Sustained activation of proton channels and NADPH oxidase in human eosinophils and murine granulocytes requires PKC but not cPLA$_2\alpha$ activity

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The prevailing hypothesis that a signalling pathway involving cPLA$_2\alpha$ is required to enhance the gating of the voltage-gated proton channel associated with NADPH oxidase was tested in human eosinophils and murine granulocytes. This hypothesis invokes arachidonic acid (AA) liberated by cPLA$_2\alpha$ as a final activator of proton channels. In human eosinophils studied in the perforated-patch configuration, phorbol myristate acetate (PMA) stimulation elicited NADPH oxidase-generated electron current (I_e) and enhanced proton channel gating identically in the presence or absence of three specific cPLA$_2\alpha$ inhibitors, Wyeth-1, pyrrolidine-2 and AACOCF$_3$ (arachidonyl trifluoromethyl ketone). In contrast, PKC inhibitors GFX (GF109203X) or staurosporine prevented the activation of either proton channels or NADPH oxidase. PKC inhibition during the respiratory burst reversed the activation of both molecules, suggesting that ongoing phosphorylation is required. This effect of GFX was inhibited by okadaic acid, implicating phosphatases in proton channel deactivation. Proton channel activation by AA was partially reversed by GFX or staurosporine, indicating that AA effects are due in part to activation of PKC. In granulocytes from mice with the cPLA$_2\alpha$ gene disrupted (knockout mice), PMA or fMetLeuPhe activated NADPH oxidase and proton channels in a manner indistinguishable from the responses of control cells. Thus, cPLA$_2\alpha$ is not essential to activate the proton conductance or for a normal respiratory burst. Instead, phosphorylation of the proton channel or an activating molecule converts the channel to its activated gating mode. The existing paradigm for regulation of the concerted activity of proton channels and NADPH oxidase must be revised.

(Resubmitted 3 November 2006; accepted after revision 18 December 2006; first published online 21 December 2006)
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Crucial to innate immunity, the NADPH oxidase complex produces the superoxide anion ($O_2^{\cdot -}$), a precursor to many other reactive oxygen species. Because NADPH oxidase is electrogenic (Henderson *et al.* 1987; Schrenzel *et al.* 1998), sustained activity requires activation of voltage-gated proton channels to compensate charge (Henderson *et al.* 1988; DeCoursey *et al.* 2003). For over a decade, it seemed fairly well established that phospholipase A_2 (PLA_2) contributed to activation of NADPH oxidase (Maridonneau-Parini & Tauber, 1986; Henderson *et al.* 1989), presumably by liberating arachidonic acid (AA). Abundant evidence (summarized by DeCoursey & Cherny, 1993; DeCoursey, 2003) established that AA is released by phagocytes during the respiratory burst and that AA itself is a powerful stimulus for $O_2^{\cdot -}$ generation, both in intact cells and in cell-free systems (the NADPH oxidase complex reconstructed from its component parts). The cell-free system requires AA or another amphiphile to activate NADPH oxidase (McPhail *et al.* 1985; Bromberg & Pick, 1985). Despite the evidence that AA *can* activate NADPH oxidase, the question whether it does so under physiological conditions has been controversial. For example, although a variety of PLA_2 inhibitors inhibit $O_2^{\cdot -}$ release (Henderson *et al.* 1989; Dana *et al.* 1994; Daniels *et al.* 1998), others do not (Tsunawaki & Nathan, 1986; Suszták *et al.* 1997; Daniels *et al.* 1998; Mollapour *et al.* 2001). In some studies, PLA_2 inhibitors, now known to be of questionable potency and specificity, prevented the respiratory burst stimulated by AA or fMetLeuPhe, but not that by PMA (Maridonneau-Parini & Tauber, 1986; O'Dowd *et al.* 2004). Several PLA_2 inhibitors were shown to act in macrophages by inhibiting glucose uptake rather

 DOI: 10.1113/jphysiol.2006.124248

than NADPH oxidase activity (Tsunawaki & Nathan, 1986). In eosinophils, PLA_2 inhibitors prevented $O_2^{\cdot -}$ production, but the response was not restored by AA (White *et al.* 1993), in contrast with neutrophils in which AA restored the burst after PLA_2 inhibition (Henderson *et al.* 1989; Dana *et al.* 1994). In the context of confusing and contradictory pharmacological studies, the use of antisense to create a $cPLA_2\alpha$ deficient PLB-985 cell line (Dana *et al.* 1998) promised to clarify the situation. The $cPLA_2\alpha$ deficient cells failed to produce $O_2^{\cdot -}$ upon stimulation, and the response was fully restored by exogenous AA (Dana *et al.* 1998), apparently confirming a requirement for $cPLA_2\alpha$ and AA to activate the respiratory burst. However, in a recent study, a completely normal respiratory burst was observed in the presence of potent and selective $cPLA_2\alpha$ inhibitors that demonstrably prevented arachidonic acid release (Rubin *et al.* 2005). Similarly, PLA_2 inhibitors did not affect $O_2^{\cdot -}$ production in *rac*2 knockout mouse neutrophils stimulated with PMA or AA (Kim & Dinauer, 2006). Finally, macrophages and neutrophils from $cPLA_2\alpha$ knockout mice have a normal respiratory burst despite reduced AA release (Gijón *et al.* 2000; Rubin *et al.* 2005). The present results extend this conclusion, showing that NADPH oxidase activation does not require $cPLA_2\alpha$ in human eosinophils stimulated by PMA or in murine granulocytes stimulated by PMA or fMetLeuPhe.

A similar, but less complete and thus far, less controversial, story exists for voltage-gated proton channels in phagocytes, the main focus of the present study. These channels mediate the proton efflux that balances the electronic charge translocated by NADPH oxidase (Henderson *et al.* 1987; Murphy & DeCoursey, 2006), preventing extreme depolarization that would otherwise abolish NADPH oxidase activity (DeCoursey *et al.* 2003). Henderson & Chappell (1992) proposed that AA was the final, necessary activator of the proton conductance, g_H, during the respiratory burst. For PMA stimulation, the proposed pathway is (Chappell & Henderson, 1991):

$$\text{PMA} \rightarrow \text{PKC} \xrightarrow{\text{phosphorylation}} \text{cPLA}_2\alpha \xrightarrow{\text{synthesis}} \text{AA} \xrightarrow{\text{activation}} \text{H}^*$$

Scheme 1

where H^* indicates the activated gating mode of proton channels (Bánfi *et al.* 1999; DeCoursey *et al.* 2000*b*). In a number of cells, PKC is immediately upstream of $cPLA_2$ (Qiu & Leslie, 1994; Xia *et al.* 1995; Han *et al.* 2004).

Substantial indirect evidence supports this hypothesis. AA enhances proton currents in whole-cell voltage-clamp studies of neutrophils (DeCoursey & Cherny, 1993), macrophages (Kapus *et al.* 1993*b*; Suszták *et al.* 1997), and eosinophils (Gordienko *et al.* 1996; Schrenzel *et al.* 1996). Using pH changes to measure proton fluxes, Kapus *et al.* (1993*b*) found that the PLA_2 inhibitor bromophenacyl bromide prevented the PMA-induced cytoplasmic alkalinization believed to be mediated by proton channels. Suszták *et al.* (1997) found that the $cPLA_2\alpha$ inhibitor $AAOCOCF_3$ prevented PMA- or fMetLeuPhe-induced pH changes, but did not inhibit NADPH oxidase, nor did it directly affect proton currents in whole-cell studies. Schrenzel *et al.* (1996) reported that proton currents were larger in cells with high $[Ca^{2+}]_i$ and that 10 μM bromophenacyl bromide inhibited proton currents, suggesting that proton current enhancement was mediated by PLA_2. Proton currents also were enhanced by AA, even after bromophenacyl bromide, leading to the conclusion that activation of the g_H during the respiratory burst is controlled by elevation of $[Ca^{2+}]_i$, which activates PLA_2 (Schrenzel *et al.* 1996). The final evidence that seemed to confirm this hypothesis was again the $cPLA_2\alpha$ deficient cell line. Levy and colleagues (Lowenthal & Levy, 1999; Levy *et al.* 2000) showed that $cPLA_2\alpha$ deficient PLB-985 cells lacked the Zn^{2+} sensitive alkalinization that is seen in normal cells after stimulation by PMA. AA restored this response. Finally, PMA stimulated proton efflux in PLB-985 cells transfected with a fragment of $gp91^{phox}$, but not in identical cells with $cPLA_2\alpha$ knocked out (Mankelow *et al.* 2003). Thus, in contrast to the NADPH oxidase story, the implication of $cPLA_2\alpha$ and its product AA as the 'final' necessary physiological activators of proton channels seemed to be clearly established.

Despite the unanimity in the literature on the requirement for $cPLA_2\alpha$ to activate the proton conductance, the revision of the NADPH oxidase story (Rubin *et al.* 2005) stimulated us to re-examine the idea that proton channels are obligatorily activated during the respiratory burst by AA generated by $cPLA_2\alpha$. The previously discussed electrophysiological studies of AA effects in phagocytes were all done using the whole-cell configuration, in which the pipette solution replaces the cytoplasm and abolishes many signalling pathways, including the activation of NADPH oxidase and H^+ channels by PMA (DeCoursey *et al.* 2000*b*). In contrast, in the perforated-patch configuration, which preserves the cytoplasm, AA activates electron currents that reflect NADPH oxidase activity, and also dramatically alters the properties of proton channels to promote their opening, which we consider a manifestation of 'activation' of these channels (Cherny *et al.* 2001). The effects of AA on H^+ currents in perforated-patch configuration were more profound than in whole-cell studies, and, except for tail current kinetics, closely resembled the constellation of effects seen with PMA stimulation (DeCoursey *et al.* 2000*b*, 2001*a*) or in cells that are spontaneously activated (perhaps by adherence) (DeCoursey *et al.* 2001*a*). Here we explore the potent and selective $cPLA_2\alpha$ inhibitors pyrrolidine-2, also known as pyrrophenone (Seno *et al.* 2001; Ono *et al.* 2002; Ni *et al.* 2006; Vandal *et al.* 2006), Wyeth-1 (Ni *et al.* 2006), and $AACOCF_3$ (Street *et al.* 1993; Bartoli *et al.* 1994; Riendeau *et al.* 1994), in human eosinophils studied in the

perforated-patch configuration. Surprisingly, we found no evidence that cPLA$_2\alpha$ is necessary for activation of proton channels. This conclusion was tested independently by examining the activation of granulocytes from cPLA$_2\alpha$ knockout mice. The phenomenology of proton channel activation in these mice was indistinguishable from that in control mice. We conclude that AA generated by cPLA$_2\alpha$ is not a necessary component of the signalling pathway that leads to activation of voltage-gated proton channels. In contrast, we show that PKC inhibition prevents the proton channel response and can also reverse the response. Thus, the activation of proton channels is stimulated and sustained by PKC via a pathway that does not require cPLA$_2\alpha$.

Methods

Isolation of human neutrophils and eosinophils

Venous blood was drawn from healthy adult donors under informed written consent in accordance with the procedures outlined by the Rush University Institutional Review Board, Federal regulations, and the *Declaration of Helsinki*. Neutrophils were isolated by density gradient centrifugation (Boyum, 1968). Blood was mixed into a sterile 0.9% sodium chloride solution with 3 mM EDTA and layered onto Lymphocyte Separation Medium (LSM, Cambrex Bioscience, Walkersville, MD, USA). The blood was then separated by centrifugation at 400 *g* at room temperature (21–24°C) for 30 min. Mononuclear cells and plasma were aspirated off and the pellet resuspended in ice-cold distilled water for 10–20 s to lyse the red blood cells. The water–cell mixture was diluted with ice-cold 2 × Hanks' balanced salt solution (HBSS) with 5 mM Hepes (adjusted to pH 7.4) and centrifuged at 350 *g* for 10 min at 4°C, and then this process was repeated. The pellet was then resuspended in 1 × HBSS with 2.5 mM Hepes.

Eosinophils were isolated from the neutrophil preparation by negative selection. After counting, 1×10^7 cells were seeded in 10 μl PBS with 2 mM EDTA and 0.5% bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) and mixed with an equal volume (10 μl) of MACS CD16 MicroBeads (Miltenyi Biotec, Auburn, CA, USA). The resulting mixture was incubated at 4°C for 45 min, mixing by pipette every 10 min. The beads–cells mixture was diluted with 0.5 ml PBS–EDTA–BSA (with EDTA and BSA as described above), and then added to a magnetized column. The column was washed twice with PBS–EDTA–BSA. Cells were kept on ice in this solution or resuspended in RPMI medium with granulocyte–macrophage colony-stimulating factor (GM-CSF) (at a working concentration of 1 ng ml^{-1}) and stored in an incubator. The eosinophils or neutrophils were suspended in PBS with 2 mM EDTA and 0.5% BSA. Neutrophil purity was routinely 95% or greater. Eosinophil purity was typically > 98% as determined by counting Wright-stained cytospin preparations.

Murine granulocytes

C57BL/6 mice with the cPLA$_2\alpha$ gene disrupted (cPLA$_2\alpha$ knockout mice) were obtained from Professor T. Shimizu (University of Tokyo, Japan). The cPLA$_2\alpha$ transgene is in a C57BL/6 background and is a targeted vector using a PGK-*neo* expression cassette to disrupt the endogenous cPLA$_2\alpha$ gene. The genotype of the mice studied was verified by using PCR to amplify the gene, as follows. One forward primer, cPLAF (5′-TTC TCT GGT GTG ATG AAG GC-3′), was designed against an exonic sequence and a reverse primer, cPLAR (5′-AAA CTG ACT GTA GCA TCA CAC-3′), was designed to recognize a downstream intron. A third primer was useful in keeping the diagnostic product bands within a range compatible with the selected polymerase. The third primer, NeoF2 (5′-ATC GCC TTC TTG ACG AGT TC-3′), was designed to recognize a sequence in the 3-prime side of the poly-A region of the PGK-*neo* cassette. In a multiplex format (all three primers in one PCR reaction), the expected PCR products are 570 bp for the knockout allele and 224 bp for the wild-type allele. PCR conditions described are for the Qiagen Hot Start TAQ Polymerase Kit (Qiagen, Valencia, CA, USA) with 2.5 mM $MgCl_2$ final concentration. After an initial denaturation step at 94°C for 15 min, 35 cycles of PCR were performed (94°C for 45 s, 55°C for 1 min, and 72°C for 1 min), with a final elongation cycle of 72°C for 10 min. PCR products were resolved on a 2% agarose gel with ethidium bromide. Further details are described elsewhere (Bonventre *et al.* 1997).

We used three strains of control mice: BALB-c, SCID mice (in BALB-c background) obtained from the National Canter Institute, and C57BL/6 mice that were littermates of the KO mice. The bulk of the control data were collected on C57BL/6 mice. All animal protocols were approved by the Rush University Medical Center Institutional Animal Care and Use Committee, and are in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Surgical procedures were performed in mice anaesthetized by ketamine (100 mg kg^{-1}) and xylazine (5 mg kg^{-1}) administered intraperitoneally. Granulocytes were isolated from blood obtained either by cardiac puncture or from the supraorbital plexus of anaesthetized mice. The mice were killed while under anaesthesia, by cervical dislocation. Granulocytes were isolated using Lympholyte-M or Lympholyte-Mammal (Cedarlane Laboratories Ltd, Burlington, NC, USA). A small volume of blood (< 0.5 ml) was diluted into 3 ml EDTA–saline solution (PBS with 2 mM EDTA and 0.5% BSA), layered onto 3 ml Lympholyte in a 15 ml tube and centrifuged at 800 *g* for 20 min at room temperature. The layer above the Lympholyte (containing lymphocytes and monocytes) was

discarded and the pellet (containing granulocytes and red cells) was resuspended in ice-cold sterile water for 10 s (to lyse the red cells), then diluted with an equal volume of 2 × HBSS (with 5 mM Hepes at pH 7.4). After mixing, the result was centrifuged at 4°C for 10 min at 400 *g*. This step was repeated and then the pellet was resuspended in PBS with 2 mM EDTA and 0.5% BSA. Cells were kept on ice until use. We selected non-adherent cells for recording.

H_2O_2 measurement

H_2O_2 release (next paragraph) was measured using an Amplex red kit purchased from Molecular Probes (Eugene, OR, USA) according to the instructions. Fluorometric measurements were done in a volume of 120 μl, with typically 2×10^4 cells per well. Absolute concentrations of H_2O_2 were determined from a calibration curve. $AACOCF_3$ produced a baseline signal that was subtracted from data at subsequent time points.

cPLA$_2\alpha$ inhibitors and other materials

Pyrrolidine-2 and Wyeth-1 were prepared as described (Ni *et al.* 2006), and used at concentrations shown previously to abolish cPLA$_2\alpha$ activity (Seno *et al.* 2001; Ono *et al.* 2002; Ni *et al.* 2006; Vandal *et al.* 2006). Aliquots of $AACOCF_3$ (Calbiochem, San Diego, CA, USA) were kept at −20°C in argon-filled vials. The AA analogue, $AACOCF_3$, reportedly prevents activation of proton flux by PMA or fMetLeuPhe (Suszták *et al.* 1997). The IC_{50} of $AACOCF_3$ for inhibiting AA release is 2–10 μM in platelets (Bartoli *et al.* 1994) and U937 cells (Riendeau *et al.* 1994). The IC_{50} of $AACOCF_3$ reported to prevent proton channel activation is also 3–5 μM (Suszták *et al.* 1997). In preliminary experiments, we examined its effects on NADPH oxidase activity, assessed as H_2O_2 production. $AACOCF_3$ alone (10–100 μM) did not elicit significant H_2O_2 production. $AACOCF_3$ inhibited PMA-stimulated H_2O_2 release significantly only at high concentration (100 μM, $n = 6$, ANOVA, $P < 0.01$). Thus NADPH oxidase activity is not inhibited at concentrations that inhibit cPLA$_2\alpha$ activity as assessed directly by AA release (Street *et al.* 1993; Bartoli *et al.* 1994; Riendeau *et al.* 1994) or proton channel activation assessed by pH changes (Suszták *et al.* 1997). Concentrations of $AACOCF_3$ used here (up to 20 μM) were sufficient to inhibit cPLA$_2\alpha$ activity (Street *et al.* 1993; Bartoli *et al.* 1994; Riendeau *et al.* 1994), but lower than those that inhibit H_2O_2 release.

Stock solutions (5 mM) of arachidonic acid (AA) and oleic acid (both from Sigma) were prepared in 50% ethanol ('vodka') and aliquots were stored in argon or nitrogen atmosphere at −20°C. PMA, DPI (diphenylene iodonium) and staurosporine were obtained from Sigma, GFX (GF109203x) was from Calbiochem, and okadaic acid was from LC Laboratories (Woburn, MA, USA)

Electrophysiology

The data collection setups and analysis software have been previously described (Morgan *et al.* 2003). Pipettes were made from 8250 glass (Garner Glass Co., Claremont, CA, USA). Seals were formed with Ringer solution (mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.4) in the bath, and the potential zeroed after the pipette was in contact with the cell. The pipette solution for perforated patch recording contained 128 mM KCH_3SO_3 or 130 mM tetramethylammonium methanesulphonate ($TMAMeSO_3$), 50 mM NH_4^+ in the form of 25 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 10 mM BES buffer and 1 mM EGTA, and was titrated to pH 7.0 with KOH or tetramethylammonium hydroxide (TMAOH). Then ∼500 μg ml^{-1} solubilized amphotericin B (∼45% purity) (Sigma) was added. The pipette was backfilled after first dipping the pipette tip in amphotericin-free solution. The bath solution contained (mM): 130 $TMAMeSO_3$, 25 $(NH_4)_2SO_4$, 2 $MgCl_2$, 1.5 $CaCl_2$, 1 EGTA, and 10 BES at pH 7.0. All solutions were roughly 300 mosmol l^{-1}. Studies were done at room temperature (20–25°C).

Most data were acquired with a sampling rate of 50–200 Hz and lowpass filtering at 10–100 Hz. For the purpose of displaying long records (e.g. Fig. 7) the data were digitally re-filtered at 2 Hz in order to show the amplitude of the H^+ current, without its being obscured by capacity transients. This filtering attenuated the capacity current spikes with little effect on the proton current.

Results

cPLA$_2\alpha$ inhibition does not prevent the response of NADPH oxidase or proton channels to PMA in human eosinophils

The activation of NADPH oxidase can be detected in individual granulocytes studied in the perforated-patch configuration as an inward electron current (I_e) (DeCoursey *et al.* 2000*b*). Figure 1*A* illustrates the response of a human eosinophil stimulated with 60 nM PMA. Robust I_e was also activated in the presence of two specific cPLA$_2\alpha$ inhibitors, 10 μM Wyeth-1 (Fig. 1*B*) and 5 μM pyrrolidine-2 (Fig. 1*C*). The mean I_e was the same in cells stimulated by PMA in the absence or presence of cPLA$_2\alpha$ inhibitors (Table 1). Although the kinetics of I_e turn-on differs in these cells, this variability occurs normally, and the time course did not noticeably depend on the cPLA$_2\alpha$ inhibitor used. That the inward current activated by PMA is I_e generated by NADPH oxidase was confirmed by its sensitivity to inhibition by 10 μM diphenylene iodonium (DPI) (e.g. Fig. 1*B* and *C*). Of 14 cells pretreated with cPLA$_2\alpha$ inhibitors, inward current that was clearly inhibited by DPI was seen in 12, with two being equivocal. Evidently, NADPH oxidase in human eosinophils can be activated by PMA without the involvement of cPLA$_2\alpha$.

This result extends similar conclusions reached in human neutrophils stimulated by PMA or opsonized zymosan (Rubin *et al.* 2005).

In contrast, pretreatment with 1 μM GFX (GF109203X), a PKC inhibitor, prevented any I_e response to PMA (Fig. 1*D*) in all 13 cells tested ($P < 0.001$ by a χ^2 test *versus* PMA stimulation after pretreatment with cPLA$_2\alpha$ inhibitors). Eosinophils studied in the perforated patch configuration occasionally convert to the whole-cell configuration via spontaneous patch rupture (Morgan *et al.* 2003). No response to PMA occurs in the whole-cell configuration (DeCoursey *et al.* 2000*b*); thus we confirmed the configuration by including Lucifer yellow in the pipette solution in some experiments. Lucifer yellow rapidly enters the cell upon patch rupture (Morgan *et al.* 2003). Absence of Lucifer yellow fluorescence in the cell confirmed that the patch was intact in three cells pretreated with 1 μM GFX that failed to respond to PMA. Pretreatment with staurosporine, another PKC inhibitor, also prevented activation by PMA in three cells. In two of these cells, after washout of staurosporine, PMA elicited a normal response (both I_e and enhanced proton current, I_H) that could then be inhibited by staurosporine (*vide infra*). The reversibility of staurosporine allows confirmation that the configuration is perforated patch and that the cell is responsive.

In addition to reliably activating NADPH oxidase, PMA profoundly alters the gating kinetics of voltage-gated proton channels, greatly enhancing their probability of opening (DeCoursey *et al.* 2000*b*, 2001*a*). Proton currents measured at various times (indicated by lower case letters) during the experiments in Fig. 1 are superimposed to the right of the I_e records. Characteristically drastic changes in proton channel gating (increased g_H, faster activation, slower deactivation) are evident in the response to PMA of the untreated human eosinophil (Fig. 1*A*) and the cells pretreated with Wyeth-1 (Fig. 1*B*) or pyrrolidine-2 (Fig. 1*C*). Evidently, the response of voltage-gated proton channels to PMA in human eosinophils does not require functional cPLA$_2\alpha$. Pretreatment with 1 μM GFX

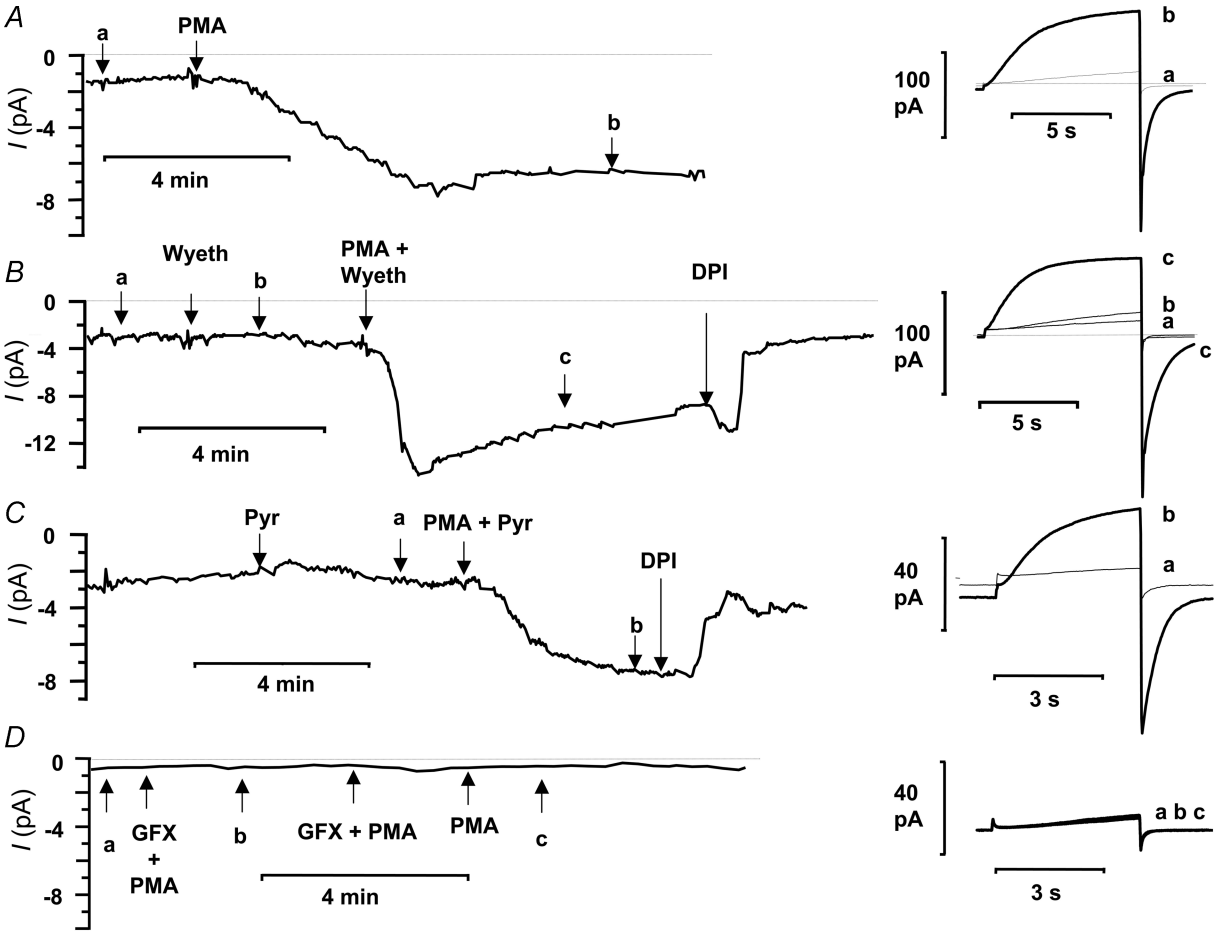

Figure 1. cPLA$_2\alpha$ inhibitors have no effect on the activation of NADPH oxidase or H$^+$ current by PMA
Records on the left show the current at −60 mV in human eosinophils in the perforated patch configuration. The traces on the right show proton currents in response to pulses to +60 mV applied at the time points indicated by lower case letters. Currents after PMA stimulation are shown as darker lines. Bath and pipette solutions consisted of TMA $MeSO_3$ at pH 7. Cells were pretreated with nothing (*A*), 10 μM Wyeth-1 (*B*), 5 μM pyrrolidine-2 (*C*), or 2 μM G109203FX (*D*) and then stimulated with 60 nM PMA.

Table 1. Comparison of PMA responses of human eosinophils in the absence or presence of cPLA$_2\alpha$ inhibitors

	PMA	PMA*	Pyrrolidine-2	Wyeth-1
I_H (PMA/control)	13.5 ± 3.9 (8)	—	12.6 ± 2.2 (4)	11.3 ± 2.1 (4)
$g_{H,max}$ (PMA/control)	3.9 ± 1.0 (4)	—	2.3 ± 0.4 (3)	2.5 ± 0.4 (3)
τ_{act} (control/PMA)	3.4 ± 0.7 (5)	4.2	3.7 ± 1.7 (4)	4.7 ± 2.1 (4)
τ_{tail} (PMA/control)	3.1 ± 0.5 (6)	5.4	4.0 ± 0.6 (5)	4.5 ± 0.5 (5)
$V_{threshold}$ shift (mV)	−36.6 ± 12.4 (6)	−40	−52.5 ± 9.6 (5)	−50 ± 8 (3)
I_e (pA)	−5.9 ± 1.1 (9)	−6.0	−5.0 ± 0.4 (5)	−5.5 ± 1.0 (4)

Most parameters (mean ± S.E.M. (*n*)) are compared by taking the ratio of values from currents elicited before and a few minutes after PMA. I_H is the amplitude of time-dependent outward current at the end of a 6–8 s pulse (usually to +40 mV or +60 mV). The $g_{H,max}$ was determined as the largest g_H in families of pulses. The rising current was fitted by a single exponential to obtain the activation time constant, τ_{act}. Tail current time constants, τ_{tail}, were measured at −60 mV. $V_{threshold}$ shift is the change after PMA stimulation in the threshold voltage at which I_H was first detectable, $V_{threshold}$. I_e is the net inward current at −60 mV elicited by PMA. Where indicated, cells were pretreated with 1–10 μM pyrrolidine-2 or 10–20 μM Wyeth-1. *Mean values from a previous study of human eosinophils under similar conditions (DeCoursey *et al.* 2001*a*). In that study, I_H and g_H were quantified differently and thus are not directly comparable with the present data.

prevented the proton channel response to PMA (Fig. 1*D*), consistent with a previous report (Bankers-Fulbright *et al.* 2001). We found previously that a lower concentration of GFX (200 nM) reduced, but did not abolish, the PMA response in human neutrophils (DeCoursey *et al.* 2000*a*). GFX, staurosporine, and cPLA$_2\alpha$ inhibitors had no detectable effect on proton currents in unstimulated, resting cells.

The several responses of proton channels to stimulation occur at different rates (DeCoursey *et al.* 2000*b*, 2001*b*) and exhibit differential sensitivity to DPI (DeCoursey *et al.* 2000*b*, 2001*a*), which might indicate that the responses are not monolithic. To evaluate the changes in proton channel gating in more detail, we compared families of H^+ currents recorded during an identical series of depolarizing pulses in each cell before and after PMA stimulation (Fig. 2*A*). Analogous measurements in an eosinophil pretreated with pyrrolidine-2 (Fig. 2*B*) produced responses similar to those in the control cell in Fig. 2*A*. Figure 3*A* summarizes these results together with data from other experiments in which cells were pretreated with AACOCF$_3$, Wyeth-1, or GFX. Average g_H–V (g_H *versus* voltage) relationships before and after PMA stimulation are plotted in Fig. 3*A* (■ and ▲, respectively), revealing a roughly 40 mV hyperpolarizing shift and an increase in the maximum g_H ($g_{H,max}$) that are characteristic of the PMA response (DeCoursey *et al.* 2000*b*, 2001*a*). The average g_H–V relationships of PMA-stimulated cells were identical whether they were pretreated with cPLA$_2\alpha$ inhibitors or not. In contrast, cells pretreated with the PKC inhibitor GFX (○) did not respond to PMA.

In Fig. 3*B*, the faster activation of PMA-stimulated proton currents is compared in the presence or absence of the signalling inhibitors. Currents were fitted by a single rising exponential to obtain the activation time constant (τ_{act}). Confirming the impression of the I_H responses in Figs 1 and 2, Fig. 3*B* shows that PMA decreased τ_{act} to a similar extent at all voltages in the presence or absence of the cPLA$_2\alpha$ inhibitors pyrrolidine-2 or Wyeth-1. Furthermore, cPLA$_2\alpha$ inhibitors did not prevent the slowing of tail current deactivation (τ_{tail}; Table 1). The responses of human eosinophils to PMA that are summarized in Table 1 indicate no effect of pretreatment with cPLA$_2\alpha$ inhibitors. Responses of all cells are similar to those previously reported in human neutrophils and eosinophils (DeCoursey *et al.* 2000*b*, 2001*a*).

GFX reverses the activation of NADPH oxidase and proton channels

Pretreatment with GFX prevented activation of NADPH oxidase and proton currents, as shown in Fig. 1*D*. GFX presumably prevents the phosphorylation of the cytosolic oxidase components, especially p47^{phox}, and thereby prevents assembly of the NADPH oxidase complex (Babior, 1999). In the absence of a known mechanism, it might be speculated that GFX prevents the phosphorylation of the proton channel itself or an activating molecule. Given these mechanisms, it was not clear whether the activation proton currents would be reversible. However, as shown in Fig. 4, addition of 3 μM GFX at the peak of the PMA response reversed the activation of both molecules. The time course of these GFX actions exhibited marked variability and differed even in the same cell. In the example in Fig. 4, there was a pronounced delay before I_e began to decline. In other cells, this delay ranged from 0 to 3 min and appeared to be independent of GFX concentration (100 nM to 3 μM).

In contrast, the proton current, I_H, usually responded almost immediately. For example, in Fig. 4, I_e had hardly changed by time point 'c', but already I_H was distinctly attenuated. Once I_e began to decay, however, it decreased marginally faster ($\tau = 99 \pm 49$ s, mean $\pm$ S.D., $n = 8$) than did I_H ($\tau = 140 \pm 59$ s, $n = 9$) ($P = 0.07$, but $P = 0.01$ for a paired t test with $n = 7$). In a minority of cells, GFX even at high concentration did not produce effects within a few minutes. Although we detected no concentration dependence of the rate of action of GFX on either molecule, low concentrations of GFX (50–200 nM) inhibited I_e only partially, whereas 2–3 μM GFX effectively abolished I_e in most cells. Proton currents appeared never to be completely restored to their preactivated state. However, the extent of deactivation is difficult to quantify, because there is a tendency for H^+ currents to increase gradually during experiments. In four cells activated by PMA, addition of 100 nM staurosporine attenuated I_H and I_e, closely resembling the effects of GFX. Overall, the effects of GFX are consistent with independent regulation of proton channels and NADPH oxidase. Bankers-Fulbright *et al.* (2001) reached a generally similar conclusion, because rottlerin inhibited NADPH oxidase activity but not the activation of the proton conductance. Taken together, the kinetics of these responses suggests that the signalling pathways immediately proximal to the activation of the two molecules may be distinct, and the regulatory mechanisms that govern the level of activation also may be distinct.

Because PMA activates PKC, it is of interest whether PKC inhibitors also inhibit the responses when the respiratory burst is triggered by other kinds of stimuli. Effects of PKC inhibition on activation by AA or oleic acid are described below. Some eosinophils become activated spontaneously, possibly in response to contact with the glass microelectrode. Figure 5 illustrates currents recorded in a cell that is representative of four spontaneously activated cells tested. Addition of 100 nM staurosporine reversed the

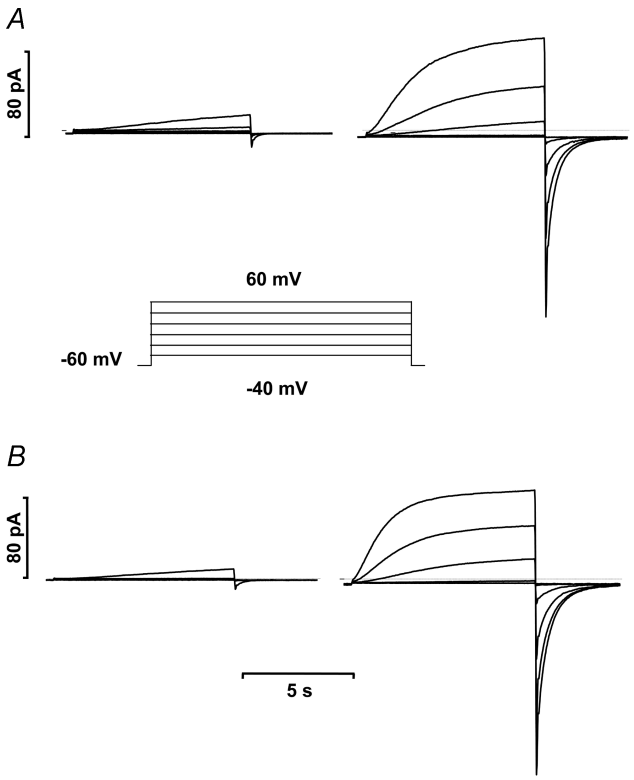

Figure 2. Proton channel 'activation' occurs despite cPLA$_2\alpha$ inhibition
Proton currents elicited by identical families of 8 s voltage pulses in 20 mV steps from −40 mV to 60 mV (inset) in human eosinophils before (left) or after (right) stimulation with 60 nM PMA. The eosinophil in *A* was untreated, that in *B* was pretreated with 10 μM pyrrolidine-2.

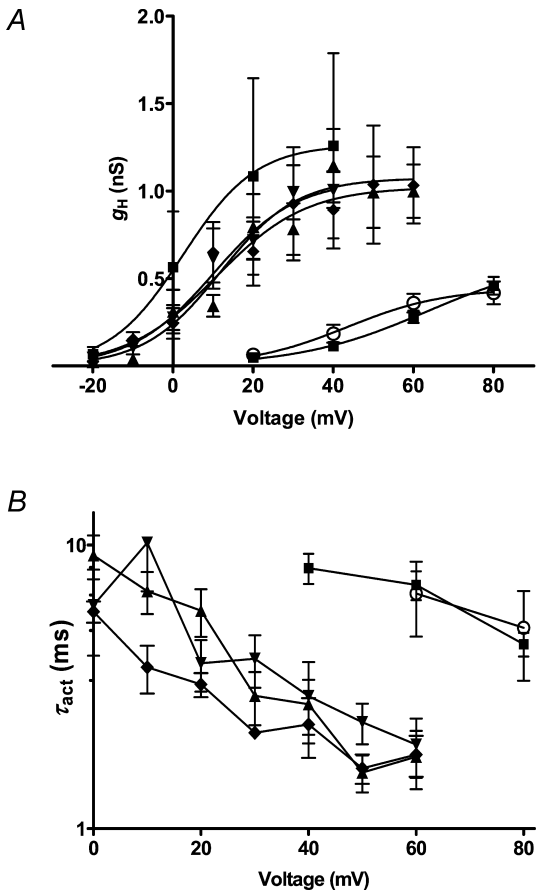

Figure 3. Enhanced proton channel gating occurs despite cPLA$_2\alpha$ inhibition
A, summary of g_H–V (mean $\pm$ S.E.M) relationships before (■, $n = 9$) or after stimulation with PMA alone (▲, $n = 9$) or following pretreatment with 10–20 μM pyrrolidine-2 (▼, $n = 5$), 1–10 μM Wyeth-1 (♦, $n = 5$), 10–20 μM AAOCOF$_3$ (■, $n = 5$), or 0.2–3 μM GFX (○, $n = 8$). Data are fitted with Boltzmann curves constrained to limit to 0. Conductance was calculated from I_H measured at the end of the pulse, with leak subtracted. In most cases, V_{rev} was measured, or was assumed to be 0 mV. *B*, the time constant of activation (τ_{act}) of proton currents in human eosinophils before (■, $n = 20$) or after stimulation with 60 nM PMA (▲, $n = 9$), in the presence of 10–20 μM pyrrolidine-2 (▼, $n = 6$), 10–20 μM Wyeth-1 (♦, $n = 5$) and 1 μM GFX (○, $n = 6$). The turn-on of proton current during depolarizing pulses was fitted by a single exponential, whose time constant is plotted here (mean $\pm$ S.E.M).

activation of both I_e and I_H. The proton current began to decrease immediately, but I_e decreased after a delay, a time course reminiscent of that for addition of GFX to PMA-stimulated cells.

Okadaic acid preserves the activated state of proton channels during GFX exposure

The failure of cPLA$_2\alpha$ inhibitors to prevent activation of proton channels rules out Scheme 1. However, proton channels might also be activated by an alternative signalling pathway, as follows:

$$\text{PMA} \rightarrow \text{PKC} \begin{cases} \rightarrow \text{cPLA}_2\alpha \rightarrow \text{AA} \xrightarrow{direct} \text{H channel}^* \\ \xrightarrow[phosphorylation]{} \; ? \; \xrightarrow[indirect]{} \text{H channel}^* \end{cases}$$

Scheme 2

Here PMA can activate proton channels either via cPLA$_2\alpha$ and AA or by phosphorylation of the channel or an intermediary.

If activation of the proton channel by PMA is due to phosphorylation, the return toward the resting state that is induced by GFX may reflect ongoing phosphatase activity. Figure 6 illustrates the average time course of deactivation of proton currents in eosinophils that were activated with PMA, then treated with GFX in the presence (□) or absence (▲) of okadaic acid, a phosphatase inhibitor (Bialojan & Takai, 1988). After a transient increase due to temperature, I_H decreased by > 60% in the presence of GFX alone. Okadaic acid greatly reduced the extent of deactivation. To the extent that okadaic acid acted by inhibiting phosphatases, this result supports the idea that the proton channel is activated by phosphorylation of either the channel itself or a regulatory intermediary. Usually okadaic acid was added simultaneously with PMA. Pretreatment of two cells with okadaic acid for 5 min did not noticeably activate either I_H or I_e, although both were activated by subsequent addition of PMA.

Activation of proton channels by AA is also sensitive to PKC inhibitors

The observation that AA has substantially greater effects on proton currents in perforated-patch than in the whole-cell configuration (Cherny *et al.* 2001) suggests that diffusible second messengers contribute to its effects in perforated-patch studies. The possibility that AA might activate PKC was explored using GFX or staurosporine. Figure 7*A* illustrates the response to AA of an eosinophil in perforated patch configuration. After addition of 5 μM AA, I_H increased progressively for several minutes. The inward current at the holding potential, − 60 mV,

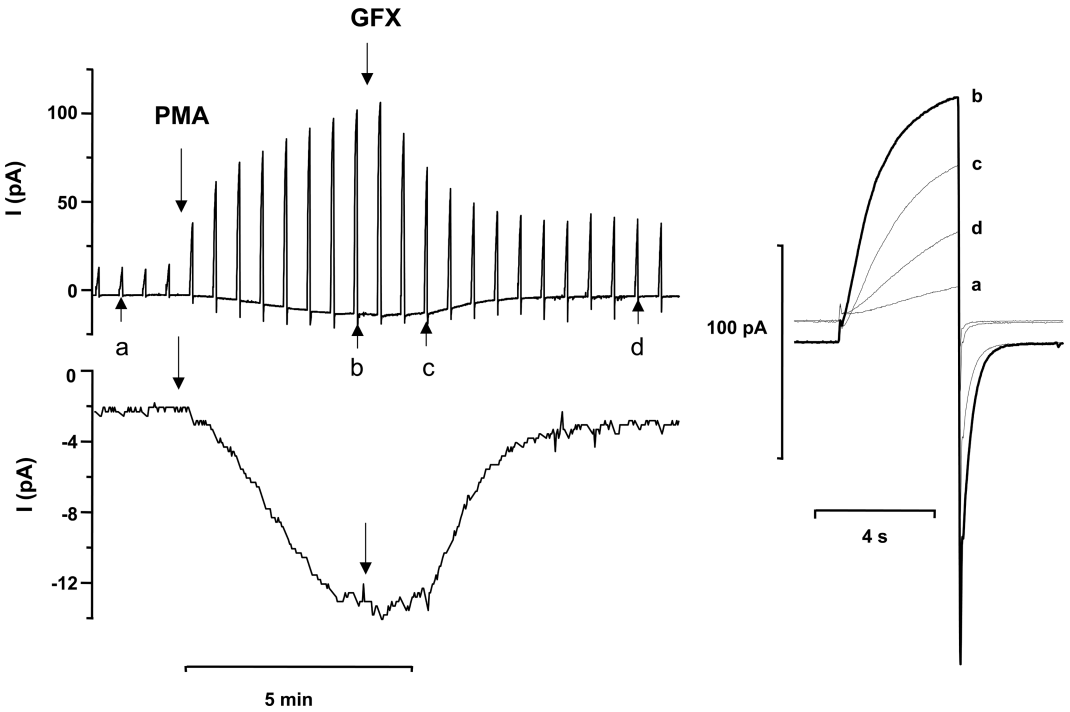

Figure 4. GFX reverses activation of both NADPH oxidase and proton channels in human eosinophils
Current at −60 mV (left records) in a human eosinophil in the perforated patch configuration stimulated with PMA and then treated with 3 μM GFX. The upper record shows currents during steps to +60 mV applied every 30 s, as well as the current at the holding potential, −60 mV. The lower record shows the same holding current at higher gain, with the currents during the test pulses blanked. On the right are proton currents during pulses to +60 mV recorded in this experiment at the times indicated by lower case letters.

also increased progressively. We interpret the increase in inward current as electron current, I_e. Addition of 100 nM staurosporine in the continued presence of the same AA concentration (followed by stirring) inhibited I_e and progressively reduced I_H, although not to the level before stimulation with AA. GFX (1.5–3.0 μM, $n = 4$) and staurosporine (100 nM, $n = 3$) had similar effects, reducing proton currents and I_e after activation with AA. Evidently, part of the effect of AA on proton currents is mediated by PKC, and is reversed by PKC inhibition. In addition, PKC inhibitors inhibited I_e despite the continued presence of AA in the bath. Evidently, both NADPH oxidase and the enhanced gating mode of proton channels are sustained by continuous phosphorylation, the prevention of which results in dephosphorylation and deactivation of both molecules.

Families of H^+ currents during the experiment in Fig. 7*A* are illustrated in Fig. 7*B–D*. AA greatly increased I_H, accelerated activation of the H^+ current during voltage pulses, and shifted the threshold for activating the conductance ($V_{threshold}$) toward more negative values. In addition, the inward current at −60 mV increased, reflecting I_e generated by NADPH oxidase activity. Each of these effects was reversed partially by 100 nM staurosporine, added to the bath in the continued presence of AA (Fig. 7*D*). The AA induced inward current cannot be ascribed to leak current because it was abolished by staurosporine in the continued presence of AA. Later, AA was washed out of the bath, and I_H decreased gradually (not shown). The effects of AA that are sensitive to PKC inhibitors presumably reflect the consequences of phosphorylation, whereas the residual effects of AA in the presence of PKC inhibitors reflect direct effects on proton channels (see Discussion).

After a response to AA and subsequent partial reversal by staurosporine (e.g. Fig. 7*A*), the bath could be washed and the entire process repeated, with similar results. Cells that had responded to AA ($n = 3$) or PMA ($n = 2$) and then were inhibited by staurosporine, responded to subsequent exposure to AA with increased I_H and activated I_e, and both responses were again partially reversed by addition of staurosporine to the bath (in the continued presence of AA).

Other unsaturated fatty acids, such as oleic acid, also activate NADPH oxidase (Badwey *et al.* 1984) and enhance proton currents (Kapus *et al.* 1994). In 12 eosinophils in the perforated patch configuration, 2.5–10 μM oleic acid elicited presumed electron current and profound, progressive increases in proton current reminiscent of AA effects (not shown). However, these responses did not stabilize, and without further treatment, all cells rapidly became leaky or spontaneously went to the whole-cell configuration, precluding further tests. In two cells, however, we added 100–300 nM staurosporine shortly after

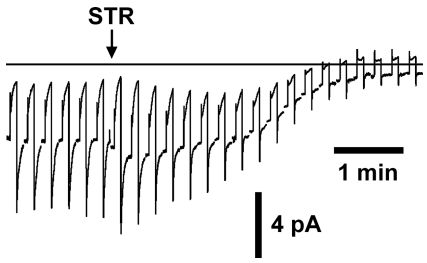

Figure 5. Staurosporine reverses spontaneous activation of both NADPH oxidase and proton channels in human eosinophils
Current recorded shortly after establishing perforated-patch recording. Voltage pulses to +20 mV were applied from a holding potential of −60 mV every 15 s. At the arrow, 100 nM staurosporine was introduced into the bath. The time-dependent outward currents are proton currents; the inward current is presumably largely I_e. The horizontal line indicates zero current. It was evident that this cell was activated because a pulse to −20 mV elicited inward proton current (not shown), something never seen in resting cells. In addition, superposition of the inward current, presumed to be I_e, and the proton current during the test pulses results in a net inward current throughout the pulses to +20 mV, despite the activation of several picoamperes of outward proton current. Capacity transients have been truncated for clarity.

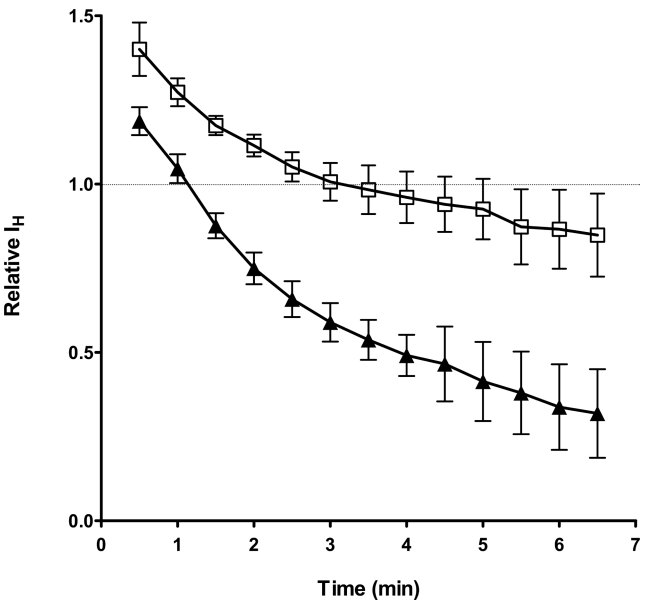

Figure 6. Okadaic acid inhibits the deactivation of proton current induced by GFX
Eosinophils were stimulated with PMA and then 2–3 μM GFX was introduced in the absence (▲) or presence of 100 nM okadaic acid (□) ($n = 5$ cells for both). Test pulses to +60 mV (+40 mV in one cell) were applied every 30 s. The mean (± S.E.M.) leak-corrected I_H (the time-dependent rising current) at the end of the 4 s test pulse is plotted, normalized to its value during the final test pulse before addition of GFX, which is indicated as a horizontal line. All I_H values obtained > 1.5 min after addition GFX in the presence of okadaic acid are significantly greater than those in its absence ($P < 0.05$, by Student's two-tailed t test). The I_H during the first few pulses after the bath change increased presumably due to a transient temperature increase. (Evaporation of the bath reduces its temperature below ambient.)

the progressive increase in I_H began. After a delay during which both I_H and I_e continued to increase progressively, both I_H and I_e stabilized and then began to decrease. Despite the harshness of oleic acid, some of its effects appear to be reversed by PKC inhibition. A minimum estimate of the magnitude of I_H enhancement by fatty acids was obtained by taking the ratio of g_H during the test pulses (I_H is defined here as the time-dependent increase in current during the pulse) in the presence of fatty acid to $g_{H,max}$ determined from the g_H–V relationship and V_{rev} measured before addition. The cells did not survive oleic acid long enough to obtain a full g_H–V relationship in its presence. By this somewhat crude measure, the mean increase in $g_{H,max}$ was 5.0 ± 1.5-fold by AA (mean ± S.D., $n = 10$) and 5.0 ± 2.5-fold by oleic acid ($n = 12$).

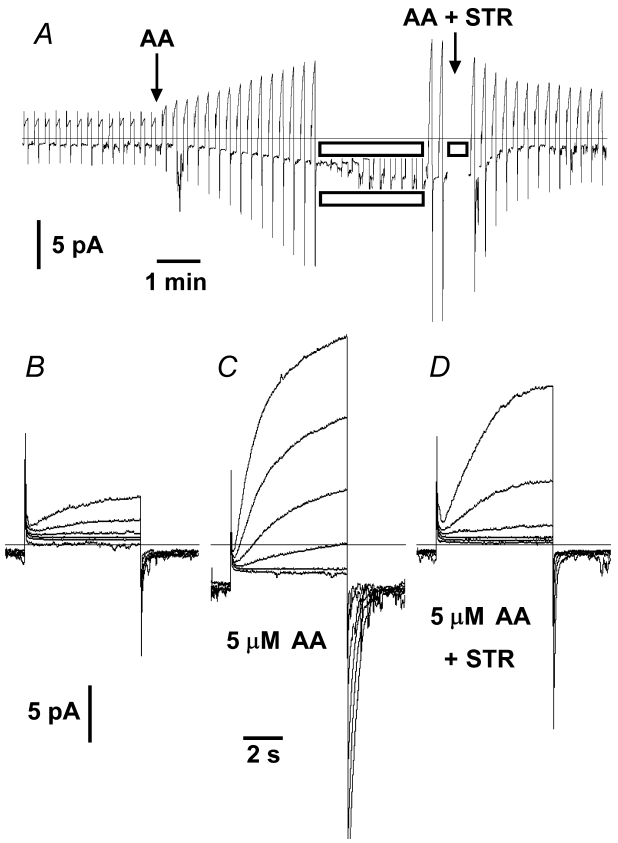

Figure 7. Staurosporine partially reverses the activation of proton currents by AA
A, a continuous record of currents during test pulses to +20 mV applied every 15 s from a holding potential of −60 mV. At the first arrow, 5 μM AA was added. I_e and H^+ currents increased progressively. The interruptions (boxes) are due to recording a family of currents (shown in *C*) and then addition of 100 nM staurosporine (STR) directly into the bath followed by stirring. The horizontal line indicates zero current. Digitally filtered at 2 Hz to reduce capacity transients. *B–D*, currents in the same cell before AA (*B*), in the presence of 5 mM AA (*C*) (the blanked family in *A*), and after addition of 100 nM staurosporine to the bath, in the continued presence of AA (*D*). Currents in *B–D* are from −20 mV to +30 mV in 10 mV increments, from a holding potential of −60 mV.

The responses of NADPH oxidase and proton channels are identical in normal and cPLA$_2\alpha$ knockout mice

The existence of mice with the cPLA$_2\alpha$ gene disrupted (cPLA$_2\alpha$ 'knockout' mice) enables a direct test of the proposed requirement for cPLA$_2\alpha$ in activating proton current during the respiratory burst. It was shown previously that NADPH oxidase activity (assessed as cytochrome *c* reduction) stimulated by PMA or zymosan was similar in normal and cPLA$_2\alpha$ knockout mouse macrophages (Gijón *et al.* 2000) and neutrophils (Rubin *et al.* 2005). Figure 8 illustrates experiments in which we examined this question in murine peripheral granulocytes, assessing NADPH oxidase activity directly in individual cells as I_e. We could detect no difference between the responses of control and knockout murine cells, whether they were stimulated with fMetLeuPhe (*vide infra*) or PMA. In both the normal mouse cell (Fig. 8*A*) and the cPLA$_2\alpha$ KO cell (Fig. 8*B*), addition of 10 μM fMetLeuPhe had no effect, but subsequent introduction of PMA produced a vigorous I_e that was inhibited by DPI. Proton currents in both normal and cPLA$_2\alpha$ KO cells were enhanced profoundly by PMA (current records on the right in Fig. 8).

No previous studies of proton currents in murine granulocytes exist, although proton currents in murine macrophages have been studied extensively (Kapus *et al.* 1993*a*, 1994; Grinstein *et al.* 1994; Suszták *et al.* 1997). That the time- and voltage-dependent outward currents in murine granulocytes were proton selective was confirmed by appropriate shifts of V_{rev} and $V_{threshold}$ (the threshold voltage at which distinct I_H is first elicited) when pH_o was changed directly or when pH_i was changed indirectly by varying the NH_4^+ concentration (data not shown). Proton currents in murine granulocytes were generally similar to those in human granulocytes in terms of gating kinetics, amplitude, and the voltage dependence and pH dependence of gating. More important for the present purposes, proton channels in murine cells responded to PMA (or to fMetLeuPhe) in a manner reminiscent of, but not identical to, the responses of human cells. Figure 9*A* and *B*, respectively, illustrate families of proton currents in a murine granulocyte before and after stimulation with PMA. All of the changes produced by PMA in human cells also occur in murine cells, but in most cases, the magnitude of the effect is smaller. It is evident in Fig. 9 that after PMA stimulation, the H^+ current is larger, activates at more negative voltages, turns on faster, and less obviously, the tail current decays more slowly. Table 2 summarizes some of the effects of PMA on proton currents in murine granulocytes. The amplitude of I_H at the end of the test pulse increased about 4-fold. Activation of proton current (τ_{act}) was faster after PMA stimulation, but only by about 2-fold. Channel closing, measured as the time constant of tail current decay (τ_{tail}), was 2- to 3-fold slower. All of these

parameters were changed significantly by PMA. There was no significant difference between control and cPLA$_2\alpha$ KO cells for any parameter, either before or after stimulation.

Figure 10 shows that PMA shifted the g_H–V relationship negatively, but only by ~20 mV, as opposed to the 40 mV shift in human cells (Table 1). All of these effects of PMA on proton channel gating in cPLA$_2\alpha$ KO murine cells were indistinguishable from those in control cells.

Stimulation of murine granulocytes with fMetLeuPhe

Although cPLA$_2\alpha$ is evidently not required for the response of murine granulocytes to PMA, it was possible that cPLA$_2\alpha$ might be required in a more physiological signalling pathway such as that triggered by fMetLeuPhe. Therefore, we examined the effects of 10 μM fMetLeuPhe in normal and cPLA$_2\alpha$ KO mice. The effect of fMetLeuPhe on murine proton currents was similar to that of PMA, although the extent of the response was more variable. Intriguingly, proton currents responded to fMetLeuPhe in all cells in which I_e was elicited, including one cPLA$_2\alpha$ KO cell in which I_e was very small. In the cells illustrated in Fig. 11*B* and *E*, the I_H in the presence of 10 μM fMetLeuPhe was approximately doubled and $V_{threshold}$ shifted about 20 mV more negative. In both cells, the inward current elicited by fMetLeuPhe was confirmed to be I_e because it was inhibited by 20 μM DPI (not shown). Subsequent addition of 100 nM PMA produced a response that was greater than that elicited by fMetLeuPhe (Fig. 11*C* and *F*).

The I_e response of murine granulocytes to fMetLeuPhe was highly variable. About half of the cells did not respond detectably (Fig. 8*A* and *B*), others exhibited a weak response (compared with the response to PMA added subsequently to the same cell), and in some cells there was a dramatic response comparable with the PMA response (e.g. Fig. 11*E* and *F*). This variability is consistent with a previous study in which only about half of murine neutrophils responded to fMetLeuPhe in an nitro blue tetrazolium (NBT) test that reflects $O_2^{\cdot -}$ production by individual cells (Kim & Dinauer, 2001). We excluded cells that did not respond to fMetLeuPhe or subsequently to PMA, because they might have spontaneously gone to the whole-cell configuration, which precludes any response (Morgan *et al.* 2003). The peak I_e elicited by fMetLeuPhe was −1.5, −7.2 and −8.0 pA in 3/5 control cells that responded, and −4.0, −1.5, −0.9 and possibly −0.2 pA in 4/7 cPLA$_2\alpha$ KO mouse cells. The small numbers of responding cells preclude quantitative comparison, but it is clear that some granulocytes from cPLA$_2\alpha$ KO mice respond to fMetLeuPhe with both I_e and enhanced proton currents.

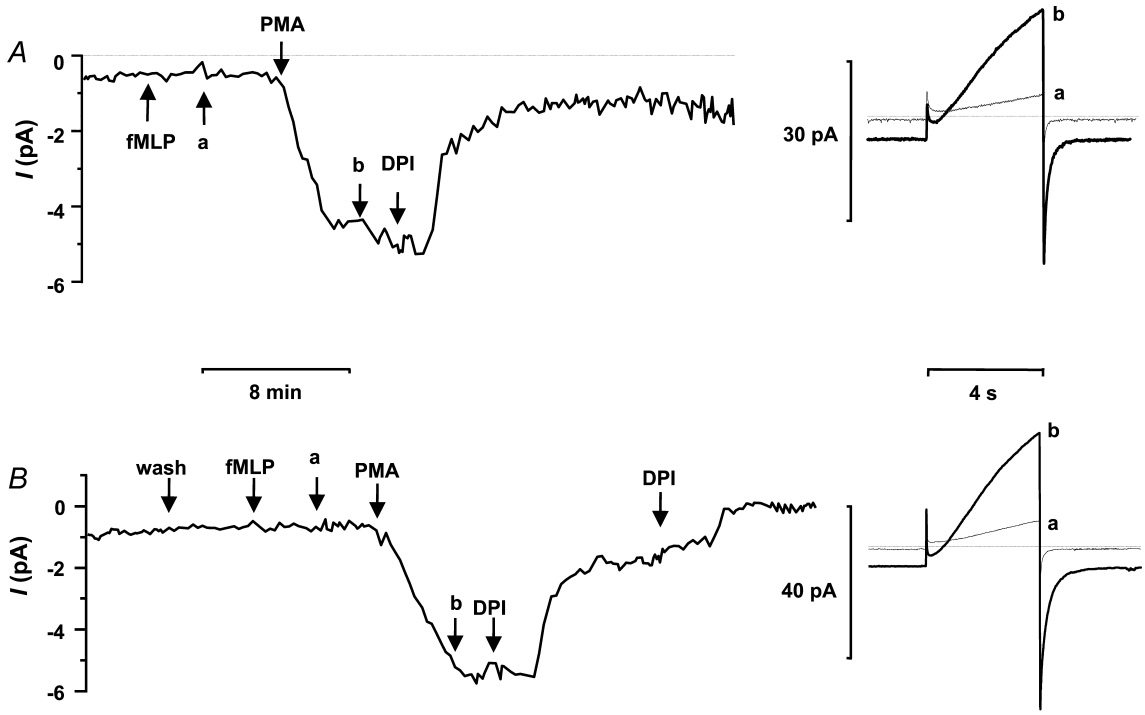

Figure 8. Similarity of the PMA responses of NADPH oxidase and H$^+$ channels in granulocytes from control or cPLA$_2\alpha$ KO mice

Currents at −60 mV (left traces) in granulocytes in the perforated patch configuration from a control mouse (*A*) and a cPLA$_2\alpha$ KO mouse (*B*). Arrows indicate addition of 10 μM fMetLeuPhe, 60 nM PMA, or simple bath exchanges ('wash'). Currents during 4 s depolarizing pulses to +60 mV (*A*) or +40 mV (*B*) (right traces) before (*a*) and after (*b*) addition of 60 nM PMA at the times indicated by lower case letters.

Discussion

cPLA$_2\alpha$ and increased AA levels are not required for activation of proton channels

The cPLA$_2\alpha$ isoform has been proposed to be required for agonist-induced AA release, NADPH oxidase activity, and proton fluxes associated with the respiratory burst (Dana *et al.* 1998; Lowenthal & Levy, 1999). This study demonstrates that the transformation of voltage-gated proton channels into their 'activated' state or 'enhanced gating mode' occurs completely normally in the absence of cPLA$_2\alpha$ activity. Furthermore, we show that cPLA$_2\alpha$ activity is not required for a normal respiratory burst stimulated by PMA in human eosinophils or stimulated by PMA or fMetLeuPhe in murine granulocytes, extending similar findings in other cells (Gijón *et al.* 2000; Rubin *et al.* 2005). The cPLA$_2\alpha$ inhibitors used here, especially pyrrolidine-2 and Wyeth-1, appear to be more selective for this isoform than are previously used inhibitors (Street *et al.* 1993; Ni *et al.* 2006). For the purpose of this study, however, perfect selectivity is not required – what is required is that the inhibitors prevent cPLA$_2\alpha$ activity at the concentrations used. The results do not categorically rule out any involvement of cPLA$_2\alpha$ in responses to agonists that were not studied. However, the studies that provided the evidence for the cPLA$_2\alpha$ hypothesis tested here all used PMA as a stimulus (Henderson *et al.* 1989; Chappell & Henderson, 1991; Kapus *et al.* 1993*b*; Suszták *et al.* 1997; Lowenthal & Levy, 1999; Levy *et al.* 2000; Mankelow *et al.* 2003).

The results in cPLA$_2\alpha$ KO mice do not in themselves rule out a role for AA in the activation of either protein. Because AA release (spontaneous or stimulated by PMA and A23187) is reduced but not abolished in cPLA$_2\alpha$ KO macrophages (Bonventre *et al.* 1997; Gijón *et al.* 2000), it could be speculated that other phospholipases might be up-regulated in the KO mice. In PLB-985 cells, reduction of cPLA$_2\alpha$ activity by antisense RNA abolished both PMA-stimulated AA production and $O_2^{\cdot -}$ release (Dana *et al.* 1998), suggesting that no compensatory up-regulation of other isoforms occurred in this system. Furthermore, acute application of specific cPLA$_2\alpha$ inhibitors abolished agonist-stimulated AA release from human phagocytes (Rubin *et al.* 2005). Consequently, the lack of effect of specific cPLA$_2\alpha$ inhibitors indicates that proton channel activation does not require newly generated AA. It remains conceivable that AA already present before stimulation may play some role or interact in some way with the proton channel, perhaps analogous to the role of AA on NADPH oxidase activity in a cell-free system. However, it seems clear that a different mechanism, most likely phosphorylation, mediates the activation of the proton channel *per se*.

Phosphorylation of proton channels and NADPH oxidase

The activation of proton efflux by PMA (Henderson *et al.* 1987; Nanda & Grinstein, 1991; Kapus *et al.* 1992) immediately suggested the possibility that proton channels might be activated directly by PKC (rather than indirectly via cPLA$_2\alpha$ and AA). Because most early studies inferred proton channel activity from pH changes, their interpretation is complicated because PMA also activates proton extrusion by Na^+/H^+-antiport and a V-type H^+-ATPase (Nanda *et al.* 1992), as well as NADPH oxidase,

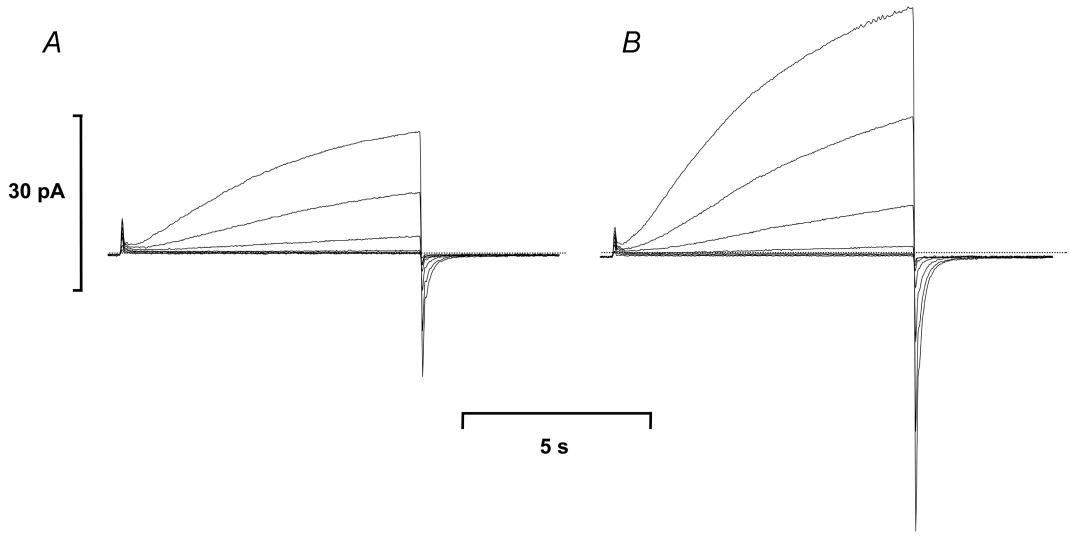

Figure 9. PMA enhances proton channel gating in murine granulocytes
Families of currents in a normal mouse granulocyte during identical pulses to −40 through +80 mV in 20 mV increments, before (left) and after stimulation with PMA (right). The dotted line shows zero current: PMA elicited inward electron current.

Table 2. Comparison of PMA responses of granulocytes from control or cPLA$_2\alpha$ knockout mice

	Control		cPLA$_2\alpha$ KO	
	Unstimulated	PMA	Unstimulated	PMA
I_H (+60 mV) (pA)	9.2 ± 3.7 (14)	41.8† ± 8.8 (12)	13.2 ± 2.6 (10)	39.8† ± 8.3 (11)
$g_{H,max}$ (pS)	180 ± 60 (13)	690† ± 140 (10)	240 ± 50 (10)	610† ± 110 (11)
τ_{act} (+60 mV) (s)	6.6 ± 1.6 (6)	3.4* ± 0.9 (6)	4.3 ± 0.6 (8)	2.8* ± 0.2 (8)
τ_{tail} (− 60 mV) (ms)	100 ± 15 (9)	330† ± 73 (9)	105 ± 16 (10)	208† ± 24 (11)
I_e (pA)	—	−2.6 ± 0.5 (11)	—	−3.0 ± 0.7 (12)

Mean ± S.E.M. (*n*) parameter values in murine granulocytes. All PMA values were significantly (*$P < 0.05$, or †$P < 0.01$) different from unstimulated cells (paired, two-tailed *t* test). I_H was measured at the end of 6–8 s pulses. Values of $g_{H,max}$ were calculated from I_H at the most positive voltage studied in each cell, usually +60 to +100 mV, and the measured H^+ current reversal potential. No parameter value differed significantly between control and KO mouse cells, either before or after PMA stimulation.

which creates a large driving force for proton extrusion. The idea that phosphorylation is a key process is supported by the prevention of activation of either NADPH oxidase or proton channels by the PKC inhibitors, GFX (Fig. 1*D*; Bankers-Fulbright *et al.* 2001) and staurosporine. GFX exhibits high selectivity for PKC compare with other protein kinases (Toullec *et al.* 1991; Davies *et al.* 2000), and inhibits most conventional and novel PKC isoforms (Martiny-Baron *et al.* 1993). In the case of NADPH oxidase, phosphorylation of its components precedes assembly of the oxidase complex (Babior, 1999). The 'activation' of proton channels may also be less straightforward than direct phosphorylation of the channel molecule by PKC. Our results do not distinguish whether the proton channel itself or an intermediate channel-activating molecule is phosphorylated.

Might the PKC inhibitors act by inhibiting cPLA$_2\alpha$? In many cells, PKC is upstream of cPLA$_2$ and PKC activation enhances cPLA$_2$ activity. However, in several systems, GFX inhibited only the PKC-dependent cPLA$_2$ activity and did not inhibit basal activity (Qiu & Leslie, 1994; Xia *et al.* 1995; Han *et al.* 2004). Similarly, staurosporine does not inhibit AA release from macrophages (Beppu *et al.* 2002). Because selective inhibitors of cPLA$_2\alpha$ do not inhibit activation of I_H or I_e, there is no reason to expect that PKC inhibitors might act in this way. The effects of the PKC inhibitors clearly are not mediated by incidental effects on cPLA$_2\alpha$.

The active states of both proton channels and NADPH oxidase, whether resulting from PMA or AA stimulation or spontaneous activation, were reversed by GFX or staurosporine. This reversibility links phosphorylation by PKC not only to the initial activation, but also to the sustained activity of both proteins. Several types of studies support the idea that NADPH oxidase activity is regulated by a balance between phosphorylation and dephosphorylation (Heyworth & Badwey, 1990), although a number of other explanations for deactivation exist (DeCoursey & Ligeti, 2005). Electron currents generated by NADPH oxidase reportedly run down more slowly with time in excised membrane patches upon addition of cytosolic ATP and GTPγs (Petheö *et al.* 2003). Although ATP has effects on proton currents that are unrelated to phosphorylation (pp. 533–534 in DeCoursey, 2003, and DeCoursey, 2006), the possibility remains that the channel may be phosphorylated in intact cells. The time constants of inhibition of I_e and I_H were not obviously dependent on GFX concentration, suggesting that when phosphorylation is interrupted, deactivation proceeds by another mechanism that becomes rate determining.

The hypothesis that the activated gating mode of proton channels reflects phosphorylation of the channel or a closely related regulatory element was supported by the observation (Fig. 6) that okadaic acid largely

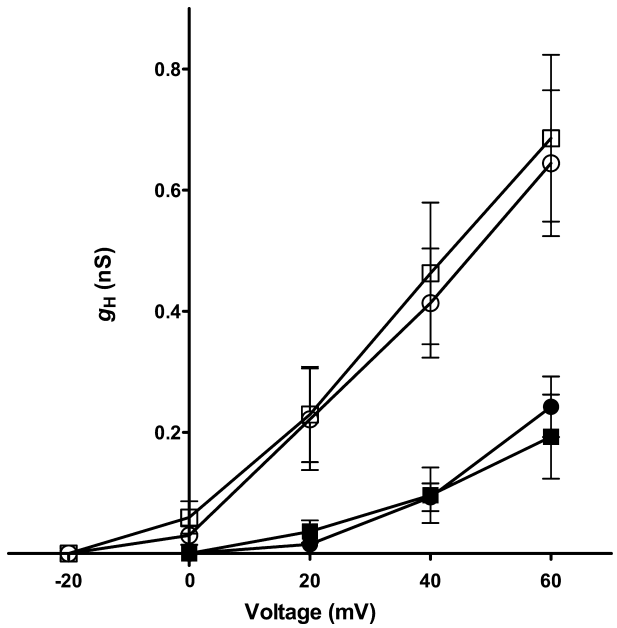

Figure 10. The g_H–V relationships before and after stimulation are similar in granulocytes from control and cPLA$_2\alpha$ KO mice
Mean ± S.E.M. values of g_H calculated from I_H at the end of 8 s pulses and measured V_{rev} values (in most cases) in control granulocytes (■) and cPLA$_2\alpha$ KO (●) mice. The open symbols indicate data after stimulation with 60 nM PMA in control (□) or cPLA$_2\alpha$ KO cells (○). All data are from 10 to 12 cells.

prevented deactivation by GFX. Because GFX simply prevents further phosphorylation, but would not be expected to affect already phosphorylated channels, the diminution of H^+ currents toward their resting condition must reflect another process, such as the activity of phosphatases. This interpretation is supported by the independence of the rate of the GFX effect on concentration. Thus, okadaic acid must interfere with the process that causes deactivation, presumably phosphatase activity. Neither staurosporine nor GFX had detectable effects on I_H in unstimulated eosinophils, suggesting that the resting level of phosphorylation of proton channels must be minimal. Similar to the lack of activation of NADPH oxidase by okadaic acid alone (Ding & Badwey, 1992; Lu *et al.* 1992; DeCoursey & Ligeti, 2005; present results), okadaic acid alone did not activate I_H significantly. Thus, neither NADPH oxidase nor proton channels are kept in their resting state by ongoing serine–threonine phosphatase activity.

Role of AA in proton channel activation

Consistent with an earlier study (Cherny *et al.* 2001), we found that AA profoundly activated both I_H and I_e in human eosinophils. Oleic acid appeared to be equally effective. Unlike AA, oleic acid is not metabolized by the lipoxygenase or cyclooxygenase pathways. Hence, the efficacy of oleic acid in activating I_H (and I_e) indicates that AA itself is active, not a metabolite. The proton channel response to AA was graded, in contrast with the PMA response that appears to be all-or-nothing. Nevertheless, it is possible to distinguish two types of AA responses, direct and indirect. The direct response is seen in whole cell configuration and consists of a modest hyperpolarizing shift in the g_H–V relationship ($\leq$ 20 mV), faster H^+ current activation ($\leq$ 2-fold smaller τ_{act}), faster deactivation (2-fold smaller τ_{tail}), and a ($\leq$ 2-fold) larger $g_{H,max}$ (DeCoursey & Cherny, 1993; Kapus *et al.* 1994; Gordienko *et al.* 1996). The AA response in perforated patch studies can be more profound and in some respects qualitatively different, strongly implicating a diffusible second messenger in the response. The perforated patch configuration preserves signalling pathways by retaining intracellular constituents that rapidly diffuse into the pipette in whole-cell configuration. The additional effects of AA that occur in perforated-patch studies thus reflect an indirect response. Even in perforated patch studies, low [AA] (1 μM) increased I_H without shifting the g_H–V relationship and hastened both τ_{act} and τ_{tail}, without activating detectable I_e (Cherny *et al.* 2001), which can be taken as the 'direct' response. However, higher [AA] (3–10 μM) induced a profound negative shift of the g_H–V relationship (resulting in distinct inward H^+ current negative to E_H), 4-fold faster τ_{act}, 4.6-fold larger I_H (measured during pulses to 40 or 60 mV), and also activated I_e (Cherny *et al.* 2001), all of which indicate an indirect response, analogous to that seen with PMA stimulation. The main difference between the responses to AA and PMA is that τ_{tail} was slowed at least 3- to 5-fold by PMA (DeCoursey *et al.* 2000*b*, 2001*a*; Table 1), but was not consistently changed by AA (Cherny *et al.* 2001). This difference can be explained by a combination of the direct effect of speeding τ_{tail} and the indirect of slowing τ_{tail}, as follows:

$$\text{AA} \xrightarrow{direct} \text{H channel (faster tails)}$$
$$\text{AA} \rightarrow \text{PKC} \xrightarrow[phosphorylation]{} \text{H}^* \text{ channel (slow tails)}$$

Scheme 3

Here we assume that phosphorylation of the proton channel or an intermediary after PMA or AA stimulation results in slowing of τ_{tail}. Because AA acts via both pathways, it simultaneously speeds and slows τ_{tail}, and the net result is little or no change. The possibility that the indirect effects of AA are mediated by PKC was suggested by the partial reversal of proton channel activation by GFX or staurosporine (Fig. 7).

Oleic acid induced profound activation of both I_H and I_e in human eosinophils studied in the perforated patch configuration. Although oleic acid increased I_H in a whole-cell study (Kapus *et al.* 1994), this effect

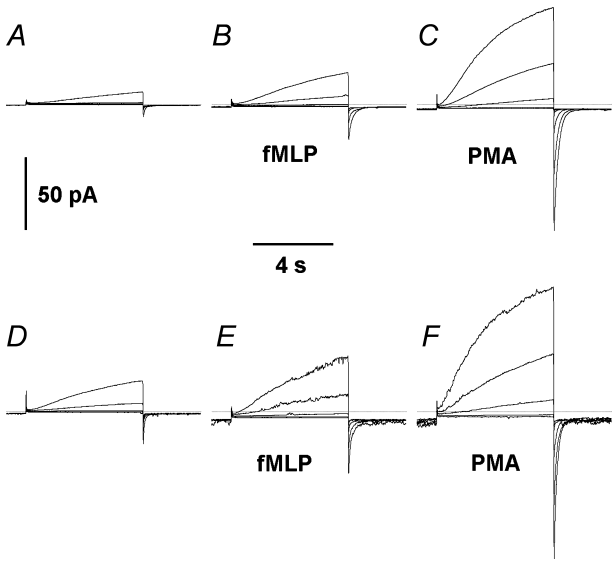

Figure 11. Responses to fMetLeuPhe are similar in granulocytes from control and cPLA$_2\alpha$ KO mice
Families of currents before (*A* and *D*) and after stimulation with 10 μM fMetLeuPhe (*B* and *E*) or 100 nM PMA (*C* and *F*) in a normal mouse cell (*A*–*C*) and a cPLA$_2\alpha$ KO cell (*D*–*F*). All families illustrated are from −20 mV through +60 mV in 20 mV increments. The horizontal line indicates zero current. Calibration bars apply to all parts.

was much weaker than that for AA in the same study (a 1.3-fold increase in I_H by oleic acid, *versus* a 3.7-fold increase by AA). In contrast, in the present measurements, oleic acid appeared to activate I_H as profoundly as did AA. Taken together, these observations suggest that AA has greater direct effects on proton channels, but oleic acid and AA are comparably effective in activating I_H by the indirect pathway (presumably via PKC activation).

One of the earliest proposed mechanisms of activation of NADPH oxidase by AA was through PKC activation (McPhail *et al.* 1984). Unsaturated fatty acids like AA and oleic acid activate PKC directly (McPhail *et al.* 1984; Murakami & Routtenberg, 1985). Subsequent studies questioned whether this mechanism occurred at physiologically relevant levels of AA (Ely *et al.* 1995), which is difficult to evaluate. The PKC inhibitor H-7 partially inhibited $O_2^{\cdot-}$ production stimulated by PMA but not by 25 μM AA in intact neutrophils (Maridonneau-Parini & Tauber, 1986). A recent study demonstrates translocation (but not activation) of PKC by 10–100 nM AA, with activation occurring at higher concentrations (O'Flaherty *et al.* 2001). Effects of AA on translocation of PKC were partially inhibited by GFX (Hii *et al.* 1998). We conclude that at least part of the effect of AA on proton currents in intact phagocytes is due to activation of PKC. This conclusion for proton channels may parallel the phenomenon that NADPH oxidase can be fully activated by high [AA] alone, or by lower [AA] in synergism with PKC (Shiose & Sumimoto, 2000). In murine neutrophils, activation of NADPH oxidase by AA requires PKCζ (Kim & Dinauer, 2006). PKC inhibitors reduced or abolished AA-induced I_e despite the continued presence of AA in the bath. Evidently, both NADPH oxidase activity and the enhanced gating mode of proton channels are sustained by continuous phosphorylation, the prevention of which results in dephosphorylation and deactivation of both molecules.

Proton and electron currents in murine granulocytes

This is the first study of proton and electron currents in murine granulocytes and their responses to stimulation. In most respects, the properties of proton currents in murine cells resembled those in their human counterparts. Stimulation with either PMA or fMetLeuPhe produced changes in proton channel gating (enhanced gating mode) that were qualitatively like those in human cells. However, most of the changes in proton channel gating were distinctly less profound. The speeding of H^+ current turn-on (τ_{act}), the slowing of deactivation (τ_{tail}), and the hyperpolarizing shift of the voltage dependence of gating were all only about half of their magnitude in human cells. In this sense, the mouse is a more difficult model system because the effects of stimulation are simply less obvious; its utility derives from the possibility of generating knockouts.

Responses to fMetLeuPhe

To confirm that the lack of a requirement for cPLA$_2\alpha$ was not restricted to activation of cells by PMA, we used fMetLeuPhe in murine granulocytes. The response of both I_e and I_H in individual cells ranged from no response to a full PMA-like response, consistent with a previous study in which only half of murine neutrophils responded to fMetLeuPhe in an NBT test that reflects $O_2^{\cdot-}$ production by individual cells (Kim & Dinauer, 2001). A response to fMetLeuPhe may require priming (DeCoursey & Ligeti, 2005), and spontaneous priming may occur in only a subpopulation of cells. In the cells in Fig. 8, there was no response to fMetLeuPhe, but subsequent introduction of PMA produced a large I_e that was inhibited by DPI. These results clearly demonstrate that the lack of fMetLeuPhe response was not due to the cells being non-viable or having already exhausted their capacity to assemble NADPH oxidase. All murine granulocytes that responded to fMetLeuPhe with I_e also exhibited enhanced proton channel gating. This result suggests that the 'activation' of H^+ channels and NADPH oxidase are mediated by common or overlapping pathways that diverge relatively late.

In this study, we found no evidence to support the prevailing hypothesis regarding the mechanism of activation of the proton conductance during the respiratory burst in human eosinophils and murine granulocytes. Despite extensive circumstantial data showing (1) production of AA during the respiratory burst, (2) stimulation of NADPH oxidase and proton channels by AA, (3) inhibition of responses by broad-spectrum PLA$_2$ antagonists, and (4) a requirement for AA or another amphiphile in the cell-free NADPH oxidase system, neither direct, specific inhibition of cPLA$_2\alpha$ (which generates AA during the respiratory burst) nor genetic knockout of cPLA$_2\alpha$ detectably impaired the enhanced gating of proton channels or NADPH oxidase activity. Instead, a role for PKC was demonstrated in activation of proton channels as well as NAPDH oxidase. Both molecules were deactivated within a few minutes of GFX or staurosporine addition, demonstrating that the signalling pathways downstream from PKC are rapidly reversible upon removal of the stimulus. Okadaic acid prevented the deactivation of proton current, implicating phosphatases in this process. Stimulatory effects of AA and oleic acid on proton currents were separated into direct and indirect components, and the indirect effects on both proton channels and NADPH oxidase are due at least in part to activation of PKC by AA.

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Acknowledgements

This work was supported in part by the Heart, Lung and Blood Institute of the National Institutes of Health (research grants HL52671 and HL61437 to T.D. and HL50040 to M.G.). The authors thank Prof T. Shimizu (University of Tokyo) for providing cPLA$_2\alpha$ KO mice, Prof Linda C. McPhail (Wake Forest University) for a helpful critique of the manuscript, and Dr Tatiana Iastrebova for excellent technical assistance.