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Potential, pH, and Arachidonate Gate Hydrogen Ion Currents in Human Neutrophils

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ABSTRACT Indirect evidence indicates that a proton-selective conductance is activated during the respiratory burst in neutrophils. A voltage- and time-dependent H^+-selective conductance, g_H, in human neutrophils is demonstrated here directly by the whole-cell patch-clamp technique. The g_H is extremely low at large negative potentials, increases slowly upon membrane depolarization, and does not inactivate. It is enhanced at high external pH or low internal pH and is inhibited by Cd^{2+} and Zn^{2+}. Arachidonic acid, which plays a pivotal role in inflammatory reactions, amplifies the g_H. The properties of the g_H described here are compatible with its activation during the respiratory burst in stimulated neutrophils, in which it may facilitate sustained superoxide anion release by dissipating metabolically generated acid.

INTRODUCTION

A fundamental response of activated neutrophils is the respiratory burst, in which NADPH oxidase reduces O_2 to superoxide anion (O_2^-), which is released to the extracellular solution or into the phagocytic vacuole which is its topological equivalent. O_2^- in turn rapidly dismutates into hydrogen peroxide, H_2O_2, and other bactericidal oxygen radicals. Activation of the hexose monophosphate shunt and the NADPH oxidase generates intracellular protons which must be efficiently secreted from the cell, where they participate in O_2^- dismutation (Borregaard et al., 1984; Grinstein and Furuya, 1986). This metabolic acid production during the respiratory burst results in transient cytoplasmic acidification (Grinstein and Furuya, 1986; Henderson et al., 1987, 1988a) and membrane depolarization (Whitin et al., 1980; Jones et al., 1980; Sklar et al., 1981; Kuroki et al., 1982; Sullivan et al., 1987; Myers et al., 1990; reviewed by Gallin, 1991), both of which are then compensated. The Na^+/H^+ exchanger contributes to pH_i homeostasis in activated neutrophils (Molski et al., 1980; Simchowitz, 1985; Grinstein and Furuya, 1986), but is electroneutral, and its inhibition by amiloride or removal of Na^+ increases the extent but not the initial rate of cytoplasmic acidification (Simchowitz, 1985; Grinstein and Furuya, 1986; Henderson et al., 1988a). Phorbol esters activate electrogenic H^+ efflux in neutrophils (Henderson et al., 1987, 1988a; Nanda and Grinstein, 1991; Kapus et al., 1992). The divalent cations Cd^{2+} and Zn^{2+}, which block H^+ currents in other cells (Thomas and Meech, 1982; Byerly et al., 1984; Barish and Baud, 1984; Byerly and Suen, 1989; Mahaut-Smith, 1989b; DeCoursey, 1991), inhibit the compensatory responses so that in Na^+ free media, pH_i can drop to 6.4 and the membrane potential can depolarize above 0 mV (Henderson et al., 1988a, 1988b; Nanda and Grinstein, 1991; Kapus et al., 1992). Taken together, these data provide compelling evidence for the existence of proton-selective channels capable of mediating H^+ efflux in human neutrophils during the respiratory burst (Henderson et al., 1987, 1988a; Nanda and Grinstein, 1991; Kapus et al., 1992).

Few voltage-clamp studies of ion channels in human neutrophils have appeared. Ca^{2+}-activated nonselective cation channels (von Tscharner et al., 1986), depolarization-activated K^+ currents, and Ca^{2+}-activated K^+ and Cl^- currents have been described (Krause and Welsh, 1990). A voltage- and time-dependent H^+-selective conductance, g_H, is demonstrated here in human neutrophils using tight-seal whole-cell recording (Hamill et al., 1981). Newt neutrophils express inwardly rectifying K^+ channels which have not been reported in human neutrophils, but also exhibit a slow decrease in input resistance at large positive potentials under current-clamp conditions, which is consistent with the slow activation of a another conductance (Kawa, 1989), perhaps a proton conductance. Voltage-activated, polyvalent cation-sensitive H^+-selective currents have been described in snail (*Lymnaea* or *Helix*) neurons (Thomas and Meech, 1982; Byerly et al., 1984), axolotl (*Ambystoma*) oocytes (Barish and Baud, 1984), rat alveolar epithelial cells (DeCoursey, 1991), and murine macrophages (Kapus et al., 1993). Evidence consistent with a g_H sharing at least some of these properties has been presented in human monocyte-derived macrophages (Nelson et al., 1990), the OK cell line (Graber et al., 1991), leech CNS neurons (Frey and Schule, 1993), and in several other cells (see discussions in Byerly and Moody (1986); Meech and Thomas (1987)). The g_H described here in human neutrophils closely resembles the g_H in other cells: it is activated by membrane depolarization, it is regulated by pH as its voltage-dependence is shifted to more negative potentials by low pH_i or high pH_o, and it is sensitive to Cd^{2+} and Zn^{2+}. The kinetics of activation appear substantially slower than in neurons and axolotl oocytes. The properties of the g_H described here, including its enhancement by arachidonic acid, resemble those of the proton conductance activated during

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the respiratory burst in intact neutrophils in the studies discussed in the previous paragraph. Part of this data was presented in abstract form (DeCoursey, 1993).

MATERIALS AND METHODS

Cells

Neutrophils were isolated from normal human blood by density gradient centrifugation (Schmeichel and Thomas, 1987), and kept on ice in RPMI 1640 media for not more than 6 h before use. Immediately before recording, neutrophils were transferred to the glass recording chamber, and superfused with Ringer's solution (in millimolar: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4). Neutrophils often adhered to the glass and exhibited shape changes and movement. While shape changes indicate that some degree of activation may have occurred in these cells, it is clear that there are several pathways of neutrophil activation, and the respiratory burst requires several specific intermediary steps including protein kinase C activation (Rossi, 1986) which are not required for simple shape changes (Baggiolini and Kernen, 1992). Cells selected for recording were sometimes adherent and sometimes nonadherent. In the latter case, the cell was approached and then suction applied to the pipette before contact with the cell was made. The maximum H^+ current recorded in cells which were tightly adherent (14.5 ± 13.9 pA/pF, mean ± S.D., n = 15) was not different from that in cells categorized as lightly attached (13.8 ± 14.9 pA/pF, n = 10) or from cells which were not attached to the chamber (19.1 ± 15.3 pA/pF, n = 12), all studied with the pH 6.0 TEA methanesulfonate pipette solution. In several experiments fresh blood from the authors was studied without purification, and cells presumed to be neutrophils were identified visually by their size (~8-μm diameter) and spherical, granular appearance. Since neutrophils are statistically the most common white blood cell in humans (60–70%) and the next most common type, lymphocytes (25–33%), are distinctly smaller, the possible contaminating cells are monocytes (2–6%), eosinophils (1–4%), and basophils (0.25–0.5% (Sherwood, 1993)); hence, most of the cells selected in this way were likely neutrophils. A g_H similar in magnitude and properties to that in purified neutrophils was observed in these fresh nonpurified cells.

Electrophysiology

Standard whole-cell patch-clamp techniques were used (Hamill et al., 1981; DeCoursey, 1991). Micropipettes were pulled in several stages using a Flaming Brown automatic pipette puller (Sutter Instruments, San Rafael, CA) from EG-6 glass (Garner Glass Co., Claremont, CA). Pipettes were coated with Sylgard 184 (Dow Corning Corp., Midland, MI), and heat-polished to a tip resistance measured in Ringer's solution ranging typically between 2 and 4 MΩ. Electrical contact with the pipette solution was achieved by a thin sintered Ag-AgCl pellet (In Vivo Metric Systems, Healdsburg, CA) attached to a silver wire covered by a Teflon tube. The pipette and the initial bath solutions were filtered at 0.1–0.2 μm (Millipore, Bedford, MA). A reference electrode made from a Ag-AgCl pellet was connected to the bath through an agar bridge saturated with Ringer's solution. The current signal from the patch clamp (List Electronic, Darmstadt, Germany) was recorded and analyzed using an Indec Laboratory Data Acquisition and Display System (Indec Corporation, Sunnyvale, CA). Currents were sometimes recorded on a Gould 220 chart recorder (Cleveland, OH) in order to appreciate slow changes occurring on a time scale of minutes. Series resistance compensation was used, although the typically small H^+ current amplitude minimized voltage errors. Solutions were made with largely impermeant ions, usually tetraethylammonium (TEA^+) methanesulfonate ($CH_3SO_3^-$), by neutralizing methanesulfonic acid with TEA hydroxide, and included 20 mM buffer (2[*N*-morpholino]ethanesulfonic acid (MES) for pH 5.5–6.6, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) for pH 7.0, HEPES for pH 7.4, 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) for pH 9.0). In some experiments other solutions were used, as indicated in figure legends. External solutions included 2 mM $CaCl_2$ and internal solutions contained 2 mM $MgCl_2$ and 5 mM EGTA, except where noted. TEA^+ was used in spite of its partial inhibition at high concentrations of the g_H in neurons (Byerly et al., 1984) in order to block K^+ conductances which are present in human neutrophils (Krause and Welsh, 1990), and to preclude interference by other ion transport systems. Except where indicated, liquid junction potentials were not corrected; the combined (measured) junction potential error for the most frequently used bath and pipette solutions was 2 mV. Experiments were done at room temperature (19–22°C), with the bath temperature monitored continuously by a thin film platinum RTD (resistance temperature detector) element (Omega Engineering, Stamford, CT) immersed in the bath solution, and the temperature stored along with each record. When bath solutions were changed, possible temperature effects were avoided by comparing temperature-matched control and test records. MES, HEPES, BES, CHES, PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]), EGTA, and sodium arachidonate were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Sodium arachidonate was dissolved in dimethyl sulfoxide (DMSO) under N_2 gas and aliquots kept at −70°C until the day of the experiment.

RESULTS

H^+ selective currents were observed in most neutrophils studied in solutions devoid of other permeant ions. Fig. 1 (*A*, *C*, and *E*) illustrates families of H^+ currents in a cell studied with pH_i 6.0 at three pH_o. The g_H was extremely low at negative potentials and increased slowly with time upon membrane depolarization. At room temperature the g_H did not reach steady-state during 4-s depolarizing pulses. The timecourse of H^+ currents was slightly sigmoid and could be fitted fairly well by a Hodgkin-Huxley parameter raised to the 1.5–2.0 power. As in other cell types (Byerly et al., 1984; Barish and Baud, 1984; Meech and Thomas, 1987; DeCoursey, 1991), lowering pH_o shifted the voltage dependence of the g_H to more positive potentials. In the experiment in Fig. 1, the g_H was first activated at −40 mV in pH_o 8.5 (not shown), at 0 mV in pH_o 7.6 (*A*), at +20 mV in pH_o 7.0 (*C*), and at +60 mV in pH_o 5.9 (*E*). There was cell-to-cell variability in the potential at which activation occurred in a given solution, but over a pH_o range from 5.9 to 9.0 lowering pH_o in a given cell consistently shifted g_H activation to more positive potentials.

Reversal potential measurements confirm the H^+ selectivity of the time-dependent outward currents in Fig. 1. A prepulse activated the g_H, and the polarity of the "tail current" upon repolarization was determined. At pH_o 7.6, 7.0, and 5.9 the observed reversal potential, V_{rev}, in this experiment was approximately −90, −45, and +15 mV (Fig. 1 (*B*, *D*, and *F*)) in good agreement with the Nernst potential for H^+, E_H, of −93, −58, and +6 mV, respectively. In most experiments, especially at high pH_o, V_{rev} was more positive than E_H. Analogous deviation of V_{rev} from E_H in H^+ currents in alveolar epithelial cells also studied using whole-cell recording was shown to be attributable mainly to H^+ depletion from the cytoplasmic compartment that inevitably results from outward H^+ current flow during the prepulse (DeCoursey, 1991). That the deviation in the present study was less pronounced may reflect the smaller H^+ current magnitude of neutrophils. The neutrophils in these experiments averaged 8.0 μm in diameter with an input capacity of 2.1 pF, and a specific capacitance of 1.07 ± 0.31 μF/cm² (mean ± S.D., n = 48). Normalized to input capacity, the maximum H^+

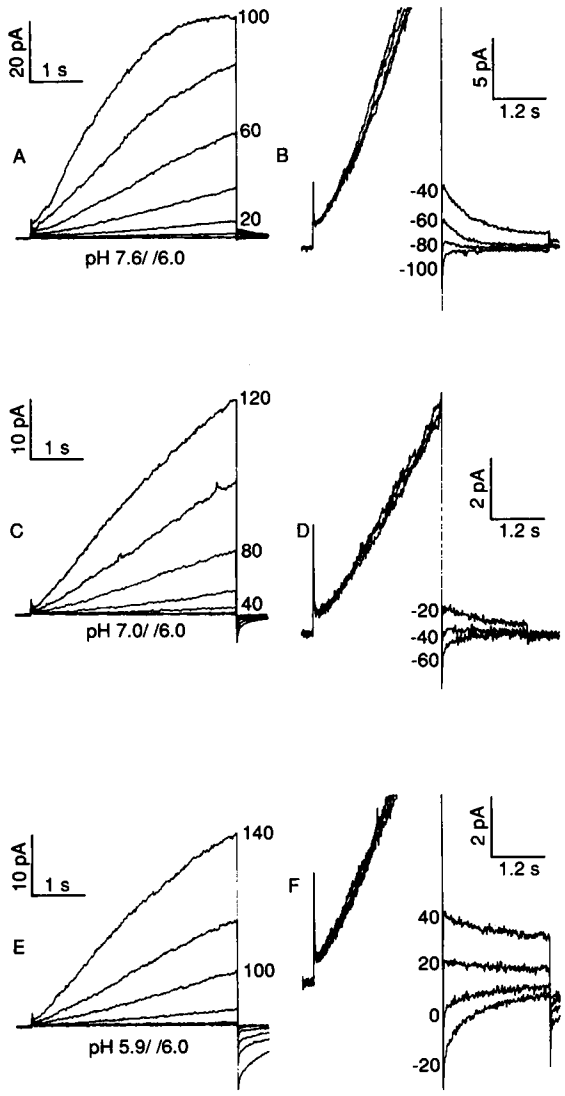

FIGURE 1 Whole-cell H^+ currents in a human neutrophil at various pH_o, indicated as $pH_o//pH_i$. (*A*) A family of superimposed currents during 4-s pulses applied every 20 s from a holding potential −60 in 20-mV increments to −40 through +100 mV. Time-dependent H^+ currents turn on at 0 mV. The bath contained sodium bicarbonate and 10 mM HEPES, the pipette TEA methanesulfonate, 19.4°C, filter 200 Hz. (*B*) Determination of reversal potential, V_{rev}, by tail currents. A prepulse to +60 mV activated the g_H (with currents off-scale), and the cell was then clamped to various potentials as indicated. The amplitude of the tail current transient at each potential was determined by fitting the decay with a single exponential. Although inward tail currents were not well-resolved at high pH_o, V_{rev} was approximately −90 mV. (*C*) H^+ currents in pH_o 7.0 TEA methanesulfonate. The holding potential was −60 mV, and pulses were applied in 20-mV increments to 0 through +120 mV. At high gain H^+ current activation was detectable at +20 mV. (*D*) V_{rev} measured with prepulses to +80 mV was −45 mV. (*E*) H^+ currents in pH_o 5.9 TEA methanesulfonate. The holding potential was −20 mV, and pulses were applied in 20-mV increments to +20 through +140 mV. H^+ current activation occurred first at +60 mV. (*F*) V_{rev} measured with prepulses to +110 mV, was +15 mV.

current with pH_i 6.0 was 17.1 ± 14.1 pA/pF (mean ± S.D., $n = 41$), which is comparable to 14.6 pA/pF in snail neurons (Byerly and Suen, 1989) and 27.3 pA/pF in alveolar epithelium (with high pipette buffer concentration) (DeCoursey, 1991).

The voltage dependence of the g_H was sensitive to pH_i as well as to pH_o. Fig. 2 illustrates current-voltage relationships in a neutrophil studied with pH_i 5.5 and pH_o 7.0 or 5.9. The g_H is detectably activated at pH_o 7.0 at −20 mV, and as shown in Fig. 1, lowering pH_o to 5.9 shifts the voltage dependence of the g_H to more positive potentials by 20–30 mV. The voltage dependence of activation of the g_H in other cells is similarly shifted toward more negative potentials as pH_i is lowered (Byerly et al., 1984; Mahaut-Smith, 1989a). This figure also illustrates that for comparable pH_o the outward currents are similar in TEA methanesulfonate, sodium methanesulfonate, and Ringer's solution in which Cl^- is the major anion. The V_{rev} in these three solutions was −15 to −25 mV. This type of experiment does not rule out possible interactions between other ions and the g_H, but shows that H^+ currents can be observed in the presence of Na^+ instead of TEA^+ or Cl^- instead of methanesulfonate$^-$.

No single channel H^+ currents could be detected. Unitary conductance was estimated from the current variance during small depolarizations above threshold after steady-state current levels were achieved. This approach is described by Hille (1992): $i = \sigma_I^2/\{I(1 - p)\}$, where i = single channel current, σ_I^2 = current variance, I = mean current, and p = open probability. The variance measured in the same solutions at subthreshold potentials was subtracted to define the excess variance attributable to the g_H. Excess variance could be detected at potentials where the g_H was activated with lowpass filtering at 200 Hz but not at 2000 Hz. Even when

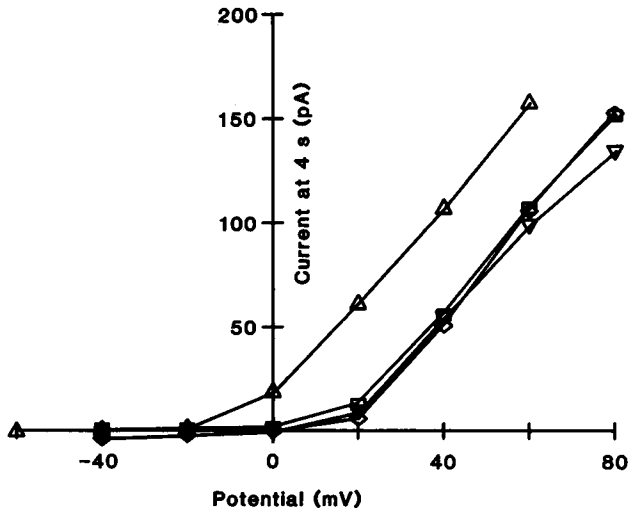

FIGURE 2 H^+ currents with pH_i 5.5, and pH_o 7.0 or 5.9. The currents were measured at the end of 4-s-long depolarizing pulses from a holding potential of −60 mV for pH_o 7.0 or −40 mV for pH_o 5.9. In pH_o 7.0 TEA methanesulfonate solution (△), the g_H was detectably activated at −20 mV. When pH_o was lowered to [illegible], the g_H was clearly activated at +20 mV, whether the bath contained TEA methanesulfonate at pH 5.87 (□), Na methanesulfonate at pH 5.77 (▽), or Ringer's solution at pH 5.85 (◇). V_{rev} in these three solutions after correction for liquid junction potentials was −16 mV (□), −11 mV (▽), or −28 mV (◇), and was −66 mV at pH_o 7.0 (△). The pipette solution contained 119 mM *N*-methyl-D-glucamine and MES buffer, in addition to 3.7 mM EGTA, 0.7 mM $CaCl_2$ and was titrated to pH 5.5 with methanesulfonic acid.

filtering at 200 Hz, the signal-to-noise ratio was poor (<0.5), and thus the estimate must be considered a rough approximation. The unitary conductance based on the observed V_{rev} was ~10 fS for H^+ currents in pH_o 9.0 at potentials just above the threshold for activating the g_H. Byerly and Suen (1989) saw no excess H^+ current variance in snail neurons and placed an upper limit of <4 fA for unitary H^+ currents. Using the estimated conductance of 10 fS, if the g_H is mediated by a channel, then given the mean maximum whole-cell H^+ current of 35 pA, there are ~35,000 channels per cell and the channel density in the membrane is ~170 μm^{-2}, based on the membrane area estimated for a smooth spherical surface. This density is comparable to that of Na^+ channels in excitable cells, and an order of magnitude lower than that of Na^+ or K^+ channels at the node of Ranvier (Hille, 1992).

The behavior of the g_H during long voltage steps is illustrated in Fig. 3 (*top panel*). When the holding potential was clamped from −40 to +20 mV outward H^+ current increased slowly, achieving a steady-state level after ~2 min. The currents recorded during 4-s-long depolarizing pulses, as in Fig. 1, clearly underestimate the H^+ current attainable at a given voltage. There was no suggestion of inactivation after 4 min at +20 mV. In other experiments, inactivation of H^+ current was not observed during *small* depolarizing voltage steps lasting up to 3–5 min. H^+ currents "droop" only in cells with large currents and at large positive potentials at which the g_H is activated extensively. In a study of analogous H^+ currents in rat alveolar epithelial cells, H^+ current saturation and droop were shown to be due to bulk depletion of H^+ and protonated buffer from the cell as a consequence of the large H^+ efflux (DeCoursey, 1991). To ensure that the steady-state H^+ current in Fig. 3 was not due to diffusion-limitation-induced saturation, the potential was stepped to +40 mV. There was an immediate jump in current reflecting the increased driving force, that was followed by a slower increase indicating that the g_H was activated more fully by further depolarization. Repolarization to 0 mV resulted in a slow decrease in current as the g_H diminished. After this first series of voltage steps, the cell was clamped at −40 mV. Stepping back to 0 mV resulted in a slowly increasing H^+ current, indicating that the g_H turned on to a finite extent at this potential. That the initial H^+ current observed after the step to 0 mV was smaller than that extrapolated from the slowly decaying current at 0 mV in the previous pulse sequence (or after a comparable time at 0 mV in the last sequence) indicates that the g_H turned off more rapidly at −40 mV than at 0 mV. During a depolarizing pulse directly to +40 mV from −40 mV the initial current was small and, after 30 s, reached the amplitude attained after 11 s at +40 mV when the membrane was stepped directly from +20 mV. To summarize this experiment: 1) the g_H is activated in a graded manner by depolarization; 2) the steady-state H^+ current during prolonged (small-amplitude) depolarizations reflects partial activation of the g_H rather than current limitation due to H^+

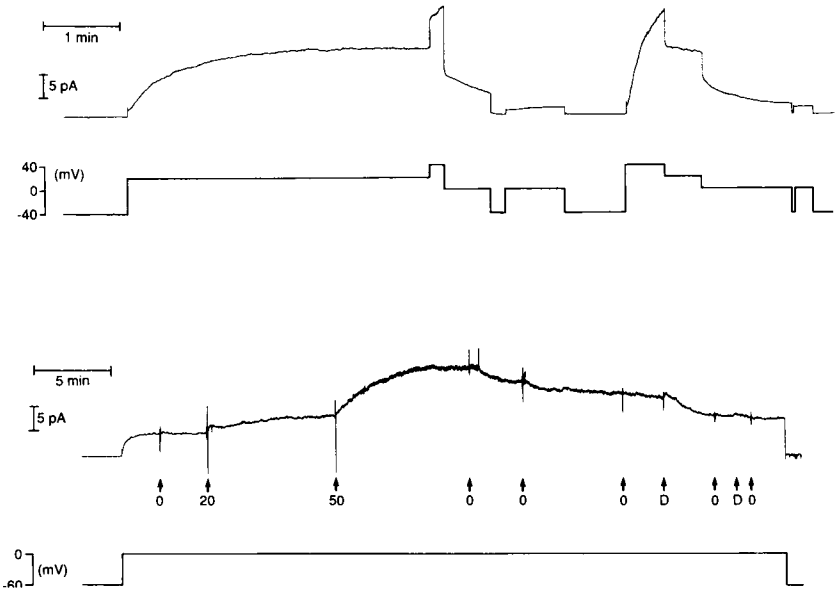

FIGURE 3 Whole-cell H^+ currents during voltage-clamp steps to various potentials as indicated (*top panels*), and during addition of arachidonic acid (*lower panels*). In both experiments (on different cells) the pipette solution was pH 6.0 TEA methanesulfonate with 20 mM MES, the bathing solution was pH 7.0 TEA methanesulfonate with 20 mM BES, and currents were filtered at 20 Hz. There was no detectable H^+ current at the holding potential. (*Lower panels*) At the arrows, the bath was replaced by the same solution with the indicated concentrations of arachidonate (in μM). Most added solutions were at room temperature (22.5°C), but the 20 μM arachidonate solution increased the bath temperature transiently by 2–3°C, and thus the initial bump of current is a temperature artifact. Arachidonate solutions were always sonicated just before addition to the bath, which tends to increase their temperature. It was attempted to cool sonicated solutions to room temperature, but this was not always accomplished. The 50 μM arachidonate solution included 1% DMSO as a vehicle; 2% DMSO was added as a control for its effects (labeled *D*). The H^+ current decreased in the presence of DMSO, but this effect was not reversed upon washout (*0*). In other experiments, 2% DMSO either had no effect on or reversibly inhibited H^+ currents assessed by 4-s depolarizing pulses. In this experiment DMSO may have accelerated the washout of arachidonate. The current returned to baseline at the end of the experiment when the potential was clamped back to −60 mV.

depletion; 3) voltage-dependent changes in g_H in neutrophils occur slowly; and 4) there is no detectable inactivation of the g_H during maintained depolarization. The lack of inactivation of H^+ currents is shared by the g_H in other cells (Thomas and Meech, 1982; Byerly et al., 1984; Byerly and Suen, 1989). The activation kinetics are comparable with or somewhat slower than those in mammalian (rat) alveolar epithelial cells (DeCoursey, 1991) but are much slower than those in *Ambystoma* (Barish and Baud, 1984) or *Lymnaea* (Byerly et al., 1984).

Arachidonic acid and its metabolites, which play a pivotal role in inflammatory reactions, are produced by activated macrophages and neutrophils (Bromberg and Pick, 1983; Galbraith, 1988; Tao et al., 1989; Grimminger et al., 1991). A variety of ion channels are activated directly by arachidonate (Ordway et al., 1991). The electrogenic proton efflux associated with NADPH-oxidase in human neutrophils is activated by arachidonate (Henderson and Chappell, 1992). Fig. 3 (*lower panel*) illustrates the effect of arachidonic acid on H^+ currents. When the membrane potential was clamped from −60 to 0 mV an outward H^+ current was activated. At the first arrow the bath was flushed with the same solution, illustrating the absence of flow effects. After addition of 20 μM arachidonate an early artifactual rise due to a transient bath temperature increase was followed by a slow increase in the outward current. Addition of 50 μM arachidonate enhanced the H^+ current to ~4 times its original value, with the effect becoming maximal after ~6 min. Washout resulted in slow reversal of the H^+ current enhancement. In some experiments the arachidonate effect was fully reversible, but in others reversal was incomplete. The enhancement of H^+ currents was not detected at 5 μM arachidonate and was small at 20 μM, and increased with concentration up to 100 μM (the highest concentration tested). Human neutrophils produce O_2^- in response to arachidonic acid with similar sensitivity (Badwey et al., 1984; Maridonneau-Parini and Tauber, 1986; Axtell et al., 1990). Free arachidonic acid levels are low in most tissues, but can increase to >100 μM in involved epidermis of psoriasis (Hammarström et al., 1975), and 50–100 μM in activated pancreatic islet cells or platelets (Sumida et al., 1993).

Fig. 4*A* illustrates that the H^+ current during a pulse to +100 mV more than doubled after addition of 50 μM arachidonic acid to the bath. Similar to the control current, the current in the presence of arachidonate is initially quite small and increases slowly during the voltage pulse, suggesting that arachidonate amplifies the voltage- and time-dependent g_H rather than introducing a separate H^+ conductance mechanism, such as a protonophore. A protonophore would be expected to produce a time-independent g_H. The rate of rise of the current was increased by arachidonate at all potentials. No large changes in the V_{rev} were detected in the presence of arachidonate; however, if arachidonate induced an additional conductance which was time-independent, for example, tail current relaxations would appear unaltered. Given the small H^+ currents in most neutrophils, changes in V_{rev} of ~10 mV would not have been distinguished. There

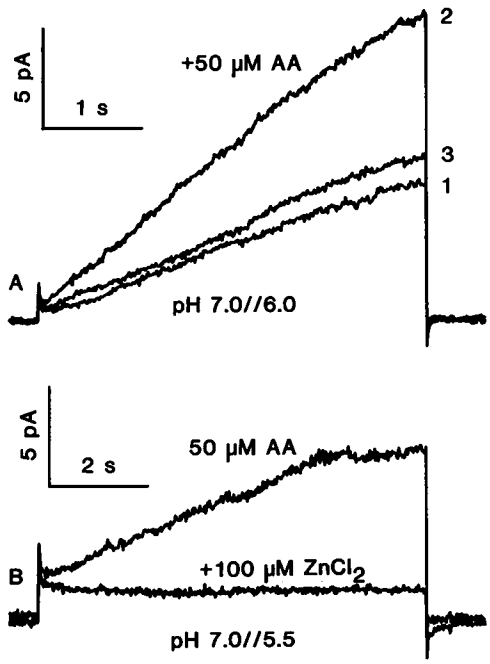

FIGURE 4 (*A*) Enhancement of the H^+ current by 50 μM arachidonic acid (*AA*). Superimposed are currents recorded during identical voltage pulses to +100 mV from a holding potential of −40 mV, applied immediately before (*trace 1*), 3 min after addition of arachidonate to the bath (*trace 2*), and after washout (*trace 3*). The arachidonate effect was maximal in this experiment at 3 min, and the H^+ current was not further increased after 8-min exposure. The bath contained pH 7.0 TEA methanesulfonate, and the bath temperature was 19.4–19.7°C. (*B*) The arachidonate-enhanced current is inhibited by $ZnCl_2$. The H^+ current during an 8-s pulse from −60 to +20 mV in the presence of 50 μM AA is shown, with the current at the same potential in the presence of both 50 μM AA and 100 μM $ZnCl_2$. The H^+ current at this potential before addition of AA was ~2 pA. The bath contained 145 mM TMA methanesulfonate and the pipette the *N*-methyl-D-glucamine solution described in Fig. 2 legend. Filter, 200 Hz; 20.0°C.

was no consistent effect of arachidonate on leak currents. In some experiments, especially after prolonged exposures, there was an irreversible increase in the holding current (i.e., leak). But as in the illustrated experiment, it was sometimes possible to observe substantial increases in H^+ currents without detectable effects on the membrane resistance at subthreshold potentials. The enhanced time- and voltage-dependent outward currents in the presence of arachidonic acid were inhibited by $CdCl_2$ and $ZnCl_2$, further supporting the interpretation that arachidonic acid modifies the g_H rather than introducing a new conductance. Fig. 4 *B* illustrates that the current at +20 mV in another experiment in the presence of 50 μM arachidonate was abolished by addition of 100 μM $ZnCl_2$ to the arachidonate-containing solution. The mechanism of inhibition was qualitatively like that in control cells in that the voltage-dependence of the g_H was shifted to more positive potentials.

The effects of arachidonic acid on the voltage-dependence of the g_H are illustrated in Fig. 5. Two families of H^+ currents were obtained in pH 7.0//6.0 solutions (labeled *1* and *2*). After addition of 50 μM arachidonate time-dependent H^+ currents were increased at all potentials (*3*), and activation

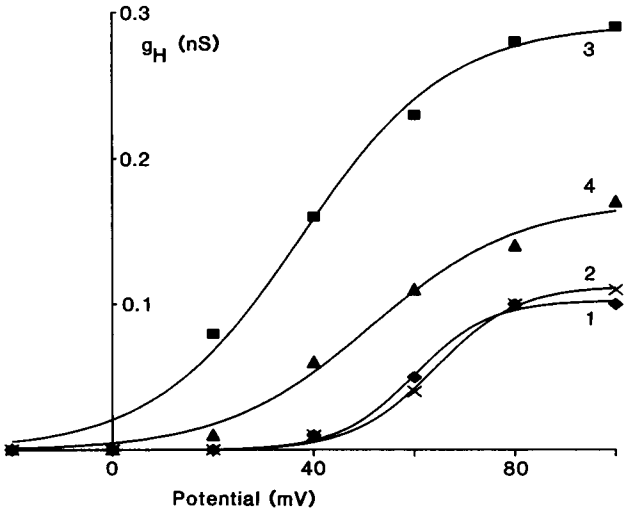

FIGURE 5 Effect of 50 μM arachidonic acid (*AA*) on the voltage dependence of the g_H in pH 7.0//6.0 TEA methanesulfonate solutions. H^+ currents were measured at the end of 4-s pulses from a holding potential of −40 mV, and chord conductance was calculated assuming a V_{rev} of −45 mV. Two control runs were made (labeled *1* and *2*), then 50 μM arachidonic acid was added (*3*), and then washed out (*4*). Curves illustrate fits of the data to a Boltzmann function: $g_H(V) = g_{H,max}/(1 + \exp((V - V_{½})/k))$, where $g_{H,max}$ is the limiting g_H, $V_{½}$ is the potential at which the g_H is half its maximum, and k is a slope factor. Fitted parameters are ($V_{½}$, k): 1 (+60 mV, −7.5 mV), 2 (+64 mV, −8.2 mV), 3 (+37 mV, −14.7 mV), 4 (+51 mV, −14.7 mV).

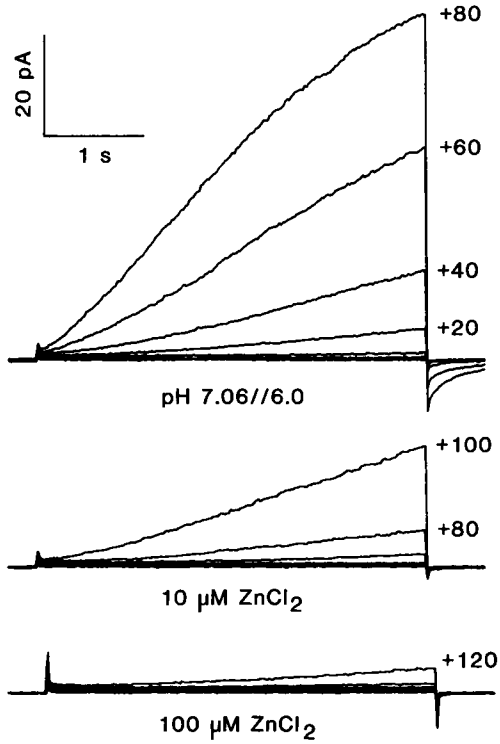

FIGURE 6 Inhibition of H^+ currents by $ZnCl_2$. A family of H^+ currents in pH_o 7.06 TEA methanesulfonate solution (*top panel*), and in the same cell in the presence of 10 μM $ZnCl_2$ (*middle panel*) or 100 μM $ZnCl_2$ (*lower panel*). Calibration bars apply to all families. The holding potential was −60 mV.

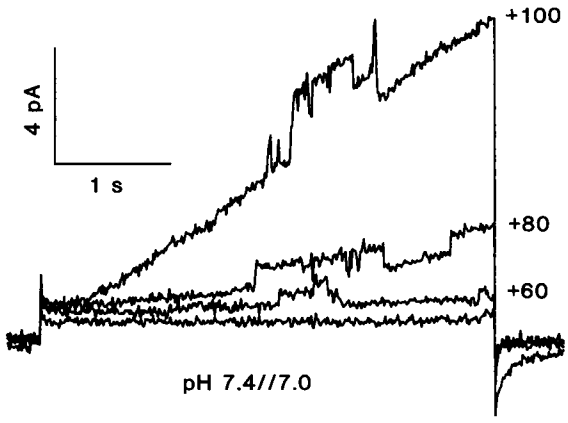

FIGURE 7 H^+ currents and Cl^- currents in a cell with Ringer's solution in the bath at pH_o 7.4 and with pH_i 7.0 (in mM: 73 PIPES, 73 TEA methanesulfonate, 12 EGTA, 1.9 $CaCl_2$, titrated to pH 7.01 with TEAOH) in the pipette. In addition to the slowly activating outward H^+ currents, single-channel outward currents can be seen. These unitary events are most likely Cl^- currents and are obvious here because the external solution contains Cl^-.

occurred at more negative potentials. The threshold typically shifted by ~20 mV to more negative potentials after addition of arachidonate. After washout (*4*) both the voltage shift and the current amplitude recovered partially. Similar effects on maximum current and voltage-dependence were also observed when arachidonate was applied in tetramethylammonium (TMA^+) methanesulfonate or Ringer's solution.

H^+ currents in neutrophils were inhibited by 10–100 μM $ZnCl_2$ or $CdCl_2$. A family of H^+ currents in pH 7.06//6.0 is shown in the top panel of Fig. 6. The g_H is just activated during a pulse to 0 mV. Addition of 10 μM $ZnCl_2$ (*middle panel*) inhibited the g_H, with more complete inhibition at 100 μM (*lower panel*). The effect of these divalent cations consists largely of a shift in the voltage-dependence of the g_H to more positive potentials. The g_H is first activated at +60 mV in 10 μM $ZnCl_2$, and at +100 mV in 100 μM. Whether the maximum g_H was reduced could not be determined, because the cells did not tolerate the extreme positive potentials required to answer this question. $CdCl_2$ was found to have similar effects. Thus, as in other cells (Thomas and Meech, 1982; Byerly et al., 1984; Barish and Baud, 1984; Byerly and Suen, 1989; Mahaut-Smith, 1989b; DeCoursey, 1991), inhibition of H^+ currents by divalent cations can be overcome by further depolarization.

H^+ currents could be detected under more nearly physiological ionic and pH conditions. A family of currents in Ringer's solution at pH_o 7.4 and with pH_i 7.0 is illustrated in Fig. 7. In other cells studied at these pH, H^+ currents were similarly small or not detectable, although even a moderate leak conductance could have obscured tiny H^+ currents. It would not be surprising if increasing pH_i from 6 to 7 reduced the amplitude of outward H^+ currents, which are already small in neutrophils at pH_i 6, due to the order-of-magnitude reduction of the ion species carrying the outward current. The voltage dependence of the g_H with pH_i 7.0 appears to be

shifted to more positive potentials compared with experiments with lower pH_i, consistent with the pattern seen at pH_i 5.5 and 6, although the small current amplitude makes quantitation only approximate.

Single-channel currents are apparent in Fig. 7 superimposed on the smoothly rising H^+ currents. These events most likely represent the opening of Cl^- channels. In most whole-cell experiments either macroscopic or single-channel Cl^- currents were observed. In some cells there was significant outward Cl^- current when the bath contained Cl^-, which was reduced but not abolished when Cl^- was replaced with $CH_3SO_3^-$. These residual anion currents were small and during pulses either decreased with time or were time-independent, so their contamination of the g_H comprised an increase in the instantaneous current during a voltage jump. Anion currents tended to disappear during experiments, presumably due to gradual depletion of Ca^{2+} from cellular stores, because the major Cl^- conductance in human neutrophils is Ca^{2+}-activated (Krause and Welsh, 1990). In many cells single-channel currents which underlie these macroscopic Cl^- currents were detected, usually early in the experiment. The open-channel (leak-corrected) current-voltage relation displayed marked outward rectification under conditions of Cl^- in the external solution and methanesulfonate in the pipette solution. The slope conductance at +100–200 mV was ~100 pS, and was reduced to ~20 pS when the Cl^- was replaced with methanesulfonate.

DISCUSSION

Evidence accumulated over the past several years has led to the proposal that H^+ selective ion channels are present in human neutrophils (Henderson et al., 1987, 1988a, 1988b; Nanda and Grinstein, 1991; Kapus et al., 1992; Henderson and Chappell, 1992). The g_H described here exhibits a number of properties which identify it as the mechanism which has been deduced in these studies. Because single-channel currents are too small to detect by direct measurement, it cannot be stated with certainty that the g_H is due to H^+-selective ion channels rather than to some other type of transport mechanism. The estimate of unitary conductance of 10 fS corresponds with a flux of ~6000 H^+/s through a channel with a 100-mV driving force, which is closer to the turnover rate of transporters than of ion channels, although few ion channels have been studied with micromolar concentrations of permeant ions. The voltage and time dependence of the g_H seem more consistent with the idea of a channel-like mechanism than of a pump, exchanger, or carrier. Pumps and exchangers seem unlikely mechanisms because H^+ currents persist in the ATP-free solutions and are not greatly affected when the logical co- or counter-ions, Cl^- and Na^+, are removed.

Seven properties of the g_H described here are consistent with its activation during O_2^- generation by human neutrophils:

1) The g_H is activated by depolarization. At negative potentials the g_H was undetectable and the whole-cell "leak" conductance was typically <10 pS, results consistent with the high membrane resistance and low g_H in unstimulated intact neutrophils (Simchowitz et al., 1982; Nanda and Grinstein, 1991; Henderson and Chappell, 1992). The membrane potential is at least transiently depolarized during activation of neutrophils (Whitin et al., 1980; Jones et al., 1980; Sklar et al., 1981; Kuroki et al., 1982; Sullivan et al., 1987; Henderson et al., 1987; Myers et al., 1990; Gallin, 1991), after which compensatory mechanisms result in repolarization. The depolarization is not affected by removal of Na^+ or by amiloride, indicating that the Na^+-H^+ antiporter is electroneutral and cannot restore the resting potential (Myers et al., 1990).

2) Raising pH_o greatly enhances the g_H by shifting its activation to more negative potentials. This shift is in the same direction observed for pH_o effects on a variety of voltage-dependent ion channels (Hille, 1992). This effect of pH_o may explain why (a) Cd^{2+} enhances the phorbol ester-induced depolarization of human neutrophils to a greater extent at high pH_o, and (b) a pH_o jump from 7.4 to 8.3 results in a Cd^{2+}-sensitive repolarization in phorbol-activated neutrophils (Henderson et al., 1987).

3) Lowering pH_i shifts activation of the g_H to more negative potentials. The initial decrease in pH_i during O_2^- generation (Grinstein and Furuya, 1986; Henderson et al., 1987, 1988a) would in itself tend to activate the g_H.

4) Arachidonate amplifies the voltage- and time-dependent g_H, consistent with its effects in intact neutrophils (Henderson and Chappell, 1992). Arachidonic acid is generated during neutrophil activation (Galbraith, 1988; Tao et al., 1989; Cockcroft and Stutchfield, 1989; Grimminger et al., 1991) and itself stimulates a variety of neutrophil responses, including O_2^- release (Badwey et al., 1984; Maridonneau-Parini and Tauber, 1986; Axtell et al., 1990).

5) H^+ currents do not inactivate. This property is essential for the proposed function of the g_H during O_2^- production by neutrophils, because sustained O_2^- production requires prolonged g_H elevation (Henderson et al., 1988b).

6) The g_H is large enough to completely dissipate the H^+ load during the respiratory burst. Activated human neutrophils generate 5–10 fmol of H^+/cell/min (Borregaard et al., 1984; Nanda and Grinstein, 1991), which corresponds with 8–16 pA of whole-cell H^+ current. The average maximum H^+ current recorded in human neutrophils is 35 pA at pH_i 6.0, and is likely smaller at pH_i 7, due to the lower permeant ion concentration. However, the H^+ current measurements likely underestimate the g_H, because the H^+ current was measured using pulses too brief (4–8 s) to achieve steady-state, the buffering capacity of cytoplasm, 28–50 mM/pH (Simchowitz and Roos, 1985; Grinstein and Furuya, 1986), is higher than that of the pipette solution, and the g_H would be substantially larger at body temperature than at 20°C. Scaled according to the Q_{10} of the g_H in snail neurons, 2.1 (Byerly and Suen, 1989), the maximum H^+ current in human neutrophils would be 124 pA at 37°C.

7) H^+ currents are inhibited by Cd^{2+} and Zn^{2+} at concentrations which enhance the depolarization and pH_i decrease in stimulated neutrophils (Henderson et al., 1987; Kapus et al., 1992), and which depress the respiratory burst (Henderson et al., 1988b).

Other membrane transporters which could influence pH_i in neutrophils include the Na^+-H^+ antiporter, the Cl^-/HCO_3^- exchanger (reviewed by Swallow et al., 1990), and possibly a H^+-ATPase (Nanda and Grinstein, 1991). A large body of evidence suggests that the activity of the Na^+-H^+ antiporter is increased in activated phagocytes, where it contributes to maintaining pH_i (Swallow et al., 1990). The H^+-ATPase in rat alveolar macrophages was recently proposed to play a larger role than the Na^+-H^+ antiporter in pH_i regulation during the respiratory burst (Murphy and Forman, 1993). As multiple membrane transporters capable of affecting pH_i are described, careful studies will have to be carried out to elucidate the situations in which each is involved.

In summary, human neutrophils express a highly H^+-selective conductance which is activated by depolarization, increased pH_o, and decreased pH_i, and is enhanced by arachidonate. The properties of this g_H appear ideally suited to mediating the conductive H^+ efflux which helps sustain the respiratory burst.

Note added in proof

A large g_H with similar properties has been reported in HL-60 cells (Demaurex et al., 1993, *J. Physiol. (Lond.)*. 466:329–344.

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