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Research Article

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Role of Protein Kinases in Stimulation of Human Polymorphonuclear Leukocyte Oxidative Metabolism by Various Agonists

Differential Effects of a Novel Protein Kinase Inhibitor

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Abstract

Isoquinoline sulfonamides have recently been shown to exert novel inhibitory effects on mammalian protein kinases by competitively binding to the ATP substrate site (Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki, 1984, *Biochemistry,* 23: 5036–5041). We synthesized a unique analog of the previously reported compounds, 1-(5-isoquinolinesulfonyl) piperazine (C-I), in order to assess the role of protein kinases in modulating the agonist-stimulated oxidative burst of human polymorphonuclear leukocytes (PMN).

Compound C-I, at micromolar concentration, markedly inhibited the release of superoxide anion from human PMN stimulated with phorbol myristate acetate or the synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol. These data are consonant with previously reported data which indicate that the calcium and phospholipid-dependent protein kinase, protein kinase C, serves as the intracellular receptor for these agonists. In contrast, superoxide anion production stimulated by the complement anaphylatoxin peptide C5a or the synthetic chemotaxin formylmethionyl-leucyl-phenylalanine were not inhibited by C-I. These data suggest that parallel pathways exist for the agonist-stimulated respiratory burst of human neutrophils, only one of which utilizes the calcium and phospholipid-dependent protein kinase.

Introduction

Stimulated polymorphonuclear leukocytes (PMN)[1] reduce molecular oxygen to reactive metabolites, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (1). This respiratory burst is crucial to adequate host defense against bacteria. The enzymatic systems responsible for activation of the respiratory burst are only currently being defined. The response occurs in PMN stimulated by a variety of agonists. Possible physiologic stimuli include C5a, bacterial chemotactic factors, and certain arachidonate metabolites such as leukotriene B_4, as well as phagocytosis (2). Stimulated neutrophils demonstrate increased oxygen consumption, release of O_2^- and H_2O_2, and oxidation of NADPH with resultant stimulation of glucose metabolism by the hexose monophosphate shunt. The enzyme (or enzyme complex) involved in the respiratory burst has been termed "NADPH oxidase", and may involve transfer of electrons from NADPH to a flavoprotein, thence to ubiquinone, thence to a b cytochrome, and finally to O_2 (3–5). Before stimulation, the components of this electron transport chain appear to be present in intracellular organelle(s) and are translocated to the membrane fraction after application of stimulating agonists (6). The biochemical mechanisms responsible for such translocation and/or activation of the oxidase complex are not well understood, but several lines of evidence have recently implicated the calcium and phospholipid-dependent protein kinase, protein kinase C (PKC), as a central component in the response (7–9).

A cell-free system for activation of NADPH oxidase has recently been developed in which activation can be induced by arachidonate or other unsaturated fatty acids in particulate fractions (containing plasma membrane and intracellular organelles) from unstimulated cells (10–13). However, this response requires an unidentified component from the cellular cytosol. In unstimulated cells PKC is located primarily in the cytosol (7, 8) and would be an appropriate candidate enzyme for the reaction. The xenobiotic, phorbol myristate acetate (PMA), directly binds and activates PKC (14, 15) and is a potent stimulant of the respiratory burst (16). Stimulation of PMN with PMA results in a rapid apparent translocation of cytosolic PKC to the particulate fraction in correlation with activation of NADPH oxidase (8). Diacylglycerols have been suggested as the natural activators of PKC (17), and the synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG) activates PKC and stimulates the respiratory burst (9). These data strongly suggest that activation of PKC can lead to activation of NADPH oxidase, possibly by phosphorylating a component of the oxidase enzyme system.

Hidaka and co-workers have recently described the synthesis and properties of a novel class of mammalian protein kinase inhibitors based on isoquinoline sulfonamides (18). These agents possess potent inhibitory activity against cyclic AMP (cAMP) and cyclic guanosine 3′5′-monophosphate–dependent protein kinases, as well as PKC, by competitively interacting with the ATP binding site of the enzymes. In contrast to the napthalene sulfonamides (e.g., W-7), the isoquinoline sulfonamides do not inhibit calmodulin-dependent kinase at low concentrations. The most potent of the inhibitors tested, H-7, has been used to examine the role of PKC in the PMA-stimulated release of platelet serotonin (19). Since PKC plays a potentially key role in modulating the oxidative response of neutrophils, it was of interest to study the pharmacologic effect of isoquinoline sulfonamides in this system. In this communication we report the synthesis and properties of an isoquinoline sulfonamide, 1-(5-isoquino-

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1. *Abbreviations used in this paper:* C-I, 1-(5-isoquinolinesulfonyl) piperazine; Cyto B, cytochalasin B; FMLP, *N*-formyl-methionyl-leucyl-phenyl-alanine; O_2^-, superoxide anion; OAG, 1-oleoyl-2-acetyl-glycerol; PKC, protein kinase C; PMA, phorbol myristate acetate.

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linesulfonyl) piperazine (C-I), as a probe of the human neutrophil respiratory burst.

Methods

Synthesis and characterization of C-I. Isoquinoline-5-sulfonic acid (1.5 g) (Pfaltz and Bauer Inc., Stamford, CT) and phosphorous pentachloride (1.75 g) (Aldrich Chemical Inc., Co., Milwaukee, WI) were combined in a round-bottomed flask with a magnetic stirring bar and a reflux condenser. The heterogeneous reaction mixture was heated under an atmosphere of nitrogen until it melted (oil bath temperature, 210°C) and reflux begun. The resulting yellow liquid was heated for an additional 15 min and then cooled to room temperature, whereupon the reaction mixture solidified. Small pieces of the resulting solid were carefully added to H_2O-ice (250 ml). The aqueous mixture was then extracted with ether (2 × 200 ml) and then with ethyl acetate (2 × 250 ml). The combined organic extracts were washed with brine, dried ($MgSO_4$), and evaporated in vacuo to yield 0.795 g of yellow crystalline solid. Recrystallization of the crude product from toluene gave 0.675 g light-yellow crystalline plates of isoquinoline-5-sulfonyl chloride. Melting point was 104–105°; precise mass spectra calculated for $C_9H_6NSClO_2$ m/e = 226.9, was 226.9.

The isoquinoline-5-sulfonyl chloride was reacted with piperazine exactly as described by Hidaka et al. (18) to yield C-I, with an overall yield of 84%. Crystalline C-I possessed a melting point of 132–134°C; mass spectra calculated as m/e = 277, found 277. The nuclear magnetic resonance and infrared spectra, as well as elemental analysis, confirmed the chemical structure (data not shown).

PKC assay and determination of inhibitor constant. PKC was assayed in subcellular fractions of human PMN as previously described (7, 8). Briefly, in a final volume of 0.25 ml, reaction mixtures contained 35 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 0.6 mM added calcium chloride, 0.4 mM EGTA, 0.4 mM phenylmethysulfonyl fluoride, 0.01% Triton X-100, 2 mM 2-mercaptoethanol, 20 μg/ml phosphatidylserine, 2 μg/ml diolein, 10 or 50 μM (γ-^{32}P)-ATP (New England Nuclear, Boston, MA), 40 μg of histone H-1, and 10–20 μg of neutrophil fraction. Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co., St. Louis, MO. Reactions were conducted for 5 min at 30°C in the presence or absence of added Ca^{2+} and lipids and terminated by the addition of ice-cold 25% trichloroacetic acid followed by the addition of albumin as a carrier. The precipitates were collected by filtration on type HA filters, 0.45 μm (Millipore Corp., Bedford, MA), washed, and subjected to scintillation counting. The inhibitor constant (K_i) was determined for compound C-I as described by Dixon (20). cAMP-dependent protein kinase was assayed under the same conditions except that added Ca^{2+} and lipids were omitted and assays were conducted in the presence or absence of 10 μM cAMP (Sigma Chemical Co.).

Isolation of human PMN neutrophils and subcellular fractions. Neutrophils were isolated from peripheral blood of healthy donors by dextran sedimentation and Ficoll-Hypaque centrifugation as previously described (21). Following hypotonic lysis of contaminating erythrocytes, cells were resuspended in Hank's balanced salt solution (HBSS) containing 4.2 mM sodium bicarbonate and 10 mM Hepes, pH 7.4, and utilized for either intact cell functional studies or for isolation of subcellular fractions.

For isolation of neutrophil subcellular fractions, cells at 5 × 10^7/ml were either not stimulated or stimulated with 1.6 × 10^{-7} M PMA for 30 s, diluted 10-fold with ice-cold HBSS, sedimented, and resuspended in an extraction buffer containing 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and 2 mM EGTA, pH 7.5 (8, 22). Cells were sonicated, centrifuged at 500 *g* to remove unbroken cells and nuclei, then centrifuged at 180,000 *g* for 90 min (7, 8). The 180,000-*g* supernatant was saved and the pellet was resuspended in the same extraction buffer plus 0.1% Triton X-100. Both fractions were stored at 0–4°C.

Measurement of superoxide anion release and oxygen uptake. The release of O_2^- by isolated intact PMN was assessed as the superoxide dismutase-inhibitable reduction of cytochrome *c* as previously described (23). Briefly, reaction mixtures containing 2.5 × 10^6 PMN in HBSS and 0.08 mM cytochrome *c* were prewarmed for 5 min at 37°C in the presence of either C-I or dimethylsulfoxide; the desired stimulus was then added and incubation continued for an additional 5 min. Where indicated, 10 μM cytochalasin B (Cyto B, Sigma Chemical Co.) was added before the 5 min prewarm. Reactions were terminated by placing tubes in melting ice and centrifuging at 500 *g* to sediment cells. The optical density at 550 nm was determined and O_2^- release was expressed as nmol/5 min per 2.5 × 10^6 cells using an extinction coefficient of 21 mM^{-1}, cm^{-1} for cytochrome *c* (24). Under conditions where the cytochrome *c* concentration could be limiting (with PMA or with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) + Cyto B as stimuli), the cell concentration was decreased to 7.5 × 10^5. O_2^- release with PMA as stimulus was linear with cell concentration in the range utilized (data not shown). Oxygen consumption by 10^7 PMN was determined polarographically in a 2-ml water-jacketed cuvette at 37°C using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). PMN were preincubated with the indicated concentrations of C-I before addition of PMA. Data are expressed as the slope of the linear portion of the curve of oxygen tension and are expressed in nmol O_2 consumed/min per 2.5 × 10^6 PMN.

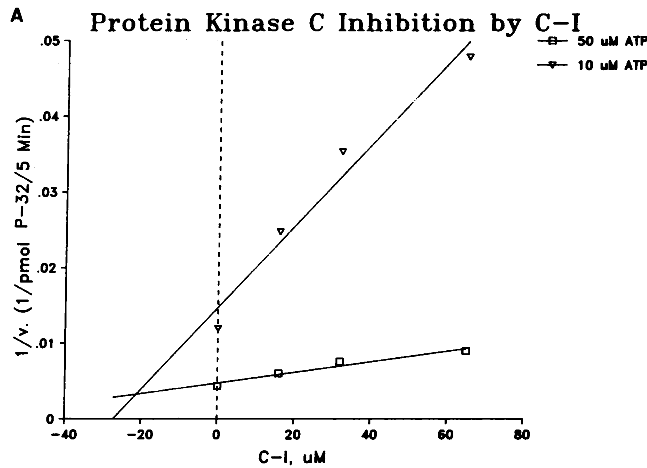
Human PMN stimuli. Human C5a was purified to homogeneity from complement activated sera as described by Hugli, et al. (25). FMLP and PMA were products of Sigma Chemical Co. The synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG), was the generous gift of Jeffrey Schmitt and Dr. Robert Wykle, Bowman Gray School of Medicine (Winston-Salem, NC).

Results

Characterization of C-I. The crystalline C-I preparation was characterized by elemental analysis, infrared spectroscopy, 1-H nuclear magnetic resonance spectroscopy, and mass spectrum analysis (see Methods). The sulfonamide was assayed according to the method of Dixon (20) as an inhibitor of human neutrophil PKC and cAMP-dependent protein kinase in cytosolic fractions prepared from unstimulated human peripheral PMN. As shown in Fig. 1, C-I was a potent inhibitor of both enzymes, with a K_i value of ~20 μM for PKC and ~3 μM for cAMP-dependent protein kinase. In addition, C-I inhibited the particulate-bound protein kinase obtained by PMA-stimulation of intact cells (8) with a 50% inhibition concentration of ~24 μM (data not shown). These results confirm and extend the observations of Hidaka et al. (18) to the kinases present in the human PMN.

Since PMA is a potent agonist of the respiratory burst of neutrophils and since it directly activates PKC, it was of interest to ascertain whether the response of PMN to PMA could be inhibited by compound C-I. As shown in Fig. 2 *A* the release of O_2^- by human PMN stimulated with 30 ng/ml PMA was inhibited in a concentration-dependent fashion by C-I. The 50% inhibition concentration value of this inhibition was 64 μM. Inhibition by the sulfonamide did not appear to be caused by competition with PMA, since the mean percentage inhibition by 103 μM C-I did not change over a 10-fold range of PMA concentrations yielding maximal O_2^- release (3 ng/ml = 50% inhibition, n = 3; 10 ng/ml = 46% inhibition, n = 6; 30 ng/ml = 51% inhibition, n = 12). This conclusion is further supported by one additional experiment, performed in triplicate, in which O_2^- release induced by PMA at concentrations of 10, 30, 100, 300, and 1,000 ng/ml was inhibited 44%, 40%, 39%, 50%, and 40%, respectively, by 103 μM C-I.

The effect of C-I on oxygen consumption induced by PMA was also examined. Results indicated that the time to onset of stimulated consumption (the lag time) was unchanged, but the



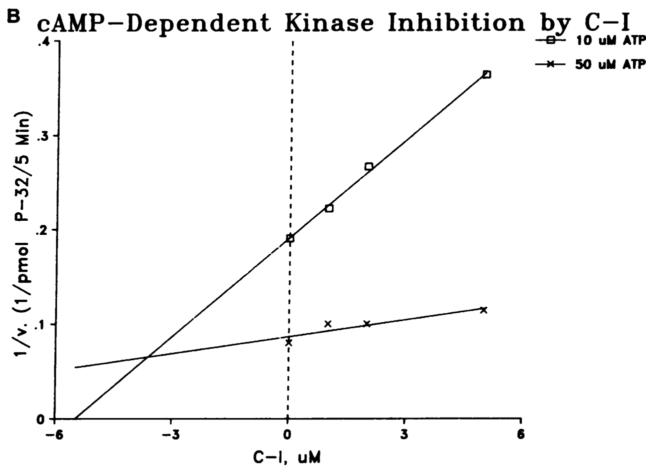
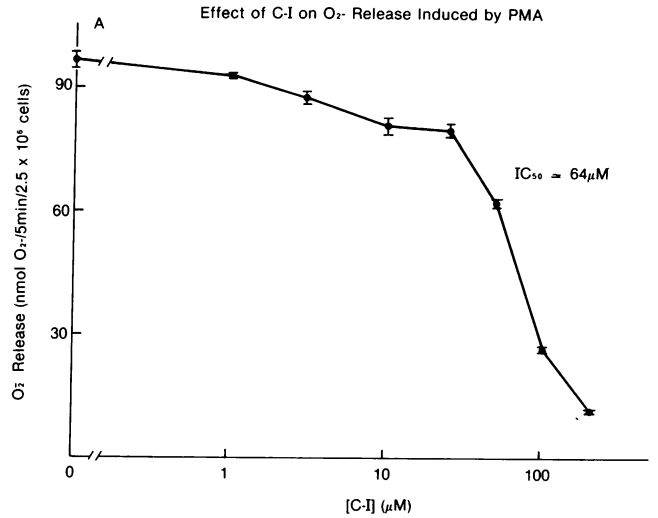


Figure 1. Dixon plot of inhibition by C-I of human PMN protein kinase C and cAMP-dependent protein kinase activities. Cytosolic fractions were assayed for PKC (*A*) or cAMP-dependent protein kinase (*B*) activity at either 10 μM (—▽—, —□—) or 50 μM (—□—, —×—) ATP in the presence of the indicated concentrations of C-I. Data were plotted and the K_i determined from the intersection of the two lines according to the method of Dixon (20).



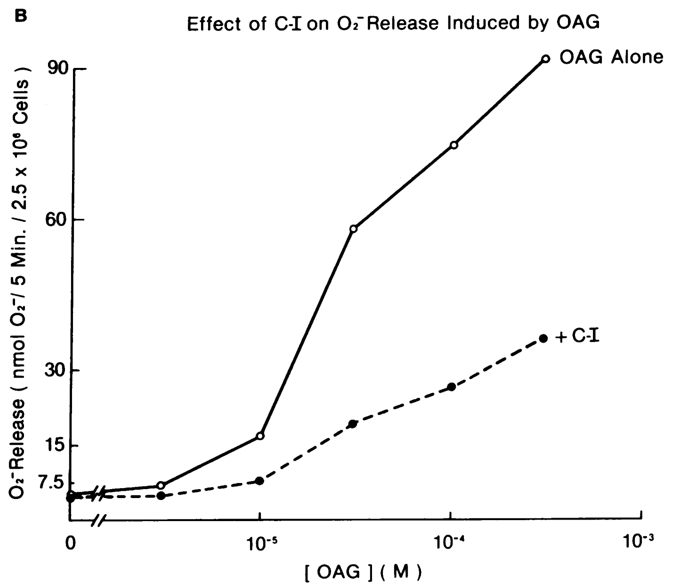


Figure 2. Inhibition by C-I of O_2^- release from PMN-stimulated with PMA or OAG. PMN were assayed for O_2^- release induced by: (*A*) 30 ng/ml PMA in the presence of the indicated concentrations of C-I, or (*B*) various concentrations of OAG in the presence (●) or absence (○) of 103 μM C-I. Shown are the means of triplicate determinations in one representative experiment with each stimulus. Means±SEM are depicted in *A*. Summarized data with each stimulus in the absence and presence, respectively, of 103 μM C-I were as follows: resting (n = 5), 5±1, 4±0; 30 ng/ml PMA (n = 5), 98±2, 44±6; 100 μM OAG (n = 3), 56±11, 23±5; nanomoles O_2^-/5 min per 2.5 × 10^6 PMN, mean±SEM, n = number of experiments.

stimulated rate of consumption was inhibited by C-I (cells + PMA, 46.8±5.8; cells + PMA + 124 μM C-I, 10.3±1.5 nmol O_2/min; means±SEM of five separate PMN preparations).

The proposed natural analogues of PMA in vivo are diacylglycerols. OAG has been particularly useful in implicating PKC in stimulus-response coupling since, unlike diolein, it can penetrate intact cells (17). Fig. 1 *B* demonstrates that the release of O_2^- by human neutrophils stimulated with various concentrations of synthetic OAG is also susceptible to inhibition with C-I. Thus, these data taken together indicate that activation of cytosolic PKC is a necessary step in the activation of the respiratory burst in human PMN induced by PMA and OAG.

Effect of C-I on stimulation of neutrophils by C5a and FMLP. C5a and FMLP are potent agonists of PMN function. Both peptides have been shown to function through binding to receptors present on the plasma membrane (26, 27). The mechanism of signal transduction is unknown; however, the binding of both peptides is followed by a rapid and brief elevation of intracellular cAMP (28). In addition, FMLP has been shown to induce a rapid rise in cytosolic calcium concentration (29) and stimulation of polyphosphoinositide turnover (30). Phosphatidylinositol 4,5 bisphosphate is apparently metabolized to inositol triphosphate and diacylglycerol. Inositol 4,5 bisphosphate may act as a secondary messenger by regulating free intracellular calcium (31); diacylglycerol may also be a secondary messenger through its direct activation of PKC. Thus, it appeared reasonable to speculate that PKC might mediate the respiratory burst stimulated by FMLP and C5a.

As presented in Fig. 3 *A*, O_2^- release in response to 300 nM FMLP was not inhibited by the isoquinoline sulfonamide, C-I (1–206 μM), either in the presence or absence of Cyto B. Similarly, the dose-response profile of C5a was unaffected by 103 μM C-I (Fig. 2 *B*). If PMN were preincubated with 103 μM C-I for 60 min, inhibition of O_2^- release induced by FMLP was still not observed (data not shown). Thus, in contrast to the clear inhibition by C-I of O_2^- release induced with the PKC ligands, these data suggest that the peptide agonists affect the oxidative response in a manner independent of PKC.

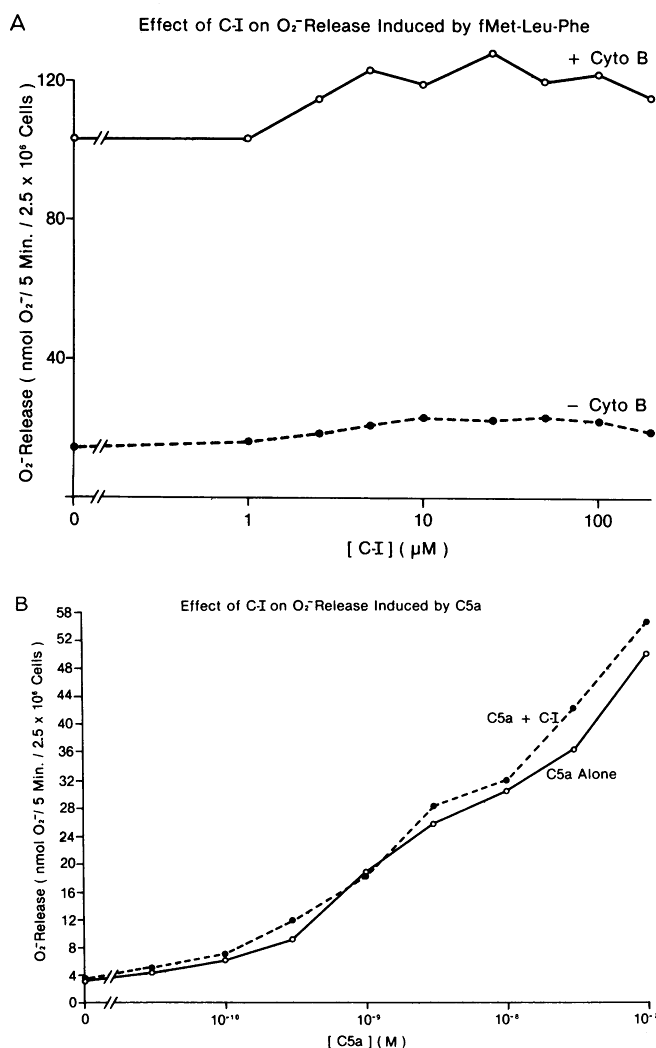


Figure 3. Effect of C-I on O_2^- release from PMN stimulated with FMLP or C5a. PMN were assayed for O_2^- release induced by: (*A*) 300 nM FMLP in the presence of the indicated concentrations of C-I and either in the presence (○) or absence (●) of 10 μM Cyto B, or (*B*) various concentrations of C5a (plus 10 μM Cyto B) in the presence (●) or absence (○) of 103 μM C-I. Shown are the means of triplicate determinations in one representative experiment with each stimulus. Summarized data with each stimulus (plus 10 μM Cyto B) in the absence and presence of 103 μM C-I were as follows: resting (n = 7), 3±0, 3±0; 300 nM FMLP (n = 5), 86±6, 96±7; 30 nM C5a (n = 2), 32±4, 38±4; nmol O_2^-/5 min per 2.5 × 10^6 PMN, mean±SEM, n = number of experiments. In four experiments with 300 nM FMLP in the absence of Cyto B, O_2^- release (nanomoles O_2^-/5 min per 2.5 × 10^6 PMN) was 16±4 and 24±6 (mean±SEM) in the absence and presence, respectively, of 103 μM C-I.

Discussion

In conclusion, our results indicate that the human PMN possesses more than one pathway by which it can mount the respiratory burst. In the case of PMA or diacylglycerol, cytosolic PKC (or a kinase inhibited by C-I) is an essential cofactor in the activation of the oxidase enzyme. Presumably phosphorylation switches on the oxidase pathway since stimulation of neutrophils induces the phosphorylation of several intracellular proteins (9). Although it is not possible to assign the relevant target protein at present, it is tempting to speculate that the terminal b cytochrome may possess both active (phosphorylated) and inactive (dephosphorylated) forms. In this scenario, it is also possible that multiple allosteric modulators may exist for the oxidase. In contrast, the peptide agonists might function either by leading to phosphorylation via a different kinase, (e.g., a calmodulin-dependent kinase or a C-type kinase which is inaccessible to C-I) or by leading to the production of another factor which allosterically activates the enzyme. In any event these data indicate the presence of dual pathways for activation of the respiratory burst in human PMN, one of which requires cytosolic PKC and one of which does not.

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