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ARACHIDONIC ACID INDUCED DEGRANULATION OF RABBIT PERITONEAL NEUTROPHILS

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SUMMARY: We have found that arachidonic acid rapidly and selectively induces the release of lysosomal enzymes from cytochalasin B treated rabbit peritoneal neutrophils. 5, 8, 11, 14-eicosatetraynoic acid inhibits the arachidonate induced release with an apparent K_D of 1.5×10^{-6}M. 5,8,11,14-eicosatetraynoic acid (2.5×10^{-5}M) also inhibits the chemotactic factors and the A23187 induced release in the presence of cytochalasin B but does not affect the degranulation induced by A23187 alone. These observations strongly suggest a role for arachidonate metabolites in rabbit neutrophil physiology.

INTRODUCTION

Interrelationships between arachidonic acid metabolites, calcium and cyclic nucleotides are at the center of the present ideas concerning non-muscle cell activation by a variety of hormonal and non-hormonal stimuli (1). Phospholipid and/or arachidonic acid metabolisms have, in particular, been postulated to be involved in the ionic gating mechanisms of a number of hormonally responsive cells (2).

In the neutrophils, changes in phospholipid metabolism (3) and in the generation of prostaglandins (4, 5, 6) and unsaturated hydroxy fatty acids (7) have been reported upon exposure to phagocytic stimuli and to the calcium ionophore A23187. PGE_1, thromboxane B_2 and hydroxy fatty acids have, in addition, been reported to possess chemotactic activity for the neutrophils (8, 9, 10).

We have previously shown that a close correlation exists between stimulated cation fluxes, especially those of Ca^{2+}, and the secretory activity of neutro-

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Abbreviations:

Formyl-Methionyl-Leucyl-Phenylalanine = F-Met-Leu-Phe .

5,8,11,14-eicosatetraynoic acid = ETYA.

Prostaglandin E_1 = PGE_1.

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phils (11). We wish now to report the results of preliminary experiments strongly implicating arachidonic acid and/or its metabolites in the mechanism of activation of neutrophils by secretory stimuli.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils obtained and treated as previously described (12) were used throughout these studies. The cells were resuspended in complete Hanks' balanced salt solution (12) in the absence of albumin. Lysosomal enzyme release was measured as described in Showell et al. (12) with particular care taken to ensure thermal equilibration. The lysosomal enzymes determined were lysozyme and β glucuronidase. Only the results with lysozyme are shown since the release of the two enzymes followed essentially the same course in the various experiments. Degranulation was induced by the addition of the cells to thermally equilibrated test tubes containing the desired fatty acids and cytochalasin B. The incubations were terminated after 5 minutes by transfer to an ice cold water bath. ETYA (a generous gift of Dr. W.E. Scott, Hoffman-La Roche) was added to the cells within the minute preceeding their transfer to the test tubes. Longer preincubation with ETYA were not found to be necessary. Lactate dehydrogenase leakage, a measure of cell death, was checked routinely and never found to be significant.

Stock solutions of the various fatty acids were made up at 10^{-1}M in dimethyl sulfoxide in the presence of 1.4×10^{-4}M butylated hydroxy toluene and stored at -70°C under nitrogen. Butylated hydroxy toluene was present to ensure the stability of the fatty acid solutions and did not in any detectable manner, affect the results described below. Routine control experiments with fresh batches of fatty acids were also performed.

The synthetic chemotactic peptide F-Met-Leu-Phe was kindly supplied by Dr. R.J. Freer, Medical College of Virginia, Richmond, Virginia. Partially purified $C5_a$ was isolated from whole serum according to the method of Fernandez and Hugli (14). The calcium ionophore A23187 was generously provided by Robert Hamil of Eli Lilly Co. Indianapolis.

Arachidic acid, linolenic acid, linoleic acid, lignoceric acid, stearic acid, homogamma linolenic acid, 11,14,17-eicosatrienoic acid and decosatetranoic acid were obtained from Nu-check Co. Elysian, Minn. Arachidonic acid was obtained from both Nu-check Co. and Sigma Chemical Co., St. Louis, Mo. and found to react indistinguishably. The purity of all of the fatty acids was greater than 99%.

RESULTS AND DISCUSSION

Three conditions represent the minimal necessary requirements for considering arachidonic acid metabolites to be involved in the secretory activity of the neutrophils:

1) Exogenously added arachidonic acid should mimic the effect of natural stimuli.

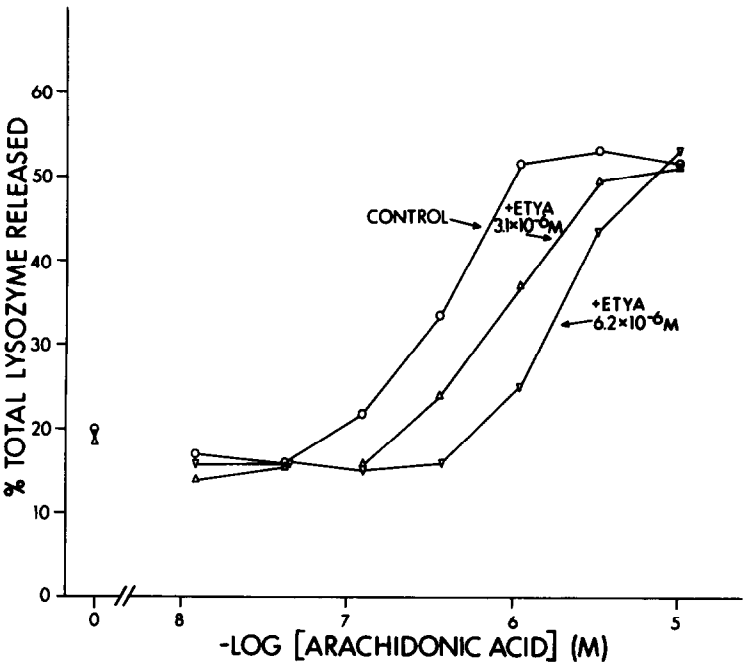

Figure 1: Effect of arachidonic acid and ETYA on lysozyme release from cytochalasin B treated rabbit neutrophils.

2) The effects of exogeneously added fatty acids should be specific to arachidonic acid.

3) Inhibitors of arachidonic acid metabolism should inhibit the response of the neutrophils to secretory stimuli.

A direct test of the first of the three listed above conditions, i.e. the ability of arachidonate to act as a secretory stimulus, is presented in Figure 1. This figure clearly shows that arachidonic acid is a potent secretagogue for cytochalasin B treated rabbit peritoneal neutrophils. The mean ED_{50} for arachidonic acid induced granule release is $2.3 \pm 0.2 \times 10^{-7}$M. Maximal activity is reached at micromolar concentrations of arachidonic acid. Extracellular calcium potentiates the arachidonic acid induced enzyme release in the same way it does the chemotactic factors, i.e., by shifting the dose-response curve towards the left (12) (results not shown)). Arachidonic acid induced degranulation is complete within less than 2 minutes. Under our experimental conditions,

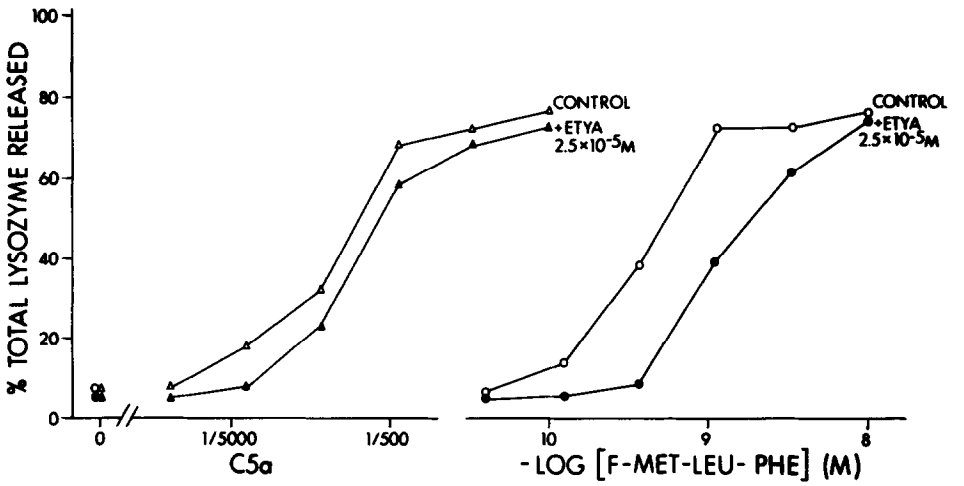

Figure 2: Effect of ETYA on the chemotactic factors induced release of lysosome from cytochalasin B treated neutrophils.

the presence of cytochalasin B appears to be an absolute requirement for the arachidonate induced release.

ETYA is an analog of arachidonic acid which has been shown to inhibit the enzymatic activities of both the prostaglandin synthetase from sheep seminal vesicles and the soybean lypoxygenase (14). ETYA as shown in Figure 1 is a potent inhibitor of arachidonic acid induced degranulation in rabbit neutrophils. The concentrations of ETYA required to inhibit the arachidonic acid induced release are smaller than those required to inhibit the other secretagogues tested as can be seen from a comparison of Figures 1, 2 and 3. From experiments such as the one shown in Figure 1, the assumption that ETYA is acting as a competitive inhibitor (an assumption supported by some published reports (15) and by the parallel nature of the shifts in the dose-response curves for lysosomal enzyme release (Figures 1, 2 and 3)), an apparent K_D for ETYA can be calculated. The average K_D of four experiments was 1.5×10^{-6}M. The inhibition of arachidonic acid induced granule release by ETYA is reversible, i.e., cells pretreated with ETYA for 10 minutes, washed free of the inhibitor and tested for secretory responsiveness to arachidonic acid behave no differently than control, untreated cells (results not shown).

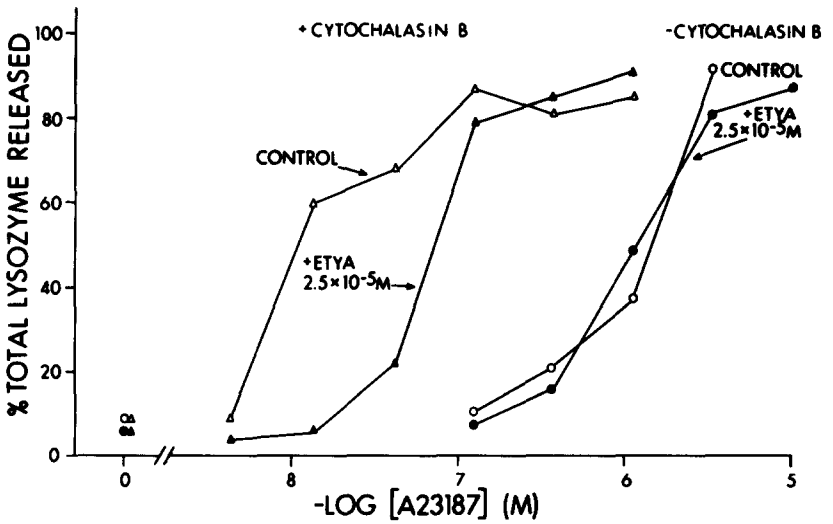

Figure 3: Effect of ETYA on the A23187 induced release of lysozyme from rabbit neutrophils in the presence and absence of cytochalasin B.

The specificity of the effect of arachidonic acid was then tested by assessing the secretory activity of various other fatty acids. None of these other fatty acids possessed any significant secretory activity for rabbit neutrophils although they share many properties with arachidonic acid and differ structurally only slightly from it (Table 1).

A satisfactory answer to the third of the three criteria listed above is provided by the results illustrated in Figure 2. This figure demonstrates that lysosomal enzyme release, as induced in the presence of cytochalasin B by either F-Met-Leu-Phe or C5a can be inhibited by 2.5 x 10^{-5}M ETYA. The inhibition is rapid, less than 1 minute preincubation with ETYA being required. The inhibition is dose dependent and can be observed at concentrations of ETYA larger than 10^{-5}M. In addition, ETYA induced inhibition of lysosomal enzyme release is reversible in the manner defined above (results not shown). It is also noteworthy to point out the lower effectiveness of ETYA when C5a instead of F-Met-Leu-Phe is used as the secretory stimulus (% inhibition of lysosomal enzyme release by 2.5 x 10^{-5}M ETYA measured by the shifts in the dose response curves, as induced by F-Met-Leu-Phe and C5a, were found to be 71 ± 6 and 37 ± 6 respectively, averages of four experiments).

oxide dismutase (30 mg/ml) or EDTA (10 mM) had little effect on the appearance of sharp signal. Further, the same spectral change was observed with α-tocopherol-treated microsomes (α-tocopherol, 25 mg/g liver wet weight, was added during homogenization). These results seem to dismiss the involvement of superoxide anion or lipid peroxidation as a possible explanation for the phenomenon.

After aerobic incubation of spin-labeled microsomes with NADPH, the membrane fraction was recovered by centrifugation. The membrane fraction showed an EPR spectrum similar to that of the original microsomes before NADPH addition except that the peak heights were decreased, while the supernatant showed only the sharp signal (Fig. 3). These results indicate that spin label is released into aqueous phase during the reaction with NADPH. Little degeneration of electron transfer system appears to occur during this treatment, since the membrane fraction responded again to NADPH with the appearance of the rapidly tumbling component in EPR spectrum.

DISCUSSION

The functional properties of microsomal cytochrome P-450 system are known to be intimately related to the microenvironment provided by membrane phospholipids (3-6). Therefore, it will naturally be expected that the phospholipids composing the microenvironment of this enzyme system would in turn, be physically perturbed during the enzyme reactions. The experiments presented in this paper were attempted to explore the latter point. The results clearly suggest that the microsomal electron transfer from NADPH to oxygen induces a physical perturbation of the lipid bilayer resulting in the release of its component into the aqueous phase.

Superoxide anion formation and lipid peroxidation, both of which are known to take place during the oxidation of NADPH by microsomes (9, 10), do not seem to be involved in this phenomenon.

A quantitative estimate of the released spin moiety was made from the peak height of high field signal using spin-labeled stearic acid in aqueous

The relative contributions of the cyclooxygenase and of the lipoxygenase pathways to the phenomena described above cannot be ascertained at present. Goldstein et al. (5) have stated that lysosomal enzyme release (experimental conditions unspecified) is not inhibited by indomethacin. Previous reports have however, indicated that under some circumstances, lysosomal enzyme release is sensitive to indomethacin (16). Our preliminary experiments have shown that relatively high concentrations of indomethacin (>10^{-5} M) do inhibit the F-Met-Leu-Phe induced release of lysosomal enzyme release from rabbit neutrophils (H.J. Showell, unpublished observations). In view of the multiplicity of effects of indomethacin, these results are however difficult to interpret mechanistically. The results presented above clearly point out however, the importance and necessity of defining the active metabolite(s) of arachidonic acid

Taken together, these results present strong evidence for a role of arachidonate metabolites in the initiation of lysosomal enzyme release in neutrophils. Whether the postulated role of arachidonate metabolites is of an essential or only of a modulatory nature in the mechanism of stimulus-secretion coupling in the neutrophils is unknown at present. In view of the interrelated nature of the various neutrophils functions (17) and of the evidence briefly summarized in the introduction it is however very likely that arachidonate metabolism may also be involved in neutrophil chemotaxis and phagocytosis.

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