Mono- and Dihydroxyeicosatetraenoic Acids Alter Calcium Homeostasis in Rabbit Neutrophils

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ABSTRACT Products of the lipoxygenation of arachidonic acid that express neutrophil chemotactic activity were examined in vitro for effects on the uptake of ^{45}Ca by rabbit peritoneal neutrophils. At optimally chemotactic concentrations, 5- and 11-hydroperoxyeicosatetraenoic acid, 11- and 12L-hydroxyeicosatetraenoic acid, and leukotriene B_4 enhanced ^{45}Ca uptake within 1 min by a mean of 212–694% of the values for control neutrophils incubated in buffer alone, as compared with 75% for 5(*S*)-hydroxyeicosatetraenoic acid and no effect for 15-hydroperoxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid. The approximate rank of potency of the factors stimulating ^{45}Ca uptake was similar to that observed for chemotaxis. Leukotriene B_4, in addition to stimulating the rate of ^{45}Ca uptake into rabbit neutrophils, also displaced intracellular calcium. The net result of the leukotriene B_4-induced changes in calcium homeostasis was to elevate transiently the intracellular level of exchangeable calcium. That neutrophil lipoxygenase metabolites of endogenous arachidonic acid rapidly enhance the influx of ^{45}Ca supports a possible role for such metabolites in general, and leukotriene B_4 in particular, in the regulation of the intracellular levels of free calcium in the neutrophils and possibly in other hormonally sensitive cells in which this cation is a second messenger.

INTRODUCTION

Polymorphonuclear leukocytes (neutrophils) have been shown to metabolize arachidonic acid mostly through the lipoxygenase pathway (1). Several of the lipoxygenase metabolites of arachidonic acids (hydroxyeicosatetraenoic acids [HETE])[1] influence diverse functions of the neutrophils. Human and rat neutrophils can be induced to move (2–7), aggregate (6), and degranulate to a limited extent (8) upon the addition of HETE in vitro, and guinea pig neutrophils accumulate in the peritoneal cavity in response to the introduction of HETE in vivo (9).

We have recently proposed that the neutrophil-directed activities of arachidonic acid and its lipoxygenase metabolites may be mediated by the ability of these fatty acids to alter the homeostatic regulation of the intracellular level of free calcium. This hypothesis was based on the results of experiments showing that calcium fluxes in rabbit neutrophils are specifically altered by exogenous arachidonic acid (10, 11) and that the capacity of rabbit and human neutrophils to migrate and to release lysosomal enzymes is dependent on the integrity of the lipoxygenase pathway (12–16).

We have therefore examined the effects of several monohydroxyperoxyeicosatetraenoic acids (HPETE) isomers, the corresponding mono-HETE, and the predominant natural di-HETE product of neutrophils, 5(*S*), 12(*R*)-dihydroxyeicosa-6,8,10 (two-*trans*, one-*cis*)-14-*cis*-tetraenoic acid (or leukotriene B_4), all of which are neutrophil chemotactic factors of differing potency, on the rate of ^{45}Ca uptake and on the steady-state level of exchangeable calcium in rabbit neutrophils. The results of these experiments are presented below.

METHODS

The experiments to be described were performed with rabbit peritoneal neutrophils collected, handled, and suspended in

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[1] *Abbreviations used in this paper*: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroxyperoxyeicosatetraenoic acid: HPLC, high-pressure liquid chromatography.

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modified Hanks' balanced salt solution, as previously reported (13). All the experiments were performed on cells thermally equilibrated at 37°C for 20 min and in the absence of added magnesium and extracellular protein. The rapid-sampling silicone oil method previously described in detail (17) was used for the calcium flux studies.

5-HPETE, 11-HPETE, and 15-HPETE were generated by incubating arachidonic acid (Supelco, Inc., Bellafonte, Pa.) with hydrogen peroxide and cupric chloride in methanol buffered with Tris–HCl (pH 7.8) as described (18). The HPETE were extracted into ethyl ether, resolved, and purified by reverse-phase high-performance liquid chromatography (HPLC), identified by mass spectrometry, and quantified by optical density at 235 nm and by the conversion of triphenylphosphine to triphenylphosphine oxide, as assessed by gas-liquid chromatography (18). 12-L-HETE was produced by incubating arachidonic acid with a partially purified preparation of lipoxygenase from human platelets (4, 9), whereas 5-HETE, 11-HETE, 15-HETE, and leukotriene B_4 were generated from arachidonic acid by neutrophils that had been stimulated with 10 μM calcium ionophore A23187 (5, 9, 14). The mono- and di-HETE were purified by sequential silicic acid column chromatography and reverse-phase HPLC, identified by mass spectrometry, and quantified by optical density at 235 and 280 nm, respectively (5, 14). 15-HPETE and 15-HETE were prepared from the soya bean lipoxygenase, extracted with ether, and fractionated by silicic acid chromatography. The fractions containing the compounds of interest were purified in two steps by HPLC on silica gel and octadecyl silica (reverse-phase). These compounds, whose identity was checked by mass spectrometry, were >95% pure as determined by gas chromatography.

RESULTS AND DISCUSSION

Inhibitors of the lipoxygenation of arachidonic acid have been shown to inhibit the functional responsiveness of rabbit and human neutrophils (12–16) and to concomitantly block both the chemotactic factor and arachidonic acid-induced stimulation of ^{45}Ca uptake in rabbit neutrophils (10, 12) and the metabolism of exogenously added arachidonic acid (14, 15). These results suggested that the lipoxygenation of endogenous arachidonic acid may represent one of the biochemical prerequisites for calcium mobilization and the presumably subsequent neutrophil activation by chemotactic factors and other agonists.

Intracellular calcium metabolism in neutrophils and its perturbation by chemotactic factors can be monitored by a variety of indirect radioisotopic tests. One of these, the rate of ^{45}Ca uptake, is a composite parameter reflecting both the plasma membrane permeability and the intracellular level of exchangeable calcium. As such it is an adequate initial test for a postulated alteration in calcium homeostasis.

As shown in Fig. 1, leukotriene B_4 significantly and rapidly increases the initial rate of ^{45}Ca uptake into rabbit neutrophils. The effects of arachidonic acid on this parameter of calcium metabolism are also illustrated in Fig. 1. Concentrations of the two fatty acids which produce about equivalent increases in ^{45}Ca uptake were chosen. Leukotriene B_4 can thus be seen to be close to 100 times more effective on a molar basis than its metabolic precursor, a result in agreement with known biological activities of these two compounds (5–7). Fig. 1, in addition, shows that neither of these two fatty acids is as effective as an optimum concentration of the synthetic chemotactic peptide formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe).

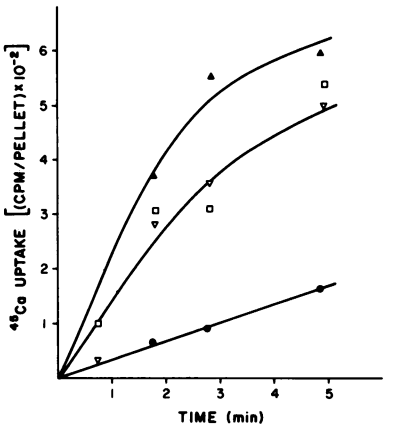

FIGURE 1 Effect of leukotriene B_4, 0.02 μg/ml (□), arachidonic acid, 1.5 μg/ml (▽), and f-Met-Leu-Phe, 1 nM (▲) on the time-course of ^{45}Ca uptake in rabbit neutrophils. ^{45}Ca and the various stimuli were added at zero time. The results shown in the figure depict those of a representative experiment (three different experiments). ●, control.

At concentrations which elicit a maximal optimal chemotactic response, 5-HPETE, 11-HPETE, 12-L-HETE leukotriene B_4 and to a lesser extent 5-HETE, but not 15-HPETE or 15-HETE, increased significantly the rate of uptake of ^{45}Ca by rabbit neutrophils (Table I). The maximal changes were observed within the 1st min of the additions, following which the rates of uptake paralleled that of unstimulated neutrophils (results not shown). The time-course of the effects of the HETE on ^{45}Ca uptake is similar to that of the formyl-methionyl peptides (17).

Leukotriene B_4 (1) exhibits a maximal effect on ^{45}Ca uptake at concentrations that are more than 20 times lower than those required for somewhat lesser effects by the HPETE and mono-HETE. In the 5- and 11-series, the HPETE is more active than the corresponding HETE. 11-HPETE and HETE showed no detectable activity on the rate of ^{45}Ca uptake at concentrations at high as 3.3 μg/ml (Table I). To the extent that such a correlation can be drawn, the approximate rank order of potencies of the HETE corresponds to their known chemokinetic activity (leukotriene B_4 > 5-HETE > 11-HETE > 12-HETE > 15-HETE; HPETE > HETE) (16, 19).

The effects of leukotriene B_4 on the net movement of ^{45}Ca in rabbit neutrophils were then examined. In these experiments, the cells were allowed to reach

TABLE I
Effect of HETE on the Initial Rate of ^{45}Ca Uptake in Rabbit Peritoneal Neutrophils

Compound	Concentration	Percent change from control
	μg/ml	
11-HPETE	1	435±51 (4)
11-HETE	2	250±90 (4)
5-HPETE	0.5	212±67 (5)
5-HETE	1	75±30 (3)
12-HETE	2	247±78 (3)
15-HPETE	3.3	32±70 (3)
15-HETE	3.3	−24±127 (2)
Leukotriene B_4	0.02	694±144 (4)

Calculated as the difference in counts between experimental and control conditions after 1 min of incubation with ^{45}Ca and the stimulus. The results are expressed as means±standard error of the mean. The numbers in parentheses refer to the number of experiments, each carried out in duplicate.

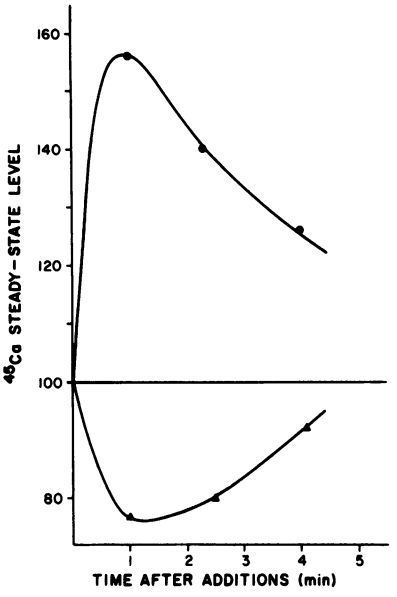

FIGURE 2 Effect of leukotriene B_4, 0.02 μg/ml, on the steady-state level of cell-associated ^{45}Ca in rabbit neutrophils. In these experiments the cells were incubated with or without "cold" $CaCl_2$ for 50 min before adding leukotriene B_4. Each point, taken in duplicate, was then normalized to its respective control. The results shown in the figure depict those of a representative experiment (three different experiments). ●, 0.05 mM Ca^{2+}; ▲, ≤5 μM.

isotopic equilibration before being exposed to leukotriene B_4 (Fig. 2). In the presence of 0.5 mM extracellular calcium (intracellularly directed electrochemical gradient for calcium), leukotriene B_4 causes a rapid and significant increase in the steady-state level of cell-associated ^{45}Ca. Leukotriene B_4 therefore unambiguously causes a net increase in the intracellular concentration of exchangeable calcium. In the absence of extracellular calcium, on the other hand, leukotriene B_4 induces a rapid and transient drop in the steady-state level of ^{45}Ca. As detailed elsewhere (23), this last result indicates that leukotriene B_4 causes an intracellular displacement of previously unexchangeable calcium.

Leukotriene B_4 therefore behaves as f-Met-Leu-Phe in causing both an intracellular redistribution of calcium and an increase in the plasma membrane permeability to calcium, the net result of which is to increase, at least transiently, the intracellular concentration of exchangeable calcium (17, 20). As the latter experiments lend themselves more readily to dose-response studies than any of the other parameters of ^{45}Ca fluxes, and because of the unique information that can be derived from these experiments concerning the intracellular status of calcium, we have examined the effects of three different concentrations of leukotriene B_4 on the steady-state level of cell-associated ^{45}Ca in the absence of extracellular calcium. The average drop in the steady-state level of ^{45}Ca 1 min after the addition of 0.0002, 0.002, and 0.02 μg/ml of leukotriene B_4 was found to be 10, 19, and 25%, respectively (means of two experiments, each carried out in duplicate).

The level of stimulation of the rate of ^{45}Ca uptake that was produced by the various HETE with the exception of leukotriene B_4 (see Fig. 1 and Table I) was significantly lower than that evoked either by f-Met-Leu-Phe or by arachidonic acid. In addition, the high concentrations required for the mono-HETE to exhibit biological activity and to alter ^{45}Ca uptake make it unlikely that these compounds are, under physiological conditions, involved in the alterations of the intracellular level of free calcium. Leukotriene B_4, on the other hand, affects ^{45}Ca metabolism at concentrations characteristic of the formyl-methionyl peptides and are likely to be attained in vivo. It is unknown at present whether the effective concentrations of these metabolites are overestimated because of the method of exogenous presentation to the neutrophils, as contrasted with the intracellular generation by physiological stimuli.

The capacity of exogenously added lipoxygenase products of arachidonic acid to enhance the rate of uptake of ^{45}Ca and to alter its steady-state level supports the hypothesis that such endogenous metabolites are at least in part responsible for the previously described perturbations of the intracellular level of calcium, the postulated second messenger in neutrophils. The recent findings that lipoxygenase inhibitors markedly diminish the anaphylactic contractile response of lung parenchymal strips from ovalbumin-sensitized guinea pigs (21) and the arachidonic acid- and $C5_a$-induced

contraction of guinea pig trachea (22), make it possible for this hypothesis to have validity in the study of other hormonally sensitive cells.

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