Nonselective Inhibition of Neutrophil Functions by Sphinganine*

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Sphinganine has been proposed to be a specific inhibitor of protein kinase C. In the present study we have evaluated whether sphinganine is a convenient tool to probe for the role of protein kinase C in neutrophil function. Human neutrophils were loaded with the fluorescent probe quin2 and then tested in parallel for cytosolic free Ca2+, [Ca2+]i, membrane potential changes, O2 production, and exocytosis in a dose-dependent manner. Sphinganine inhibited exocytosis elicited by the calcium ionophore ionomycin. Although sphinganine blocked O2 production due to phorbol 12-myristate 13-acetate, the most striking finding was that the drug rendered the cells leaky. Thus, at similar concentrations as those inhibiting cellular functions, sphinganine was shown to lead to cell permeabilization, as assessed by release of quin2 and cytoplasmic markers into the extracellular medium, and changes in plasma membrane potential.

We conclude, therefore, that sphinganine does not appear to be a suitable compound for the evaluation of the involvement of protein kinase C in neutrophil activation.

Activating protein kinase C by the chemotactic peptide fMLP causes cleavage of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (Ins-1,4,5-P3). Ins-1,4,5-P3 production leads to a rise in the cytosolic free Ca2+ concentration, [Ca2+]i. A rise in [Ca2+]i, is a potent intracellular signal in neutrophils (3); however, activation of neutrophils by the chemotactic peptide creates at least one additional signal, whose nature remains unclear (4). For several reasons, protein kinase C is the most attractive candidate as, on the one hand, diacylglycerol, the endogenous activator of protein kinase C, is generated during cell activation (5) and on the other hand, phorbolesters, known to directly activate protein kinase C, are potent stimulators of various functions of neutrophils (6). However, major methodological problems preclude the definitive demonstration of this scheme. The most significant elevations of diacylglycerol can be detected only at late times of neutrophil activation (7, 8) and there are no direct methods presently available to assess protein kinase C activity in intact cells. For these reasons, a specific inhibitor of protein kinase C could be an extremely useful research tool. It has been reported that the C kinase inhibitor 1-(5-isouquinolinesulfonyl) piperazine (C-1) inhibits neutrophil activation by PMA but not by fMLP (9). The authors concluded that protein kinase C does not mediate the effect of chemotactic peptide. However, the reliability of compound C-1 as a specific protein kinase C inhibitor is questionable, since it also inhibits CAMP-dependent protein kinases.

Recent studies demonstrated that certain long-chain (sphingoid) bases, namely sphinganine and sphingosine, are potent inhibitors of the oxidative burst in neutrophils in response to a variety of stimuli (10). As these compounds have been shown in an in vitro assay to inhibit protein kinase C activation (11), the authors proposed that the suppression of the oxidative burst is due to selective inhibition of the enzyme (10, 11). A major argument for the selectivity of this inhibition was shown by studies of [Ca2+]i; it was found that inhibitory concentration of sphinganine did not significantly affect basal or fMLP-stimulated [Ca2+]i levels (10). If this were the case, then fMLP-induced phospholipase C activation and subsequent generation of Ins-1,4,5-P3 and diacylglycerol are unimpaired, whereas more distally the activity of protein kinase C is inhibited (12).

In the present study we have evaluated the specificity of the inhibitory effect of sphinganine. In contrast to the study of Wilson et al. (10), we demonstrate here that sphinganine as well as sphingosine interfere with various steps of neutrophil activation, and most importantly increase the plasma membrane permeability. Thus, long-chain (sphingoid) bases cannot be used as selective inhibitors of protein kinase C in intact human neutrophils.

EXPERIMENTAL PROCEDURES

The materials and their sources were as follows: trans-DL-erythro-1,3-dihydroxy-2-amino-octadecane (erythrodihydroxyphosphogine) referred to as sphinganine (batches S-0523 and 115 C-003), trans-DL-erythro-2-amino-4-octadecane-1,3-diol referred to as sphingosine or its sulfate salt D-sphingosine sulfate, N-palmitoyldihydrosphingosine referred to as sphinganine (batches 58 C-0232 and 115 C-003), trans-DL-erythro-2-amino-4-octadecane-1,3-diol referred to as sphingosine or its sulfate salt D-sphingosine sulfate, N-hexadecanoyl-DL-dihydroxyphosphogine referred to as N-palmitoyldihydroxyphosphogine, ceramides preparation from bovine brain sphingomyelin (type III), N-formyl-methionyl-leucyl-phenylalanine (fMLP), dihydrosphingosine referred to as sphinganine, tetraacylphosphatidylcholine (PC) (Sigma), N, N-diethylethlenediamine pentacetic acid (DTPA) (Sigma), BSA, bovine serum albumin.
Preparation of Human Neutrophils—Neutrophils were prepared from blood samples (usually 90 ml) obtained from healthy volunteers. Briefly, fresh neutrophils were purified by dextran sedimentation followed by centrifugation through a layer of Ficoll-Paque as previously described (4). In one control experiment, the entire supernatant of dextran sedimentation was used (to eliminate the Ficoll step of otherwise described (4)). In another control experiment, neutrophils were also prepared by Percoll density centrifugation as described for guinea pig neutrophils (14), using a discontinuous Percoll gradient (53 and 67%). The cells were suspended in a medium containing 138 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.1 mM CaCl₂, 100 mM EGTA, 1 mM NaHPO₄, 5 mM NaHCO₃, 5.5 mM glucose, and 20 mM Hepes, pH 7.4. (This medium will be referred to as Ca²⁺ medium.)

Experimental Conditions—The various experiments were performed under the following standardized conditions to allow comparison: (a) a single batch of quin2-loaded cells was used to perform in parallel the various experiments on a given day; (b) if not stated otherwise, the cells were preincubated for 5 min with the indicated concentration of sphinganine or the other tested substances before addition of the stimulus; (c) since it has been suggested that the cell/sphingoid ratio is important (10, 12), the same cell concentration (2 × 10⁶/ml) was used for all experiments (except for depolarization, where 0.5 × 10⁶ cells/ml were used, as changing the cell concentration interferes with the assay).

Superoxide Production—Superoxide production was monitored continuously in a double beam spectrophotometer, thermostated at 37 °C as previously described (15). Data are shown as percentage of control. For control cells, absolute values are given in the figure legend.

Reversibility Experiments—Neutrophils (2 × 10⁶ cells/ml) were incubated at 37 °C for 10 min in Ca²⁺ medium in the presence of MeSO₄ or sphinganine (3, 6, or 12 μM). Treated and control cells were tested for superoxide production, quin2 release, and trypan blue exclusion before and immediately after treatment. Thereafter, cells were washed twice in Ca²⁺ medium containing 0.5% bovine serum albumin (BSA) or 0.3% defatted BSA (10) and restocked again.

Degranulation—Primary granule release was assessed as previously described (4), measuring released β-glucuronidase with 4-methylumbelliferyl substrate (16). Values are given as percentage of total cellular content.

Measurement of Cytosolic Free Ca²⁺—Quin2/AM loading, fluorescence measurement, and calibration were performed as previously described (3). Cells were equilibrated at 37 °C for 5 min. Quin2/AM was added to a final concentration of 20 μM from a 10 mM stock solution in MeSO₄.

Depolarization—Changes in membrane potential were measured by a fluorometric assay, using the membrane potential-sensitive cyanine dye Di-O-C₆ (3) (100 nM final concentration) as described previously by Seligman et al. (17).

Measurement Release of Lactic Dehydrogenase and [³H]Adenine Derivatives—Cells (2 × 10⁶/ml) were incubated for 1 h with 2 μM [³H]Adenine (13.8 Ci/mmol), in certain experiments in parallel to quin2 loading. After two washes and preincubation at 37 °C for 5 min, sphinganine (3 to 50 μM) or MeSO₄ (0.4% final concentration) were added. Incubation was terminated by rapid cooling on ice and centrifugation (800 × g for 10 min). Lactic dehydrogenase, β-glucuronidase, quin2, and [³H]adenine derivatives were measured in the supernatant and calculated as percentage of total cellular content released from an aliquot of the same cell suspension treated with 0.1% Triton X-100. Basal values (control absence of sphinganine) were subtracted.

Lactic dehydrogenase activity was evaluated according to the method described by Bergmeyer et al. (18) and expressed in Wroelewski units (19). For assessment of release of [³H]adenine derivatives, 1 ml of the supernatant was mixed with 10 ml of Hydro Luma and subjected to liquid scintillation counting.

RESULTS AND DISCUSSION

Superoxide Production and Quin2 Fluorescence—Fig. 1A shows the effect of preincubation with increasing concentrations of sphinganine on the initial rates of superoxide production in response to a maximally stimulatory dose of fMLP or PMA. A concentration-dependent inhibition by sphinganine was observed. The concentration for half-maximal inhibition was 5.7 ± 0.5 μM and 5.1 ± 0.1 μM for fMLP- and PMA-induced superoxide production, respectively (mean ± S.D., LOG/LOGIT computer program (20) for 19 determinations on 5 different experiments). Fig. 1B depicts a typical trace of a continuous monitoring of O₂ production, using the lowest concentration of sphingamine that fully inhibited this process (12 μM). This confirms the observation by Wilson et al. (10) that sphingamine blocks superoxide production in response to various stimuli in neutrophils. In order to study the mechanism of this inhibition, we assessed in parallel, under identical conditions, changes in quin2 fluorescence on the same batch of cells. As previously stated, a specific inhibitor of protein kinase C should inhibit neutrophil responses without affecting basal [Ca²⁺]; levels or stimulus-induced [Ca²⁺] rises. Quin2

FIG. 1. A, effect of different sphingamine concentrations on stimulated superoxide production. Superoxide production was continuously monitored as reduction of cytochrome c (monitored at 551 nm). The initial rates of superoxide production in response to 1 μM fMLP or 50 nM PMA were measured. Control cells generated 10.2 and 5.7 nmol of O₂/10⁶ cells/min upon stimulation with PMA and fMLP, respectively (mean of two determinations). B, effect of sphinganine (SA) on superoxide production. Experiment performed as described in A. Neutrophils were preincubated for 3 min in the absence (−SA) or presence (+SA) of 12 μM sphingamine. 1 μM fMLP and 50 nM PMA were added where indicated. The traces are typical for 15 determinations on 7 different days. C, effect of sphingamine (SA) on fMLP-induced increase in quin2 fluorescence. Quin2-loaded human neutrophils were preincubated for 3 min in the absence or presence of 12 μM sphingamine. Where indicated, 1 μM fMLP was added. The traces are typical for 25 determinations on 8 different days. A–C show a typical experiment, where superoxide production and quin2 traces were assessed in parallel, the same day, with the same batch of quin2 loaded cells. DMSO, dimethyl sulfoxide.
traces obtained in the presence and absence of sphinganine are shown in Fig. 1C. In the absence of sphinganine, a stable baseline was observed and fMLP led to a transient increase of quin2 fluorescence. In contrast, in the presence of sphinganine (12 nM), after a lag time of a few seconds, a progressive increase of basal fluorescence was seen and addition of fMLP was without effect.

Thus, sphingamine led to a slow increase in quin2 fluorescence and an abolishment of fMLP-induced quin2 responses. An increase in quin2 fluorescence reflects a rise in \([\text{Ca}^{2+}]_i\), provided that the integrity of the plasma membrane of the quin2-loaded cell is preserved. If the membrane becomes leaky, it simply reflects a release of quin2 into the extracellular space where it binds extracellular \(\text{Ca}^{2+}\) ions or entry of \(\text{Ca}^{2+}\) into the cell. In order to distinguish between these possibilities, we assessed the presence of extracellular quin2 by various methods after incubation of cells with sphingamine.

**Release of Quin2 and Cytoplasmic Markers**—Fig. 2A shows a slow, concentration-dependent increase in quin2 fluorescence in response to two different concentrations of sphingamine. Maximal increase in quin2 fluorescence was observed already after 6 min. No further rise was seen after 30 min of exposure to 50 \(\mu\text{M}\) sphingamine. At this point, the addition of the \(\text{Ca}^{2+}\)-chelator EGTA led to an instantaneous drop of quin2 fluorescence, which was further pronounced by increasing the binding capacity of EGTA following the addition of Tris base (final pH \(> 8\)). Finally, cell lysis by Triton X-100 only had a small additional effect on quin2 fluorescence, indicating that most of the quin2 was extracellular. Similar results were obtained at lower sphingamine concentrations. Experiments were also performed in a \(\text{Ca}^{2+}\)-free medium (nominally \(\text{Ca}^{2+}\)-free medium with addition of 1 mM EGTA, \([\text{Ca}^{2+}]_{\text{out}} < 20 \text{ nM}\)) and showed a slow decrease of quin2 fluorescence in response to sphingamine, as opposed to control cells in which stable fluorescence was observed (not shown, see Ref. 4), again indicating that quin2 was released into the extracellular medium.

The presence of extracellular quin2 can also be assessed by the quenching of its fluorescence by Mn\(^{2+}\) and the reversal by DTPA (21). Quin2 has a higher affinity for Mn\(^{2+}\) than for \(\text{Ca}^{2+}\), and Mn\(^{2+}\) quenches quin2 fluorescence (21, 22, 23). DTPA, a nonpermeant high affinity metal ion chelator, can reverse the effect of Mn\(^{2+}\), provided that the quin2 is extracellular. Only a small effect of Mn\(^{2+}\) and DTPA was detected in control cells, whereas the presence of extracellular quin2 was revealed by the drop of quin2 fluorescence in response to Mn\(^{2+}\) and, in particular, its subsequent reversal upon DTPA addition (Fig. 2B). It can be seen that the fluorescence level reached with DTPA in the presence of sphingamine fell on the line obtained by extrapolation of the fluorescence prior to addition of Mn\(^{2+}\). This demonstrates that quin2 continuously leaked out during the 8.5 min of the experiment (Fig. 2B).

In addition, the presence of extracellular quin2 was also assessed by pelleting the cells and measuring the percentage of quin2 that remained in the supernatant. In control cells 15 ± 2% (mean ± S.D., \(n = 8\)) of the total cellular quin2 was found in the supernatant after a 10-min incubation at 37 °C. In contrast, after 10-min incubation with 12 and 25 \(\mu\text{M}\) sphingamine, respectively, 61 ± 25% and 80 ± 13% (mean ± S.D., \(n = 8\)) were found in the supernatant (see also Fig. 2C). Cells samples, collected from these experiments, were also examined for trypan blue exclusion. A dose- and time-dependent decrease in trypan blue exclusion was observed in cells incubated in the presence of sphingamine. Uptake of trypan blue was 2.5 ± 1% in controls, 25.5 ± 3% at 12 \(\mu\text{M}\), and 62 ± 8% at 25 \(\mu\text{M}\) sphingamine after 3-min incubation.

Fig. 2. A, effect of different concentrations of sphingamine on quin2 fluorescence. Quin2 traces are recorded in \(\text{Ca}^{2+}\)-medium in the presence (SA 12 \(\mu\text{M}\) and SA 50 \(\mu\text{M}\)) or the absence (−SA) of sphingamine. The traces were done on the same day (with the same batch of quin2-loaded cells) and are typical for at least six other experiments. EGTA, 4 mM; Tris, 30 mM, and Triton, 0.1%. B, effect of Mn\(^{2+}\) and DTPA on quin2 fluorescence. Quin2-loaded human neutrophils were preincubated in the absence (−SA) or presence (+SA) of 12 \(\mu\text{M}\) sphingamine. 0.5 mM Mn\(^{2+}\) and 1 mM DTPA were added, where indicated. This represents a typical experiment, repeated at least three times. C, effect of different concentrations of sphingamine on quin2 (●), lactic dehydrogenase (○), β-glucuronidase (□), and [3H]adenine derivatives (△) release into the extracellular medium. Basal values were 15 ± 2%, 12 ± 0.5 units/100 \(\mu\text{l}\), 5.3 ± 0.4 nmol/min, and 15,856 ± 340 cpm and maximal values were 100 ± 2%, 141 ± 8 units/100 \(\mu\text{l}\), 71.6 ± 4 nmol/min, and 58,100 ± 440 cpm, for quin2, lactic dehydrogenase, β-glucuronidase, and [3H]adenine derivatives, respectively. Results are mean ± S.D. of triplicate determinations for one experiment. Similar data were obtained in two other experiments. Note that in contrast to Table I the experiment was performed in the absence of cytochalasin B (which is usually added to enhance secretion). D, effect of sphingamine (SA) on membrane potential. Membrane potential changes in response to two different concentrations of sphingamine were recorded using the membrane potential sensitive fluorescent dye Di-O-C6(3). A typical experiment is shown which was repeated at least four times. DMSO, dimethyl sulfoxide.
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(\text{mean} \pm \text{S.D.}, n = 4). Even higher values were found after 10-min incubation with the respective concentrations of sphinganine (n = 20).

Quin2 release is a sensitive measure of plasma membrane permeabilization. This is demonstrated in the experiments shown in Fig. 2C where the release of two cytoplasmic markers ([3H]adenine derivatives and lactic dehydrogenase) and a primary granule marker (β-glucuronidase) was measured in the same supernatants of sphinganine-treated cells. Lactic dehydrogenase was slightly less sensitive when compared to quin2 or [3H]adenine derivatives release as indicator of cytoplasmic leakage. This is in agreement with findings in other cellular systems, where [3H]adenine derivatives and lactic dehydrogenase release were used to assess different levels of plasma membrane permeabilization (24). Release of β-glucuronidase contained in primary granules was minimal, confirming that most of the effects of sphinganine were at the level of the plasma membrane. An identical dose-response was obtained in cells not loaded with quin2 (not shown).

In conclusion, these experiments demonstrate that there is a permeabilization of the plasma membrane of neutrophils after incubation with sphinganine, which leads to a release of quin2 and cytoplasmic markers into the extracellular medium. Reversibility—Wilson et al. (10) found reversibility of the effects of sphinganine and took this in support of a specific, and against a cytotoxic, effect of sphinganine. We examined the reversibility of sphingamine effects under our experimental conditions and could never find total restoration of initial functional activity, even at subinhibitory concentrations of sphingamine in six independent experiments. In these experiments, cells were incubated in the presence and absence of sphingamine and tested for superoxide production and trypan blue exclusion before and after two washes in a 50-fold excess of Ca2+ medium containing 0.5% BSA or 0.3% defatted BSA (10).

Out of a total of six experiments, two showed irreversible cell clamping and, therefore, could not be evaluated for O2− production. In the remainder, despite a partial reversibility as evaluated by decrease in trypan blue uptake, PMA-induced superoxide production was maximally restored to 21% of control (average 8% for 12 determinations) using 12 μM sphingamine.

In one experiment, exposure of the cells to 6 μM sphingamine for 10 min partially inhibited superoxide production (56 ± 17% (mean ± S.D., n = 3) of control values); trypan blue uptake was not increased, whereas quin2 release was clearly detectable. Thus, quin2, which has a lower molecular weight than trypan blue, is a more sensitive tool for the assessment of cell leakiness. Inhibition of O2− production under these conditions was not reversible; after washing and resuspension, cells generated 66.6 ± 2.5% (mean ± S.D., n = 3) of control values.

Structural Analogues of Sphingamine—The above observed cell permeabilization by sphingamine might have other structural requirements than the inhibitory effect on superoxide production. We therefore tested the ability of several structural analogues of sphingamine to inhibit superoxide production on the one hand and to permeabilize neutrophils on the other. The closely related structural analogue D-sphingosine and its sulfate salt (D-sphingosine sulfate) inhibited PMA-induced superoxide production at slightly higher concentrations than observed for sphingamine (half-maximal inhibition about 12 μM, n = 3). Both analogues also permeabilized neutrophils at these concentrations as assessed by increases in quin2 fluorescence (n = 3–6), depolarization, and uptake of trypan blue (n = 4). In contrast, the less closely related analogues, N-palmitoyldihydrosphingosine and ceramides, did not affect FMLP- and PMA-induced superoxide production, nor increase quin2 fluorescence or uptake of trypan blue (n = 3), at doses as high as 100 μM. It seems, therefore, that the cytotoxic effect of sphingamine and D-sphingosine and their inhibitory effects on superoxide production are closely linked.

Additional Control Experiments—To ascertain that the cytotoxic effects of sphingamine are indeed due to the action of the drug and not to uncontrolled experimental conditions, the following tests were performed: we investigated the effect of preparation of the sphingamine stock solution in different solvents, the effect of preparation of neutrophils by different techniques, as well as the application of two different batches of sphingamine.

Similar concentration dependency in regard to cytotoxicity as well as to inhibition of superoxide production was obtained when sphingamine was prepared, at equimolar concentrations either with fatty acid-free BSA, in 50% ethanol (yielding a final ethanol concentration of 0.5%) (10, 11, 12) or in MeSO (yielding a final MeSO concentration of 0.2%). For example, after 3-min incubation with sphingamine diluted in BSA at concentrations of 0, 12, and 25 μM, respectively, 1 ± 1, 54 ± 15, and 92 ± 6% (mean ± S.D., n = 3) of the cells were trypan blue-positive.

In one experiment, neutrophils were prepared by three different methods: the usual dextran/Ficol method, only dextran sedimentation, or by Percoll gradient centrifugation (see "Experimental Procedures"). The three cell preparations gave identical results: sphingamine (12 μM) and D-sphingosine (25 μM) induce cell permeabilization as assessed by quin2 release (n = 2–4) and trypan blue exclusion (80–100% trypan blue-positive cells, n = 2–4 for each cell preparation).

Two different batches of sphingamine were tested and no differences could be observed (not shown).

Membrane Potential—Stimulation of protein kinase C by PMA induces depolarization of neutrophils (25). Theoretically, it would be expected, therefore, that an inhibitor of protein kinase C should increase slightly or not affect the resting potential, whereas the depolarization in response to activators of protein kinase C should be inhibited. We assessed the membrane potential, using the membrane potential-sensitive cyanine dye Di-O-C3(3) (Fig. 2D). Sphingamine caused a transient hyperpolarization followed by a depolarization. These membrane potential variations were concentration-dependent. Because of the depolarization by sphingamine alone, a possible inhibition of depolarizing agents such as PMA could not be assessed. The depolarization can be regarded as a further indication of cell permeabilization, since permeabilized cells are not capable of maintaining ionic gradients across the plasma membrane.

Degranulation—In neutrophils PMA is a potent trigger of the oxidative burst but not of primary granule release (26). This suggests that only the former process is tightly coupled to protein kinase C activation. Conversely, Ca2+ ionophores such as ionomycin hardly elicit any oxidative burst but are potent activators of primary granule release (27), suggesting that this event is tightly coupled to the Ca2+-dependent pathway of exocytosis in human neutrophils.

In addition, a dose-dependent release of granular content
by sphinganine itself was observed. This was most probably due to the cell permeabilization by sphinganine.

CONCLUSION

Sphinganine and other long-chain (sphingoid) bases have been shown to be potent inhibitors of protein kinase C in a cell-free system (11). The use of inhibitors in intact cells, however, is always complicated by the possible interference with cellular activation at various levels. Here we demonstrate that, in studies with intact neutrophils, sphinganine has effects which cannot be explained by inhibition of protein kinase C.

Sphinganine leads to permeabilization of neutrophils, demonstrated as release of quin2 and cytoplasmic markers, increased permeability to trypan blue, depolarization, and release of primary granule contents at the minimal concentration which completely inhibits O2 production. In addition, sphinganine inhibited calcium ionophore-stimulated granule secretion and appeared to abolish agonist-induced Ca2+ rises but this was hard to assess quantitatively because of the continuous quin2 leakage. A recent study performed with human neutrophils (10) led to opposite conclusions: while O2 production was entirely blocked, agonist-induced [Ca2+]i rises were not. However, while that study clearly demonstrates the efficacy of sphinganine as inhibitor of the respiratory burst, little effort was made to test for its nonspecific actions. Thus, only mean values of experiments performed with quin2-loaded cells are given, but no individual traces are shown. In addition, the time of preexposure to sphinganine of the quin2-loaded cells is not indicated. Therefore, it is impossible to gain sufficient information from the results of Wilson et al. (10), providing an explanation of the difference between their and our results.

We conclude that, in addition to its inhibitory effect on the protein kinase C pathway, sphinganine leads to (i) cell permeabilization, (ii) inhibition of chemoattractant-induced Ca2+ rises and thus interference with signal transduction at a step not involving protein kinase C, and (iii) inhibition of exocytosis induced by Ca2+ ionophore. Therefore, sphinganine and other long-chain (sphingoid) bases do not appear to be suitable compounds for the assessment of the involvement of protein kinase C in the activation of neutrophils and possibly in that of other cellular systems.

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