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Arachidonic acid-induced mobilization of calcium in human neutrophils: evidence for a multicomponent mechanism of action

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1 The mechanism(s) involved in the mobilization of calcium induced by arachidonic acid in human neutrophils was investigated.

2 The addition of arachidonic acid to a suspension of human neutrophils led to a time- and concentration-dependent mobilization of calcium which was the result of two separate and experimentally differentiable processes. The latter consisted of a rapid and transient phase followed by a slower and more sustained response.

3 The initial phase of calcium mobilization elicited by arachidonic acid was decreased in the presence of EGTA, inhibited by pertussis toxin as well as by nordihydroguaiaretic acid (NDGA), and diminished following a pre-incubation with leukotriene B_4, but not platelet-activating factor.

4 The characteristics of the first phase of the mobilization of calcium were consistent with an interaction of the fatty acid with the leukotriene B_4 receptors, either directly or indirectly following the synthesis of leukotriene B_4, as well as with a release of internal calcium.

5 The second, slower and more sustained phase of calcium mobilization was more apparent at high concentrations ($\geqslant 8$–$16\,\mu M$) of arachidonic acid, and was relatively insensitive to pertussis toxin, EGTA or NDGA.

6 The characteristics of the 'slow' phase of calcium mobilization by arachidonic acid are consistent with its being associated primarily with a release of calcium from internal storage pools.

7 The data presented indicate that the mechanism of mobilization of calcium by arachidonic acid in human neutrophils is complex and involves specific activation pathways employed, in part at least, by other neutrophil agonists. These findings may have relevance to various inflammatory situations in which the elevated levels of extracellular arachidonic acid known to be present could modulate the functional responsiveness of the neutrophils to other stimuli.

Introduction

Arachidonic acid is a multifunctional agonist for neutrophilic polymorphonuclear leukocytes (neutrophils). The addition of the fatty acid stimulates several of the characteristic functions of the cells including: the mobilization of calcium (Volpi *et al.*, 1980; Sha'afi *et al.*, 1980; Naccache *et al.*, 1983), the polymerization of actin (Yassin *et al.*, 1985), degranulation (Naccache *et al.*, 1979), aggregation (Naccache *et al.*, 1983; O'Flaherty *et al.*, 1979), and the stimulation of superoxide production (Badwey *et al.*, 1981; 1987). Furthermore, arachidonic acid has been found to augment the activity of purified protein kinase C (McPhail *et al.*, 1984; Murakami *et al.*, 1986; Morimoto *et al.*, 1988), an enzyme potentially involved in the initiation and/or regulation of neutrophil responsiveness (White *et al.*, 1984; Naccache *et al.*, 1985; Rossi, 1986; Tauber, 1987), as well as that of partially purified NADPH-oxidase preparations from these cells (Rossi, 1986; Tauber, 1987; Curnutte *et al.*, 1987; Clark *et al.*, 1987). These various biological activities of arachidonic acid are generally assumed to support the concept that the fatty acid is an important modulator of the pathophysiological functions of the neutrophils.

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However, whereas the mode of action of 'classical' neutrophil stimuli such as the small molecular weight fragment of the fifth component of complement, C5a, and of the formylated chemotactic peptides such as fMet-Leu-Phe are relatively well established (for reviews, see Snyderman & Verghese, 1987; Sha'afi & Molski, 1988), little attention has been paid to the mechanism of activation of the neutrophils by arachidonic acid. This problem is compounded by the fact that arachidonic acid is metabolized by the 5-lipoxygenase system of the neutrophils (Borgeat & Samuelsson, 1979; Borgeat *et al.*, 1985) resulting in the generation of neutrophil active molecules such as leukotriene B_4 (Ford-Hutchinson *et al.*, 1980; Palmer *et al.*, 1980; Naccache *et al.*, 1988).

We have recently observed that the synthesis of lipoxygenase products in response to the addition of arachidonic acid is the result of more than the simple provision of substrate to the 5-lipoxygenase enzyme system. This conclusion was supported in part by the finding that the synthesis of lipoxygenase products observed following the addition of arachidonic acid was sensitive to inhibition by pertussis toxin, even though the latter did not affect directly the activity of the 5-lipoxygenase (McColl *et al.*, 1989). Since the neutrophil functional responses stimulated by arachidonic acid (e.g., degranulation, superoxide production, protein kinase C translocation and leukotriene synthesis) are calcium-dependent, we have attempted to determine the mechanism(s) by which arachidonic acid mobilizes calcium in these cells. The results are supportive of a model of the mechanism of action of arachidonic acid involving at least three separate sites of action, two of which are closely associated with the activation of receptor-dependent events and which collectively contribute to the mobilization of calcium induced by the fatty acid.

Methods

The human neutrophils used in this study were isolated following dextran sedimentation on Ficoll-Paque cushions. The remaining erythrocytes were lysed by hypotonic shock (20 s at room temperature). Neutrophils represented at least 97% of the cell suspensions. Cell viability, as estimated by trypan blue exclusion, was better than 98%. The cells were isolated and resuspended in Hanks' balanced salt solution (HBSS) (Gibco, Burlington, Ontario, Canada), pH 7.4, containing 1.6 mM $CaCl_2$ but no added $MgCl_2$.

Intracellular free calcium was monitored with the fluorescent probe fura-2 as described in Grynkievicz *et al.* (1985) and Faucher & Naccache (1987). Briefly, neutrophil suspensions (1×10^7 cells ml^{-1}) were incubated with 1 μM fura-2/AM for 30 min at 37°C. The cells were then washed free of the extracellular probe, resuspended at 5×10^6 cell ml^{-1} and allowed to re-equilibrate for 10 min at 37°C. They were then transferred to the thermostatted cuvette compartment (37°C) of the fluorimeter and the fluorescence monitored (excitation and emission wavelengths, 340 and 510 nm respectively). The internal calcium concentrations were calculated as described in Tsien *et al.* (1982) by individually calibrating each run.

Formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), platelet-activating factor (Paf), nordihydroguaiaretic acid (NDGA) and cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ficoll-Paque was from Pharmacia (Dorval, Québec, Canada), fura-2/AM was purchased from Molecular Probes (Junction City, OR, U.S.A.), and pertussis toxin was from List Biochemical Laboratories (Campbell, CA, U.S.A.). Leukotriene B_4 was generously provided by Dr J. Rokach (Merck-Frosst Canada, Pointe-Claire, Dorval, Canada). Arachidonic acid was purified by silicic acid chromatography or by r.p.-h.p.l.c. prior to use. The purity of the leukotriene B_4 was determined by r.p.-h.p.l.c., and the concentration of its stock solutions by u.v. spectrophotometry (270 nm). All the other reagents were analytical grade.

Results

Concentration-dependence of the mobilization of calcium induced by arachidonic acid and its sensitivity to NDGA

The addition of micromolar concentrations of arachidonic acid to human neutrophils elicited a rapid and concentration-dependent increase in the concentration of cytoplasmic free calcium (as monitored by the fluorescent calcium probe fura-2) (Figure 1). The threshold concentration of arachidonic acid required consistently to elicit a calcium response from the neutrophils was found to be about 1 μM. The magnitude of the calcium signal increased with the concentration of arachidonic acid added and showed signs of saturation at or above 33 μM. The fura-2 response to relatively low concentrations of arachidonic acid ($\leqslant 8\ \mu$M) characteristically comprised a rapid and transient peak that decayed to resting or higher than resting level of cytoplasmic free calcium.

The potential involvement of 5-lipoxygenase products in the mediation of the calcium mobilizing properties of arachidonic acid was tested with the help of the 5-lipoxygenase inhibitor NDGA (Tappel

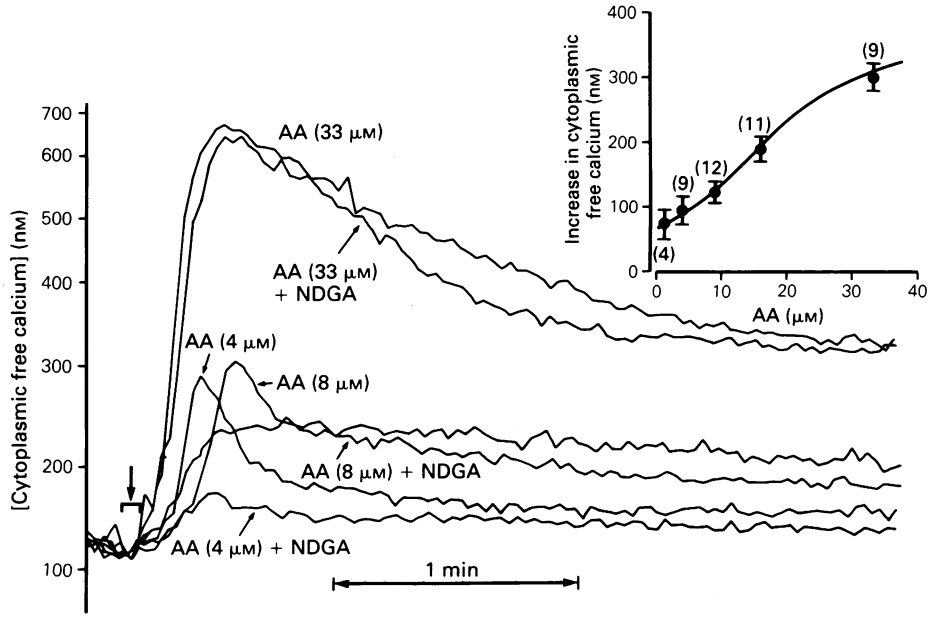

Figure 1 Arachidonic acid-mobilization of calcium in human neutrophils. The levels of calcium were measured as described in Methods. Arachidonic acid (AA), at the indicated concentrations, was added at the arrow and where indicated, cells were pre-incubated with NDGA (10 μM) for 3 min before the addition of the fatty acid. The results shown are representative of at least three such experiments. The concentration–response curve for increases in cytoplasmic free calcium induced by arachidonic acid is inset. The values represent the mean of the number of experiments indicated in parentheses with s.e.mean shown by vertical bars.

et al., 1953; Hamberg, 1976; Walenga *et al.*, 1980; Salari *et al.*, 1984). The pre-incubation of the cells with NDGA was found to have differential effects depending on the concentration of arachidonic acid used (Figure 1). The calcium responses to low concentrations of arachidonic acid ($\leqslant 4\,\mu$M) were found to be significantly reduced in the presence of the inhibitor, while those of high concentrations of ($\geqslant 33\,\mu$M) were essentially unaffected. At intermediate concentrations of arachidonic acid (about 8 μM), NDGA preferentially inhibited the initial spike in calcium but had little effect on the raised level of cytoplasmic free calcium that was maintained during the course of these experiments.

Dependence on extracellular calcium

The contribution of extracellular calcium to the increase in cytoplasmic free calcium was evaluated by adding the calcium chelator EGTA. Under these conditions, little, if any, extracellular calcium is available to diffuse into the cells, and the changes in the fluorescence of fura-2 that are recorded can be assumed to be due to calcium released from internal stores (Molski *et al.*, 1983). The results of one such experiment are shown in Figure 2. EGTA reduced by similar amounts (about 50 nM) the increase in calcium induced by 2 and 33 μM arachidonic acid. However, the calcium chelator had, on a relative

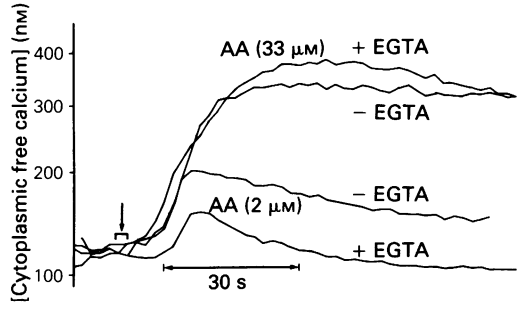

Figure 2 Effect of EGTA on the increase in cytoplasmic free calcium elicited by arachidonic acid (AA). The experimental conditions were as described in Methods. The arrow represents the time of addition of arachidonic acid (at the indicated concentrations) and, where indicated, EGTA (3 μM) was added 1 min before arachidonic acid. The concentration of free calcium in the buffer in the presence of the calcium chelator was calculated to be 4.1×10^{-8} M. The result is representative of at least three such experiments.

basis, a much larger effect on the response to 2 μM arachidonic acid than on 33 μM.

Sensitivity to pertussis and cholera toxin

Pre-incubation of human neutrophils with pertussis toxin (0.5 μg ml^{-1}, 2 h at 37°C) decreased the magnitude of the calcium response to low concentrations of arachidonic acid principally by affecting the initial calcium spike (Figure 3a). In the presence of this toxin, arachidonic acid (8 μM) caused a slow increase in cytoplasmic calcium that levelled off at a value close to the sustained plateau level achieved in control, untreated cells. The calcium response to 33 μM arachidonic acid was, on the other hand, unaffected by pertussis toxin. Pre-incubation with cholera toxin (0.5 μg ml^{-1}, 2 h at 37°C) did not affect significantly, either the magnitude or the shape of calcium responses to arachidonic acid 2 μM (Figure 3b) or 33 μM (results not shown).

Cross-desensitization of the calcium responses to arachidonic acid, leukotriene B_4 or platelet-activating factor

Potential interrelationships among the mechanism(s) of mobilization of calcium by arachidonic acid and either leukotriene B_4 or platelet-activating factor were investigated by examining the effects of pre-exposure to the cells to the latter two lipid mediators before stimulating with arachidonic acid. The results of these experiments are depicted in Figure 4. Note that the ordinate axis of the three panels in this Figure are slightly different from each other due to the individual calibrations that were performed on each run. In these experiments, the response to arachidonic acid in control cells (Figure 4c) is compared to that observed in cells pre-treated with either leukotriene B_4 or platelet-activating factor (Figure 4a and b). In each case the calcium response to the first stimulus was allowed to decay as close as possible to its resting level before adding arachidonic acid. The data shown are taken from successive runs in the same experiment. Figure 4a and c demonstrates that pre-incubation of the cells with leukotriene B_4 essentially abolished the rapid calcium spike induced by 8 μM arachidonic acid. However, a slow increase in calcium was still observed in response to arachidonic acid in the cells pretreated with leukotriene B_4. In contrast, the response to arachidonic acid was essentially unchanged by previous exposure to platelet-activating factor at concentrations that increased intracellular calcium to the same extent as leukotriene B_4 (Figure 4b and c).

The pre-incubation of the cells with arachidonic acid, on the other hand, inhibited in a concentration-dependent manner, the calcium response to platelet-activating factor as well as to leukotriene B_4 (results not shown). These results are reminiscent of the previously documented inhibition by arachidonic acid of the mobilization of calcium induced by fMet-Leu-Phe (Naccache *et al.*, 1983). The study of the biochemical mechanism(s) underlying this heterologous desensitization is beyond the aims of the present investigation.

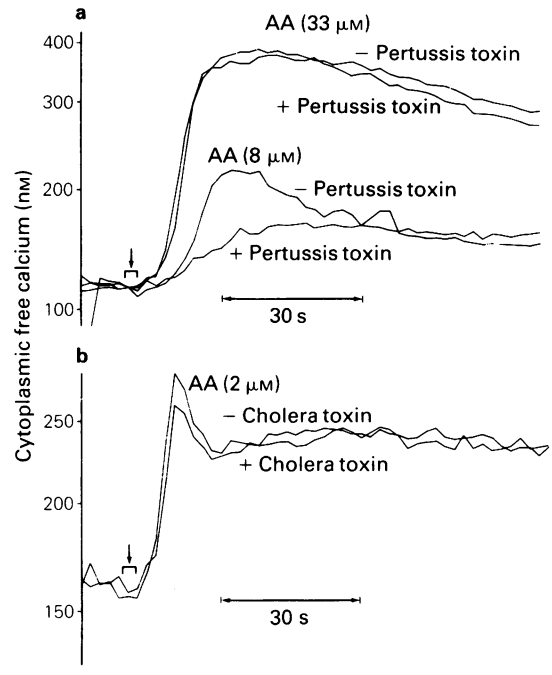

Figure 3 Effect of pertussis toxin and cholera toxin on the mobilization of calcium induced by arachidonic acid (AA) in human neutrophils. The experimental conditions are as described in Methods. Where indicated, the cells were incubated with 0.5 μg ml^{-1} of the toxins for 2 h at 37°C. The arrow indicates the time of addition of arachidonic acid. Note that the graphs in (a) and (b) were drawn to different scales in order to optimize the display of the data. The result is representative of at least three experiments.

Discussion

Arachidonic acid is one of the most commonly used stimuli for neutrophils, having been shown to stimulate several of these cells' characteristic functions and biochemical correlates (see references in Introduction). In the present paper, we have examined the

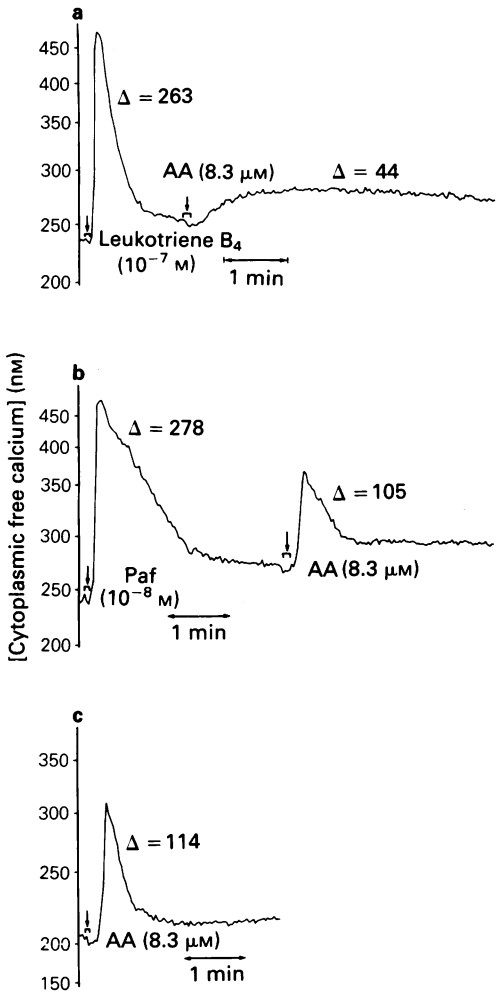

Figure 4 Cross-densitization of the mobilization of calcium induced by pre-stimulation of human neutrophils with leukotriene B_4 or arachidonic acid (AA). The experimental conditions are as described in Methods. Leukotriene B_4 (10^{-7} M), platelet-activating factor (Paf 10^{-8} M), or arachidonic acid (8 μM) were added at the arrows as indicated. The 'deltas' represent the increases in the concentrations of cytoplasmic free calcium calculated from the differences between the prestimulation levels of calcium and the peaks attained after the addition of the respective stimuli. The result is representative of at least three experiments.

calcium mobilizing ability of arachidonic acid in order to characterize the mechanism of action of the fatty acid. The results indicate that the changes in intracellular calcium of human neutrophils in response to arachidonic acid are complex and result from interactions at several distinct sites of action that are likely to include: (1) internal calcium storage sites, (2) direct or indirect receptor-activation mediated, in part at least, through the formation of biologically active metabolites such as leukotriene B_4.

As previously observed in rabbit peritoneal neutrophils (Volpi *et al.*, 1980; Sha'afi *et al.*, 1980; Naccache *et al.*, 1983), and more recently, in the presence of cytochalasin B, in human neutrophils (Smith *et al.*, 1987), the addition of arachidonic acid elicits a rapid mobilization of calcium. As described in the current paper, both the magnitude and the shape of the fura-2 signals induced by arachidonic acid were concentration-dependent. Low concentrations of the fatty acid induced a transient increase in the fluorescence signal, high concentrations a much more lasting response, while at intermediate concentrations the effect was composed of the sum of these two effects. This summation resulted in an initial spike of calcium followed by a plateau at a level significantly higher than the resulting levels of calcium. It should be noted that, while this general pattern was quite reproducible, the relative magnitude of the two phases of calcium mobilization was found to vary among different cell preparations. It is unknown at present whether this variability represents inherent differences in the neutrophils or was induced by uncontrolled influences affecting the cells during the isolation or incubation procedures, e.g., endogenous liberation and metabolism of arachidonic acid (Gorman *et al.*, 1985).

Several lines of evidence indicate that the biphasic shape of the calcium response to arachidonic acid is a reflection of at least two independent sites of action. Indeed, these two phases were differentially sensitive to a variety of pharmacological and experimental manipulations.

The calcium response to arachidonic acid was decreased in the presence of the calcium chelator EGTA, thus indicating its partial dependence on extracellular calcium. The sensitivity of the initial calcium transient to inhibition by pertussis toxin implies that a GTP binding protein (and by implication, a specific receptor site) is involved in the mediation of this response. This interpretation was supported by the finding that the pre-exposure of the cells to leukotriene B_4 led to a significant decrease in the magnitude of the initial phase of the calcium response to arachidonic acid, even though concentrations of the latter close to 100 fold larger than those of leukotriene B_4 were used. The lack of effect of platelet-activating factor on the ability of arachidonic acid to mobilize calcium underlines the specificity of this desensitization. These results make it likely that part of the effects of arachidonic acid are due to direct or indirect interactions with the leukotriene B_4 receptors (see Figure 5). It is possible that the amount of leukotriene B_4 generated as a result of

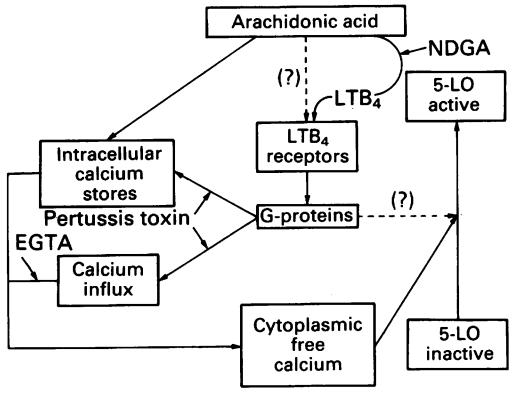

Figure 5 Schematic representation of the events initiated by arachidonic acid in human neutrophils. The complete lines represent pathways, the existence of which is relatively well-established; the dotted lines indicate potential regulatory mechanisms that remain to be firmly demonstrated. The primary site of action of the inhibitors NDGA, pertussis toxin and EGTA are also indicated: LTB_4 = leukotriene B_4;5-LO = 5-lipoxygenase.

the presentation of substrate (arachidonic acid) to the 5-lipoxygenase (8.5, 3.5 and 1.0 ng of leukotriene B_4 and its Ω-oxidation products per 10^6 neutrophils after a 15 min incubation with 33, 10 and 3 μM arachidonic acid, respectively (McColl *et al.*, 1989) would account for the initial phase of the mobilization of calcium by arachidonic acid. The sensitivity of the arachidonic acid-induced initial calcium spike to inhibition by NDGA, as well as that of the arachidonic acid stimulated influx of ^{45}Ca in rabbit neutrophils (Volpi *et al.*, 1980) support this conclusion.

The second phase of calcium mobilization that is observed upon the addition of arachidonic acid is likely to be due to direct effects of the fatty acid on intracellular pools of calcium (see Figure 5). It is most apparent at high concentrations of arachidonic acid, under which conditions the direct release of internal calcium overwhelmed the contribution of the receptor-activated mechanisms that are more prominent at lower concentrations of the fatty acid. Arachidonic acid has previously been shown to elicit a release of calcium from non-mitochondrial calcium storage pools in detergent-permeabilized neutrophils (Beaumier *et al.*, 1987), as well as in isolated sarcoplasmic reticulum of striated myocytes (Cheah, 1981), pancreatic islets (Wolf *et al.*, 1986) and rat liver microsomes (Chan & Turk, 1987). The ability of arachidonic acid to induce a release of calcium from intracellular stores in permeabilized cells has been found to be independent of its metabolism by either 5-lipoxygenase or the cyclo-oxygenase (Beaumier *et al.*, 1987; Chan & Turk, 1987). The relative intensitivity of the slow phase of the arachidonic acid-induced mobilization of calcium in human neutrophils to the presence of extracellular calcium chelators as well as to pertussis toxin, NDGA and to preincubation with leukotriene B_4 are all consistent with this interpretation. The present results thus demonstrate that the mobilization of internal calcium by arachidonic acid, an effect previously only described in permeabilized cell preparations, can also be observed in intact cells. A corollary of this interpretation is that the effect of arachidonic acid on the internal calcium pools is independent of the pertussis toxin-sensitive G protein, and is not shared by leukotriene B_4.

The question remains as to whether the mobilization of calcium by arachidonic acid represents a causal event in the initiation of the physiological responses of the neutrophils to the fatty acid. Arachidonic acid, as previously mentioned, has been shown to stimulate neutrophil aggregation, degranulation, superoxide production and the synthesis of 5-lipoxygenase products. It is noteworthy that all of these functional responses of the cells have been shown to be calcium-dependent. Moreover, the activation of 5-lipoxygenase by arachidonic acid has recently been found to be pertussis toxin-sensitive (McColl *et al.*, 1989). Taken together with the results presented above, these data imply that receptor occupation and activation play a pivotal, and yet to be described, role in the activation of the 5-lipoxygenase enzymatic pathway.

In summary, the present results demonstrate that arachidonic acid, in the low micromolar range, elicits a significant mobilization of calcium that is the result of its direct and indirect interactions with specific receptor sites, as well as with the internal calcium storage pools of the neutrophils. A schematic representation of the events initiated by arachidonic acid that are supported by the present data is shown in Figure 5. These observations are relevant to the patho-physiological behaviour of the neutrophils at inflammatory sites in which levels of free arachidonic acid similar to those used in this study have been observed (Hammarstrom *et al.*, 1975; Greaves, 1986). Indeed, under those conditions, arachidonic acid may modulate, positively as well as negatively, the responses of the neutrophils to other agonists such as C5a, platelet-activating factor, leukotriene B_4 or fMet-Leu-Phe. According to this view, arachidonic acid would be considered as an autocoid in its own right, and not simply as a substrate for a series of metabolizing enzymes.

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