Regulation of eosinophil membrane depolarization during NADPH oxidase activation

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Summary

Protein kinase C (PKC) activation in human eosinophils increases NADPH oxidase activity, which is associated with plasma membrane depolarization. In this study, membrane potential measurements of eosinophils stimulated with phorbol ester (phorbol 12-myristate 13-acetate; PMA) were made using a cell-permeable oxonol membrane potential indicator, diBAC$_4$(3). Within 10 minutes after PMA stimulation, eosinophils depolarized from –32.9±5.7 mV to +17.3±1.8 mV. The time courses of depolarization and proton channel activation were virtually identical. Blocking the proton conductance with 250 μM $ZnCl_2$ (+43.0±4.2 mV) or increasing the proton channel activation threshold by reducing the extracellular pH to 6.5 (+44.4±1.4 mV) increased depolarization compared with PMA alone. Additionally, the protein kinase C (PKC) δ-selective blocker, rottlerin, inhibited PMA-stimulated depolarization, indicating that PKCδ was involved in regulating depolarization associated with eosinophil NADPH oxidase activity. Thus, the membrane depolarization that is associated with NADPH oxidase activation in eosinophils is sufficient to produce marked proton channel activation under physiological conditions.

Key words: Membrane potential, PKCδ, superoxide, H^+ channel, NADPH oxidase

Introduction

NADPH oxidase generates superoxide anions in several cell types by transferring electrons from intracellular NADPH to extracellular molecular oxygen (reviewed by Babior, 1999). Cytosolic protons are generated directly by NADPH oxidation and indirectly by regeneration of NADPH via the hexose monophosphate shunt (Borregaard et al., 1984; Lukacs et al., 1993). Associated with NADPH oxidase is an outwardly rectifying proton channel that is functionally required for continued oxidase activity (Henderson et al., 1988; Gordienko et al., 1996; Schrenzel et al., 1996). This NADPH oxidase-associated proton channel is ligand- and voltage-regulated and has a predicted reversal potential that is nearly equal to the equilibrium potential for protons (E_H). The voltage dependence of the proton channel is such that membrane depolarization is required during NADPH oxidase activation to allow proton efflux from the cell. Under normal physiological conditions (intracellular pH=7.0; extracellular pH=7.4) the activation voltage of the channel would be approximately –23 mV (Cherny et al., 1995).

Membrane depolarization after NADPH oxidase activation has been reported in neutrophils (Seligmann and Gallin, 1980; Whitin et al., 1980; Henderson et al., 1987; Demaurex et al., 1993; Åhlin et al., 1995; Susták et al., 1997; Jankowski and Grinstein, 1999) and appears to be produced in part by NADPH oxidase activity itself. Electron transfer through NADPH oxidase may depolarize the cell, and concomitant proton efflux attenuates this depolarization. Neutrophils from patients with chronic granulotomatous disease (CGD), in which NADPH oxidase is not functional, do not depolarize in response to various stimuli (Seligmann and Gallin, 1980; Cohen et al., 1981; Castranova et al., 1981; Åhlin et al., 1995). Similarly, neutrophils treated with the NADPH oxidase electron transport inhibitor diphenyleneiodonium (DPI) have attenuated depolarization (Henderson et al., 1987; Jankowski and Grinstein, 1999). When proton efflux is inhibited during oxidase activation, eosinophils (Bánfi et al., 1999) and neutrophils (Henderson et al., 1987; Suszták et al., 1997) depolarize to a substantially greater degree. Likewise, depolarization is attenuated when NADPH oxidase is activated under conditions that favor increased proton efflux (Henderson et al., 1987). Thus, activation of the electrogenic NADPH oxidase assures coordination of proton efflux that is required for continued superoxide production.

Jankowski and Grinstein (Jankowski and Grinstein, 1999) have confirmed that neutrophils depolarize sufficiently during activation to promote the efflux of protons through the NADPH oxidase-associated proton channel. After phorbol ester stimulation, neutrophils depolarized from a resting membrane potential of –58 mV to +58.6±6 mV, a magnitude consistent with a physiological role for the proton conductance during NADPH oxidase activity. Eosinophil membrane potential has been measured in both resting and activated states. Roberts and Gallin (Roberts and Gallin, 1985) used di-O-C_5(3), a membrane potential-sensitive carboxycyanine dye, to document relative eosinophil depolarization after phorbol 12-myristate 13-acetate (PMA) stimulation; however, when loaded with the same dye, eosinophils depolarized less than

neutrophils. Subsequently, current clamp recordings on eosinophils suggested an absolute resting membrane potential of approximately –60 to –80 mV (Gordienko, 1996; Tare, 1998), and an increase of 30-40 mV following NADPH oxidase activation with NADPH and GTPγS (Bánfi et al., 1999). Therefore, on the basis of current data describing eosinophil plasma membrane potential, the NADPH oxidase-associated proton conductance is unlikely to be activated in eosinophils. This is in contrast to what has been described for neutrophils and to circumstantial evidence for proton efflux after NADPH oxidase stimulation in eosinophils.

We have previously reported that PMA-stimulated human eosinophils produce superoxide and activate the NADPH oxidase-associated proton conductance (Bankers-Fulbright, 2001). Here, we describe the effects of PMA on the membrane potential of eosinophils using a cell-permeable oxonol membrane potential indicator, bis-barbituric acid oxonol [diBAC$_4$(3)], to determine the extent of depolarization (Krasznai et al., 1995). Like neutrophils, eosinophils depolarize to a membrane potential sufficient to allow activation of the NADPH oxidase-associated proton channel under conditions where superoxide is produced. Additionally, protein kinase C δ (PKCδ) appears to be crucial for PMA-induced depolarization.

Materials and Methods

Reagents

Unless indicated otherwise, all reagents were purchased from Sigma Chemical (St Louis, MO). PMA was purchased from Calbiochem (La Jolla, CA) and was diluted to a stock concentration of 5 mg/ml in DMSO, aliquoted and stored at –30°C.

Eosinophil isolation

Peripheral blood human eosinophils were isolated as described (Hansel et al., 1991; Ide et al., 1994). Briefly, heparinized blood was collected from atopic and nonatopic volunteers, an equal volume of 1× PIPES was added (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4), and the diluted blood was layered onto Percoll (density 1.085 g/ml). After centrifugation at 1000 ***g*** for 30 minutes at 4°C, the plasma and Percoll layers were removed by aspiration. Tubes were wiped to remove contaminating leukocytes, and red cells were lysed by osmotic shock. The remaining pellet, containing neutrophils and eosinophils, was incubated with an equal volume of anti-CD16 magnetic beads (Miltenyi Biotec, Auburn, CA) on ice for 30 minutes. After incubation the cell mixture was diluted with 1× PIPES + 1% α calf serum (αCS) (HyClone Laboratories, Logan, UT) and eluted through a steel wool column suspended in a strong magnet. Column eluate (14 ml) was collected and the number of eosinophils was determined by staining with Randolph's stain. Eosinophil purity was always greater than 93% and the major contaminating cells were neutrophils.

Transmembrane potential measurements

Eosinophil transmembrane potential was determined using diBAC$_4$(3), a ratiometric fluorescent membrane potential-sensitive dye (Molecular Probes, Eugene, OR) based on a protocol by Krasznai et al. (Krasznai et al., 1995). Cells were resuspended at 0.5×10^6 cells/ml in HybriCare media (no bicarbonate buffer, with 25 mM HEPES) (ATCC, Rockville, MD) in HSA-blocked FACS tubes (Falcon 2052, Fisher Scientific, Lincoln Park, NJ). For the standard curve, cells were resuspended at room temperature with varying concentrations of diBAC$_4$(3) (5000 nM to 300 nM). Cells to be stimulated were resuspended with 600 nM diBAC$_4$(3). Additionally, control cells were fixed for 1 hour on ice with ice-cold 2% formaldehyde, washed with HybriCare and incubated for 1 hour at room temperature with 600 nM diBAC$_4$(3). All samples were run on a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ) at room temperature. Cells were stimulated with 800 nM PMA and fluorescence was measured at time 0 and every minute thereafter for 10 minutes. Inhibitors were incubated with cells at least 10 minutes before PMA stimulation. A linear calibration curve was determined from the cells incubated with varying concentrations of diBAC$_4$(3), and this equation was used to calculate the absolute transmembrane potential of the cells as described by Krasznai et al. (Krasznai et al., 1995).

Confocal microscopy

Eosinophils were resuspended to 0.5×10^6 cells/ml in HybriCare media with 600 nM diBAC$_4$(3) and loaded into eight-chambered coverglass slides (Nalge Nunc International Corporation, Naperville, IL) at room temperature. Cells were stimulated with 800 nM PMA and examined using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss, Oberkochen, Germany). An excitation wavelength of 488 nm was used and emission was detected using a 505LP filter.

Superoxide assay

Superoxide assays were performed at 37°C in HBSS buffer (Hank's balanced salt solution) supplemented with 10 mM HEPES. Cytochrome c was used to detect the production of extracellular superoxide as previously described (Bankers-Fulbright et al., 1998). Inhibitors were added immediately before stimulation unless otherwise noted. Stock solutions of diphenyleneiodonium chloride (DPI), Gö6976, GF109203X and rottlerin (all from Calbiochem, LaJolla, CA) were diluted in HBSS so that the final concentration of DMSO or ethanol was less than 0.5%. The rate of superoxide production was calculated using the linear part of the superoxide production curve (usually 20 to 50 minutes following stimulation) and is presented as nanomoles of superoxide produced per minute.

Intracellular pH measurements

Intracellular pH was measured as described previously (Boyer and Hedley, 1994), with the following changes. Purified eosinophils were resuspended to 0.5×10^6 per ml in physiological saline solution [140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM 2-[*N*-morpholino]ethanesulfonic acid (MES)] supplemented with 1% α calf serum. Eosinophils were loaded with 5 μM BCECF-AM [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein (acetoxymethyl ester derivative)] (Molecular Probes) for 30 minutes at 37°C in the dark, washed once, resuspended in HBSS (with 25 mM PIPES, pH 7.4) and plated at 5×10^5 eosinophils per well in an albumin-blocked, 96-well plate (final volume per well=200 μl). Plates were read at 37°C on a CytoFluor Series 4000 (perSeptive Biosystems, Framingham, MA) fluorescent plate reader with excitation at 485 nm and 450 nm and emission at 535 nm. Calibration of the BCECF-AM dye and calculation of intracellular pH was performed as described earlier (Boyer and Hedley, 1994).

Perforated whole-cell patch clamp technique

The amphotericin perforated whole-cell patch configuration was used. Pipette electrodes were pulled to a resistance of 2-4 MΩ from 7052 glass (Garner Glass, Claremont, CA). The pipette tip was filled with $KMeSO_4$ Ringer solution (130 mM potassium $MeSO_3$, 5 mM NaCl, 1 mM $CaCl_2$, 25 mM $(NH_4)_2SO_4$, pH 7.0). The pipette was then backfilled with the same solution containing 10 μM amphotericin B.

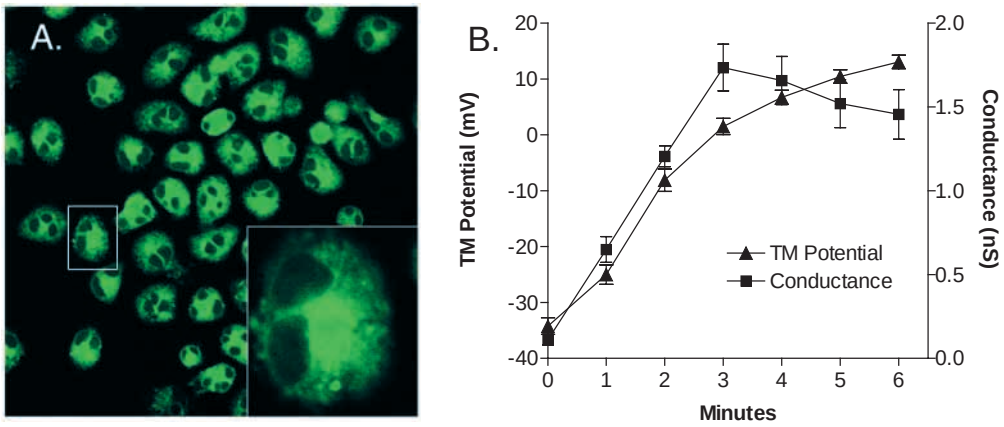

Fig. 1. The time course of eosinophil depolarization correlates with activation of the proton conductance. (A) Eosinophils were loaded with 600 nM diBAC$_4$(3) and analzyed at room temperature by confocal microscopy (magnification=630×). Inset is a 3× electronic magnification of the area in the box. A representative experiment is shown (n=4). (B) Eosinophils were stimulated with 800 nM PMA at room temperature. Depolarization (▲; n=26) was assayed using diBAC$_4$(3) and flow cytometry as described in Materials and Methods. Conductance values (■; n=6) were calculated from current measurements following a voltage step from the holding potential (–60) to +80 mV.

High resistance seals (>5 GΩ) were formed between the pipette and the cell membrane, and amphotericin was allowed to partition into the membrane to obtain the whole-cell configuration before currents were recorded. Typical series resistances ranged from 8 to 20 MΩ. Compensation for series resistance was accomplished using the series resistance compensation circuitry available with the Axopatch 1D amplifier. The standard recording solution for patch-clamp experiments consisted of HybriCare media (ATCC) supplemented with 2 mM glutamine, 50 μg/ml gentamicin sulfate, 7.5 mM $(NH_4)_2SO_4$ 0.3% serum albumin and buffered with 10 mM HEPES, pH 7.4. An Axopatch 1D voltage clamp and Digidata 1322 interface were used (both from Axon Instruments, Union City, CA). P-CLAMP 8.0 software was used to generate the voltage-step commands and to record the resulting currents. Analysis of the whole-cell current traces was performed using AxoGraph 3.0.3 software from Axon Instruments.

Results

DiBAC$_4$(3) labeling of eosinophils resulted in diffuse staining of the eosinophil cytosol and apparent exclusion from secondary granules (Fig. 1A). This observation is consistent with the interpretation that diBAC$_4$(3) fluorescence reflects the eosinophil plasma membrane potential and is presumably not influenced by the membrane potentials of intracellular organelles. As described in Materials and Methods, we used diBAC$_4$(3) to determine the resting membrane potential of eosinophils, which was –32.9±5.7 mV (Fig. 1B). Under the conditions used in our experiments, the eosinophil transmembrane potential must reach a voltage greater than –23 mV (the proton equilibrium potential) to allow even minimal NADPH oxidase-associated proton efflux. After PMA stimulation, eosinophils rapidly depolarized to +17.3±1.8 mV within 10 minutes, approximately 40 mV above the activation threshold. Thus, under conditions where superoxide is produced, PMA-stimulated eosinophils depolarize sufficiently to allow substantial activation of the proton channel. Additionally, the time courses of proton channel activation under patch-clamp conditions and eosinophil depolarization as measured by flow cytometry are nearly identical (Fig. 1B). This observation is consistent with reports that depolarization and NADPH oxidase activity are tightly linked in granulocytes (Seligmann and Gallin, 1980; Whitin et al., 1980; Castranova et al., 1981; Cohen et al., 1981; Henderson et al., 1987; Åhlin et al., 1995; Susták et al., 1997; Jankowski and Grinstein, 1999).

One consequence of proton channel activation in eosinophils should be attenuation of NADPH oxidase-dependent depolarization. If the proton channel is activated, then blocking proton efflux should increase the magnitude of depolarization. To test this, we stimulated eosinophils with PMA in the presence of 250 μM $ZnCl_2$; this concentration of $ZnCl_2$ fully blocks PMA-stimulated eosinophil proton currents but does not fully inhibit PMA-stimulated NADPH oxidase activity (Bankers-Fulbright, 2001). As predicted, treatment with $ZnCl_2$ enhanced the magnitude of eosinophil depolarization (+43.0±4.2 mV; P=0.0006), in contrast to stimulation with PMA alone (+16.2±1.3 mV) (Fig. 2).

To confirm that the outcome observed with $ZnCl_2$ was due to effects on proton transport, we altered the proton channel activation threshold by changing the extracellular pH (pH_e). If proton efflux plays a role in regulating eosinophil depolarization, increasing the activation threshold (lowering pH_e) should increase the magnitude of depolarization and

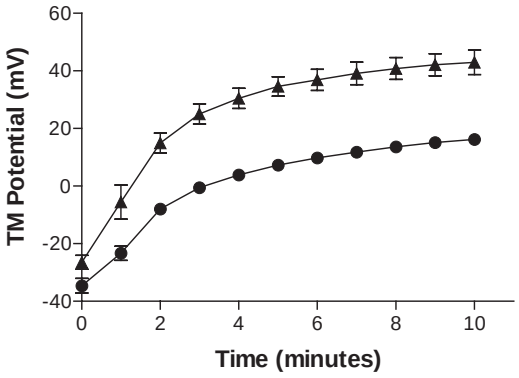

Fig. 2. $ZnCl_2$ potentiates PMA-stimulated eosinophil depolarization. Transmembrane potential was determined using diBAC$_4$(3) and flow cytometry. Absolute membrane potential was calculated as described in Materials and Methods. Eosinophils were stimulated at room temperature with 800 nM PMA in the absence (●; n=4) or presence (▲; n=4) of 250 μM $ZnCl_2$. $ZnCl_2$ was added immediately before stimulation. Mean values±s.e.m. are shown; where the error bars are not visible they are smaller than the symbol.

decreasing the threshold (increasing pH_e) should attenuate depolarization (Cherny et al., 1995; Åhlin et al., 1995; Henderson et al., 1987). Notably, the voltage threshold for activation of the proton conductance depends primarily on the difference between, not the absolute values of, intracellular and extracellular pH (Cherny et al., 1995). Thus, we first measured eosinophil intracellular pH (pH_i) under different pH_e conditions to document this difference (Fig. 3A). Eosinophils in standard buffer (pH 7.4) have a resting pH_i of 7.12±0.30. Decreasing pH_e to 7.0, 6.5 and 6.0 induces corresponding significant decreases in pH_i (7.00±0.05, 6.71±0.04 and 6.53±0.04, respectively; all $P<0.00001$ compared with pH_e 7.4). Eosinophils stimulated with PMA for up to 2 hours consistently maintain their resting pH_i at all pH_e (data not shown). Thus, at pH_e greater than 7.0, the ratio of intracelluar to extracellular proton concentration favors efflux; at pH_e less than 7.0, proton efflux is diminished. PMA-stimulated NADPH oxidase activity is also affected by changing pH_e (Fig. 3B). Interestingly, the rate of superoxide production is not significantly affected by decreasing pH_e to 7.0 ($P=0.48$) or 6.5 ($P=0.065$). However, the PMA-stimulated NADPH oxidase rate is inhibited by approximately 50% at pH_e 6.0 ($P=0.005$).

To document the effect of pH_e on eosinophil depolarization, we analyzed diBAC$_4$(3)-loaded eosinophils at pH_e between 6.0 and 8.0. Membrane potential measurements of eosinophils maintained at pH_e 6.0 and pH_e 8.0 showed some variability, but no significant differences in resting membrane potential (–19.8 vs –33.2, respectively; $P=0.06$) (Fig. 3C). As predicted, eosinophils at pH_e 8.0 showed a decreased magnitude of PMA-stimulated depolarization (+8.9±1.4 mV) compared with cells stimulated at pH_e 7.4 (+19.2±2.2 mV; $P=0.002$). Similarly, decreasing pH_e to 7.0, 6.5 and 6.0 (reducing proton efflux) increased the magnitude of depolarization to +33.9±2.6 mV ($P=0.0001$), +44.4±1.4 mV ($P=0.004$) and +42.2±4.6 mV ($P=0.010$), respectively. There was no significant difference between the rate ($P=0.082$) or magnitude ($P=0.092$) of PMA-stimulated eosinophil depolarization at pH_e 6.0 vs 6.5. In addition, these results were not significantly different from the values obtained following zinc inhibition of the proton channel. Thus, eosinophil depolarization is predictably affected by changing proton efflux through modifications of the proton gradient, as well as through blocking the proton channel with $ZnCl_2$.

The NADPH oxidase and its associated proton channel are often activated by the same stimuli (DeCoursey et al., 2000; DeCoursey et al., 2001b; Cherny et al., 2001). We have previously shown that superoxide production by PMA-stimulated eosinophils is inhibited by the selective PKCδ

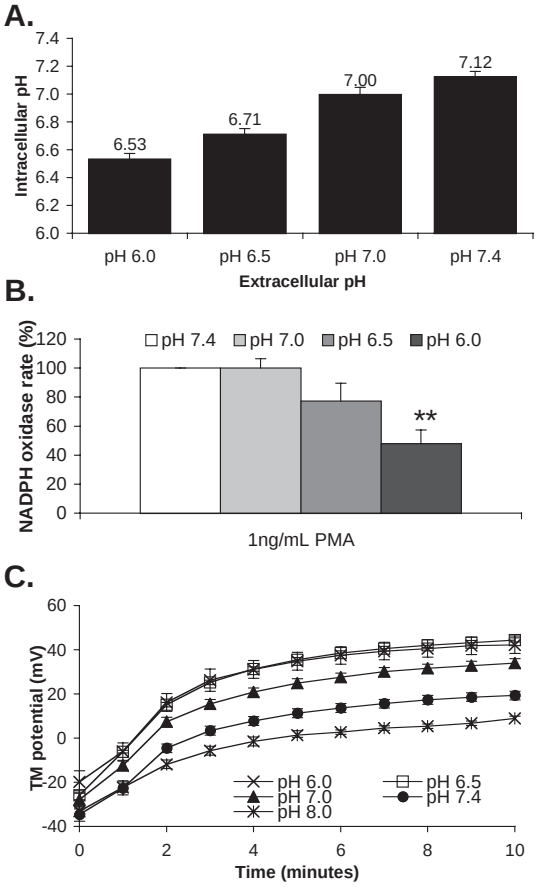

Fig. 3. Varying extracellular pH predictably affects eosinophil depolarization. (A) Eosinophil intracellular pH was determined using BCECF/AM and calibrated as described in Materials and Methods. Buffer pH was verified and adjusted before each experiment. Unstimulated eosinophils were resuspended at 37°C at pH 6.0 (n=8), pH 6.5 (n=8), pH 7.0 (n=8) and pH 7.4 (n=8). The mean is stated above each bar (mean±s.e.m.). (B) Superoxide production was determined using the cytochrome c colorimetric assay as described in Materials and Methods. Buffer pH was verified and adjusted before each experiment. Cells were stimulated with 2 nM PMA at 37°C at pH 6.0 (n=5), pH 6.5 (n=5), pH 7.0 (n=5) and pH 7.4 (n=5). NADPH oxidase rate was calculated as described in Materials and Methods (means±s.e.m.). **$P<0.01$. (C) Eosinophil membrane potential was measured using diBAC$_4$(3) and flow cytometry. Media pH was verified and adjusted before each experiment. Eosinophils were stimulated with 800 nM at room temperature at pH 6.0 (×; n=3), pH 6.5 (□; n=4), pH 7.0 (▲; n=8), pH 7.4 (●; n=8) and pH 8.0 (*; n=4). Means±s.e.m. are shown.

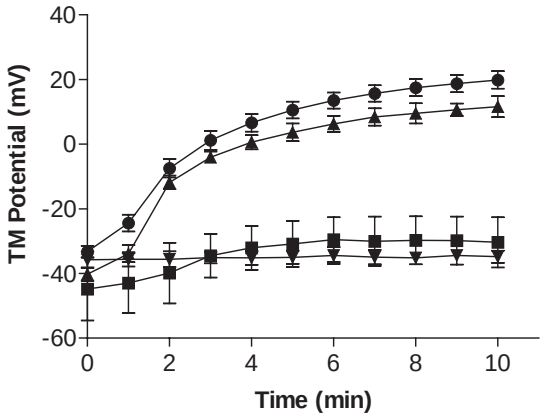

Fig. 4. PKC blockers prevent PMA-induced eosinophil depolarization. Eosinophils were resuspended in diBAC$_4$(3) and stimulated with 800 nM PMA at room temperature. Absolute transmembrane potential was determined by flow cytometry as described in Materials and Methods. Eosinophils were stimulated at room temperature with 800 nM PMA alone (●; n=9) or in the presence of 5 μM Gö6976 (▲; n=3), 1 μM GF109203X (▼; n=3), or 10 μM rottlerin (■; n=4). PKC inhibitors were added immediately before stimulation with PMA. Means±s.e.m. are shown.

blocker, rottlerin (Bankers-Fulbright, 2001). However, proton channel activation in these cells is not blocked by rottlerin alone, although the pan-specific PKC blocker, GF109203X, does block proton channel activity. To determine which PKC isoforms were modulating eosinophil depolarization, we stimulated cells with PMA in the presence of different PKC inhibitors (Fig. 4). None of the PKC inhibitors significantly affected eosinophil resting membrane potential. The pan-specific PKC blocker, GF109203X, completely inhibited PMA-induced depolarization (P=0.00006). Additionally, the PKCδ-selective blocker, rottlerin, significantly inhibited PMA-stimulated depolarization (P=0.02), and a blocker of Ca^{2+} dependent PKC isoforms, Gö6976, had a minimal but statistically significant (P=0.03) effect.

Discussion

The results of this study show that membrane depolarization associated with NADPH oxidase activation in eosinophils is sufficient to produce a substantial level of proton channel activation. This is consistent with recent results obtained in neutrophils (Jankowski and Grinstein, 1999). Although several groups have described the presence of proton channels in eosinophils (Gordienko et al., 1996; Schrenzel et al., 1996; Bánfi et al., 1999; Bankers-Fulbright et al., 2001; Cherny et al., 2001; DeCoursey et al., 2001a; DeCoursey et al., 2001b), the membrane potential determined by current clamp measurements indicated that depolarization following activation of NADPH oxidase was not sufficient to activate the proton channel (Gordienko et al., 1996; Tare et al., 1998; Bánfi et al., 1999). Eosinophil resting membrane potential has been reported to be between –60 and –80 mV (Gordienko et al., 1996; Tare et al., 1998). After activation of eosinophil NADPH oxidase with intracellular NADPH and GTPγS and after blocking the inwardly rectifying K^+ channel, Bánfi et al. (Bánfi et al., 1999) reported a 40 mV depolarization. This suggests that eosinophils have a final membrane potential between –20 and –40 mV when the NADPH oxidase is activated. This voltage range is at the threshold of proton channel activation. Our results using an anionic membrane potential-sensitive dye agree reasonably well with the magnitude of depolarization described by Bánfi et al. (Bánfi et al., 1999) after NADPH oxidase activation, but they indicate that the resting membrane potential of human eosinophils is approximately 30 mV to 50 mV more positive than previously reported (Fig. 1B). The discrepancy between our results and previous resting membrane potential determinations made under current clamp conditions is probably related to differences in methodology (current clamp vs anionic dye) and the extracellular media composition (buffered salt solution vs supplemented media). We specifically used a membrane potential dye so that we could keep the eosinophil as close to its normal physiological state as possible.

Although both neutrophils and eosinophils are granulocytes and use the same NADPH oxidase to generate superoxide, the amount and location of the oxidase is different in these cell types. Thus, extrapolating membrane potential results from recent neutrophil data is not necessarily predictive of the depolarization magnitude or time course in eosinophils. Eosinophils express more NADPH oxidase than neutrophils in both humans and guinea pigs (Yagisawa et al., 1996; Someya et al., 1997) and the majority of the oxidase is located on the plasma membrane (Lacy et al., 2003). By contrast, the vast majority of neutrophil oxidase is located intracellularly. This difference in location is consistent with the distinct functional roles of neutrophils and eosinophils. Neutrophils kill primarily by phagocytosing their targets, whereas eosinophils kill non-phagocytosable targets by secreting toxic agents.

In these experiments, membrane potential measurements were made using the cell-permeable fluorescent dye, $diBAC_4(3)$. A primary concern when working with membrane potential-sensitive dyes is the possibility of reporting intracellular compartment membrane potentials instead of the plasma membrane potential and the dye's possible sensitivity to the oxygen radicals. Our data probably reflect the plasma membrane potential for several reasons. First, although the sulfur-containing, cationic carbocyanine dyes have been reported to be quenched during superoxide production, this is unlikely to occur with $diBAC_4(3)$, an anionic, non-sulfur-containing oxonol dye (Whitin et al., 1981). Second, eosinophils, in contrast to neutrophils or monocytes, are not primarily phagocytic and thus are less likely to accumulate extracellular dye in phagosomes. Third, confocal microscopy documents that the dye is localized to the cytosol of the eosinophil and not sequestered in the secondary granules or other intracellular organelles. Fourth, altering the external conditions of the cell by adding $ZnCl_2$ or changing the pH_e affects $diBAC_4(3)$ fluorescence as would be predicted if the dye was measuring the plasma membrane potential. Additionally, the effects of $ZnCl_2$ and pH_e changes on eosinophil membrane potential are consistent with previous reports (Henderson et al., 1987; Susztak et al., 1997; Banfi et al., 1999).

Proton efflux through the NADPH oxidase-associated proton channel appears to be required for continued superoxide production by neutrophils and eosinophils. Previous studies, using $ZnCl_2$ to block proton efflux, document inhibited superoxide production (Henderson et al., 1988a; Bankers-Fulbright et al., 2001). This is probably due to the lack of electrogenic compensation for electron transfer through the membrane by NADPH oxidase, allowing unattenuated depolarization and proton accumulation at the inner membrane surface (Henderson et al., 1988a). Although originally thought to be part of the NADPH oxidase complex, the proton channel now appears to be a distinct protein that is, nevertheless, concomitantly regulated by PKC (DeCoursey et al., 2001a). Stimuli such as arachidonic acid and PMA, which activate the proton channels in neutrophils and eosinophils, also stimulate electron transport through the oxidase (DeCoursey et al., 2000; DeCoursey et al., 2001b; Cherny et al., 2001). Although PMA-stimulated NADPH oxidase activity can be blocked with the PKCδ selective inhibitor, rottlerin, the proton conductance is insensitive to PKCδ inhibition (Bankers-Fulbright, 2001). Consistent with our previous data, we report here that rottlerin also inhibits PMA-stimulated eosinophil depolarization (Fig. 4). Thus, the proton channel and electron transport appear to be regulated independently by different PKC isoforms.

Pharmacological specificity is always a concern when using inhibitors. At the concentrations used in this paper, rottlerin is selective for PKCδ and does not inhibit the activity of any other known PKC isoforms. However, rottlerin can block calmodulin-dependent (CaM) kinase III (Geschwendt et al., 1994). CaM kinase III is unlikely to be the target of rottlerin

in PMA-stimulated eosinophils because stimulation with PMA does not increase intracellular calcium concentrations (data not shown). Additionally, we have previously shown that PKCδ is indeed activated in PMA-stimulated eosinophils, and this activation is inhibited by rottlerin (Bankers-Fulbright, 2001).

In summary, we have shown that PMA-stimulated eosinophils depolarize to voltages sufficient to activate the proton conductance, and proton efflux can regulate the extent of depolarization. PKCδ appears to be necessary for PMA-induced depolarization and superoxide production, but not for proton channel activation in human eosinophils.

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