The gp91<sub>phox</sub> Component of NADPH Oxidase Is Not the Voltage-gated Proton Channel in Phagocytes, but It Helps*

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During the “respiratory burst,” the NADPH oxidase complex of phagocytes produces reactive oxygen species that kill bacteria and other invaders (Babior, B. M. (1999) Blood 93, 1464–1476). Electron efflux through NADPH oxidase is electrically dissipating (Henderson, L. M., Chappell, J. B., and Jones, O. T. G. (1987) Biochem. J. 246, 325–329) and is compensated by H<sup>+</sup> efflux through proton channels that reportedly are contained within the gp91<sub>phox</sub> subunit of NADPH oxidase. To test whether gp91<sub>phox</sub> functions as a proton channel, we studied H<sup>+</sup> currents in granulocytes from X-linked chronic granulomatous disease patients lacking gp91<sub>phox</sub> (X-CGD), the human myelocytic PLB-985 cell line, PLB-985 cells in which gp91<sub>phox</sub> was knocked out by gene targeting (PLB<sub>KO</sub>), and PLB-985 knockout cells re-transfected with gp91<sub>phox</sub> (PLB<sub>B91</sub>). H<sup>+</sup> currents in unstimulated PLB<sub>KO</sub> cells had amplitude and gating kinetics similar to PLB<sub>B91</sub> cells. Furthermore, stimulation of the phorbol ester phorbolester 12-myristate-13-acetate increased H<sup>+</sup> currents to a similar extent in X-CGD, PLB<sub>KO</sub>, and PLB<sub>B91</sub> cells. Thus, gp91<sub>phox</sub> is not the proton channel in unstimulated phagocytes and does not directly mediate the increase of proton conductance during the respiratory burst. Changes in H<sup>+</sup> channel gating kinetics during NADPH oxidase activity are likely crucial to the activation of H<sup>+</sup> flux during the respiratory burst.

A voltage-gated proton conductance is activated during the respiratory burst in human neutrophils (1–6). The resulting H<sup>+</sup> efflux compensates for the electric action of NADPH oxidase (1). Several lines of evidence have suggested that the gp91<sub>phox</sub> component of the NADPH oxidase complex might be the proton channel that is activated during the respiratory burst (2, 3, 7, 8). The presence of H<sup>+</sup> currents in granulocytes from gp91<sub>phox</sub>-deficient CGD patients appeared to refute this idea (9), but Henderson and Chappell (10) argued that these data were inconclusive. Furthermore, it has been reported that heterologous expression of gp91<sub>phox</sub> results in the appearance of proton fluxes or proton currents resembling those activated during the respiratory burst (8, 11–15). The expression systems employed to date provide ambiguous results, because CHO and HEK-293 cells express endogenous voltage-gated proton channels (14–17) and mRNA for gp91<sub>phox</sub> and four gp91<sub>phox</sub> homologs have been detected by reverse transcriptase polymerase chain reaction in HEK-293 cells (18). An increase in H<sup>+</sup> currents after transfection might reflect expression of channels formed by the transfected gene product but could simply reflect up-regulation of constitutively expressed H<sup>+</sup> channels. It is also possible that expression of gp91<sub>phox</sub> in a background lacking p22<sub>phox</sub> might induce non-physiological behavior that is not exhibited in phagocytes. The stability of gp91<sub>phox</sub> and p22<sub>phox</sub> expression in phagocytes is enhanced by the formation of heterodimers of these two components of flavocytochrome b<sub>558</sub> (19, 20). We therefore studied stable PLB-985 cell lines with gp91<sub>phox</sub> genetically knocked-out and with gp91<sub>phox</sub> re-expressed in the same background (21).

EXPERIMENTAL PROCEDURES

Cells—The PLB-985-derived cell lines were developed by Dinauer and colleagues (21). Wild-type PLB-985 cells (PLB<sub>WT</sub>), PLB<sub>KO</sub> (PLB-985 cells genetically knocked-out and with gp91<sub>phox</sub> cDNA) were all induced by incubation with 0.5% N,N-dimethylformamide (DMF, Sigma) for 4–7 days. Some whole-cell studies were done on PLB<sub>KO</sub> cells before DMF induction, designated PLB<sub>KO</sub><sup>*</sup>. The absence of gp91<sub>phox</sub> expression in the PLB<sub>KO</sub> granulocytes is well documented (20–23). X-CGD granulocytes (mainly neutrophils) were isolated by density gradient centrifugation as described (24) from three patients with CGD, all of whom had documented absent neutrophil superoxide production and mutations that would prevent stable expression of gp91<sub>phox</sub> (25). The specific mutations were (a) Cys<sup>1347</sup> → Ala in exon 11, changing the codon for Cys<sup>1347</sup> to a premature STOP codon (b) deletion of Cys<sup>1106</sup> in exon 9, leading to a frameshift after Pro and a premature STOP codon after Gly<sup>1106</sup> in exon 3, leading to a frameshift after Leu and a premature STOP codon in exon 5. In patient c, the absence of cytochrome b<sub>558</sub> was demonstrated spectrophotometrically in neutrophil extracts. Blood from patient c was refrigerated overnight before use, and most surviving granulocytes were identified as eosinophilic in a Wright-stained cytopsin preparation.

Electrophysiology—Whole-cell and permesibilized patch voltage-clamp recordings were done as described (26, 27) with micropipettes pulled from 7052 glass (Garner Glass). Whole-cell solutions (pipette and bath) included 100 mM KCl buffer near its pK<sub>i</sub> and tetramethylammonium<sup>+</sup> and methanesulfonate<sup>−</sup> as the main ions, 1 mM EGTA, and 1–2 mM

1 The abbreviations used are: CGD, chronic granulomatous disease; DMF, N,N-dimethylformamide; gp91<sub>phox</sub> proton conductance; I<sub>H</sub>, H<sup>+</sup> current amplitude; pH<sub>i</sub>, intracellular pH; pH<sub>e</sub>, extracellular pH; PLB<sub>KO</sub>, PLB-985 cells with gp91<sub>phox</sub> knocked out by gene targeting; PLB<sub>B91</sub>, PLB-985 knockout cells before induction with DMF; PLB<sub>B91</sub><sup>ko</sup>, PLB-985 knockout cells with gp91<sub>phox</sub> restored; PMA, phorbol 12-myristate-13-acetate; τ<sub>act</sub>, time constant of H<sup>+</sup> current activation; τ<sub>rec</sub>, time constant of H<sup>+</sup> channel closing (tail current decay); V<sub>1/2</sub>, threshold to 50% activation; X-CGD, X-linked chronic granulomatous disease; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PLB<sub>WT</sub>, wild-type PLB-985 cells; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid.
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Fig. 1. Whole-cell proton currents do not require gp91<sub>phox</sub>. Families of currents in PLB<sub>WT</sub> (A and B), PLB<sub>91</sub> (C and D), and PLB<sub>KO</sub> cells (E and F) in whole-cell configuration at pH 7.0 (A, C, and E) and pH 5.5 (B, D, and F), all with pipette pH 5.5. Currents are in 20-mV increments, from a holding potential of −60 mV (A, C, E, and F), −40 nV (D), or −20 mV (B). The capacities were 6, 5, and 8.1 pF, respectively. Pulse duration was adjusted to minimize pH depletion due to H<sup>+</sup> efflux.

CaCl<sub>2</sub> or MgCl<sub>2</sub>. For permeabilized patch recording, all solutions contained 50 mM NH<sub>4</sub><sup>+</sup>, 2 mM MgCl<sub>2</sub>, 5 mM BES, 1 mM EGTA, titrated to pH 7.0 with tetramethylammonium hydroxide. The symmetrical NH<sub>4</sub><sup>+</sup>-clamped pH near 7.0 (22). Currents are shown without correction for leak or liquid junction potentials. Data were collected at 20–21 °C or at room temperature.

RESULTS

PLB<sub>KO</sub> Cells, Which Lack the gp91<sub>phox</sub> Protein (21, 22), Express Large Voltage-gated Proton Currents—PLB-985 cells induced by DMF to granulocyte differentiation express all NADPH oxidase components and are capable of a respiratory burst (21). PLB<sub>WT</sub> cells had large voltage-gated proton currents (Fig. 1, A and B) that resemble those in other phagocytes and related cells (17). Proton currents in DMF-induced PLB<sub>91</sub> cells (Fig. 1, C and D) were similar to those in DMF-induced PLB<sub>WT</sub> cells, as expected. PLB<sub>KO</sub> cells, which do not express gp91<sub>phox</sub> protein (21, 22), also had large H<sup>+</sup> currents both before (Fig. 1, E and F) and after induction with DMF. These results demonstrate unequivocally that gp91<sub>phox</sub> is not the voltage-gated proton channel in unstimulated phagocytes.

The Selectivity and Gating Kinetics of Voltage-gated Proton Channels Are Identical Regardless of Whether gp91<sub>phox</sub> Is Present—To explore whether expression of gp91<sub>phox</sub> might alter the properties of H<sup>+</sup> channels, we characterized the H<sup>+</sup> currents thoroughly. Tail currents reversed near the Nernst potential for H<sup>+</sup> in the three PLB lines (Fig. 2A), confirming that protons carry these currents. The slope of the data is 51.8 mV/Unit pH, which is close to the 58.2 mV given by the Nernst equation. The largest deviation from the Nernst prediction indicates that H<sup>+</sup> is >10<sup>6</sup> more permeant than tetramethylammonium<sup>+</sup>, the main cation present. Like other H<sup>+</sup> channels (17), those in PLB cells are essentially perfectly H<sup>+</sup>-selective.

The voltage dependence of H<sup>+</sup> current activation was very similar in PLB<sub>WT</sub>, PLB<sub>91</sub>, and PLB<sub>KO</sub> cells, as evident in average H<sup>+</sup> chord-conductance voltage (g<sub>H</sub>) data (Fig. 2B). H<sup>+</sup> currents in PLB knockout cells studied before (PLB<sub>KO</sub>) and after induction with DMF (PLB<sub>KO</sub>) were identical. The effects of changing pH from 7.0 (Fig. 1, A, C, and E) to 5.5 (Fig. 1, B, D, and F) were similar in all cell types and to effects reported previously (17, 28).

The voltage dependence of H<sup>+</sup> activation (g<sub>H</sub>) is the Nernst potential for H<sup>+</sup> (V<sub>0</sub>) data: g<sub>H</sub>[max] = [1 + exp((V<sub>0</sub> − V)/kT)]<sup>-1</sup>, with fitted parameters k = 1.97, 2.94, 3.22, and 2.86 nano Siemens; V<sub>0</sub> = −9.2, −9.8, −6.2, and −12.4 mV; and k = −10.3, −10.4, −10.3, and −11.5 mV for PLB<sub>WT</sub>, PLB<sub>91</sub>, PLB<sub>KO</sub>, and PLB<sub>KO</sub>, respectively. C, average tail measured at pH 7.0 and pH 5.5 (symbols defined in B). The average slope is 54 mV/10<sup>−6</sup> change in tail. Average tail from single exponential fits measured at pH 7.0 and pH 5.5 (symbols defined in B). The average slope is 41 mV/10<sup>−6</sup> change in tail. Data are from four to six cells for each set in B–D.
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The X-CGD cells had normal or larger than normal H+ currents, and their response to PMA was similar to that of PLBKO cells. Although the mean change in $\tau_{act}$ was larger in PLBKO than PLB91 or X-CGD cells, our exclusion from analysis of PLB91 cells without electron currents may account for this difference, because this criterion could not be used to exclude non-responding PLBKO or X-CGD cells. The PMA-induced changes in H+ currents in the eight PLB91 cells with no detectable electron currents (not shown) were identical to those in PLBKO cells. Because $I_H$ increased to a similar extent in PLBKO, X-CGD, PLB91 cells, and human neutrophils, the increased $g_h$ during the respiratory burst (1, 2, 4, 5) is not because of the appearance of proton currents conducted through the gp91phox molecule.

**PMA Elicits Fewer Changes in Gating Kinetics of Proton Channels in gp91phox-deficient Cells**—Although $I_H$ increased to the same extent after PMA stimulation, the response of H+ currents to PMA was different in cells expressing or lacking gp91phox. The slowing of $\tau_{tail}$ and large hyperpolarizing shift of $V_{threshold}$ were not observed in PLBKO or X-CGD cells (Fig. 3C). The slowing of $\tau_{tail}$ was less pronounced in PLB91 cells than in neutrophils and eosinophils. In most cells there was a distinct but relatively subtle slowing. The hyperpolarizing voltage shift was almost as large in PLB92 cells (−32 mV) as in neutrophils (−39 mV) and in eosinophils (−43 mV) stimulated with PMA under similar conditions (24, 27). This voltage shift was sufficient to result in the appearance of inward H+ currents in some cells, a hallmark property of the NADPH oxidase-related H+ channel (29).

**DISCUSSION**

The presence of robust H+ currents in PLBKO cells demonstrates unequivocally that the voltage-gated proton channel in unstimulated phagocytes is not gp91phox nor does it require gp91phox expression. Similarly, granulocytes (this study) or monocytes (9) from CGD patients lacking gp91phox exhibit normal levels of H+ currents. Furthermore, genetic knockout of gp91phox did not detectably alter the amplitude or behavior of whole-cell H+ currents. Voltage-gated proton channels in whole-cell studies of unstimulated phagocytes function independently of gp91phox.

Bánfi et al. (29) proposed that there were two types of H+ channels in eosinophils, one in resting cells and a novel variety that is observed only under conditions that permit NADPH oxidase function. This novel channel reportedly differs from that in resting cells in (a) activating at more negative voltages, (b) activating more rapidly, (c) deactivating more slowly, and (d) being more sensitive to inhibition by Zn2+. We observed novel H+ channel gating behavior during NADPH oxidase function in human neutrophils and eosinophils stimulated with PMA or arachidonic acid in permeabilized patch studies (24, 27, 31). However, we saw no evidence of multiple kinetic components in stimulated phagocytes, no correlation between the amplitude of the NADPH oxidase-generated electron currents and the amplitude of PMA-activated H+ currents (27), and identical Zn2+ sensitivity of H+ currents in resting and activated cells displaying both types of channel behavior (24). We conclude that there is one type of H+ channel in phagocytes, whose properties are greatly altered during the respiratory burst.

Here we examined whether the increased H+ conductance in stimulated cells is because of the appearance of additional channels formed by gp91phox. PMA stimulation clearly increased $I_H$ in cells that lack gp91phox (PLBKO and X-CGD). This increase was not statistically different from that in cells expressing gp91phox (PLB91 and neutrophils). If a small gp91phox-mediated H+ conductance were also activated in neutrophils...
and PLB_{91} cells, it could be only a small fraction of the total $g_H$. It is conceivable that under some conditions, such as heterologous expression in non-phagocytes, gp91^{phox} might function as a proton channel, but the evidence presented here indicates that it does not contribute significantly to the total proton conductance of phagocytes.

In CHO cells transfected with gp91^{phox}, arachidonic acid stimulated larger proton fluxes than in control cells (8, 11, 12). Although suggestive of enhanced H$^+$ channel activity, these measurements are indirect. It is difficult to determine which part of these H$^+$ fluxes was mediated by H$^+$ channels, because suppression of flux by the H$^+$ channel inhibitor Zn$^{2+}$ was not demonstrated. Patch-clamp studies of CHO cells transfected with gp91^{phox} (13) reveal a large conductance with properties fundamentally different from H$^+$ channels in native cells. A quintessential feature of H$^+$ channels is poten inhibition by Zn$^{2+}$, which slows $\tau_{act}$ (17, 26). The conductance in CHO cells was weakly inhibited by Zn$^{2+}$, and no slowing of activation was evident at 200 $\mu M$ Zn$^{2+}$ (13), whereas even 1 $\mu M$ Zn$^{2+}$ slows $\tau_{act}$ 3–10-fold in cells expressing voltage-gated proton channels (24, 26). In all cells with H$^+$ channels, increasing pH$_i$ shifts the voltage-activation curve by ~40 mV/unit pH (16, 17, 28). In contrast, the conductance in CHO cells was activated at ~20 mV at pH$_i$ 6.9, but no H$^+$ current was seen at pH$_i$ 7.5 at voltages up to +140 mV (13). The failure to see H$^+$ current at pH$_i$ 7.5 is especially surprising, because the currents at pH$_i$ 6.9 are an order of magnitude greater than in any mammalian cell. Finally, the outward currents in CHO cells activate anomalously rapidly, within ~100 ms, whereas $\tau_{act}$ for phagocyte H$^+$ channels is typically seconds (9, 17, 24, 27, 29, 31). It was reported recently that transient gp91^{phox} expression in COS-7 cells results in voltage-gated proton currents (15). However, the currents shown appear to reverse roughly near 0 mV at pH$_o$ 7.5 and pH$_i$ 5.7, where the Nernst potential for H$^+$ is ~105 mV; thus, this conductance is not H$^+$-selective. Evidently, expression of gp91^{phox} in alien cell lines can induce novel conductances that differ markedly from H$^+$ currents in resting or activated phagocytes or any cell studied to date.

The gating kinetics of H$^+$ channels responds differently to PMA in cells lacking gp91^{phox}. Although it is possible that gp91^{phox} itself modulates H$^+$ channels, we propose that these modulations of H$^+$ channel function occur only in the presence of a functioning NADPH oxidase complex. The properties that are influenced by NADPH oxidase function, slower $\tau_{tail}$ and hyperpolarization of the $g_H$-V relationship, promote activation of the $g_H$ at membrane potentials that might occur in intact phagocytes. The alterations in H$^+$ channel gating during NADPH oxidase activity probably contribute more to activating H$^+$ flux during the respiratory burst than does the increase in $g_H$. 

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