Na⁺/H⁺ exchange modulates the production of leukotriene B₄ by human neutrophils

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Human neutrophils produce various compounds of the 5-lipoxygenase pathway, including (5*S*)-hydroxyeicosatetraenoic acid, leukotriene B₄, its 6-*trans* isomers and ω -oxidation metabolites of LTB₄, when the cells are stimulated with the Ca²⁺ ionophore A23187. The elevation in the extracellular pH (pH_o) facilitated the cytoplasmic alkalization induced by the ionophore as determined fluorometrically using 2',7'-bis(carboxyethyl)carboxyfluorescein and enhanced the production of all the 5-lipoxygenase metabolites. The production decreased when the alkalization was blocked by the decrease in the pH_o, the removal of the extracellular Na⁺ or the addition of specific inhibitors of the Na⁺/H⁺ exchange, such as 5-(*NN*-hexamethylene)amiloride, 5-(*N*-methyl-*N*-isobutyl)amiloride and 5-(*N*-ethyl-*N*-isopropyl)amiloride. The alkalization of the cytoplasm with methylamine completely restored the production suppressed by the removal of Na⁺ from the medium. These findings suggest that the change in the cytoplasmic pH (pH_i) mediated by the Na⁺/H⁺ exchange regulates the production of the lipoxygenase metabolites. The site of the metabolism controlled by the pH_i change seemed to be the 5-lipoxygenase, because the production of all the metabolites decreased in parallel and the release of [³H]arachidonic acid from the neutrophils in response to the ionophore was not affected by the pH_i change. Furthermore, the production of the 5-lipoxygenase metabolites stimulated by A23187 with or without exogenous arachidonic acid showed a similar pH_o-dependence and the production induced by *N*-formylmethionyl-leucylphenylalanine (chemotactic peptide) with exogenous arachidonic acid also decreased when the cytoplasmic alkalization was inhibited.

INTRODUCTION

Leukotriene B₄ (LTB₄), a metabolite of arachidonic acid formed by the 5-lipoxygenase pathway, has potent chemotactic and chemokinetic activities for human neutrophils [1,2]. It elicits aggregation, lysosomal-enzyme release and, to a lesser extent, superoxide production of the neutrophils [3–6]. These activities suggest that the LTB₄ is important in inflammation and hypersensitivity reactions [7]. The leukotriene has been detected in inflammatory sites of some diseases [8,9].

Human neutrophils respond to LTB₄ and also produce this factor when exposed to the Ca²⁺ ionophore A23187 [10]. The pathway of the production is as follows. Arachidonic acid is released from the membrane phospholipids mainly by the action of the phospholipase A₂ [11]. 5-Lipoxygenase adds molecular oxygen to the acid forming (5*S*)-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5-HPETE), which is either dehydrated by the lipoxygenase to leukotriene A₄ (LTA₄) or reduced to (5*S*)-hydroxyeicosa-6,8,11,14-tetraenoic acid (5-HETE) [12–14]. LTA₄ is hydrolysed by a specific hydrolase to LTB₄ or non-enzymically to 6-*trans* isomers of LTB₄ [15]. LTB₄ is metabolized via ω -oxidation [16–18]

by a neutrophil-specific cytochrome *P*-450 [19] and then NAD⁺-dependent dehydrogenases [20] to its 20-hydroxy and 20-carboxy derivatives.

Little information is available concerning the mechanism by which the production of the 5-lipoxygenase metabolites, including LTB₄, is regulated. Two enzymes, namely phospholipase A₂ and 5-lipoxygenase, may be activated by stimulation, because these enzymes show minimal activities in resting cells. The elevation in the intracellular concentration of Ca²⁺ may be responsible for the increased production of the 5-lipoxygenase metabolites. The production by neutrophils is stimulated by the Ca²⁺ ionophore, and the activities of phospholipase A₂ and 5-lipoxygenase preparations are strongly dependent on the concentration of Ca²⁺ [21–24]. The involvement of protein kinase C in the arachidonic acid release has been proposed [25,26], but little is known about the regulation of the 5-lipoxygenase in intact cells.

In the present study we provide several lines of evidence suggesting that the Na⁺/H⁺ exchange regulates, via the cytoplasmic alkalization, the production of the 5-lipoxygenase metabolites by human neutrophils and that the regulation occurs at the level of the 5-lipoxygenase.

Abbreviations used: LTB₄, leukotriene B₄ or (5*S*,12*R*)-dihydroxyeicosa-6,8,10,14-*ZZZZ*-tetraenoic acid; 5-HPETE, (5*S*)-hydroperoxyeicosa-tetraenoic acid; LTA₄, leukotriene A₄; 5-HETE, (5*S*)-hydroxyeicosatetraenoic acid; 20-OH-LTB₄, 20-hydroxy-LTB₄; 20-COOH-LTB₄, 20-carboxy-LTB₄; RP-h.p.l.c., reverse-phase h.p.l.c.; pH_o, extracellular pH; pH_i, cytoplasmic pH; FMLP, *N*-formylmethionyl-leucylphenylalanine (chemotactic peptide); PG, prostaglandin; BCECF, 2',7'-bis(carboxyethyl)carboxyfluorescein; A23187, Ca²⁺ ionophore A23187; [Na⁺]_o, extracellular concentration of Na⁺; IC₅₀, concentration causing half-maximal inhibition.

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MATERIALS AND METHODS

Materials

Ca^{2+} ionophore A23187 was purchased from Calbiochem-Behring, San Diego, CA, U.S.A., *N*-formylmethionyl-leucylphenylalanine (FMLP) from the Peptide Institute, Osaka, Japan, and synthetic LTB_4 from Paesel G.m.b.H., Frankfurt, Germany. Authentic 20-hydroxy- LTB_4 (20-OH- LTB_4) and 20-carboxy- LTB_4 (20-COOH- LTB_4) were prepared by the incubation of the leukotriene with sonicated human neutrophils and purified by reverse-phase-h.p.l.c. (RP-h.p.l.c.). The purities were checked by g.c.-m.s. analyses as described previously [20,27]. Prostaglandin B_2 (PGB_2) and authentic 5-HETE were generously given by Ono Pharmaceutical Co., Osaka, Japan. [5,6,8,9,11,12,14,15- ^3H]-arachidonic acid was obtained from New England Nuclear, Boston, MA, U.S.A.; unlabelled arachidonic acid from Nakarai Chemicals, Kyoto, Japan; *N*-methyl-D-glucamine and methylamine from Wako Pure Chemicals, Osaka, Japan; 2',7'-bis(carboxyethyl)-carboxyfluorescein (BCECF) acetoxymethyl ester from Dojindo Laboratories, Kumamoto, Japan. 5-(*NN*-Hexamethylene)amiloride, 5-(*N*-methyl-*N*-isobutyl)-amiloride, and 5-(*N*-ethyl-*N*-isopropyl)amiloride were synthesized as previously described [28,29]. All other reagents were of the highest purity commercially available.

Incubation media

The standard medium used throughout the present study had the following composition: 135 mM-NaCl/5 mM-KCl/0.6 mM- CaCl_2 /1 mM- MgSO_4 /2 mM-glucose/20 mM-Hepes, pH 7.40. Media containing various concentrations of Na^+ were prepared by equimolar substitution of *N*-methyl-D-glucamine chloride for NaCl.

Cell preparation

Human neutrophils were isolated from the blood of healthy volunteers by dextran sedimentation, hypo-osmotic lysis, and the Conray-Ficoll method as described by Böyum [30]. The cell populations contained more than 98% neutrophils and less than 2% lymphocytes, monocytes or eosinophils.

Incubation and extraction

Human neutrophils (1.0×10^7 cells/ml) were pre-incubated for 5 min at 37°C and then stimulated with either 5 μM -A23187 or 1 μM -FMLP (plus 50 μM -arachidonic acid). After incubation for the indicated time at 37°C, the reaction was stopped by the addition of 3 ml of ice-cold ethanol containing 700 pmol of PGB_2 as an internal standard. The incubation mixture was centrifuged at 1000 *g* for 30 min, and the supernatant was evaporated under a stream of N_2 . The aqueous residue was acidified to pH 3 with 100 mM- H_3PO_4 and extracted three times with an equal volume of ethyl acetate. The extract was evaporated under N_2 , and the residue was dissolved in 200 μl of methanol/acetonitrile/water/acetic acid (2000:1250:3250:1, by vol.) and subjected to RP-h.p.l.c.

H.p.l.c. analysis

RP-h.p.l.c. was performed on a Shimadzu model LC-6A gradient system with Chemcosorb 5-ODS-H (5 μm

particle size; 4.6 mm $\times$ 150 mm; Chemco Scientific Co. Ltd., Osaka, Japan), and the flow rate was 1 ml/min. Solvents A and B consisted of methanol/acetonitrile/water/acetic acid (2000:1250:3250:1, by vol.) and methanol/acetonitrile (7:3, v/v) respectively. Fig. 1*b* (below) shows the solvent programme used. After equilibrating the column with solvent A, the elution was performed isocratically with the solvent for the first 6 min, followed by a linear increase in solvent B from 0 to 40% for the next 6 min. From 12 to 24 min, the column was eluted isocratically with 60% solvent A and 40% solvent B. From 24 to 25.5 min solvent B was linearly increased from 40 to 55%, followed by an isocratic elution with 45% solvent A and 55% solvent B for the next 20 min. After 40.5 min, solvent B was increased to 100% at a rate of 10%/min. Lipids were monitored by u.v. absorbance at 270 nm for the first 34 min and at 235 nm from 34 to 45 min. Typical retention times for 20-COOH- LTB_4 , 20-OH- LTB_4 , 6-*trans*- LTB_4 , 6-*trans*-12-*epi*- LTB_4 , LTB_4 , (5*S*,12*S*)-diHETE and 5-HETE were 8.7, 9.6, 20.9, 21.6, 22.7, 23.2 and 38.2 min respectively (Fig. 1*a*, below). 15-HETE and 12-HETE were eluted at 35.1 and 36.2 min respectively, well separated from 5-HETE. Recoveries of the arachidonic acid metabolites studied throughout the extraction and chromatographic procedures were between 91 and 96%. The metabolites were quantified by comparison of their peak areas with that of the internal standard, PGB_2 .

Identification of 5-lipoxygenase metabolites

LTB_4 , its 20-oxidized metabolites and 5-HETE were identified by co-elution with the authentic ones on RP-h.p.l.c., u.v. absorption spectra and g.c.-m.s. (5*S*, 12*S*)-diHETE, 12-HETE, 15-HETE, and 6-*trans* isomers of LTB_4 were identified by u.v. spectra and g.c.-m.s. For g.c.-m.s. analyses, lipids were converted into their methyl ester trimethylsilyl ether derivatives with ethereal diazomethane and hexamethyldisilazane/trichloromethylsilane.

Arachidonic acid release

The release of arachidonic acid from human neutrophils was assayed as previously described [31]. Briefly, neutrophils (1.0×10^8 cells/ml) were incubated for 15 min at 37°C with 1 μM -[^3H]arachidonic acid (1.0 Ci/mmol). After washing the cells, 1 μM -[^3H]arachidonic acid was loaded again. The cells were washed three times with the Ca^{2+} + Mg^{2+} -free standard medium containing 1 mg of bovine serum albumin/ml. The labelled cells were suspended in 1.0 ml at a concentration of 1.0×10^7 cells/ml. After preincubation for 5 min at 37°C, the cells were treated with 5 μM -A23187 for 5 min and sedimented by centrifugation. The radioactivity in 500 μl of the supernatant was determined by liquid-scintillation counting.

Cytoplasmic pH (pH_i) measurement

pH_i was determined fluorometrically using BCECF as previously described [32]. Human neutrophils were loaded with the probe by the incubation with the precursor BCECF acetoxymethyl ester for 30 min at 37°C with gentle stirring. The loaded cells were transferred to the thermostatically controlled stirred cuvette of a Hitachi F-7000 fluorescence spectrophotometer and preincubated for 5 min at 37°C. The

Fig. 1. RP-h.p.l.c. profile of the 5-lipoxygenase products from A23187-stimulated neutrophils

Human neutrophils (1.0×10^7 cells/ml) were stimulated with $5 \mu\text{M}$ -A23187 for 5 min at 37°C . The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. (a) RP-h.p.l.c. chromatogram of the 5-lipoxygenase products. Elution was monitored at 270 nm and then changed to 235 nm at 34 min. (b) The gradient program of RP-h.p.l.c. Details are given in the Materials and methods section.

fluorescence intensity was monitored with emission at 500 nm and excitation at 530 nm, and the pH_i was calculated by the nigericin/ K^+ method of Thomas *et al.* [33].

RESULTS

Production of 5-lipoxygenase metabolites

When human neutrophils (1.0×10^7 cells/ml) were stimulated with $5 \mu\text{M}$ -A23187 for 5 min at 37°C , they formed various 5-lipoxygenase products, namely LTB₄, its 6-*trans* isomers, 5-HETE and 20-OH and 20-COOH derivatives of LTB₄ as detected by the u.v. absorption on separation by RP-h.p.l.c. (Fig. 1). No detectable amount of the metabolites was produced without the addition of the ionophore (results not shown).

Fig. 2 shows the time course of the appearance of the 5-lipoxygenase metabolites. 5-HETE and LTB₄ appeared immediately after stimulation, and amounts became maximal at 2.5 and 5 min respectively and then decreased rapidly (Fig. 2). The rapid disappearance of 5-HETE may be due to its utilization for the esterification of the cellular glycerolipids and ω -oxidation [34]. The synthesis

Fig. 2. Time course of the formation of the 5-lipoxygenase metabolites by A23187-stimulated neutrophils

Human neutrophils (1.0×10^7 cells/ml) were incubated with $5 \mu\text{M}$ -A23187 for the indicated times at 37°C . The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. $\circ$, LTB₄; $\triangle$, 6-*trans*-isomers of LTB₄; $\square$, 20-OH-LTB₄ and 20-COOH-LTB₄; $\bullet$, 5-HETE.

of 5-HETE may also rapidly decelerate or terminate because the arachidonate 5-lipoxygenase reaction by neutrophil preparations is self-limiting and continues for only a few minutes [22,23]. The sum of LTB₄ and its ω -oxidized metabolites represents the net production of the leukotriene, because LTB₄ is converted into 20-OH-LTB₄ and 20-COOH-LTB₄ via ω -oxidation by human neutrophils [16–20]. The production rapidly proceeded for 5 min and almost ceased at 10 min (Fig. 3a). The production of LTA₄ may be represented by the sum of LTB₄, its ω -oxidized metabolites and 6-*trans* isomers of LTB₄, because LTA₄ is converted into LTB₄ or 6-*trans* isomers of LTB₄ [15]. The 6-*trans* isomers were metabolized very slowly (Fig. 2). The rapid production of LTA₄ was observed for 5 min and a negligible amount was produced after 10 min (Fig. 3b), which is consistent with the observation that 5-lipoxygenase reaction terminates within a few minutes [22,23].

Effects of pH_i

As shown in Fig. 3, the decrease in pH_i from 7.4 to 7.0 suppressed the production of LTA₄ and LTB₄ by about 50%. The amount of 5-HETE at pH_i 7.0 also decreased to about half of that at pH_i 7.4 at all the times studied (Fig. 3c), whereas its disappearance rate was not affected by pH_i (Fig. 4). The findings indicate that the production of 5-HETE decreased at pH_i 7.0. Similarly, the increase

Fig. 3. Effect of varying the pH_o on the production of the 5-lipoxygenase products

Human neutrophils (1.0×10^7 cells/ml) were suspended in the medium at pH_o 7.0 (●), 7.4 (○) and 7.8 (□). The cells were incubated with $5 \mu\text{M}$ -A23187 for the indicated times at 37°C . The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. (a) the sum of LTB_4 and its ω -oxidized metabolites; (b) the sum of LTB_4 , its 6-*trans* isomers and ω -oxidized LTB_4 ; (c) 5-HETE.

of the pH_o to 7.8 enhanced the amounts of the leukotrienes (Figs. 3a and 3b). The net production of 5-HETE may increase at increased pH_o , because its appearance rate increased while its disappearance rate was not affected (Fig. 4). Thus the effects of the pH_o change on the formation of LTA_4 paralleled those of LTB_4 and 5-HETE. When neutrophils suspended in media of various pH_o (from 6.6 to 8.2) were preincubated for 10 min at 37°C , washed with the standard medium (pH 7.4) and stimulated with $5 \mu\text{M}$ -A23187 at pH_o 7.4, the production of the 5-lipoxygenase metabolites was essentially the same as that observed in the cells preincubated in a medium of pH_o 7.4 (results not shown). The observation suggests that the effects of pH_o are reversible and not due to the damage to the cells. At all the pH_o values studied, no detectable amount of the metabolites was formed without the addition of the ionophore (results not shown).

Effects of extracellular concentration of Na^+

The production of the 5-lipoxygenase metabolites was also dependent on the extracellular concentration of Na^+ ($[\text{Na}^+]_o$) (Fig. 5). The decrease in the $[\text{Na}^+]_o$ led to a decrease in the production. In the absence of the extracellular Na^+ , the production decreased to about 40% of that at an $[\text{Na}^+]_o$ of 135 mM. The production of all three metabolites, 5-HETE, LTA_4 and LTB_4 , decreased in parallel when $[\text{Na}^+]_o$ was lowered. The production blocked by the removal of the extracellular Na^+ was completely restored by resuspending the cells in the standard medium (135 mM- Na^+) (results not shown), which shows that the cells were not damaged by the treatment.

Effects of inhibitors of the Na^+/H^+ exchange

When human neutrophils were stimulated with A23187 in the presence of 5-(*NN*-hexamethylene)-amiloride, a potent and reversible inhibitor of Na^+/H^+ exchange [35], the production of LTB_4 was inhibited with an IC_{50} of $2 \mu\text{M}$ (Fig. 6). The inhibition by the amiloride derivative was readily reversible when the cells were

Fig. 4. Effect of pH_o on the disappearance of 5-HETE in A23187-stimulated neutrophils

Human neutrophils (1.0×10^7 cells/ml) were incubated with $5 \mu\text{M}$ -A23187 at 37°C as described in Fig. 3. The time at 2.5 min after the stimulation was set as zero time and the HETE concentration at zero time was set as 100%. ●, pH_o 7.0; ○, pH_o 7.4; □, pH_o 7.8.

washed and then exposed to the ionophore in the absence of the drug (results not shown). Other inhibitors of Na^+/H^+ exchange, namely the 5-(*N*-methyl-*N*-isobutyl) and 5-(*N*-ethyl-*N*-isopropyl) derivatives of amiloride, blocked the production with IC_{50} values of $5 \mu\text{M}$ and $4 \mu\text{M}$ respectively (results not shown). The order of their inhibitory potencies paralleled that for the inhibition of the Na^+/H^+ exchange [35]. The formation of LTA_4 and 5-HETE was also diminished by the inhibitors with similar IC_{50} values (results not shown).

Fig. 5. Effect of varying $[Na^+]_o$ on the production of the 5-lipoxygenase metabolites

Human neutrophils (1.0×10^7 cells/ml) were suspended in medium including the indicated concentration of Na^+ and incubated with $5 \mu M$ -A23187 for 5 min at $37^\circ C$. The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. $\circ$, Sum of LTB_4 and its ω -oxidized metabolites; $\square$, sum of LTB_4 , its 6-*trans* isomers and ω -oxidized LTB_4 ; $\bullet$, 5-HETE.

Fig. 6. Effect of an inhibitor of Na^+/H^+ exchange on LTB_4 production

Human neutrophils (1.0×10^7 cells/ml) were incubated with $5 \mu M$ -A23187 for 5 min at $37^\circ C$ in the presence of the indicated concentration of 5-(*NN*-hexamethylene)amiloride. The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. Values represent the percentage of LTB_4 produced (the sum of LTB_4 and its ω -oxidized metabolites) in the absence of the inhibitor.

Fig. 7. A23187-induced pH_i change in neutrophils

Isolated neutrophils were loaded with BCECF as described in the Materials and methods section, and suspended at a concentration of 3×10^6 cells/ml in (a) the standard medium (135 mM- Na^+ , pH 7.4) or the medium (135 mM- Na^+) of the indicated pH, (b) Na^+ -free medium (135 mM-*N*-methyl-D-glucamine⁺, pH 7.4) or (c) the standard medium including $10 \mu M$ -5-(*NN*-hexamethylene)amiloride. The cells were preincubated for 5 min at $37^\circ C$. Where indicated by the arrows, the cells were activated by the addition of $5 \mu M$ -A23187. The traces are representative of at least four experiments.

A23187-induced pH_i changes

We studied the pH_i changes of neutrophils induced by A23187 using a fluorescent pH_i indicator, BCECF. As shown in Fig. 7, the addition of A23187 induced a biphasic change of the pH_i of neutrophils suspended in the standard medium (135 mM- Na^+ , pH 7.4): an initial acidification of 0.06–0.08 pH unit followed by an alkalization of 0.09–0.12 unit above the resting level of pH_i , 7.22. The maximum pH_i decrease occurred at 40–50 s after the stimulation and the subsequent alkalization phase was observed for 2–3 min. Extracellular acidification and alkalization caused slight changes in resting pH_i in the same direction (Fig. 7a). The alkalization was facilitated by the elevation in the pH_o to 7.8 and inhibited by the decrease in the pH_o to 6.6 (Fig. 7a), the removal of the extracellular Na^+ (Fig. 7b) or the addition of $10 \mu M$ -5-(*NN*-hexamethylene)amiloride (Fig. 7c), indicating that the alkalization evoked by A23187 is mediated by Na^+/H^+ exchange. The blocking of the exchange prolonged the acidification, and the new steady state of pH_i (6.9–7.0) was attained within 3 min.

Effect of methylamine on the metabolite production in the absence of extracellular Na^+

The findings presented above led us to postulate that Na^+/H^+ exchange may control the production of

Table 1. Reversal by methylamine of the inhibited production of 5-lipoxygenase products

Human neutrophils (1.0×10^7 cells/ml) suspended in the standard medium (135 mM- Na^+) or Na^+ -free medium were stimulated for 5 min at 37°C with $5 \mu\text{M}$ -A23187 in the presence or absence of 20 mM-methylamine. The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 3$).

[Na^+] (mM)	[Methylamine] (mM)	Product formed (pmol/ 10^7 cells)		
		5-HETE	ΣLTA_4^*	$\Sigma\text{LTB}_4^\dagger$
135	0	280 ± 8	838 ± 15	571 ± 22
0	0	149 ± 2	347 ± 9	195 ± 2
0	20	270 ± 17	841 ± 41	494 ± 36

* Sum of LTB_4 , its 6-*trans* isomers and ω -oxidized LTB_4 .
† Sum of LTB_4 and its ω -oxidized metabolites.

Table 2. Release of [^3H]arachidonic acid from human neutrophils

Neutrophils (1.0×10^7 cells/ml) loaded with [^3H]-arachidonic acid were incubated with A23187 ($5 \mu\text{M}$) for 5 min at 37°C in 1.0 ml of the standard medium (135 mM- Na^+ , pH 7.4) in the presence or absence of $10 \mu\text{M}$ -5-(*NN*-hexamethylene)amiloride (HMA) or the medium containing the indicated concentration of Na^+ (substituted with equimolar *N*-methyl-D-glucamine $^+$) or H^+ (the indicated pH), but otherwise identical with the standard medium. The suspension was centrifuged, and the radioactivity in a $500 \mu\text{l}$ aliquot of the supernatant was determined with a liquid-scintillation counter. Details are given in the Materials and methods section. The value was obtained by subtracting the radioactivity without A23187 from that with the ionophore (values are means $\pm$ S.E.M., $n = 4$).

Conditions	Arachidonic acid release (c.p.m.)
Control	3799 ± 461
pH 7.0	3522 ± 545
pH 6.6	2411 ± 248
Na^+ -free	3873 ± 274
+HMA	3787 ± 166

5-lipoxygenase metabolites by A23187-stimulated neutrophils via cytoplasmic alkalization. To test this hypothesis, we blocked cytoplasmic alkalization by removing Na^+ from the medium and determined whether artificial alkalization of the cytoplasm by the addition of methylamine could restore the suppressed production. The addition of 20 mM-methylamine to the extracellular medium caused a rapid and sustained rise in the pH_i from 7.2 to about 7.6, but no change in the pH of the medium (pH_o) (results not shown). As shown in Table 1, the addition of methylamine restored the A23187-stimulated production of the 5-lipoxygenase metabolites in the Na^+ -free medium to a level comparable with that in the standard medium (135 mM- Na^+). These findings are consistent with the hypothesis that the cytoplasmic alkalization by the Na^+/H^+ exchange is important in

Fig. 8. Effect of exogenous arachidonic acid on the production of 5-lipoxygenase metabolites by neutrophils suspended in medium at various pH_o values

Human neutrophils (1.0×10^7 cells/ml) suspended in the medium at the indicated pH_o were incubated for 5 min at 37°C with $5 \mu\text{M}$ -A23187 in the presence or absence of $50 \mu\text{M}$ -arachidonic acid. The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. $\circ$, $\bullet$, Sum of LTB_4 and its ω -oxidized metabolites; $\square$, $\blacksquare$, sum of LTB_4 , its 6-*trans* isomers and ω -oxidized LTB_4 ; $\triangle$, $\blacktriangle$, 5-HETE; $\circ$, $\square$, $\triangle$, in the absence of arachidonic acid; $\bullet$, $\blacksquare$, $\blacktriangle$, in the presence of arachidonic acid.

regulation of the production and suggests that the entry of Na^+ into the cells does not directly affect production. The addition of methylamine alone did not stimulate production (results not shown), suggesting that an alkalization of the pH_i itself does not trigger production.

Arachidonic acid release

Our next aim was to study the site where production is regulated by the cytoplasmic alkalization. The observation that the inhibition of the alkalization decreased the production of all the 5-lipoxygenase metabolites in parallel suggests that the release of arachidonic acid or the subsequent steps catalysed by 5-lipoxygenase may be regulated by pH_i change. We examined the effects of the blockade of the exchange on the release of arachidonic acid from neutrophils labelled with [^3H]arachidonic acid. The release was not affected by the decrease in the pH_o to 7.0, the removal of the extracellular Na^+ or the addition of $10 \mu\text{M}$ -5-(*NN*-hexamethylene)amiloride (Table 2). When neutrophils suspended in the medium at pH_o 6.6 were stimulated with ionophore, the arachidonic acid release was decreased to two thirds of that at pH_o 7.4 (Table 2). Nevertheless, production of the 5-lipoxygenase metabolites by neutrophils stimulated with A23187 in the presence of exogenous arachidonic acid showed a pH_o -dependence similar to that observed in the absence of the exogenous acid (Fig. 8).

Table 3. Production of 5-lipoxygenase metabolites by FMLP and exogenous arachidonic acid

Human neutrophils (1.0×10^7 cells/ml) were suspended in the standard medium (135 mM-Na⁺, pH 7.4) with or without 10 μ M-5-(*NN*-hexamethylene)amiloride (HMA), the Na⁺-free medium (135 mM-*N*-methyl-D-glucamine⁺, pH 7.4) or the medium at pH 6.7 (135 mM-Na⁺). The cells were incubated for 5 min at 37 °C with 1 μ M-FMLP and 50 μ M-arachidonic acid. The products were extracted and analysed by RP-h.p.l.c. as described in the Materials and methods section. Values are means $\pm$ S.D. for three experiments.

Conditions	Products formed (pmol/10 ⁷ cells)		
	5-HETE	Σ LTA ₄ *	Σ LTB ₄ †
Control	1139 $\pm$ 115	466 $\pm$ 46	268 $\pm$ 27
pH 6.7	580 $\pm$ 104	268 $\pm$ 12	135 $\pm$ 7
Na ⁺ -free	236 $\pm$ 19	54 $\pm$ 16	19 $\pm$ 4
+HMA	355 $\pm$ 59	164 $\pm$ 50	65 $\pm$ 21

* Sum of LTB₄, its 6-*trans* isomers and ω -oxidized LTB₄.
† Sum of LTB₄ and its ω -oxidized metabolites.

Production of 5-lipoxygenase metabolites by neutrophils stimulated with FMLP in the presence of arachidonic acid

The regulation of the 5-lipoxygenase activity in neutrophils stimulated with a receptor agonist FMLP was studied. Neither FMLP (1 μ M) nor arachidonic acid (50 μ M) induced the production of the 5-lipoxygenase metabolites (results not shown), but a simultaneous addition of both FMLP and arachidonic acid to the cells resulted in a marked production of the metabolites (Table 3). The 5-lipoxygenase may be activated by FMLP, as pointed out by Clancy *et al.* [36]. The production decreased by the blockade of the Na⁺/H⁺ exchange, i.e. the decrease in the pH_o, the removal of the extracellular Na⁺ or the addition of 5-(*NN*-hexamethylene)amiloride (Table 3). They are known to inhibit the cytoplasmic alkalization by the exchange which is activated by FMLP [35,37].

DISCUSSION

A putative Na⁺/H⁺ exchanger on the plasma membrane exerts pH_i change and thereby regulates the cellular processes that are dependent on the pH_i in various types of cells [38]. The modulation of the superoxide generation and chemotaxis of the FMLP-stimulated neutrophils by the cytoplasmic alkalization via the activation of the Na⁺/H⁺ exchange has been shown, although the site regulated by the pH_i change has not been elucidated [39,40]. Sweatt and co-workers have reported that blocking Na⁺/H⁺ exchange inhibits the arachidonic acid release from human platelets, which results in a diminution in thromboxane synthesis [41,42]. Nothing, however, has been reported about the role of exchange in arachidonic acid metabolism in other types of cells.

In the present study we provide several lines of evidence suggesting that the cytoplasmic alkalization by the activation of Na⁺/H⁺ exchange regulates the production of 5-lipoxygenase metabolites, including LTB₄, in human

neutrophils: (1) effect of pH_o; (2) concentration-dependent effect of [Na⁺]_o; (3) effect of amiloride derivatives; and (4) effect of methylamine. The cytoplasmic alkalization *per se*, however, does not trigger production, because an alkalization by addition of methylamine alone or exposure of the cells to FMLP, which led to an alkalization of the pH_i to about 7.4 [32,37], did not cause synthesis.

The finding of parallel changes in the production of all the 5-lipoxygenase metabolites with the pH_i change suggest that the regulated enzyme(s) may be phospholipase A₂, 5-lipoxygenase, or both. The contribution of phospholipase A₂ in regulation may be ruled out by the following observations: (1) the inhibition of the alkalization by the blockade of the Na⁺/H⁺ exchange did not affect the release of arachidonic acid evoked by A23187; (2) the production of all the metabolites induced by A23187 with exogenous arachidonic acid showed a pH_o-dependence similar to that without the acid; (3) FMLP-induced production in the presence of the exogenous acid was also blocked by the inhibition of the cytoplasmic alkalization. Especially, the production of LTA₄ in the presence of exogenous arachidonic acid strongly indicates the importance of 5-lipoxygenase in regulation, because the formation of LTA₄ reflects the 5-lipoxygenase activity. LTA₄ is formed from arachidonic acid by the two-step catalysis of the lipoxygenase [12–14], and the arachidonic acid moiety is preferentially converted by the lipoxygenase into LTA₄ in a concerted reaction without dissociation of a 5-HPETE intermediate from the enzyme [43,44]. A possibility that the decrease in the LTA₄ production is due to the enhancement in the reduction of 5-HPETE to 5-HETE with no change in 5-lipoxygenase activity can be ruled out by the finding that the net formation of 5-HETE was also decreased by the inhibition of the alkalization (Figs. 3 and 4). It has been reported that the synthesis of LTA₄ by neutrophil 5-lipoxygenase is not affected by the change in the conversion of 5-HPETE into 5-HETE [44]. The possibility that the LTA₄ hydrolase is also regulated by the pH_i change remains, but the primary regulation site in the LTB₄ formation may be 5-lipoxygenase, because the effects of the pH_i change on the production of LTB₄ paralleled those on the formation of its precursor, LTA₄.

The mechanism whereby the pH_i change controls lipoxygenase is not known. A simple explanation is the direct effect of the pH_i on the enzyme, because the activity of human neutrophil 5-lipoxygenase is strongly dependent on pH, with a maximal pH at above 7.5 and decreased to about half at pH 7.0 [45]. Rouzer & Samuelsson [24] have reported that the purified 5-lipoxygenase of human leucocytes requires multiple stimulatory factors for its maximal activity, in addition to a requirement for Ca²⁺ and ATP. Changes in pH_i may modulate the interaction of the enzyme with these factors.

Activation of Na⁺/H⁺ exchange is considered to be achieved by several pathways, including the protein kinase C-dependent and the Ca²⁺-dependent ones, depending on the cell type [46]. Platelets may be an example in which Ca²⁺ ionophores do not cause the cytoplasmic alkalization [47]. The latter pathway may be involved in the activation of human neutrophils by A23187, because the drug had no activity in the absence of extracellular Ca²⁺ (results not shown). Since LTB₄ activates the Na⁺/H⁺ exchange [32], the leukotriene

produced may increase its production via activation of the exchange by a positive-feedback mechanism.

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