Reactive oxidants regulate membrane repolarization and store-operated uptake of calcium by formyl peptide-activated human neutrophils

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Abstract

Although the rapid and considerable membrane depolarization response which accompanies activation of the phagocyte NADPH oxidase is due to transmembrane electron fluxes, little is known about the involvement of reactive oxidant species (ROS) in the subsequent repolarization response. In the current study, we have investigated the effects of superoxide dismutase (SOD), catalase, methionine, and the myeloperoxidase (MPO) inhibitors, sodium azide and 4-aminobenzoyl hydrazide (ABAH), as well as those of H_2O_2 and HOCl (both at 100 μM) on the alterations in membrane potential which accompany activation of human neutrophils with the chemoattractant, FMLP (1 μM), and on store-operated uptake of Ca^{2+}. The generation of ROS by FMLP-activated neutrophils was monitored according to the magnitude of oxygen consumption and autoiodination, while spectrofluorimetric procedures were used to measure alterations in membrane

potential and influx of Ca^{2+}. Treatment of the cells with H_2O_2, and HOCl, significantly impeded membrane repolarization, while sodium azide, ABAH, methionine, and catalase exerted the opposite effects, potentiating both the rates and the magnitudes of membrane repolarization and store-operated uptake of Ca^{2+}. These observations demonstrate that NADPH oxidase regulates neutrophil membrane potential and Ca^{2+} influx not only via its electrogenic activity, but also as a consequence of the generation of ROS.

Abbreviations: ABAH, 4-aminobenzoyl hydrazide; ATP, adenosine 5-triphosphate; di-O-C_5(3), dipentyloxacarbocyanine; DMSO, dimethyl sulfoxide; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks' balanced salt solution; LECL, lucigenin-enhanced chemiluminescence; MPO, myeloperoxidase; PBS, phosphate-buffered saline; RMP, resting membrane potential; ROS, reactive oxidant species; SOD, superoxide dismutase

Article Outline

Introduction

In addition to its microcidal role, NADPH oxidase, the electron-transporting, superoxide-generating complex of phagocytes, protects neutrophils from calcium flooding of the cytosol during exposure of the cells to calcium-mobilizing stimuli such as formyl peptides (FMLP). Following neutrophil activation, the oxidase mediates the electrogenic transfer of electrons to molecular oxygen, resulting in depolarization of the membrane potential, which generates an electrical gradient unfavorable for Ca^{2+} entry [1]. Store-operated Ca^{2+} reuptake is delayed by this depolarizing action of the oxidase and occurs during the subsequent phase of membrane repolarization [2]. Importantly, the rate of Ca^{2+} entry is regulated by that of membrane repolarization as these two events are functionally interdependent [3].

Although the electrogenic properties of NADPH oxidase are primarily responsible for membrane depolarization and exclusion of extracellular Ca^{2+}, little is known about the involvement of NADPH oxidase-derived reactive oxygen species (ROS) in regulating membrane depolarization/repolarization and uptake of Ca^{2+}.

In the current study, we have investigated the effects of neutrophil-derived ROS, as well as inhibitors of their generation or reactivity, on the rates of membrane repolarization and store-operated Ca^{2+} reuptake in chemoattractant-activated neutrophils. Our results suggest that H_2O_2 and especially HOCl restrict both membrane repolarization and Ca^{2+} uptake. This novel regulatory role of NADPH oxidase, acting in concert with

myeloperoxidase (MPO), is likely to complement the electrogenic activity of the oxidase in regulating the influx of Ca^{2+} into activated neutrophils.

Materials and methods

Chemicals and reagents

Sodium azide was purchased from UniLab, SAARCHEM (Pty) Ltd (Krugersdorp, South Africa), while the myeloperoxidase inhibitor, 4-aminobenzoyl hydrazide (ABAH), and catalase (extract of human erythrocytes) were purchased from Calbiochem, EMD, Biosciences, Inc. (La Jolla, CA), and methionine (scavenger of HOCl), from the Sigma Chemical Co. (St. Louis, MO); these agents were made to stock concentrations of 76 mM, 50 mM, 200 U/μl, and 10 mM, respectively. Appropriate solvent controls were included when dimethyl sulfoxide (DMSO) was used as solvent.

Neutrophils

Purified human neutrophils were prepared from heparinized venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400*g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) before sedimentation with 3% gelatin in order to remove most of the erythrocytes. Following centrifugation (280 g at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (> 90%) and viability (> 95%), were resuspended to 1×10^7/ml in PBS and held on ice until used.

Oxygen consumption

This was measured using a three-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils (2×10^6) were preincubated for 8 min at 37°C in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) in the presence or absence of sodium azide (760 μM), or ABAH (50 μM), or methionine (100 μM), followed by activation of the cells with the chemoattractant, *N*-formyl-L-

methionyl-L-leucyl-L-phenylalanine (FMLP; 1 μM final, Sigma). The PO_2 of the cell suspending medium was monitored prior to and for 10 min following addition of FMLP.

Myeloperoxidase (MPO) activity

Autoiodination of neutrophils was used to measure the activity of MPO following activation of neutrophils with FMLP (1 μM final) in the absence and presence of sodium azide (760 μM), ABAH (50 μM), and methionine (100 μM). Briefly, neutrophils (6×10^6) were preincubated for 10 min at 37°C in 5.94 ml HBSS containing 1 μCi/ml of iodine-125 (as $Na^{125}I$, specific activity 17.4 Ci/mg, Perkin Elmer Life and Analytical Sciences, Boston, MA) and 25 nmol of cold carrier NaI. Following preincubation with the test agents, 60 μl of HBSS (unstimulated systems) or 60 μl FMLP was added to each tube. The tubes, which contained a total volume of 6 ml, were incubated for 5 min at 37°C, and then the reactions were terminated by the addition of ice-cold PBS. The neutrophils were pelleted by centrifugation and washed once with PBS, the levels of radioactivity in the pellets measured, and the results expressed as nmol $^{125}I/10^7$ neutrophils.

Membrane potential

The potential sensitive fluorescent dye, dipentyloxacarbocyanine (di-O-C_5(3)), was used to measure changes in membrane potential in activated neutrophils. The cells (1×10^6/ml) were preincubated for 10 min at 37°C in HBSS containing 80 nM (final) di-O-C_5(3), after which they were transferred to disposable reaction cuvettes and held at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 and 510 nm, respectively. The neutrophils were then activated with FMLP (1 μM final), and the subsequent alterations in fluorescence intensity monitored over a 5- to 10-min period. The final volume in each cuvette was 3 ml containing a total of 3×10^6 neutrophils.

To calibrate alterations in membrane potential, as described previously [3], neutrophils were bathed in HBSS containing Na^+, K^+, and Cl^- ions at fixed concentrations of 141, 5.7, and 145 mM, respectively. The intracellular concentrations of these ions were assumed to

be 20, 130, and 80 mM, for Na^+, K^+, and Cl^-, respectively, taking into consideration reported estimations of intracellular concentrations in neutrophils varying from 13.5 to 26 mM for Na^+, 120 to 138 mM for K^+, and 80 mM for Cl^- [4], [5], [6], [7] and [8]. The neutrophil permeability coefficients for sodium (P_{Na}), potassium (P_K), and chloride (P_{Cl}) ions are approximately 5×10^{-9}, 5×10^{-8}, and 5×10^{-9} cm/s, respectively [8]. This information allows the resting membrane potential (RMP) of neutrophils in HBSS to be calculated according to the constant-field equation of Goldmann [8]. The RMP was manipulated in the presence of valinomycin (12 μM) by altering the extracellular concentration of K^+ by addition of KCl to give final extracellular potassium concentrations of 31, 56, 80, and 106 mM. The constant-field equation was used to calculate the estimated new RMP for each extracellular concentration of K^+ and this was correlated with the measured decrease (cm) in RMP which allowed estimation of the magnitude of alterations in fluorescence intensity in units of mV/cm. The results of three separate experiments for each concentration of extracellular K^+ were used to calculate the mean value for changes in fluorescence intensity and membrane potential as about 10 mV/cm.

This procedure was used to determine the effects of each of the following on RMP, as well as on alterations in membrane potential following activation of the cells with FMLP: (i) catalase (500 U/ml) and superoxide dismutase (SOD) (100 mU/ml); (ii) methionine (100 μM); and (iii) sodium azide (760 μM) and ABAH (50 μM).

Additional experiments were performed to determine the effects of HOCl (100 μM) and H_2O_2 (100 μM) (pH of stock solutions adjusted to 6.8) on the rates of membrane repolarization in FMLP-activated neutrophils, as well as in neutrophils pretreated with sodium azide or ABAH. According to the results of preliminary experiments it was determined that the effects of these agents were minimal at concentrations below 100 μM. In order to evaluate the effects of the test agents on membrane repolarization, uncomplicated by alterations in the RMP, HOCl and H_2O_2 were added about 30–40 s after FMLP at the point of maximal membrane depolarization and subsequent changes in fluorescence intensity monitored over 5 min.

Mn^{2+} quenching of fura-2 fluorescence

Fura-2/AM (Sigma) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments [9]. Neutrophils (1×10^7/ml) were preloaded with fura-2/AM (2 μM) for 30 min at 37°C in phosphate-buffered saline (0.15 M, pH 7.4), washed twice, and resuspended in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) containing 1.25 mM $CaCl_2$. The fura-2 loaded cells (2×10^6/ml) were then preincubated for 5 min at 37°C followed by the addition of $MnCl_2$ (300 μM) for a further 3 min, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in the fluorescence spectrophotometer. After a stable baseline was obtained (1 min), the neutrophils were activated by the addition of FMLP (1 μM) and fluorescence quenching as a measure of Ca^{2+} influx was monitored at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm [1]. This procedure was used to investigate the effects of sodium azide (760 μM) and ABAH (50 μM) added 1 and 8 min before FMLP, respectively, on the rates of Mn^{2+} quenching of fura-2 fluorescence as a surrogate marker of Ca^{2+} influx. Additional experiments were performed to determine the effects of HOCl (100 μM) and H_2O_2 (100 μM) added 1 min after and 1 min before FMLP, respectively, on Mn^{2+} quenching of fura-2 fluorescence in sodium azide-treated neutrophils, with an equal volume of H_2O added to the control system. This experimental design was used to minimize the contribution from endogenously generated HOCl.

Spectrofluorimetric measurement of cytosolic Ca^{2+}

Neutrophils (2×10^6/ml) loaded with fura-2/AM as described above (Mn^{2+} quenching of fura-2 fluorescence) were preincubated for 8 min at 37°C in the presence or absence of sodium azide (760 μM) and ABAH (50 μM), after which they were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (± 1 min), the neutrophils were activated by the addition of FMLP (1 μM) and the subsequent alterations in fura-2 fluorescence intensity were monitored over a 5-min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils.

Cellular ATP levels

Intracellular ATP concentrations in resting and FMLP-activated neutrophils were measured in the lysates of unstimulated (1×10^6 cells/ml) and FMLP-activated neutrophils in the absence or presence of sodium azide (760 μM) and ABAH (50 μM) immediately prior to, and at 3 and 5 min following exposure of the cells to the chemoattractant, using a luciferin/luciferase procedure [10].

Expression and statistical analysis of results

The results of each series of experiments (*n* values represent the number of different donors used for each series of replicated experiments) are expressed as the mean values ± SE. Levels of statistical significance were calculated using analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple comparisons and a P value < 0.05 was considered significant.

Results

Oxygen consumption

These results are shown in Table 1. Activation of neutrophils with FMLP was accompanied by an abrupt, transient increase in the rate of oxygen consumption by the cells, which was linear for 1–2 min and subsided thereafter. FMLP-activated oxygen consumption was enhanced in the presence of the MPO inhibitors, while methionine did not significantly alter the magnitude of O_2 consumption.

Table 1.

Effects of methionine (100 μM), sodium azide (760 μM), and ABAH (50 μM) on oxygen consumption by, and on MPO-mediated autoiodination of FMLP-activated neutrophils

	Oxygen consumption	**MPO activity**
Methionine	98 ± 3	23 ± 2*
Sodium azide	122 ± 5*	4 ± 1*
ABAH	108 ± 3*	3 ± 1*

The results of each series of experiments ($n = 3$) are expressed as the mean percentage ± SE of the corresponding control system. The actual rate of oxygen consumption by control FMLP-activated cells was 19 ± 0.7 nmol/min/10^6 neutrophils during the period when the reaction was linear. For the MPO-mediated iodination experiments, the absolute values for the unstimulated and FMLP-activated control systems were 0.65 ± 0.06 and 2.33 ± 0.1 nmol ^{125}I/10^7 cells, respectively. *$P < 0.05$ for comparison with the untreated control system.

Activity of myeloperoxidase

These results are also shown in Table 1. MPO-mediated autoiodination following activation of neutrophils with FMLP was almost completely inhibited in the presence of sodium azide and ABAH, with marked attenuation observed for methionine-treated cells.

FMLP-activated neutrophil membrane depolarization/repolarization

These results are shown in Fig. 1 and Fig. 2, and Table 2. Exposure of the cells to FMLP was accompanied by a characteristic abrupt decrease in membrane potential which leveled off at around 1–2 min after addition of the chemoattractant and was followed by a gradual recovery of the membrane potential toward resting levels over 5–10 min. The effects of sodium azide, ABAH, methionine and catalase added to the cells prior to FMLP on the resting membrane potential and FMLP-activated membrane depolarization/repolarization are shown in Fig. 1. Treatment of neutrophils with these agents did not affect the RMP or the magnitude of depolarization following activation of

the cells with FMLP (87 ± 2 mV for control cells and 89 ± 2, 93 ± 2, 92 ± 3, and 88 ± 2 mV for azide-, ABAH-, methionine-, and catalase-treated neutrophils, respectively). However, as shown in Fig. 1 and Table 2, both the rate and the magnitude of repolarization were significantly increased by these agents, while neither of these was affected by SOD.

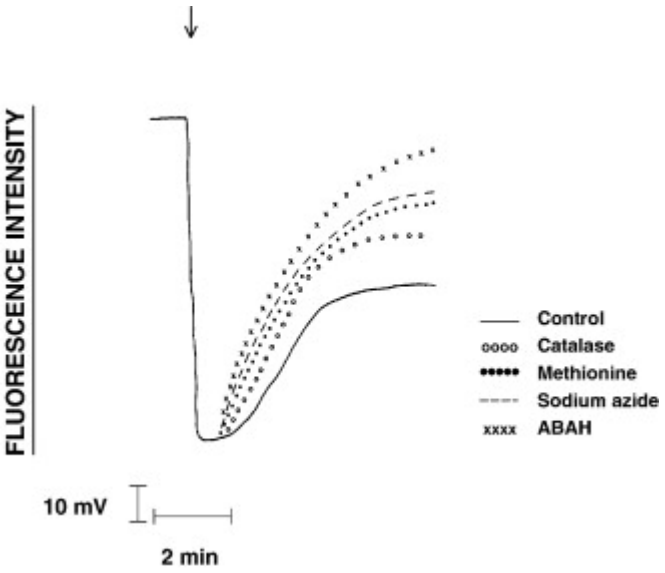

Fig. 1. Effects of catalase (500 U/ml), methionine (100 μM), sodium azide (760 μM), and 4-aminobenzoyl hydrazide (ABAH) (50 μM) on the rate and magnitude of recovery of the membrane potential following addition of FMLP (1 μM) as indicated (↓). The traces shown are those from a single representative experiment (6–20 in this series).

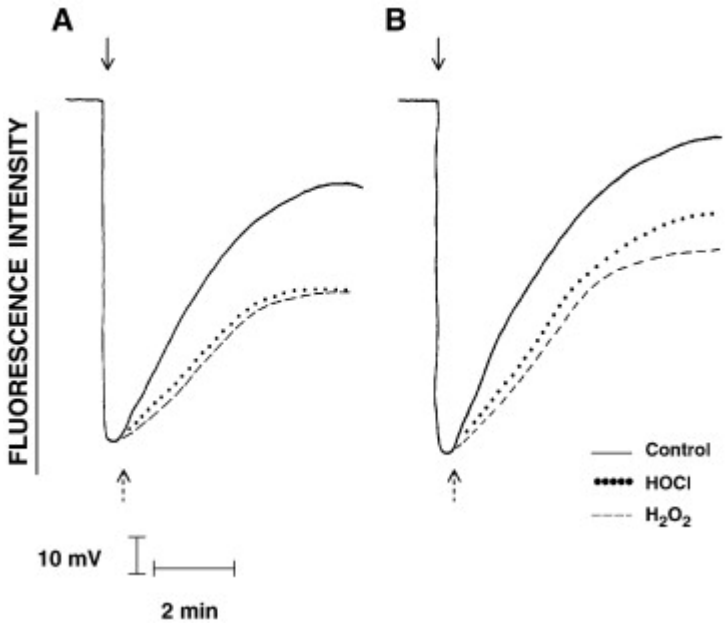

Fig. 2. Effects of H_2O_2 (100 μM) and HOCl (100 μM) on the rate and magnitude of membrane potential recovery in FMLP (1 μM)-activated neutrophils. FMLP was added as indicated (↓) to neutrophils pretreated with sodium azide (760 μM) (A) or ABAH (50 μM) (B) followed by addition of H_2O_2 or HOCl at the point of maximum membrane depolarization (↑). The traces shown are those from a single representative experiment (9 in the series).

Table 2.

Effects of SOD (100 mU/ml), catalase (500 U/ml), methionine (100 μM), sodium azide (760 μM), and ABAH (50 μM), with and without catalase, on the rates of membrane repolarization in FMLP-activated neutrophils measured at 3 and 5 min following addition of the chemoattractant, as well as the effects of HOCl (100 μM) and H_2O_2 (100 μM) on the rate of membrane repolarization in the presence and absence of sodium azide or ABAH

	Magnitude of membrane repolarization (mV)	
	3 min	**5 min**
Control (*n* = 6)	35 ± 2	42 ± 2
SOD (*n* = 3)	34 ± 2	40 ± 2
Catalase (*n* = 3)	48 ± 1*	57 ± 1*
Methionine (*n* = 3)	50 ± 1.3*	64 ± 1*
Sodium azide (*n* = 6)	51 ± 2*	64 ± 2*
ABAH (*n* = 3)	54 ± 0.9*	74 ± 1.4*
ABAH + catalase (*n* = 3)	55 ± 0.7	70 ± 0.9
HOCl (*n* = 3)	27 ± 1.3*	34 ± 1.4*
H_2O_2 (*n* = 3)	21 ± 1.6*	26 ± 1.8*
Sodium azide + HOCl (*n* = 3)	32 ± 0.5^{+}	40 ± 1.2^{+}
Sodium azide + H_2O_2 (*n* = 3)	30 ± 0.5^{+}	39 ± 1.1^{+}
ABAH + HOCl (*n* = 3)	43 ± 1.7°	57 ± 0.7°
ABAH + H_2O_2 (*n* = 3)	37 ± 1.9°	50 ± 3.7°

The results are expressed as the magnitude of recovery (mV) of the membrane potential from the point of maximum depolarization ± SE.

*$P < 0.05$ for comparison with the untreated control system and $^{+}P < 0.05$ or °$P < 0.05$ for comparison with neutrophils pretreated with sodium azide or ABAH, respectively.

Conversely, addition of HOCl or H_2O_2 to FMLP-activated neutrophils at the point of maximum membrane depolarization significantly attenuated the rates of membrane repolarization and antagonized the effects of the MPO inhibitors (Fig. 2 and Table 2).

Influx of Ca^{2+} using Mn^{2+} quenching of fura-2 fluorescence

The effects of sodium azide (760 μM) and ABAH (50 μM) added 1 min before FMLP on the Mn^{2+} quenching of fura-2 fluorescence assay are shown in Table 3 and Fig. 3A. In control cells, the decrease in fluorescence intensity, which indicates influx of the cation, was delayed for about 30 s after the addition of FMLP, followed by an almost linear decrease over 3–4 min, while in neutrophils pretreated with the MPO inhibitors the onset of the decrease in fluorescence intensity occurred slightly earlier (about 20 s) following the addition of FMLP, while the magnitude of decline in fluorescence intensity was significantly greater than that observed with control cells. The effects of HOCl (100 μM) and H_2O_2 (100 μM) added 1 min after and 1 min before FMLP, respectively, on Mn^{2+} quenching of fura-2 fluorescence in sodium azide (760 μM)-pretreated neutrophils are shown in Table 3 and Fig. 3B. Both HOCl and H_2O_2 significantly attenuated the magnitude of the decrements in fura-2 fluorescence intensity measured 2 and 4 min following the addition of the chemoattractant.

Table 3.

Effects of sodium azide (760 μM) and ABAH (50 μM) on Mn^{2+} quenching of fura-2 fluorescence in FMLP-activated neutrophils measured at 1 and 3 min following addition of the chemoattractant, as well as the effects of HOCl (100 μM) and H_2O_2 (100 μM) in the presence of sodium azide (760 μM) on Mn^{2+} quenching of fura-2 fluorescence measured at 2 and 4 min after addition of FMLP

	Magnitude of the decrement in fura-2 fluorescence intensity (cm)	
	1 min	**3 min**
Control (*n* = 4)	1.1 ± 0.03	3.7 ± 0.2
Sodium azide (*n* = 4)	1.4 ± 0.04*	4.7 ± 0.2*
ABAH (*n* = 3)	1.6 ± 0.05*	4.3 ± 0.2*
	2 min	4 min
Sodium azide + H_2O (*n* = 3)	2.8 ± 0.1	5.3 ± 0.17
Sodium azide + HOCl (*n* = 3)	2.5 ± 0.09	4.6 ± 0.1^{+}
Sodium azide + H_2O_2 (*n* = 3)	1.9 ± 0.06^{+}	4.3 ± 0.13^{+}

The results are expressed as the magnitude of the decrement (cm) in fura-2 fluorescence intensity ± SE.

*$P < 0.05$ for comparison with the untreated control system and $^{+}P < 0.05$ for comparison with neutrophils pretreated with sodium azide.

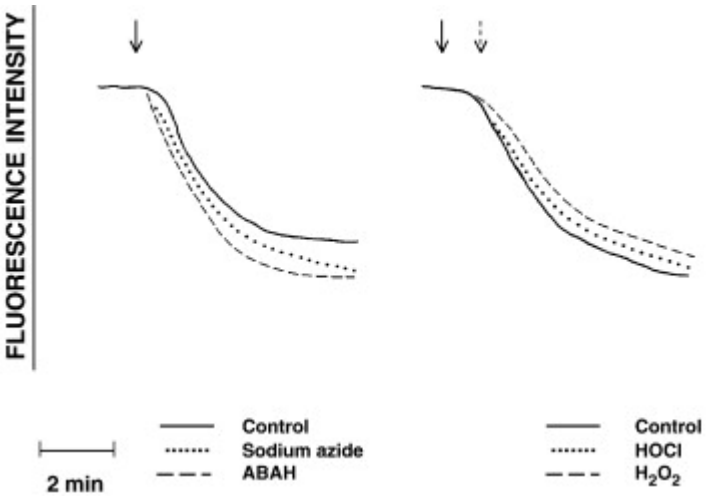

Fig. 3. Effects of sodium azide (760 μM) and 4-aminobenzoyl hydrazide (ABAH) (50 μM) on the Mn^{2+} quenching of fura-2 fluorescence responses (A), as well as the effects of HOCl (100 μM) (⇣) and H_2O_2 (100 μM) on the quenching of fura-2 fluorescence in sodium azide (760 μM)-treated neutrophils (B), activated with FMLP (1 μM) as indicated (↓). The results shown are typical traces from a single representative experiment (9–12 in the series).

Effects of the MPO inhibitors on the fura-2 fluorescence responses of FMLP-activated neutrophils.

The results shown in Fig. 4 are typical traces of the FMLP-activated fura-2 responses of control and sodium azide- or ABAH-treated neutrophils. Addition of FMLP to the cells was accompanied by the characteristic, abrupt increase in fura-2 fluorescence concomitant with the release of the cation from intracellular stores. In the case of control neutrophils, attainment of peak fluorescence was followed by a rapid decline in fluorescence intensity for about 1½ min which subsequently slowed forming a transient plateau before subsiding to basal levels. Treatment of neutrophils with sodium azide or ABAH did not alter the magnitude of the peak fluorescence responses, nor the initial rapid rate of decline in fluorescence intensity. However, the onset of the plateau phase occurred slightly earlier and was prolonged in the presence of the MPO inhibitors.

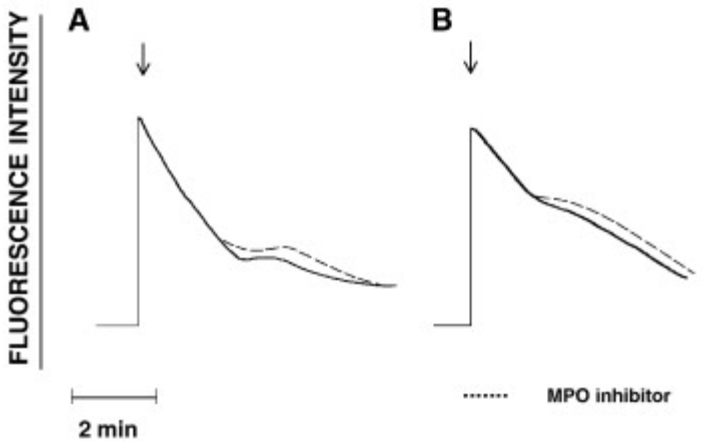

Fig. 4. Effects of sodium azide (760 μM) (A) and 4-aminobenzoyl hydrazide (ABAH) (50 μM) (B) on the fura-2 fluorescence responses of neutrophils activated with FMLP (1 μM) (↓). The results shown are typical traces from 2 separate donors (4 in the series).

Cellular ATP levels

Pretreatment of neutrophils with sodium azide or ABAH did not significantly alter intracellular ATP concentrations measured at rest (28.9 ± 2.5 nmol ATP/10^7 cells) and over 5 min following exposure to FMLP. The values for control cells and those treated with sodium azide or ABAH measured 3 min following addition of FMLP were 17.9 ± 2.9, 18.1 ± 3.5, and 17.8 ± 3.5 nmol ATP/10^7 cells, respectively, reflecting turnover of ATP, while the corresponding values measured at 5 min were 15 ± 1.8, 15.3 ± 2.6, and 15 ± 1.5 nmol ATP/10^7 cells. These observations appear to exclude protection against oxidant-mediated depletion of ATP as a potential mechanism of sodium azide/ABAH-mediated potentiation of membrane repolarization on FMLP-activated neutrophils.

Discussion

Notwithstanding the crucial role of calcium as an intracellular messenger and activator of the proinflammatory responses of human neutrophils, dysregulation of Ca^{2+} homeostasis may be associated with Ca^{2+} overload and hyperactivity of these cells [11]. In this regard, the membrane-associated, electron-transporting NADPH oxidase plays a vital role. The

membrane depolarizing action of the oxidase limits Ca^{2+} influx, and cells with impaired function of this electron-transporting complex (such as those from patients with chronic granulomatous disease) are inherently predisposed to flooding of the cytosol by Ca^{2+} [1] and [12]. In contrast, following activation with FMLP, the uptake of extracellular Ca^{2+} into neutrophils with a functional oxidase is a delayed event, proceeding gradually over a 5-min time course which is superimposable on that of membrane repolarization, suggestive of a mechanistic interrelationship between these two events [3] and [13]. Although activation of NADPH oxidase results in membrane depolarization by promoting the outward movement of electrons, the involvement of ROS, resulting from oxidase activation, in regulating membrane depolarization/repolarization has not been established. In the current study, addition of HOCl and H_2O_2 (100 μM) to neutrophils resulted in attenuation of both the rate and the magnitude of the membrane repolarization response of FMLP-activated neutrophils, compatible with a role for ROS in counteracting the recovery of membrane potential. To probe the role of endogenously generated HOCl in regulating membrane depolarization/repolarization in FMLP-activated neutrophils, we used inhibitors of MPO and a scavenger of HOCl, while catalase and SOD were used to probe the involvement of H_2O_2 and superoxide, respectively. The failure of SOD to affect either the membrane depolarization or the repolarization responses of FMLP-activated neutrophils excluded involvement of superoxide in either of these processes.

The most compelling evidence in support of the involvement of neutrophil-derived HOCl in attenuating both the rate and the magnitude of membrane repolarization in FMLP-activated cells, but not depolarization, was derived from experiments using the inhibitors of MPO, ABAH and sodium azide, as well as the HOCl-scavenger, methionine. All three of these agents, especially ABAH and sodium azide, potentiated the membrane repolarization responses of chemoattractant-activated neutrophils in the setting of protection of the cells against MPO-mediated autooxidation, while addition of HOCl to ABAH-treated, FMLP-activated neutrophils restored the repolarization response to that of control cells. This latter observation also demonstrates that ABAH acts primarily as an inhibitor of MPO, and not as a scavenger of HOCl.

Although hypochlorous acid may act in concert with other oxidants to counter recovery of membrane potential, as evidenced by the similar restraining action of added H_2O_2 on membrane repolarization, we believe that only HOCl is of physiological significance in the case of FMLP-activated neutrophils. Taken together with the observations that oxygen consumption by FMLP-activated neutrophils, and by implication H_2O_2 production, was increased by sodium azide [14] and ABAH, this contention is based on two lines of evidence. First, although the H_2O_2 scavenger, catalase, accelerated the rate of membrane repolarization, the effects of this agent were less pronounced than those observed with the MPO inhibitors. Secondly, catalase, which retains its activity in the presence of ABAH [15], did not potentiate the effects of the MPO inhibitor, suggesting that when the oxidase is activated for brief periods, as is the case with FMLP, only small amounts of H_2O_2 are generated and the rate of membrane repolarization is primarily modulated by the more potent MPO/H_2O_2-derived oxidant, HOCl. However, in the setting of prolonged activation of the oxidase, or in the presence of the catalase inhibitor sodium azide, H_2O_2 may also contribute to negative regulation of membrane repolarization.

Several mechanisms have been reported to mediate membrane repolarization in activated neutrophils. Proton efflux [4] and possibly activation of high conductance Ca^{2+}-sensitive K^+ channels [16] are reported to be operative in phorbol ester-activated cells, while charge compensation in FMLP-activated neutrophils may also involve upregulation of the activity of the electrogenic Na^+/Ca^{2+} exchanger operating in reverse mode [3]. The well-recognized oxidant sensitivity of the Na^+/Ca^{2+} exchanger [17], [18] and [19] implicates this cation transporter as a putative target of HOCl and H_2O_2.

Following activation of neutrophils with FMLP, Ca^{2+} mobilized from intracellular storage compartments is cleared from the cytosol by the plasma membrane and endomembrane Ca^{2+}-ATPases operating in unison [20]. The efficiency of these Ca^{2+} clearance mechanisms is potentiated by NADPH oxidase-mediated membrane depolarization which prevents influx of extracellular Ca^{2+} [21]. Given the well-recognized dependence of capacitative Ca^{2+} entry on membrane repolarization in various

cell types, including neutrophils, we also investigated the effects of the MPO inhibitors on the store-operated uptake of the cation by FMLP-activated neutrophils. Pretreatment of neutrophils with both ABAH and sodium azide shortened the time to onset of influx, prolonged the linear phase of influx, and increased the magnitude of store-operated uptake of Ca^{2+} by the cells, observations which are compatible with a regulatory role for HOCl in controlling the tempo of influx of store-operated Ca^{2+} entry. This contention is further supported by the restraining actions of exogenous HOCl and H_2O_2 on the magnitude of Ca^{2+} reuptake by formyl peptide-activated neutrophils. The effects of the MPO inhibitors on cytosolic Ca^{2+} concentrations in FMLP-activated neutrophils are consistent with the earlier onset and greater magnitude of Ca^{2+} influx observed with the Mn^{2+} quenching of fura-2 fluorescence assays. Somewhat paradoxically, this represents a physiological, anti-inflammatory activity of HOCl, because Ca^{2+} influx is required for sustaining several proinflammatory functions of the cells such as activity of phospholipase A_2 [22] and activation of β2-integrins [23]. Moreover, refilling of stores enables the cells to reinitiate Ca^{2+}-dependent inflammatory responses on reexposure to a Ca^{2+}-mobilizing stimulus.

In conclusion, the current study has identified a previously unrecognized role for MPO and HOCl in regulating both membrane repolarization and store-operated Ca^{2+} entry in chemoattractant-activated neutrophils. Acting in concert, NADPH oxidase and MPO are likely to fulfil a physiologically important, anti-inflammatory function by preventing Ca^{2+} overload and hyperreactivity of neutrophils. Aside from physiological relevance, this has potential significance for the design of pharmacological anti-inflammatory strategies which target inhibition of either or both of NADPH oxidase and MPO, since these may be counteracted by inadvertent potentiation of Ca^{2+} dependent, oxygen-independent, proinflammatory activities of phagocytes.

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