

Activation of NADPH oxidase-related proton and electron currents in human eosinophils by arachidonic acid

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1. Effects of arachidonic acid (AA) on proton and electron currents in human eosinophils were studied using the permeabilized-patch voltage-clamp technique, using an applied NH_4^+ gradient to control pH_i .
2. Superoxide anion (O_2^-) release was assessed by cytochrome *c* reduction in human eosinophils. Significant O_2^- release was stimulated by 5–10 μM AA.
3. AA activated diphenylene iodonium (DPI)-inhibitable inward current reflecting electron efflux through NADPH oxidase. These electron currents (I_e) were elicited in human eosinophils at AA concentrations (3–10 μM) similar to those that induced O_2^- release.
4. The voltage-gated proton conductance (g_{H}) in eosinophils stimulated with AA was profoundly enhanced: H^+ current amplitude (I_{H}) increased 4.6 times, activation was 4 times faster, and the H^+ conductance–voltage ($g_{\text{H}}-V$) relationship was shifted to substantially more negative voltages. The electrophysiological effects of AA resembled those reported for PMA, except that AA did not consistently slow τ_{tail} (deactivation of H^+ currents).
5. The stimulation of both proton and electron currents by AA was reversible upon washout. Repeated exposure elicited repeated responses. The activation of H^+ currents by AA was dissociable from its activation of NADPH oxidase; H^+ currents were enhanced at low concentrations of AA that did not elicit detectable I_e or when NADPH oxidase was inhibited by DPI.
6. Most of the effects of AA on H^+ currents qualitatively resemble those reported in whole-cell studies, reflecting a more direct action than PMA. The results are compatible with AA being an immediate activator of both NADPH oxidase and proton channels in human eosinophils.

The importance of arachidonic acid (AA) during the respiratory burst – the rapid conversion of oxygen to superoxide anion (O_2^-) and other cytotoxic reactive oxygen species to kill bacteria and other invaders – is well established in neutrophils (Badwey *et al.* 1981; Curnutte *et al.* 1984; Henderson *et al.* 1989; Henderson & Chappell, 1992). Arachidonic acid is released from phagocytes during the respiratory burst, upon challenge with a wide variety of agonists including chemotactic peptides and phorbol myristate acetate (PMA) (Walsh *et al.* 1981; Bromberg & Pick, 1983; Galbraith, 1988; Tao *et al.* 1989). In addition, AA is itself a potent agonist that can trigger O_2^- release from human neutrophils (Badwey *et al.* 1981; Henderson *et al.* 1989, 1993), porcine macrophages (Bromberg & Pick, 1983), guinea-pig eosinophils (Lindsay *et al.* 1998) and, as demonstrated here, from human eosinophils. In fact, it has been proposed that AA is the immediate activator of NADPH oxidase (Bromberg &

Pick, 1983; Henderson *et al.* 1989, 1993). Mechanisms of action proposed for AA include activation of a GTP binding protein (Abramson *et al.* 1991), increasing the activity of the assembled NADPH oxidase complex (Rubinek & Levy, 1993), and stimulation of the translocation of p47^{phox} and its assembly with cytochrome b_{558} (Uhlinger *et al.* 1993; Cross *et al.* 1999). In a cell-free system, both AA and PKC-mediated phosphorylation of p47^{phox} induce identical conformational changes in p47^{phox} that result in NADPH oxidase activity (Swain *et al.* 1997; Park & Babior, 1997).

NADPH oxidase is electrogenic (Henderson *et al.* 1987, 1988*a, b*; Nanda & Grinstein, 1991); the flow of electrons along the transport chain and out of the cell comprises a measurable electrical current (Schrenzel *et al.* 1998; Bánfi *et al.* 1999; DeCoursey *et al.* 2000, 2001). During the respiratory burst, electrogenic H^+ efflux, proposed to be

mediated by proton channels, compensates the charge translocation by the oxidase complex (Henderson *et al.* 1987, 1988*a,b*; Nanda & Grinstein, 1991). Although AA-sensitive voltage-gated proton channels were observed in human neutrophils (DeCoursey & Cherny, 1993) and eosinophils (Gordienko *et al.* 1996; Schrenzel *et al.* 1996), their mediation of the H⁺ efflux that occurs during the AA-stimulated respiratory burst has not been demonstrated directly. AA enhances voltage-gated proton currents in whole-cell studies: the maximum H⁺ conductance ($g_{H,max}$) is increased, the H⁺ chord conductance–voltage (g_{H-V}) relationship is shifted ~20 mV toward more negative potentials, and both activation (channel opening) and deactivation (channel closing) are roughly twice as fast (DeCoursey & Cherny, 1993; Kapus *et al.* 1994; Schrenzel *et al.* 1996; Gordienko *et al.* 1996; Suszták *et al.* 1997). Here we explore the effects of AA on voltage-gated proton currents in human eosinophils studied in the permeabilized-patch configuration, which allows the simultaneous monitoring of the activity of NADPH oxidase as electron current (DeCoursey *et al.* 2000, 2001). We demonstrate that AA activates NADPH oxidase in human eosinophils, measured both as O₂⁻ release and as electron current. We confirm that AA enhances voltage-gated proton currents. Although there is an intimate relationship between NADPH oxidase and voltage-gated proton channels, the enhancement of H⁺ current by AA was largely dissociable from the activation of NADPH oxidase.

A preliminary account of this work has been published (Cherny *et al.* 2001).

METHODS

Venous blood was drawn from healthy adult volunteers under informed consent according to procedures approved by our Institutional Review Board and in accordance with Federal regulations. Unless stated otherwise, the Methods are identical to those described in the accompanying paper (DeCoursey *et al.* 2001). Studies using AA are complicated by its tendencies to oxidize, to adhere to surfaces, to form micelles, and to damage cell membranes (Meves, 1994). The sodium salt of AA was purchased from Sigma Chemical Co. (St Louis, MO, USA). Immediately upon opening the container, AA was dissolved at 5 mM in 50% ethanol, aliquotted into small glass vials, and gassed with nitrogen. Vials were stored at -20°C for no more than 2–3 weeks before use. Once opened, a vial was used only for a few hours.

Eosinophils were positioned on glass coverslip chips precoated with bovine serum albumin, to reduce adherence-induced activation (DeCoursey *et al.* 2001). In light of the high affinity of AA for albumin (Ashbrook *et al.* 1975; Berde *et al.* 1979; Badwey *et al.* 1984), the local concentration of AA near the cell may have been reduced by binding to albumin. In some cells simply exchanging the bath with the same AA-containing solution produced additional effects. We attribute this result to the tendency of AA to adhere to surfaces, so that a bath exchange at the same nominal concentration transiently increased the local AA concentration.

Production of O₂⁻ was measured as described in the companion paper (DeCoursey *et al.* 2001) with three modifications. (1) Albumin was omitted from the incubation buffer and the 96-well plates were not pretreated with albumin. (2) Eosinophil concentration was increased

to 10⁶ cells ml⁻¹. (3) The cells were pre-warmed in Hepes (10 mM)-buffered HBSS, pH 7.4, containing 50 μM cytochrome *c* for 3 min at 37 °C in a flat-bottom 96-well tissue culture plate (Costar, Acton, MA, USA) before addition of AA. The indicated concentrations of AA were added, and the contents were incubated for 14 min at 37 °C. Absorbance at 550 nm was recorded at 1 min intervals, and O₂⁻ production was calculated as described previously (Horie & Kita, 1994).

RESULTS

Superoxide anion release induced by AA in eosinophils

Although widely studied in neutrophils, AA has been evaluated only rarely as a stimulus for O₂⁻ production by eosinophils (Lindsay *et al.* 1998). We therefore examined AA-stimulated O₂⁻ release by eosinophils using some of the same eosinophil preparations used for patch-clamp studies. The mean dose–response relationship is plotted in Fig. 1 (○). We observed donor-to-donor variability, but 5 μM AA consistently stimulated O₂⁻ release as measured by cytochrome *c* reduction. The concentration–response relationship is steep, with no further increase in O₂⁻ generation at AA concentrations higher than 10 μM AA.

Electron current (I_e) elicited by AA

Because NADPH oxidase is electrogenic, its activity can be detected as a sustained inward current that presumably would be suppressed by extreme depolarization. Figure 1 shows the average peak I_e (■) elicited by various AA concentrations. The concentration–response relationship is steep, with little I_e activated by 1 μM AA and a strong response at 3 μM AA. Determining the concentration dependence was complicated by the tendency of the cells to become non-specifically leaky within a few minutes of addition of high concentrations of AA (> 5 μM). The average amplitude of I_e elicited by 3–10 μM AA was -7.4 ± 3.8 pA (mean \pm s.d., $n = 22$), slightly larger than the -6.0 pA elicited by PMA under identical conditions (DeCoursey *et al.* 2001). The concentration of AA required to stimulate I_e is similar to that required to stimulate O₂⁻ release.

Figure 2 illustrates I_e responses of three human eosinophils in continuous chart records of the holding current at -60 mV. The frequent interruptions are the result of depolarizing voltage pulses, which were applied to elicit H⁺ currents that were recorded separately. In the cell illustrated in Fig. 2A, soon after achieving a seal and establishing permeabilized patch configuration, the inward current increased spontaneously, reaching an amplitude of approximately -10 pA. The tail current decay during this time became slower, typical of the responses seen with PMA (DeCoursey *et al.* 2000, 2001), and suggesting that the cell was becoming activated. Spontaneous activation of putative I_e was reported previously in human eosinophils dialysed with a pipette solution containing NADPH (Schrenzel *et al.* 1998). At the time indicated by the first arrow, 6 μM DPI was added. The holding current decreased to approximately -2 pA and tail current decay became

faster. DPI is an inhibitor of NADPH oxidase (Robertson *et al.* 1990); thus the DPI-sensitive fraction of inward current at the holding potential (V_h) is attributable to electron currents flowing through NADPH oxidase. Typical for non-activated eosinophils, the background holding current at -60 mV was approximately -2 pA, was not affected by DPI, and is presumably attributable to non-specific leakage. When 60 nM PMA was added several minutes later, it elicited an I_e that was inhibited by subsequent addition of DPI. As in this cell, the I_e elicited by AA or PMA was generally several times larger than the non-specific leak current at V_h . After washing DPI out of the bath, 5 μ M AA was added, which elicited I_e that was inhibited by DPI. DPI was added together with AA as a control for possible non-specific leakage current that might be induced by AA. In the experiment shown in Fig. 2A, I_e attributable to NADPH oxidase was activated 3 times in the same eosinophil, first spontaneously, then by PMA, and finally by AA.

The inhibition of I_e by DPI was slower when PMA was the agonist than when AA was the agonist. The average time required for DPI to reduce I_e to half its peak value was 1.8 ± 1.2 min (mean \pm s.d.) in nine cells stimulated with PMA, but only 0.34 ± 0.51 min in eight cells stimulated by AA. The difference in time course is significant at $P < 0.01$ by Student's two-tailed t test.

The time course of the activation of I_e by AA in another cell is illustrated in Fig. 2B. The I_e began to increase within seconds after addition of AA to the bath, increased for 2–3 min, then reached a maximum and began to decline slowly. Spontaneous decline in I_e elicited by AA was often observed. DPI added together with AA rapidly eliminated most of the inward current. That the inward current did not return completely to the previous baseline might reflect a small component of AA-induced leak, or it may reflect that NADPH oxidase continues to function at a greatly reduced level in the presence of AA and DPI together.

Figure 2C illustrates a biphasic response to AA in another eosinophil. This type of response was seen occasionally in response to either AA or PMA. In this experiment, the AA-induced inward current was identified as NADPH-generated I_e by addition of DPI alone (i.e. without AA). Subsequent addition of AA together with DPI elicited little or no inward current. This result rules out the possibility that the AA-induced current was non-specific membrane leak. Later in this experiment AA was applied and DPI-inhibitable I_e was again stimulated (not shown), confirming that the failure of the cell to respond to AA when added together with DPI was due to DPI inhibition rather than to 'desensitization' or some other process.

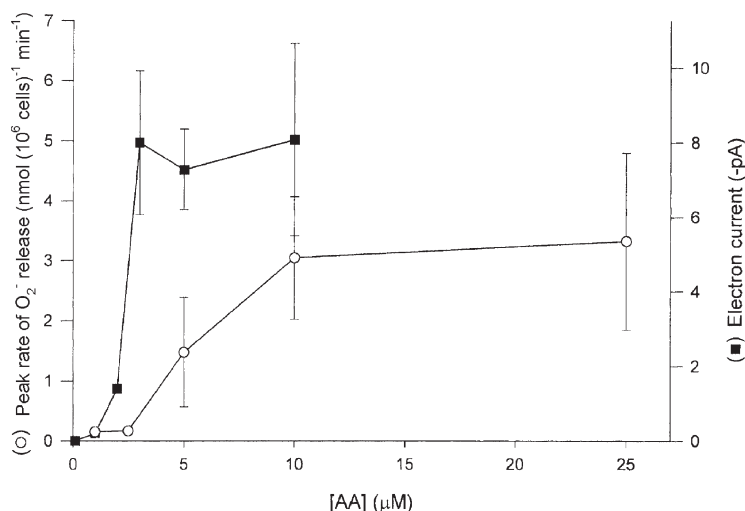


Figure 1. Comparison of the dose–response relationships for superoxide anion release and electron currents stimulated by AA in human eosinophils

The background level of cytochrome c reduction has been subtracted to give the net O_2^- release which is plotted (\circ , left axis) as mean \pm S.E.M. for $n = 3$ experiments. Measurements were made every 1 min, and the maximum increase in any 1 min time period was determined for each AA concentration. For low AA concentrations that induced a small response compared with background, the average over 2 min was determined. The peak I_e measured at -60 mV in single cells in the permeabilized patch configuration (\blacksquare , $n = 2$ – 12 , right axis) is plotted for comparison. The values used for I_e are the maximum I_e measured in a given cell at each concentration; thus if a given concentration of AA was applied more than once, the largest response was selected. The ordinates have been scaled so that the O_2^- release rate corresponds with the I_e that would be measured in an average cell, assuming that each translocated electron results in the generation of one O_2^- molecule.

Effects of AA on H^+ currents

Figure 3A illustrates the electrophysiological effects of AA on human eosinophils. From $V_h = -60$ mV, 8 s test pulses to +40 mV were applied at 30 s intervals. The H^+ current elicited by the first pulse after 5 μ M AA addition was already increased. I_H continued to increase over the 4 min illustrated. The inward current at -60 mV also increased progressively over this time period. The activation time constant, τ_{act} , decreased from 4.4 to 1.5 s. In contrast, although the tail current amplitude increased substantially, τ_{tail} did not change (215 ms before and after AA). The main difference in the response to AA compared with PMA (DeCoursey *et al.* 2001) is that τ_{tail} was slowed markedly by PMA, but was minimally affected by AA.

Figure 3B illustrates the concentration dependence of AA effects. A test pulse to +40 mV elicited a small H^+ current ('0'). After addition of 1 μ M AA, I_H increased and τ_{act} became faster but no I_e was detected. Similar effects were seen in four other cells treated with low AA concentrations. Addition of 3 μ M AA not only produced a large additional increase in I_H , but also activated I_e at -60 mV. Thus H^+ current was enhanced by AA at lower concentrations than those required to elicit distinct I_e . This experiment further illustrates the complex effects of AA on tail current kinetics. Initially τ_{tail} was 230 ms and after addition of 1 μ M AA decreased to 160–200 ms. In six of eight cells stimulated with AA at concentrations too low to activate I_e , τ_{tail} became faster. After addition of 3 μ M AA τ_{tail} first decreased to 150 ms, but while I_e was activated maximally τ_{tail} slowed to 355 ms. It appears that AA has a tendency to speed up tail current decay, but also that activation of I_e by AA is associated with a slowing of τ_{tail} , as observed with PMA stimulation.

Plotted in Fig. 3C are g_H-V relationships measured at 0 μ M AA (\bullet), 1 μ M AA (\square), and 3 μ M AA (Δ) in the same eosinophil shown in Fig. 3B. The g_H calculated from the amplitude of a single exponential fitted to the currents was more than doubled at 1 μ M AA at all voltages. When these g_H data are normalized (Fig. 3D) it becomes clear that there was no change in the voltage dependence of gating in the presence of 1 μ M AA. However, in the presence of 3 μ M AA (Δ) there was a 20–30 mV shift. Intriguingly, I_e was observed at 3 μ M AA but was not detectable at 1 μ M AA. In other cells, voltage shifts were quantified by estimating the threshold voltage for activating the g_H . By this criterion, in five cells treated with low AA concentrations that did not activate I_e no voltage shift was detected, whereas in seven eosinophils in which 3–10 μ M AA activated I_e voltage shifts in the range 20–50 mV were observed. In summary, at low concentrations AA increased g_H and sped up τ_{act} and at higher concentrations AA had these effects but also activated I_e and shifted the g_H-V relationship toward more negative voltages.

As observed with PMA stimulation (DeCoursey *et al.* 2001), V_{rev} measured after AA stimulation tended to be more negative than in the same cell before AA. In 13 eosinophils exposed to 3–5 μ M AA, V_{rev} shifted -5.3 ± 1.2 mV (mean \pm S.E.M.), corresponding to a drop in the apparent pH_i of ~ 0.1 unit. Thus, at most only a small part of the hyperpolarizing shift of the g_H-V relationship can be ascribed to uncompensated cytoplasmic acidification.

The constellation of effects of AA is illustrated in more depth in Fig. 4. Test pulses to +40 mV were applied periodically. Figure 4A shows test currents before and

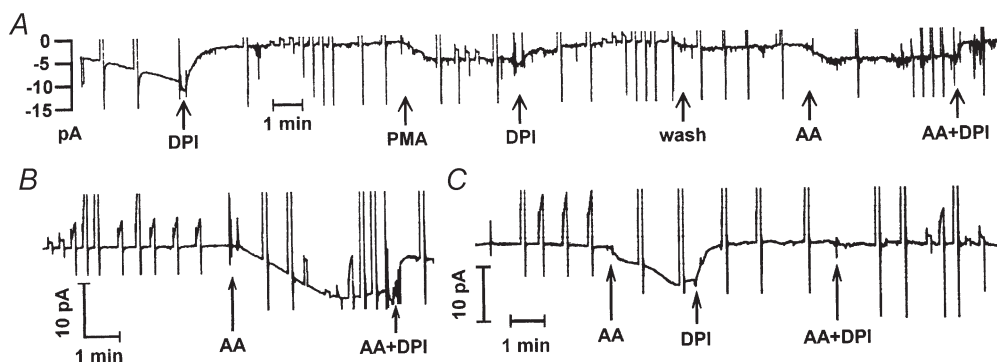


Figure 2. Examples of I_e in three different eosinophils

The holding current at -60 mV was recorded continuously on a chart recorder during each experiment. The frequent interruptions are the result of voltage pulses, which produced currents that were off-scale in most cases. All chart records were low-pass filtered at 4 Hz. *A*, this cell appeared to become activated spontaneously. At the times indicated by arrows, DPI (6 μ M), PMA (60 nM), or AA (5 μ M) was introduced. Additions were made by completely changing the bath solution. The bath changes took 10–20 s and the arrows indicate the middle of this time. *B*, activation of I_e by AA in another eosinophil. *C*, activation of I_e by AA. This is an example of a cell in which the I_e time course was biphasic. This pattern was seen with some cells stimulated with AA and others stimulated with PMA. Subsequent addition of AA together with DPI did not elicit detectable I_e .

after the first addition of $5 \mu\text{M}$ AA. The H^+ current was greatly enhanced, with both increased amplitude (I_{H}) and faster activation (smaller τ_{act}). These parameters are plotted in Fig. 4D as a function of time during the experiment. Soon after AA was added, the current at -60 mV (+ in Fig. 4D) increased from -1.6 to -9.4 pA , a net change of -7.8 pA that we attribute to I_{e} generated by NADPH oxidase. Addition of DPI and removal of AA (Fig. 4B) not only eliminated I_{e} , but also resulted in a slowing of H^+ current activation (Fig. 4Bb and c). This

result is in apparent contrast to the behaviour of PMA-stimulated neutrophils (DeCoursey *et al.* 2000) in which τ_{act} became faster after PMA, but this response was not reversed by DPI. However, we attribute the slowing of τ_{act} in Fig. 4Bc to removal of AA rather than to the effects of DPI, because addition of AA together with DPI accelerated τ_{act} (Fig. 4C and D, \blacktriangle). It appears that the faster activation is a relatively direct effect of AA on voltage-gated proton channels that occurs independently of NADPH oxidase activity.

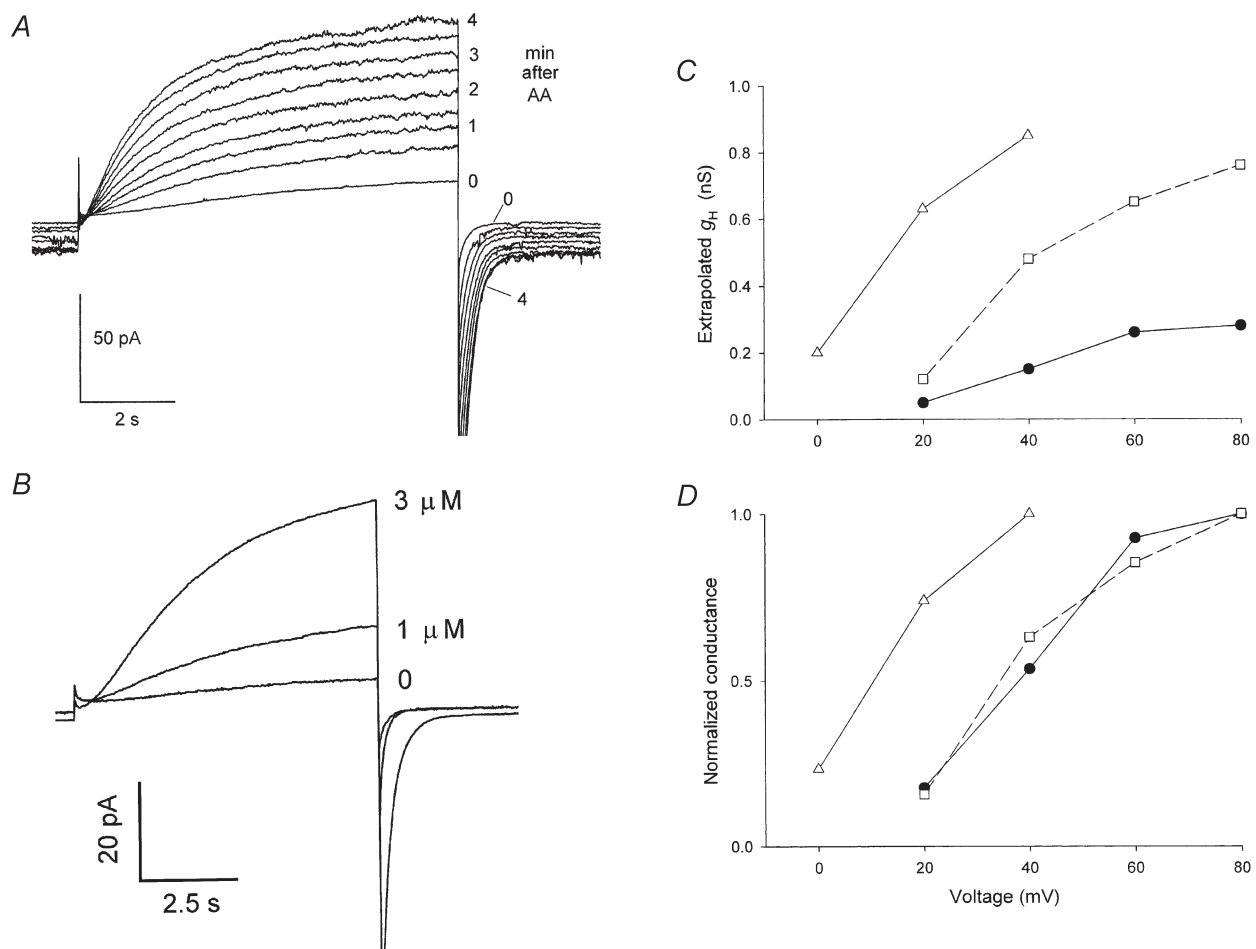


Figure 3. Effects of AA on H^+ currents

A, from $V_{\text{h}} = -60 \text{ mV}$, test pulses 8 s in duration to $+40 \text{ mV}$ were applied at 30 s intervals. The smallest current is the control. The first pulse after $5 \mu\text{M}$ AA addition elicited a larger H^+ current, and I_{H} continued to increase over the 4 min illustrated. The inward current at -60 mV also increased progressively. The larger tail currents were truncated. *B*, currents during pulses to $+40 \text{ mV}$ from $V_{\text{h}} = -60 \text{ mV}$ in an eosinophil in the presence of 0, 1 and $3 \mu\text{M}$ AA are superimposed. Note that in the presence of $3 \mu\text{M}$ AA there is distinct inward I_{e} not only at -60 mV , but also at $+40 \text{ mV}$ at the start of the test pulse. The peak tail current in $3 \mu\text{M}$ AA was off-scale. *C*, measurements were made in the same cell shown in *B* before exposure (●), and in the presence of $1 \mu\text{M}$ AA (□), and $3 \mu\text{M}$ AA (△). Because the currents did not reach steady state during the pulses, we fitted them with a single exponential after a delay to obtain the steady-state amplitude. The chord conductance g_{H} plotted here was calculated from the extrapolated currents using V_{rev} measured in each condition. The g_{H} values plotted in *A* were normalized (arbitrarily) to the largest value in each condition and are replotted in *D* to illustrate the voltage dependence.

Addition of AA increased I_H in this experiment (Fig. 4D, ○), and this increase was not reversed within a few minutes after the first addition of DPI and washout of AA. AA added together with DPI increased I_H further even though I_e was not activated. Eventually, after AA was washed out during the final DPI addition, I_H began to decrease. In other experiments, washout of AA usually resulted in a gradual decrease in H^+ current. Our impression is that, similar to its acceleration of τ_{act} , AA increased I_H amplitude independently of NADPH oxidase activity, and that this increase was at least partially reversible upon washout. In most experiments, I_H and τ_{act} changed in parallel. When AA increased I_H it usually also decreased τ_{act} , and when I_H decreased after washout of AA, τ_{act} usually slowed with a similar time course.

Response of eosinophils to repeated stimulation

The ability of eosinophils to respond to repeated stimuli is illustrated in Figs 2A and 5. As can be seen in Fig. 5A, distinct I_e (+) was activated by each of three consecutive

additions of 3 μM AA. The amplitudes and time courses of the three responses were not identical, but in each there was a tendency toward spontaneous turn-off. The continuous records of holding current (Fig. 2) show that DPI turns off AA-induced I_e rapidly. There was a clear response to PMA when applied after washout of the third application of AA.

In this experiment, τ_{tail} became slower during the first application of AA. This slowing reversed in parallel with the shut-off of I_e , consistent with the parallel responses of these parameters during PMA application (DeCoursey *et al.* 2001). During subsequent applications of AA no further changes in τ_{tail} were observed. This cell remained responsive, however, because addition of PMA did result in a distinct slowing of τ_{tail} that occurred simultaneously with the induction of DPI-sensitive I_e . The slowing of τ_{tail} by PMA was reversed by DPI. The effects of DPI in this experiment taken together are consistent with the idea that DPI does not directly change τ_{tail} , but only reverses

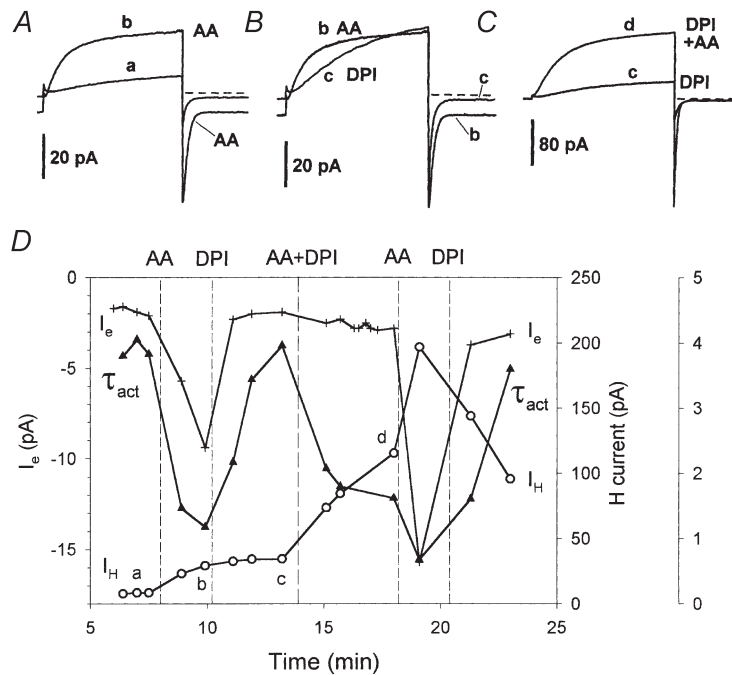


Figure 4. Electrophysiological effects of AA and DPI on an eosinophil

Current during 8 s pulses to +40 mV from $V_h = -60$ mV are superimposed. The raw current records plotted in A–C are labelled with lower case letters that also appear in D showing the time point of each illustrated record. A, currents before and ~ 2 min after addition of 5 μM AA. The H^+ current activated more rapidly – note the smaller activation time constant τ_{act} in D (▲) – and the H^+ current amplitude I_H (○) was several times larger, but τ_{tail} was not markedly changed (not plotted). The inward current at $V_h = -60$ mV (+ in D) increased by -7.8 pA after AA addition, attributed to I_e generated by NADPH oxidase. B, the same current record in the presence of AA (b) is superimposed on the current after replacement of AA with 6 μM DPI in the bath (c). Activation is slower and the putative I_e is eliminated. C, re-addition of 5 μM AA together with 6 μM DPI greatly enhanced I_H (note different current calibration bar) but did not activate distinct I_e . D, the time course of the experiment is illustrated. After addition of AA and DPI together, 5 μM AA alone was added, and again I_e was activated, which was eliminated by further addition of 6 μM DPI in the absence of AA. A continuous record of I_e in this cell is also shown in Fig. 2C. Note that the total current at V_h is plotted (+) and labelled I_e , but we interpret only the AA-activated and DPI-inhibited fraction as genuine (net) I_e .

Table 1. Comparison of the magnitude and time course of effects of AA and PMA in human eosinophils

	τ_{act}	τ_{tail}	I_H	I_e (pA)
AA/control	0.25 ± 0.12 (7)	1.0 ± 0.4 (6)†	4.6 ± 1.5 (7)	-7.4 ± 3.8 (22)
PMA/control	0.24 ± 0.09 (12)	5.4 ± 3.4 (14)	4.7 ± 2.8 (12)	-6.0 ± 2.8 (23)
$t_{1/2}$ AA (min)	0.1 ± 0.2 (7)†	—	0.7 ± 1.1 (7)	1.2 ± 1.1 (17)*
$t_{1/2}$ PMA (min)	1.0 ± 0.6 (12)	1.6 ± 0.7 (14)	2.0 ± 1.5 (12)	2.5 ± 2.1 (17)

The average ratio of each parameter, except I_e , measured after treatment with 3–10 μM AA to its value before treatment. Mean \pm S.D. is given for (*n*) observations. Usually I_H and τ_{act} were measured during pulses to +40 or +60 mV, and τ_{tail} and I_e at -60 mV. I_e is taken as the average peak increase in inward current after PMA. The $t_{1/2}$ value is the time required for the change to reach half-completion. The difference between AA and PMA is significant at * $P < 0.05$, † $P < 0.01$ by Student's *t* test. The PMA values are for human eosinophils, taken from DeCoursey *et al.* (2001).

slowing that occurs in conjunction with NADPH oxidase activation. In most experiments in which I_e was elicited upon AA addition, slowing of τ_{tail} was observed. Subsequently, however, τ_{tail} often became progressively faster. Our impression is that AA has two competing effects on H^+ current deactivation.

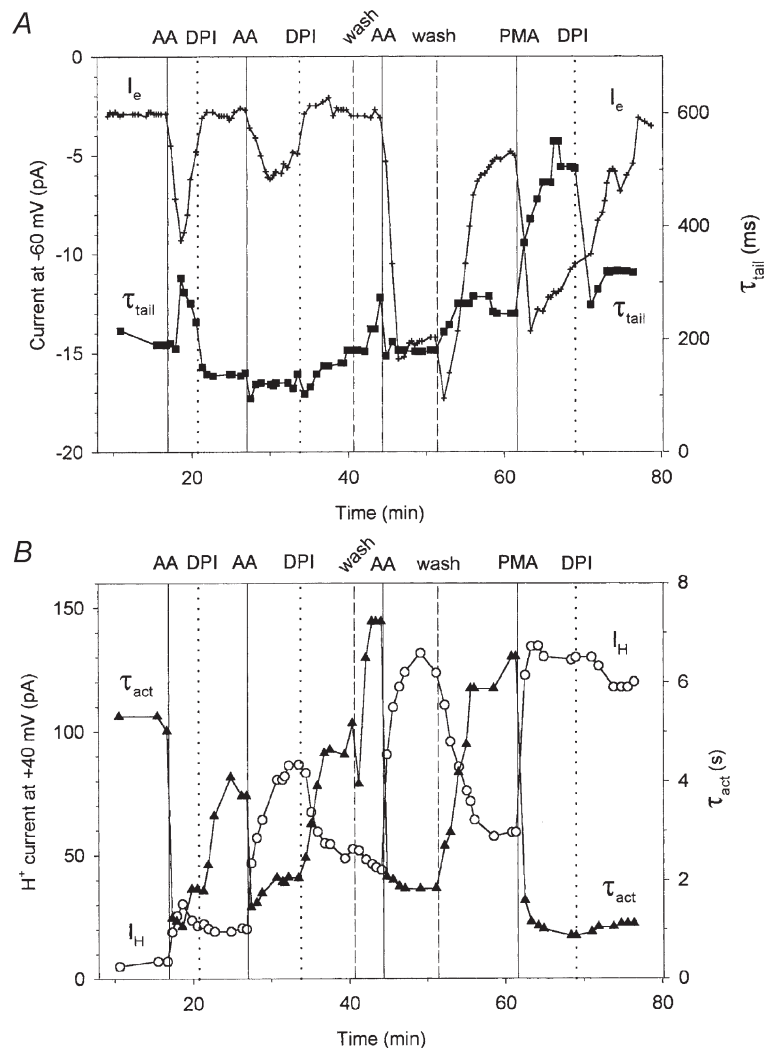
Like PMA, AA increased I_H amplitude and made activation faster (smaller τ_{act}). Unlike PMA, these changes

usually reversed at least partially when AA was washed out of the bath (Fig. 5B). Addition of DPI also appeared to reverse these changes, but it is likely that the simultaneous washout of AA was responsible.

The time course and amplitude of the effects of AA are summarized and compared with those of PMA in Table 1. Except for the lack of net effect on τ_{tail} , the magnitudes of the effects of AA and PMA were identical. The time

Figure 5. Time course of the response to multiple application of agonists

Temporal changes in H^+ current parameters and I_e during repeated application of 3 μM AA, 3 μM DPI, 60 nM PMA, and after washout, as indicated. Parameters from the same experiment are plotted separately in *A* and *B* for clarity. *A*, inward I_e (+) was activated during each of three applications of 3 μM AA. There were only minor changes in τ_{tail} (■) after AA treatment, but PMA caused distinct slowing, which was reversed by DPI. *B*, the amplitude of the H^+ current at +40 mV is plotted (○) along with τ_{act} (▲) during the same experiment as in *A*. Note that although DPI appears to reverse the AA-induced changes, similar reversal is seen with simple washout of AA. In contrast, the changes in these parameters induced by PMA were not reversed by DPI.



course of the changes was consistently faster for AA than for PMA. The three parameters in Table 1 that were changed by AA all changed at least twice as fast as they did after PMA stimulation. As was the case with PMA, the most rapid response was the faster activation rate of H^+ currents. Because τ_{act} was reduced before I_e had turned on significantly, it cannot be a consequence of NADPH oxidase activity.

DISCUSSION

AA elicits O_2^- release and I_e in human eosinophils

Here we demonstrate that AA is a potent and effective activator of NADPH oxidase in human eosinophils. O_2^- release was observed at $5 \mu M$ AA and at $10 \mu M$ AA, the response was near maximal. Although the maximum rate of O_2^- generated by human eosinophils in response to AA ($3.3 \text{ nmol } (10^6 \text{ cells})^{-1} \text{ min}^{-1}$) was lower than that elicited by PMA (DeCoursey *et al.* 2001), the maximal rate of AA-stimulated O_2^- release averaged 70% of that elicited by an optimal concentration of PMA under identical conditions (data not shown). To our knowledge, the only other study of AA activation of NADPH oxidase in eosinophils is of guinea-pig eosinophils (Lindsay *et al.* 1998), in which the maximum AA-stimulated H_2O_2 generation rate was $6.15 \text{ nmol } (10^6 \text{ cells})^{-1} \text{ min}^{-1}$.

In eosinophils studied with permeabilized-patch recording, AA at $3 \mu M$ or higher elicited distinct inward currents (I_e) that were largely inhibitable by DPI and were partially reversed by washout of AA. The dose–response relationship for I_e , like that for O_2^- release, was steep, with little or no activation by $1 \mu M$ AA and a maximal response at 5 – $10 \mu M$ AA. A similarly steep concentration–response relation has also been reported in human neutrophils (Henderson *et al.* 1993) and in guinea-pig eosinophils (Lindsay *et al.* 1998). Importantly, the concentration–response relationship for O_2^- release measured by cytochrome *c* reduction closely paralleled that observed for putative electron current (I_e). This similarity reinforces the identification of the observed inward currents as I_e that is generated by functioning NADPH oxidase. The correspondence between I_e and the electron flux rate calculated from O_2^- generation studies indicates that I_e as measured electrically under the conditions of this study is a good model system to study AA effects at the level of individual cells.

The I_e elicited by AA averaged -7.4 pA , and was similar in amplitude to that elicited by PMA, which averaged -6.0 pA (Table 1). This result is consistent with the finding that AA and PMA stimulate comparable maximal levels of O_2^- release in human neutrophils (Curnutte *et al.* 1984; Steinbeck *et al.* 1991). The maximum AA-stimulated H_2O_2 generation rate in guinea-pig eosinophils studied at $37^\circ C$ corresponds to -19.7 pA of I_e (Lindsay *et al.* 1998).

A surprising result was that DPI shut down I_e more rapidly when AA was the stimulus than when PMA was

the stimulus or in spontaneously activated eosinophils. The most obvious interpretation of this result is that the NADPH oxidase complex activated by AA is functionally different from that activated by PMA or spontaneously. There is some evidence that AA and PMA might lead to two distinct forms of activated NADPH oxidase. At high concentrations (50 – $100 \mu M$) AA alone (i.e. without phosphorylation of p47^{phox}) is sufficient to induce the required interaction between cytosolic p47^{phox} and p22^{phox} of the membrane-bound cytochrome *b*, but at low concentrations (1 – $5 \mu M$) AA acts synergistically with phosphorylation of p47^{phox} to activate the NADPH oxidase (Shiose & Sumimoto, 2000). In this view, the more rapid inhibition of the I_e by DPI may indicate that the AA-activated NADPH oxidase complex is less stable than that assembled by the action of PMA.

AA causes membrane damage at high concentrations

High concentrations of AA induce non-specific membrane damage in many cell types (Meves, 1994). High concentrations of AA ($> 5 \mu M$), or long and repeated exposure to lower concentrations of AA, often resulted in non-specific increases in membrane permeability (leak) in individual eosinophils under voltage clamp. Similar sensitivity of the membrane to AA-induced damage was reported previously in murine macrophages studied in the whole-cell configuration (Kapus *et al.* 1994). AA partitions strongly into cell membranes, with a partition coefficient of $\sim 2 \times 10^4$; at $5 \mu M$ applied AA, 3.6% of the membrane is composed of AA molecules intercalated between membrane phospholipids (Hutter, 1997). Micelle formation at high concentrations of AA has been suggested as a mechanism for non-specific damage (Meves, 1994).

As a control for non-specific membrane damage, we generally added AA together with DPI (Figs 2 and 4). That little inward current appeared under these conditions eliminates the possibility that the inward current during the addition of AA alone was due to non-specific leakage. Further evidence that the inward current reflects NADPH oxidase activity (rather than leak) is provided by the finding that the AA-activated current is also inward at $+40 \text{ mV}$, and is visible as net inward current at the start of the test pulses (Figs 3A, 3B, 4A and 4B). The zero-current potential of NADPH oxidase (viewed as an electron pump) is likely to be large and positive, and thus the I_e that it generates should be inward over a wide voltage range. In contrast, a non-specific leak current would be expected to reverse near 0 mV . In cells that became leaky, either spontaneously or after addition of high concentrations of AA, the inward current at -60 mV and outward current at $+40 \text{ mV}$ both increased. In summary, genuine I_e could be distinguished from non-specific membrane damage: I_e was reversible upon washout of AA or addition of DPI, and I_e was inward at the test potentials of $+40$ or $+60 \text{ mV}$, whereas leak was outward.

AA promotes opening of voltage-gated proton channels

AA profoundly enhanced voltage-gated proton currents in eosinophils studied in permeabilized-patch conditions: $g_{H,max}$ increased several-fold, activation was faster, and the g_H-V relationship was shifted toward more negative voltages. Because these effects were concentration dependent and, as can be seen in Figs 4D and 5, repeated applications of the same concentration of AA produced cumulative effects on I_H , quantifying them is somewhat arbitrary. Nevertheless, the magnitude of the changes produced by 3–10 μM AA was identical to that seen after PMA stimulation (Table 1). Like the effects of PMA, all of the effects of AA on H^+ channels lead to enhanced H^+ current. Although the probability and rate of H^+ channel opening at any voltage is increased by AA or PMA, the g_H remains voltage gated. Thus, H^+ channels in intact cells will open only with an appropriate combination of pH changes and depolarization.

The response of phagocytes to AA differs in four respects from the response to PMA. (1) PMA was completely ineffective in eosinophils (data not shown) or neutrophils (DeCoursey *et al.* 2000) studied in the whole-cell configuration, stimulating neither NADPH oxidase (I_e) nor voltage-gated proton channels. In contrast, AA enhances H^+ currents even in whole-cell configuration (see next paragraph). (2) The increase in I_H , speeding of τ_{act} , and activation of I_e all occurred more rapidly when AA was the stimulus. (3) I_e was inhibited by DPI much more rapidly when stimulated by AA than by PMA. (4) τ_{tail} was slowed profoundly by PMA but not by AA. AA appears to speed τ_{tail} in general, but in some experiments a slowing (< 2-fold) was observed (e.g. Fig. 5A), usually in conjunction with the appearance of I_e . These results can be explained if AA has two distinct effects on H^+ channels, both slowing and speeding τ_{tail} . In spontaneously activated or PMA-treated eosinophils, τ_{tail} is profoundly slowed. If both effects occur in permeabilized patch conditions, they will counteract each other, resulting in little or no net change.

Whole-cell studies of AA effects on voltage-gated proton channels in human eosinophils (Gordienko *et al.* 1996; Schrenzel *et al.* 1996), human neutrophils (DeCoursey & Cherny, 1993), and murine macrophages (Kapus *et al.* 1994) described a hyperpolarizing shift of 15–20 mV in the g_H-V relationship, an increase in the limiting g_H ($g_{H,max}$), and ~ 2 -fold faster activation and deactivation kinetics. It appears that AA increased $g_{H,max}$ more in permeabilized patch conditions. The hyperpolarizing shift in the g_H-V relationship also appears to be greater than in whole-cell studies. Activation of H^+ currents negative to V_{rev} after AA treatment was not reported in the whole-cell studies just cited. In the present study, AA shifted the voltage-activation curve sufficiently to activate the g_H negative to V_{rev} in some cells and, consequently, produced inward currents over a small voltage range. Most of the effects of AA on H^+ currents can be elicited in whole-cell configuration. Thus, although

the PMA response of eosinophils requires diffusible second messengers, much of the AA enhancement of H^+ currents does not. Hence, at least part of the enhancement of H^+ currents by AA reflects a relatively direct action on the channel. The more rapid response to AA than to PMA is consistent with this conclusion.

The properties of H^+ currents during NADPH oxidase activity differ from those in resting cells. Bánfi *et al.* (1999) suggested that two types of voltage-gated proton channels exist, one that is active in resting eosinophils, and another that becomes active during the respiratory burst. We prefer the simpler interpretation of a single population of H^+ channels that is modified extensively by NADPH oxidase activators like PMA (DeCoursey *et al.* 2000, 2001) and AA. With the exception of τ_{tail} , all the effects of AA on H^+ currents were similar to those elicited by PMA, and are also similar to the properties of voltage-gated proton channels in eosinophils dialysed with NADPH in the whole-cell configuration (Bánfi *et al.* 1999).

The enhancement of voltage-gated proton currents by AA was dissociable from the activation of NADPH-oxidase-mediated I_e . Low concentrations of AA enhanced I_H without activating detectable I_e (Fig. 3B). Also, addition of AA in the presence of DPI did not activate detectable I_e but enhanced I_H substantially (Fig. 4). These results are consistent with the conclusion of Nanda *et al.* (1994) that the activation of H^+ efflux by PMA does not require functional NADPH oxidase. Similarly, AA activates channel-mediated H^+ efflux in PLB cells deficient in phospholipase A_2 , even in the presence of DPI (Lowenthal & Levy, 1999).

Response to repetitive stimulation

After the I_e elicited by AA was inhibited by DPI or by simple washout of AA, I_e could again be activated by subsequent application of AA. DPI is believed to inhibit NADPH oxidase by an irreversible chemical reaction (O'Donnell *et al.* 1994). However, DPI and the related compound iodonium biphenyl appear to interact with NADPH oxidase only under reducing conditions (i.e. when the oxidase is functioning) – no inhibition occurs if these drugs are applied when the oxidase is not active (Doussiere & Vignais, 1991; Doussiere *et al.* 1999). This mechanism of action of DPI can be reconciled with our observation of repetitive responses to AA if only a fraction of the total oxidase components in the cell are assembled during each AA application. Because I_e measured electrically reflects only the active NADPH oxidase complexes present in the plasma membrane, the observation of repetitive responses further suggests that not all of the available oxidase components are translocated to the plasma membrane (and inhibited) during the first exposure to AA. This conclusion is not surprising considering that sustained O_2^- generation requires continuous translocation of the cytosolic NADPH oxidase components to the membrane (Quinn *et*

al. 1993), and that in intact cells O_2^- release persists for 20 min at 37 °C (DeCoursey *et al.* 2001).

In contrast to the repeated responses of eosinophils to AA described here, re-application of PMA generally elicited a weaker response or no response (DeCoursey *et al.* 2001). However, some eosinophils previously exposed to AA responded to PMA. Also, eosinophils that had recovered from spontaneous activation usually were capable of responding to either PMA or AA. Thus, it appears that PMA produces some changes that are not completely reversible either by DPI or by time, at least within the duration of these experiments. In contrast, AA is capable of activating I_e and increasing I_H repeatedly. Even in cells in which previous exposure to AA had greatly enhanced I_H , additional AA produced even greater responses. This pattern suggests that at least one step in the PMA signalling pathway is saturable. For example, once PKC has phosphorylated a target, additional PMA cannot produce a further response. In contrast, there appears to be no saturable intermediate or irreversible step in the AA response pathway.

Is AA the immediate activator of NADPH oxidase?

It has been proposed that in neutrophils AA is the immediate activator of NADPH oxidase (Henderson *et al.* 1989, 1993), and that AA is required for activation of H^+ efflux during the respiratory burst (Henderson & Chappell, 1992; Suszták *et al.* 1997). Several of the present results impact on these hypotheses. We demonstrate that AA is a potent activator both of voltage-gated proton channels and also of the NADPH oxidase in human eosinophils. It is clear that AA alters the functional properties of the H^+ channel, and that these effects are not simply the consequence of cytoplasmic acidification and/or membrane depolarization that tend to occur when NADPH oxidase is functioning. Activation occurs as a result of modification of the properties of the H^+ channels themselves. Teleologically, this result is in keeping with the idea that voltage-gated proton channels provide a mechanism of H^+ extrusion during the respiratory burst. The dual action of AA raises a question. Is it simply a coincidence that AA activates both NADPH oxidase and H^+ channels? Two explanations are possible: NADPH oxidase and H^+ channels might be activated by a common signalling pathway or they are activated by separate pathways that act concurrently and independently or perhaps in a coordinated fashion (Henderson & Chappell, 1992).

Overall, the most remarkable result of this study may be that AA, PMA, and spontaneous activation of eosinophils all produce the same constellation of effects. Both qualitatively and quantitatively, the speeding of τ_{act} , the increase in I_H , the hyperpolarizing voltage shift in the g_H-V relationship, and even the amplitude of I_e elicited by PMA or AA were quite similar (Table 1). This similarity in the responses to AA and PMA lends support to the common pathway idea.

Several distinct variants of common pathway mechanisms can be envisioned. The most extreme is that NADPH oxidase and voltage-gated proton channels are one and the same molecule. Although this idea has been discussed for some time (Henderson & Chappell, 1992, 1996; Nanda *et al.* 1994), our data do not resolve the question so we will not discuss it further. Another form of the common pathway idea is that AA is the immediate activator of NADPH oxidase (Henderson *et al.* 1993), whether it is stimulated by PMA or directly by AA. In this view, the effects of PMA on voltage-gated proton channels are mediated by AA that is released as a consequence of the action of PMA. That the action of AA is faster than PMA is consistent with this notion, as is the observation that much of the stimulation of H^+ channels by AA occurs in whole-cell configuration, and thus does not require diffusible second messenger pathways. The failure of AA to change τ_{tail} consistently is more difficult to explain. AA might have two opposing effects on τ_{tail} , namely a slowing as seen with PMA, and an acceleration that is also seen in whole-cell studies. Even so, it is not clear why the acceleration would be seen when AA is applied as the sole stimulus, but not if AA is the final step in the PMA-induced cascade. Other evidence seems to argue against an absolute requirement for AA to activate NADPH oxidase in eosinophils (White *et al.* 1993). Phorbol 12,13-dibutyrate inhibits H_2O_2 generation by ~80% at concentrations that reduce AA release by leukotriene B_4 by only ~40% (Lindsay *et al.* 1998). Evidently, PMA initiates responses via multiple pathways that overlap partially with the effects of AA.

The failure of AA to stimulate NADPH oxidase in whole-cell studies might indicate that diffusible second messengers are involved in the response. However, activation of NADPH oxidase requires assembly of at least six components, four of which in unstimulated cells are located in the cytoplasm. Diffusion of any required cytosolic component into the pipette would preclude NADPH oxidase assembly in whole-cell configuration. Overall, our data are most compatible with the idea that AA can directly enhance H^+ channel function, but that the complete physiological response involves additional modulation by a pathway that requires a diffusible intermediary, and may be linked to NADPH oxidase function.

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