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LEUKOTRIENE PRODUCTION IN RAT PERITONEAL LEUKOCYTES REQUIRES INTACT ENERGY METABOLISM

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Abstract—Compounds which inhibit cellular production of ATP either by uncoupling of oxidative phosphorylation (valinomycin, carbonylcyanide-4-trifluoromethoxphenylhydrazone, and 2,4-dinitrophenol), glycolytic phosphorylation (2-deoxy-D-glucose) or by inhibiting respiratory-chain reactions (antimycin A) were all shown to inhibit calcium-ionophore A23187-induced leukotriene synthesis in rat peritoneal leukocytes at concentrations closely correlating with those needed to block ATP synthesis. In contrast, none of the compounds interfered with cyclo-oxygenase or other enzymes involved in arachidonate metabolism in these cells. Two well-known inhibitors of 5-lipoxygenase, nordihydroguaiaretic acid and phenidone, blocked LTB_4 synthesis without affecting ATP production. In conclusion, rat peritoneal leukocyte leukotriene synthesis depends on intact energy metabolism.

Stimulation of arachidonic acid release in inflammatory cells, e.g. macrophages and granulocytes, results in the rapid formation of prostanoids and leukotrienes, which mediate and modulate many of the pathophysiological reactions involved in the clinical manifestation of inflammation. For example, prostaglandin E_2 and prostacyclin are potent dilators of the microvasculature [1] and they sensitize peripheral pain receptors [2]. The peptido-leukotrienes (LTC_4, LTD_4 and LTE_4) produce extravasation of macromolecules [3] and enhance mucus secretion [4]. Chemotaxis, degranulation and aggregation of neutrophils are produced by the hydroxyeicosatetraenoic acids (HETEs), in particular LTB_4 [5].

Inhibition of prostaglandin synthesis is characteristic of non-steroidal anti-inflammatory drugs [6]. However, a clinically acceptable drug acting by specific inhibition of 5-lipoxygenase, the enzyme catalysing the formation of leukotrienes [7], has not yet been introduced. The interest for such compounds is considerable not only because of their anti-inflammatory potential, but also because they may be of value in the treatment of asthma, peptido-leukotrienes being extremely potent bronchospasmogenic hormones [8].

The enzyme, 5-lipoxygenase, is relatively unstable in a cell-free medium but has been recovered in an active form from rat basophilic leukaemia (RBL) cells [9, 10] and guinea-pig neutrophils [11]. It requires Ca^{2+} for expression of activity, and the activity is enhanced by ATP and other nucleotides. The study of drug effects on leukotriene production is, however, more easily performed using intact cells as the source of 5-lipoxygenase. The rat leukocytes obtained by lavage of the peritoneal cavity consist mainly of macrophages and are well equipped for such studies [12] as they release arachidonic acid, prostacyclin, thromboxanes, prostaglandins, HETEs and leukotrienes in response to calcium ionophore A23187.

Metabolic energy is required for stimulus-secretion coupling in inflammatory cells [13]. However, the specific requirement of energy for eicosanoid production has never been studied in detail. The question is highly relevant when intact cell preparations are used to screen compounds for 5-lipoxygenase inhibitory activity. If, indeed, the enzyme depends on ATP for expression of activity, the risk of false identification as 5-lipoxygenase inhibitors of compounds, which in fact interfere with cellular energy metabolism, is evident. Such compounds should be excluded from successive *in vivo* screens, as they are probably of little therapeutic value.

The present report describes the effect on eicosanoid production in rat peritoneal leukocytes of compounds known to interfere with normal ATP synthesis. Uncouplers of oxidative phosphorylation, valinomycin, a carbonylcyanide phenylhydrazone (FCCP) and 2,4-dinitrophenol, an inhibitor of electron transport in the respiratory chain, antimycin A, and an inhibitor of glycolysis, 2-deoxyglucose, were used to deplete the cells of ATP.

MATERIALS AND METHODS

Preparation and incubation of cell suspensions. Resident rat peritoneal leukocytes were harvested by injection of 20 ml Hank's balanced salt solution (HBSS) containing 12.5 units/ml heparin into the peritoneal cavity of female Sprague–Dawley rats, weighing 125–150 g, killed immediately before by CO_2 asphyxia. Cells from 10 rats were pooled and washed 3 times in HBSS. In some experiments the cells were then labelled for 90 min at 30° with 5 μCi [1-^{14}C]arachidonic acid, sp. act. ~ 60 mCi/mmole (New England Nuclear, Dreieich, F.R.G.), in 10 ml

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HBSS containing 0.5% bovine serum albumin. The residual radioactivity was then eliminated by repeated washings in the same medium, and the labelled cell suspension was finally adjusted to 1×10^7 cells/ml in HBSS. The cells were pre-incubated with drugs for 5 min at 37° before addition of A23187 (CalBiochem, San Diego, CA) and Ca^{2+} at final concentrations of 2×10^{-6} and 2×10^{-3} M, respectively, and at a final volume of 0.5 ml. In other experiments the labelling was omitted and the cells were simultaneously challenged with 0.5 μCi of the ^{14}C-labelled arachidonic acid, A23187 and Ca^{2+}.

Work up, quantification and identification of eicosanoids. In both types of experiments the incubations were stopped after 5 min by transfer to an ice bath and the cells were removed by centrifugation at 4°. An aliquot of the supernatant was counted to determine the free radioactivity and another was extracted twice with 4 volumes of ethyl acetate at neutral pH, then the pH was lowered to 3.0 with 1 N HCl and the extraction was repeated twice. The four extracts were combined, an aliquot was optionally counted to determine the efficiency of the extraction and the rest was evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of methanol and automatically spotted on a silica thin-layer plate fitted with a polar concentrating zone (E. Merck, Darmstadt, F.R.G.) by means of an Autospotter® (Desaga, Heidelberg, F.R.G.). The extracted eicosanoids were separated by TLC in the organic layer of the solvent mixture ethyl acetate:iso-octane:acetic acid:water (55:25:10:50) (solvent B). An autoradiogram was produced (Agfa-Gaevert Osray RPI X-ray film, Mortsel, Belgium) and the radioactive metabolites were quantified by laser densitometry using an LKB Ultroscan® (LKB, Bromma, Sweden) in combination with a computing integrator, SP 4100 (Spectra-Physics, San Jose, CA) as previously described [12].

The identification of radioactive metabolites was established by co-chromatography with authentic, tritiated standards in two solvent systems, system B (see above) and system A [Chloroform:methanol: acetic acid:water (90:9:1:0:65)], and by the use of the following specific inhibitors to block specific arachidonate metabolic pathways: NDGA, REV 5901 [14], indomethacin and imidazole.

Other procedures. ATP was measured at the time immediately before the addition of A23187 in 0.5-μl aliquots of the cell suspensions. After lysis of the cells the nucleotide was determined by the luciferin-luciferase method using a commercial kit LUMIT-PM® and automatic equipment for detection of bioluminescence (LUMAC® Biocounter Model 2010, Schaesberg, The Netherlands).

The effects of test compounds are expressed as percent of solvent-exposed control sample values. The solvent was dimethylsulfoxide (DMSO) except in the case of 2-deoxyglucose (water). All experiments were performed in duplicate with pools of cells from at least 10 rats. The results shown are means of the duplicate determinations, and the technical variation was insignificant.

The following compounds were investigated with regard to inhibition of leukotriene formation and ATP synthesis: Valinomycin (prepared at Leo Pharmaceutical Products by P. Rasmussen, lic. techn.), antimycin A, nordihydroguaiaretic acid (NDGA) and 2-deoxy-D-glucose (Sigma Chemical Co., St. Louis, MO), carbonylcyanide-4-trifluoromethoxy-phenylhydrazone (FCCP) (Fluka, Bucks, Switzerland), 2,4-dinitrophenol (Eastman Kodak, Rochester, NY) and 1-phenyl-3-pyrazolidone (Phenidone) (Ilford, London, U.K.). In addition indomethacin (Dumex A/S, Copenhagen, Denmark), imidazole (E. Merck, Darmstadt, F.R.G.) and REV 5901 were used to characterize the metabolism of arachidonic acid. REV 5901, a specific 5-lipoxygenase inhibitor [14], was a gift from Revlon Health Care Group (Tuckahoe, NY). Tritiated standards of 5-HETE, LTB_4, LTD_4, 6-keto-$PGF_{1\alpha}$, TXB_2, PGD_2 and PGE_2 (The Radiochemical Centre, Amersham, U.K.) were used for the identification of cellular metabolites of arachidonic acid.

RESULTS

Work up, identification and quantification of eicosanoids

Suspensions of washed peritoneal leukocytes incorporated approximately 50% (range 47–55% [^{14}C]arachidonic acid during the 90-min labelling period and stimulation with A23187 resulted in a

Table 1. Eicosanoid release from rat peritoneal leukocytes exposed to 2×10^{-6} M ionophore A23187

Metabolic pathway	Eicosanoid	Radioactivity recovered in each eicosanoid species (%) Prelabelled cells*	Unlabelled cells†
Cyclo-oxygenase products	6-keto-$PGF_{1\alpha}$	24.5	22.6
	TXB_2	17.2	17.2
	PGD_2	9.3	10.5
	HHT	12.6	15.2
5-lipoxygenase products	LTB_4	19.5	10.8
	5-HETE	12.7	12.5
	Unknowns	4.2	8.5
	C-20:4	<1	2.7

Results are means of duplicate determinations in pools of cells from at least 10 rats.

* Cells were labelled with [^{14}C]20:4 for 90 min before challenge with A23187.

† Washed cells were challenged with A23187 and at the same time exposed to [^{14}C]20:4 (0.5 μCi/10^6 cells).

release of 8–13% of the label. The radioactive metabolites were identified by cochromatography with authentic, ^{3}H-labelled standards in two different solvent systems and also by observing the effects on their synthesis of well-established, specific inhibitors or arachidonate metabolic pathways.

The radioactive standards chromatographed with the following R_f values in the two solvent systems A and B, respectively: arachidonic acid, 0.68, 0.70; 5-HETE, 0.49, 0.51; LTB_4, 0.38, 0.37; PGD_2, 0.42, 0.28; PGE_2, 0.38, 0.16; TXB_2, 0.28, 0.17; 6-keto-$PGF_{1\alpha}$, 0.37–0.39, 0.06. Solvent B was preferred for the present study since it separates all eicosanoids of interest except PGE_2 and TXB_2. As PGE_2 was formed in insignificant quantities under the present experimental conditions (see below) this did not, however, present a problem. The lipoxygenase products, 5-HETE and LTB_4, and the cyclo-oxygenase products, 6-keto-$PGF_{1\alpha}$ (the stable prostacyclin metabolite), TXB_2, PGD_2 and HHT, were tentatively identified by their chromatographic properties as the major metabolites of A23187-stimulated resident-rat peritoneal leukocytes. Laser densitometry of autoradiograms showed that these metabolites accounted for 96% of the radioactivity found in the supernatants after 5 min incubation and the amount of each eicosanoid was determined (Table 1). The residual 4% metabolic radioactivity was mainly located with an R_f value similar to that of 15-HETE, but no further attempt was made to identify this. This metabolite identified as LTB_4 occasionally appeared as a blurred spot on the autoradiogram, which may indicate the presence of other diastereomeric 5,12-di-HETEs [15].

The identification of metabolites representing 5-lipoxygenase and cyclo-oxygenase activity was then finally established by the use of specific inhibitors. Two lipoxygenase inhibitors, REV 5901 (10^{-6} M) and NDGA (10^{-5} M), completely prevented the formation of the compounds identified as 5-HETE and LTB_4. Those compounds showed no inhibitory action on the cyclooxygenase-initiated pathways. In contrast, indomethacin (10^{-6} M) completely blocked the formation of 6-keto-$PGF_{1\alpha}$, TXB_2, PGD_2 and HHT, stimulating the 5-lipoxygenase-related metabolism to LTB_4 and 5-HETE, which proved the absence of 5-lipoxygenase-derived products co-chromatographing with any of the cyclo-oxygenase products. Finally, imidazole (10^{-3} M) a specific thromboxane-synthetase inhibitor [16], abolished the formation of TXB_2 and HHT, redirecting the conversion of prostaglandins towards PGD_2, 6-keto-$PGF_{1\alpha}$ and, to a small degree, PGE_2. A similar approach was used to identify and quantify the arachidonate metabolism in unlabelled cells (Table 1).

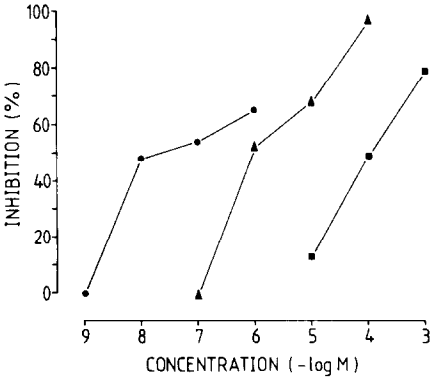

Fig. 1. Effect of uncouplers of oxidative phosphorylation on LTB_4 synthesis in rat peritoneal leukocytes prelabelled with [^{14}C]arachidonic acid. A pool of cells from 10 rats were labelled with 5 μCi of the acid, washed, resuspended at 10^7 cells/ml and preincubated with valinomycin (●) FCCP (▲), or 2,4-dinitrophenol (■) for 5 min before exposure to 2 μM A23187. The solvent (1% DMSO) was added to the control tubes. LTB_4 was isolated by ethyl acetate extraction and TLC and quantified by laser densitometric analysis of autoradiograms. Results shown are mean values of duplicate determinations.

The formation of peptido-leukotrienes, LTC_4 and LTD_4, was not investigated in the present study as these eicosanoids were too polar even to migrate out of the concentrating zone of the TLC plates as shown by application of a [^{3}H]LTD_4 standard. Furthermore, the recovery of LTD_4 in ethyl acetate extracts was only 55.7 ± 2.5 (mean ± S.D., N = 6), in contrast to the overall efficiency of extraction which was 81.9 ± 3.7 (N = 20) as calculated from the radioactivity present in the cell supernatants and in the combined extracts. A small part of the non-extractable radioactivity released after incubation might thus represent peptido-leukotrienes. Extraction at neutral pH prior to acidification was included because it yields a better recovery of lipophilic eicosanoids (HETEs and arachidonic acid).

Experiments with [^{14}C]arachidonate labelled cells

Valinomycin, FCCP and 2,4-dinitrophenol, three different uncouplers of oxidative phosphorylation, all inhibited the A23187-induced formation of LTB_4 from rat peritoneal leukocytes prelabelled with [^{14}C]-arachidonic acid in a dose-dependent manner (Fig. 1). The inhibition of 5-HETE closely parallelled that of LTB_4 and the decrease in lipoxygenase product formation was compensated for by an increase in cyclo-oxygenase products, in the metabolite tentatively identified as 15-HETE, and in free arachidonic acid. The total release of radioactivity was thus not affected by the three compounds tested. The cell viability, estimated by the eosine dye-eclusion method, was 79 ± 7% (mean ± S.D., N = 7) and was not affected by either compound.

Experiments with unlabelled cells

The assay using unlabelled cells was then employed in order to further investigate the apparent specific effect on 5-lipoxygenase activity by ATP inhibitors, as this assay bypasses the steps involving activation of phospholipase A_2. In addition to valinomycin, FCCP and 2,4-dinitrophenol, the *Streptomyces* antibiotic, antimycin A, the glycolysis inhibitor, 2-deoxyglucose and the 2 anti-oxidant-type inhibitors of lipoxygenase, NDGA and phenidone, were tested for interference with eicosanoid synthesis in unlabelled rat leukocytes.

All of the compounds inhibited LTB_4 formation in a dose dependent manner (Fig. 2), the order of

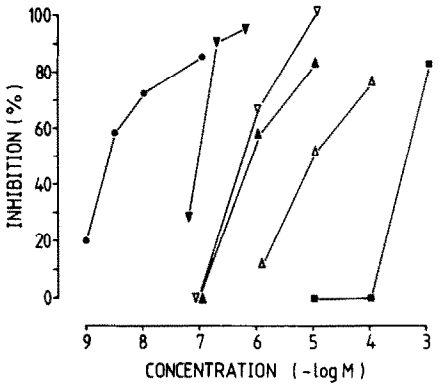

Fig. 2. Inhibition of [^{14}C]LTB production in rat peritoneal leukocytes by compounds interfering with ATP synthesis [valinomycin (●), antimycin A (▼), FCCP (▲), 2,4-dinitrophenol (■)], and anti-oxidants [NDGA (▽) and phenidone (△).] The cells were preincubated for 5 min with the compounds before addition of A23187 and [^{14}C] arachidonic acid. The amount of radioactivity associated with LTB_4 was determined by densitometry of autoradiograms after extraction and separation by TLC. Results shown are mean values of duplicate determinations using a pool of cells from at least 10 rats.

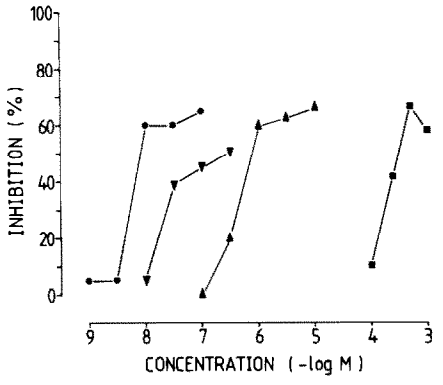

Fig. 3. Reduction of ATP levels in rat peritoneal leukocytes by compounds interfering with ATP synthesis [valinomycin (●), antimycin A (▼), FCCP (▲) and 2,4-dinitrophenol (■)]. The cells were incubated for 5 min with the compounds and the intracellular concentration of ATP was then determined by an automatic luciferin-luciferase method.

potency being valinomycin > antimycin > NDGA = FCCP > phenidone > 2,4-dinitrophenol. 2-Deoxyglucose also exerted a dose-dependent inhibition of 5-lipoxygenase reaching a value of 30% at 10^{-2} M (data not shown). Solubility problems excluded experiments at higher concentrations. 5-HETE was also inhibited by all compounds at similar concentrations indicating an effect at the initial step of the reaction, i.e. inhibition of 5-lipoxygenase activity. The inhibition led to a diversion of arachidonate metabolism towards the cyclo-oxygenase pathway and an increase in free arachidonic acid. The increased formation of the metabolite tentatively identified as 15-HETE was also occasionally observed. When indomethacin (10^{-6} M) was added to cells preincubated with valinomycin (10^{-7} M) at a concentration sufficient to substantially inhibit the amount of radioactive LTB_4 and 5-HETE, the cyclo-oxygenase products were completely inhibited (as in experiments with indomethacin alone). This excludes the possibility that the decrease in LTB_4 after valinomycin was caused by an increase in ω-oxidation of LTB_4 to more polar products co-chromatographing with cyclo-oxygenase metabolites such as TXB_2. The extraction recovery was not altered in the presence of valinomycin (79.7 ± 2.8 vs 80.0 ± 3.3, N = 3 in each group), excluding the possibility of a diversion towards peptide-leukotrienes.

Effects on ATP *concentration*

The effects of the test compounds on intracellular ATP concentrations were measured by the bioluminescence method. A concentration-dependent decrease in ATP was observed with valinomycin, antimycin A, FCCP and 2,4-dinitrophenol (Fig. 3). It was not, however, possible to reduce ATP by more than 50–70% with these compounds, indicating that other mechanisms providing the cells with ATP were operating, such as glycolytic phosphorylation. The half-maximal inhibition of ATP with inhibitors of oxidative phosphorylation in these cells is thus approximately 30% and the concentrations necessary to obtain this reduction in ATP (IC_{30}) were compared with the IC_{50} for inhibition of LTB_4-synthesis (Table 2). A highly significant correlation was present between the inhibition of LTB_4 synthesis and the reduction in ATP by these compounds ($r = 1.0000$).

The glycolysis inhibitor, 2-deoxyglucose, at 10^{-2} M decreased ATP by 18% and inhibited 5-lipoxygenase by 30%, which, by extrapolation to higher concentrations, indicates an effect similar to that of the

Table 2. Inhibition of leukotriene synthesis in rat peritoneal leukocytes partially depleted of ATP by oxidative phosphorylation inhibitors

Compound	Inhibition of LTB_4 synthesis IC_{50} (M)	Reduction in intracellular ATP concentration IC_{30} (M)
Valinomycin	3.2×10^{-9}	5.3×10^{-9}
Antimycin A	8.4×10^{-8}	2.4×10^{-8}
FCCP	7.3×10^{-7}	4.5×10^{-7}
2,4-Dinitrophenol	4.0×10^{-4}	1.8×10^{-4}

Results shown are means of duplicate determinations in pools of cells from at least 10 rats.
IC_{30} is the drug concentration necessary for half-maximal reduction in intracellular ATP concentration.
IC_{50} is the drug concentration necessary for half-maximal reduction in 5-lipoxygenase activity.

oxidative phosphyration inhibitors. NDGA and phenidone had no effect on ATP at concentrations relevant to 5-lipoxygenase inhibition (data not shown).

DISCUSSION

The resident rat peritoneal leukocytes constitute an ideal model for the study of drug effects on arachidonic acid metabolism as they produce a variety of eicosanoids representative of both cyclo-oxygenase and lipoxygenase pathways in response to calcium ionophore A23187. In the present study [^{14}C]arachidonic acid was used as substrate for these enzymes, and the eicosanoids were extracted, separated by TLC, and quantified by laser densitometry of autoradiograms as previously described [12].

The arachidonate metabolites were identified by two different methods, co-chromatography with tritiated standards in different solvent systems and manipulation with the metabolism using specific inhibitors of discrete enzymatic pathways, i.e. 5-lipoxygenase, cyclo-oxygenase and thromboxane synthetase. By these methods it was possible to establish the identity of the 5-lipoxygenase products, 5-HETE and LTB_4 (and possibly diastereomeric 5,12-di-HETEs), and the cyclo-oxygenase products 6-keto-$PGF_{1\alpha}$, TXB_2, PGD_2 and HHT. PGE_2 was formed in insignificant quantities under the present experimental conditions.

LTB_4 may be further metabolized by ω-oxidation, but the more polar metabolites 20-OH-LTB_4 and 20-COOH-LTB_4 were probably not formed in sufficient quantities for detection after 5 min of incubation, as indomethacin, a well-known cyclo-oxygenase inhibitor, was able to inhibit completely the formation of eicosanoids more polar than 5,12-di-HETE. This is in agreement with previously published results [17], showing LTB_4 production in the same cells to reach a maximum 5 min after challenge with A23187. Only at later times will ω-oxidation be of significant importance.

Peptido-leukotrienes were probably a product of the cells investigated, but the strong polarity of these eicosanoids prevented their analysis by the present method. By the indirect method of studying the extraction recovery in the absence and presence of valinomycin, however, circumstantial evidence was provided to exclude an effect of this agent on the balance between LTB_4, LTC_4 and LTD_4. Thus the formation of LTB_4 and 5-HETE, which was inhibited by the specific lipoxygenase inhibitions REV 5901 [14] and NDGA, could be safely used as a marker of 5-lipoxygenase activity.

Eucaryotic cells obtain most of their energy by respiration and glycolysis. Energy is conserved through generation of ATP by the processes known as oxidative phosphorylation and glycolytic phosphorylation, respectively. In the present experiments we have used compounds which affect these processes by a number of different mechanisms in order to reduce the available energy in rat peritoneal leukocytes.

Valinomycin, FCCP and 2,4-dinitrophenol are all uncouplers of oxidative phosphorylation, i.e. they prevent the phosphorylation of ADP without affecting respiration; valinomycin differs from the other agents in respect of its requirement for K^+ to exert the uncoupling effect. Antimycin A inhibits the transfer of electrons from cytochrome *b* to *c*, thereby partially blocking the respiratory chain and the corresponding building up of energy. Finally, 2-deoxy-D-glucose, is a competitive inhibitor of glycolysis.

All of the compounds mentioned were able to reduce the endogenous ATP concentration of rat peritoneal leukocytes as measured by the luciferin-luciferase method, thus decreasing the cellular energy level. Their potency varied over a wide concentration range (10^{-9}–10^{-2} M) in the following order: Valinomycin > antimycin A > FCCP > 2,4-dinitrophenol > 2-deoxyglucose. A strong correlation between the reduction in cellular ATP and inhibition of 5-lipoxygenase activity was observed with all five agents independently of the mechanism of action by which ATP was reduced.

It has been reported that phosphatidyl inositol turnover and arachidonate liberation in human platelets require metabolic energy and are strongly inhibited by antimycin A and 2-deoxyglucose [18]. However, the present results showed a reduction in 5-lipoxygenase activity of rat leukocytes in the presence of metabolic inhibitors, whether the substrate, arachidonic acid, was released from endogenous phospholipids, or was present in the medium as a free fatty acid. This strongly suggests an absolute requirement for ATP to express 5-lipoxygenase activity in these cells. In contrast, prostaglandin synthetase was completely unaffected by all inhibitors of ATP-generation.

A marked stimulation by ATP of partly purified 5-lipoxygenase protein has previously been reported [10, 11]. The mechanism of action was not elucidated by these authors. However, superoxide anion generation with ATP as a substrate for xanthine oxidase, phosphorylation of 5-lipoxygenase by protein kinase or protection by ATP of the enzyme from self-catalysed inactivation were possible mechanisms that were all excluded by Ochi *et al.* [11]. The present results emphasize a role for ATP at a physiological co-factor involved in the regulation of leukotriene synthesis *in vivo*. An implication of this finding is the possibility that, during activation of inflammatory cells which elicits a transient depletion of cellular ATP, leukotriene production may be a self-limited process.

The present observations may also explain why some non-steroidal anti-inflammatory drugs, such as indomethacin and benoxaprofen, have been reported to inhibit leukotriene synthesis *in vitro* [19, 20], since at high concentrations these compounds also act as inhibitors of oxidative phosphorylation [21].

From the drug development point of view, in the search for selective inhibitors of arachidonate 5-lipoxygenase it is important to exclude compounds that interfere with normal cellular energy metabolism. In the light of the present results such compounds would appear as specific inhibitors of leukotriene synthesis in screening assays using A23187-stimulated rat peritoneal leukocytes. As shown here with the well-known lipoxygenase inhibitors, NDGA

and phenidone, it is possible to select more specific compounds for further evaluation *in vivo* by including a rapid assay for ATP in the primary screening.

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