0026-895X/93/010030-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:30-36

Crystal-Induced Protein Tyrosine Phosphorylation in Neutrophils and the Effect of a Tyrosine Kinase Inhibitor on Neutrophil Responses

HELEN M. BURT, JOHN K. JACKSON, PETER DRYDEN, and HASSAN SALARI

Faculty of Pharmaceutical Sciences (H.M.B., J.K.J.) and the Department of Medicine (P.D., H.S.), University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada

Received July 7, 1992; Accepted October 14, 1992

SUMMARY

A specific tyrosine kinase inhibitor, methyl 2,5-dihydroxycinnamate (mDHC), has been used to investigate the role of tyrosine kinases in monosodium urate monohydrate and calcium pyrophosphate dihydrate (CPPD) crystal-induced neutrophil activation. Both uncoated and plasma protein-coated CPPD crystals increased protein tyrosine phosphorylation in human neutrophils. Neutrophils pretreated with mDHC or control neutrophils were stimulated by plasma-opsonized CPPD, uncoated CPPD, or uncoated monosodium urate monohydrate, and chemiluminescence, superoxide generation, intracellular calcium concentration, degranulation (myeloperoxidase and lysozyme release), and protein tyrosine phosphorylation were monitored. mDHC strongly inhibited all neutrophil responses and tyrosine phosphorylation was reduced to the basal levels seen in control unstimulated neutrophils. The possible role of tyrosine kinases in the regulation of crystal-induced neutrophil activation is discussed.

Recent reports have described tyrosine phosphorylation of several distinct proteins in activated neutrophils (1–10). It has been proposed that the balance between stimulation and inhibition of tyrosine kinase and phosphatase enzymes may play a critical role in stimulus-response coupling in neutrophils (4, 5). Furthermore, there is speculation that tyrosine kinases may be involved in the regulation of the signal transduction pathway leading to neutrophil activation. All these studies describing the involvement of tyrosine kinases have used neutrophil stimulation by agonists with known receptors on the plasma membrane, such as FMLP, leukotriene B_4, or platelet-activating factor.

The inflammatory diseases known as acute gouty arthritis and acute pseudogout are caused by the deposition of MSUM and CPPD crystals in the synovial joints of humans (11, 12). These crystals are known to be strong activators of neutrophil respiratory burst activity and lysosomal enzyme release (11), but it is not known by what mechanism these crystals stimulate neutrophils.

There are no reports to date of the involvement of post-receptor signaling systems such as tyrosine kinases mediating the activation of neutrophils by crystals. The involvement of a pertussis toxin-sensitive guanine nucleotide-binding protein (G_i) is unclear (13–15), and studies with inhibitors of PKC have shown that such inhibitors are ineffective in suppressing crystal-induced neutrophil activation (13, 15).

The tyrosine kinase inhibitor erbstatin has been shown to block FMLP-induced neutrophil activation (6, 16). α-Cyano-3,4-dihydroxycinnamamide, a specific protein tyrosine kinase inhibitor, was shown to suppress a wide range of neutrophil responses to a variety of soluble agonists (10). The action of these inhibitors is thought to be at the cellular second messenger level, which may be dependent on tyrosine phosphorylation (6, 10).

Because the mechanism of neutrophil activation by crystals is not yet characterized we have investigated the role of tyrosine kinases in this process by using a novel specific protein tyrosine kinase inhibitor, mDHC. mDHC has been reported to have greater stability and activity than erbstatin (17). Evidence of increased protein tyrosine phosphorylation in neutrophils stimulated with particulate agonists is presented. It is proposed that cellular activation by MSUM and CPPD crystals utilizes pathways in neutrophils that are partially dependent on tyrosine

This work was supported by a grant from the Medical Research Council of Canada.

ABBREVIATIONS: FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; MSUM, monosodium urate monohydrate; CPPD, calcium pyrophosphate dihydrate; mDHC, methyl-2,5-dihydroxycinnamate; HBSS, Hanks' balanced salt solution; TNB, tetrazolium nitro blue; MPO, myeloperoxidase; TBS, Tris-buffered saline; LDH, lactate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP_3, inositol triphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; PLC, phospholipase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C.

kinase activation and thus inhibitors of tyrosine kinases are able to inhibit neutrophil activation and the generation of toxic oxygen species.

Materials and Methods

Chemicals. The tyrosine kinase inhibitor mDHC was synthesized as reported (17). The purity of the compound was confirmed by mass spectroscopy. mDHC is a more stable analogue of erbstatin (17), which has previously been reported to be a potent and specific inhibitor of tyrosine kinase (6, 17). HBSS, pH 7.4, was used throughout this study. Stock concentrations of drug were made in dimethylsulfoxide and the serial dilutions were made in HBSS. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. All experiments were performed at 37° unless otherwise stated.

Preparation and characterization of crystals. MSUM crystals were prepared and characterized by X-ray diffraction and scanning electron microscopy as reported previously (18). Crystals were used unheated in all studies. Over 90% of the crystals were <20 μm in size (as measured along the long needle axis). CPPD triclinic crystals were prepared and characterized as reported previously (19). Over 90% of the crystals were <20 μm in size. Plasma precoating of crystals was done with 50% heparinized plasma, using 50 mg of CPPD/0.66 ml of plasma. Crystals were incubated with plasma for 30 min at 37° and then washed in excess HBSS.

Neutrophil preparation. Neutrophils were prepared from freshly collected, citrate-anticoagulated, whole human blood. Briefly, 400 ml of blood were mixed with 100 ml of 3% dextran T500 (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) in HBSS and allowed to settle for 1 hr. Plasma was collected continuously and 5 ml were applied to 5 ml of Ficoll Paque (Pharmacia) in 15-ml polypropylene tubes (Corning, NY). After centrifugation at 500 × *g* for 30 min, the neutrophil pellets were washed free of erythrocytes by 20 sec of hypotonic shock. Neutrophils were resuspended in HBSS, kept on ice, and used for experiments within 3 hr. Neutrophil viability and purity was always >90%.

Chemiluminescence assay. Cells at 5 × 10^6/ml in HBSS were preincubated with drug (2–150 μM) for 2 min at 37°. To 1 ml of cells were added 50 mg of CPPD or 5 mg of MSUM crystals, in 1.5-ml capped Eppendorf tubes. Luminol in dimethylsulfoxide was added to a final concentration of 1 μM and the samples were mixed to initiate neutrophil activation by the crystals. Chemiluminescence was monitored using an LKB luminometer (model 1250) at 37°, with shaking immediately before measurements to resuspend the crystals. Control tubes contained cells, drug, and luminol (crystals absent).

Superoxide anion generation. Superoxide anion concentration was measured using modifications of the TNB assay as described elsewhere (20). TNB was dissolved in HBSS at 3.3 mg/ml, filtered, and pipetted onto the CPPD crystals in 1.5 ml Eppendorf tubes. Cells were incubated with the drug at 37° for 2 min before addition to crystals. Final concentrations were as follows: TNB, 0.3 mg/ml; cells, 5 × 10^6/ml; and CPPD crystals, 50 mg/ml. Replicate tubes were also set up containing superoxide dismutase at 600 units/ml. The tubes were tumbled gently at 37° to ensure crystal-neutrophil contact and interaction. At given times 0.5 ml of 1 M HCl was added and the tubes were centrifuged in a microfuge for 10 sec. Pyridine (1 ml) was added to the crystal-cell pellet and vortexed. The tubes were boiled for 20 min. Tubes were centrifuged in a microfuge and 0.8 ml of supernatant was used for assay of superoxide anion using visible spectroscopy at 515 nm, against pyridine as blank.

Neutrophil degranulation. Cells at 5 × 10^6/ml were incubated with the drug for 2 min at 37° before the addition to CPPD crystals. Reactions were started by mixing of the tubes followed by end-over-end tumbling at 37°. At appropriate times, tubes were centrifuged in a microfuge for 10 sec and 0.4 ml of supernatant was stored at −20° for later assay.

Lysozyme concentration was measured by the decrease in absorbance at 450 nm of a *Micrococcus lysodeikticus* suspension. *M. lysodeikticus* was suspended at 0.1 mg/ml in 0.65 M potassium phosphate buffer, pH 6.2, and the absorbance at 450 nm was adjusted to 0.7 units by dilution. The crystal-cell supernatant (100 μl) was added to 2.5 ml of the *Micrococcus* suspension and the decrease in absorbance was monitored. Lysozyme standards (chicken egg white) in the range of 0 to 2000 units/ml were prepared and a calibration graph of lyzozyme concentration versus the rate of decrease in the absorbance at 450 nm was obtained.

MPO activity was measured by the increase in absorbance at 450 nm that accompanys the oxidation of *o*-dianisidine. Dianisidine was dissolved at 3.2 mM in 0.1 M citrate buffer, pH 5.5, by sonication. To a 1-ml cuvette 0.89 ml of the dianisidine solution was added, followed by 50 μl of 1% Triton X-100 and 50 μl of crystal-cell supernatant. MPO activity was determined from the change in absorbance per minute, ΔA_{450}, using the following equation:

$$\text{Dianisidine oxidation (nmol/min)} = 50 \times \Delta A_{450} \quad (1)$$

Intracellular calcium. Intracellular calcium concentrations were determined using the calcium probe fluo-3/AM as reported (21). Cells (5 × 10^6/ml) were incubated with fluo-3/AM at 2 μM at room temperature for 30 min. Cells were centrifuged at 1000 × *g*, washed once in HBSS, resuspended at 5 × 10^6/ml, and stored on ice for use within 3 hr. In separate tubes, 5 ml of cells were coincubated with fluo-3/AM at 2 μM and BAPTA/AM at 30 μM, as described for fluo-3/AM alone. The cell suspension (1.5 ml) was added to a fluorescence cuvette, equilibrated at 37°, and stirred using a micro-stir bar in a spectrofluorophotometer (Shimadzu RF540). Resting calcium levels were determined as described elsewhere (21), using the following equation:

$$\text{calcium concentration} = \frac{K_d[F - F_{min}]}{[F_{max} - F]} \quad (2)$$

where F is the fluorescence intensity using 480-nm (excitation) and 525-nm (emission) wavelengths, F_{max} and F_{min} are the fluorescence intensities at maximal (0.33% Triton X-100) and minimal (50 mM EGTA) calcium concentrations, respectively, and K_d is the dissociation constant of fluo-3 with calcium and is equal to 864 nM (21). Calcium concentrations in the presence of the drug and/or 7.5 mg of MSUM crystals were determined as outlined above. Drug was added to the cells 2 min before the addition of MSUM crystals. Background scatter due to the crystals was subtracted from the changes in fluo-3 fluorescence intensities. Fluorescence was monitored for 5 min and the peak change in calcium concentration at approximately 1 min was determined from eq. 2.

Neutrophil viability. To determine the effect of the drug on neutrophil viability the release of the cytoplasmic marker enzyme LDH was measured as described elsewhere (22). Control tubes containing cells with drug (crystals absent) from degranulation experiments were also assayed for LDH.

Tyrosine kinase assay. Fractions were prepared using modifications of the method of Berkow *et al.* (9). Neutrophils (1 × 10^7/ml) and MSUM (10 mg/ml) or CPPD (100 mg/ml) were shaken in 1.5-ml Eppendorf tubes for 1 min at 37°. Tubes were immediately centrifuged at 10,000 × *g* for 5 sec, supernatants were discarded, and the crystal-cell pellet was frozen in liquid nitrogen and stored at −80° for 1 day before further preparation. Tube contents were thawed in the presence of 500 μl of fractionation buffer consisting of 20 mM HEPES, pH 7.5, 2 mM β-mercaptoethanol, 0.34 M sucrose, 100 μM sodium vanadate, 10 μg/μl leupeptin, 1 μM pepstatin, 10 μg/μl soybean trypsin inhibitor, 2 mM phenylmethylsulfonyl fluoride, and 1 mM diisopropyl fluorophosphate. Tubes were sonicated at 4° for 30 sec using a micro-sonic probe sonicator and were centrifuged at 2000 × *g* to pellet unbroken cells, nuclei, and crystals. Supernatants were transferred to fresh Eppendorf tubes and centrifuged at 200,000 × *g* in a Beckman TL-100 ultracentrifuge for 15 min. The supernatant was saved and termed the cytosolic fraction. The pellet (particulate fraction) was resuspended and sonicated for 30 sec in fractionation buffer that also contained 1% Nonidet

P-40, 0.5 mM EGTA, and 2 mM EDTA. All cytosolic and particulate fractions were then assayed for protein content using the Bradford protein assay (9).

Cytosolic and particulate fractions were assayed for tyrosine kinase activity by measuring the phosphorylation of a synthetic copolymer of glutamine/tyrosine $[(4:1)_n]$ (9). Cytosolic and particulate fractions were diluted to a final protein concentration of 0.5 mg/ml in a reaction buffer (pH 7.5) that consisted of 20 mM HEPES, 10 mM magnesium chloride, 10 mM manganese chloride, and 100 μM sodium vanadate. Phosphorylation reactions were performed in 1.5-ml Eppendorf tubes in a reaction volume of 55 μl. Quadruplicate tubes containing 20 μl of neutrophil fraction, *p*-nitrophenyl phosphate (as a competitive substrate for phosphotyrosine phosphatases) at 7 mg/ml, and copolymer substrate at 5 mg/ml in the reaction buffer were prepared. Where necessary tubes also contained the tyrosine kinase inhibitor. Reactions were started by the addition of ATP (2000–4000 cpm/pmol [γ-^{32}P] ATP) to a final concentration of 8 μM, followed by vortexing and incubation at 30°. Control tubes contained 20 μl of reaction buffer in place of the neutrophil fraction. After 10 min 40 μl of solution were spotted onto 1-inch^2 pieces of filter paper (Whatman P81 ion exchange paper), which were then air dried for 2 min. Filter papers were immersed in 1% phosphoric acid for 4 hr, with many changes of acid wash. Papers were then counted for radioactivity using a Beckman model LS 5000 scintillation counter.

Tyrosine phosphorylation. Neutrophils (10^7 cells/ml) in HBSS were stimulated with crystals for 1 min in the presence or absence of drug at 37°. The crystal-cell suspension was rapidly pelleted by 5-sec centrifugation in a microfuge and the pellet was immediately solubilized with 3% Triton X-100 and sonicated in buffer composed of 50 mM Tris·HCl, pH 7.7, 5 mM β-methylaspartate, 150 mM sodium chloride, 0.2 mM sodium vanadate, 10 mM sodium fluoride, 1 mM sodium molybdate, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride and 1 mM diisopropyl fluorophosphate. Insoluble cellular and crystal debris was removed by pelleting in a microfuge (10 sec), and soluble cell supernatants were combined with SDS-PAGE sample buffer, boiled, and electrophoresed by discontinuous SDS-PAGE. Separated proteins were transferred to nitrocellulose sheets and blocked overnight in TBS (0.02 M Tris·HCl, pH 7.5, 0.05 M NaCl) containing 5% bovine serum albumin and 1% ovalbumin. Blots were washed in TBS and probed with antiphosphotyrosine specific antibody PY-20 in TBS with 1% bovine serum albumin and 0.02% sodium azide. After washing, blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG in TBS with 0.05% Nonidet NP-40 for 2 hr at room temperature before color development with 5-bromo-4-chloro-3-indolyphosphate and TNB. The specificity of antibody PY-20 has been demonstrated by competition with phosphotyrosine or phenylphosphate but not phosphoserine, phosphothreonine, or free phosphate (23). The density of the immunoblot protein bands was determined using a densitometer (Bio-Rad, Richmond, CA).

Results

At a cell concentration of 5×10^6/ml it was determined that optimal crystal-neutrophil responses were obtained with crystal concentrations of 50 mg/ml and 5 mg/ml for CPPD and MSUM, respectively. All of the neutrophil responses measured in this study were enhanced by precoating of CPPD crystals with plasma. However, there was no significant effect of precoating MSUM with plasma on levels of neutrophil activation achieved, compared with uncoated MSUM crystals. Hence, CPPD crystals were always precoated with plasma and MSUM crystals were left uncoated for use in these studies.

Cytochalasin B (an inhibitor of phagocytosis) is often included in neutrophil response studies with soluble stimuli to increase the extracellular release of granule enzymes (for example). It was not included in neutrophil incubations in these studies because this agent is reported to increase tyrosine phosphorylation independently (3) and to have differential effects on neutrophil superoxide release in response to nonsoluble stimuli (24, 25).

Neutrophils treated with the tyrosine kinase inhibitor up to a concentration as high as 250 μM did not show any increased level of LDH above controls, indicating that mDHC did not cause cell death. Furthermore, cells treated with the tyrosine kinase inhibitor and then washed free of the drug with HBSS regained the ability to generate superoxide anion in response to crystals. Therefore, the drug does not seem to induce irreversible toxicity on the cells.

Chemiluminescence. Chemiluminescence largely measures the activity of the MPO-hydrogen peroxide system (26) and is a reliable but nonspecific measure of the production of reactive oxygen species in neutrophils. Neutrophils produced an extensive chemiluminescence response to both plasma-coated CPPD and uncoated MSUM crystals. The tyrosine kinase inhibitor mDHC has been shown to be a potent inhibitor of this chemiluminescence response to both crystals (Figs. 1 and 2). Chemiluminescence values from control samples never exceeded 5 mV in intensity and the addition of the drug up to concentrations of 250 μM had no effect on the chemiluminescence values of controls.

Superoxide generation. The time course of plasma-coated CPPD crystal-induced superoxide anion production, as measured by the superoxide dismutase-inhibitable reduction of TNB, is shown in Fig. 3. Treatment of cells with 100 μM mDHC produced a decrease in the amount of superoxide generated at all times.

Neutrophil degranulation. Neutrophil degranulation was monitored by the plasma-coated CPPD crystal-induced release of MPO and lysozyme. Fig. 4 shows the time course of the release of MPO (Fig. 4A) and lysozyme (Fig. 4B) from neutro-

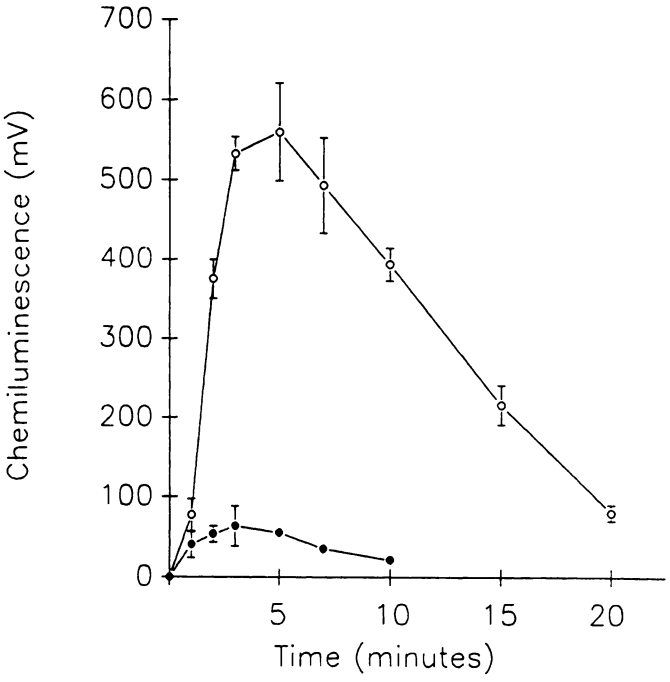

Fig. 1. ●, Effect of mDHC at 100 μM on the time course of MSUM (5 mg/ml)-induced neutrophil (5×10^6/ml) chemiluminescence (three experiments). ○, No drug.

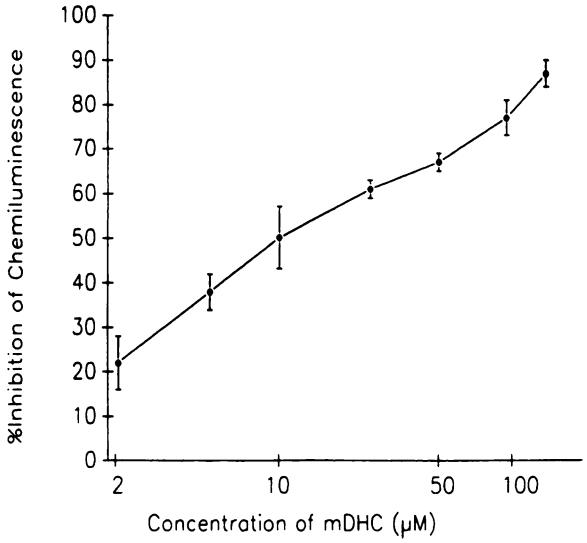

Fig. 2. Concentration dependence of the inhibition of plasma-coated CPPD (50 mg/ml)-induced neutrophil (5×10^6/ml) chemiluminescence by mDHC (three experiments).

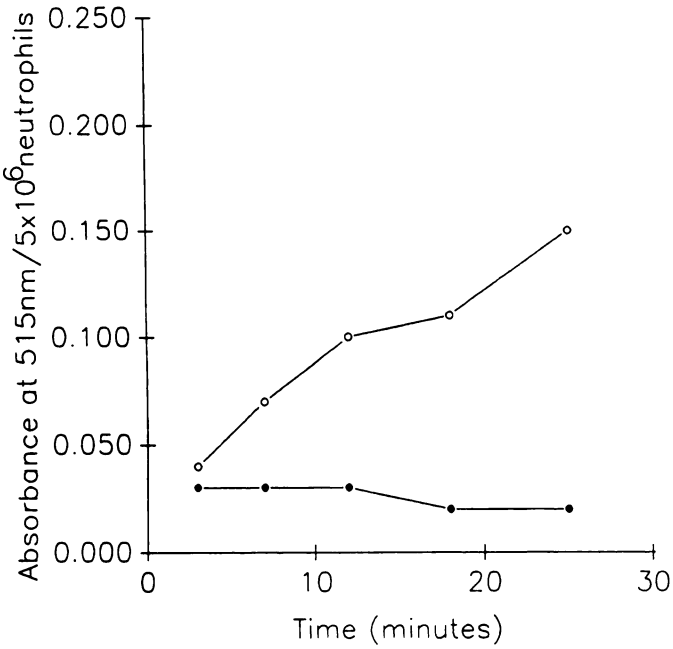

Fig. 3. Time course of superoxide production (expressed as the absorbance at 515 nm of TNB) by neutrophils (5×10^6/ml) stimulated with plasma-coated CPPD (50 mg/ml). ●, Inhibition by mDHC at 50 μM; ○, no drug.

phils stimulated by plasma-coated CPPD. The pretreatment of cells with 50 μM mDHC resulted in decreases in MPO and lysozyme release at all times measured. The concentration dependence of the inhibition of MPO release is shown in Fig. 5 for neutrophils stimulated with plasma-coated CPPD (25-min incubations). It was not possible to measure MPO release when neutrophils were activated by MSUM crystals, because it was found that MSUM strongly adsorbs all MPO released from neutrophils.[1] However, adsorption of lysozyme to MSUM was found to be <5%, and the inhibition of MSUM-induced lysozyme release by the drug was measured. mDHC at 100 μM was found to inhibit lysozyme release by 44 ± 12% (three experiments; significant at $p < 0.05$, unpaired Student's t test) after a 25-min MSUM-neutrophil incubation.

Intracellular calcium determinations. Intracellular calcium concentrations were determined using the fluorescence probe fluo-3/AM. It was determined that there was no fluorescence interference from mDHC at the wavelengths used with fluo-3. The drug produced interfering fluorescence at the wavelengths used for calcium concentration determinations with other probes such as fura-2 or indo-1. The wavelengths for maximal fluo-3 fluorescence, i.e., 505 nm (excitation) and 525 nm (emission), were so close that scattered light from the crystals in the cuvette exceeded the 525-nm fluo-3 emission. Therefore, fluo 3 was excited at 480 nm, compromising the fluo-3 intensity in favor of a major reduction in scatter to minimal levels. To ensure that the probe was correctly monitoring intracellular calcium concentrations, the response of the cells to 10 μM FMLP was studied. FMLP is known to induce an immediate increase in intracellular calcium concentrations upon addition to neutrophils, with a slow decay back to resting levels within 2 min (13, 15). We found that in fluo-3-loaded neutrophils FMLP induced an immediate increase in calcium concentration of 500 nmol, with a decay back to resting levels within 100 sec (Fig. 6). MSUM crystals were used to activate neutrophils in these studies because CPPD crystals produced only slow and small increases in neutrophil intracellular calcium concentrations, as similarly reported by others (13, 27). Cells coincubated with fluo-3/AM and the calcium chelator BAPTA/AM showed no changes in fluo-3 fluorescence when stimulated with FMLP (10 μM) or MSUM (5 mg/ml), showing that fluo-3 fluorescence was correctly monitoring intracellular calcium levels.

The addition of uncoated MSUM (5 mg/ml) to neutrophils produced a large, rapid, and transient change in intracellular calcium levels, from a resting concentration of approximately 200 nM to a maximum of approximately 600 nM, within 1 min (Fig. 6). Cells pretreated with mDHC at 100 μM produced significantly smaller increases ($p < 0.05$) in intracellular calcium concentration, compared with untreated cells, when stimulated with MSUM crystals. A typical time course of intracellular calcium concentration changes is shown in Fig. 6. In four separate experiments the inhibition of MSUM-induced calcium concentration changes, determined at 40 sec after the addition of MSUM, was 88% (one experiment), 80 ± 20% (three experiments), 80 ± 20% (three experiments), and 67 ± 11% (four experiments).

Tyrosine kinase activity determination. The use of synthetic copolymers of glutamine/tyrosine as substrates in the assay of tyrosine kinase activity in neutrophil extracts has been described previously (4, 9). Using the same concentration of ATP (8 μM) (4, 9), we optimized the concentration of copolymer so that the kinase assay produced a linear increase in phosphorylation up to 30 min with our neutrophil extracts (Fig. 7). We have found that neutrophils incubated for 1 min with either plasma-coated or uncoated crystals of MSUM or CPPD showed elevated tyrosine kinase activity. Both cytosolic and particulate fractions of neutrophils showed elevated levels of activity, relative to control neutrophil fraction levels (data not shown). The cytosolic or particulate fractions that showed the greatest elevations in tyrosine kinase activity were used to investigate the effect of mDHC on tyrosine kinase activity. mDHC inhib-

[1] H. M. Burt and J. K. Jackson, unpublished observations.

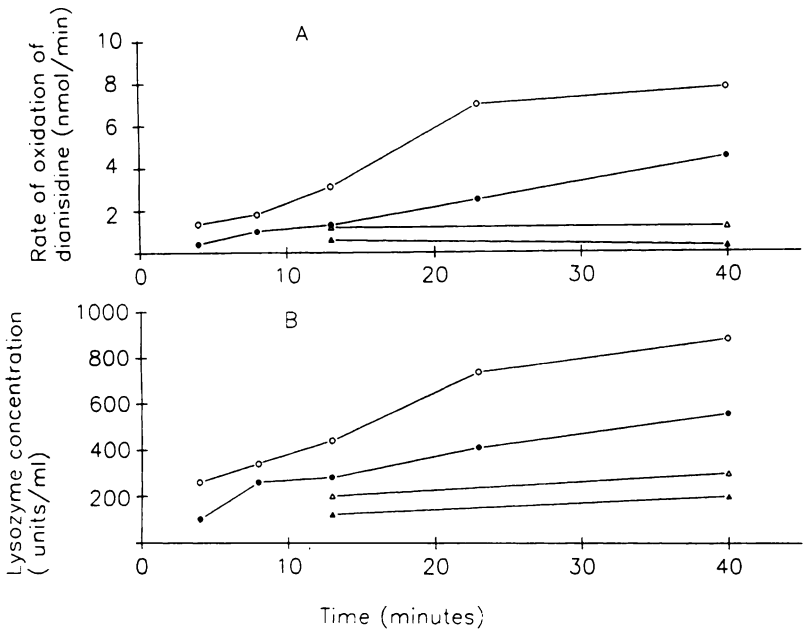

Fig. 4. Time course of neutrophil (5 × 10^6/ml) degranulation, as measured by MPO release (MPO activity expressed as the rate of oxidation of dianisidine, in nmol/min) (A) or lysozyme release (B), due to plasma-coated CPPD (50 mg/ml) stimulation. ●, mDHC at 50 μM, ○, no drug; △, control with no drug; ▲, control with mDHC at 50 μM.

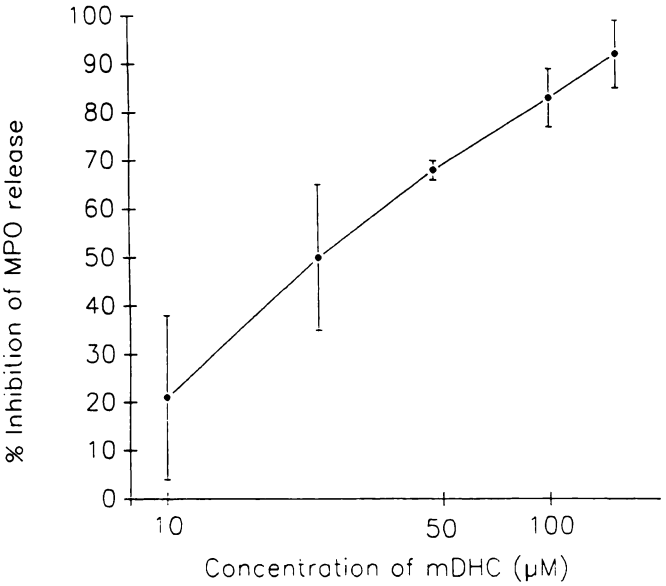

Fig. 5. Concentration dependence of the percentage of inhibition by mDHC of plasma-coated CPPD (50 mg/ml)-induced neutrophil (5 × 10^6/ml) degranulation, as measured by MPO release. Twenty-five-minute crystal-cell incubation (three experiments).

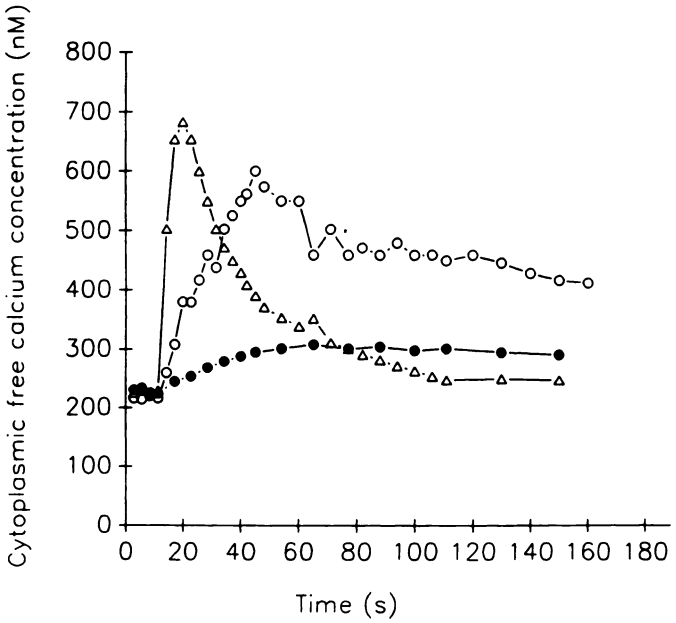

Fig. 6. Time course of neutrophil (5 × 10^6/ml) cytoplasmic free calcium concentration changes, as measured with the fluorescent calcium probe fluo-3. Excitation at 480 nm; emission at 525 nm. △, FMLP (1 μM); ○, MSUM (5 mg/ml) with no drug; ●, MSUM (5 mg/ml) and mDHC (100 μM).

ited tyrosine kinase activity in all fractions, as can be seen in Fig. 7. The concentration dependence of this inhibition can be seen in Fig. 7C. In all experiments, tubes that did not contain copolymer substrate showed <5% of the radioactivity of tubes with substrate.

Neutrophil protein tyrosine phosphorylation. The effect of mDHC on tyrosine phosphorylation in human neutrophils after stimulation for 1 min by plasma-coated or uncoated CPPD crystals was studied on three separate occasions. Fig. 8 (a representative experiment) shows that unstimulated cells contained tyrosine-phosphorylated proteins (Fig. 8, *lane 1*). Both uncoated (Fig. 8, *lane 2*) and plasma-coated (Fig. 8, *lane 4*) CPPD crystals resulted in an increase in the tyrosine phosphorylation of many proteins in the molecular mass range of 44 to 60 kDa. Densitometry measurements on the protein bands of 44, 49, 52, and 60 kDa showed increases of 60%, 50%, 75%, and 150%, respectively, for uncoated CPPD (Fig. 8, *lane 2*) and 40%, 150%, 420%, and 950%, respectively, for plasma-coated CPPD (Fig. 8, *lane 4*), compared with control values (Fig. 8, *lane 1*). Preincubation of the cells with mDHC at 100 μM for 2 min decreased the extent of tyrosine phosphorylation of all these proteins when neutrophils were activated with plasma-coated CPPD (Fig. 8, *lane 3*). Tyrosine phosphorylation was reduced by drug treatment to the basal levels seen in control unstimulated neutrophils. Control cells treated with mDHC at 100 μM (crystals absent) (Fig. 8, *lane 5*) showed levels of tyrosine phosphorylation similar to those of control cells (no drug) (Fig. 8, *lane 1*).

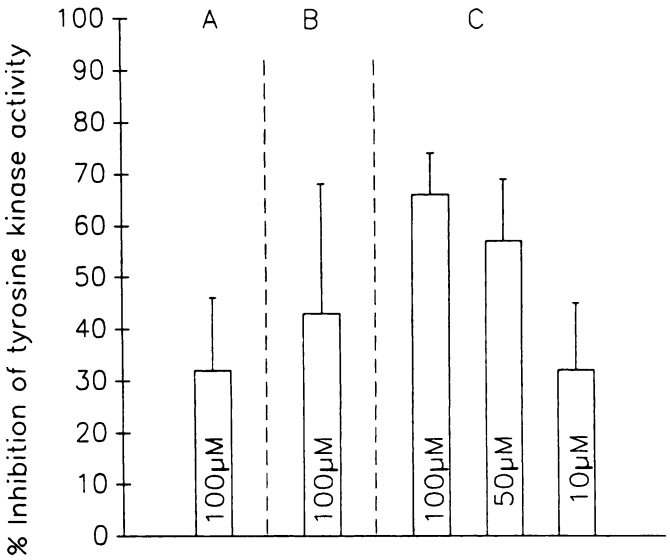

Fig. 7. Percentage of inhibition of neutrophil tyrosine kinase activity by mDHC. One-minute crystal-cell incubations. Cytosolic fractions from uncoated CPPD (100 mg/ml) (A), plasma-coated CPPD (100 mg/ml) (B), and MSUM (10 mg/ml) (C) neutrophil (1 × 10^7/ml) incubations. mDHC concentrations are shown in *bars*. Four experiments.

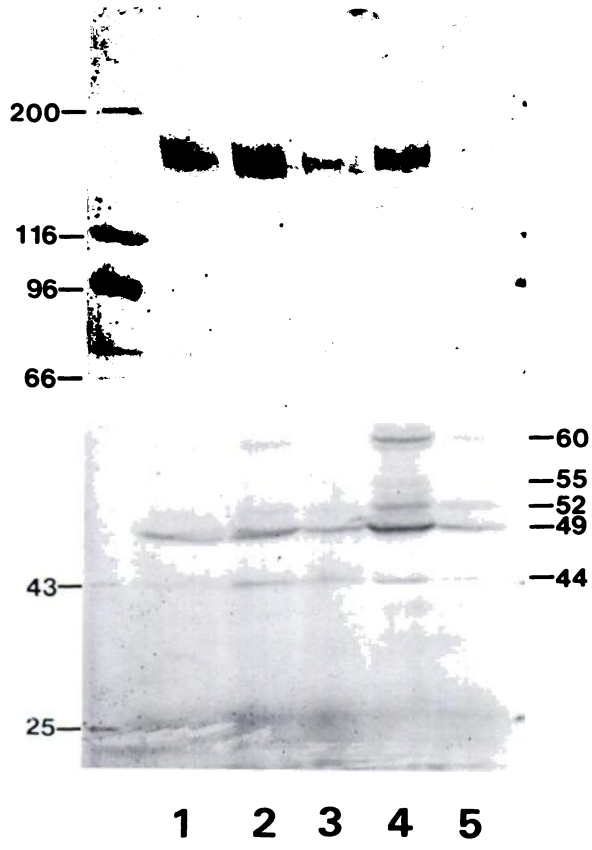

Fig. 8. Tyrosine phosphorylation stimulated by crystals in human neutrophils. Neutrophils (1 × 10^7/ml) were incubated with (*lanes 3* and *5*) or without (*lanes 1*, *2*, and *4*) mDHC (100 μM) for 2 min. Neutrophils were then stimulated with uncoated CPPD (*lane 2*) or plasma-coated CPPD (100 mg/ml) (*lanes 3* and *4*) for 1 min. *Lane 1*, control cells without mDHC. *Lane 5*, control cells with mDHC (100 μM). Tyrosine phosphorylation was determined by immunoblotting as described in Materials and Methods.

Discussion

The generation of highly reactive oxygen radicals and degranulation events in the neutrophil are brought about by a signal transduction system that starts with receptor-ligand binding on the plasma membrane extracellular surface. The pathway leading to receptor activation by the chemottractant FMLP is well characterized (28, 29). FMLP receptor-linked activation involves a pertussis toxin-sensitive guanine nucleotide-binding protein (G_i) that is linked to PLC. PLC activation leads to the formation of IP_3 and 1,2-diacylglycerol. IP_3 is a second messenger molecule that results in increases in the intracellular concentration of calcium, which acts synergistically with 1,2-diacylglycerol to activate PKC. PKC then activates a membrane-bound NADPH-oxidase system that generates superoxide anions. The enzyme MPO is concomitantly released and converts superoxide anions to other toxic oxygen species. The full activation pathway leading to MPO release is unknown, but degranulation has been reported to have some dependance on an increase in calcium concentration (30).

The neutrophil activation pathway involved in phagocytosis is only partly elucidated. There are receptors on the neutrophil plasma membrane for initiation of this process. They recognize the F_c domain of IgG or the C3bi fragment of complement, which may be adsorbed to the surface of bacteria or other foreign matter. It is now apparent that signal transduction leading to phagocytosis, degranulation, and respiratory burst activity by the neutrophil in response to these ligands is varied and does not rely on the full involvement of all the components of the FMLP activation pathway (31, 32). Similarly, it has been reported that crystal-induced neutrophil activation may or may not depend on G_i (13–15), that there are increases in intracellular calcium concentration (13, 14, 15, 27), and that the role of PKC is uncertain (13, 15). It has been suggested that crystals may activate neutrophils via nonspecific interactions that lead to perturbations of the membrane or cross-linking of the membrane glycoproteins (14, 15).

Recently a number of studies have proposed the involvement of protein tyrosine phosphorylation in the regulation of neutrophil activation by a variety of stimuli (1, 2, 7, 9, 10, 16). Using soluble agonists such as FMLP, leukotriene B_4, or platelet-activating factor with known receptors on the neutrophil, the peak levels of increased protein tyrosine phosphorylation were shown to occur within 2 min of addition of these agents to neutrophils (1, 8). Tyrosine kinase inhibitors such as ST638 (2, 9), genistein (16), erbstatin (6, 16), or 3,4-dihydroxycinnamamide (10) have been shown to inhibit superoxide anion generation by neutrophils in response to soluble agonists, presumably by inhibition of the tyrosine kinases. ST638 (9) and 3,4-dihydroxycinnamamide (10) are also reported to be potent inhibitors of calcium mobilization and degranulation. Increases in neutrophil intracellular calcium concentration in response to receptor-linked stimulation by agents such as FMLP are the result of the PLC-directed generation of IP_3 (28, 29). PKC activation and the subsequent activation of the NADPH-oxidase superoxide anion-generating complex require increases in intracellular calcium concentration (28, 29). It has been shown that there are increases in IP_3 levels in neutrophils stimulated with MSUM (14) or quartz crystals (33) (an agent that may act analogously to MSUM or CPPD). It is therefore possible that the inhibition of crystal-induced calcium mobilization and superoxide anion generation by neutrophils caused by mDHC

is due to the initial inhibition of PLC and that PLC may be the substrate for tyrosine phosphorylation. Recently it has been reported that PLC may be the substrate protein for phosphorylation by tyrosine kinases in B cells (34) and NIH 3T3 cells (35). Furthermore, Dryden *et al.* (10) have reported that 3,4-dihydroxycinnamamide inhibits IP_3 formation in FMLP-stimulated neutrophils, suggesting that PLC is the site of the inhibition of tyrosine kinase phosphorylation processes.

Our results show that both uncoated and plasma-opsonized crystals induce the increased tyrosine phosphorylation of many proteins in the molecular mass range of 40 to 60 kDa. We have shown that mDHC virtually abolishes the tyrosine phosphorylation of the prominant protein bands at 52, 55, and 60 kDa and inhibits the phosphorylation of the 44- and 49-kDa proteins.

mDHC has also been shown to inhibit crystal-induced neutrophil responses such as superoxide generation, calcium mobilization, and degranulation. The differences in the concentration dependence of the mDHC inhibition of MPO release (Fig. 5) and the inhibition of chemiluminescence (Fig. 2) indicate that the activation pathways leading to degranulation and superoxide anion generation may be dependent to different degrees on protein tyrosine phosphorylation. The inhibition of tyrosine phosphorylation, tyrosine kinase activity, and neutrophil responses reported in this study leads us to suggest that tyrosine kinases may be involved to some degree in the regulation of crystal-induced neutrophil activation.

Acknowledgments

The authors wish to thank Dr. Roger Brownsey for his technical assistance with densitometry determinations.

References

1. Berkow, R. L., and R. W. Dobson. Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood* **75**:2445–2452 (1990).
2. Gomez-Cambronero, J., C. H. Huang, V. A. Bonak, E. Wang, J. E. Casnellie, T. Shiraishi, and R. I. Shaafi. Tyrosine phosphorylation in human neutrophils. *Biochem. Biophys. Res. Commun.* **162**:1478–1485 (1989).
3. Huang, C. K., G. R. Laramee, and J. E. Casnellie. Chemotactic factor induced tyrosine phosphorylation of membrane associated proteins in rabbit peritoneal neutrophils. *Biochem. Biophys. Res. Commun.* **151**:794–801 (1988).
4. Kraft, A. S., and R. L. Berkow. Tyrosine kinase and phosphotyrosine phosphatase activity in human promyelocytic leukemia cells and human polymorphonuclear leukocytes. *Blood* **70**:356–362 (1987).
5. Grinstein, S., W. Furuya, D. J. Lu, and G. B. Mills. Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electropermeabilized human neutrophils. *J. Biol. Chem.* **265**:318–327 (1990).
6. Naccache, P. H., C. Gilbert, A. C. Caon, M. Gaudry, C. K. Huang, V. A. Bonak, K. Umezawa, and S. R. McColl. Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. *Blood* **76**:2098–2104 (1990).
7. McColl, S. R., J. F. Dipersio, A. C. Caon, P. Ho, and P. H. Naccache. Involvement of tyrosine kinases in the activation of human peripheral blood neutrophils by granulocyte-macrophage colony stimulating factor. *Blood* **78**:1842–1852 (1991).
8. Gomez-Cambronero, J., E. Wang, G. Johnson, C. K. Huang, and R. I. Shaafi. Platelet-activating factor induces tyrosine phosphorylation in human neutrophils. *J. Biol. Chem.* **266**:6240–6245 (1991).
9. Berkow, R. L., R. W. Dodson, and A. S. Kraft. Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation. *Biochim. Biophys. Acta* **997**:292–301 (1989).
10. Dryden, P. J., V. Duronio, L. Martin, A. T. Hudson, and H. Salari. Inhibition of human neutrophil responses by *alpha*-cyano-3,4-dihydroxythiocinnamamide, a protein-tyrosine kinase inhibitor. *Br. J. Pharmacol.* **106**:656–664 (1992).
11. McCarty, D. J. Pathogenesis and treatment of crystal-induced inflammation, in *Arthritis and Allied Conditions* (D. J. McCarty, ed.). Lea and Febiger, Philadelphia, 1495–1514 (1985).
12. Terkeltaub, R., L. K. Curtiss, A. J. Tenner, and M. Ginsberg. Lipoproteins containing apoprotein B are a major regulator of neutrophil responses to monosodium urate crystals. *J. Clin. Invest.* **73**:1719–1730 (1984).
13. Naccache, P. H., M. Grimard, C. J. Roberge, C. Gilbert, A. Lussier, R. De Medicis, and P. E. Poubelle. Crystal-induced neutrophil activation. 1. Initiation and calcium mobilization and superoxide production by microcrystals. *Arthritis Rheum.* **34**:333–342 (1991).
14. Terkeltaub, R. A., L. A. Sklar, and H. Mueller. Neutrophil activation by inflammatory microcrystals of monosodium urate monohydrate utilizes pertussis toxin-insensitive and -sensitive pathways. *J. Immunol.* **144**:2719–2724 (1990).
15. Onello, E., A. Trayor-Kaplan, L. Sklar, and R. Terkeltaub. Mechanism of neutrophil activation by an unopsonized inflammatory particulate: monosodium urate crystals induce pertussis toxin-insensitive hydrolysis of phosphatidylinositol 4,5-biphosphate. *J. Immunol.* **146**:4289–4294 (1991).
16. Kusunoki, T., H. Higashi, S. Hosoi, D. Hata, K. Sugie, M. Mayami, and H. Mikawa. Tyrosine phosphorylation and its possible role in superoxide production by human neutrophils stimulated with FMLP and IgG. *Biochem. Biophys. Res. Commun.* **183**:789–796 (1992).
17. Umezawa, K., T. Hori, T. Hirohisa, I. Masaya, K. Isshiki, and T. Takeuchi. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Lett.* **260**:198–200 (1990).
18. Burt, H. M., P. H. Kalkman, and D. Mauldin. Membranolytic effects of crystalline monosodium urate monohydrate. *J. Rheumatol.* **10**:440–448 (1983).
19. Burt, H. M., and J. K. Jackson. Characterization and membranolytic effects of calcium pyrophosphate dihydrate crystals. *J. Rheumatol.* **14**:968–973 (1987).
20. Simchowitz, L., J. P. Atkinson, and I. Spilberg. Stimulation of the respiratory burst in human neutrophils by crystal phagocytosis. *Arthritis Rheum.* **25**:181–188 (1982).
21. Merritt, J. E., S. A. McCarthy, M. P. Davies, and K. E. Moores. Use of fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils. *Biochem. J.* **269**:513–519 (1990).
22. Burt, H. M., J. K. Jackson, and J. Rowell. Calcium pyrophosphate and monosodium urate crystal interactions with neutrophils: effect of crystal size and lipoprotein binding to crystals. *J. Rheumatol.* **16**:809–817 (1989).
23. Glenny, J. R., L. Zokus, and M. P. Kamps. Monoclonal antibodies to phosphotyrosine. *J. Immunol. Methods* **109**:277–285 (1988).
24. Hed, J., and O. Stendahl. Differences in the ingestion mechanism of IgG and C3b particles in phagocytosis by neutrophils. *Immunology* **45**:727–736 (1982).
25. Lucas, M., and F. Solano. Opposite effect of cytochalasin B on agonist-induced respiratory burst in neutrophils and monocytes. *Biochem. Pharmacol.* **42**:425–430 (1991).
26. Nurcombe, H. L., and S. W. Edwards. Role of myeloperoxidase in intracellular and extracellular chemiluminescence of neutrophils. *Ann. Rheum. Dis.* **48**:56–62 (1989).
27. Poubelle, P. E., R. De Medicis, and P. H. Naccache. Monosodium urate and calcium pyrophosphate crystals differentially activate the excitation-response coupling sequence of human neutrophils. *Biochem. Biophys. Res. Commun.* **149**:649–657 (1987).
28. Christiansen, N. O. Pertussis toxin inhibits the FMLP-induced membrane association of protein kinase C in human neutrophils. *J. Leuk. Biol.* **47**:60–63 (1990).
29. Liang, S., T. J. Woodlock, J. C. Whitin, M. A. Lichtman, and G. B. Segal. Signal transduction in *N*-formyl-methionyl-leucyl-phenylalanine and concanavalin A stimulated neutrophils: superoxide production without a rise in intracellular free calcium. *J. Cell. Physiol.* **145**:295–302 (1990).
30. Niessen, H. W. M., T. W. Kuijpers, D. Roos, and A. J. Verhoeven. Release of azurophilic granule contents in fMLP-stimulated neutrophils requires two activation signals, one of which is a rise in cytosolic free Ca^{2+}. *Cell. Signalling* **3**:625–633 (1991).
31. Fallman, M., G. Martin, C. Hellberg, and T. Andersson. Complement receptor-mediated phagocytosis is associated with accumulation of phosphatidylcholine-derived diglyceride in human neutrophils. *J. Biol. Chem.* **267**:2656–2663 (1992).
32. Rosales, C., and E. J. Brown. Signal transduction by neutrophil immunoglobulin G Fc receptors. *J. Biol. Chem.* **267**:5265–5271 (1992).
33. Tuomala, M. H., M. R. Hirvonen, and K. M. Savolainen. Production of inositol phosphates and reactive oxygen metabolites in quartz-dust-stimulated human polymorphonuclear leukocytes. *FEBS Lett.* **296**:57–60 (1992).
34. Roifman, C. M., and G. Wang. Phospholipase C-γ1 and phospholipase C-γ2 are substrates of the B cell antigen receptor associated protein tyrosine kinase. *Biochem. Biophys. Res. Commun.* **183**:411–416 (1992).
35. Kim, H. K., J. W. Kim, A. Zilberstein, B. Margolis, J. G. Kim, J. Schlessinger, and S. W. Rhee. PDGF stimulation of inositol phospholipid hydrolysis requires PLC-γ1 phosphorylation on tyrosine residues 783 and 1254. *Cell* **65**:435–441 (1991).

Send reprint requests to: Helen M. Burt, University of British Columbia, Faculty of Pharmaceutical Sciences, 2146 East Mall, Vancouver, B.C., V6T 1Z3, Canada.