A Noninvasive Fluorimetric Procedure for Measurement of Membrane Potential

QUANTIFICATION OF THE NADPH OXIDASE-INDUCED DEPOLARIZATION IN ACTIVATED NEUTROPHILS*

(Received for publication, June 1, 1999)

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The electrogenic activity of the NADPH oxidase is associated with depolarization of the plasma membrane in activated neutrophils. The magnitude and consequences of this depolarization, however, remain unknown. Neutrophils are not amenable to electrophysiological determinations of membrane potential by current clamp. Instead, the occurrence of depolarization has been inferred from the use of potential-sensitive fluorescent dyes. However, such dyes partition into intracellular organelles and may yield erroneous results, particularly because the NADPH oxidase resides largely in secretory granules, where it has been claimed to become activated. We confirmed the intracellular generation of oxidase products using dihydrorhodamine, which is converted to the fluorescent rhodamine 123 when oxidized. Rhodamine 123 accumulated inside endomembrane organelles in both neutrophils and in differentiated HL60 cells, where it co-localized with the primary granule marker CD63. To estimate the surface membrane potential without interference from organelles, we devised a method based on the voltage-driven uptake of Mn2+ across the plasmalemma. The uptake of Mn2+ through calcium release-activated channels was measured as the rate of Indo-1 fluorescence quenching in thapsigargin-treated cells. The rate of Mn2+ influx was found to vary when the membrane potential was manipulated using conductive ionophores and also when the NADPH oxidase was activated. A calibration curve in the positive potential range was constructed using the Na+ ionophore SQ-2Pr. Using this calibration, the membrane potential of phorbol ester-activated neutrophils was found to reach +58 ± 4 mV, a sustained depolarization of over 100 mV compared with the resting potential. The depolarization was greatly diminished when the NADPH oxidase was inhibited with diphenylene iodonium. Together, these results indicate that the NADPH oxidase can generate a large depolarization of the plasmalemma, which should suffice to activate a variety of voltage-gated channels, including the outwardly rectifying H+ conductance.

Neutrophils are essential contributors to host defense against invading microorganisms. They circulate in the bloodstream in a quiescent state but are rapidly recruited to sites of infection upon activation by chemoattractants produced by the microbes and/or by the surrounding tissues. Following diapedesis across the endothelium and chemotaxis to the site of infection, neutrophils employ phagocytosis to internalize the invading microorganisms. These are subsequently eliminated by a combination of microbicidal mechanisms that include phagosomal acidification, secretion of lytic enzymes and cationic peptides, and generation of reactive oxygen species (1, 2).

The primary source of reactive oxygen metabolites is the NADPH oxidase, an enzymatic complex consisting of both membrane-bound and cytosolic subunits. The former include flavocytochrome b558, which is a heterodimer of gp91phox and p22phox. The cytosolic subunits include p47phox, p67phox, and the recently discovered p40phox, as well as the GTPase Rac (see Refs. 3 and 4 for reviews). Assembly of the active oxidase, which involves translocation of the cytosolic subunits to the membrane, facilitates transfer of one electron from intracellular NADPH to molecular oxygen. The resulting massive consumption of oxygen and concomitant production of superoxide is called the respiratory burst.

Other products of the reduction of oxygen by the oxidase are NADP+ and protons, which are seemingly released intracellularly. Intracellular release of H+ (equivalents) is suggested by the large cytosolic acidification that accompanies the respiratory burst when Na+/H+ exchange is precluded (5). Because superoxide anions are delivered to the extracellular (or intraphagosomal) space while NADP+ and H+ are released into the cytosol, separation of charges must occur across the plasmalemma. Accordingly, a depolarization of the membrane potential was reported to accompany the activation of neutrophils, which was absent in patients with chronic granulomatous disease that lack a functional oxidase (6, 7). Moreover, in activated eosinophils, which also express the NADPH oxidase, a transmembrane electron current was recorded electrophysiologically by Schrenzel et al. (8).

Although these observations confirm the predicted occurrence of an oxidase-associated membrane potential change, neither the magnitude of the depolarization nor its functional consequences have been established. The precise magnitude of the membrane potential change (E_m) has been difficult to determine electrophysiologically because: (i) the input resistance of the neutrophil membrane is inordinately high, precluding accurate current clamp determination of E_m; and (ii) neutrophils are activated by contact with the glass micropipette used for patch clamping, complicating the determination of the resting potential. As an alternative, indirect methods have been applied to estimate E_m in activated neutrophils. These have included isotopic (9, 10) and fluorescence determinations of the...
partition of lipid-soluble ions (6, 11, 12). Although qualitatively informative, these approaches have proved unsuitable for precise quantitative determinations for several reasons. Some of the probes used undergo chemical conversion when exposed to the products of the NADPH oxidase (7). More importantly, the lipophilic ions can traverse not only the plasma membrane but also the membranes of intracellular organelles, where they partition in accordance with the prevailing organellar potential. Because a large fraction of the cellular volume of neutrophils is occupied by endomembrane granules and because their content changes during the course of activation, the determinations of $E_m$ using partition dyes are inherently inaccurate.

An added complication of the methods employing lipophilic ions is introduced by the possibility that the organellar potential may itself change during the course of neutrophil stimulation. In this regard, the group of Dahlgren has provided evidence that activation of the oxidase occurs not only on the plasma membrane but also in intracellular granules (13–15). If separation of charges is also part of the reaction in the granules, a sizable potential change is predicted to occur across the membrane of these organelles.

The objective of the experiments reported here was 2-fold. We initially wanted to establish whether superoxide was in fact produced intracellularly and whether such production is associated with changes in organellar potential. Secondly, we attempted to devise a method for measurement of the plasma membrane potential of activated neutrophils that would not be contaminated by the contribution of intracellular organelles.

**EXPERIMENTAL PROCEDURES**

**Materials**—Gramicidin D and thapsigargin were purchased from Calbiochem. Sodium azide was from Fisher, DHFR, DSC(3), (3), rhodamine 123, valinomycin, Indo-1, and its acetoxymethyl ester form were purchased from Molecular Probes (Eugene, OR). Antimycin A, ATP (K+ salt), dimethyl sulfoxide, NADPH, GTP (Li+ salt), PBS,1 and TPA were from Sigma-Aldrich. The sodium ionophore SQi-Pr was from Testlabs Inc. (Austin, Texas). DPI was synthesized in our laboratory as described (16). Monoclonal antibodies against CD63 and CD66b were obtained from Caltag Laboratories (Burlingame, CA) and Serotec Ltd. (Oxford, UK), respectively. Cy3-conjugated donkey anti-mouse antibodies were obtained from Jackson Immunoresearch (West Grove, PA).

**Media**—Medium RPMI 1640 (bicarbonate-free) was purchased from Sigma. Na+–rich medium consisted of 140 mM NaCl, 3 mM KCl, 1 mM MgCl2, 10 mM glucose, 20 mM HEPES (pH 7.3). K+–rich medium (maintained 140 mM KCl, 1 mM MgCl2, 10 mM glucose, 20 mM HEPES (pH 7.3). To calibrate $E_m$ to the indicated values using valinomycin, Na+– and K+–rich media were mixed in the appropriate proportions. NMG medium consisted of 143 mM NMG-Cl, 1 mM MgCl2, 10 mM glucose, 20 mM HEPES (pH 7.3). In all cases the osmolality was adjusted to 290 ± 5 mOsm.

**Cell Isolation and Permeabilization**—Neutrophils were isolated from heparinized whole blood obtained by venipuncture, using dextran sedimentation, followed by Ficol-Hypaque gradient centrifugation as described (17). After isolation, cells were suspended in 1 × 107 cells/ml in bicarbonate-free, HEPES-buffered RPMI 1640 and rotated at room temperature until used (less than 4 h). HL60 cells obtained from the American Tissue Culture Collection were cultured and differentiated using dimethyl sulfoxide as described previously (18).

Where indicated, cells were permeabilized by electroporation, essentially as described (19). Briefly, neutrophils were suspended in 1 ml of ice-cold K+–rich medium at 107 cells/ml and placed in a Bio-Rad electroporation cuvette. Three pulses of 5 kV/cm were applied, mixing the cells gently between pulses. After electroporation, the cells were resuspended in K+–rich medium supplemented with 300 μM NADPH, 1 mM ATP, and 0.1 mM GTP. Where specified, NADPH was omitted. Electroporation efficiency, assessed by exclusion of trypan blue, was found to be ~95%.

**DHR Conversion Assay**—Cells suspended in Na+–rich medium at a concentration of 107/ml were incubated with 200 nM DHR with or without 100 nM TPA for 15 min at 37°C. Where indicated, 4 μg/ml antimycin A, 4 μM valinomycin, or 10 μM DPI were also present. At the end of this incubation, the cells were rapidly sedimented, resuspended in PBS and layered on a 25-mm coverslip mounted in a Leiden chamber for direct analysis by epifluorescence microscopy.

Alternatively, fluorescence was analyzed by cytometry using a Beck-Dickinson HP FACScan flow cytometer. In this case 250–μl samples containing 107 cells/ml were incubated with DHR plus or minus TPA as above. The samples were then diluted with 500 μl of PBS and used immediately for cytometry. When using permeabilized cells, K+–rich solution was used as the diluent.

**Immunofluorescence**—Cells were incubated with DHR and TPA as detailed above, sedimented, and layered onto a coverslip to allow spreading, facilitating visualization of granules. Epifluorescence and differential interference contrast images were acquired on a Leica DM-IRB microscope with a MicroMax 2 cooled charge-coupled device camera (Princeton Instruments), using WinView software and a PC-compatible computer. The region of interest was marked on the coverslips using a diamond pencil, and the cells were then fixed for 30 min using 4% paraformaldehyde in PBS at room temperature. Next, samples were permeabilized with 0.1% Triton X-100 in PBS containing 0.1% bovine serum albumin and 5% donkey serum for 1 h at room temperature. The same medium was used to dilute the primary antibodies (monoclonal antibodies against CD63 and CD66b, used at a 1:1000 dilution) as well as the secondary antibody (Cy3-labeled donkey anti-mouse IgG, used at 1:1500 dilution). Incubation with the antibodies was for 1.5 h at room temperature, followed by washing and finally mounting using Dako mounting medium. The cells that had been photographed live after reaction with DHR were located using the marks on the coverslips and analyzed by epifluorescence microscopy, using the same setup as above.

**Spectrofluorimetry**—Aliquots of the neutrophil stock suspension (107 cells/ml) were loaded with Indo-1 by incubation with 2 μM of the precursor acetoxymethyl ester for 15 min at 37°C. Unreacted ester was removed by washing the cells twice in 0.5 ml of NaCl medium with 25 μM EGTA, and the cells were eventually resuspended in 1.3 ml of the appropriate medium for fluorimetry. Indo-1 fluorimetry was performed in a cuvette using a thermostatted Hitachi F-4000 spectrofluorimeter with magnetic stirring. Following addition of thapsigargin (77 nM), depletion of endomembrane Ca2+ stores was monitored with excitation at 331 nm, while recording emission at 410 nm, using 5-nm slits. When depletion was found to be complete (i.e. when [Ca2+]i returns to baseline levels), the excitation and emission wavelengths were switched to 335 and 450 nm, respectively, corresponding to the isosbestic point of Indo-1 toward Ca2+. Measurements of Mn2+ influx were then initiated by adding to the cuvette either 38 or 380 μM MnCl2 (final) from an aqueous stock. Where specified, TPA or DPI were also added. For calibration of flux versus $E_m$, the cells were suspended in the appropriate medium, and valinomycin or SQi-Pr were added 2 min prior to the introduction of Mn2+.

To measure the activity of SQi-Pr, 2 × 106 cells were suspended in 1.7 ml of Na+–rich medium and allowed to equilibrate with 25 mM DSC3 (5) in a fluorimeter cuvette. Fluorescence was measured as above with excitation at 651 nm and emission at 675 nm. Next, 765 mM SQi-Pr was added, followed lastly by 5.9 μM gramicidin D.

**Measurement of Cellular Na+ and K+ Content**—Cellular content of Na+ and K+ was determined by flame photometry, using Li+ as an internal standard. Cells were washed three times in ice-cold medium containing 140 mM NaCl, 10 mM HEPES, and 10 mM LiCl, and then resuspended in Tris-Cl. An aliquot was taken for the determination of cell number and volume using a Coulter counter-channelizer (Coulter Inc., Hialeah, FL), and the remaining cells were resuspended in 1 ml of 15 mM Li+ standard solution. Samples were analyzed in a model 443 flame photometer (Instrumentation Laboratories, Lexington, MA) and compared with Na+ and K+ standards.

**RESULTS**

**Generation of Intracellular Superoxide in Activated Neutrophils**—We used dihydrorhodamine (DHR) to assess whether reactive oxygen intermediates are generated intracellularly by activated neutrophils. The cell-permeant nonfluorescent substrate DHR is converted into rhodamine 123 (Rh123), a brightly fluorescent lipophilic cation when oxidized. The latter can be directly visualized by fluorescence microscopy, affording a convenient means of assessing intracellular accumulation of the reaction product. Cells were incubated in suspension with 2 The abbreviations used are: PBS, phosphate-buffered saline; DHR, dihydorhodamine; DPI, diphenylene iodonium; NMG, N-methyl-D-glucammonium; Rh123, rhodamine 123; SOC, store-operated channels; TPA, 12-O-tetradecanoylphorbol 13-acetate.
with DHR.

Human neutrophils were incubated with 200 nM DHR for 15 min at 37°C in the absence (A and B) or presence (C and D) of 100 nM TPA. The cells were then sedimented, resuspended in medium without DHR, and plated on coverslips for observation by fluorescence (A and C) or differential interference contrast (DIC) microscopy (B and D). The results are representative of 20 experiments. Bar, 10 μm.

DHR in the presence and absence of the soluble agonist TPA, sedimented to remove unreacted DHR, and plated onto glass coverslips to allow cell spreading, which facilitates visualization of intracellular organelles. As shown in Fig. 1 (A and B), no conversion of DHR to Rh123 was detectable for up to 15 min at 37°C in unstimulated cells. Notice that activation of the oxidase by spreading (20) was not detected under these conditions, because of prior removal of DHR. By contrast, cells incubated in suspension with TPA and DHR showed distinct accumulation of Rh123 in punctate intracellular structures (Fig. 1, C and D), despite removal of the excess DHR before adherence to the coverslips. These observations imply that stimulation by TPA induces the oxidation of DHR, with intracellular accumulation of Rh123.

Intracellular accumulation of Rh123 does not necessarily imply that the fluorescent product was generated within the cell. Because it is a lipophilic cation, Rh123 synthesized extracellularly may have diffused back into the neutrophils, traversing the plasma and organelar membranes. To exclude this possibility, samples were incubated with DHR and TPA in the presence of superoxide dismutase and catalase at concentrations that rapidly eliminate the products of the NADPH oxidase (21), thereby precluding the extracellular oxidation of DHR. Under these conditions, the intracellular accumulation of Rh123 was unaffected (not illustrated), implying that oxidation of DHR occurred within the cells.

In other cell types, small amounts of superoxide can be generated by the respiratory chain of mitochondria (22) and also by the endoplasmic reticulum (23). Although these systems would not be expected to activate upon addition of TPA, it was nevertheless imperative to demonstrate that the intracellular formation of Rh123 was mediated by the NADPH oxidase. This was confirmed by studying the NADPH dependence and DPI sensitivity of the accumulation of Rh123. NADPH is the physiological substrate of the oxidase (3), whereas DPI has been shown to be a potent inhibitor (24). The cytosolic content of NADPH was manipulated by electroporation of the plasma-membrane, as described earlier (19). Fig. 2 (A and B) illustrates that formation and intracellular accumulation of Rh123 persisted in electroporated neutrophils but only when NADPH was present in the solution. Accumulation of fluorescence was not only visualized microscopically but was also quantified by flow cytometry. As summarized in Fig. 2 (C and D), fluorescence in the absence of NADPH was insignificant (≤3.5% of control). Moreover, DPI virtually eliminated the fluorescence observed in the presence of NADPH. Together, these findings confirm that intracellular accumulation of Rh123 results from activation of the NADPH oxidase by TPA.

Identification of the Subcellular Compartment(s) That Accumulate Rh123—Having established the source of oxidants that convert DHR, we attempted to identify the intracellular site of accumulation of Rh123. Because Rh123 is a lipophilic cation, it tends to accumulate in organelles with negative (inside) membrane potential, such as mitochondria. In fact, Rh123 has been extensively used as a mitochondrial marker in a variety of cells (25, 26) and accumulates readily in the mitochondria of unstimulated neutrophils (Fig. 3B). It was therefore conceivable that, although generated elsewhere, Rh123 might accumulate within mitochondria in activated neutrophils. The following findings argue against this possibility. First, the elongated shape of mitochondria (visualized adding Rh123 to unstimulated cells) is different from the more punctate distribution of Rh123 generated from DHR when the oxidase is activated (cf. Fig. 3, A and B). Secondly, treatment with DPI eliminated the conversion of DHR to Rh123 but only partially inhibited the accumulation of exogenous Rh123 in mitochondria (Fig. 3, C and D). Conversely, antimycin A, which blocks cytochrome c1 of the mitochondrial respiratory chain, obliterated staining of mitochondria by added Rh123 but had little effect on the

![Fig. 1. Intracellular accumulation of Rh123 in cells incubated with DHR. Human neutrophils were incubated with 200 nM DHR for 15 min at 37°C in the absence (A and B) or presence (C and D) of 100 nM TPA. The cells were then sedimented, resuspended in medium without DHR, and plated on coverslips for observation by fluorescence (A and C) or differential interference contrast (DIC) microscopy (B and D). The results are representative of 20 experiments. Bar, 10 μm.](image)

![Fig. 2. DHR oxidation in electroporabilized cells. Human neutrophils were electroporabilized and suspended in medium with (A) or without (B) NADPH. The cells were then stimulated with TPA and analyzed by fluorescence as in Fig. 1. C and D, permeabilized cells were suspended with or without NADPH and DPI, as indicated, and stimulated with TPA. Rh123 generation was then assessed by flow cytometry. A typical frequency histogram (C) and the summary of four similar experiments (D) are presented. RFI, relative fluorescence intensity.](image)

2 DPI can partially dissipate the mitochondrial membrane potential by interaction with flavoprotein components of the respiratory chain.
mation of Rh123 from DHR and on its accumulation in the punctate organelles within neutrophils (Fig. 3, E and F). Similarly, the conductive ionophore valinomycin abrogated the staining of mitochondria by shunting the potential across their inner membrane, whereas oxidation of DHR and accumulation of the resultant Rh123 were unaffected (Fig. 3, G and H). These findings imply that mitochondria are not the site of accumulation of Rh123 derived from DHR. Moreover, they suggest that organellar membrane potential does not play an important role in Rh123 accumulation in TPA-stimulated neutrophils, inasmuch as valinomycin had no effect.

The punctate nature of the stained compartment suggested accumulation in secretory granules, which are abundant in neutrophils. Two types of granules are likely candidates to accumulate Rh123. Secondary granules are the most likely site of generation, because subcellular fractionation studies revealed that they are rich in flavocytochrome b$_{558}$, the integral membrane component of the NADPH oxidase (27, 28). Primary granules could conceivably also accumulate Rh123 because they contain myeloperoxidase, which has been claimed to be required for conversion of DHR to its fluorescent product (21). We attempted to identify the site of Rh123 accumulation using specific immunological markers: CD63, a marker of primary (azurophilic) granules, and CD66b, which is present exclusively in secondary (specific) granules (see Ref. 29 for review). Cells were incubated with TPA and DHR, then fixed, and photographed to locate the sites of Rh123 accumulation. Because Rh123 is poorly retained in the cells following permeabilization, despite fixation, the location of this dye had to be established prior to immunostaining. To localize the same cells after staining for CD63 or CD66b, the coverslips were scored with a diamond pen to identify the area used for analysis of Rh123. The same area was then scanned after immunostaining to localize the cells that were photographed earlier. As shown in Fig. 4 (A and B), there was good correspondence between CD63 and the Rh123 fluorescence, suggesting that the product of DHR oxidation accumulates in primary granules. In contrast, the distribution of CD66b differed from that of Rh123, suggesting little accumulation in secondary granules.

To confirm the presence of Rh123 in primary but not secondary granules we used differentiated HL60 cells. Unlike mature neutrophils, differentiated HL60 cells contain a substantial number of primary but not secondary granules (30). We initially confirmed using the cytochrome c assay that HL60 cells differentiated by addition of dimethyl sulfoxide effectively generate superoxide in response to TPA (not shown). We next established that under the conditions used for our study, differentiated HL60 cells had numerous CD63-positive granules but no detectable CD66b (Fig. 5). More importantly, we found that these cells converted DHR into Rh123, which accumulated in a punctate pattern, despite the absence of secondary granules. These findings are consistent with the notion that Rh123 accumulates in primary granules.

Method for Selective Determination of $E_m$—In view of the fact that oxygen radicals appear to be generated intracellularly, it is conceivable that endomembrane potential may change during neutrophil activation. Moreover, because granule secretion accompanies stimulation of the oxidase, changes in the volume trapped by endomembranes also occur, affecting the overall distribution of lipophilic ions. Therefore, the partition methods used heretofore for the estimation of $E_m$ in activated neutrophils are inaccurate. We therefore sought a novel approach to the measurement of $E_m$ that would selectively probe the plasma membrane potential while excluding organellar potential. We devised a method that has the following central features: (i) determination of $E_m$ is based on measurements of the
rate of influx of an ion across the plasmalemma as opposed to its intracellular/extracellular partition, (ii) the ion of choice is hydrophilic to preclude rapid penetration into endomembrane compartments, and (iii) a divalent cation, namely Mn$^{2+}$, was chosen to magnify the effect of $E_{m}$ on the rate of influx.

The procedure involves estimation of the rate of Mn$^{2+}$ influx into suspended neutrophils. Mn$^{2+}$ is advantageous because it is not extruded from the cells by divalent cation pumps that eject Ca$^{2+}$. The rate of entry of Mn$^{2+}$ was monitored using Indo-1, which is effectively quenched by the divalent cation (31). Use of the cytosolic dye Indo-1 implied that the cation would be detected and chelated immediately upon entry to the cells, ensuring minimal contribution by endomembrane compartments. It is also noteworthy that, because of the high sensitivity of Indo-1, minute amounts of Mn$^{2+}$ entering the cell can be detected. This feature is essential to ensure that the flux of the probe would not by itself significantly alter the membrane potential.

The rate of entry of Mn$^{2+}$ is normally limited by the low endogenous permeability of the plasma membrane to divalent cations, which may vary during stimulation. To circumvent this limitation, we enhanced the cation permeability by activation of an endogenous plasmalemmal conductive pathway, specifically the store-operated channels (SOC), which are permeable to both Ca$^{2+}$ and Mn$^{2+}$ and are comparatively voltage-insensitive (32). Systematic activation of the SOC was accomplished by prior depletion of the intracellular calcium stores with thapsigargin (33). Changes in Indo-1 fluorescence because of alterations in [Ca$^{2+}$], were obviated by using nominally Ca$^{2+}$-free solutions and measuring the emission at the isosbestic point. Under these conditions the rate of entry of Mn$^{2+}$ was dictated primarily by the transmembrane potential.

A representative experiment is illustrated in Fig. 6. When measuring Indo-1 emission at 410 nm, a Ca$^{2+}$-sensitive wavelength, addition of thapsigargin induced a rapid and transient increase in fluorescence because of release of Ca$^{2+}$ from intracellular stores (33), followed by extrusion across the plasma membrane. When depletion of the stores was complete, i.e. when the SOC were fully activated, the emission wavelength was switched to the isosbestic point (450 nm), and extracellular Mn$^{2+}$ was added shortly thereafter. Under these conditions the addition of 38 $\mu$M Mn$^{2+}$ produced a rapid quenching of Indo-1 in otherwise untreated cells (not shown) as well as in cells treated with valinomycin to bring $E_{m}$ to the K$^{+}$ equilibrium potential (Fig. 6). The rate of quenching, a measure of the influx of the cation, was exquisitely sensitive to Mn$^{2+}$, as shown in Fig. 7. When $E_{m}$ was gradually depolarized by incremental changes in the concentration of extracellular K$^{+}$, the rate of Mn$^{2+}$ uptake declined in parallel (inset to Fig. 7). This enabled us to construct a calibration curve relating $E_{m}$ to the rate of Indo-1 quenching (Fig. 7). An intracellular K$^{+}$ concentration of 138 mM was used for the calculation of $E_{m}$. This concentration was determined by flame photometry (see “Experimental Procedures”) using a mean cell volume of 300 fl/cell, estimated in parallel using the Coulter-Channelyzer. Using the $E_{m}$ calibration we calculated the membrane potential of unstimulated neutrophils$^{3}$ to average ~58 mV, which is in the range of estimates made by other methods.

Having established a calibration procedure, we tested the effect of activation of the NADPH oxidase on $E_{m}$. As shown in Fig. 6, the rate of Mn$^{2+}$ uptake was much decreased in the stimulated cells. According to the curve in Fig. 7, the potential increased above 0 mV. Two lines of evidence indicate that the decreased rate of influx of Mn$^{2+}$ is indeed a consequence of an oxidase-mediated depolarization, as opposed to decreased permeability of the SOC or other nonspecific effects induced by TPA. First, the apparent depolarization was virtually eliminated in cells treated with DPI. Secondly, the effect of TPA was negated by the addition of valinomycin, which clamped the $E_{m}$.

The data in Fig. 7 suggest that in activated neutrophils $E_{m}$ may overshoot to positive potentials. Unfortunately, calibration of $E_{m}$ using valinomycin/K$^{+}$ is limited to the (inside) negative range, because osmotic considerations preclude increasing extracellular K$^{+}$ beyond the cytosolic concentration. We therefore used a novel conductive Na$^{+}$ ionophore, SQI-Pr, to calibrate the fluxes of Mn$^{2+}$ in the positive potential range. That SQI-Pr selectively increases Na$^{+}$ conductance in human neutrophils is demonstrated in Fig. 8, where the effect of the

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$^{3}$Although not stimulated by phorbol esters or chemoattractants, the cells used for this calculation had been pretreated with thapsigargin.
where indicated, 765 nM SQI-Pr was added. Finally, 60.9 fluorescence recorded as described under "Experimental Procedures." Protein was maintained constant using NMG. Representative traces are shown in the inset. A typical calibration curve relating the initial rate of Mn\textsuperscript{2+}-induced Indo-1 quenching versus \( E_m \) is illustrated in the main panel (squares). \( E_m \) was calculated using an intracellular \([\text{K}^+]\) of 138 mM determined by flame photometry. The line was fitted by least squares. Mn\textsuperscript{2+} influx in cells treated with TPA is also shown (top trace in inset and cross in main panel). The results are representative of three similar experiments.

Ionophore on \( E_m \) was measured fluorimetrically using DiSC\textsubscript{3} (5). In cells suspended in physiological (Na\textsuperscript{+}-rich) solution, addition of SQI-Pr elicited a large increase in fluorescence, indicative of depolarization. Subsequent addition of gramicidin, which forms channels permeable to monovalent cations, reduced the fluorescence. By increasing the permeability to both Na\textsuperscript{+} and K\textsuperscript{+}, gramicidin brings \( E_m \) to nearly 0 mV. The finding that the depolarization induced by SQI-Pr exceeds the 0 mV level implies that this ionophore must have preferentially increased the permeability of the neutrophil membrane to Na\textsuperscript{+}, in accordance with the manufacturer’s report that the Na\textsuperscript{+} to K\textsuperscript{+} permeability ratio can approach 100 (Teflabs).

We next utilized SQI-Pr to calibrate the influx of Mn\textsuperscript{2+} versus \( E_m \) in the positive (inside) range. Solutions containing increasing concentrations of Na\textsuperscript{+} were used to induce progressive depolarization with SQI-Pr while keeping the osmolarity constant with NMG\textsuperscript{3+}. A representative experiment is shown in the inset of Fig. 9, whereas the main panel illustrates the calibration curve. As for K\textsuperscript{+}, the intracellular Na\textsuperscript{+} concentra-

**Discussion**

Our results confirm that products of the oxidation of DHR accumulate in endomembrane compartments in activated neutrophils. Several lines of evidence indicate that oxidation is mediated by products of the NADPH oxidase. First, in electroneutralized cells the conversion of DHR to Rh123 was found to depend on the supply of exogenous NADPH. Secondly, in both permeabilized and intact cells the generation of Rh123 was obliterated by DPI, a potent inhibitor of the NADPH oxidase. Lastly, we had earlier noted that organelar accumulation of Rh123 was absent in cells obtained from chronic granulomatous disease patients (20).

Rh123 has been shown to enter intact cells and to accumulate in mitochondria and possibly other negatively charged organelles (25). Thus, it is conceivable that superoxide generated extracellularly might form Rh123, which would in turn permeate into the cells. However, our findings suggest that the reactive oxygen species responsible for the conversion of DHR are generated intracellularly. This was concluded from the failure of extracellular addition of superoxide dismutase and catalase to prevent the accumulation of Rh123. Moreover, our results argue against electrophoretic accumulation of Rh123 in mitochondria because (i) dissolution of the mitochondrial potential by antimycin A or by valinomycin had no effect on intracellular accumulation of Rh123 and (ii) intracellular Rh123 co-localized with CD63, a marker of primary granules that is not present in mitochondria. Taken together, these observations support the notion that reactive oxygen intermediates formed by the oxidase are released intracellularly, in agreement with the interpretation of Dahlen and colleagues (14, 15).

The finding that Rh123 accumulates within primary (azurophilic) granules was unexpected, because these organelles are not believed to express the flavocytochrome component of the NADPH oxidase (28). Several mechanisms can be envisioned to
account for this observation. Negatively charged superoxide may be attracted electrorepulsively to the interior of the granules, which express an electrogenic vacuolar H⁺-ATPase (34). However, the failure of valinomycin to alter Rh123 accumulation makes this alternative unattractive. Instead, it is possible that hydrogen peroxide diffuses electroneutrally across the granule membrane and that preferential Rh123 accumulation results from the intragranular presence of myeloperoxidase, which has been suggested to catalyze the conversion of DHR to Rh123 (21). Intracellular hydrogen peroxide may originate from dismutation of superoxide formed in the lumen of secondary granules, a model that would be compatible with Dahlgren’s concept that the oxidase can become activated in granules (14, 15). However, the finding that HL60 cells, which lack secondary granules (30), also accumulate Rh123 in endomembranes argues against this interpretation. Alternatively, hydrogen peroxide may emanate from the plasmalemmal NADPH oxidase by one of two processes: by invagination and detachment of surface-derived vesicles containing active oxidase or by diaphragm activity of the plasmalemmal NADPH oxidase. The latter appears more likely, considering that externally added dismutase and catalase were without effect (see above). These enzymes would have been trapped in the lumen of invaginating vesicles, destroying oxygen radicals generated therein.

Regardless of the precise underlying mechanism, it is clear that the intracellular generation of oxygen radicals, together with the active membrane remodelling reported to occur in activated neutrophils, compromise the accuracy of earlier $E_m$ measurements using fluorescent and other partition probes (see Ref. 35 for review). For this reason, we devised a novel approach to measure $E_m$ in small cells not amenable to electrophysiological analysis. The method, based on estimation of the rate of Mn$^{2+}$ uptake via endogenous SOC, was initially calibrated and validated using ionophores. It was then applied to the measurement of $E_m$ in activated neutrophils, yielding values of approximately +58 mV. These estimates exceed the magnitude of the depolarization determined in preceding studies, where values of −40 to 0 mV were reported (35). The difference can be attributed to (i) the inability of cationic cyanine dyes to effectively monitor $E_m$ in the positive range, (ii) the concomitant oxidation of the dyes reported in some instances, (iii) the earlier of studies to calibrate $E_m$ in the positive range, and (iv) the sensitivity of earlier methods to remodel secondary intracellular compartments during secretion. Taking the shortcomings of earlier methods into account, we feel that the Mn$^{2+}$ flux rate method provides a more accurate estimation of the true $E_m$ reached by neutrophils during the course of activation.

What are the possible functional consequences of this overshoot of $E_m$ during activation of the NAPDH oxidase? It has already been reported that the depolarization curtails the entry of Ca$^{2+}$ via the SOC during activation by chemotaxants (36, 37). In this regard, the differential ability of several agonists to stimulate the NAPDH oxidase may explain their paradoxical effects on [Ca$^{2+}$]. Comparatively weak stimulants, such as platelet-activating factor and LTB$_4$ produce greater [Ca$^{2+}$], transients than do chemotactic peptides (38). The latter are also effective stimulants of inositol 1,4,5-trisphosphate production but induce a smaller calcium influx via SOC because they are also potent activators of the depolarizing NAPDH oxidase. A second possible consequence of the large depolarization reported here is the activation of the outwardly rectifying H$^+$ conductance. This pathway serves to eliminate excess metabolic acid generated during the respiratory burst but in the physiological pH range is active only when $E_m$ overshoots 0 mV (39, 40). Our data provide the first evidence that under physiological conditions, $E_m$ can reach the positive levels required for effective activation of the H$^+$ conductance, an important contributor to the regulation of pH.

Lastly, inasmuch as the NADPH oxidase itself is electrogenic, the depolarization it generates could exert a negative feedback on its own activity, preventing excessive activation (10). Future studies should define the contribution of the electrical gradient on the thermodynamic control of the activity of the oxidase.

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