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Source of endogenous arachidonate and 5-lipoxygenase products in human neutrophils stimulated by bradykinin and A23187

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SUMMARY **The lipoxygenase products of arachidonic acid (AA) metabolism, 5-hydroxyeicosatetraenoic acid (5-HETE) and leucotriene B_4 (LTB_4), are considered to have an important pathophysiological role in inflammatory bowel disease by stimulating the inflammatory response and by contributing to the diarrhoea. The present studies were designed to investigate the effect of the physiological stimulants bradykinin (BK) and 5-hydroxytryptamine (5-HT), in addition to the influence of the calcium ionophore A23187, on the source of AA release and 5-lipoxygenation in human neutrophils (PMNs) *in vitro*. This was done to elucidate the specificity of the mechanism by which PMNs respond to physiological, extracellular Ca^{2+} dependent agonists. The results of the study indicate that stimulation of 1-^{14}C-AA-prelabelled PMNs with BK liberates AA mainly from phosphatidylinositol, while A23187 causes release of AA from phosphatidylcholine, phosphatidylethanolamine, and possibly phosphatidylserine. Furthermore BK (10^{-9}–10^{-6}M) dose-dependently stimulated the formation of 5-HETE and LTB_4, reaching a maximum at 10^{-7}M, while 5-HT (10^{-8}–10^{-4}M) released only negligible amounts of eicosanoids, similar to those observed in control experiments. Stimulation with A23187 (10^{-5}M) caused a high release of both 5-HETE and LTB_4. These results offer evidence that BK, but not 5-HT, initiates formation of lipoxygenase products by binding to specific receptors on the external surface of PMNs, whereas A23187 accelerates 5-lipoxygenation through mechanisms which do not involve a cell surface receptor.**

In the diseased colon eicosanoids may potentially contribute to inflammation, in addition to alterations in fluid and electrolyte transport, intestinal motor activity, and mucosal blood flow.[1] Thus previous studies have observed increased local formation of cyclooxygenase – and lipoxygenase products of arachidonic acid (AA) metabolism in experimental models of colonic inflammation[2,3] and in man with ulcerative colitis (UC).[4–7]

Although neutrophils (PMNs), which are abundant in the inflamed colonic mucosa,[8] are considered to metabolise AA *via* the cyclooxygenase pathway, their major AA metabolites are the lipoxygenase products, 5-hydroxyeicosatetraenoic acid (5-HETE) and leucotriene B_4.[9,10] The latter substance is a potent chemotactic agent that promotes migration and stimulates aggregation and degranulation of neutrophils, in addition to release of lysosomal enzymes and superoxide production.[11,12]

Arachidonic acid is liberated not only by phospholipase A_2, however, but also by phospholipase C.[13] Thus the stimulation of AA metabolism by physical (mechanical, irradiation) or chemical (hypoxia, 'free radicals') damage, which does not require a specific receptor, is associated with an increased turnover of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) and catalysed by phospholipase A_2.[9,14,15] On the other hand, the mucosal infiltrate *per se* may also promote eicosanoid formation and diarrhoea by releasing agents, such as bradykinin (BK) and 5-hydroxytryptamine (5-HT), that function as extracellular stimulants of membrane bound phospholipase C, which hydrolyses PI by a series of reactions involving diacylglycerol and monoacylglycerol lipases.[15–17] This receptor linked breakdown

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of PI leads to formation of diacylglycerol, inositoltriphosphate, as well as AA metabolism,[13 15 18] which appears to be integrated with calcium mobilisation and cyclic GMP production in a single receptor cascade system,[19] without involving cyclic AMP. Diacylglycerol acts as an essential cofactor for protein kinase C,[19] while inositoltriphosphate functions as a messenger to mobilise calcium from an intracellular site,[13] and AA metabolites, such as prostaglandins of the E type, act as calcium ionophores to increase intracellular calcium by opening calcium channels.[15 20]

The present study was carried out to study the effect of physiologic Ca^{2+} dependent agonists on the source of endogenous AA and 5-lipoxygenation in human PMNs *in vitro*. 1-^{14}C-AA-prelabelled PMNs were stimulated, therefore, by BK and 5-HT and the results were compared with those obtained by using the calcium ionophore, A23187, to elucidate the specificity of the mechanism by which PMNs respond to extracellular, Ca^{2+} dependent agonists.

Methods

CELL ISOLATION

Venous blood (EDTA 10 mM) was obtained from the cubital vein of healthy volunteers who had taken no drugs for at least two weeks. Purification of the cells were initiated within 30 minutes after blood sampling.

Leucocytes were isolated by a modification of the method described by Böyum:[21] (1) erythrocytes were sedimented for 45 minutes at 20°C with methylcellulose (0·8%), (2) the 'buffy coat' leucocytes were washed twice in Gey's solution in human albumin (2%), (3) mononuclear leucocytes and platelets were separated from neutrophils by gradient centrifugation using Lymfoprep^{R} (Nygaard and Co, Oslo, Norway), and (4) contaminating erythrocytes were removed by hypotonic lysis with NaCl (0·2%).

Isolated PMNs (median recovery 44%, more than 97% purity) were washed twice and resuspended in the culture medium RPMI 1640 (Gibco Ltd, Renfrewshire, Scotland) with hepes buffer (25 mM) and glutamine (2 mM) under a stream of 5% carbondioxide and 95% atmospheric air.

AGONISTS

Solutions of BK or 5-HT (Sigma Inc, St Louis, MO, USA) were prepared in Gey's solution shortly before use in physiologically relevant concentrations: 10^{-9} to 10^{-6} M for BK[22] and 10^{-8} to 10^{-4} M for 5-HT.[23]

The calcium ionophore, A23187 (Calbiochem, La Jolla, CA, USA) (10^{-5} M, 15 min), was included as a positive control and a comparator of eicosanoid production and AA mobilisation, respectively.

ARACHIDONIC ACID METABOLISM

Neutrophils (5×10^{6} cells/ml) were incubated with 1-^{14}C-AA (37×10^{3} Becquerel (Bq)/5×10^{6} PMNs, $2{\cdot}2\times10^{9}$ Bq/mmol, Amersham International, Buckinghamshire, England) for five hours at 37°C to achieve steady state conditions for the labelling of intracellular pools of AA. Excess AA was removed by washing with Gey's solution. Bradykinin or 5-HT were added after a 15 minute equilibration period.

Time course experiments were carried out to determine the lag until optimum stimulation. Isolation of extracellular radiolabelled metabolites was carried out by centrifugation (8×10^{3} G, 1 min) of the cells through dibutyl-phthalate:dinonyl-phthalate; 3:1 (density 1·033 g/ml), and extraction according to Folch.[9] The radioactive metabolites, 5-HETE and LTB_4, were separated by thin layer chromatography and quantified by autoradiography and laser densitometry as previously described.[9]

To define the phospholipid groups, from which AA was mobilised, thin layer chromatography analysis of lipid extracts from total cell suspensions was carried out as previously described in detail.[9] Shortly, the suspensions were extracted *ad modum* Folch[9] and the organic phases evaporated in a stream of nitrogen. The residues were applied to thin-layer chromatography (TLC) using CH_2Cl_2:methanol:2-propanol:0·25%KCl:ethylacetate (30:9:25:6:18) as a developing solvent. After autoradiography of the TLC-plates, which were exposed for one week, quantification of the radioactive spots were performed by laser densitometry. Identification of the different phospholipids was performed by cochromatography with pure standards.[9]

The specific activity of released LTB_4 was determined by high pressure liquid chromatography as previously described.[7 9]

ETHICS

Informed consent was obtained from all healthy volunteers and the study was approved by the Scientific Ethics Committee of the Copenhagen County.

STATISTICAL ANALYSIS

Results are given as medians with ranges in brackets. Wilcoxon's test for paired and unpaired observations were applied. A p value of less than 0·05 (2α) was considered significant.

Results

In a preliminary series of experiments stimulation with BK for 10 minutes was found to cause optimum conditions (Fig. 1). For BK the release of radioactivity from 1-^{14}C-prelabelled PMNs, in the concen-

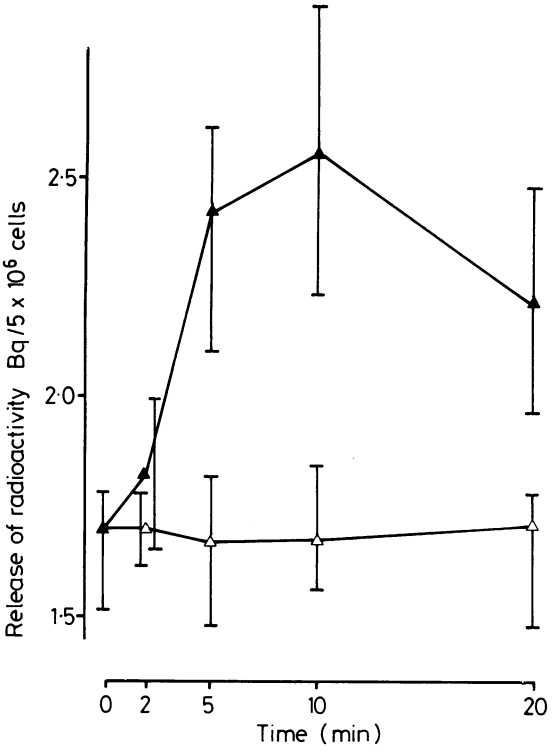

Fig. 1 *Time course experiments for stimulation of 1-^{14}C-arachidonic acid labelled neutrophils with bradykinin (10^{-7} M) (▲) or 5-hydroxytryptamine (10^{-7} M) (△). Values are given as medians with Q_{50} ranges for 6 experiments.*

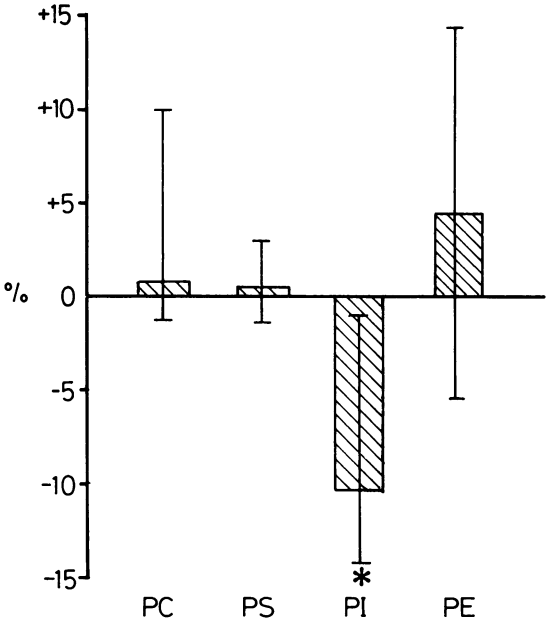

Fig. 2 *Change in relative distribution of arachidonic acid in the different phospholipid fractions after stimulation with bradykinin (10^{-7} M, 10 min). Phosphatidylcholine (PC), phosphatidylserine (PS), Phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Values are given as medians with ranges (bars) for 10 experiments. *p<0·03.*

tration range tested, reached a maximum at 10^{-7} M (Table 1). The total amount of radioactivity released – that is, AA and its metabolites, was $2{\cdot}6\times10^2$ Bq/5×10^6 cells ($2{\cdot}0$–$3{\cdot}5\times10^2$ Bq/5×10^6 cells) corresponding to 7·8% (5·0–11·7%) of the 1-^{14}C-AA incorporated into phospholipids compared with a release of $1{\cdot}7\times10^2$ Bq/5×10^6 cells or approximately 5% of the 1-^{14}C-AA incorporated into phospholipids in non-stimulated cells ($p<0{\cdot}01$) (Table 2), whereas stimulation with the calcium ionophore, A23187, resulted in a total release of $6{\cdot}7\times10^2$ Bq/5×10^6 cells ($2{\cdot}6$–$9{\cdot}9\times10^2$ Bq/5×10^6 cells) corresponding to 20·3% (15·4–24·3%) of the 1-^{14}C-AA incorporated into phospholipids.

Analysis of the distribution of radioactivity within the cells before and after stimulation with BK revealed that AA was mobilised primarily from PI (Fig. 2) ($p<0{\cdot}03$), whereas no detectable alterations in PC, PE, or phosphatidylserine (PS) were found (Fig. 2). By contrast, A23187 caused a relative decrease in radioactivity within PC, PE, and PI by 32% (18–50%; $p<0{\cdot}01$), 27% (12–45%; $p<0{\cdot}02$),

Table 1 *Total amount of radioactivity released from 1-^{14}C-arachidonic acid prelabelled neutrophil granulocytes (10^2 Bq/5×10^6 cells) after stimulation with bradykinin. Values are given as medians with ranges in brackets*

	Bradykinin concentration (M)				
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	$10^{-\infty}$
Radioactivity	1·8 (1·5–2·5)	2·6 (2·0–3·5)	1·9 (0·9–2·3)	1·6 (0·7–2·0)	1·7 (0·7–2·1)

Table 2 *Total amount of radioactivity (arachidonic acid and its metabolites) released from phosphatidylinositol in 1-^{14}C-arachidonic acid labelled neutrophils during control conditions and after challenge with bradykinin (10^{-7} M), or 5-hydroxytryptamine (10^{-7} M) for 10 minutes (n=10). Values are given as medians with ranges in brackets*

	n	*Released radioactivity (10^2 Bq/5×10^6 cells)*
Controls	10	1·7 (1·1–2·2)
Bradykinin	10	2·6* (2·0–3·5)
5-hydroxytryptamine	10	1·6 (0·8–1·9)

*$p<0{\cdot}01$ compared with controls and 5-hydroxytryptamine.

and 11% (0–28%; $p<0.05$), respectively, indicating that AA was released from these three phospholipid pools. Because only small amounts of PS were detected it cannot be excluded that this pool may be responsible for AA release as well.

In non-stimulated PMNs there was no detectable amount of LTB_4, but trace amounts of 5-HETE was observed.

Bradykinin ($10^{-9}-10^{-6}$ M) caused a dose dependent increase in the release of both lipoxygenase products, which reached a maximum at 10^{-7} M (Fig. 3). The release corresponded to 2·8% (2·2–6·6%) and 2·3% (0·7–4·9%) of the total radioactivity released as far as 5-HETE and LTB_4 are concerned, whereas A23187 resulted in formation of 5-HETE and LTB_4 corresponding to 14·9% (9·5–19%) and 5·3% (2·0–7·6%), respectively, of the total radioactivity released.

5-HT ($10^{-8}-10^{-4}$ M) caused no detectable release of lipoxygenase products (Fig. 1).

The difference in specific activity of ^{14}C-metabolites, as assessed by high-pressure liquid chromatography for evaluation of the homogeneity of radiolabelling, was negligible wiz $5{\cdot}4\times10^8$ Bq/mmol ($5{\cdot}1–6{\cdot}9\times10^8$ Bq/mmol) and $6{\cdot}3\times10^8$ Bq/mmol ($4{\cdot}8–7{\cdot}4\times10^8$ Bq/mmol) for BK and A23187, respectively.

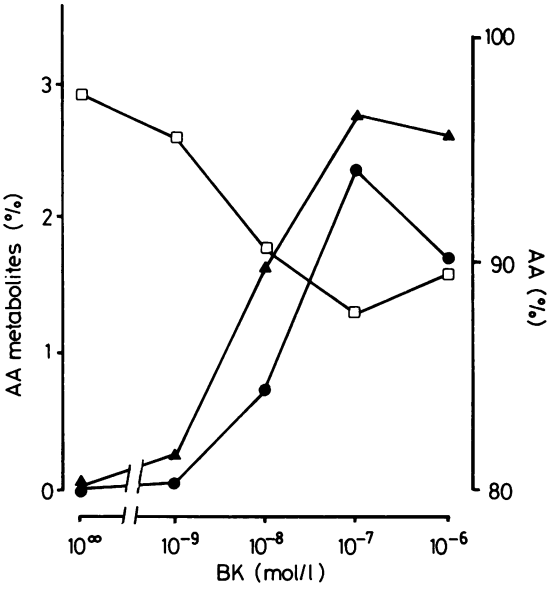

Fig. 3 *Relative amounts of radioactive arachidonic acid (□), leukotriene B_4 (●), and 5-hydroxyeicosatetraenoic acid (▲) released during incubation of 1-^{14}C-arachidonic acid labelled neutrophils with increasing concentrations of bradykinin for 10 minutes (n=10).*

Discussion

These results show that concentrations of BK, comparable with those measured in plasma ($10^{-9}-10^{-6}$ M), stimulates 5-lipoxygenation of AA in human PMNs, in addition to increasing the release of AA from PI. By contrast, the calcium dependent neurotransmitter, 5-HT, has no effect on AA release and 5-lipoxygenation in the concentration range used. The amount of LTB_4 produced by 5×10^6 PMNs, in response to optimal challenge with BK, resulted in a median concentration – that is, 11·1 nM, within the range which stimulates chemotaxis,[12] whereas the less potent chemoattractant, 5-HETE, did not reach concentrations range sufficiently high to stimulate chemotaxis.[12]

The mechanism by which BK stimulates the release and metabolism of AA in human PMNs has not yet been defined, but is probably dependent of stimulation of specific receptors on the external surface of the cells.[24 25 26] 5-HT, which has been reported earlier to increase PI turn-over in other cell types,[27] had no effect on AA release and metabolism in human PMNs, which may be caused by lack of specific receptors.

On the other hand, the calcium ionophore, A23187, caused a high release of radio-labelled AA originating in both PI, PC, PE, and probably also PS. At maximum stimulation with A23187 (10 μM, 15 min) the synthesis of LTB_4 was approximately 13 times higher than that obtained with BK 10^{-7} M.

Conflicting reports concerning BK induced release of AA from phospholipids have been published. Thus it has been reported that AA is released from PI, PC, and PE in tumour cells,[28] and from cells of kidney cortex and medulla,[29] while others have found that BK-induced AA release arise from mainly PI[30] or PC synthesised by the methylation pathway.[31]

As the present study suggests that BK initiates formation of lipoxygenase products in human PMNs by a specific mechanism, whereas A23187 accelerates 5-lipoxygenation through a mechanism, which does not require a specific receptor, these results may be relevant for the understanding of the pathophysiology of inflammatory disorders, such as the inflammatory bowel diseases. In these conditions plasma concentrations of kinins are closely related to the onset of vasomotor and gastrointestinal symptoms[32–35] and it is well established that inflammation is associated with the formation of kinins.[36 37] Accordingly, many factors may contribute to raised kinin concentrations. Thus it has been shown that the inflamed colonic tissue from patients with ulcerative colitis contains abnormally high concentrations of the BK releasing enzyme, kallikrein,[38] and that plasma and tissue concentrations of the kinin degrading

enzyme, peptidyl-dipeptidase, are depressed in patients with Crohn's disease.[39] Furthermore, plasma concentrations of α_2-macroglobulin, which inhibits kallikrein, are low in patients with Crohn's disease.[40]

We would tentatively conclude, therefore, that the results of the present study offer evidence for the view that BK, but not 5-HT, adds to the formation of lipoxygenase products, such as LTB_4, by binding to specific receptors on the external surface of PMNs, thus promoting inflammation as well as diarrhoea in inflammatory bowel disease.

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