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HUMAN NEUTROPHIL ACTIVATION WITH INTERLEUKIN-1

A ROLE FOR INTRACELLULAR CALCIUM AND ARACHIDONIC ACID LIPOXYGENATION

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Abstract—Human monocyte-derived interleukin-1 (IL-1) stimulated the selective extracellular release of cytoplasmic granule-associated elastase from human neutrophils. Although extracellular calcium (Ca^{2+}) enhances but is not required for the expression of granule exocytosis, IL-1 did induce the mobilization of previously sequestered intracellular Ca^{2+} as measured with the highly selective fluorescent Ca^{2+} indicator, Quin 2. Further, IL-1 stimulated the mobilization of cell membrane-associated Ca^{2+} as monitored by a decrease in fluorescence of chlorotetracycline (CTC)-loaded neutrophils. W-7, a calmodulin antagonist, and TMB-8[8(*N*,*N*-diethylamino)-octyl-(3,4,5-trimethoxy)benzoate hydrochloride], an intracellular Ca^{2+} antagonist, inhibited the Quin 2 fluorescent response by neutrophils to IL-1. TPCK (*N*-α-*p*-tosyl-L-lysine chloromethylketone), a serine protease inhibitor, suppressed IL-1-induced Quin 2 and CTC fluorescence. Exposure of neutrophils to IL-1 resulted in a concentration-dependent production of the 5-lipoxygenase product, LTB_4 [5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid] which was enhanced in the presence of arachidonic acid (AA). LTB_4 production by IL-1-activated neutrophils was suppressed by the lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA) and piriprost potassium [6,9,deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1], and a cyclooxygenase/lipoxygenase inhibitor, 5,8,11,14-eicosatetraynoic acid (ETYA), whereas a cyclooxygenase inhibitor, flurbiprofen, was inactive. These data indicate that cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and a metabolite(s) of AA lipoxygenation mediate granule exocytosis elicited with IL-1.

The neutrophil represents the predominant fluid phase cell type in the rheumatoid arthritic joint. Contact between neutrophils and a number of soluble and particulate stimuli eventuates in the extracellular release of granule constituents including proteolytic enzymes which participate in the destruction of joint cartilage and bone matrices. Interleukin-1 (IL-1), a 17 kD polypeptide(s) synthesized by monocytes/macrophages [1], endothelial cells [2] and neutrophils [3] and found in arthritic joints [4], has been reported recently to stimulate neutrophil degranulation [5]. To the extent that receptors for IL-1 have been identified on several cell types [6, 7], it is conceivable that a receptor–ligand interaction constitutes the initial event in the activation of neutrophils with IL-1. A rise in cytosolic free calcium ($[Ca^{2+}]_i$) has been demonstrated in neutrophils subsequent to receptor occupancy [8–10], and $[Ca^{2+}]_i$ is, therefore, believed to be a signal for the expression of neutrophil effector functions including granule exocytosis [11–15] and oxygen-derived free radical production [16, 17]. The purpose of the studies reported here was to examine the requirements for $[Ca^{2+}]_i$ in the sequence of events underlying neutrophil degranulation elicited with IL-1. Further, in that secretory stimuli have been demonstrated to induce arachidonic acid (AA) lipoxygenation resulting in the generation of putative mediators of physiological responses expressed by neutrophils, we also investigated the capacity of IL-1 to induce AA lipoxygenation and the role of lipoxygenase metabolites in the mechanism of neutrophil activation with this monokine.

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MATERIALS AND METHODS

Purification of human neutrophils. Blood from normal human donors was drawn by venipuncture into 0.1 vol. of 3.8% citrate in conical plastic tubes. Neutrophils were purified using standard techniques of dextran sedimentation, centrifugation on Hypaque/Ficoll, and hypotonic lysis of red blood cells. Final cell suspensions contained a minimum of 98% neutrophils, and cell viability always exceeded 98% as determined by trypan blue exclusion.

Preparation of IL-1. Human adherent blood monocyte-derived IL-1 was obtained from Dr. Charles Dinarello (Tufts University, School of Medicine) and was purified according to previously published methods which entail membrane filtration, immunoadsorption and gel filtration [18–20]. The final IL-1 preparation contained approximately 90%

IL-1-β(pl 7 form) and 10% IL-1-α(pl 5 form). Both forms of IL-1 are biologically active, but IL-1-β is the predominant form in human monocytes. IL-1 activity is expressed as thymocyte proliferation units (LAF units) as determined in a standard murine thymocyte co-stimulator assay. One LAF unit is defined as the amount of IL-1 which doubles the mitogen response [18–20]. The specific activity of the IL-1 preparation used in these studies was approximately 10^7 units/mg protein. IL-1 was prepared in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin.

Incubation conditions. Neutrophils suspended in PBS, pH 7.4, containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.1% glucose were incubated at 37° in a Dubnoff shaking incubator according to the various protocols described under results. After incubation the samples were centrifuged at 750 *g* (4°) for 3 min, and the clear supernatant fractions were assayed for lactate dehydrogenase (LDH) activity and arachidonic acid metabolites.

Measurement of Quin 2 fluorescence. Neutrophils were suspended in a 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer, pH 7.45, containing 150 mM NaCl, 5 mM KCl, 1.29 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 10 mM Hepes. Quin 2 loading and fluorescence measurements were performed according to a modification of the methods of Tsien *et al.* [21] and Korchak *et al.* [10]. Neutrophils (10^8 ml) in Hepes buffer were loaded with 50 μM Quin 2 acetoxymethyl ester and incubated for 20 min at 37°. The cells were then diluted 10-fold and incubated at 37° for an additional 30 min. After loading, the cells were centrifuged at 150 *g* (25°) for 8 min in a clinical centrifuge and resuspended in Hepes buffer at a final concentration of 7.5×10^6 cells in 2.9 ml buffer. For fluorescence measurements, 2.9 ml of cells (containing 7.5×10^6 cells) were placed in a thermostated 1-cm^2 quartz cuvette. IL-1 was added to the cell suspensions in 100-μl aliquots via a direct injection system using a Hamilton syringe to bring the final volume to 3 ml. Fluorescent changes were monitored without stirring at 37° with an excitation wavelength of 339 nm and an emission wavelength of 492 nm, utilizing a SLM 4800S fluorescence spectrophotometer.

Measurement of CTC fluorescence. Neutrophils (2×10^7 ml) in Hepes buffer were incubated with 100 μM chlorotetracycline (CTC) at 37° for 15 min. The cells were then washed once and resuspended to a final concentration of 2.5×10^6 ml. The experimental conditions involving CTC-loaded cells were the same as those described for Quin 2 fluorescence. Fluorescent changes were monitored without stirring with an excitation wavelength of 370 nm and and emission wavelength of 560 nm.

Enzyme and B_{12}-BP assays. LDH (EC 1.1.1.27) activity was determined as previously described [22].

Elastase activity was monitored continuously by determining the release of *p*-nitrophenol from the substrate *N-t*-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester according to previously described methods [10, 23]. Briefly, neutrophils (1.25×10^6) were preincubated with cytochalasin B (5 μg/ml) at 37° for 5 min followed by the addition of elastase substrate (150 μM) to both reference and sample cuvettes. The reaction was initiated by the addition of IL-1 to the sample cuvette, and changes in absorbance (400 nm) were followed in an AMINCO DW-2a twin-beam spectrophotometer.

Generation, isolation and quantification of leukotriene B_4. Neutrophils (5×10^6 ml) in PBS were incubated with IL-1 according to the procedures described under Results. After incubation, the clear supernatant fractions were assessed for LTB_4 content with a specific and sensitive radioimmunoassay as previously described for other leukotrienes [24]. The data are expressed as picograms of LTB_4 per 10^6 cells.

Source and preparation of reagents. Cytochalasin B (Aldrich Chemical Co., Milwaukee, WI), W-7 (Seikagaku America, Inc., St. Petersburg, FL) and TMB-8 (The Upjohn Co.) were dissolved in ethanol, distilled water and PBS respectively. 5,8,11,14-eicosatetraynoic acid (ETYA) (The Upjohn Co.), piriprost potassium (The Upjohn Co.), flurbiprofen (The Upjohn Co.) and nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co., St. Louis, MO) were prepared in dimethyl sulfoxide (DMSO) Ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA) (Sigma), CTC hydrochloride (Sigma), and Quin 2/acetoxymethyl ester (Lancaster Synthesis, Ltd., Windham, NH) were dissolved in 50 mM Tris buffer, PBS, and DMSO respectively. *N-t*-butoxycarbonyl-L-alanine-*p*-nitrophenyl ester (Sigma) and *N-α-p*-tosyl-L-lysinechloromethylketone (TPCK) (Sigma) were prepared in ethanol and DMSO respectively. Arachidonic acid (Nu-Chek-Prep, Inc., Elysian, MN) was dissolved in methanol. [^{3}H]LTB_4 (5 μCi/0.25 ml/vial; 220 Ci/mol) was purchased from the Amersham Corp. (Arlington Heights, IL). The small amounts of ethanol, methanol and DMSO (final concentration of 0.05%) employed as vehicles did not affect cell viability or granule exocytosis.

RESULTS

IL-1-induced changes in fluorescence of Quin-2-loaded human neutrophils. IL-1 stimulated a concentration-dependent increase in fluorescence of Quin-2-loaded neutrophils with a maximum fluorescent response occurring with an IL-1 concentration of 2000 LAF units (Fig. 1). The fluorescent response of neutrophils to IL-1, which reflects the mobilization of $[Ca^{2+}]_i$, was quite rapid with no measurable lag period between the time of cell contact with IL-1 and the elicitation of a quantifiable increase in fluorescence.

Effects of cytochalasin B and extracellular calcium on IL-1-stimulated changes in fluorescence of Quin-2-Loaded human neutrophils. The rate and magnitude of the fluorescent response of Quin-2-loaded neutrophils to IL-1 were essentially the same whether or not cells were preincubated with cytochalasin B (CB) prior to exposure to IL-1 (Fig. 2). In contrast to this observation, we previously reported granule exocytosis from IL-1-treated neutrophils to be markedly attenuated in the absence of CB [5]. Therefore, the present findings suggest that the capacity of CB to enhance, and in some situations be required for, degranulation may not be mediated by an effect on

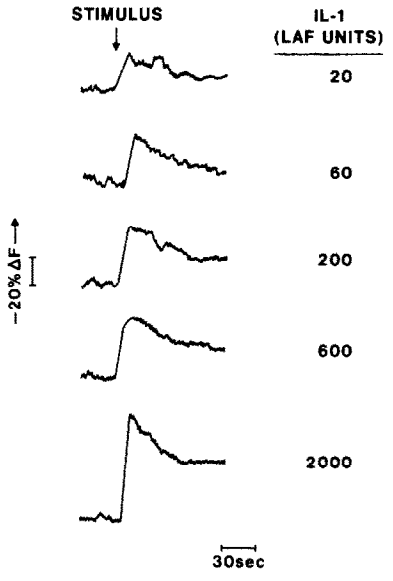

Fig. 1. Concentration dependence of the effect of Interleukin-1 on the fluorescent response of Quin-2-loaded human neutrophils. Quin-2-loaded neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 5 min at 37°. The respective concentrations of IL-1 were added at the *arrow*. The data constitute the results of a single experiment, representative of a minimum of five separate experiments.

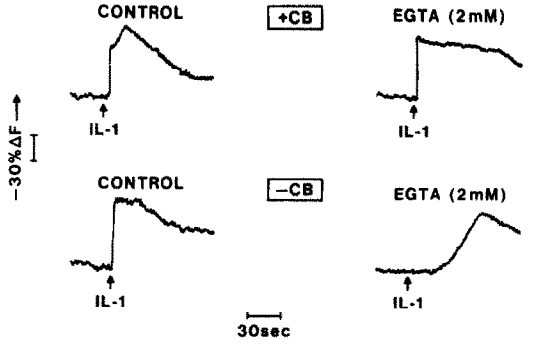

Fig. 2. Effects of cytochalasin B and extracellular calcium on Interleukin-1-stimulated changes in fluorescence of Quin-2-loaded human neutrophils. Quin-2-loaded neutrophils (7.5×10^6) were preincubated with or without cytochalasin B (5 μg/ml) for 4 min followed by incubation in the presence or absence of EGTA (2 mM) for 1 min. IL-1 (600 LAF units) was added at the *arrows*. The data constitute the results of a single experiment, representative of a minimum of four separate experiments.

$[Ca^{2+}]_i$ mobilization. Nevertheless, whereas a gradual loss in fluorescence was demonstrated in the absence of CB, a rapid loss in fluorescence was consistently observed in cells pretreated with CB. This finding can best be explained by the enhanced autofluorescence observed in CB-treated neutrophils that were not loaded with Quin 2. A similar finding has been reported for FMLP-induced fluorescent changes in Quin-2-loaded neutrophils [10].

Exposure of neutrophils to EGTA immediately before cell contact with IL-1 caused a modest decrease in the Quin 2 fluorescent response both in the presence (10%) and the absence (15%) of CB (Fig. 2). As was previously indicated, the recovery phase was more rapid in the presence than in the absence of CB. However, in the presence of CB, EGTA-treated cells demonstrated a slower loss in fluorescence than neutrophils pretreated with EGTA in the absence of CB. A measurable lag time in the fluorescent response of Quin-2-loaded cells to IL-1 was observed when cells were treated with EGTA in the absence of CB (Fig. 2).

Effects of TMB-8, W-7 and TPCK on IL-1-induced changes in fluorescence of Quin-2-loaded human neutrophils. TMB-8, an intracellular Ca^{2+} antagonist, and W-7, an inhibitor of calmodulin, exerted a significant inhibitory effect on the fluorescent change in Quin-2-loaded neutrophils in response to IL-1 (Fig. 3). The percent inhibition of IL-1-elicited Quin 2 fluorescence caused by TMB-8 and W-7 was 54 and 52% respectively. TPCK, a serine protease inhibitor, caused a significant inhibition (70%) of the fluorescent response by Quin-2-loaded neutrophils to IL-1 (Fig. 4).

Concentration dependence of the effects of TMB-8, W-7 and TPCK on IL-1-stimulated human neutrophil degranulation. TMB-8 (100–400 μM) and W-7 (50–200 μM) caused a concentration-related inhibition of granule exocytosis from neutrophils activated with IL-1 (data not shown). The IC_{50} (concentration causing 50% inhibition of the maximal secretory response) values for TMB-8 and W-7 were 251 and 82 μM respectively. TPCK also caused a concentration-related inhibition of IL-1-induced granule exocytosis from neutrophils [25]. The IC_{50} for TPCK was 3 μM.

Concentration dependence of the effect of IL-1 on the fluorescence of CTC-loaded human neutrophils. A decrease in CTC fluorescence, which signifies the mobilization of membrane-associated Ca^{2+}, was demonstrated by CTC-loaded neutrophils exposed to IL-1 (Fig. 5). This effect of IL-1 was concentration dependent with a maximum decrease in the CTC fluorescent response of neutrophils observed with an IL-1 concentration of 60 LAF units.

Effect of cytochalasin B on IL-1-induced changes in fluorescence of CTC-loaded human neutrophils. The capacity of IL-1 to stimulate the mobilization of

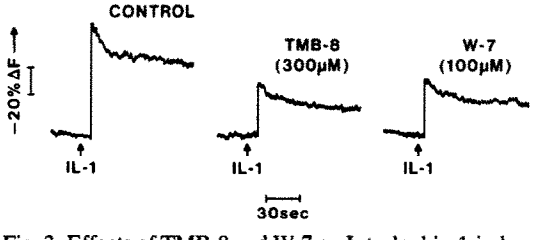

Fig. 3. Effects of TMB-8 and W-7 on Interleukin-1-induced changes in fluorescence of Quin-2-loaded human neutrophils. Quin-2-loaded neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) in the presence and absence of TMB-8 or W-7 for 5 min at 37°. IL-1 (600 LAF units) was added at the *arrows*. The data constitute the results of a single experiment, representative of a minimum of five separate experiments.

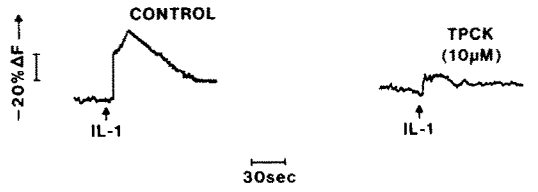

Fig. 4. Effect of TPCK on Interleukin-1-elicited changes in fluorescence of Quin-2-loaded human neutrophils. Quin-2-loaded neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) in the presence and absence of TPCK for 5 min. IL-1 (600 LAF units) was added at the *arrows*. The data constitute the results of a single experiment representative of a minimum of five separate experiments.

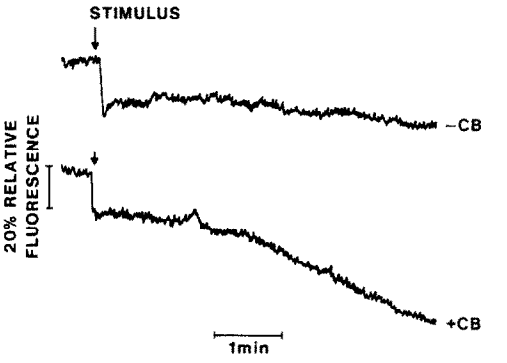

Fig. 6. Effect of cytochalasin B on Interleukin-1-stimulated changes in fluorescence of CTC-loaded human neutrophils. CTC-loaded neutrophils (7.5×10^6) were preincubated in the presence and absence of cytochalasin B (5 μg/ml) for 5 min. IL-1 (600 LAF units) was added at the *arrows*. The data constitute the results of a single experiment, representative of a minimum of five separate experiments.

membrane-associated Ca^{2+} was diminished significantly if cells were not pretreated with CB prior to contact with IL-1 (Fig. 6).

Effect of extracellular calcium on IL-1-stimulated changes in fluorescence of CTC-loaded human neutrophils. The rate and magnitude of the decrease in fluorescence of CTC-loaded neutrophils stimulated with IL-1 were essentially the same in the presence and absence of extracellular Ca^{2+} (1.29 mM). However, these studies could not be performed with EGTA in that it interfered with the fluorescent properties of CTC. The concentration of Ca^{2+} in the Ca^{2+}-free Hepes buffer is approximately 10^{-8} M as determined by atomic absorption. We have found this concentration of Ca^{2+} to have no measurable effect on neutrophils. Nevertheless, the possibility that this level of extracellular Ca^{2+} could influence IL-1-mediated fluorescent changes in CTC-loaded cells cannot be dismissed.

Effects of TMB-8 and TPCK on IL-1-elicited changes in fluorescence of CTC-loaded human neutrophils. Pretreatment of CTC-loaded neutrophils with TMB-8 virtually eliminated the IL-1-induced decrease in fluorescence of these cells (Fig. 7). TPCK also caused a significant inhibition of the rate and magnitude (40%) of the IL-1-induced decrease in fluorescence of CTC-loaded neutrophils (Fig. 7).

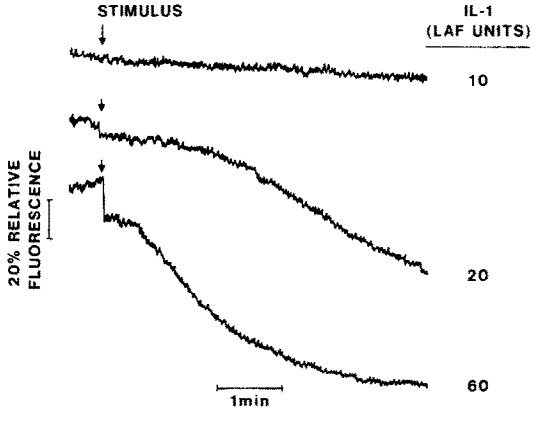

Fig. 5. Concentration dependence of the effect of Interleukin-1 on the fluorescence of CTC-loaded human neutrophils. CTC-loaded neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 5 min. The respective concentrations of IL-1 were added at the *arrows*. The data constitute the results of a single experiment, representative of a minimum of seven separate experiments.

Kinetics of IL-1-induced elastase release from and Quin 2/CTC fluorescent responses by human neutrophils. The temporal relationships amongst the kinetics of granule exocytosis, intracellular calcium mobilization and translocation of cell membrane-associated calcium in neutrophils activated with IL-1 are illustrated in Fig. 8. An increase in Quin 2 fluorescence indicating a rise in $[Ca^{2+}]_i$ was the earliest measurable event following cell contact with IL-1. Maximum fluorescence was observed between 15 and 30 sec following neutrophil exposure to stimulus, and the entire Quin 2 fluorescent response was completed during 60 sec of cell contact with IL-1. Elastase release from IL-1 activated cells occurred after a lag period of 12–15 sec and increased during the 5-min incubation period. A decrease in CTC fluorescence began after 15 sec of neutrophil interaction with IL-1, and the fluorescent response had a duration of approximately 4 min. This kinetic analysis of neutrophil activation with IL-1 revealed a sequence of events which confirm and extend observations regarding the stimulation of neutrophils with other secretagogues [26].

Less than 4% of total cell LDH activity was released under the defined experimental conditions which demonstrates neutrophil activation with IL-1 to be a selective and noncytotoxic process.

IL-1-stimulated leukotriene B_4 production by human neutrophils in the presence and absence of arachidonic acid. IL-1-induced a concentration-dependent generation of LTB_4 by human neutrophils (Fig. 9A). Concentrations of IL-1 in excess of 600 and 60 LAF units did not stimulate the generation of quantities of LTB_4 in excess of those demonstrated for these concentrations of IL-1 in the absence and presence of AA respectively. The EC_{50} for IL-1, in the absence of AA, was approximately 135 LAF units. AA (10 μM) significantly enhanced the LTB_4-generating activity of neutrophils stimulated with IL-

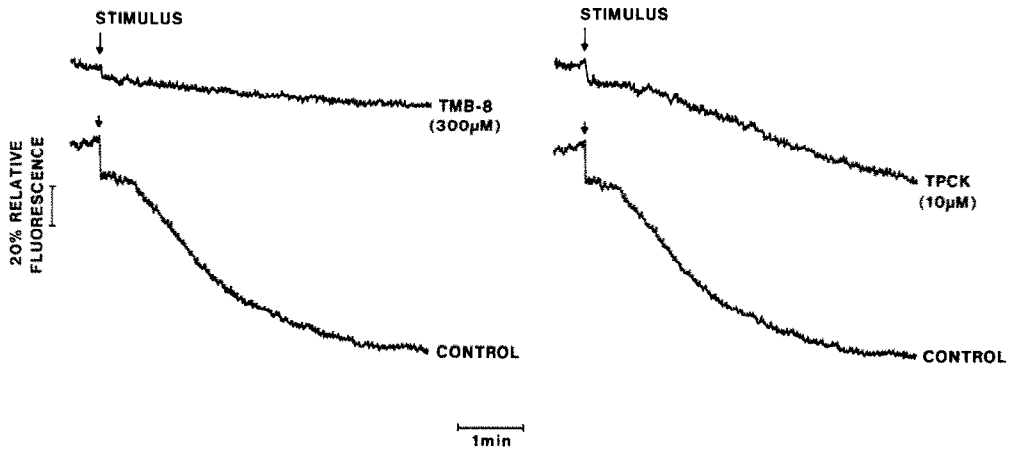

Fig. 7. Effects of TMB-8 and TPCK on Interleukin-1-elicited changes in fluorescence of CTC-loaded human neutrophils. CTC-loaded neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 5 min in the presence and absence of TMB-8 or TPCK. IL-1 (600 LAF units) was added at the *arrows*. The data constitute the results of a single experiment, representative of a minimum of four separate experiments.

1 (Fig. 9B). In the presence of AA, there was a marked shift to the left in the dose–response curve for IL-1-induced LTB_4 production (EC_{50} ~2 LAF units).

Concentration dependence of the effects of inhibitors of arachidonic acid metabolism on IL-1-stimulated LTB_4 production by human neutrophils. The lipoxygenase inhibitors, NDGA and piriprost potassium, was well as the cyclooxygenase/lipoxygenase inhibitor, ETYA, caused a concentration-dependent suppression of IL-1-induced LTB_4 production by neutrophils (Fig. 10). The IC_{50} values were 0.03, 0.28, and 5 μM for NDGA, ETYA, and piriprost potassium, respectively. Flurbiprofen, a cyclooxygenase inhibitor, had no effect on IL-1-induced LTB_4 production.

DISCUSSION

Neutrophil activation has been described as a mechanism of "Stimulus–Response Coupling" wherein $[Ca^{2+}]_i$ functions as a signal in mediating the expression of various cell responses to a number of stimuli [8–17]. We report here that exposure of human neutrophils to IL-1 resulted in an immediate rise in $[Ca^{2+}]_i$ as measured with Quin 2. Further, a decrease in fluorescence of CTC-loaded neutrophils in contact with IL-1 indicates that this cytokine induces the mobilization of cell-membrane-associated Ca^{2+} which may contribute to the rise in $[Ca^{2+}]_i$ observed in Quin-2-loaded neutrophils. Secretory stimuli such as FMLP activate neutrophils via receptor-mediated activation of a signal transduction sys-

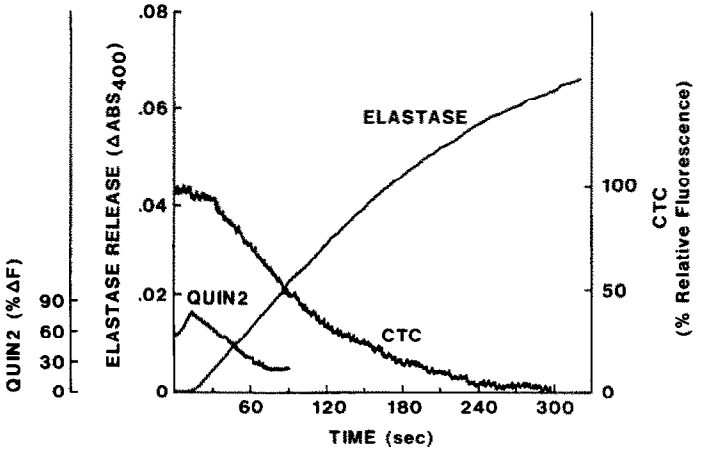

Fig. 8. Kinetics of Interleukin-1-induced elastase release from and Quin 2/CTC fluorescence responses by human neutrophils. Neutrophils (7.5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 5 min prior to the addition of IL-1 (600 LAF units) at zero time. The data constitute the results of single experiments for elastase release, Quin 2 fluorescence, and CTC fluorescence. However, each assay was performed a minimum of five times.

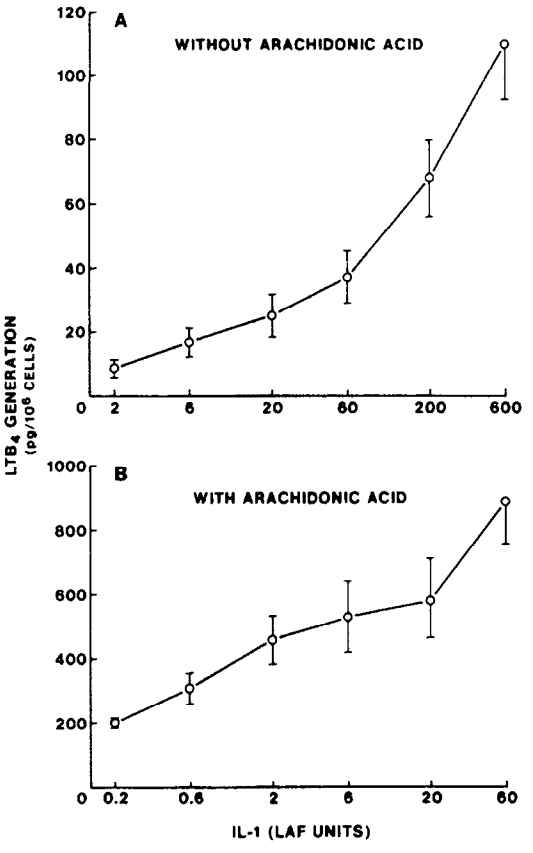

Fig. 9. Interleukin-1-stimulated leukotriene B_4 production by human neutrophils in the presence and absence of arachidonic acid. Neutrophils (10^7) were preincubated with cytochalasin B (5 μg/ml) for 10 min followed by a 15-min incubation with the specified concentrations of IL-1. The cells were then incubated with or without arachidonic acid (10 μM) for 5 min. Data represent the mean ± SEM of six separate experiments performed in duplicate.

tem which generates two products, myo-inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (1,2-DAG), which serve as endogenous messengers of Ca^{2+}-mediated events involved in "Stimulus–Response Coupling" [27–30]. IP_3 stimulates the release of sequestered Ca^{2+} from endoplasmic reticulum (ER) [28] leading to elevated levels of $[Ca^{2+}]_i$, and 1,2-DAG activates a Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) [31]. Calmodulin (CaM), a Ca^{2+}-binding protein which regulates numerous enzymatic activities [32], is found in neutrophils [33], and our finding that W-7, a selective CaM inhibitor [34], suppressed the fluorescent response by Quin-2-loaded neutrophils to IL-1 indicates that W-7 may interfere with the IP_3-mediated mechanism for releasing Ca^{2+} from the ER. CaM-dependent protein kinase activity has also been identified in neutrophils [31], and the substrate(s) for this enzyme differs from those of protein kinase C [35]. Therefore, W-7, by inhibiting the CaM-dependent protein kinase, could function to suppress IL-1-induced neutrophil degranulation by interfering with the phosphorylation of certain components of the granule exocytotic process. In any event, the capacity of W-7 to inhibit IL-1-induced increases in $[Ca^{2+}]_i$ correlates with its suppressive effect on IL-1-induced degranulation (IC_{50} values ~80–100 μM). TMB-8, an intracellular Ca^{2+} antagonist, has been reported to inhibit caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum in striated muscle [36]. By analogy our data suggest that TMB-8 may interfere with Ca^{2+} efflux from the ER resulting in decreased levels of $[Ca^{2+}]_i$ which is represented by an attenuation of the Quin 2 response by IL-1-activated neutrophils. By decreasing the levels of $[Ca^{2+}]_i$, TMB-8 could function to inhibit granule exocytosis [5] by making $[Ca^{2+}]_i$ less available to protein kinase C and/or the CaM-dependent protein kinase. TMB-8 also suppresses the fluorescent response by CTC-loaded neutrophils to IL-

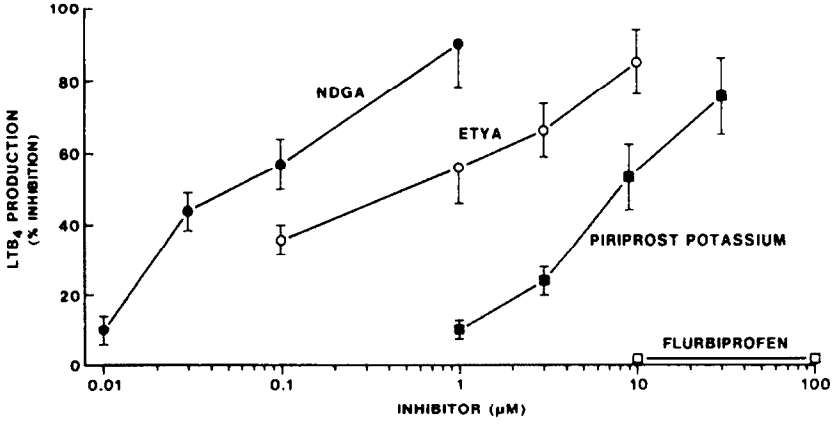

Fig. 10. Concentration dependence of the effects of inhibitors of arachidonic acid metabolism on Interleukin-1-stimulated leukotriene B_4 generation by human neutrophils. Neutrophils (10^7) were preincubated with cytochalasin B (5 μg/ml) for 9 min followed by a 1-min incubation with or without the indicated concentrations of the respective inhibitors. The cells were then incubated with IL-1 (100 LAF units) and arachidonic acid (10 μM) for 15 min. Data represent the mean ± SEM of five separate experiments performed in duplicate.

1, suggesting that cell membrane-associated Ca^{2+} is made less available for mobilization to an intracellular site.

TPCK, a serine protease inhibitor, suppressed the Quin 2 and CTC fluorescent responses by neutrophils exposed to IL-1. Consistent with these findings is the suppression of calcium uptake by neutrophils activated with various stimuli in the presence of TPCK [37]. To the extent that a TPCK-sensitive chymotrypsin-like esterase has been identified in cell membrane preparations of human neutrophils [38], our data suggest that a membrane-associated chymotrypsin-like esterase mediates, in part, Ca^{2+} mobilization in response to IL-1. The finding of Duque *et al.* [39] that TPCK inhibits membrane potential changes in activated neutrophils, which together with Ca^{2+} mobilization constitute two of the early steps in the activation sequence, further supports a role for cell membrane-associated esterases in neutrophil activation with IL-1 [25] and other secretory stimuli [40].

We have provided the first demonstration of the 5-lipoxygenation of arachidonic acid (AA) in human neutrophils activated with IL-1. The data show that LTB_4 (the major 5-lipoxygenase product in human neutrophils) production by IL-1 activated cells was enhanced in the presence of AA; and the dose–response curve for IL-1-stimulated LTB_4 production in the presence of AA was shifted to the left. However, the generation of LTB_4 in the absence of AA indicates that IL-1 stimulates AA lipoxygenation via a phospholipase A_2-mediated pathway. In this regard, IL-1 has been shown to activate phospholipase A_2 activity in rabbit chondrocytes [41]. The added AA constitutes substrate for the 5-lipoxygenase enzyme and together with the phospholipase A_2-generated AA is converted to quantities of LTB_4 which exceed those produced by IL-1-stimulated neutrophils in the absence of added AA. We have also demonstrated the inhibition of IL-1-elicited LTB_4 production by the 5-lipoxygenase inhibitors, piriprost potassium and NDGA, as well as by the lipoxygenase/cyclooxygenase inhibitor, ETYA. The order of potency of these LTB_4 synthesis inhibitors—NDGA > ETYA > piriprost potassium—parallels their relative potencies with respect to suppression of IL-1-induced granule exocytosis [5]. Further, flurbiprofen, a cyclooxygenase inhibitor, which has no effect on neutrophil degranulation elicited with IL-1 [5], is also inactive against LTB_4 production by cells exposed to this cytokine.

We have shown IL-1-induced LTB_4 generation to correlate with a rise in $[Ca^{2+}]_i$ on a concentration basis. To the extent that the neutrophil 5-lipoxygenase and phospholipase A_2 are Ca^{2+}-dependent enzymes [42, 43], it is possible that $[Ca^{2+}]_i$ serves to activate 5-lipoxygenase and phospholipase A_2. Accordingly, TMB-8 and W-7, which we have demonstrated to inhibit IL-1-stimulated intracellular Ca^{2+} mobilization, were tested for their effects on LTB_4 production by IL-1 activated neutrophils and found to be inactive (R. J. Smith, unpublished observation). We conclude, therefore, that $[Ca^{2+}]_i$ and possibly CaM mediate aspects of the IL-1-associated activation pathway independent of the 5-lipoxygenation of AA. Nevertheless, it must be noted that nonsecretory concentrations of IL-1 (<60 LAF units) elicited LTB_4 generation as well as intracellular and membrane-associated Ca^{2+} mobilization. Thus, Ca^{2+} mobilization and AA lipoxygenation constitute but two of an as yet undetermined number of components of the granule exocytotic pathway activated with IL-1.

We have presented data demonstrating that IL-1-induced neutrophil degranulation is preceded and mediated by a rise in the $[Ca^{2+}]_i$. Thus, $[Ca^{2+}]_i$ appears to function as an IL-1 receptor-derived signal for the expression of this cell activity. Our findings also support a role for calmodulin and cell membrane-associated esterases as components of the secretory process. In addition, the participation of an endogenous 5-lipoxygenase product in the sequence of events underlying neutrophil activation with IL-1, a stimulus of pathogenetic significance, remains a viable possibility.

REFERENCES

1. C. A. Dinarello, *Rev. infect. Dis.* **6**, 51 (1984).
2. P. Miossec, D. Cavender and M. Ziff, *J. Immun.* **136**, 2486 (1986).
3. K. Tiku, M. L. Tiku and J. L. Skosey, *J. Immun.* **136**, 3677 (1986).
4. D. D. Wood and D. Hamerman, in *Symposium on The Physiologic, Metabolic, and Immunologic Actions of Interleukin-1* (Eds. M. L. Kluger, J. J. Oppenheim and M. C. Powanda), *Prog. Leuk. Biol.*, Vol. 2, pp. 63–71. Alan R. Liss, New York (1985).
5. R. J. Smith, B. J. Bowman and S. C. Speziale, *Int. J. Immunopharmac.* **8**, 33 (1986).
6. S. K. Dower, S. R. Kronheim, C. J. March, P. J. Conlon, T. P. Hopp, S. Gillis and D. L. Urdal, *J. exp. Med.* **162**, 501 (1985).
7. S. K. Dower, S. M. Call, S. Gillis and D. L. Urdal, *Proc. natn. Acad. Sci. U.S.A.* **83**, 1060 (1986).
8. T. Pozzan, D. P. Lew, C. B. Wollheim and R. Y. Tsien, *Science* **221**, 1413 (1983).
9. H. M. Korchak, L. E. Rutherford and G. Weissmann, *J. biol. Chem.* **259**, 4070 (1984).
10. H. M. Korchak, K. Vienne, L. E. Rutherford, C. Wilkenfeld, M. C. Finkelstein and G. Weissmann, *J. biol. Chem.* **259**, 4076 (1984).
11. R. J. Smith and S. S. Iden, *Biochem. biophys. Res. Commun.* **91**, 263 (1979).
12. R. J. Smith, W. Wierenga and S. S. Iden, *Inflammation* **4**, 73 (1980).
13. G. Weissmann, J. E. Smolen and H. M. Korchak, *New Engl. J. Med.* **303**, 27 (1980).
14. R. J. Sha'afi and P. H. Naccache, in *Advances in Inflammation Research* (Ed. G. Weissmann), Vol. 2, pp. 115–48. Raven Press, New York (1981).
15. R. J. Smith, S. S. Iden and B. J. Bowman, *Inflammation* **8**, 365 (1984).
16. R. J. Smith and S. S. Iden, *J. reticuloendothel. Soc.* **29**, 215 (1981).
17. R. J. Smith, B. J. Bowman, S. S. Iden, G. J. Kolaja and S. K. Wiser, *Immunology* **49**, 367 (1983).
18. C. A. Dinarello, L. Reufer and S. M. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4264 (1977).
19. L. J. Rosenwasser and C. A. Dinarello, *Cell. Immun.* **63**, 134 (1981).
20. C. A. Dinarello, H. A. Bernheim, J. G. Cannon, G. LoPreste, S. J. C. Warner, A. C. Webb and P. E. Auron, *B. J. Rheumatol.* **24**(Suppl. 1), 59 (1985).
21. R. Y. Tsien, T. Pozzan and T. J. Rink, *J. Cell Biol.* **94**, 325 (1982).

22. H. U. Bergmeyer, E. Bernt and B. Hess, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), pp. 736–43. Verlag Chemie, Academic Press, New York (1965).
23. A. Baici, P. Salgam, K. Fehr and A. Boni, *Biochem. Pharmac.* **30**, 703 (1981).
24. M. A. Wynalda, J. R. Brashler, M. K. Bach, D. R. Morton and F. A. Fitzpatrick, *Analyt. Biochem.* **56**, 1862 (1984).
25. R. J. Smith, S. C. Speziale and B. J. Bowman, *Biochem. biophys. Res. Commun.* **130**, 1233 (1985).
26. G. Weissmann, C. Serhan, H. M. Korchak and J. E. Smolen, *Ann. N.Y. Acad. Sci.* **389**, 11 (1982).
27. M. J. Berridge, *Biochem. J.* **220**, 345 (1984).
28. M. J. Berridge and R. F. Irvine, *Nature, Lond.* **312**, 315 (1984).
29. C. D. Smith, B. C. Lane, I. Kusaka, M. W. Verghese and R. Synderman, *J. biol. Chem.* **260**, 5875 (1985).
30. C. D. Smith, C. C. Cox and R. Snyderman, *Science* **232**, 97 (1986).
31. C-K, Huang, J. M. Hill, B. J. Bowmann, W. M. Mackin and E. L. Becker, *Biochem. biophys. Res. Commun.* **760**, 126 (1983).
32. W. Y. Cheung, *Science* **207**, 19 (1980).
33. J. G. Chafouleas, J. R. Dedman, R. P. Munjaal and A. R. Means, *J. biol. Chem.* **254**, 10262 (1979).
34. H. Hidaka, Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii and T. Nagata, *Proc. natn. Acad. Sci. U.S.A.* **78**, 4354 (1981).
35. D. M. Helfman, B. D. Appelbaum, W. R. Vogler and J. F. Kuo, *Biochem. biophys. Res. Commun.* **111**, 847 (1983).
36. C. Y. Chiou and M. H. Malagodi, *B. J. Pharmac.* **53**, 279, (1975).
37. H. J. Showell, P. H. Naccache, R. W. Walenga, M. Dalecki, M. B. Feinstein, R. I. Sha'afi and E. L. Becker, *J. reticuloendothel. Soc.* **30**, 167 (1981).
38. M-F. Tsan and M-S. Jiang, *Inflammation* **9**, 113 (1985).
39. R. E. Duque, S. M. Phan, M. C. Sulavik and P. A. Ward, *J. biol. Chem.* **258**, 8123 (1983).
40. R. J. Smith, S. C. Speziale, L. M. Sam, J. M. Justen and B. J. Bowman, *Inflammation* **10**, 257 (1986).
41. J. Chang, S. C. Gilman and A. J. Lewis, *J. Immun.* **136**, 1283 (1986).
42. C. A. Rouzer and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **82**, 6040 (1985).
43. F. Marki and R. Franson, *Biochim. biophys. Acta* **879**, 149 (1986).