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

Gábor L. PETHEŐ*†¹, Nathalie C. GIRARDIN*, Nicolas GOOSSENS*, Gergely Z. MOLNÁR* and Nicolas DEMAUREX*

*Department of Cell Physiology and Metabolism, University of Geneva Medical School, 1 Michel-Servet, CH-1211 Geneva 4, Switzerland, and †Department of Physiology and Laboratory of Cellular and Molecular Physiology, Faculty of Medicine, Semmelweis University, P.O. Box 259, H-1444 Budapest, Hungary

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 ($\tau \approx 6$ min) and was maximally stabilized by the combined addition of 5 mM ATP and 50 μ M of the non-hydrolysable GTP analogue GTP[S] (guanosine 5'-[γ -thio]triphosphate) ($\tau \approx 57$ 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 μ M PI(3,4)P₂ (phosphoinositide 3,4-bisphosphate) to the cytosolic side of the patch.

PI(3,4)P₂ 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'-[γ -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)P₂, a protein known to anchor the cytosolic phox subunit p47^{phox} 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 gp91^{phox} that contains the electron transport chain, and p22^{phox}. Activation of the oxidase requires the membrane translocation of the small G-protein Rac and of three cytosolic subunits, p67^{phox}, p47^{phox} and p40^{phox}, which form a heterotrimeric complex that can be extensively phosphorylated on p47^{phox}. Upon phosphorylation, the 'organizer' p47^{phox} 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' p67^{phox} 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 gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox} 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'-[γ -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 gp91^{phox} or p47^{phox} 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 gp91^{phox} 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'-[γ -thio]triphosphate; CGD, chronic granulomatous disease; DPI, diphenyleneiodonium; GAP, GTPase-activating protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; Hv1/VSOP, voltage-gated hydrogen channel 1/voltage sensor domain-only protein; PI(3,4)P₂, phosphoinositide 3,4-bisphosphate; PIP, phosphoinositide phosphate; PX domain, phox homology domain.

¹ To whom correspondence should be addressed (email petheo@puskin.sote.hu).

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 (Treff) at approx. 500 cells/ μ l 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 $\times$ 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 μ m of the bottom. The initial volume of the bath solution was 50 μ l and drugs were added directly into the bath and thoroughly mixed (pipetting five times with a 20 μ l 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 G Ω 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 M Ω 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 Hepes (GibcoTM, Invitrogen) and L-glutamine-free RPMI 1640 medium (GibcoTM) supplemented with 5 mM Na₂EDTA and 2% (v/v) fetal calf serum (BioConcept). The recording solutions contained (mM): CsCl 1, tetraethylammonium chloride 1, MgCl₂ 2, EGTA 1, *N*-methyl-D-glucamine base 101 and either 200 Mes (pH 6.15) or 200 Hepes (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 ZnCl₂ (free [Zn²⁺] $\approx$ 1 mM). ATP[S] (adenosine 5'-[γ -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 μ M. DiC₈-phosphoinositides (Echelon), MgATP and Na₄NADPH were dissolved in pH 7.55 solution to give a stock solution of 1.25, 30 and 80 μ M respectively. Na₂EDTA 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 preparations. Results are presented as means $\pm$ S.E.M. For statistical analysis the non-parametric Mann–Whitney test was applied using 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 I_e in inside-out patches from human eosinophils treated with PMA (200–400 nM for 10–15 min). I_e was recorded at a constant voltage of 0 mV, and 1 mM Zn^{2+} was added to the pipette solution to block proton currents [24]. Figure 1(A) shows a typical recording of I_e evoked by bath application of 0.8 mM NADPH, a near saturating concentration ($\sim 90\%$, [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], I_e ran down within minutes after the addition of NADPH when no nucleotide was present in the recording buffer.

The time course of I_e 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), I_e decay was not significantly reduced by the addition of 5 mM ATP or 50 μ M 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 μ M GTP[S], the run-down was minimal and stable electron currents could be recorded for more than 30 min (Figures 1B and 1C, $\tau = 57 \pm 6$ min, $n = 11$, $P < 0.0001$). Increasing the GTP[S] concentration to 250 μ M 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 μ M GTP[S] had a slight, but significant, effect on current stability ($\tau = 35.8 \pm 6.5$ min, $n = 7$, $P < 0.05$).

To verify that I_e decline was not due to the presence of Zn^{2+} in the pipette solution, we recorded I_e in the absence of Zn^{2+} 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 combination of ATP and GTP[S] also stabilized I_e recorded at -60 mV. These results indicate that the combination of ATP and of the non-

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 Zn^{2+} to block proton currents. (A) Original current recording from a representative patch. The electron current (I_e) 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 μ M GTP[S] ('GTP γ 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 I_e . (C) Effects of ATP, GDP, GTP[S] and ATP + GTP[S] on the time constant of I_e 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.

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 2 Effects of nucleotides and PI(3,4)P₂ on the stability of inward proton currents

Inside-out patches were excised into a NADPH free bath solution and inward proton currents (I_H) were elicited by applying a voltage pulse to 0 mV from the -60 mV holding potential. Pipettes were devoid of Zn²⁺, pH in/out 7.55/7.05. **(A)** Original current recording from a representative patch. H⁺ currents ran down within minutes following the voltage step to 0 mV. **(B)** Normalized currents recorded in a nucleotide-free bath solution (control) or in a bath solution containing 5 mM ATP, 50 μ M GTP[S] (GTP γ S) and 25 μ M PI(3,4)P₂. The currents were normalized to the peak inward current recorded within 2 min following the voltage jump. **(C)** Effects of ATP, GTP[S], ATP + GTP[S], PI(3,4)P₂ and ATP + GTP[S] + PI(3,4)P₂ on the time constant of proton current rundown. The current traces were fitted with a single exponential decay function. ATP + GTP[S] or PI(3,4)P₂ significantly prevented I_H decay, but the combination of ATP, GTP[S] and PI(3,4)P₂ was most effective in stabilizing I_H (* P < 0.01, ** P < 0.001, and *** P < 0.0001 versus control, n values are given below each condition).

systematically the effects of ATP and GTP[S] on the stability of the inward proton current, I_H . An inward pH gradient of 0.5 was imposed to allow the influx of H⁺ from the pipette to the cytosolic side of the patch (pH in/out 7.55/7.05, calculated E_H = +30 mV; pipette was devoid of Zn²⁺), and I_H was elicited by a long-lasting voltage pulse to 0 mV. A typical recording of the H⁺ current evoked by a voltage step to 0 mV is shown in Figure 2(A). The time course of I_H was also biphasic with an activation phase followed by a short peak period and a subsequent current decay that was well described by a single exponential component. In a nucleotide-free recording bath solution, I_H decayed with

an average time constant of 2.4 ± 0.3 min, i.e. slightly, but not significantly, faster than I_e (Figures 2B and 2C). Neither ATP nor GTP[S] significantly slowed I_H decline, but in the presence of 5 mM ATP the kinetics of I_H decay was very variable, with time constants ranging from 1.4 to 46.1 min ($\tau = 9.5 \pm 5.3$ min, $n = 8$, Figure 2C). As observed for I_e , the combination of 5 mM ATP and 50 μ M GTP[S] significantly prevented the current decline, but the effect was less dramatic with only a 6-fold increase in current stability ($\tau = 14.4 \pm 5.7$ min, $n = 17$, $P = 0.0005$). Thus I_H was also stabilized by the combination of ATP and GTP[S], but less efficiently than I_e .

Effect of phosphoinositides on the stability of proton currents

The activity of several ion channels and transporters is promoted by membrane PIPs (phosphoinositide phosphates), primarily PI(4,5)P₂ (phosphoinositide 3,4-bisphosphate), and application of PI(4,5)P₂ to inside-out patches can prevent or reverse the run-down of K⁺ and Ca²⁺ 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)P₂ or PI(3)P [26,27]. To test whether PIPs are involved in the regulation of the activity of the phox-associated proton channel, we assayed the effect of different PIPs on the run-down kinetics of I_H . To allow the exogenous PIPs to integrate into the plasma membrane of eosinophils, 25 μ M of short-chain fatty acid C₈ PIP analogues were added to the bath solution 10–15 min before patch excision. Pilot experiments indicated that application of PI(3,4)P₂ to the bath solution slightly, but significantly, slowed the run-down process ($\tau = 4.8 \pm 0.7$ min, $n = 5$, $P < 0.01$, Figure 2C). The effect was much more dramatic when PI(3,4)P₂ was added together with ATP and GTP[S], with a 16-fold increase in I_H stability ($\tau = 38.9 \pm 9.3$ min, $n = 17$, $P < 0.0001$, Figure 2C). In contrast, PI(3,4)P₂ had no effect on the run-down of I_e 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)P₂ respectively, $P = 0.12$].

To determine which phosphoinositide was involved in the stabilization of I_H , we systematically tested the effects of different PIPs on I_H . As shown in Figure 3(A), only PI(3,4)P₂ significantly increased I_H stability, while PI, PI(3)P, PI(4,5)P₂ and PI(3,4,5)P₃ 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)P₂ significantly decreased I_H 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_H stability. Cytosolic application of both PI(3)P and PI(3,4)P₂ was significantly better than extracellular application in promoting I_H stability, but the effect was highly significant for PI(3,4)P₂ and marginal for PI(3)P ($P < 0.0001$ and $P < 0.05$ respectively, Figure 3B). These results indicate that PI(3,4)P₂ is only effective when added from the cytosolic side, and has deleterious effects on I_H stability when added to the extracellular side.

Because I_H still declined more rapidly than I_e even in the presence of PI(3,4)P₂, we tested whether I_H 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 μ M. As shown in Figure 3(C), addition of either PI(3)P or PI(4,5)P₂ (12.5 μ M each) to a bath solution containing ATP, GTP[S] and PI(3,4)P₂ (12.5 μ M) did not further increase I_H stability, while addition of PI(3)P (25 μ M) to the pipette side of the patch significantly decreased I_H stability ($\tau = 9.7 \pm 5.4$ min, $n = 6$, $P < 0.05$,

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 μ M GTP[S] ('GTP γ S') (control, $n = 17$) supplemented with 25 μ M of each different PIP [$n = 8, 8, 17, 7$ and 10 for PI, PI(3)P, PI(3,4)P₂, PI(4,5)P₂ and PI(3,4,5)P₃ respectively; * $P < 0.05$ 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 μ M GTP[S] in all cases [$n = 17, 8, 8$ and 6 for bath and pipette application of 25 μ M PI(4,5)P₂ and PI(3)P respectively; * $P < 0.05$ and ** $P < 0.002$ 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 μ M GTP[S], and either 12.5 μ M (for PIPs mixtures) or 25 μ M PI(3,4)P₂ (control, $n = 17$) supplemented with 12.5 μ M PI(3)P ($n = 16$), 12.5 μ M PI(4,5)P₂ ($n = 7$), 100 nM arachidonic acid ($n = 9$) or 0.8 mM NADPH and 10 μ M DPI ($n = 8$). None of these agents further stabilized I_H , while the addition of PI(3)P to the pipette solution accelerated I_H decay ($n = 6$, * $P < 0.05$ 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 .

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 nM) or cyclosporine (100 nM) 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 μ M 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 μ M) 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.

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 activity: 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, and I_e run-down reflects the progressive loss of redox activity of the enzyme. I_H is only observed in cells with an activated oxidase and reflects the modulation of H^+ channel(s) that might be contained within the phox complex [23]. Importantly, the appearance and maintenance of I_H require the assembly of the phox but not its redox function, because 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).

Figure 4 Effect of GTP and ATP hydrolysis 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 reducing the ATP concentration on the time constant of I_H decay. The bath solution contained 50 μ M GTP[S] ('GTP γ S'), 25 μ M PI(3,4) P_2 and either 5 mM ATP ($n=17$), 0.5 mM ATP ($n=7$) or no ATP ($n=6$); * $P < 0.02$ and ** $P < 0.0001$ versus 5 mM ATP. **(B)** Effects of phosphatase inhibitors and of ATP[S] on I_H decay. The bath solution contained 50 μ M GTP[S], 25 μ M PI(3,4) P_2 and either 1 mM orthovanadate ($n=6$), 10 mM fluoride ($n=7$), 100 nM okadaic acid ($n=7$), 100 nM cyclosporine A ($n=6$) or a combination of 0.5 mM ATP and 0.5 mM GTP[S] ($n=10$). Only ATP[S] significantly prevented I_H decay (* $P < 0.01$ versus control). **(C)** Effects of GTP analogues on the time constant of I_H decay. The bath solution contained 5 mM ATP, 25 μ M PI(3,4) P_2 and either 50 μ M GTP[S] (control, $n=17$), 50 μ M GTP ($n=10$), or no GTP analogue ($n=6$); * $P < 0.02$ and ** $P < 0.002$ versus 50 μ M GTP[S].

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^{phox}–p67^{phox} 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 tetratricopeptide repeat domain of p67^{phox}, and the suppression of the Rac2–p67^{phox} interaction prevents electron transfer from FAD to oxygen [40]. The formation of the Rac2–p67^{phox} complex thus 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 RacGTP 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_e , 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 and I_H run-down was only partially prevented by the combination of ATP and GTP[S]. Instead, we found that a specific phosphoinositide, PI(3,4) P_2 , was required for maximal stabilization

of I_H . PI(3,4)P₂ 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)P₂ was only effective when added from the cytosolic side. When added to the extracellular side, PI(3,4)P₂ was detrimental and accelerated I_H decay. Despite its pronounced and specific effects on I_H stability, PI(3,4)P₂ had no stabilizing effects on I_e , indicating that PI(3,4)P₂ specifically modulates the voltage-sensitivity of proton channels under our experimental conditions. PI(3,4)P₂ is not a classical modulator of ion channels, in contrast with PI(4,5)P₂ [25], and the epithelial Na⁺ channel ENaC is the only channel reportedly modulated by PI(3,4)P₂ [43]. PI(3,4)P₂ 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)P₂, while p47^{phox} preferentially binds PI(3,4)P₂ [27]. Our observation that PI(3,4)P₂ 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)P₂ 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 inward I_H , because the addition of ATP, GTP[S] and PI(3,4)P₂ 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 Arg²⁰¹ produced inward proton currents, suggesting that modification of this critical residue might account for the inward I_H of activated phagocytes. However, the Arg²⁰¹ 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 Arg²⁰¹ 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_e and I_H 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)P₂. 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_e . However, decreasing the ATP concentration from 5 to 0.5 mM significantly increased I_e decay (from 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)P₂ [47]. Non-hydrolysable ATP can also activate the Na⁺/H⁺ antiporter [48] and this activation is stronger than that of PI(4,5)P₂ [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)P₂, a phospholipid that recruits p47^{phox} to the plasma membrane.

This work was supported by grant number 31-068317.02 from the Swiss National Science Foundation.

REFERENCES

- Babior, B. M. (2004) NADPH oxidase. *Curr. Opin. Immunol.* **16**, 42–47
- Groemping, Y. and Rittinger, K. (2005) Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem. J.* **386**, 401–416
- Henderson, L. M., Chappell, J. B. and Jones, O. T. (1987) The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H⁺ channel. *Biochem. J.* **246**, 325–329
- Demaurex, N., Grinstein, S., Jaconi, M., Schlegel, W., Lew, D. P. and Krause, K. H. (1993) Proton currents in human granulocytes: regulation by membrane potential and intracellular pH. *J. Physiol.* **466**, 329–344
- DeCoursey, T. E. (2003) Interactions between NADPH oxidase and voltage-gated proton channels: why electron transport depends on proton transport. *FEBS Lett.* **555**, 57–61
- Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G., Bolsover, S., Gabella, G., Potma, E. O., Warley, A., Roes, J. and Segal, A. W. (2002) Killing activity of neutrophils is mediated through activation of proteases by K⁺ flux. *Nature* **416**, 291–297
- Ahluwalia, J., Tinker, A., Clapp, L. H., Duchon, M. R., Abramov, A. Y., Pope, S., Nobles, M. and Segal, A. W. (2004) The large-conductance Ca²⁺-activated K⁺ channel is essential for innate immunity. *Nature* **427**, 853–858

- 8 Demaurex, N., Schrenzel, J., Jaconi, M. E., Lew, D. P. and Krause, K. H. (1993) Proton channels, plasma membrane potential, and respiratory burst in human neutrophils. *Eur. J. Haematol.* **51**, 309–312
- 9 Banfi, B., Schrenzel, J., Nusse, O., Lew, D. P., Ligeti, E., Krause, K. H. and Demaurex, N. (1999) A novel H(+) conductance in eosinophils: unique characteristics and absence in chronic granulomatous disease. *J. Exp. Med.* **190**, 183–194
- 10 Decoursey, T. E. (2003) Voltage-gated proton channels and other proton transfer pathways. *Physiol. Rev.* **83**, 475–579
- 11 DeCoursey, T. E., Cherny, V. V., Zhou, W. and Thomas, L. L. (2000) Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6885–6889
- 12 Henderson, L. M. and Meech, R. W. (1999) Evidence that the product of the human X-linked CGD gene, gp91-phox, is a voltage-gated H(+) pathway. *J. Gen. Physiol.* **114**, 771–786
- 13 Maturana, A., Arnaudeau, S., Ryser, S., Banfi, B., Hossle, J. P., Schlegel, W., Krause, K. H. and Demaurex, N. (2001) Heme histidine ligands within gp91(phox) modulate proton conduction by the phagocyte NADPH oxidase. *J. Biol. Chem.* **276**, 30277–30284
- 14 Murillo, I. and Henderson, L. M. (2005) Expression of gp91phox/Nox2 in COS-7 cells: cellular localization of the protein and the detection of outward proton currents. *Biochem. J.* **385**, 649–657
- 15 Nanda, A., Romanek, R., Curnutte, J. T. and Grinstein, S. (1994) Assessment of the contribution of the cytochrome b moiety of the NADPH oxidase to the transmembrane H⁺ conductance of leukocytes. *J. Biol. Chem.* **269**, 27280–27285
- 16 Morgan, D., Cherny, V. V., Price, M. O., Dinauer, M. C. and DeCoursey, T. E. (2002) Absence of proton channels in COS-7 cells expressing functional NADPH oxidase components. *J. Gen. Physiol.* **119**, 571–580
- 17 Sasaki, M., Takagi, M. and Okamura, Y. (2006) A voltage sensor-domain protein is a voltage-gated proton channel. *Science* **312**, 589–592
- 18 Ramsey, I. S., Moran, M. M., Chong, J. A. and Clapham, D. E. (2006) A voltage-gated proton-selective channel lacking the pore domain. *Nature* **440**, 1213–1216
- 19 Shiose, A., Kuroda, J., Tsuruya, K., Hirai, M., Hirakata, H., Naito, S., Hattori, M., Sakaki, Y. and Sumimoto, H. (2001) A novel superoxide-producing NAD(P)H oxidase in kidney. *J. Biol. Chem.* **276**, 1417–1423
- 20 Decoursey, T. E. and Ligeti, E. (2005) Regulation and termination of NADPH oxidase activity. *Cell. Mol. Life Sci.* **62**, 2173–2193
- 21 Schrenzel, J., Serrander, L., Banfi, B., Nusse, O., Fouyoudi, R., Lew, D. P., Demaurex, N. and Krause, K. H. (1998) Electron currents generated by the human phagocyte NADPH oxidase. *Nature* **392**, 734–737
- 22 Petheő, G. L., Maturana, A., Spat, A. and Demaurex, N. (2003) Interactions between electron and proton currents in excised patches from human eosinophils. *J. Gen. Physiol.* **122**, 713–726
- 23 Demaurex, N. and Petheő, G. L. (2005) Electron and proton transport by NADPH oxidases. *Philos. Trans. R. Soc. London Ser. B* **360**, 2315–2325
- 24 Petheő, G. L. and Demaurex, N. (2005) Voltage- and NADPH-dependence of electron currents generated by the phagocytic NADPH oxidase. *Biochem. J.* **388**, 485–491
- 25 Hilgemann, D. W., Feng, S. and Nasuhoglu, C. (2001) The complex and intriguing lives of PIP2 with ion channels and transporters. *Science STKE* 2001, RE19
- 26 Ellson, C. D., Gobert-Gosse, S., Anderson, K. E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J. W., Cooper, M. A., Lim, Z. Y., Holmes, A. B. et al. (2001) PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40(phox). *Nat. Cell Biol.* **3**, 679–682
- 27 Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsuo, T., Brown, G. E., Cantley, L. C. and Yaffe, M. B. (2001) The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat. Cell Biol.* **3**, 675–678
- 28 Cherny, V. V., Henderson, L. M., Xu, W., Thomas, L. L. and DeCoursey, T. E. (2001) Activation of NADPH oxidase-related proton and electron currents in human eosinophils by arachidonic acid. *J. Physiol.* **535**, 783–794
- 29 Hilgemann, D. W. and Ball, R. (1996) Regulation of cardiac Na⁺/Ca²⁺ exchange and KATP potassium channels by PIP2. *Science* **273**, 956–959
- 30 Aharonovitz, O., Zaun, H. C., Balla, T., York, J. D., Orlowski, J. and Grinstein, S. (2000) Intracellular pH regulation by Na(+)/H(+) exchange requires phosphatidylinositol 4,5-bisphosphate. *J. Cell Biol.* **150**, 213–224
- 31 Peveri, P., Heyworth, P. G. and Curnutte, J. T. (1992) Absolute requirement for GTP in activation of human neutrophil NADPH oxidase in a cell-free system: role of ATP in regenerating GTP. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2494–2498
- 32 Bromberg, Y. and Pick, E. (1984) Unsaturated fatty acids stimulate NADPH-dependent superoxide production by cell-free system derived from macrophages. *Cell. Immunol.* **88**, 213–221
- 33 Abo, A., Boyhan, A., West, I., Thrasher, A. J. and Segal, A. W. (1992) Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21rac1, and cytochrome b-245. *J. Biol. Chem.* **267**, 16767–16770
- 34 Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T. and Bokoch, G. M. (1991) Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science* **254**, 1512–1515
- 35 Akard, L. P., English, D. and Gabig, T. G. (1988) Rapid deactivation of NADPH oxidase in neutrophils: continuous replacement by newly activated enzyme sustains the respiratory burst. *Blood* **72**, 322–327
- 36 Tamura, M., Takeshita, M., Curnutte, J. T., Uhlinger, D. J. and Lambeth, J. D. (1992) Stabilization of human neutrophil NADPH oxidase activated in a cell-free system by cytosolic proteins and by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. *J. Biol. Chem.* **267**, 7529–7538
- 37 Ebisu, K., Nagasawa, T., Watanabe, K., Kakinuma, K., Miyano, K. and Tamura, M. (2001) Fused p47phox and p67phox truncations efficiently reconstitute NADPH oxidase with higher activity and stability than the individual components. *J. Biol. Chem.* **276**, 24498–24505
- 38 Knaus, U. G., Heyworth, P. G., Kinsella, B. T., Curnutte, J. T. and Bokoch, G. M. (1992) Purification and characterization of Rac 2. A cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. *J. Biol. Chem.* **267**, 23575–23582
- 39 Bokoch, G. M. and Diebold, B. A. (2002) Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* **100**, 2692–2696
- 40 Diebold, B. A. and Bokoch, G. M. (2001) Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. *Nat. Immunol.* **2**, 211–215
- 41 Geiszt, M., Dagher, M. C., Molnar, G., Havasi, A., Faure, J., Paclet, M. H., Morel, F. and Ligeti, E. (2001) Characterization of membrane-localized and cytosolic Rac-GTPase-activating proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase. *Biochem. J.* **355**, 851–858
- 42 Moskwa, P., Dagher, M. C., Paclet, M. H., Morel, F. and Ligeti, E. (2002) Participation of Rac GTPase activating proteins in the deactivation of the phagocytic NADPH oxidase. *Biochemistry* **41**, 10710–10716
- 43 Tong, Q., Gamper, N., Medina, J. L., Shapiro, M. S. and Stockand, J. D. (2004) Direct activation of the epithelial Na(+) channel by phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate produced by phosphoinositide 3-OH kinase. *J. Biol. Chem.* **279**, 22654–22663
- 44 Xu, Y., Seet, L. F., Hanson, B. and Hong, W. (2001) The Phox homology (PX) domain, a new player in phosphoinositide signalling. *Biochem. J.* **360**, 513–530
- 45 Mizrahi, A., Molshanski-Mor, S., Weinbaum, C., Zheng, Y., Hirshberg, M. and Pick, E. (2005) Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase. *J. Biol. Chem.* **280**, 3802–3811
- 46 Harbecke, O., Liu, L., Karlsson, A. and Dahlgren, C. (1997) Desensitization of the fMLP-induced NADPH-oxidase response in human neutrophils is lacking in okadaic acid-treated cells. *J. Leukocyte Biol.* **61**, 753–758
- 47 Shyng, S. L., Barbieri, A., Gumusboga, A., Cukras, C., Pike, L., Davis, J. N., Stahl, P. D. and Nichols, C. G. (2000) Modulation of nucleotide sensitivity of ATP-sensitive potassium channels by phosphatidylinositol-4-phosphate 5-kinase. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 937–941
- 48 Demaurex, N., Romanek, R. R., Orlowski, J. and Grinstein, S. (1997) ATP dependence of Na⁺/H⁺ exchange. Nucleotide specificity and assessment of the role of phospholipids. *J. Gen. Physiol.* **109**, 117–128
- 49 Fuster, D., Moe, O. W. and Hilgemann, D. W. (2004) Lipid- and mechanosensitivities of sodium/hydrogen exchangers analyzed by electrical methods. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10482–10487

Received 19 April 2006/7 July 2006; accepted 14 July 2006

Published as BJ Immediate Publication 14 July 2006, doi:10.1042/BJ20060578