Naphthalenesulphonamides block neutrophil superoxide production by intact cells and in a cell-free system: is myosin light chain kinase responsible for these effects?

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Selective antagonists of myosin light chain kinase (MLCK) [e.g. ML-7; 1-(5-iodonaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride] were found to inhibit superoxide (O_2^-) release from stimulated neutrophils. The concentrations of ML-7 that were inhibitory were substantially lower than those reported for a selective antagonist of protein kinase C [i.e. H-7; 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride]. ML-7 also reduced the phosphorylation of the 47 kDa subunit of the NADPH-oxidase system (p47-*phox*) and blocked translocation of this protein to the Triton X-100-insoluble fraction in stimulated cells. Interestingly, ML-7 also inhibited

O_2^- production in a cell-free system derived from neutrophils at concentrations similar to those that were effective *in vivo*. This cell-free system does not require ATP and is insensitive to all other inhibitors of protein kinases tested, including some highly effective against MLCK (i.e. staurosporine). Thus, the data suggest that ML-7 does not block O_2^- release by inhibiting a protein kinase but instead may interact directly with a subunit of the oxidase. The binding site for ML-7 may provide a valuable target for inhibiting the inflammatory properties of phagocytic leucocytes by naphthalenesulphonamides designed to lack activity against protein kinases.

INTRODUCTION

Neutrophils stimulated with activators of protein kinase C (PKC) [e.g. 4 β -phorbol 12-myristate 13-acetate (PMA)] or other agonists [e.g. formylmethionyl-leucyl-phenylalanine (fMLP)] release large quantities of O_2^- , an antimicrobial agent. The multicomponent NADPH-oxidase system that catalyses the generation of O_2^- is dormant and dissociated in unstimulated cells and consists of cytosolic (i.e. p47-*phox*, Rac-1 and/or -2), cytoskeletal-associated (p67-*phox*) and integral membrane proteins (flavocytochrome b_{558}). Upon stimulation, there is a translocation of p47-*phox*, p67-*phox* and Rac to the inner surface of the plasmalemma where the active oxidase is assembled (for a review see [1,2]). The C-terminal region of p47-*phox* undergoes continual, multisite phosphorylation in PMA-stimulated cells that appears to be necessary for both the translocation of this protein and for maintaining the oxidase complex in the assembled/active state (e.g. [3–7]). A large body of evidence supports a major role for PKC in some of the signal transduction pathways of these cells (for a review see [8]). For example, certain synthetic isoquinoline-sulphonamides that are selective antagonists of PKC (i.e. H-7 [9]) block phosphorylation and translocation of p47-*phox* along with O_2^- generation in cells stimulated with some agonists (e.g. [5,7,10]).

Certain naphthalenesulphonamides (ML-7, ML-9) have recently been designed as selective antagonists of myosin light chain kinase (MLCK) [11]. These compounds have also been utilized to implicate this kinase in a variety of phenomena associated with phagocytic leucocytes. For example, ML-7 has

been shown to effect differentiation of leukaemic cells (e.g. [12,13]) and to block activation of phospholipase D [14] and O_2^- release [15] in mature neutrophils triggered with certain agonists. The multiplicity of these effects is surprising since the consensus sequence recognized by MLCK is extremely restrictive (e.g. [16]) and is not present in p47-*phox* (or any other component of the oxidase) (the sequences of these proteins are presented in [1]).

In this paper, we compare the abilities of ML-7 and ML-9 to inhibit O_2^- release from stimulated neutrophils with that of H-7. In addition, we show that ML-7 blocks the phosphorylation and translocation of p47-*phox* in PMA-stimulated cells. However, we present evidence that the effects of ML-7 on intact neutrophils may not be due to a blockade of either MLCK or PKC. Finally, we show that ML-7 can also inhibit O_2^- generation in a cell-free system derived from neutrophils. To our knowledge, ML-7 is the first antagonist of this cell-free system described to date which is neither a peptide, sulphhydryl nor flavin inhibitor. Problems associated with the use of naphthalenesulphonamides as antagonists of MLCK in neutrophils are discussed and the potential utility of derivatives of these compounds as direct inhibitors of the NADPH-oxidase complex is intimated.

MATERIALS AND METHODS

Materials

The protein kinase inhibitors ML-7, ML-9, H-7 and KN-62 were obtained from Seikagaku America, Inc., St. Petersburg, FL, U.S.A. Staurosporine was from Kamiya Biochemical Co., Thou-

Abbreviations used: PKC, protein kinase C; MLCK, myosin light chain kinase; O_2^- , superoxide; PMA, 4 β -phorbol 12-myristate 13-acetate; fMLP, N-formylmethionyl-leucyl-phenylalanine; GTP[S], guanosine 5'-[γ -thio]triphosphate; p47-*phox*, the 47 kDa protein component of the phagocyte oxidase; p67-*phox*, the 67 kDa protein component of the phagocyte oxidase; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; ML-7, 1-(5-iodonaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride; ML-9, 1-(5-chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine hydrochloride; diC8, 1,2-dioctanoyl-*sn*-glycerol; diC8PA, 1,2-dioctanoyl-*sn*-phosphatidate; AMP-PNP, 5'-adenylylimidodiphosphate.

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sand Oaks, CA, U.S.A. 1,2-Dioctanoyl-*sn*-glycerol (diC8) was purchased from Calbiochem. Calyculin A and okadaic acid were obtained from LC Services Corporation, Woburn, MA, U.S.A. 1,2-Dioctanoyl-*sn*-phosphatidate (diC8PA) was a product of Avanti Polar Lipids, Alabaster, AL, U.S.A. 5'-Adenylylimidodiphosphate (AMP-PNP) and FAD were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Anti-peptide antibodies to p47-*phox* and p67-*phox* were raised in rabbits using synthetic peptides corresponding to amino acids 340–355 of p47-*phox* and 437–450 of p67-*phox*, as previously described [7,17]. Sources of all other materials are described elsewhere [5,10,18].

Preparation of neutrophils

Guinea-pig peritoneal neutrophils and human blood neutrophils were prepared as described in refs. [19] and [20] respectively. Cell preparations from guinea-pigs and humans contained $\geq 90\%$ and $\geq 95\%$ neutrophils respectively, with viabilities always $\geq 90\%$.

Superoxide release

Superoxide release was measured as described earlier [18]. Cells $[(1.0\text{--}1.5) \times 10^6/\text{ml}]$ were incubated in the reaction mixture at 37°C with or without the inhibitor for 3 min before O_2^- release was initiated by the addition of the stimulus.

Stock solutions of ML-7 (40 mM) and ML-9 (40 mM) were prepared in DMSO and stored at 4°C . These compounds were diluted with DMSO so that the final amount of solvent in each assay was either 0.25 or 0.50% (v/v) [this includes the 0.25% (v/v) added with the stimulus]. These amounts of solvent did not cause any of the effects noted. All other stock solutions were prepared as described in refs. [5,18].

Labelling of neutrophils with $^{32}\text{P}_i$ and SDS/PAGE for studies on protein phosphorylation

These techniques were performed as described previously [10], except the amount of radioactivity was increased to 1.0 mCi of $^{32}\text{P}_i/\text{ml}$ and the labelling time was extended to 1 h.

Isolation of the Triton X-100 fractions of neutrophils and immunoblotting

These experiments were performed exactly as described in [7].

Superoxide generation by a cell-free system from human neutrophils

This assay was performed as described elsewhere [17,21].

RESULTS

Effects of ML-7 and ML-9 on stimulated neutrophils

Certain synthetic sulphonamides (H-7, ML-7) can inhibit a variety of protein kinases by competing for the ATP-binding sites on these enzymes [9,11]. The inhibitors ML-7 and H-7 were designed as selective antagonists of MLCK (ML-7 > H-7) and PKC (H-7 > ML-7) respectively [9,11]. The isoquinoline-sulphonamide H-7 (100–200 μM) is known to substantially block O_2^- release ($\geq 80\%$) from guinea-pig or human neutrophils if added to the cells either prior to or after stimulation (e.g. [5,7,22,23]; Figure 1, curves e and f). Half-maximal inhibition (i.e. IC_{50}) occurred at approx. 60 μM H-7 when either PMA (50 nM) or fMLP (1.0 μM) was utilized as the stimulating agent

Figure 1 Effects of the naphthalenesulphonamide ML-7 on superoxide release by stimulated neutrophils

Reaction progress curves demonstrate the effects of ML-7 and H-7 on superoxide release from guinea-pig neutrophils. Cells ($10^6/\text{ml}$) were incubated in the reaction mixture for 3 min at 37°C prior to the addition of 50 nM PMA, which was added last to initiate the reactions. Curve a shows a portion of the curve for stimulated cells. Curve b is for unstimulated cells. ML-7 (20 μM) completely blocked O_2^- release if added to the cells 3 min prior to stimulation (curve c) or to cells already stimulated for 1 min (curve d). Similarly, H-7 (200 μM) substantially blocked O_2^- release if added to the cells 3 min prior to stimulation (curve e) or to cells already stimulated for 1 min (curve f). Reaction curves are continuous; symbols do not represent time points and are used only to distinguish between curves.

[24]. These results are consistent with a variety of other evidence that implicated PKC in a signal transduction pathway for O_2^- production with certain agonists (for a review see [8]).

Surprisingly, we have now observed that the naphthalene-sulphonamide derivative ML-7 completely blocked O_2^- release (100%) from guinea-pig neutrophils at a concentration of 20 μM (Figure 1, curves c and d). Complete dose-response curves for ML-7 are presented in Figure 2(a). Half-maximal inhibition in cells stimulated with 50 nM PMA or 1.0 μM fMLP occurred at $4.6 \pm 0.7 \mu\text{M}$ (range, $n = 2$) and $4.9 \pm 0.8 \mu\text{M}$ ML-7 (S.D., $n = 3$) respectively. Similarly, treatment of human neutrophils with 25 μM ML-7 for 3 min at 37°C inhibited O_2^- release upon subsequent stimulation with 50 nM PMA or 1.0 μM fMLP by $100 \pm 0.5\%$ and $80 \pm 16\%$ (S.D., $n = 4$) respectively.

The naphthalenesulphonamide ML-9 is also a selective antagonist of MLCK but is considerably less potent than ML-7. The K_i values for ML-7 and ML-9 with respect to ATP are 0.30 and 3.8 μM respectively [11]. Interestingly, the concentrations of ML-9 which produced 50% inhibition of O_2^- release were only about 2-fold higher than those observed with ML-7 (Figures 2a and 2b). The IC_{50} values for ML-9 in cells stimulated with 50 nM PMA or 1.0 μM fMLP were $7.8 \pm 1.2 \mu\text{M}$ (S.D., $n = 6$) and $9.2 \pm 1.5 \mu\text{M}$ (S.D., $n = 4$) respectively. The Hill plots of the data for ML-7 and ML-9 were complex and appeared to contain two linear regions, both of which exhibited slopes > 1.0 (Figures 2a and 2b, insets). The concentrations of ML-7 and ML-9 utilized did not affect cell viability, as measured by the exclusion of Trypan Blue, nor did they affect O_2^- release in an O_2^- -generating system (xanthine oxidase plus purine) (results not shown).

Previous studies have shown that 200 μM H-7 substantially reduced both the phosphorylation of p47-*phox* [25] and the translocation of this protein to the Triton X-100-insoluble

Figure 2 Effects of the naphthalenesulphonamides ML-7 and ML-9 on superoxide release by neutrophils

Dose-response curves demonstrate the inhibition of O_2^- release from guinea-pig neutrophils by different concentrations of ML-7 (a) and ML-9 (b). The data shown are for cells stimulated with 50 nM PMA (○) or 1.0 μM fMLP (▲). Cells were incubated with the inhibitors for 3 min at 37 °C in the standard assay mixture prior to the initiation of the reactions by the addition of the stimulus. The insets show the Hill plots of these data, where V is the activity without inhibitor, and v is the activity with inhibitor. These concentrations of ML-7 and ML-9 did not affect cell viability, as measured by exclusion of Trypan Blue, nor did they affect O_2^- release in an O_2^- -generating system (i.e. xanthine oxidase plus purine).

fraction of neutrophils stimulated with PMA [7]. This Triton X-100-insoluble fraction contains the assembled oxidase of stimulated cells (e.g. [6]). Figure 3 demonstrates the effects of ML-7 on protein phosphorylation in human neutrophils. Cells stimulated with PMA exhibited a rapid incorporation of $^{32}P_i$ into a 47 kDa protein ([26,27]; Figure 3, lane b, arrow). Significantly less phosphorylation of this 47 kDa protein occurred if the cells were treated with 25 μM ML-7 for 3 min at 37 °C prior to stimulation (Figure 3, lane c). The extent of phosphorylation of this 47 kDa protein in the presence of 25 μM ML-7 was estimated by densitometry (e.g. Figure 3b) and found to be inhibited in human and guinea-pig neutrophils by $53 \pm 7\%$ (S.D., $n = 3$) and $52 \pm 12\%$ (S.D., $n = 4$) respectively. This 47 kDa protein appeared to be p47-phox since it was not observed in PMA-stimulated neutrophils from patients with chronic granulomatous disease deficient in this protein (cf. [1,2,27]). Neutrophils also exhibited a pair of proteins with molecular masses of approx. 67 kDa that underwent an increased incorporation of $^{32}P_i$ upon stimulation with PMA (Figure 3, lane b, area designated 'S'). Labelling of the upper band in this area was also markedly reduced by treating the cells

Figure 3 Effects of ML-7 on protein phosphorylation in human neutrophils stimulated with PMA

^{32}P -labelled neutrophils (1.5×10^6 /ml) were treated with 25 μM ML-7 for 3 min prior to stimulation with 50 nM PMA for 2 min. The autoradiograms in (a) are from: lane a, unstimulated cells [i.e. treated with 0.25% (v/v) DMSO for 1 min]; lane b, stimulated cells; and lane c, stimulated cells treated with ML-7. The positions of p47-phox and a 67 kDa protein that undergoes increased phosphorylation upon cell stimulation are designated by an arrow and an arrowhead respectively. (b) presents the densitometric scans of p47-phox under the various conditions of (a). This pattern was observed in three separate experiments performed on different preparations of cells.

Figure 4 Effects of ML-7 on the translocation of p47-phox to the Triton X-100-insoluble fraction in human neutrophils stimulated with PMA

Immunoblots show the distribution of p47-phox and p67-phox in the Triton X-100 soluble (a) and insoluble fractions (b) from: lane a, unstimulated cells [i.e. treated with 0.25% (v/v) DMSO for 3 min]; lane b, stimulated cells; and lane c, stimulated cells treated with ML-7. The locations of p67- and p47-phox are indicated by the broken and unbroken arrows, respectively. Neutrophils (10^6 /ml) were treated with 25 μM ML-7 for 3 min at 37 °C prior to stimulation with 50 nM PMA for 3 min. This pattern was observed in two separate experiments performed on different preparations of cells.

with ML-7 (Figure 3, lane c, arrowhead). The identities of these two phosphoproteins are not known. However, studies on neutrophils from three patients with chronic granulomatous disease deficient in p67-phox indicate that neither protein is p67-phox (P. G. Heyworth, J. Ding, R. W. Erickson, D. J. Lu, J. T. Curnutte and J. A. Badwey, unpublished work).

When unstimulated human neutrophils are solubilized in Triton X-100, p67- and p47-phox are located by Western blotting primarily in the insoluble and soluble fractions, respectively ([4]; Figure 4). Stimulation of the cells with PMA is known to result in approx. 50% of p47-phox undergoing translocation to the Triton X-100-insoluble fraction ([4]; Figure 4, lane b). Treatment of unstimulated cells with ML-7 (25 μM) for 3 min at 37 °C

Figure 5 Effects of calyculin A on superoxide release from stimulated neutrophils

Reaction progress curves demonstrate the effects of calyculin A on superoxide release by stimulated guinea-pig neutrophils treated with H-7 or ML-7. Cells ($10^6/\text{ml}$) were incubated in the reaction mixture for 10 min at 37°C with 20 nM calyculin A prior to the addition of 50 nM PMA, which was added last to initiate the reactions. Superoxide release from cells treated with calyculin A was largely insensitive to H-7 (200 μM) when added to the reaction mixture 1 min after PMA (curve c; compare with curve f in Figure 1). In contrast, superoxide release from cells treated with calyculin A was highly sensitive to inhibition by ML-7 (25 μM) when added to the reaction mixture 1 min after PMA (curve d; compare with curve d in Figure 1).

blocked this translocation of p47-phox upon subsequent stimulation with PMA (Figure 4, lane c).

The isoquinolinesulphonamide KN-62 {1-[*N,O*-bis(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine} is a potent ($K_i = 0.9 \mu\text{M}$) and specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinase 2 [28]. Treatment of neutrophils with KN-62 (25–100 μM) for 3 min at 37°C did not appreciably inhibit O_2^- release (<20%) upon subsequent stimulation of the cells with either 50 nM PMA or 1.0 μM fMLP ($n = 2$; results not shown).

Differential effects of ML-7 and H-7 on O_2^- release from stimulated neutrophils

As noted above, the addition of H-7 to guinea-pig neutrophils that are already stimulated with PMA results in a rapid cessation of O_2^- release ([5,7]; Figure 1, curve f). Calyculin A (20 nM), an antagonist of type-1 and -2A protein phosphatases [29,30], can prevent this inhibition ([5,7]; Figure 5, curve c). These data strongly indicated that type-1 and/or -2A protein phosphatases were involved in the termination of O_2^- release under these circumstances [5,7,31]. In contrast, calyculin A (20 nM) did not prevent the termination of O_2^- release when ML-7 was added to stimulated cells (i.e. compare curves d in Figures 1 and 5). Similar effects were observed in human neutrophils (results not shown). These data suggest that if ML-7 was blocking O_2^- release by inhibiting a protein kinase, that kinase was likely to be different from the one inhibited by H-7 (and phosphorylating different sites) since the phosphatases active against the products of these kinases were different.

Figure 6 Effects of protein kinase inhibitors on superoxide release from neutrophils stimulated with 1,2-dioctanoyl-*sn*-glycerol (diC8)

Reaction progress curves demonstrate the effects of staurosporine, H-7 and ML-7 on O_2^- release from guinea-pig neutrophils. Cells ($10^6/\text{ml}$) were incubated in the reaction mixture at 37°C with or without the inhibitor for 3 min prior to the addition of 15.6 μM diC8, which was added last to initiate the reactions. The curves shown are for: curve a, stimulated cells without an inhibitor; curve b, unstimulated cells; curve c, stimulated cells treated with 150 nM staurosporine; curve d, stimulated cells treated with 200 μM H-7; and curve e, stimulated cells treated with 20 μM ML-7.

Previous studies have demonstrated that neutrophils stimulated with high concentrations of diC8 release massive quantities of O_2^- that is largely insensitive to H-7 (200 μM) and staurosporine (150 nM) ([32]; Figure 6, curves c and d). These inhibitors did, however, increase the 'lag period' of this response (cf. [32]). In contrast, 20 μM ML-7 completely inhibited O_2^- release from neutrophils stimulated with 15.6 μM diC8 (Figure 6, curve e; $n = 4$). Staurosporine is a very potent competitive inhibitor of the ATP-binding site of MLCK ($K_i = 0.0013 \mu\text{M}$) (e.g. [33,34]). The concentrations of ML-7 and staurosporine used in this experiment were 67 and 150 times the K_i values for these inhibitors with MLCK respectively. Moreover, this amount of staurosporine (150 nM) produced optimal inhibition of O_2^- release (>80%) when either PMA or fMLP was the agonist [24,32].

Effects of ML-7 on O_2^- production in a cell-free system derived from human neutrophils

The studies described above suggested that ML-7 may inhibit O_2^- release from neutrophils by binding to a target other than a protein kinase. This possibility was explored by examining the effects of ML-7 on O_2^- generation in a cell-free system that consisted of cell membranes, cytosol, endogenous GTP and an anionic lipid (e.g. SDS, diC8PA) (Figure 7). Previous studies have established that this system does not require ATP [35,36] and is refractory to antagonists of protein kinases (e.g. H-7, staurosporine) [37,38]. ML-7 substantially reduced O_2^- production by the cell-free system (>80%) under conditions where only minor inhibition (<20%) was observed with H-7 (Figures 7 and 8). ML-7 inhibited O_2^- production in this system if added to the reaction mixture either prior to (Figure 7) or after (Figure 8) the initiation of O_2^- generation by SDS. Increasing the concentration of H-7 in the cell-free system to 200 μM did not increase the percentage inhibition by more than 35% (results not shown; $n = 3$). The concentrations of ML-7 that were effective in

Figure 7 Effects of ML-7 and H-7 on the production of superoxide by a cell-free system derived from human neutrophils

Dose-response curves are shown for the inhibition of O_2^- generation from a cell-free system by ML-7 (triangles) and H-7 (circles). Superoxide production in these systems was triggered by either 40 μ M SDS (Δ , $\circ$) or 100 μ M 1,2-dioctanoyl-*sn*-phosphatidate ($\blacktriangle$, $\bullet$). Data points represent the mean values $\pm$ S.D. from three separate experiments. The reaction mixtures were incubated with different concentrations of ML-7 or H-7 for 3 min prior to the initiation of O_2^- production by the addition of the anionic lipid. The rates of O_2^- production by this cell-free system activated with either SDS or 1,2-dioctanoyl-*sn*-phosphatidate were 24.7 ± 5.5 and 48.0 ± 6.1 nmol of O_2^- /min per 10^7 cell-equivalents respectively. Increasing the concentration of H-7 to 200 μ M did not increase the percentage inhibition by more than 35% (results not shown; $n = 3$). The inset demonstrates the effects of different concentrations of GTP[S] on the inhibition by ML-7 for the cell-free system activated with 40 μ M SDS. The curves shown are for the system: ($\circ$) without GTP[S] (i.e. the endogenous amount of GTP present was 2.8 μ M); ($\blacktriangle$) plus 10 μ M GTP[S] and ($\bullet$) plus 50 μ M GTP[S].

Figure 8 Effect of adding ML-7 after the initiation of NADPH oxidase activity in a cell-free system

Superoxide production was monitored by following the reduction of cytochrome *c* at 550 nm. ML-7 was added to give a final concentration of 40 μ M ($\blacksquare$) or 100 μ M ($\blacktriangle$, $\bullet$), either 2 min before the initiation of O_2^- production with SDS (curve d, $\bullet$) or at 5 min, as indicated by the arrow, when the system was fully activated and producing O_2^- at a maximal rate (curves b and c; $\blacksquare$, $\blacktriangle$). In the control reaction (curve a, $\circ$) DMSO was added at 5 min to give a final concentration equivalent to that in the reactions containing ML-7 (0.5%, v/v). Although the microplate reader collects data every 6 s, data points at 12 s intervals are shown for clarity. For the same reason, only data points between 5 and 10 min are shown for curves b and c. The data shown are representative of three experiments.

the cell-free system triggered with diC8PA were similar to those observed in intact neutrophils (Figure 2a).

One of the cytosolic components of the cell-free system is the small GTP-binding protein Rac-1 or -2 [39,40]. It is possible that ML-7 may inhibit O_2^- production in this system by competing with endogenous GTP for the nucleotide-binding site on this protein. However, the inhibition by ML-7 was not appreciably affected by the addition of either 10 or 50 μ M guanosine 5'-[γ -thio]triphosphate (GTP[S]) to the assay (Figure 7, inset), which argues against this possibility. Recent studies have suggested that p47-*phox* and p67-*phox* may possess nucleotide triphosphate-binding sites [41,42]. ML-7 may bind at these locales or at the FAD-binding site on cytochrome *b*₅₅₈ [43,44]. However, the addition of exogenous ATP, AMP-PNP or FAD to the cell-free system prior to the initiation of O_2^- release with SDS did not significantly diminish the inhibition by ML-7. The 'percentage inhibition' by 20 μ M ML-7 in the absence of these compounds and in the presence of 0.40 mM ATP, 1.0 mM ATP, 1.0 mM AMP-PNP or 60 μ M FAD was $63 \pm 7\%$ (S.D., $n = 3$), $72 \pm 5\%$, $70 \pm 7\%$, $68 \pm 3\%$ and $61 \pm 3\%$ (range, $n = 2$) respectively. Thus, no serious competition between these compounds and ML-7 was apparent. Addition of ATP, AMP-PNP, or FAD to the cell-free system did not significantly alter the rates of O_2^- release. Finally, the effect of ML-7 on the K_m for NADPH in the cell-free system was investigated. The K_m for NADPH in the presence of 0.0 and 20 μ M ML-7 was 46.0 ± 4 μ M (S.D., $n = 3$) and 47.0 ± 6 μ M (range, $n = 2$) respectively. Thus, ML-7 does not compete for the NADPH-binding site.

DISCUSSION

Two novel and related observations are described in this paper. First, we report that the naphthalenesulphonamides ML-7 and ML-9, selective antagonists of MLCK, inhibit a number of responses in stimulated neutrophils. The pattern of inhibition with these compounds initially suggested the involvement of MLCK in O_2^- production. Secondly, we show that ML-7 inhibits O_2^- generation in a cell-free system in which protein kinases are not required. This and other studies seriously call into question the involvement of MLCK in these phenomena. The significance of these observations is developed below.

Continuous, multisite phosphorylation of p47-*phox* is necessary for O_2^- release in neutrophils stimulated with PMA (e.g. [3–7]). This phosphorylation may result in a conformational change in p47-*phox*, exposing the protein's two SH3 domains which would then be free to interact with proline-rich regions in p67-*phox* and cytochrome *b*₅₅₈ [45–48]. Interaction with the cytochrome is required for the translocation of p47-*phox* to the cytoskeleton/plasmalemma [49]. In this paper, we report that ML-7 is considerably more potent than H-7, a selective antagonist of PKC [9], in inhibiting O_2^- release (e.g. Figures 1 and 2). Both ML-7 and H-7 inhibit MLCK, in the order ML-7 > H-7 [11]. ML-7 also significantly reduced the phosphorylation of p47-*phox* (Figure 3) and blocked translocation of this protein to the Triton X-100-insoluble fraction (Figure 4). Thus, the involvement of MLCK in a stimulatory pathway for O_2^- production would be suspected by a number of criteria.

While it is difficult to prove a negative effect, several lines of evidence argue against this possibility. For example, the consensus sequence recognized by MLCK is not present in p47-*phox* (e.g. [1]) and is thought to occur only in the light chains of myosin (e.g. [16]). No role has been established to date for myosin light chain in O_2^- production. In addition, calyculin A, an antagonist of type-1 and -2A protein phosphatases [29,30],

was found to prevent inhibition of O_2^- release by stimulated neutrophils treated with H-7 but not ML-7 (Figure 5). These results suggest that if ML-7 was inhibiting a protein kinase, that kinase must be different from that inhibited by H-7 since the product(s) were obviously different. It is interesting to note in this context that the known phosphatases active against MLCK are sensitive to antagonists of type-1 and -2A protein phosphatases (e.g. calyculin A, okadaic acid; [50,51]). Similarly, ML-7 completely blocked O_2^- release from neutrophils stimulated with high concentrations of diC8 (15.6 μ M) (Figure 6). In contrast, O_2^- release from cells stimulated under these circumstances was largely insensitive to concentrations of H-7 (200 μ M) and staurosporine (150 nM) which caused maximal inhibition when PMA or fMLP was the agonist ([32]; Figure 6). Staurosporine is a very potent inhibitor of both PKC and MLCK and has similar affinities for these enzymes (e.g. [33,34]). Finally, it is particularly noteworthy that ML-7 blocked O_2^- release in the cell-free system (Figure 7). While we cannot exclude the possibility that neutrophils may contain a highly unusual MLCK that recognizes a unique consensus sequence and is insensitive to staurosporine, several studies have established that the cell-free system does not require ATP (e.g. [35–38]). Thus, there is no required role for protein phosphorylation in this system and ML-7 must be functioning in a manner other than by inhibiting a protein kinase. The effective concentrations of ML-7 when diC8PA was the anionic lipid in the cell-free system were similar to those observed in whole cells (compare Figures 2a and 7).

What, then, is the mechanism whereby ML-7 inhibits O_2^- production? A modest amount of speculation may be appropriate here. The NADPH-oxidase activity of the cell-free system can be fully reconstituted with purified or recombinant cytochrome b_{558} , p47-phox, p67-phox and Rac [43,52]. Thus, there are four proteins which may serve as possible targets for this drug. Evidence presented herein suggests that ML-7 does not compete for the GTP-binding site on Rac (Figure 7, inset), at the FAD- or NADPH-binding sites on cytochrome b_{558} , or at the putative nucleotide triphosphate sites on p47-phox or p67-phox (see the Results section). Therefore, the evidence suggests that ML-7 binds at a novel site on the oxidase. Certain synthetic peptides whose amino acid sequences correspond to regions of cytochrome b_{558} , p47-phox and Rac inhibit O_2^- production by the cell-free system only if added to the assay prior to the initiation of O_2^- release (e.g. [53–55]). While some of these peptides may function by non-specific mechanisms [56,57], these data indicate that once the oxidase is formed in the cell-free system, certain sites become inaccessible. In contrast, ML-7 can inhibit O_2^- release by intact neutrophils (Figures 1 and 5) and in the cell-free system (Figure 8) even when added after the initiation of O_2^- production. This suggests that the binding site for ML-7 is accessible in the assembled oxidase. One possibility is that ML-7 induces a conformational change in p47-phox which prevents this protein from promoting electron flow through cytochrome b_{558} (cf. [58]). Perhaps this conformational change in p47-phox also reduces its interaction with PKC. This situation would inhibit the phosphorylation (Figure 3) and subsequent translocation of p47-phox (Figure 4) and could thus also account for the results presented herein on intact cells.

Finally, it is noteworthy that while the K_i values exhibited by MLCK for ML-7 and ML-9 differ by more than an order of magnitude [11], these compounds exhibit similar IC_{50} values for the inhibition of O_2^- release from stimulated neutrophils (Figure 2). Thus, ML-7 and ML-9 may serve as excellent starting points or 'lead compounds' for the development of specific inhibitors of the oxidase that lack activity towards protein kinases.

These studies were supported by N.I.H. grants AI-24838 (to J.T.C.), AI-23323 (to J.A.B.) and RR-00833 (to the General Clinical Research Centre at Scripps), and by a grant from the Council for Tobacco Research, U.S.A. (to P.G.H.).

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