Hepoxilin A₃ induces changes in cytosolic calcium, intracellular pH and membrane potential in human neutrophils

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The effects of hepoxilin A₃ (HxA₃), a 12-lipoxygenase metabolite of arachidonic acid, on cytosolic calcium ([Ca²⁺]_i), intracellular pH (pH_i), transmembrane potential and right-angle light scattering in human neutrophils were investigated. A rapid, transient elevation of [Ca²⁺]_i was observed with HxA₃ which was dependent on the concentration used. The effect of HxA₃ on [Ca²⁺]_i was blocked by pertussis toxin, suggesting involvement of receptors coupled to GTP-binding proteins. Experiments in Ca²⁺-free medium and using intracellular Ca²⁺ chelators indicated that HxA₃ mobilized Ca²⁺ from intracellular stores. At similar concentrations, HxA₃ altered pH_i, producing an initial acidification followed by an alkalization. The initial acidification was decreased in cells loaded with a Ca²⁺ chelator. In the presence of *N*-ethyl-*N*-(1-methylethyl)amino amiloride, an inhibitor of the Na⁺/H⁺ antiport, HxA₃ induced a greater acidification but failed to elicit the recovery phase, suggesting that the latter is due to activation of the antiport. HxA₃ also depolarized the membrane potential, although this effect was small. A decrease in right-angle light scattering, qualitatively similar to that observed with chemotactic peptides, was seen with HxA₃, indicating that the 12-lipoxygenase metabolite can induce shape changes in neutrophils. At the concentrations used for the above effects, HxA₃ was unable to generate a respiratory burst. These findings suggest that hepoxilins, which are formed by stimulated neutrophils, may have a role as messengers in neutrophil activation.

INTRODUCTION

Arachidonic acid stimulates a number of responses in neutrophils, including Ca²⁺ mobilization (Volpi *et al.*, 1984; Lew *et al.*, 1984), increased superoxide formation (Badwey *et al.*, 1984), secretion (Smith *et al.*, 1987) and actin polymerization (Yassin *et al.*, 1985). However, the mechanism by which these effects occur remains unclear. Arachidonic acid may act directly, as in the case for mobilization of intracellular Ca²⁺ pools (Beaumier *et al.*, 1987; Naccache *et al.*, 1989). Alternatively, the effects may be indirect, due to the formation of products of arachidonate metabolism. In neutrophils, this occurs primarily via the lipoxygenase pathway (Sha'afi & Molski, 1988). Hence, some responses of neutrophils to arachidonic acid (Naccache *et al.*, 1979, 1989; O'Flaherty *et al.*, 1979) and chemotactic factors (Bokoch & Reed, 1979; Naccache *et al.*, 1979; Smith *et al.*, 1982) can be modulated by inhibitors of the lipoxygenase pathway. In particular, the 5-lipoxygenase metabolite leukotriene B₄ has been implicated in the stimulation of neutrophils by arachidonic acid (Naccache *et al.*, 1989).

Here we have examined the possibility that other lipoxygenase products may be involved in stimulation of neutrophils, in particular the hepoxilin A₃ (HxA₃). Hepoxilins are hydroxyepoxide derivatives of arachidonic acid which are formed via the 12-lipoxygenase pathway through an intramolecular rearrangement of 12-HPETE [12(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid], catalysed by haemoglobin or haematin (Pace-Asciak *et al.*, 1983; Pace-Asciak, 1984). Hepoxilins

have been shown to be formed in cells and tissues possessing 12-lipoxygenase, such as platelets (Jones *et al.*, 1978; Bryant & Bailey, 1979; Bryant *et al.*, 1982; Pace-Asciak *et al.*, 1983), lung (Pace-Asciak *et al.*, 1983), brain (Pace-Asciak, 1988) and pancreatic islets (Pace-Asciak *et al.*, 1985). Hepoxilins are also formed *in vivo* after a bolus intravenous injection of arachidonic acid (Pace-Asciak *et al.*, 1988). Here we report that HxA₃ was able to stimulate Ca²⁺ mobilization in neutrophils in a dose-dependent manner, as well as stimulating changes in pH_i, membrane potential and right-angle light scattering, an indirect measure of cell shape.

MATERIALS AND METHODS

Materials

HxA₃ was prepared chemically as described (Corey & Su, 1984) and dissolved in dimethyl sulphoxide at a concentration of 2 mg/ml.

Solutions

HCO₃⁻-free RPMI 1640 was buffered to pH 7.3 using 10 mM-Na Hepes. The Na⁺ medium contained (in mM): NaCl (140), KCl (5), MgCl₂ (1), CaCl₂ (1), Hepes (10) and glucose (10), pH 7.3, at 37 °C. Ca²⁺-free Na⁺ medium was prepared by omission of CaCl₂ and the addition of 1 mM-EGTA. Where indicated, Na⁺ was replaced by equimolar amounts of K⁺ or *N*-methylglucammonium⁺. The osmolarity of the media was adjusted to 290 ± 5 mosm. HxA₃, in its methyl ester form, was dissolved

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)-ethane-*NNN'*-tetra-acetic acid; BCECF, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein; EMA, *N*-ethyl-*N*-(1-methylethyl)amino amiloride; G-protein, GTP-binding protein; HxA₃, hepoxilin A₃ (8-hydroxy-11,12-epoxyeicosa-5Z, 9E, 14Z-trienoic acid; 12-HPETE, 12(*S*)-hydroperoxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid.

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in dimethyl sulphoxide at a concentration of 2 mg/ml and portions of this solution were used.

Preparation of neutrophils

Neutrophils were separated from fresh human blood by dextran sedimentation followed by centrifugation on either a Ficoll-Hypaque gradient (Boyum, 1968) or a discontinuous plasma-Percoll gradient (Downey *et al.*, 1988). Residual red blood cells were removed by lysis with NH_4Cl . Washed cells were maintained at room temperature in Hepes-buffered RPMI at 10^7 cells/ml until use.

Where indicated, cells $[(1-2) \times 10^7$ cells/ml] were loaded with the Ca^{2+} chelator BAPTA by incubation with its acetoxymethyl ester ($10 \mu\text{M}$) in Hepes-buffered RPMI for 30 min at 37°C . For some experiments, the cells were pretreated with 500 ng of pertussis toxin/ml for 3-4 h at 37°C .

Measurement of cytosolic Ca^{2+} and pH

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and pH (pH_i) were measured using the fluorescent indicators Indo-1 and BCECF respectively. Loading with the acetoxymethyl ester form of these probes and calibration of the fluorescence signal were carried out essentially as described previously (Thomas *et al.*, 1979; Grinstein & Furuya, 1986; Nasmith & Grinstein, 1987). For each measurement, 2×10^6 cells were added to 1 ml of the indicated medium in a temperature-controlled cuvette at 37°C . Arachidonate metabolites and other compounds were added to the cuvette as a 1000 times concentrate in dimethyl sulphoxide.

Measurement of membrane potential

Bis-oxonol, an anionic, potential-sensitive, fluorescent dye was used to estimate the membrane potential. Excitation was at 540 nm (3 nm slit) and the emission was measured at 580 nm (15 nm slit). Neutrophils (2×10^6 /ml) were resuspended in Na^+ medium and $0.15 \mu\text{M}$ -bis-oxonol was added. The cells were allowed to equilibrate at 37°C until a stable baseline was attained (approx. 15 min). The fluorescence signal was calibrated as described previously by Rink *et al.* (1980).

Measurement of right-angle light scattering

To determine changes in right-angle light scattering, neutrophils (3×10^6 cells/ml) were excited at 340 nm in the cuvette of a Perkin-Elmer spectrofluorimeter, and the emission was measured at the same wavelength.

Measurement of HxA_3 and other eicosanoids by g.c./m.s.

Eicosanoids released during incubation of neutrophils were measured by g.c./m.s. using the negative-ion chemical-ionization mode and the deuterium isotope dilution method, employing the mass-fragmentographic technique as described previously (Pace-Asciak *et al.*, 1985; Pace-Asciak, 1987). Samples were spiked with tetra-deuterated internal standards (10 ng each of prostaglandins F_{2x} , E_2 , 6-oxo- F_{1x} , 6-oxo- E_1 , thromboxane B_2 and trioxilin A_3) and purified through a combination of extraction with ethyl acetate, C_{18} Sep-Pak chromatography, derivatization into the pentafluorobenzyl esters and *O*-methyl oximes, further extraction followed by chromatography on C_{18} Sep-Pak and silicic acid chrom-

atography. Final derivatization into the trimethyl silyl ether derivatives was achieved before analysis by g.c./m.s. Specific fragment ions were selected for each product of interest, i.e. m/z 569 and 573 for the undeuterated and tetradeuterated prostaglandins F_{2x} , 6-oxo- E_1 and trioxilin A_3 , m/z 524 and 528 for prostaglandin E_2 , m/z 614 and 618 for thromboxane B_2 and 6-oxo- F_{1x} , and m/z 479 for leukotriene B_4 . The latter product was quantified on the basis of the area of the tetradeuterated prostaglandin F_{2x} peak, since an appropriate deuterated form of leukotriene B_4 was not available. The analysis was performed on a Hewlett-Packard combined gas chromatograph mass spectrometer (Model 5988A) equipped with a 60 m fused silica capillary column (DB-1; dimethyl silicone; J & W Scientific) and H_2 as carrier gas. The mass spectrometer was operated in the negative-ion chemical-ionization mode using methane as reagent gas (source pressure 1.33×10^3 kPa). Mass fragmentography (selected ion monitoring mode) was run at 2900 electron multiplier voltage.

Statistics

All data represent the means $\pm$ S.E.M. of the indicated numbers of experiments. Statistical comparisons were made using Student's *t*-test with a significance level of $P \leq 0.05$.

RESULTS

Effects of HxA_3 on $[\text{Ca}^{2+}]_i$

HxA_3 stimulated a rapid, transient, increase in $[\text{Ca}^{2+}]_i$ which was dose-dependent (Fig. 1*a*). Fig. 1*a* shows the effect of $2 \mu\text{g}$ of HxA_3 /ml on cells suspended in Ca^{2+} -containing medium. At this concentration, the heptoxilin elevated $[\text{Ca}^{2+}]_i$ from the resting level of 236 ± 10 nM to a mean peak value of 726 ± 87 nM ($n = 5$). The initial spike was followed by a slower decline in Ca^{2+} which in four out of five experiments attained a new steady state which was 36 ± 9 nM above the level observed under resting conditions. In one experiment, $[\text{Ca}^{2+}]_i$ returned to the original resting value.

As shown in Table 1, the increase in $[\text{Ca}^{2+}]_i$ elicited by HxA_3 was greatly inhibited by pretreatment of the cells with pertussis toxin. This toxin is known to catalyse the ADP-ribosylation of certain GTP-binding (G-) proteins, thereby inhibiting most of the receptor-mediated responses induced in neutrophils by soluble activators (Sha'afi & Molski, 1988). The fractional inhibition of the HxA_3 response was comparable with that observed in cells simulated with fMetLeuPhe, a potent chemo-attractant (Table 1). This suggests that the two responses are produced by similar mechanisms, i.e. receptor-induced activation of G-proteins, and rule out a possible direct Ca^{2+} ionophoric effect of HxA_3 .

A number of experimental manipulations were carried out to determine the source of Ca^{2+} mobilized by HxA_3 . To determine whether influx of Ca^{2+} from the extracellular medium contributed to the response, cells were resuspended in a Ca^{2+} -free medium. This decreased the resting $[\text{Ca}^{2+}]_i$ to 119 ± 7 nM (Fig. 1*b*). Under these conditions, the addition of $2 \mu\text{g}$ of HxA_3 /ml caused an increase in $[\text{Ca}^{2+}]_i$ similar to that seen in Ca^{2+} -containing media. Thus, although the absolute level of the peak response attained in Ca^{2+} -free medium (497 ± 98 nM; $n = 3$) was significantly less than that in Ca^{2+} -containing medium, the change in $[\text{Ca}^{2+}]_i$ induced by the heptoxilin

Fig. 1. Effect of HxA₃ on [Ca²⁺]_i.

Neutrophils (2×10^6 cells/ml), loaded with Indo-1, were suspended in medium containing 1 mM-Ca²⁺ (a), or in Ca²⁺-free medium containing 1 mM-EGTA (b-d). The cells used in (c) were preloaded with BAPTA as described in the Materials and methods section. Where indicated, 2 μg of HxA₃/ml or 1 μM-ionomycin was added. The traces are representative of at least three similar experiments with blood from different donors. The inset in (a) shows the dependence of the [Ca²⁺]_i increase on the concentration of HxA₃. Each data point was taken from the same experiment, which was representative of five others.

did not differ significantly: 379 ± 97 nM compared with 483 ± 77 nM in Ca²⁺-containing media. These results suggest that, for the most part, the initial rise in [Ca²⁺]_i stimulated by HxA₃ is due to redistribution of cellular Ca²⁺, rather than to entry of extracellular Ca²⁺ into the cells. The removal of extracellular Ca²⁺ had a greater effect on the [Ca²⁺]_i plateau that followed the initial spike. In this case [Ca²⁺]_i did not remain elevated, but declined rapidly towards the initial resting level, indicating that maintenance of the secondary phase required entry of extracellular Ca²⁺.

Table 1. Effect of pertussis toxin on the HxA₃-stimulated rise in [Ca²⁺]_i.

Neutrophils (10^7 cells/ml) were incubated with 500 ng of pertussis toxin/ml for 3–4 h at 37 °C and loaded with Indo-1 for [Ca²⁺]_i measurement as described in the Materials and methods section. Cells were stimulated either with 2 μg of HxA₃/ml or with 20 nM-fMetLeuPhe and the change in [Ca²⁺]_i recorded. The inhibition caused by pertussis toxin is presented as percentage of the control. Data represent means $\pm$ S.E.M. of three separate experiments using blood from different donors.

	Increase in [Ca ²⁺] _i (nM)	Inhibition (%)
HxA ₃	336 ± 107	—
HxA ₃ + pertussis toxin	24 ± 5	92 ± 1
fMetLeuPhe	1549 ± 72	—
fMetLeuPhe + pertussis toxin	48 ± 27	98 ± 1

BAPTA, a Ca²⁺ chelator, was used to demonstrate that the Ca²⁺ responsible for the initial rise in [Ca²⁺]_i originated from a limited internal pool and that the fluorescence increase is not artifactual. Neutrophils were simultaneously loaded with Indo-1 and BAPTA and then resuspended in Ca²⁺-free medium for fluorescence measurement. Under these conditions, the HxA₃-stimulated increase in Ca²⁺ was markedly blunted (Fig. 1c). There was no rapid increase in [Ca²⁺]_i and a maximum change of only 14 ± 5 nM ($n = 4$) was recorded. Subsequent addition of 1 μM-ionomycin raised [Ca²⁺]_i only modestly, indicating that sufficient BAPTA was present to chelate most of the Ca²⁺ available for mobilization. To determine whether the initial rise in [Ca²⁺]_i was caused by release of Ca²⁺ from a membrane-bound intracellular compartment, cells were resuspended in Ca²⁺-free medium and 1 μM-ionomycin was added (Fig. 1d). The ionophore, which is expected to deplete intracellular compartments, induced a large, transient elevation of [Ca²⁺]_i. After [Ca²⁺]_i had returned to near resting levels, 2 μg of HxA₃/ml was added. Under these conditions, the heparin produced a marginal change in [Ca²⁺]_i, equivalent to only 15 ± 7 nM ($n = 5$).

Effects of HxA₃ on pH_i

The addition of 2 μg of HxA₃/ml to neutrophils suspended in an Na-containing medium caused an initial acidification of 0.04 ± 0.004 pH units ($n = 6$), followed by a recovery leading to an increase in pH_i to 0.02 ± 0.01 pH units above the initial resting pH_i (Fig. 2a). To determine whether this pH_i recovery was due to activation of the Na⁺/H⁺ exchanger, cells were treated with HxA₃ either in the presence of EMA (Fig. 2b), a potent inhibitor of the exchanger, or in the absence of extracellular Na⁺. In the presence of 2.5 μM-EMA, HxA₃ induced a significantly greater decrease in pH_i (0.08 ± 0.01 pH units; $n = 4$) compared with untreated cells. This acidification was not followed by a recovery of pH_i over the time period examined. Similar results were obtained when cells were stimulated in Na⁺-free medium (N-methylglucammonium⁺ substitution; results not shown), suggesting that the Na⁺/H⁺ exchanger is, in fact, responsible for the observed pH_i recovery.

The HxA₃-stimulated pH_i changes occur concomitantly with the rise in [Ca²⁺]_i. To determine whether the initial acidification and/or the subsequent activation of the exchanger were dependent on the increased [Ca²⁺]_i, BAPTA-loaded neutrophils were resuspended in Ca²⁺-free medium and used for pH_i determination (Fig. 2c). In this case, the initial fall in pH_i (0.02 ± 0.01 pH units; $n = 5$) appeared to be less than that observed under control conditions (see Fig. 2a), although the difference was not statistically significant. The steady-state pH_i reached following activation of the Na⁺/H⁺ exchanger was 0.09 ± 0.04 pH units above the initial resting pH_i. This increase is significantly greater than that seen in the absence of the chelator.

The acidification observed in BAPTA-loaded cells was very small, making accurate measurement of the pH_i change difficult. To inhibit the antiport and thereby unmask the acidifying component, similar experiments were undertaken in the presence of EMA (2.5 μM). Under these conditions, the initial fall in pH_i was 0.02 ± 0.003 pH units ($n = 3$). This change is significantly smaller than that observed when HxA₃ was added to EMA-treated neutrophils not loaded with BAPTA (cf. Figs. 2b

Fig. 2. Effect of HxA_3 on pH_i .

BCECF-loaded neutrophils (2×10^6 cells/ml) were suspended in medium with 1 mM- Ca^{2+} (a and b) or in Ca^{2+} -free medium with 1 mM-EGTA (c and d). The cells in (c) and (d) were preloaded with BAPTA, as described in the Materials and methods section. Where indicated, $2 \mu\text{g}$ of HxA_3 /ml or $2.5 \mu\text{M}$ -EMA were added. The traces are representative of at least three experiments with blood from different donors.

and 2d). Taken together, these experiments indicate that HxA_3 induces a cytosolic acidification that is largely dependent on the elevation of $[\text{Ca}^{2+}]_i$, followed by a recovery and slight alkalosis, which are attributable to activation of the Na^+/H^+ exchanger and are independent of Ca^{2+} .

Effect of HxA_3 on membrane potential

HxA_3 ($2 \mu\text{g}/\text{ml}$) caused a small, transient, membrane depolarization of 6 ± 2 mV (Fig. 3a), as indicated by an increase in the fluorescence of bis-oxonol. No increase in fluorescence was observed when the same concentration of HxA_3 was added to cells previously depolarized with either gramicidin (500 nM) or high K^+ (140 mM-KCl substituted for NaCl), arguing against an interaction between the heptoxilin and the dye as the cause of the increase in fluorescence.

Effect of HxA_3 on right-angle light scattering

A decrease in light scattering by activated neutrophils has been shown to be associated with actin polymerization and the resulting changes in cell shape (Sklar *et al.*, 1985). Fig. 3(b) shows that $2 \mu\text{g}$ of HxA_3 /ml stimulated a rapid decrease in light scattering to $92 \pm 1\%$ ($n = 3$) of the control (untreated) value. This was followed by a slower recovery to the initial scattering level. The rapid change in light scattering is smaller than but qualitatively similar to that observed using fMetLeuPhe as the stimulus (Fig. 3b).

Effect of HxA_3 on oxygen consumption

HxA_3 did not have a measurable effect on oxygen consumption, nor did it increase the responsiveness of cells to subsequent stimulation with 20 nM-fMetLeuPhe (results not shown).

Endogenous formation of HxA_3

To establish if HxA_3 could play a role as a second messenger or as an autocrine during neutrophil stimulation, we determined whether this compound is synthesized by activated neutrophils. In cells stimulated at 37°C under conditions that induce optimal release of

Fig. 3. Effect of HxA_3 on membrane potential and right-angle light scatter.

(a) Neutrophils (2×10^6 cells/ml) suspended in Na^+ medium were equilibrated with the potential-sensitive dye, bis-oxonol ($0.15 \mu\text{M}$); $2 \mu\text{g}$ of HxA_3 /ml was added where indicated. The chemotactic tripeptide fMetLeuPhe (20 nM) was added for comparison. Membrane potential was calibrated as described in the Materials and methods section. (b) Neutrophils (3×10^6 cells/ml) were suspended in Na^+ medium and used for measurement of light scattering. Where noted, the cells were stimulated with $2 \mu\text{g}$ of HxA_3 /ml, followed by stimulation with fMetLeuPhe (20 nM). The decrease in light scattering is represented as a percentage of the initial, baseline fluorescence. The traces in (a) and (b) are representative of at least three similar experiments.

arachidonate, i.e. with 12-*O*-tetradecanoylphorbol 13-acetate (10^{-7} M) and ionomycin (10^{-6} M), generation of HxA_3 could be detected as its stable derivative trioxilin A_3 by g.c./negative-ion chemical-ionization mass spectrometry, using the deuterium isotope dilution technique. In three determinations, the production of HxA_3 amounted to 61.1 ± 9.5 ng/5 min per 1.6×10^7 cells in stimulated neutrophils, compared with a resting level of 0.02 ± 0.01 ng/5 min per 1.6×10^7 cells in unstimulated cells. For comparison, leukotriene B_4 , a well-established mediator of neutrophil activation, was accumulated to a level of 40.6 ± 16.3 ng/5 min per 1.6×10^7 cells in the stimulated cells, compared with 0.12 ± 0.05 ng/5 min per 1.6×10^7 cells in unstimulated controls.

DISCUSSION

HxA_3 , at a concentration which did not affect cell viability, stimulated a rapid and transient increase in $[\text{Ca}^{2+}]_i$. This was primarily the result of release of Ca^{2+} from intracellular stores, although influx of extracellular Ca^{2+} may also have contributed to a small extent. The initial spike of intracellular $[\text{Ca}^{2+}]_i$ was followed by a decrease to a level which was in most cases sustained above the resting value. This plateau phase of the response was more sensitive to removal of extracellular Ca^{2+} in that, under such conditions, $[\text{Ca}^{2+}]_i$ was not sustained but decreased towards the resting concentration, suggesting that Ca^{2+} influx across the plasma membrane may be involved.

HxA_3 , at the same concentration which stimulated Ca^{2+} mobilization, caused a rapid cytosolic acidification followed by a return towards the resting pH_i and, in most cases, by a slight alkalization. The recovery phase was

prevented by addition of EMA or by omission of Na⁺, implicating the involvement of the Na⁺/H⁺ antiport. A similar pattern of pH_i changes is observed when neutrophils are stimulated by fMetLeuPhe (Molski *et al.*, 1980; Grinstein & Furuya, 1984) or by other activators (Volpi *et al.*, 1985; Nasmith & Grinstein, 1988). Activation of the antiport did not require an increase in [Ca²⁺]_i, as it was still observed in BAPTA-loaded cells suspended in Ca²⁺-free medium. In contrast, the initial acidification appears to be, in part, dependent on Ca²⁺. The occurrence of a Ca²⁺-dependent acidification, followed by a Ca²⁺-independent stimulation of Na⁺/H⁺ exchange, has also been reported in other cell types stimulated with hormones or growth factors (Mitsuhashi & Ives, 1988).

As well as changes in [Ca²⁺]_i and pH_i, HxA₃ caused a decrease in right-angle light scattering by the neutrophils, indicative of a shape change associated with actin polymerization (Sklar *et al.*, 1985) and stimulated a small, but reproducible, depolarization of the membrane potential. In contrast, HxA₃ did not induce a measurable increase in the rate of O₂ consumption, nor was it able to prime the cells to subsequent stimulation with fMetLeuPhe. In this regard, HxA₃ is an incomplete activator, resembling leukotriene B₄, another arachidonate metabolite which produces substantial changes of [Ca²⁺]_i (Volpi *et al.*, 1984; Lew *et al.*, 1987) and pH_i (Nasmith & Grinstein, 1988; Sumimoto *et al.*, 1988) while having relatively little effect on the NADPH oxidase (Palmblad *et al.*, 1981; Sumimoto *et al.*, 1984). This raises the possibility that HxA₃ may be stimulating leukotriene B₄ accumulation. This is not without precedent. Maclouf *et al.* (1982) have presented evidence that the HxA₃ precursor 12-HPETE, derived from platelets, stimulated the synthesis of 5-lipoxygenase products in human blood leukocytes. One of these products was leukotriene B₄. The use of specific inhibitors of the 5-lipoxygenase pathway for arachidonic acid metabolism is required to confirm or discount this possibility.

Could HxA₃ and/or other hepoxilins mediate the activation of neutrophils by physiological stimuli? It is well established that activation of these cells by chemoattractants and other stimuli is associated with elevated concentrations of arachidonate (Bokoch & Reed, 1980; Rubin *et al.*, 1981). Moreover, it has been demonstrated that some of the effects of arachidonate on neutrophils are eliminated by nordihydroguaiaretic acid and other lipoxygenase-blocking drugs (Naccache *et al.*, 1979, 1989; Bokoch & Reed, 1979). Initial experiments indicate that human neutrophils produce a large amount of HxA₃ upon activation with calcium ionophore and phorbol ester (61.1 ± 9.5 ng/5 min per 1.6 × 10⁷ cells; see the Results section). Assuming that the HxA₃ formed remains largely inside the cells, and considering that the cellular volume averages 350 μm³/cell, the endogenously generated hepoxilin can reach a concentration of 10.9 μg/ml. This is severalfold higher than the exogenous concentration required to elicit [Ca²⁺]_i, pH_i and shape changes, as reported above. Moreover, it should be noted that HxA₃ has been shown to be formed *in vivo* after intravenous injection of arachidonate (Pace-Asciak *et al.*, 1988). Hence it is conceivable that some of the actions of arachidonic acid may be due to its conversion into the hydroxyepoxide metabolites via the 12-lipoxygenase pathway in the neutrophils themselves

and/or in other cells, such as platelets. How these novel metabolites signal cell activation remains to be determined, but both Ca²⁺-dependent and Ca²⁺-independent processes seem to be involved.

In conclusion, it appears that the novel 12-lipoxygenase metabolite, HxA₃, can elicit some physiological responses in human neutrophils. Recent evidence by Piomelli *et al.* (1989) in *Aplysia* neurons and by Carlen *et al.* (1989) in mammalian CA1 neurons suggests that hepoxilins also modulate the electrical activity of these cells. It is therefore possible that these compounds play a role as second or third messengers in a wide variety of biological systems.

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