MEDIATED UPTAKE OF ARACHIDONIC ACID BY RABBIT NEUTROPHILS

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1. Introduction

Rabbit neutrophils metabolize exogenous arachidonic acid to potent, biologically active compounds, the leukotrienes [1]. Previous work has shown that arachidonic acid stimulates neutrophil functions such as the release of lysosomal enzymes [2], concomitant with its metabolism [3]. A number of inhibitors of arachidonic acid metabolism, such as nordihydroguaiaretic acid and eicosatetraynoic acid, inhibit these responses even when induced by chemotactic factors rather than by arachidonic acid [2]. Other inhibitors of neutrophil response such as quercetin and cytocholasin A have been found to inhibit the metabolism of exogenous arachidonic acid [4]. In order to determine whether these inhibitors act directly on the metabolism of arachidonic acid or on its uptake from the medium, we have investigated the mechanism of entry of arachidonic acid into the cell. An energy-independent component of this uptake was identified using 2-deoxyglucose and antimycin A. This component of uptake appeared to be mediated by a carrier with specificity for unsaturated fatty acids and a 'K_m' for arachidonic acid between 10^{-6} and 10^{-5} M. Inhibitors of arachidonic acid metabolism in neutrophils did not significantly inhibit its uptake, with the exception of the structural analog eicosatetraynoic acid.

Abbreviations: PMN, polymorphonuclear leukocyte; NDGA, nordihydroguaiaretic acid; BPB, bromophenacyl bromide; CYTA, cytochalasin A; CYTB, cytochalasin B; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; INDO, indomethacin; QUER, quercetin

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2. Materials and methods

Throughout these studies rabbit peritoneal neutrophils were obtained and treated as in [3]. The cells were resuspended in complete Hank's balanced salt solution supplemented with dextrose (1 mg/ml). Extracellular lactic dehydrogenase, assayed by standard procedures was ≤5% throughout the experiments reported, demonstrating integrity of the PMNs.

[1-^{14}C]Arachidonic acid (55 Ci/mol, Amersham, St Louis MO) pre-purified by a silicic acid chromatography was added to 2×10^7 cells/ml at 37°C at the indicated concentrations for the indicated times. Uptake was terminated by dilution of an 0.1 ml aliquot with 1.0 ml of 5% bovine serum albumin (Sigma) at 4°C followed by centrifugation at 4°C in a Brinkman Model 3200 centrifuge. Pellets obtained from centrifugation were either dissolved in 0.2 ml 10% SDS for measurement of the total radioactivity or extracted with 4.0 ml chloroform:methanol (2:1) plus 1.0 ml 1 M KCl to analyze radioactive lipid components. Phospholipids were resolved by thin-layer chromatography on LHP-K plates (Whatman, Clifton NJ) in chloroform:ethanol:triethylamine:water (30:34:35:8) [5]. Arachidonic acid and its metabolites were resolved on silica gel G plates (Analtech) in ether:petroleum ether:acetic acid (100:50:1) [6]. Appropriate standards were visualized with 1% iodine spray. Radioactivity was monitored by autoradiography on XR-1 Xomat film (Kodak, Rochester NY). Radioactivity of labeled lipid components was measured by liquid scintillation counting of scraped TLC bands in Liquiscint (National Diagnostics, Rochester NY) containing 10% water and 5% methanol.

Stock solutions of eicosatetraynoic-, arachidonic-, linolenic-, linoleic-, lignoeric-, stearic-, dihomo-γ-linolenic-, eicosatrienoic- and decosatetranoic-acids (NU-Check, Elysian MN) were made up to 0.1 M in

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dimethylsulfoxide and stored under nitrogen at −70°C. Stock solutions of bromophenacylbromide, indomethacin, L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK), nordihydroguaiaretic acid and ricinoleic acid, (Sigma Chemical Co., St Louis MO) were made up to 0.1 M in ethanol and stored under nitrogen at −20°C. All other reagents were analytical grade.

3. Results and discussion

Arachidonic acid uptake by rabbit polymorphonuclear leukocytes was initially measured by incubation of cells at 37°C with 3.3×10^{-6} M [^{14}C]arachidonic acid, a level shown to stimulate lysosomal enzyme secretion in the presence of cytochalasin B [2]. Investigations of fatty acid uptake by mammalian cells had normally been done in the presence of albumin [10] which mimics the physiological situation for free fatty acids in serum. However, the binding of arachidonic acid to albumin complicates uptake analysis by presenting an additional variable. Moreover, studies on arachidonic acid metabolism by and stimulation of neutrophils by this group and others have been done in the absence of albumin. Therefore, in this study albumin was not present during the uptake period. Rather albumin was employed to complex free arachidonate during the cold centrifugation used to terminate the rapid uptake process.

When the time-dependence of this uptake was studied, [^{14}C]arachidonic acid was seen to become rapidly cell associated with a corresponding loss of label from the medium. Lines A and B of fig.1 represent data from 2 different rabbits and exemplify the variation found in the uptake of arachidonic acid among 24 individual rabbits tested. (Standard deviation of extent of uptake = ± 15%.)

Arachidonic acid taken into a cell becomes rapidly esterified into glycerolipids and is also converted into oxygenated metabolites. To characterize the uptake of arachidonic acid it was necessary to distinguish between uptake and metabolism. Since the incorporation of fatty acids into glycerolipids requires ATP for their conversion to coenzyme A thioesters, an attempt was made to uncouple uptake from esterification by treating neutrophils with inhibitors of energy metabolism, 2-deoxyglucose plus antimycin A. Fig.2 shows that the association of [^{14}C]arachidonic acid with neutrophils thus treated occurred at a slower rate than seen in untreated cells. The association of label with

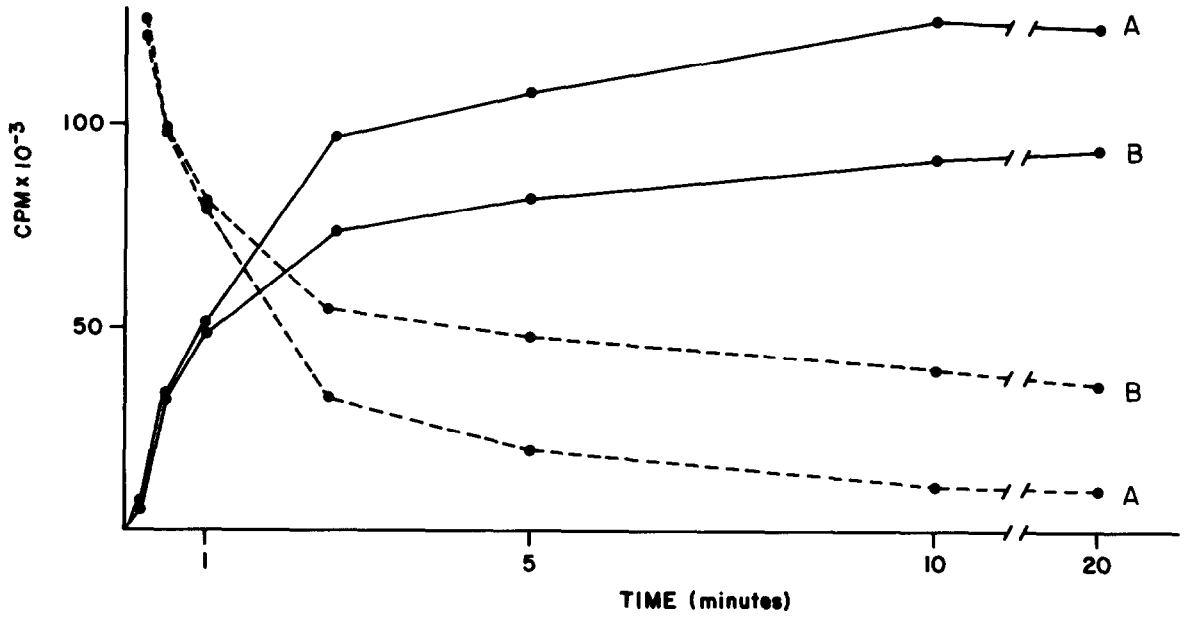

Fig.1. Rapid association of arachidonic acid with rabbit neutrophils. PMNs were incubated for the indicated times as in section 2: (A,B) results (means of duplicate determinations) obtained with PMNs from 2 different rabbits; (——) cell-associated radioactivity; (– – –) media radioactivity.

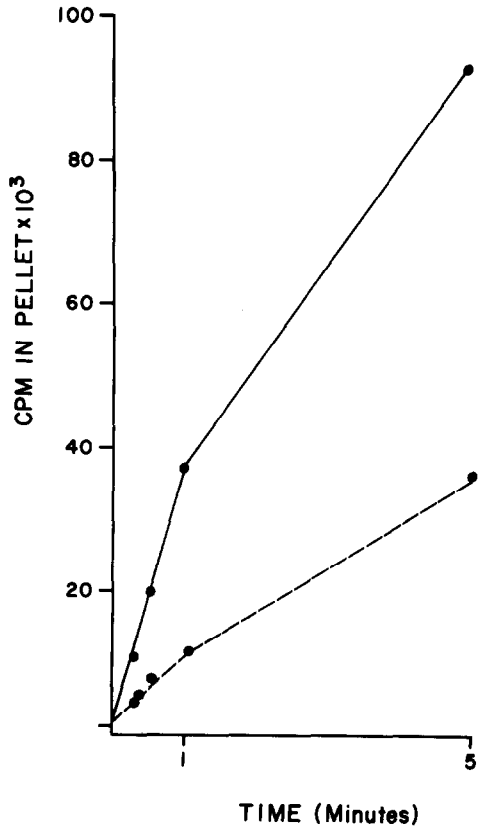

Fig.2. Uptake of arachidonic acid in the presence of 2-deoxyglucose plus antimycin A. PMNs were incubated with 2 mM 2-deoxyglucose plus 15 mM antimycin A (– – –) or dextrose (1 mg/ml) (——) at 37°C for 30 min prior to the addition of [^{14}C]arachidonic acid. Means of duplicate determinations.

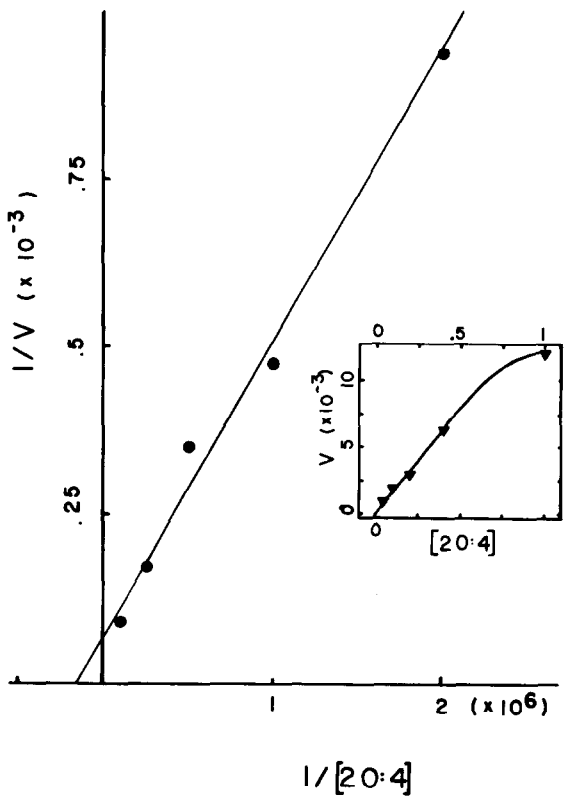

Fig.3. Concentration dependence of arachidonic acid uptake. PMNs were pretreated with antimycin A and 2-deoxyglucose described in fig.2 and incubated with the indicative concentration of arachidonic acid for 15 s. The apparent $-1/K_m$ for the data shown is -0.17×10^6 M^{-1} ($K_m = 5.9 \times 10^{-6}$ M). Points shown are the means of 3 expt.

Table 1
Distribution of [^{14}C]arachidonic acid among PMN lipids

Treatment	cpm (% total cpm): [^{14}C]Arachidonic acid in			
	Arachidonic acid	Hydroxy-fatty acids	Phospholipids	Triglycerides
None	21 200 (21.0)	7400 (7.3)	48 900 (48.4)	23 500 (23.3)
2DG + ANTA	44 000 (60.2)	7100 (9.7)	15 600 (21.3)	6400 (8.8)

Distribution of [^{14}C]arachidonic acid among lipid classes after 15 s of uptake was determined on cells pretreated as in fig.2

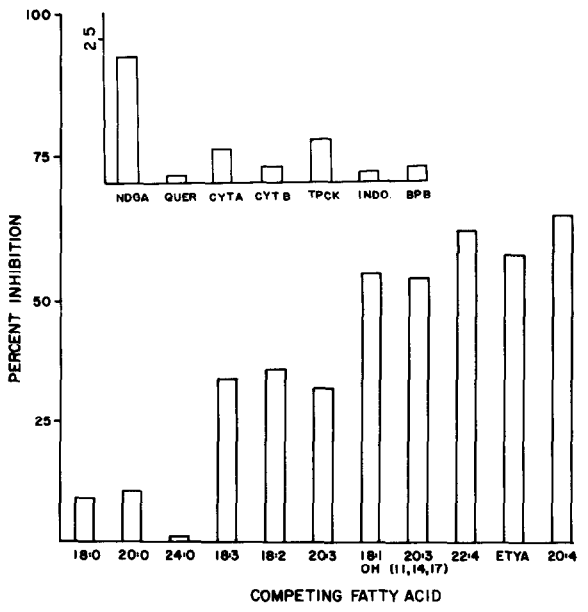

Fig.4. Inhibition of the uptake of arachidonic acid by analogs and inhibitors of PMN function. Uptake of 10^{-6} M [^{14}C]-arachidonic acid was measured at 15 s in the presence of 10^{-5} M of the indicated unlabelled fatty acid. Each bar represents the mean of 3 expt. Insert: PMNs were preincubated for 5 min in the presence of 2×10^{-5} M of the indicated inhibitor prior to the determination of [^{14}C]arachidonic acid uptake.

cells increased linearly for $\geqslant$1 min. Neither inhibitor alone inhibited uptake by >30% (not shown).

The distribution of radioactivity in lipid extracts from such cells was investigated to determine if inhibition of esterification was, in fact, responsible for the reduced labeling. The results presented in table 1 show that in cells treated with deoxyglucose plus antimycin A, 70% of the radioactivity remained non-esterified, as opposed to only 28% in untreated cells. These results demonstrate that the radioactivity which becomes associated with the treated cells can provide a reasonable estimate of the initial velocity of uptake of arachidonic acid by rabbit neutrophils.

The dependence of the initial velocity of this energy-independent component of uptake on concentration of arachidonic acid was studied. Results with neutrophils from one rabbit are presented in fig.3. The double reciprocal analyses generated a 'K_m'-value of 5.9×10^{-6} M for these neutrophils. Values of 1.7, 3.1, 3.4 and 5.4×10^{-6} M were obtained with neutrophils from 4 other rabbits, while cells from one rabbit produced a double-reciprocal plot with its intercept at the origin. We were not able to demonstrate saturation of uptake unequivocally however (fig.3, insert) as arachidonic acid $>10^{-5}$ M led to rapid cell lysis, evidenced by an increase in extracellular lactic dehydrogenase. Failure to observe curvilinear kinetics at or below the K_m does not exclude a carrier mediated mechanism of uptake [11]. A more sensitive test for a mediated uptake system is competitive inhibition by substrate analogs. The ability of various fatty acids to inhibit the uptake of arachidonic acid was therefore studied. All unsaturated fatty acids studied effectively inhibited the uptake of exogenous [^{14}C]-arachidonic acid (fig.4). Docosatetraenoate, dihomo-γ-linolenate, and the acetylenic analog eicosatetraynoic acid were nearly as potent inhibitors as unlabelled arachidonate itself. Eighteen carbon unsaturated fatty acids were slightly less potent inhibitors and saturated fatty acids were ineffective. The hydroxylated fatty acid, ricionoleate, was also a potent inhibitor of arachidonic acid uptake. Extensive kinetic analyses will be necessary to prove the competitive nature of this analog inhibition but the sensitivity of arachidonic acid uptake to a variety of substrate analogs and the selectivity for unsaturated over saturated analogs is suggestive of a mediated transport event.

The effects of inhibitors of neutrophil function which antagonize exogenous arachidonic acid metabolism were also tested. Of the inhibitors studied, only NDGA produced measurable inhibition of uptake and inhibition was only 20% (fig.4 inset). At these concentrations, NDGA, quercetin, or cytochalasin A inhibited the metabolism of exogenous arachidonic acid by >95% [4]. Thus these inhibitors appear to directly inhibit metabolism of arachidonic acid by lipoxygenases of the neutrophil, and that inhibition may be presumed to hold for endogenously released as well as exogenously provided, arachidonate.

Acknowledgements

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