concentration of cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, is dependent on both release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane [17, 18]. Indeed, in our studies pretreatment of the cells with TMB-8, a known antagonist of intracellular Ca^{2+} release [19, 20], produced a concentration-dependent inhibition of the fura-2 signal (Fig. 6), while a short pretreatment with EGTA led to a 30% reduction in the response (data not shown). In agreement with previous reports [17], this Ca^{2+} influx in PMNLs was apparently not due to activation of voltage-dependent calcium channels since addition of 40 mM KCl to the cell suspension did not produce an increase in $[Ca^{2+}]_i$ (data not shown). Furthermore 10^{-5} M nifedipine and 10^{-5} M verapamil, concentrations known to block voltage-dependent calcium channels, had no effect on fMLP-induced increases in $[Ca^{2+}]_i$.

Stimulation of $[Ca^{2+}]_i$ by fMLP was a concentration-dependent response with an EC_{50} of approximately 1×10^{-9} M (Fig. 2). These data

Table 1. Lactate dehydrogenase release from human neutrophils

[fMLP]	I.U./million cells (×100)	[Manoalide]	I.U./million cells (×100)
Vehicle	1.3	0.1 μM	1.2
0.1 μM	0.8	0.3 μM	1.4
1.0 μM	0.9	1.0 μM	1.9
10.0 μM	0.7	3.0 μM	4.6
0.02% Triton	9.1	10.0 μM	7.8

PMNLs were incubated in the presence of vehicle or increasing concentrations of fMLP or manoalide. LDH activity released into the medium was determined spectrophotometrically by the reduction of NAD, as described in Materials and Methods. Maximum enzyme release was determined by incubating the cells in the presence of 0.02% Triton X-100.

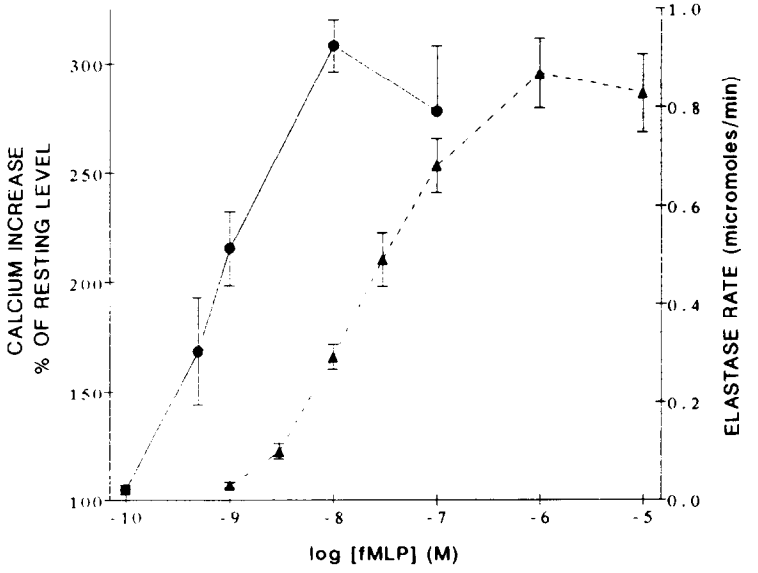

Fig. 2. Activation of human neutrophils by the chemotactic stimulus fMLP. Increases in $[Ca^{2+}]_i$ (●) were measured in fura-2 loaded cells as described in the legend of Fig. 5, while measurement of elastase release (▲) was as described in Fig. 1. Cell suspensions were incubated in the presence of vehicle or increasing concentrations of fMLP. Values are the means ± SE of 3–4 experiments.

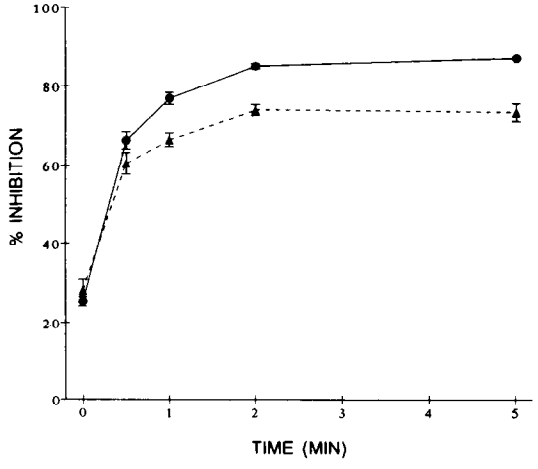

Fig. 3. Effect of preincubation time on the inhibition by manoalide of elastase release. PMNLs were incubated with vehicle or manoalide at two concentrations, 0.3 μM (●) or 0.1 μM (▲), for various times before the addition of 1 μM fMLP. The measurement of elastase release was as described in Materials and Methods. Values are the means ± SE of 3 experiments.

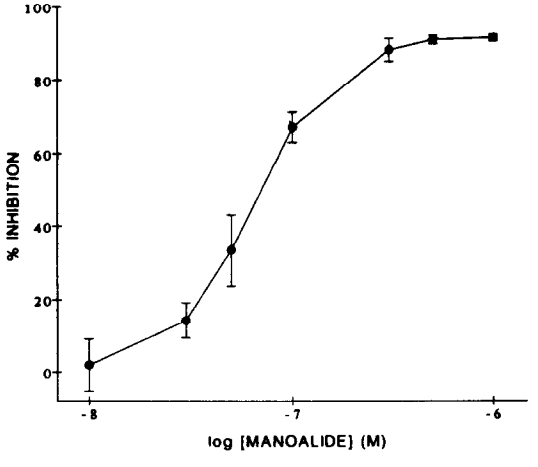

Fig. 4. Inhibition of elastase release by manoalide. Cell suspensions were preincubated with vehicle or increasing concentrations of manoalide for 5 min at 37° before the addition of fMLP. Measurement of elastase release was as described in Materials and Methods. Values are the means ± SE of 3–4 experiments.

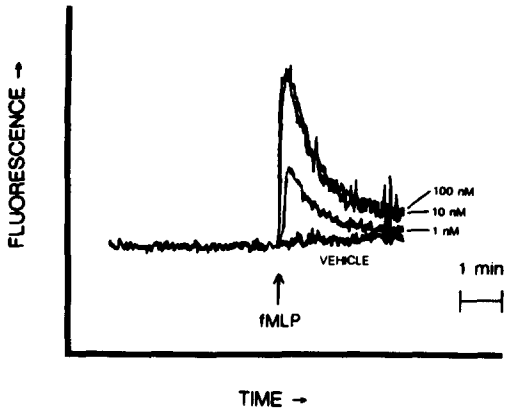

Fig. 5. fMLP-induced increase in $[Ca^{2+}]_i$ in human neutrophils. PMNLs were isolated and loaded with fura-2 as described in Materials and Methods. Approximately 5×10^5 cells/mL were suspended in physiological buffer containing 1.0 mM $CaCl_2$ and incubated in a fluorometer at 37° with continuous stirring. At the indicated time, vehicle or various concentrations of fMLP were added to the cell suspension. The resting $[Ca^{2+}]_i$ was determined to be 206 ± 10 nM (N = 10).

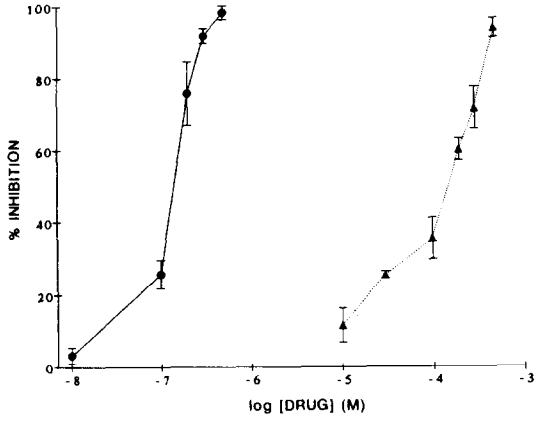

Fig. 6. Inhibition of calcium mobilization in human neutrophils. PMNL cell suspensions were preincubated with vehicle or increasing concentrations of manoalide (●) or TMB-8 (▲) for 5 min at 37° before the addition of fMLP. Values are the means ± SE of 3–4 experiments.

demonstrated that fMLP was more than 10-fold more potent in stimulating an increase in $[Ca^{2+}]_i$ than in eliciting elastase release from PMNLs. Pretreatment of PMNLs with manoalide led to an inhibition of the fMLP-induced Ca^{2+} signal. This was a concentration-dependent effect, with an IC_{50} of approximately 1.5×10^{-7} M (Fig. 6). Manoalide alone had no effect on the fura-2 signal at concentrations less than 1.0 μM. However, concentrations equal to or greater than 3 μM appeared to be cytotoxic, leading to prolonged, maximal fluorescence signals and reduced cell number as determined by FDA uptake. Manoalide appeared to be equipotent in inhibiting both Ca^{2+} mobilization and enzyme release.

Inositol phosphate production. Mobilization of cellular calcium in PMNLs by fMLP is believed to be mediated through stimulation of phospholipase C and the resulting generation of IP_3 [11]. To determine at what point in this signal transduction path-

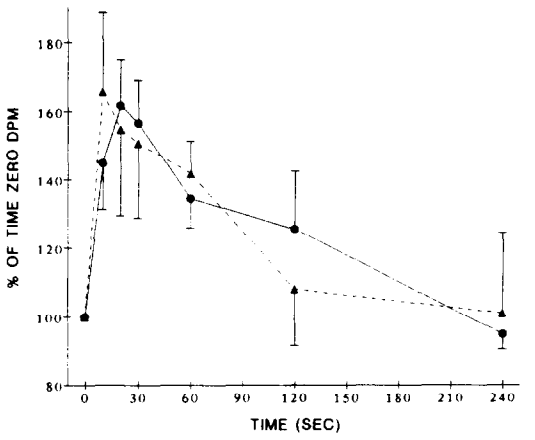

Fig. 7. Effect of manoalide on IP_3 production in human neutrophils. Cell suspensions (5×10^6 cells/mL) were pulsed with [^{3}H]inositol (100 μCi/mL) for 4 hr at 37°. Labeled cells were then preincubated with vehicle (●) or manoalide (3×10^{-7} M) (▲) for 5 min at 37° prior to the addition of fMLP (1×10^{-8} M). IP_3 production was measured as described in Materials and Methods. Values are the means ± SE of 4 experiments.

way manoalide might be acting, we examined inositol phosphate production. We found that production of IP_3 in response to fMLP was transient, with a peak occurring at about 20 sec (Fig. 7). This was approximately a 50–60% increase over basal levels. The production of IP and IP_2 showed much different response patterns. IP accumulated more slowly, with peak levels not being observed until 120 sec after stimulation. The level of IP_2 was more variable with no obvious peak in concentration over the period studied (data not shown).

Pretreatment of PMNLs with manoalide at 3×10^{-7} M (a concentration which completely blocked the Ca^{2+} signal) had no effect on IP_3 production induced by fMLP at 1×10^{-8} M (Fig. 7). Similarly, manoalide had no effect on the levels of IP or IP_2. These data indicate that the inhibition by manoalide of fMLP-induced increases in $[Ca^{2+}]_i$ in PMNLs is not related to an effect on inositol phosphate production nor, therefore, on phospholipase C.

Phospholipase C activity. In the 100,000 *g* membrane fractions of PMNLs, phospholipase C activity (using phosphatidylinositol-4,5-bisphosphate as the substrate) was approximately 8.6 nmol/mg protein/min. Preincubation of these fractions with manoalide led to a concentration-dependent inhibition of the enzyme (Fig. 8). Under these conditions, manoalide had an IC_{50} of approximately 80 μM against phospholipase C. Previous studies have demonstrated that the amount of protein in the assay affects the potency of manoalide as a phospholipase inhibitor [5]. Since we used a membrane preparation in the enzyme assay equivalent to eight times the number of cells in our Ca^{2+} mobilization experiments, we examined the effect of such a difference in protein content on the potency of manoalide against PI-PLC. We found that a decrease in protein content to the levels used in the Ca^{2+} experiments would be expected to increase the potency of manoalide by

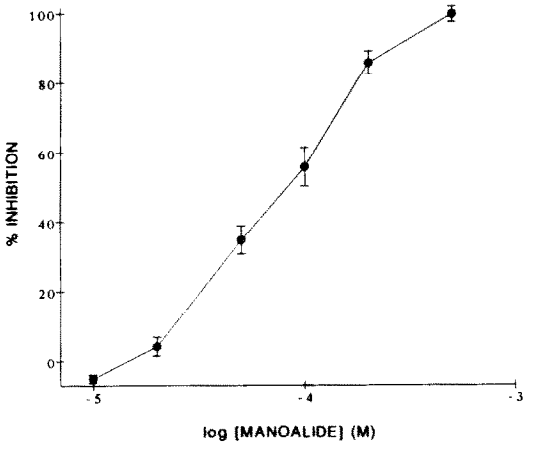

Fig. 8. Inhibition of phospholipase C by manoalide. The 100,000 *g* pellet fraction from human neutrophil homogenates was preincubated with vehicle or increasing concentrations of manoalide for 20 min at 37°. Enzyme activity was determined as described in Materials and Methods. The data are expressed as percent inhibition of basal activity of approximately 8.6 nmol/mg protein/min. Values are the means ± SE of 3 experiments.

10-fold (data not shown). This would give an IC_{50} of approximately 8 μM against PIP_2 hydrolysis, which is still at least 50-fold less potent than as an inhibitor of Ca^{2+} mobilization. These data, therefore, are in keeping with the lack of effect of manoalide on IP_3 production.

DISCUSSION

The demonstrated inhibition by manoalide of elastase release from human neutrophils suggests that the drug is potentially useful as an anti-inflammatory agent in humans. Neutrophils are a major component of the cellular infiltrate accumulating at sites of inflammation, and degranulation is a prominent feature of the overall response. Neutral proteases, such as collagenase and elastase, have been shown to play a significant role in the development of tissue damage in a number of diseases, including pulmonary emphysema and rheumatoid arthritis [21, 22]. Of particular interest to us, however, is the mechanism of action for manoalide's inhibition of enzyme release.

Degranulation of PMNLs in response to fMLP has been shown to be dependent on Ca^{2+} mobilization [23] which, in turn, reflects a release of Ca^{2+} from cytoplasmic stores, changes in membrane-associated Ca^{2+} as measured by chlorotetracycline fluorescence and an uptake of Ca^{2+} from the extracellular environment [24]. Previous studies of A431 and GH_3 cells have shown that manoalide can block the release of Ca^{2+} from intracellular stores, as well as the influx of Ca^{2+} through both hormone-operated and voltage-dependent Ca^{2+} channels [7]. One or more of these effects may be responsible for inhibition of the Ca^{2+} signal observed in human PMNLs.

The source of calcium giving rise to the signal in stimulated neutrophils must be found principally in intracellular pools since previous studies have shown that removal of extracellular Ca^{2+} has only a small effect on the stimulated increase in $[Ca^{2+}]_i$ [23]. This is supported by our own observation that treatment of the cells with 5 mM EGTA produced only a 30% reduction in the fMLP-induced Ca^{2+} transient. Since mobilization of intracellular pools has been reported to be critical for inducing extracellular Ca^{2+} uptake [25], however, inhibition of intracellular release alone could completely block the fMLP-stimulated response. This is what we observed when PMNLs were treated with TMB-8 (the effect of TMB-8 on elastase release was not measurable, however, because the drug had a direct inhibitory effect on enzyme activity). Inhibition of intracellular calcium release by manoalide, therefore, would be sufficient to completely block degranulation. The mechanism of action for manoalide's inhibition of intracellular Ca^{2+} release, however, is not clear.

Stimulation of human neutrophils by fMLP appears to be mediated through activation of a GTP-dependent phospholipase C, resulting in the production of DAG and IP_3 [26, 27]. In permeabilized PMNs, IP_3 has been shown to release Ca^{2+} from intracellular stores, leading to an increase in $[Ca^{2+}]_i$ [28]. Since manoalide has been reported to inhibit phospholipase C in both purified and cytosolic preparations [8], we examined the effect of the drug on inositol phosphate production. At a concentration of manoalide which completely blocked fMLP-induced Ca^{2+} mobilization, however, there was no effect on IP_3 levels in the cell. This suggests that in PMNLs the drug is not acting through inhibition of phospholipase C. Consistent with this idea is our observation that PLC activity in neutrophil membrane preparations was not inhibited significantly by manoalide at concentrations below 20 μM. This is at least fifty times higher than the concentration needed to completely block calcium flux in these cells. Manoalide appears to be acting, therefore, at some point beyond the release of IP_3. We have observed similar results in previous studies of A431 and GH_3 cells, where the inhibition of Ca^{2+} responses by manoalide also could be dissociated from inositol phosphate formation [7]. Inositol trisphosphate receptors have been identified in a number of cell types, including neutrophils [29–31]. Whether manoalide could be affecting the interaction of IP_3 with these receptors or acting at some other site requires further study.

In addition to Ca^{2+} mobilization, it is apparent that other factors also are important in the activation of neutrophils. The concentration–response curves for fMLP indicate that maximal increases in intracellular Ca^{2+} are not sufficient by themselves to induce maximum enzyme release. Similar findings were reported by Korchak *et al.* [32] where doses of fMLP which evoked a full calcium signal were much less than those required to elicit degranulation. In a like manner, Pozzan and co-workers [33, 34] observed that cells treated with the calcium ionophore ionomycin show an increase in $[Ca^{2+}]_i$ but no myeloperoxidase release. The subsequent addition of fMLP produced no further rise in $[Ca^{2+}]_i$, but it did result in enzyme secretion. Furthermore, buffering of the fMLP-induced Ca^{2+} signal with the EGTA analog MAPTAM inhibits degranulation by only 50% [35]. These studies suggest that some

additional factor(s), or signal transduction pathways, may be involved in modulating neutrophil activation. What pharmacological effects manoalide may have on these processes is the subject of ongoing investigation.

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