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European Journal of Pharmacology 384 (1999) 183–190

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Role of arachidonic acid in leukotriene B_4-induced guinea-pig eosinophil homotypic aggregation

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Received 14 June 1999; received in revised form 23 September 1999; accepted 1 October 1999

Abstract

The activation of eosinophils with the lipid mediator, leukotriene B_4, induces their homotypic aggregation. Upon activation with leukotriene B_4, eosinophils release a significant amount of arachidonic acid, a process dependent on the activation of phospholipase A_2. Here, we have evaluated whether arachidonic acid could induce aggregation of eosinophils and whether the release of arachidonic acid mediated the aggregation induced by leukotriene B_4. The exogenous administration of arachidonic acid induced a concentration-dependent eosinophil homotypic aggregation. Pretreatment of eosinophils with a 5-lipoxygenase inhibitor or a leukotriene B_4 receptor antagonist abrogated arachidonic-acid-induced aggregation. Arachidonic acid induced a significant increase in leukotriene B_4 levels and desensitised leukotriene B_4-induced aggregation in a dose-dependent manner. Moreover, this desensitisation was effectively reversed by a 5-lipoxygenase inhibitor. However, arachidonic acid failed to induce a rise in intracellular Ca^{2+} in eosinophils and failed to desensitise these cells to rises in intracellular Ca^{2+} induced by leukotriene B_4. Pretreatment of eosinophils with the phospholipase A_2 inhibitor, mepacrine, inhibited the aggregation responses induced by 1 nM leukotriene B_4 by approximately 50% but had no significant effect on the other concentrations of leukotriene B_4 tested (0.1 to 100 nM). In conclusion, arachidonic acid stimulates eosinophil aggregation indirectly via the release of leukotriene B_4. Although a significant amount of arachidonic acid is released in response to activation of eosinophils with leukotriene B_4, the arachidonic acid released does appear to play a major role in mediating leukotriene B_4-induced eosinophil aggregation.

Keywords: Eosinophil; Aggregation; Arachidonic acid; Signal transduction; Leukotriene B_4; Phospholipase A_2; 5-Lipoxygenase

1. Introduction

There is much evidence suggesting a role for eosinophils in the pathogenesis of allergic diseases such asthma and atopic dermatitis (Djukanovic et al., 1990; Bruijnzeel-Koomen et al., 1992). Eosinophils are typically tissue-dwelling cells and, in allergic disorders, an increased number of activated eosinophils is found in the submucosa and mucosa of affected tissues (Djukanovic et al., 1990; Giembycz and Lindsay, 1999). The current paradigm explaining the migration of eosinophils predicts that there are at least three steps prior to their entry into tissue: initially, eosinophils roll on endothelial cells, a process which is mediated by the selectin family of adhesion molecules and their carbohydrate ligands; the rolling leukocyte can then be activated in a G-protein-dependent manner via seven-transmembrane spanning receptors on the leukocyte surface (e.g., leukotriene B_4 and chemokine receptors); this activation leads to upregulation of integrin function on the leukocyte membrane and firm adhesion to endothelial cells (Teixeira et al., 1995b). Recently, we have shown that the paradigm for eosinophil migration can be mimicked in vitro using an assay which measures guinea-pig eosinophil homotypic aggregation. Thus, eosinophil aggregation is dependent on L-selectin, activation of G-protein-coupled receptors and on subsequent activation of β_2 integrins (Teixeira and Hellewell, 1997; Teixeira et al., 1995a, 1996; Giembycz and Lindsay, 1999). Eosinophil–eosinophil interactions have been demonstrated in tissues around larvae of migrating parasites and after the intrader-

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00697-4

mal injection of the chemokine, RANTES (regulated upon activation, normal T cell expressed and secreted) (McLaren, 1980; Meurer et al., 1993). Moreover, leukocyte rolling on already tethered leukocytes may facilitate their accumulation at sites of inflammation (Bargatze et al., 1994). Therefore, understanding of the signaling pathways leading to eosinophil aggregation in vitro may be helpful in the development of novel therapies aimed at inhibiting eosinophil migration in vivo and activation in the tissue.

We have previously shown that the lipid mediator, leukotriene B_4, can induce the homotypic aggregation of eosinophils (Teixeira et al., 1995a). Leukotriene B_4-induced eosinophil aggregation is pertussis-toxin-sensitive (Teixeira and Hellewell, 1997) and dependent on β_2 integrins (Teixeira et al., 1995a). In contrast, we have excluded a major role for protein kinase C (Teixeira and Hellewell, 1997), mitogen-activated protein kinases (MAPKs) and tyrosine kinases (Lindsay et al., 1998a) in mediating this response. Upon activation with leukotriene B_4, eosinophils release a significant amount of arachidonic acid, a process dependent on the activation of phospholipase A_2 (Lindsay et al., 1998b). Arachidonic acid has been shown to activate, either directly or via release of 5-lipoxygenase products, neutrophil functions, such as degranulation and integrin receptor expression (Badwey et al., 1984; Bates et al., 1993; Capodici et al., 1998). Interestingly, it has recently been shown that the activation of β_2 integrins by arachidonic acid was accompanied by the aggregation of human neutrophils in vitro (Capodici et al., 1998). In as much as kinetic studies demonstrated that the onset and peak of leukotriene B_4-induced arachidonic acid release preceded the onset of eosinophil aggregation (Lindsay et al., 1998a,b), this study was conducted to evaluate whether arachidonic acid could induce the aggregation of eosinophils and whether the release of arachidonic acid mediated the aggregation induced by leukotriene B_4. To investigate the latter, we used the phospholipase A_2 inhibitor, mepacrine, at a concentration we have previously shown to block the release of arachidonic acid from leukotriene B_4-stimulated eosinophils (Lindsay et al., 1998b).

2. Material and methods

2.1. Purification of guinea pig peritoneal eosinophils

The method is described in detail elsewhere (Teixeira et al., 1994). The methods described in this study were subject to Home Office (UK) approval and were performed according to the guidelines under the Animals (Scientific Procedure) Act, 1986. Briefly, ex-breeder female guinea pigs (Harlan, Oxon; 700–800 g) were treated with undiluted horse serum (1 ml, i.p.) every other day for 2–3 weeks and the cells harvested by peritoneal lavage with heparinized saline (10 IU ml^{-1}) 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-Hanks balanced salt solution (Ca^{2+}- and Mg^{2+}-free) gradient followed by centrifugation ($1500 \times g$, 25 min at 20°C). Eosinophils (> 95% pure, > 98% viable) were collected from the 1.090/1.095 and 1.095/1.100 g ml^{-1} density interfaces. The cells were then washed twice in phosphate-buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free, pH 7.4) to which glucose (10 mM) was added.

2.2. Eosinophil aggregation

Aggregation experiments were carried out as previously described (Teixeira et al., 1995a). Briefly, guinea-pig eosinophils were resuspended (5×10^6 cells ml^{-1}) in the above assay buffer (PBS), $CaCl_2$ and $MgCl_2$ (final concentrations 1.0 mM and 0.7 mM, respectively) added and the cells kept on ice. Five minutes before use, the cells were warmed to 37°C and aliquots (300 μl) of cells were dispensed into siliconized cuvettes which were placed into a dual-channel platelet aggregometer (Chronolog 440 VS) linked to a dual pen recorder (Chronolog 707). The cells were incubated for at least 5 min at 37°C with continuous stirring at 700 rpm prior to stimulation with the indicated agonist. The reference cuvette contained buffer alone. Responses were allowed to develop for at least 3 min and were measured at the peak of aggregation. Results are expressed as the percentage of maximal aggregation induced by 10^{-7} M phorbol myristate acetate (PMA). Eosinophils were incubated with the various pharmacological agents (mepacrine, ZM230487, LY255283 and flurbiprofen) for 2–3 min prior to stimulation. The following stimuli were used: C5a (10^{-7} M), leukotriene B_4 (10^{-10}–10^{-7} M) and arachidonic acid (10^{-6}–10^{-5} M). For the desensitization experiments, eosinophils were initially pretreated for 2 min with ZM 230487 (10^{-6} M) or vehicle. Increasing concentrations of arachidonic acid (10^{-6}–10^{-5} M) or vehicle were then added for a further 2 min followed by activation of eosinophils with leukotriene B_4 (10^{-8} M). None of the agonists or drugs tested had any significant effect on eosinophil viability as assessed by Trypan blue exclusion (data not shown).

2.3. Intracellular Ca^{2+} experiments

Purified guinea-pig eosinophils (5×10^6 cells ml^{-1} in PBS with 0.25% bovine serum albumin) were loaded with fura-2-acetoxymethyl ester (2.5 μM, 30 min at 37°C). After two washes, eosinophils were resuspended at 10^6 cells ml^{-1} in PBS buffer containing 10 mM HEPES, 0.25% bovine serum albumin and 1 mM Ca^{2+} and stored on ice. Ten minutes prior to their use, eosinophils were warmed to 37°C and 300 μl aliquots dispensed into quartz cuvettes. Changes in fluorescence were monitored at 37°C using a spectrometer (LS50; Perkin-Elmer, Beaconsfield, Bucks) at excitation wavelengths 340 and 380 nm and emission wavelength 510 nm. $[Ca^{2+}]_i$ levels were calcu-

lated using the ratio of the two fluorescence readings and a K_d for Ca^{2+} binding at 37°C of 224 nM (Teixeira et al., 1997). Fura-2-loaded eosinophils were activated with arachidonic acid (10^{-5} M), leukotriene B_4 (10^{-8} M) or vehicle as shown in Section 3. When two stimuli were used sequentially, there was at least a 2-min wait between each addition.

2.4. Measurement of leukotriene B_4 levels

Purified eosinophils (5×10^6 cells ml^{-1}) were resuspended in buffer as for the aggregation experiments and aliquots (300 μl) stimulated with buffer or arachidonic acid (10 μM). One or 5 min after addition of the stimuli, ice-cold buffer (600 μl) was added to the cell suspension and the cells immediately spun (10,000 × *g*, 15 s). The cell pellet was then resuspended in aggregation buffer containing Triton (0.2%) and underwent two freeze–thawing cycles. The lysed cells were then centrifuged (10,000 × *g*, 10 min). Supernatants obtained just after aggregation and after cell lysis were stored at −20°C until measurement of leukotriene B_4 following the recommendations of a commercially available enzymatic immunoassay (R&D Systems, Oxon, UK). Leukotriene B_4 levels in the supernatants following aggregation and cell lysis are shown as nanogram of leukotriene B_4 per milliliter and picogram of leukotriene B_4 per 1.5×10^6 eosinophils, respectively

2.5. Materials

The following reagents were purchased from Sigma (Poole): arachidonic acid, bovine serum albumin, D-glucose, dimethyl sulphoxide, flurbiprofen, mepacrine, PMA. Horse serum, Dulbecco's PBS (Ca^{2+}- and Mg^{2+}-free, pH 7.4) and Hanks balanced salt solution were from Life Technologies (Paisley). Percoll was from Pharmacia (Milton Keynes, England). Leukotriene B_4 was from Cascade (Reading). The 5-lipoxygenase inhibitor, ZM230487 (6-[3-fluoro-5-[4-methoxy-3,4,5,6-tetrahydro-2*H*-pyran-4yl]phenoxymethyl-1-ethyl-2-quinolone) (Kusner et al., 1994; Teixeira et al., 1994), was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire) and the leukotriene B_4 receptor antagonist, LY255283 (1-(5-ethyl-hydroxyl-4-(6-methyl-6-(1*H*-tetrazol-5yl)heptyloxy)phenyl)ethanone) (Silbaugh et al., 1992) was a gift from Eli Lilly (IN, USA). None of the drug vehicles had any significant effect on aggregation induced by any of the agonists tested (data not shown).

2.6. Statistical analysis

Results were analysed using analysis of variance (ANOVA) with the statistical program, Instat (GraphPad Software V2.03). When only two groups were compared, Student's *t*-test was carried out. Results were considered significant when $P < 0.05$. Data are presented as the mean ± S.E.M. of *n* experiments.

3. Results

3.1. Arachidonic-acid-induced eosinophil aggregation

The exogenous administration of arachidonic acid induced a concentration-dependent eosinophil homotypic aggregation (Fig. 1). Arachidonic-acid-induced aggregation was fast in onset and partially reversible (Fig. 1A). At the highest concentration tested (10 μM), arachidonic-acid-induced aggregation amounted to 25%–30% of that evoked by PMA (100%) (Fig. 1B). This compares favourably with the percentage aggregation seen with maximally effective concentrations of platelet-activating factor and leukotriene B_4 (Teixeira et al., 1995a). This concentration of arachidonic acid (10 μM) was used in all further experiments.

Treatment of eosinophils with the 5-lipoxygenase inhibitor, ZM230487, prior to the addition of arachidonic acid virtually abolished the homotypic aggregation of eosinophils induced by arachidonic acid and maximal inhibition occurred at 1 μM concentration of ZM230487 (Fig. 2). The leukotriene B_4 receptor antagonist, LY255283, but

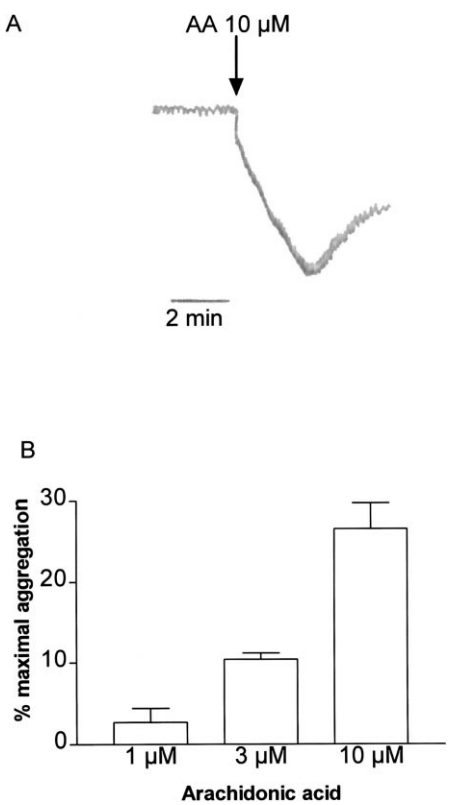

Fig. 1. Effect of arachidonic acid on the homotypic aggregation of guinea-pig eosinophils. In (A), a typical aggregation trace in response to 10 μM arachidonic acid (AA) is shown. In (B), the concentration-dependent effects of arachidonic acid (1–10 μM) are depicted. Results are expressed as the percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four experiments.

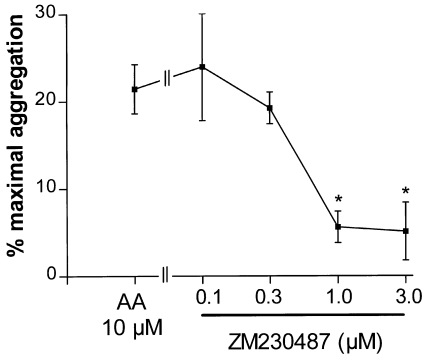

Fig. 2. Effect of the 5-lipoxygenase inhibitor, ZM230487, on arachidonic-acid-induced homotypic aggregation of eosinophils. Eosinophils were pretreated with ZM230487 (0.1–3 μM) for 3 min prior to the addition of arachidonic acid (AA, 10 μM). Results are expressed as the percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four experiments. $^{*}P < 0.01$ when compared to responses in the presence of arachidonic acid alone.

not the cyclooxygenase inhibitor, flurbiprofen, abrogated eosinophil aggregation induced by arachidonic acid (Fig. 3). Pretreatment of eosinophils with ZM230487 (1 μM) had no significant effect on the aggregation response induced by leukotriene B_4 or C5a (Table 1). Pretreatment with LY255283 failed to affect C5a-induced aggregation, while effectively inhibiting responses induced by arachidonic acid (Fig. 3) or leukotriene B_4 (Table 1).

Five minutes after stimulation of eosinophils with arachidonic acid, there was a significant increase in the levels of leukotriene B_4 in the supernatant [vehicle stimulation, below detection limit of the assay (< 200 pg ml^{-1}); arachidonic acid 10 μM, 13.1 ± 3.4 ng ml^{-1}] and cell pellet (vehicle, 1409 ± 193 pg/1.5×10^6 eosinophils; arachidonic acid 10 μM, 2593 ± 214 pg; $P < 0.01$) of aggregating cells. At 1 min, there was no increase in the levels of LTB_4 in the supernatant (bellow the detection

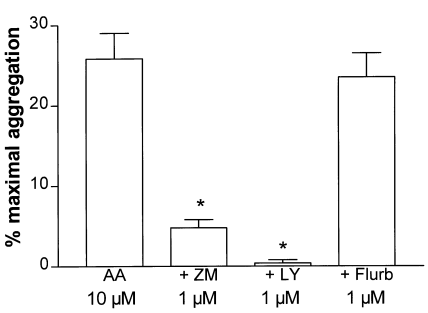

Fig. 3. Effect of the 5-lipoxygenase inhibitor, ZM230487, the leukotriene B_4 receptor antagonist, LY255283, and the cyclooxygenase inhibitor, flurbiprofen, on arachidonic-acid-induced homotypic aggregation of eosinophils. Eosinophils were pretreated with ZM230487 (ZM, 1 μM), LY255283 (LY, 1 μM) or flurbiprofen (flurb, 1 μM) for 3 min prior to the addition of arachidonic acid (AA, 10 μM). Results are expressed as the percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four to five experiments. $^{*}P < 0.01$ when compared to responses in the presence of arachidonic acid alone.

Table 1
Effects of a 5-lipoxygenase inhibitor, ZM230487, and a leukotriene B_4 receptor antagonist, LY255283, on eosinophil aggregation induced by leukotriene B_4 and C5a[a]

	Percent maximal aggregation		
	Alone	+ ZM230487	+ LY255283
LTB_4 10 nM	31.7 ± 2.8	35.0 ± 2.1	8.9 ± 2.6[b]
C5a 100 nM	38.8 ± 2.8	36.4 ± 3.6	30.7 ± 3.6

[a] Eosinophils were pretreated with ZM230487 (1 μM) or LY255283 (1 μM) for 3 min and then stimulated with C5a or leukotriene B_4 (LTB_4). Data are shown as percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four experiments.

[b] $P < 0.01$ when compared to responses in the presence of LTB_4 alone.

limit of the assay, data not shown), but there was a significant increase in the cell-pellet-associated LTB_4 (vehicle, 1708 ± 80 pg/1.5×10^6 eosinophils; arachidonic acid 10 μM, 2760 ± 105 pg; $P < 0.01$).

We have previously shown that pretreatment with leukotriene B_4 desensitised eosinophils to a further administration of the same stimulus (Teixeira et al., 1995a). Because arachidonic acid appears to stimulate eosinophil homotypic aggregation via the release of leukotriene B_4, we tested whether arachidonic acid would also desensitise responses to leukotriene B_4. As shown in Fig. 4, arachidonic acid induced a concentration-dependent desensitisation of eosinophil aggregation induced by leukotriene B_4. Interestingly, pretreatment of eosinophils with ZM230487 prior to addition of arachidonic acid partially reversed the ability of this lipid to desensitise to leukotriene B_4-induced eosinophil aggregation (Fig. 4). Eosinophil aggregation

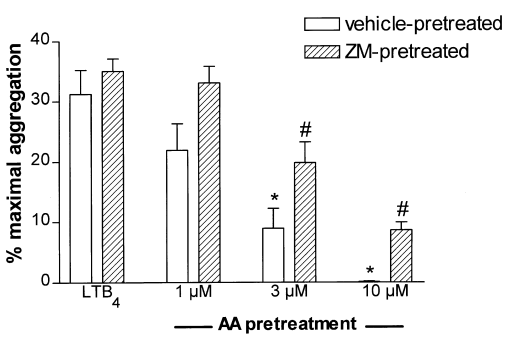

Fig. 4. Effects of the 5-lipoxygenase inhibitor, ZM230487, on the ability of arachidonic acid to desensitise the eosinophil aggregation induced by leukotriene B_4. Eosinophils were initially pretreated for 2 min with ZM 230487 (1 μM) or vehicle. Then, increasing concentrations of arachidonic acid (AA, 1 to 10 μM) or vehicle were added followed, after a further 2 min, by the activation of eosinophils with leukotriene B_4 (LTB_4, 10 nM). Results are expressed as the percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four to five experiments. $^{*}P < 0.01$ when compared to responses in eosinophils stimulated with leukotriene B_4 but not pretreated with arachidonic acid. $^{\#}P < 0.05$ when comparing responses in the absence or presence of ZM230487.

induced by C5a was not altered by pretreatment with arachidonic acid (data not shown). Collectively, the data presented above are consistent with arachidonic acid inducing the release of leukotriene B_4, which acts on leukotriene B_4 receptors to mediate eosinophil homotypic aggregation.

In contrast to its effect on eosinophil aggregation, arachidonic acid failed to induce a rise in intracellular Ca^{2+} in eosinophils and failed to desensitise these cells to rises in intracellular Ca^{2+} induced by leukotriene B_4 (Fig. 5).

3.2. Effects of the phospholipase A_2 inhibitor, mepacrine, on eosinophil aggregation

The role of arachidonic acid in leukotriene B_4-induced eosinophil homotypic aggregation was evaluated by the use of mepacrine, an inhibitor of phospholipase A_2. Mepacrine was used at a concentration (50 μM) previously shown to inhibit maximally the release of arachidonic acid by leukotriene B_4-stimulated guinea pig eosinophils (Lindsay et al., 1998b). At the concentration used, mepacrine did not induce eosinophil aggregation (data not shown). Mepacrine inhibited the aggregation responses induced by 1 nM leukotriene B_4 by approximately 50% ($P < 0.05$) but had no significant effect on the other concentrations of leukotriene B_4 tested (Fig. 6).

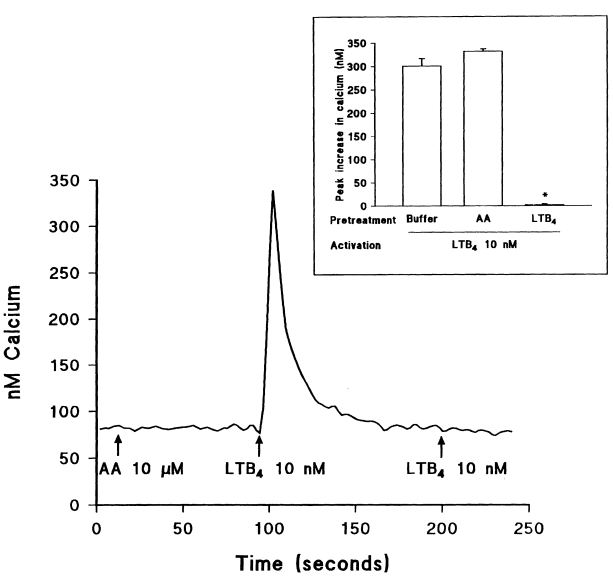

Fig. 5. Typical trace showing the inability of arachidonic acid to induce an elevation in the levels of intracellular Ca^{2+} and to desensitise the leukotriene B_4-induced responses in eosinophils. Eosinophils were pretreated for 2 min with arachidonic acid (AA, 10 μM) followed by activation with leukotriene B_4 (LTB_4, 10 nM). Results are expressed as the increase in the concentration of intracellular Ca^{2+} (in nanomolar) as a function of time (in seconds). In the insert, eosinophils were pretreated with buffer, arachidonic acid (AA, 10 μM) or leukotriene B_4 (LTB_4, 10 nM) prior to addition of leukotriene B_4 (10 nM). Data are shown as the peak increase in the concentration of intracellular Ca^{2+} (in nM) and are the means ± S.E.M. of three experiments. $^{\#}P < 0.01$ when comparing cells pretreated with buffer or leukotriene B_4.

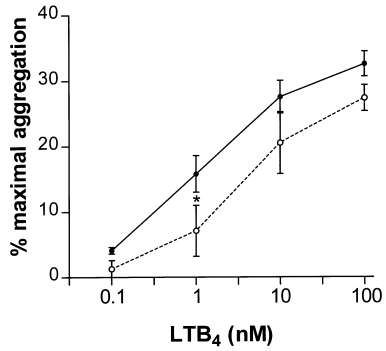

Fig. 6. Effect of the phospholipase AA_2 inhibitor, mepacrine, on leukotriene B_4-induced eosinophil homotypic aggregation. Eosinophils were pretreated with buffer (solid line) or mepacrine (50 μM, dashed line) for 3 min prior to the addition of leukotriene B_4 (0.1 to 100 nM). Results are expressed as the percent maximal aggregation in response to PMA (100 nM) and are the means ± S.E.M. of four experiments. $^{*}P < 0.05$ when comparing cells pretreated with buffer or mepacrine.

4. Discussion

The activation of eosinophils with chemoattractant molecules, such as eotaxin and leukotriene B_4, induces their homotypic aggregation which can be measured on-line as the increase in light transmittance through a stirred suspension of cells (Teixeira et al., 1995a). Previous studies from our laboratories have shown that eosinophil aggregation is Ca^{2+}- and Mg^{2+}-dependent and blocked by pretreatment with anti-L-selectin or anti-β_2 integrin monoclonal antibodies (Teixeira et al., 1995a, 1996, 1997). The signal transduction mechanisms involved in the homotypic aggregation of eosinophils in response to chemoattractant agents, such as leukotriene B_4, is largely unknown. We have previously shown that leukotriene B_4-induced eosinophil aggregation is pertussis-toxin-sensitive but independent of the activation of protein kinase C, tyrosine kinases and MAPKs (Teixeira and Hellewell, 1997; Lindsay et al., 1998a). In neutrophils, arachidonic acid activates β_2 integrins and induces their homotypic aggregation (Bates et al., 1993; Capodici et al., 1998). In as much as leukotriene B_4 induces a significant release of arachidonic acid from guinea-pig eosinophils, which precedes the onset of homotypic aggregation, we investigated whether arachidonic acid also activated eosinophils and whether it was involved in leukotriene B_4-induced aggregation.

Arachidonic acid induced a concentration-dependent homotypic aggregation of eosinophils which was fast in onset and demonstrated a similar profile to that after activation with leukotriene B_4 or C5a (Teixeira et al., 1995a). In some leukocytes, arachidonic acid interacts directly with cellular systems (e.g., NADPH oxidase) to induce physiological responses (McPhail et al., 1984; Smith

et al., 1987). Alternatively, arachidonic acid can be metabolised by 5-lipoxygenase and give rise to leukotrienes or 5-hydroxy-5,8,14-*cis*-10-trans-eicosatetraenoic acid (5-HETE), which, in turn, activate leukocyte function (Chun et al., 1997; Capodici et al., 1998). In order to assess whether arachidonic acid was metabolised by 5-lipoxygenase in our system, we pre-treated eosinophils with the 5-lipoxygenase inhibitor, ZM230487 (Kusner et al., 1994; Teixeira et al., 1994). This drug abolished arachidonic-acid-induced eosinophil aggregation but had no effect on the functional responses to leukotriene B_4 or C5a. Next, we examined whether the release of leukotriene B_4 or another 5-lipoxygenase-derived lipid was the main metabolite which accounted for arachidonic-acid-induced eosinophil aggregation. Two approaches were used to examine this issue: use of the leukotriene B_4 receptor antagonist, LY255283, and desensitisation experiments with leukotriene B_4. LY255283, at a concentration that blocked leukotriene B_4-induced responses by over 90%, virtually abolished the eosinophil aggregation induced by arachidonic acid. Similarly, desensitisation with arachidonic acid prevented the ability of leukotriene B_4 to induce the aggregation of eosinophils. Interestingly, the desensitisation of eosinophils by arachidonic acid was partly prevented by pretreatment with ZM230487, demonstrating that arachidonic acid was metabolised by 5-lipoxygenase to a lipid that activated the leukotriene B_4 receptor. In addition, eosinophils produced a significant amount of leukotriene B_4 upon stimulation with arachidonic acid. Overall, our results demonstrate the ability of arachidonic acid to stimulate eosinophil aggregation via the release of 5-lipoxygenase-derived leukotriene B_4. These results are consistent with an earlier report demonstrating the ability of guinea-pig eosinophils to produce leukotriene B_4 upon stimulation (Hirata et al., 1990). Finally, the lack of effect of the 5-lipoxygenase inhibitor on leukotriene B_4- and C5a-induced responses suggests that the autocrine production of leukotriene B_4, or other 5-lipoxygenase products, by eosinophils, does not play a role in the aggregation following activation with these stimuli.

Two unexpected observations were the lack of effect of arachidonic acid on the levels of intracellular Ca^{2+} in eosinophils and the inability of arachidonic acid to desensitise the leukotriene B_4-induced elevation in intracellular Ca^{2+} in eosinophils. Since arachidonic acid is metabolised into leukotriene B_4 in eosinophils (see above) and leukotriene B_4 induces an elevation in intracellular Ca^{2+}, it would be reasonable to suppose that arachidonic acid would also induce a similar response. For example, in human neutrophils, arachidonic acid stimulated a significant mobilisation of intracellular Ca^{2+} (Smith et al., 1987). Similarly, arachidonic acid induced a fast, transient rise of cytosolic free Ca^{2+} concentration in human eosinophils (Kok et al., 1989). In as much as arachidonic acid induced the release of leukotriene B_4, it would be reasonable to suppose that arachidonic acid would desensitise the leukotriene B_4-induced elevation of intracellular Ca^{2+}. However, neither a Ca^{2+} influx nor desensitization of leukotriene B_4-induced responses was observed. Our data are consistent with that of Subramanian (1992) who showed that the ability of leukotriene B_4 to induce Ca^{2+} changes in guinea-pig eosinophils was not related to the ability of this agonist to induce the production of superoxide anion. Only one cloned receptor has been found in guinea pigs but the expressed receptor seems to differ in its ability to activate intracellular signals depending on the ligand to which it binds (Masuda et al., 1999). In addition, leukotriene B_4 binds to guinea-pig eosinophils with two distinct binding affinities, which led the authors to suggest the existence of two leukotriene B_4 receptors (Sehmi et al., 1992). Thus, it is possible that the effects of arachidonic acid on eosinophil aggregation are mediated by leukotriene B_4 acting on a receptor distinct (or at a different affinity state) from the receptor responsible for the leukotriene B_4-induced elevation in intracellular Ca^{2+} (Giembycz and Lindsay, 1999). Furthermore, our results show that the levels of leukotriene B_4 in the cell pellet increased prior to their increase in the supernatant. In as much as the leukotriene B_4 receptor may have two distinct localisations (cell surface and nuclear receptors, described in human cell lines) (Yokomizo et al., 1997; Giembycz and Lindsay, 1999), one alternative possibility is that the leukotriene B_4 may be acting at these different locations to mediate Ca^{2+} influx and aggregation. We are presently investigating these possibilities in our laboratory.

Our present results also demonstrate that an elevation in intracellular Ca^{2+} is not essential for the homotypic aggregation of eosinophils induced by arachidonic acid or leukotriene B_4. This statement is based on the following findings: (i) arachidonic acid induced significant aggregation (Fig. 1) but no calcium response (Fig. 5); and (ii) LTB_4 induced both aggregation and calcium influx (Figs. 3 and 5). Nevertheless, arachidonic acid desensitized the LTB_4-induced aggregation, but not the Ca^{2+} response (Figs. 3 and 5). One possibility is that, like in human eosinophils, Mg^{2+} could substitute for Ca^{2+} (Koenderman et al., 1991). Nevertheless, we have previously reported that the aggregation of guinea-pig eosinophils was completely dependent on Ca^{2+} and was blocked by EGTA (Teixeira et al., 1995a,b). Thus, although the presence of extracellular Ca^{2+} is essential for the aggregation process of guinea-pig eosinophils (selectin- and integrin-dependent adhesions are Ca^{2+}-dependent; Teixeira et al., 1995a,b), elevated levels of intracellular Ca^{2+} do not appear to play a major role, at least following activation with leukotriene B_4.

Experiments were then designed to examine the role of the release of arachidonic acid on the ability of leukotriene B_4 to induce the aggregation of eosinophils. For these, we used the phospholipase A_2 inhibitor, mepacrine, at a concentration which effectively blocked the leukotriene B_4-induced release of [^{3}H]arachidonic acid from guinea-pig

eosinophils (Lindsay et al., 1998b). Moreover, mepacrine has been previously shown to modulate endogenous phospholipase A_2 and degranulation in human eosinophils at similar concentrations (White et al., 1993). Here, we showed that mepacrine only inhibited the aggregation induced by low doses of arachidonic acid and had no significant effect on the aggregation induced by 10 and 100 nM leukotriene B_4. This lack of effect of mepacrine on leukotriene B_4-induced aggregation suggests that although leukotriene B_4 induces the release of a significant amount of arachidonic acid from eosinophils, the arachidonic acid released does not play a major role in mediating eosinophil homotypic aggregation. Similarly, we found that arachidonic acid release did not play a central role in leukotriene B_4-induced activation of the NADPH oxidase in guinea-pig eosinophils (Lindsay et al., 1998b). In addition to mepacrine, we tested the effects of the reportedly more specific cytosolic phospholipase A_2 inhibitor arachidonic acid, $COCF_3$ (arachidonyltrifluoromethyl ketone) (e.g., Wissing et al., 1997). However, this compound induced significant aggregation of eosinophils and the release of [^{3}H]arachidonic acid on its own (data not shown). In human neutrophils, inhibition of phospholipase A_2 plays an important role in the regulation of the expression of β_2 integrins and integrin-dependent function (Jacobson and Schrier, 1993; Arai et al., 1998). The importance of phospholipase A_2-derived arachidonic acid for the adhesive function of human eosinophils is not known and is currently the subject of active research in our laboratories.

This is the first study to demonstrate that arachidonic acid is an effective stimulant of eosinophil homotypic aggregation. Arachidonic acid appears to stimulate eosinophils indirectly via the release of 5-lipoxygenase-derived leukotriene B_4. Although a significant amount of arachidonic acid is released in response to activation of eosinophils with leukotriene B_4, inhibition of arachidonic acid release by a phospholipase A_2 inhibitor does not have a major effect on leukotriene B_4-induced eosinophil aggregation. Further studies are needed to elucidate the mechanism(s) underlying the primary signaling pathways recruited by leukotriene B_4 to induce the homotypic aggregation of eosinophils.

Acknowledgements

We are grateful to CNPq, the Wellcome Trust and FAPEMIG for financial support. M.M.T. is a recipient of a CNPq Scientific Development grant.

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