Arachidonic acid-induced release of calcium in permeabilized human neutrophils

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The addition of arachidonic acid to a suspension of digitonin-permeabilized human neutrophils was found to induce, in a dose-dependent manner (ED_{50} about 15 μM), the release of calcium from internal stores. Arachidic acid was without effect, while linoleic acid and linolenic acid were (on a concentration basis) at least 5-times less active than arachidonic acid. The activity of arachidonic acid appears to be due to the fatty acid itself and not to one of its metabolites. The pool of calcium mobilized by arachidonic acid includes that sensitive to inositol 1,4,5-trisphosphate. These results demonstrate a significant intracellular role for arachidonic acid at the level of the internal mobilization of calcium in human neutrophils.

Arachidonic acid; Neutrophil; Inositol 1,4,5-trisphosphate; Ca^{2+} mobilization; Unsaturated fatty acid; Fura-2

1. INTRODUCTION

Neutrophil stimulation by chemotactic factors is accompanied by the activation of the phospholipases C and A_2. Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, the products of phospholipase C, are involved in the liberation of calcium from the endoplasmic reticulum and the activation of protein kinase C, respectively [1]. Arachidonic acid, the principal fatty acid liberated by phospholipase A_2, possesses biological activities of its own and is the parent molecule for the prostaglandins, prostacyclins and leukotrienes [2].

The roles of IP_3 and of arachidonic acid and/or its metabolites are generally thought of as being distinct. However, several lines of evidence indicate that the lipid mediators play internal roles in neutrophil activation. For example, arachidonic acid and lipoxin A have been shown to activate directly purified preparations of protein kinase C [3,4]. Furthermore, Wolf et al. [5] have demonstrated that arachidonic acid was capable of causing a release of calcium in permeabilized preparations of pancreatic islets.

The present studies were instigated in order to test for physiological evidence of intracellular effects of arachidonic acid in human neutrophils. The results to be presented demonstrate that arachidonic acid mobilizes internal calcium in permeabilized neutrophils and HL-60 cells and that it does so at physiologically relevant concentrations and in a relatively specific manner.

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2. MATERIALS AND METHODS

Human peripheral blood neutrophils isolated following dextran sedimentation on Hypaque Ficoll gradients were used throughout these experiments. The cells were resuspended in Hanks' balanced salt solution containing 10 mM Hepes, pH 7.0, in the absence of added calcium or magnesium.

Calcium release was monitored with the fluorescent indicator fura-2 [6]. Briefly, neutrophils were resuspended in a high potassium buffer containing

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110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 25 mM Hepes, pH 7.0, at $1-3 \times 10^7$ cells/ml. At the appropriate times, 1 mM ATP (Tris salt), 8 mM creatine phosphate, 16 μg/ml creatine phosphokinase and 2 μM fura-2 were added. The final cell concentration was 10^7 cells/ml. The cells were transferred to the cuvette compartment of the spectrofluorometer (SLM Instruments, Urbana, IL) and the fluorescence monitored (excitation, 350 nm; emission, 510 nm) at 37°C. Cell permeabilization was initiated by the addition of digitonin (20 μM, from a stock in ethanol at 10 mM maintained at 37°C during the course of the experiment).

The promyelocytic leukemia cells HL-60 were maintained in culture as in [7].

Fura-2 was purchased from Molecular Probes (Eugene, OR). Tissue culture supplies were obtained from Gibco. All the other chemicals came from Sigma (St Louis, MO).

3. RESULTS AND DISCUSSION

The addition of digitonin to neutrophil suspensions causes a rapid and ATP-dependent (not shown) drop in fluorescence of fura-2 which corresponds to a drop in the ambient levels of calcium due to accumulation of the cation by cell organelles. The permeabilized cells did not respond to addition of the chemotactic peptide fMet-Leu-Phe. Furthermore, IP_3 did not increase the fluorescence of fura-2 in non-permeabilized cells (not shown). The addition of arachidonic acid (20 μM) to digitonin-treated neutrophils causes an increase in the fluorescence of fura-2 (fig.1). A subsequent addition of the fatty acid is significantly less effective. The fluorescence signal returns to the prestimulation level within the next 10–15 min (not shown). Arachidonic acid causes no increase in fluorescence if added after the ionophore A23187, and the latter elicits a correspondingly smaller increase if added after arachidonic acid (not shown).

The effect of arachidonic acid is dose-dependent and is detectable at, or below, 5 μM (fig.2). Maximal release is observed at 50 μM and the ED_{50} is approx. 10–20 μM. Direct effects of the fatty acid on the probe or generalized disruption of cellular membranes are ruled out by the observation that arachidonic acid did not cause any increase in the fluorescence of extracellular fura-2 when added to intact, non-permeabilized cells (not shown).

The ability of arachidonic acid to induce a release of calcium in the permeabilized neutrophils was unaffected by the inclusion in the incubation medium of the mitochondrial inhibitor antimycin A (1 μM) (not shown). These results indicate that the previously observed mobilization of calcium by arachidonic acid [8] may be due, at least in part, to a direct effect of the fatty acid on a non-mitochondrial calcium store.

The pool of calcium mobilized by arachidonic acid includes that which is sensitive to IP_3. The addition of IP_3 after that of a maximal dose of

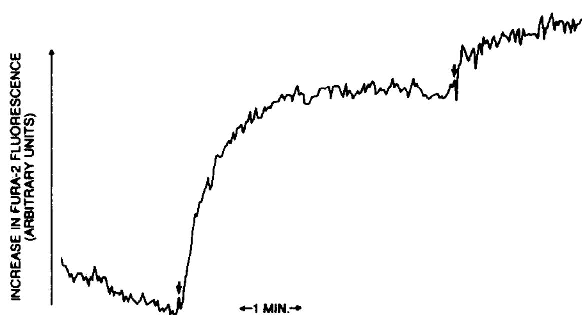

Fig.1. Effect of arachidonic acid on the fura-2 fluorescence of a suspension of permeabilized neutrophils. Arachidonic acid (20 μM) was added successively at the arrows to a suspension of neutrophils permeabilized as described in section 2. Representative of four such experiments.

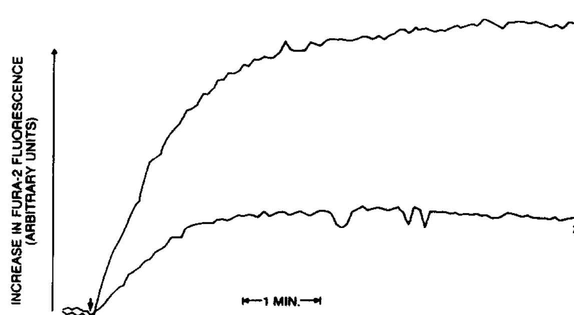

Fig.2. Dose dependence of the effect of arachidonic acid on the fluorescence of fura-2 of a suspension of permeabilized neutrophils. Arachidonic acid was added at the arrow at the following concentrations: (1) 10 μM, (2) 5 μM. Representative of three other determinations.

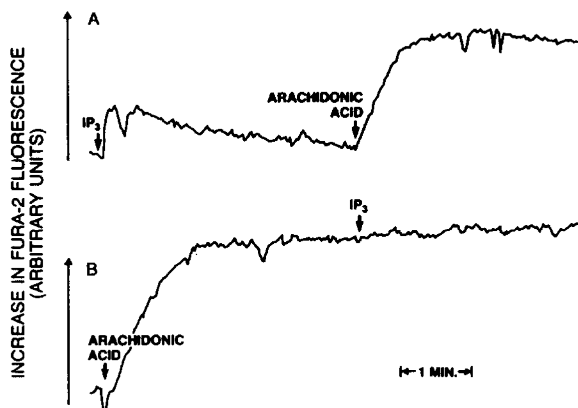

Fig.3. Sequential additions of IP_3 and arachidonic acid to suspensions of permeabilized neutrophils in the presence of fura-2. (A) IP_3 (5 μM) was added at the first arrow; arachidonic acid (5 μM) at the second arrow. (B) Arachidonic acid (5 μM) was added at the first arrow; IP_3 (5 μM) at the second arrow. The two tracings are taken from sequential runs in the same experiments. Representative of three determinations.

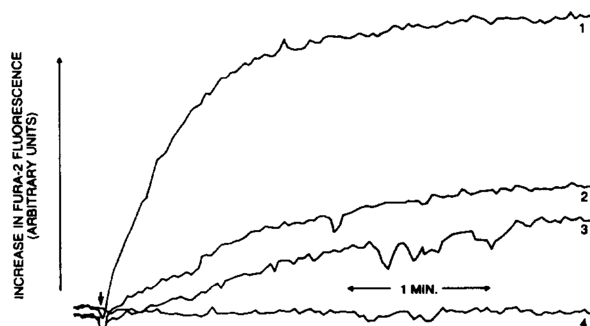

Fig.4. Effect of various fatty acids on the fura-2 fluorescence of a suspension of permeabilized neutrophils. The additions were made at the arrow: (1) arachidonic acid (10 μM), (2) linoleic acid (10 μM), (3) linolenic acid (10 μM), and (4) arachidic acid (10 μM). Representative of three other determinations.

arachidonic acid is essentially without effect. On the other hand, arachidonic acid induces the release of significant amounts of calcium even when added after IP_3 (fig.3). These data indicate that arachidonic acid has access to a larger pool of calcium than does IP_3, and conversely, that the pool of calcium affected by IP_3 represents a subset of that sensitive to the fatty acid. It has been shown that IP_3 releases only a fraction of the endoplasmic reticulum-associated calcium [9]. It should be pointed out that one cannot rule out at present the possibility that arachidonic acid inhibits directly the effects of IP_3, possibly in a manner reminiscent of the previously described antagonism of the functional responsiveness of the neutrophils [10].

The fatty acid specificity of the effect just described was ascertained by examining the ability of arachidic acid (the saturated counterpart of arachidonic acid), and of linoleic and linolenic acid (two long-chain *cis* unsaturated fatty acids) to induce the release of calcium in the permeabilized neutrophil preparation. The results of these studies are summarized in fig.4. Arachidic acid at concentrations up to 50 μM was found not to cause any significant release of calcium. Linoleic and linolenic acid, on the other hand, did increase the fluorescence of fura-2 although these two fatty acids required at least 5-times higher concentrations than arachidonic acid to induce effects of a similar magnitude. Thus arachidonic acid displays a significant, though not absolute, specificity among the fatty acids as far as its ability to mobilize internal calcium is concerned. These results approximate those previously published concerning the effects of *cis* unsaturated fatty acid on the physical organization of the lipid domains of biological membranes [11], and on the functional responsiveness of the neutrophils [12]. They suggest that the inhibition of the functional responsiveness of the neutrophils by the fatty acids may be the result of the dissipation of the functionally relevant internal calcium pool.

Several lines of evidence indicate that the release of calcium induced by arachidonic acid is due to the fatty acid itself and not to one of its metabolites. Firstly, the permeabilization is likely to result in a dramatic dilution of the metabolizing enzymes (lipoxygenase and cyclooxygenase) and their necessary cofactors [13]. Secondly, unstimulated human neutrophils metabolize exogenous arachidonic acid only very poorly. Thirdly, linoleic and linolenic acid elicit a release of calcium. Fourthly, IP_3 and arachidonic acid exhibit similar activities in the HL-60 promyelocytic leukemia cells (not shown) which are known to be deficient in arachidonic acid-metabolizing enzymes [14,15]. Finally, the lipoxygenase inhibitor nordihydroguaiaretic acid [16] failed to decrease the magnitude of the fura-2 response to arachidonic

acid (not shown).

In summary, the results presented above demonstrate that arachidonic acid is an effective agent for the mobilization of internal calcium in the neutrophils and the HL-60 cells. This effect is demonstrable at concentrations of the fatty acid that are comparable to those of IP_3 and the former releases significantly larger amounts of calcium than the latter mediator. By doing so, these studies tie together and establish a new level of interrelationship between two previously relatively independent biochemical pathways called upon during neutrophil activation, namely the phospholipases C and A_2. The determination of the relative contributions of the products of these two pathways to the increase in calcium that accompanies the physiological stimulation of the neutrophils, and of the possible effects of the lipoxygenase-derived metabolites of arachidonic acid in this response, 'remain to be determined.

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