Role of nucleotides and phosphoinositides in the stability of electron and proton currents associated with the phagocytic NADPH oxidase

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The phagocytic NADPH oxidase (phox) moves electrons across cell membranes to kill microbes. The activity of this lethal enzyme is tightly regulated, but the mechanisms that control phox inactivation are poorly understood for lack of appropriate assays. The phox generates measurable electron currents, \( I_e \), that are associated with inward proton currents, \( I_H \). To study the inactivation of the phox and of its associated proton channel, we determined which soluble factors can stabilize \( I_e \) (induced by the addition of NADPH) and \( I_H \) (initiated by small depolarizing voltage steps) in inside-out patches from PMA-activated human eosinophils. \( I_e \) decayed rapidly in the absence of nucleotides (\( r \approx 6 \) min) and was maximally stabilized by the combined addition of 5 mM ATP and 50 \( \mu \)M of the non-hydrolysable GTP analogue GTP[S] (guanosine 5'-[\( \gamma \)-thio]triphosphate) (\( r \approx 57 \) min), but not by either ATP or GTP[S] alone. \( I_H \) also decayed rapidly and was stabilized by the ATP/GTP[S] mixture, but maximal stabilization of \( I_H \) required further addition of 25 \( \mu \)M PI(3,4)P_2 (phosphoinositide 3,4-bisphosphate) to the cytosolic side of the patch. PI(3,4)P_2 had no effect on \( I_e \), and its stabilizing effect on \( I_H \) could not be mimicked by other phosphoinositides. Reducing the ATP concentration below millimolar levels decreased \( I_H \) stability, an effect that was not prevented by phosphatase inhibitors but by the non-hydrolysable ATP analogue ATP[S] (adenosine 5'-[\( \gamma \)-thio]triphosphate). Our data indicate that the assembled phox complex is very stable in eosinophil membranes if both ATP and GTP[S] are present, but inactivates within minutes if one of the nucleotides is removed. Stabilization of the phox-associated proton channel in a highly voltage-sensitive conformation does not appear to involve phosphorylation but ATP binding, and requires not only ATP and GTP[S] but also PI(3,4)P_2, a protein known to anchor the cytosolic phox subunit p47phox to the plasma membrane.

Key words: cytochrome, eosinophil, NADPH oxidase, patch-clamp, proton channel, phosphoinositide.

INTRODUCTION

The phagocyte NADPH oxidase (phox) is a transmembrane enzyme complex that translocates electrons from cytosolic NADPH to extracellular oxygen, generating superoxide anions used to kill bacteria. The activity of this enzyme is crucial for efficient killing of invading micro-organisms during phagocytosis, and individuals carrying a loss-of-function mutation in one of the main subunits of the complex suffer from severe recurrent bacterial and fungal infections, a condition known as CGD (chronic granulomatous disease). The core subunit of the oxidase is a transmembrane flavocytochrome composed of two proteins, the glycoprotein gp91phox, which form a heterotrimeric complex that contains the electron transport chain, and p22phox. Activation of the oxidase requires the membrane translocation of the small G-protein Rac and of three cytosolic subunits, p67phox, p47phox and p40phox, which form a heterotrimeric complex that can be extensively phosphorylated on p47phox. Upon phosphorylation, the ‘organizer’ p47phox unfolds from an auto-inhibitory conformation and exposes a lipid-binding PX domain (Phox homology domain) as well as SH3 (Src homology 3) domains, recruiting the ‘activator’ p67phox and the small G-protein Rac2 to the plasma or phagosomal membrane where the cytochrome is located (for recent reviews on phox activation see [1,2]). Loss-of-function mutations in gp91phox, p22phox, p67phox, p47phox and Rac cause CGD, indicating that these five proteins are absolutely required for the proper assembly and function of the enzyme [1,2].

The phox is electrogenic and its sustained activity requires a compensating charge. A large body of evidence indicates that the charge is provided by protons flowing through voltage-gated proton channels [3,4], reviewed in [5], but it has also been suggested that K⁺ ions can provide the compensating charge and mediate bacterial killing by releasing bactericidal enzymes from the phagolysosome matrix [6,7]. The phox is functionally linked to the activity of voltage-gated proton channels [8], and phox activation induces a large (40–60 mV) negative shift in the voltage dependence of proton currents [9]. The large shift in voltage dependence allows the influx of H⁺ across proton channels, which are otherwise strictly outward rectifying [10]. The voltage shift requires phox pre-activation and is more pronounced in cells dialysed with GTP[S] (guanosine 5'-[\( \gamma \)-thio]triphosphate) and NADPH in the whole-cell configuration than in cells recorded in the perforated patch configuration, which retain an intact cytosol [11]. The shift is absent from phagocytes from CGD patients lacking gp91phox or p47phox but can be observed in the absence of oxygen and in the presence of the oxidase inhibitor DPI (diphenyleneiodonium), indicating that channel modulation requires phox assembly, but not its redox function [9].

The voltage-gated proton channel was originally suggested to be contained within the phox complex [3] and expression of gp91phox alone is sufficient to generate voltage-gated proton currents in multiple expression systems [12–14]. Whether the phox is a proton channel or a proton channel modulator remains controversial however, because residual proton currents persist in

Abbreviations used: ATP[S], adenosine 5'-[\( \gamma \)-thio]triphosphate; CGD, chronic granulomatous disease; DPI, diphenyleneiodonium; GAP, GTPase-activating protein; GTP[S], guanosine 5'-[\( \gamma \)-thio]triphosphate; Hv1/VTSP, voltage-gated hydrogen channel 1/voltage sensor domain-only protein; PI(3,4)P_2, phosphoinositide 3,4-bisphosphate; PI(3,4,5)P_3, phosphoinositide 3,4,5-trisphosphate; PX domain, phox homology domain.

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cells lacking phox [15] and because one phox-expressing cell line failed to generate proton currents [16]. Recently, very large proton currents were observed upon expression of the ‘voltage-sensing only’ proteins Hv1 (voltage-gated hydrogen channel 1)/VSOP (voltage sensor domain-only protein), the structure of which resembles the voltage sensor domain of K$_v$ channels [17,18]. This suggests that Hv1/VsOP might be the long sought-after proton channel, but the available data are equally compatible with Hv1/VsOP functioning as a voltage sensor within a proton channel complex, because (i) the Hv1/VsOP-induced currents were measured in cells that express the phox homologue NOX4 [19] and (ii) the putative proton pathway could not be mapped by mutagenesis in Hv1/VsOP [17,18]. Interestingly, inward $I_H$ were recorded when a critical arginine residue was replaced within Hv1/VsOP [17], suggesting that modulation of Hv1/VsOP could account for the inward $I_H$ observed in cells with an active oxidase.

The activity of the phox is tightly regulated, because the superoxide anions generated by the active enzyme are highly reactive and potentially harmful to the host cell(s). Although tremendous efforts have been focused on the mechanisms involved in phox activation, the factors that maintain the complex active and control its inactivation are poorly understood. This lack of knowledge is partially explained by the lack of appropriate methods. Most of the data available to date were obtained either in cell-free systems or in intact cells stimulated with agonists or phorbol esters, by measuring the consumption of oxygen or the production of superoxide. These assays do not provide time-resolved measurements of the oxidase activity and do not discriminate between factors that control phox assembly, catalytic activity and inactivation (reviewed in [20]). The redox activity of the oxidase can be measured directly by recording electron currents from single cells or from membrane patches excised from stimulated cells [21,22]. This last approach is very powerful, because it allows one to study in real time the changes in activity of the oxidase and of its associated proton channel [23]. Because of the large negative shift in voltage-dependent activation, the proton current associated with an active oxidase can be isolated as inward proton current, $I_H$, and the amplitude of $I_H$ correlates well with the amplitude of the electron current recorded in the same patch [22]. Unlike cell-free systems, excised patches contain the native phox complex that has been pre-assembled in its original membrane, and only lacks small diffusible molecules required for phox activity. The inside-out configuration also allows us to discriminate between compounds acting from the cytosolic or extracellular side of the membrane.

We previously showed that the amplitude of $I_e$ and $I_H$ co-distribute in excised patches, and that both currents are stabilized by the addition of micromolar concentrations of GTP[S] and millimolar concentrations of ATP to the cytosolic (bath) side of the patch [22]. Here we study systematically the factors that control the stability of $I_e$ and $I_H$ in patches excised from human eosinophils.

**EXPERIMENTAL**

**Cell preparation and culture**

Preparation of human eosinophils was performed as previously described [22] except for minor modifications, as follows. Cells were prepared from venous blood drawn from non-atopic, healthy adult men after obtaining their informed consent. Cells were kept at 4°C in an airtight, closed, polypropylene Eppendorf tube (Trefil) at approx. 500 cells/µl density in culture media (composition given below). Eosinophils stored this way were able to respond to PMA and produced electron current in inside-out patches even after 96 h of storage. Studies conformed to the standards set by the Declaration of Helsinki, and the procedures have been approved by the ethics committee of the University of Geneva.

**Patch-clamp measurements**

Cells were deposited on a glass coverslip attached to the bottom of a 0.5-mm-thick polypropylene disc placed on an inverted microscope (Axiovert 10, Zeiss). A rhombus-shaped hole (9 mm × 10 mm) in the middle of the disc served as a recording chamber. After patch excision, the pipette electrode tip was positioned in the centre of the chamber, within 150 µm of the bottom. The initial volume of the bath solution was 50 µl and drugs were added directly into the bath and thoroughly mixed (pipetting five times with a 20 µl pipette). Drug applications caused at most a 3% cumulative error (dilution) in the concentration of purine nucleotides or phosphoinositides. Experiments were finished within 40 min after the chamber was filled with the bath solution, and the chamber was surrounded by wet tissue to avoid significant evaporation. Voltage-clamp recordings were performed with an Axopatch-1D patch-clamp amplifier (Axon Instruments) equipped with a CV-4-1/100U headstage (50 GΩ feedback resistor). Pipettes were pulled from borosilicate glass tubing (type GC150F-10; Harvard Apparatus) using a P-87 puller (Sutter Instrument Co.). After fire polishing, the pipette resistance was 7–13 MΩ when filled with the recording solution. The bath was grounded using an Ag/AgCl pellet. Current signals were low-pass filtered at 20–50 Hz (−3 dB, 8-pole Bessel filter) and sampled at 100 Hz. For data analysis and Figure preparation, software-based (pClamp 8, Axon Instruments) noise reduction was performed offline (simulating an 8-pole Bessel filter). Traces in Figures are low-pass filtered offline at 0.5–1 Hz (−3 dB) and re-sampled at 4 Hz. Data acquisition was performed using the pClamp 6 software and pClamp 8 was used for data analysis. Online compensation for the full electrode capacitance was not performed.

**Solutions**

The cell storage medium was a 2:1 (v/v) mixture of Medium 199 with Earl’s, L-glutamine and L-amino acids containing 25 mM Hesper (Gibco™ Invitrogen) and L-glutamine-free RPMI 1640 medium (Gibco™) supplemented with 5 mM Na$_2$EDTA and 2% (v/v) fetal calf serum (BioConcept). The recording solutions contained (mM): CsCl 1, tetrathyrammonium chloride 1, MgCl$_2$ 2, EGTA 1, N-methyl-d-glucamine base 101 and either 200 MES (pH 6.15) or 200 Hesper (pH 7.55). To establish a pH 7.05 solution, pH 7.55 and pH 6.15 solutions were mixed at a volume ratio of 3:2 (pH 7.55 versus 6.15 respectively). For electron current measurements at 0 mV the pipette solution (pH 7.05) was supplemented with 2 mM ZnCl$_2$ (free [Zn$^{2+}$] ≈1 mM), ATP[S] (adenosine $5'$$\gamma$-thio)triphosphate), GDP, GTP and GTP[S] were dissolved in water at 10 mM, PMA was dissolved in DMSO at 1 mM. Okadaic acid and cyclosporin A were dissolved in DMSO at 100 µM, DiC$_8$-phosphoinositides (Echelon), MgATP and Na$_2$NADPH were dissolved in pH 7.55 solution to give a stock solution of 1.25, 30 and 80 µM respectively. Na$_2$EDTA was dissolved in water at 250 mM. Chemicals were obtained from Sigma–Aldrich unless otherwise specified. All manipulations were performed at room temperature (22–26°C).

**Conventions**

The sign of the originally recorded inside-out patch current signal is inverted in the Figures, and positive values indicate outward current to comply with classical whole-cell experiments.
Data analysis

The number of cells (n) indicates the cumulative number of measurements performed on at least two independent cell prepa-

results are presented as means ± S.E.M. For statistical analy-

sis the non-parametric Mann–Whitney test was applied us-

ing the Statistica software (version 4.5; Statsoft). A value of

P < 0.05 was considered statistically significant. Exponential fits

were calculated with the built-in algorithm of the pClamp 8

software, using the sum of squared error minimization method.

The amplitudes of electron and proton currents at infinite time

points were fixed to 0 pA, and currents smaller than 0.5 pA were

excluded from analysis. Four patches had no measurable proton

current run-down and their decay time constant was set to the

largest measured value (116 min) to include these recordings in

the analysis.

RESULTS

Effects of nucleotides on the stability of electron currents in

inside-out patches

To study the effects of nucleotides on phox stability we recorded

Ie in inside-out patches from human eosinophils treated with

PMA (200–400 nM for 10–15 min). Ie was recorded at a constant

voltage of 0 mV, and 1 mM Zn2+ was added to the pipette

solution to block proton currents [24]. Figure 1(A) shows a typical

recording of Ie evoked by bath application of 0.8 mM NADPH, a

near saturating concentration (∼90%, [24]), to a patch excised

into a nucleotide-free recording buffer. Although PMA induces

a practically irreversible stimulation of oxidase activity in intact

phagocytes [20], Ie ran down within minutes after the addition

of NADPH when no nucleotide was present in the recording buffer.

The time course of Ie was biphasic, with an activation phase

followed by a short period of peak current amplitude lasting up to

170 s after NADPH addition (∼50 s on average) and a subsequent

exponential decline the time constant (τ) which averaged 5.6 ±

1.7 min (n = 10, Figures 1B and 1C, control). This indicates

that electron transport by the phox inactivates or deactivates

spontaneously in the absence of small cytosolic molecules. To
determine if ATP- or GTP-binding proteins are involved in this

deactivation process, we added ATP, GDP and GTP[S] to the bath

solution. As shown in Figure 1(C), Ie decay was not significantly

reduced by the addition of 5 mM ATP or 50 μM GTP[S] to the

bath solution. When ATP was applied along with GDP, the
duration of the activation phase was increased up to 455 s (∼215 s

on average), but the time-constant of the subsequent exponential
decay was not significantly reduced (Figure 1C). Remarkably,

when the patch was excised into a bath solution that contained

a combination of 5 mM ATP and 50 μM GTP[S], the run-down

was minimal and stable electron currents could be recorded for

more than 30 min (Figures 1B and 1C, τ = 57 ± 6 min, n = 11,

P < 0.0001). Increasing the GTP[S] concentration to 250 μM

reduced the current stability (20.0 ± 4.0 min, n = 9, P < 0.002),

while decreasing the ATP concentration from 5 to 0.5 mM in the

presence of 50 μM GTP[S] had a slight, but significant, effect on

current stability (τ = 35.8 ± 6.5 min, n = 7, P < 0.05).

To verify that Ie decline was not due to the presence of Zn2+ in

the pipette solution, we recorded Ie in the absence of Zn2+ at

−60 mV, below the threshold potential of H+ channels. The

electron current declined more slowly at −60 mV than at 0 mV

under all conditions (results not shown), but patches tended to

be less stable and the kinetics were complicated by a slow but

progressive decline in seal quality. Importantly, the combina-

tion of ATP and GTP[S] also stabilized Ie recorded at −60 mV. These

results indicate that the combination of ATP and of the non-

hydrolysable GTP analogue GTP[S] greatly stabilizes electron

currents in excised patches.

Effects of nucleotides on the stability of voltage-activated

proton currents

To evaluate whether similar mechanisms regulate the inactivation

of the phox and of its associated proton channel, we analysed

Figure 1 Effects of nucleotides on the stability of electron currents in inside-out patches

Inside-out patches were excised into a NADPH-free bath solution and electron currents were

evoked by the addition of 0.8 mM NADPH to the bath, at a constant holding voltage of 0 mV.

The pipette contained 1 mM Zn2+ to block proton currents. (A) Original current recording from

a representative patch. The electron current (Ie) decayed within minutes after the application of

NADPH. (B) Normalized currents recorded in a nucleotide-free bath solution (control) or in a bath

solution containing 5 mM ATP and 50 μM GTP[S] (GTP[S]). The currents were normalized to

the peak inward current recorded within 2 min of NADPH application. The combination of

ATP and GTP[S] greatly stabilized Ie. (C) Effects of ATP, GDP, GTP[S] and ATP + GTP[S] on the

time constant of Ie decay. The current traces were fitted with a single exponential decay function

(n = 10 for control and n = 8 for each of the other conditions). Only the combination of ATP

and GTP[S] significantly stabilized electron currents.

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The activity of several ion channels and transporters is promoted by membrane PIPs (phosphoinositide phosphates), primarily PI(4,5)P$_2$ (phosphoinoside 3,4-bisphosphate), and application of PI(4,5)P$_2$ to inside-out patches can prevent or reverse the rundown of K$^+$ and Ca$^{2+}$ channels (reviewed in [25]). On the other hand, the assembly of phox at the cell membrane requires the presence of PIPs phosphorylated at the third position, such as PI(3,4)P$_2$ or PI(3)P [26,27]. To test whether PIPs are involved in the regulation of the phox-associated proton channel, we assayed the effect of different PIPs on the rundown kinetics of $I_{hi}$. To allow the exogenous PIPs to integrate into the plasma membrane of eosinophils, 25 µM of short-chain fatty acid C$_6$ PIP analogues were added to the bath solution 10–15 min before patch excision. Pilot experiments indicated that application of PI(3,4)P$_2$ to the bath solution slightly, but significantly, slowed the rundown process ($\tau = 4.8 \pm 0.7$ min, $n = 5$, $P < 0.01$, Figure 2C). The effect was much more dramatic when PI(3)P$_2$ was added together with ATP and GTP[S], with a 16-fold increase in $I_{hi}$ stability ($\tau = 38.9 \pm 9.3$ min, $n = 17$, $P < 0.0001$, Figure 2C). In contrast, PI(3,4)P$_2$ had no effect on the rundown of $I_{hi}$ when applied together with ATP and GTP[S] ($\tau = 40.1 \pm 9.5$, $n = 6$ versus 56.7 ± 6.2, $n = 11$ with and without PI(3,4)P$_2$ respectively, $P = 0.12$).

To determine which phosphoinositide was involved in the stabilization of $I_{hi}$, we systematically tested the effects of different PIPs on $I_{hi}$. As shown in Figure 3(A), only PI(3,4)P$_2$ significantly increased $I_{hi}$ stability, while PI(3)P, PI(3,5)P$_2$, and PI(3,4,5)P$_3$ had no significant beneficial effect. Next, we tested whether PIPs could act from the extracellular side of the plasma membrane by including PIPs into the pipette solution. As shown in Figure 3(B), PI(3,4)P$_2$ significantly decreased $I_{hi}$ stability when applied to the pipette side of the patch ($\tau = 3.6 \pm 1.0$ min, $n = 8$, $P < 0.05$), an effect opposite to the one observed for bath application, whereas pipette application of PI(3)P only slightly decreased $I_{hi}$ stability.

Cytosolic application of both PI(3)P and PI(3,4)P$_2$ was significantly better than extracellular application in promoting $I_{hi}$ stability, but the effect was highly significant for PI(3,4)P$_2$, and marginal for PI(3)P ($P < 0.0001$ and $P < 0.05$ respectively, Figure 3B). These results indicate that PI(3,4)P$_2$ is only effective when added from the extracellular side, and does not affect $I_{hi}$ stability when added to the cytosolic side.

Because $I_{hi}$ still declined much more rapidly than $I_{hi}$ even in the presence of PI(3,4)P$_2$, we tested whether $I_{hi}$ could be further stabilized by supplementing the bath solution with mixtures of different PIPs at a 1:1 ratio and a final concentration of 25 µM. As shown in Figure 3(C), addition of either PI(3)P or PI(4,5)P$_2$ (12.5 µM each) to a bath solution containing ATP, GTP[S] and PI(3,4)P$_2$ (12.5 µM) did not further increase $I_{hi}$ stability, while addition of PI(3)P (25 µM) to the pipette side of the patch significantly decreased $I_{hi}$ stability ($\tau = 9.7 \pm 5.4$ min, $n = 6$, $P < 0.05$).
Role of GTP and ATP hydrolysis on the stability of proton currents

High concentrations of ATP prevent the run-down of ion channels and transporters by sustaining the activity of membrane-bound phosphoinositide kinases, which maintain the membrane PIP pool [29,30]. To evaluate whether PI(3,4)P2 is able to eliminate the need for millimolar ATP concentrations in stabilizing \( I_H \), we decreased the [ATP] to 0.5 or 0 mM in bath solutions containing GTP[S] and PI(3,4)P2. As shown in Figure 4(A), decreasing the ATP concentration to 0.5 mM significantly reduced \( I_H \) stability despite the presence of GTP[S] and PI(3,4)P2 (\( \tau = 7.0 \pm 2.0 \) min, \( n = 7 \), \( P < 0.01 \)). In the absence of ATP, \( I_H \) declined at rates identical with the ones observed in solutions lacking nucleotides and PIPs (\( \tau = 2.7 \pm 0.4 \), \( n = 6 \)). This indicates that an ATP-dependent process other than PI(3,4)P2 conservation is rate-determining. To assess whether protein phosphorylation is involved in the ATP effect, we tested the effects of various phosphatase inhibitors on \( I_H \) decay. As shown in Figure 4(B), neither orthovanadate (1 mM), fluoride (10 mM), okadaic acid (100 mM) or cyclosporine (100 mM) had any effect on \( I_H \) decline when added to a bath solution containing GTP[S] and PI(3,4)P2. We then tested whether the hydrolysis-resistant ATP analogue ATP[S] could substitute for ATP. As shown in Figure 4(B), the combination of 0.5 ATP[S] and 0.5 mM ATP was as efficient as 5 mM ATP in stabilizing \( I_H \) (\( \tau = 17.8 \pm 4.4 \) min, \( n = 10 \), \( P = 0.26 \)). Finally, we assessed whether GTP hydrolysis is required for the stabilization of \( I_H \) by comparing the effects of GTP and GTP[S] in bath solutions containing 5 mM ATP and 25 mM PI(3,4)P2. As shown in Figure 4(C), the hydrolysable, physiological compound GTP was less efficient in stabilizing \( I_H \) than the same concentration (50 mM) of the hydrolysis-resistant GTP[S] (\( \tau = 8.3 \pm 2.9 \) min, \( n = 10 \), \( P < 0.02 \)). When neither GTP nor GTP[S] was present in the bath solution, \( I_H \) decayed as rapidly as in solutions devoid of nucleotides and PIPs, indicating that the combination of ATP and PI(3,4)P2 is not sufficient to stabilize \( I_H \) (Figure 4C). Thus the maintenance of an inward \( I_H \) in excised patches requires the presence of a non-hydrolysable GTP analogue, of millimolar ATP concentrations and of PI(3,4)P2 on the cytosolic side of the patch.

**Figure 3** Effects of phosphoinositides on the stability of \( I_H \)

Recordings were performed as in Figure 2, and current traces fitted with a single exponential decay function. (A) Effects of different phosphoinositides on the time constant of \( I_H \) decay. The bath solution contained 5 mM ATP and 50 \( \mu \)M GTP[S] (control, \( n = 17 \)) supplemented with 25 \( \mu \)M of each different PIP \( [n = 8, 8, 17, 7 \& 10 \text{ for } \text{PI}, \text{PI}(3)P, \text{PI}(3,4)P_2, \text{PI}(4,5)P_2 \text{ and } \text{PI}(3,4,5)P_3 \text{, respectively; } * P < 0.05 \text{ versus ATP + GTP[S]}]. \) (B) Effects of cytosolic (bath) and extracellular (pipette) application of PIPs on \( I_H \) decay. The bath solution contained 5 mM ATP and 50 \( \mu \)M GTP[S] in all cases \( [n = 17, 8, 8 \& 6 \text{ for bath and pipette application of } 25 \mu \text{M } \text{PI}(4,5)P_2 \text{ and PI}(3)P \text{, respectively; } * P < 0.05 \text{ and } ** P < 0.002 \text{ for cytosolic versus extracellular application}]. \) (C) Effects of addition of mixtures of PIPs, arachidonic acid (AA) and of the oxidase substrate NADPH on \( I_H \) decay. The bath solution contained 5 mM ATP, 50 \( \mu \)M GTP[S], and either 12.5 \( \mu \)M (for PIPs mixtures) or 25 \( \mu \)M PI(3,4)P2 (control, \( n = 17 \)) supplemented with 12.5 \( \mu \)M PI(3)P \( [n = 16], 12.5 \mu \text{M PI}(4,5)P_2 \text{, } (n = 7), 100 \text{ nM arachidonic acid (n = 9) or } 0.8 \text{ mM NADPH and } 10 \text{ mM DPI (n = 8). None of these agents further stabilized } I_H \text{, while the addition of PI(3)P to the pipette solution accelerated } I_H \text{ decay (n = 6, } ^* P < 0.05 \text{ versus control}. \)

**Figure 3C.** Furthermore, neither arachidonic acid nor the oxidase substrate NADPH, agents known to modulate the oxidase-associated \( H^+ \) channel \([22,28]\), had any effect on \( I_H \) stability when added to the bath solution (Figure 3C). Thus, the combination of ATP, GTP[S] and PI(3,4)P2 was optimal in stabilizing \( I_H \).

**DISCUSSION**

In the present study, we have used the inside-out configuration of the patch-clamp technique to explore the mechanism of inactivation of the phagocytic NADPH oxidase. We studied the disappearance, or “run-down”, of two currents coupled with the phox activation: the electron current, \( I_e \), and the inward proton current, \( I_H \). \( I_e \) is a direct real-time readout of the electron transport rate, \( I_H \) can be recorded in the absence of oxygen or NADPH and in the presence of DPI \([9]\). The disappearance of \( I_H \) is thus expected to correlate with the disassembly or inactivation of the oxidase complex, rather than with the loss of redox activity of its core enzyme. Our recordings of \( I_e \) and \( I_H \) in excised patches thus provide a new window to study the mechanisms of phox inactivation, as the loss of redox activity of the enzyme (\( I_e \)) can be correlated with the loss of a current associated with its assembly and activation (\( I_H \)).
The kinetics of $I_e$ activation and decay provide novel and important information on the initiation and maintenance of electron transport. Whereas the presence of GTP or GTP[S] is absolutely required for phox activity in cell-free assays [31], $I_e$ could be evoked in the absence of exogenous ATP and GTP in excised patches (Figure 1A). In cell-free assays, the complex is reconstituted by mixing neutrophil membranes with purified or recombinant cytosolic proteins [32,33], and the small GTP-binding protein Rac2 is absolutely required for phox assembly [34]. In our patches excised from cells stimulated with PMA for 10–15 min, the pre-assembled phox complex contains all the necessary components and initiation of electron transport does not require additional factors. However, whereas neither ATP nor GTP was necessary for $I_e$ initiation, both nucleotides were absolutely required for the maintenance of $I_e$: in a buffer devoid of nucleotides $I_e$ decayed with a time constant of approx. 6 min, indicating that electron transport, once initiated, has a built-in capacity to inactivate. Addition of ATP and GTP[S] almost completely prevented $I_e$ inactivation, indicating that a GTP-binding protein is required to stabilize the complex in its active conformation. Under these conditions, electron currents could be recorded for more than 30 min, indicating that the native phox complex is very stable when locked in its active conformation.

Early studies suggested that the assembled phox complex is highly labile in PMA-stimulated neutrophils, because activity terminated within seconds when oxidase assembly was abruptly stopped by N-ethylmaleimide [35]. In cell-free assays, the oxidase inactivates with a half-life of 2 min when the cytosolic components are removed [36] and a half-life of 16 min when the cytosolic proteins remain present [37]. In vitro, the activity of the complex is greatly stabilized by replacing the native proteins with a fused p47<sub>phox</sub>–p67<sub>phox</sub> chimera [37] and further stabilized by the addition of a constitutively active Rac [20]. Our results indicate that the presence of ATP and GTP[S] is sufficient to stabilize the native phox complex for hours, and suggest that the rapid deactivation observed in intact cells or in patches lacking exogenous nucleotides is due to the hydrolysis of GTP.

Numerous biochemical and genetic data indicate that the small GTPase Rac is required for phox activation [34]. Recombinant Rac2 can support oxidase activation in vitro [38], and phox activity requires the GTP-bound form of Rac2 [39]. Rac-GTP binds via its switch 1 domain to the tetrastricopeptide repeat domain of p67<sub>phox</sub>, and the suppression of the Rac2–p67<sub>phox</sub> interaction prevents electron transfer from FAD to oxygen [40]. The formation of the Rac2–p67<sub>phox</sub>-GTP complex stabilizes the phox in an active conformation. Because small GTPases such as Rac function as on/off switches, Rac2 has been postulated to regulate not only the activation, but also the inactivation of the oxidase [20]. The rapid decay of $I_e$ that we observed in the absence of GTP analogues strongly suggests that GTP hydrolysis by Rac2 indeed inactivates the oxidase. The rapid loss of activity further suggests that membranes from activated eosinophils have high GTPase activity, and that upon induction of electron flow Rac-GTP is rapidly hydrolysed to its inactive, GDP-bound form. GAPs (GTPase-activating proteins) increase the rate of GTP hydrolysis severalfold when bound to GTP-binding proteins such as Rac. Three Rac GAP proteins have been reported both in the membrane (p50RhoGAP) and in the cytosol (Bcr and p190RhoGAP) of neutrophils [41], and the membrane-localized GAP is present and functional within the assembled complex [42]. Increased hydrolysis of Rac-GTP by the membrane-localized p50RhoGAP might account for the rapid $I_e$ decay observed in our excised patches in the absence of GTP.

Comparison of the kinetics of $I_{H}$ and $I_e$ revealed significant differences. As with $I_{H}$, $I_{H}$ could be activated in the absence of nucleotides and was not stabilized by the addition of ATP or GTP[S] but only by the combined addition of these two nucleotides. However, $I_{H}$ was inactivated faster than $I_e$ under all conditions. $I_{H}$ run-down was only partially prevented by the combination of ATP and GTP[S]. Instead, we found that a specific phosphinositide, PI(3,4)P₂, was required for maximal stabilization...
of I_{H}, PI(3,4)P_{2} significantly stabilized I_{H} even in the absence of nucleotides and maximally stabilized I_{H} when added together with ATP and GTP[S]. No other PIP had any positive effect on I_{H} stability, and PI(3,4)P_{2} was only effective when added from the cytosolic side. When added to the extracellular side, PI(3,4)P_{2} was detrimental and accelerated I_{H} decay. Despite its pronounced and specific effects on I_{H} stability, PI(3,4)P_{2} had no stabilizing effects on I_{N}, indicating that PI(3,4)P_{2} specifically modulates the voltage-sensitivity of proton channels under our experimental conditions. PI(3,4)P_{2} is not a classical modulator of ion channels, in contrast with PI(4,5)P_{2} [47], and the epithelial Na⁺ channel ENaC is the only channel reportedly modulated by PI(3,4)P_{2} [43]. PI(3,4)P_{2} interacts preferentially with proteins bearing a PX domain, first identified in the two oxidase components p40^{phox} and p47^{phox} and since then in approx. 50 mammalian and yeast proteins involved in signalling, protein sorting and trafficking (reviewed in [44]). Most PX domains bind to PI(3)P and, among oxidase proteins, p40^{phox} binds to PI(3)P but not PI(3,4)P_{2}, while p47^{phox} preferentially binds PI(3,4)P_{2} [27]. Our observation that PI(3,4)P_{2} but not PI(3)P could stabilize I_{H} strongly suggests that p47^{phox}, rather than p40^{phox}, is implicated. The PX domain of p47^{phox} is exposed upon phosphorylation, targeting this protein and its associated partners to the membrane. The stabilizing effects of PI(3,4)P_{2} on I_{H} raises the possibility that association of p47^{phox} with membrane lipids maintains the H⁺ channel in an active conformation.

The inward I_{H} studied here was previously shown to require phox activation [9], but it was unclear whether the change in voltage-sensitivity of proton channels was induced by the binding of soluble factor(s) to the channel or by the assembly of the phox complex. Our excised patch data now indicate that pre-assembly and activation of the phox is absolutely required for I_{H}, because the addition of ATP, GTP[S] and PI(3,4)P_{2} to patches excised from resting cells did not elicit inward I_{H}. Thus these factors do not shift the voltage sensitivity of pre-existing proton channels, but only stabilize inward currents in cells that contain a pre-assembled oxidase. The identity of the modulated channel remains open. Both phox and the newly cloned 'voltage-sensing only' protein Hv1/VSOP have been shown to generate voltage-dependent proton currents upon heterologous expression [12–14,17,18]. However, neither phox nor Hv1/VSOP has been reconstituted into artificial lipid bilayers, and thus both proteins can function either as channels or as channel regulators. Both phox and Hv1/VSOP are expressed in phagocytes and might contribute to the currents observed in resting and activated phagocytes. Mutation of the Hv1/VSOP voltage sensor residue Arg^{201} produced inward proton currents, suggesting that modification of this critical residue might account for the inward I_{H} of activated phagocytes. However, the Arg^{201} residue in Hv1/VSOP is unlikely to mediate the effects of nucleotides and PIPs on the stability of the inward I_{H}, because (i) the Arg^{201} residue is predicted to be buried in the membrane and should not be readily accessible to nucleotides and PIPs and (ii) as discussed above, nucleotides and PIPs do not shift the voltage-sensitivity of pre-existing proton channels but only stabilize inward currents in cells that contain a pre-assembled oxidase. Thus, regardless of the identity of the proton channel, our results indicate that nucleotides and/or PIPs modulate the channel indirectly, probably by stabilizing the phox complex.

Unexpectedly, the stabilization of both I_{H} and I_{N} required millimolar ATP concentrations. This is surprising, because the fluxes of electrons and protons across the membrane are driven by the redox and pH differences and do not directly require ATP hydrolysis to energize the transport process. The ATP-dependence cannot be attributed to dissipation of the pH gradient or to substrate limitation, since the pH and NADPH concentrations are clamped in our patches. ATP can have indirect effects on oxidase activity by regenerating GTP [31,45], and modulate the activity of ion transporters by altering PIPs phosphorylation [29,30]. These indirect effects can be ruled out in our case, because millimolar ATP concentrations were required for sustained activity despite the presence of maximal concentrations of GTP[S] and PI(3,4)P_{2}. Loss of phosphates from p47^{phox} or from another phox component due to endogenous phosphatase activity in the patch is another possibility, and phosphatase inhibitors have been shown to prolong the respiratory burst stimulated by fMLP (N-formyl-methionyl-leucylphenylalanine) or PMA in intact cells [46]. Three lines of evidence argue against a role for dephosphorylation in I_{H} run-down: (i) submillimolar ATP concentrations failed to sustain I_{H}, (ii) phosphatase inhibitors did not increase I_{H} stability, and (iii) the non-hydrolysable ATP analogue ATP[S] stabilized I_{H}. We did not test the effects of ATP[S] or of phosphatase inhibitors on the stability of I_{N}. However, decreasing the ATP concentration from 5 to 0.5 mM significantly increased I_{N} decay (56.7 ± 6.2, n = 11 to 35.8 ± 6.5, n = 7, P < 0.05), indicating that millimolar ATP concentrations are also required to stabilize electron flow. The positive effect of ATP[S] suggests that the hydrolysis of the γ phosphate is not required to stabilize I_{H}, but that the binding of ATP to a component of the oxidase complex might stabilize I_{H}. Binding of ATP controls the activity of K_{ATP} channels, and the affinity of the channel for ATP is modulated by PI(4,5)P_{2} [47]. Non-hydrolysable ATP can also activate the Na⁺/H⁺ antiporter [48] and this activation is stronger than that of PI(4,5)P_{2} [49]. Sustained activation of the proton channel might involve a similar mechanism, requiring both a specific phospholipid as well as the binding of ATP to the channel.

In summary, we showed that the native phox complex remains active for hours in excised patches if both ATP and a non-hydrolysable GTP analogue are present. The phox inactivates rapidly in the absence of GTP[S], suggesting that Rac GAP proteins are present in the phox complex and increase the rate of GTP hydrolysis by Rac. Millimolar ATP concentrations are required to stabilize the activity of the phox and of its associated proton channel, an effect that does not appear to involve phosphorylation but ATP binding. Sustained activation of proton channels in a 'low-threshold' conformation not only requires ATP and GTP[S] but also PI(3,4)P_{2}, a phospholipid that recruits p47^{phox} to the plasma membrane.

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