Pergamon

Biochemical Pharmacology, Vol. 48, No. 1, pp. 31–39, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0139-C

Ca^{2+} IONOPHORE A23187-STIMULATED SECRETION OF AZUROPHIL GRANULES IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES IS LARGELY MEDIATED BY ENDOGENOUSLY FORMED LEUKOTRIENE B_4

A. HATZELMANN,*† R. FRUCHTMANN,* K.-H. MOHRS,‡ S. RADDATZ‡ and R. MÜLLER-PEDDINGHAUS*§

Bayer AG, Pharma Research Center, *Institute for Cardiovascular and Arteriosclerosis Research, ‡Chemistry Science Laboratories, Wuppertal, F.R.G.

(*Received* 3 *September* 1993; *accepted* 18 *March* 1994)

Abstract—The mode of action of the new leukotriene synthesis inhibitor BAY X1005 ((*R*)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid) and structurally-related quinoline derivatives is reflected by the binding to a high-affinity binding site presumably identical to FLAP (five lipoxygenase activating protein). In addition to FLAP, we have identified a second BAY X1005 (low-affinity) binding site localized in the granule fraction of human PMNL (polymorphonuclear leukocytes). Based on the hypothesis that the corresponding target protein might be involved in the regulation of granule release, the influence of the leukotriene synthesis inhibitors BAY X1005 and MK-886 and the direct 5-LOX (5-lipoxygenase, EC 1.13.11.34) inhibitor A-64077 on the A23187- and fMLP (*N*-formyl-methionyl-leucyl-phenylalanin)-stimulated release of β-glucuronidase (as a marker for azurophil granules) and vitamin B_{12}-binding protein (as a marker for specific granules) was investigated. In contrast to MK-886, neither BAY X1005 nor A-64077 significantly affected fMLP-stimulated granule release. This was also true for the A23187-stimulated release of specific granules; however, under the same conditions the A23187-stimulated release of azurophil granules was almost totally inhibited by all three compounds. No obvious relationship between the corresponding IC_{50} values and the ability of these compounds to compete for BAY X1005 binding at the low-affinity binding site existed. Instead, by extending these studies to additional inhibitors, a correlation between the IC_{50} values for inhibition of A23187-stimulated (i) β-glucuronidase release and (ii) LTB_4 (leukotriene B_4) synthesis was found ($r = 0.969$, $N = 7$). This relationship was independent of the mode of action of the compounds, namely direct 5-LOX inhibition or indirect 5-LOX inhibition mediated via binding to FLAP. These results suggest that 5-LOX metabolites may be involved in A23187-stimulated azurophil granule release. Of the two main biologically active 5-LOX metabolites synthesized under these conditions (LTB_4 and 5-hydroxyeicosatetraenoic acid), only LTB_4 stimulated β-glucuronidase release to nearly the same extent as A23187. In addition, this metabolite significantly enhanced A23187-stimulated β-glucuronidase release, but only at A23187 concentrations (≥0.25 μmol/L) which by themselves were not sufficient to trigger LTB_4 formation. Moreover, the inhibition of A23187-stimulated β-glucuronidase release by BAY X1005 or A-64077 was totally reversed by the addition of LTB_4. Taken together, these results indicate that: (i) the BAY X1005 low-affinity binding site does not play a crucial role in the granule release process; (ii) compounds interfering with 5-LOX activity inhibit the release of azurophil granules upon A23187 stimulation; and (iii) this effect is largely due to the inhibition of endogenously synthesized LTB_4.

Key words: Ca^{2+} ionophore; degranulation; human polymorphonuclear leukocytes; leukotriene B_4; leukotriene synthesis inhibitors

The leukotrienes, which are synthesized along the 5-LOX‖ (EC 1.13.11.34) pathway of arachidonic acid metabolism, have attracted attention as mediators of several inflammatory and allergic conditions because of their various proinflammatory properties [1]. We have recently described the selective action of BAY X1005 ((*R*)-2-[4-quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid) on the formation of 5-LOX metabolites in various *in vitro* systems including human PMNL [2]. The inhibition of 5-LOX activity by BAY X1005 and other structurally-related quinoline derivatives has

† Present address: Byk Gulden Pharmaceuticals, Konstanz, F.R.G.

§ Corresponding author: Bayer AG, Pharma Research Center, Institute for Cardiovascular and Arteriosclerosis Research, P.O. Box 101709, D-42096 Wuppertal, F.R.G. Tel. (0202) 36-4090; FAX (0202) 36-8009.

‖Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanin; FLAP, five lipoxygenase activating protein; HETE, hydroxyeicosatetraenoic acid; LDH, lactate dehydrogenase; LOX, lipoxygenase; LTB_4, leukotriene B_4; PBS, Dulbecco's phosphate-buffered saline; PMNL, polymorphonuclear leukocytes.

been demonstrated to be linked to the binding of these compounds to a BAY X1005 high-affinity binding site located in the microsomal fraction of human PMNL; indeed, we have obtained several lines of evidence indicating that the corresponding target protein is identical to FLAP [3]. While the precise function of FLAP remains undefined, it is clear that this protein is necessary for leukotriene synthesis in intact cells [4]. Compounds acting via FLAP exemplified by the prototype MK-886 [5] have been termed "leukotriene synthesis inhibitors" so as to differentiate between the indirect inhibition of 5-LOX by such compounds and the direct inhibition of 5-LOX by compounds such as A-64077 [6] or AA-861 [7].

Besides the high-affinity binding site (FLAP) a second BAY X1005 binding site with an approx. 10-fold lower affinity for BAY X1005 was identified in the granule fraction of human PMNL [3] suggesting that the corresponding target protein might be involved in the regulation of the granule release process. The secretion of granule constituents represents one of the major functions of PMNL in the course of an acute inflammatory response, this in addition to its ability to synthesize and release reactive oxygen species as well as several inflammatory mediators including leukotrienes [8, 9].

The present study was undertaken to test the influence of BAY X1005 and various reference compounds on the release of β-glucuronidase and vitamin B_{12}-binding protein which served as marker proteins for azurophil and specific granules, respectively [10]. To this end, the Ca^{2+} ionophore A23187 and the tripeptide fMLP (*N*-formyl-methionyl-leucyl-phenylalanin) as a representative receptor agonist were chosen as stimuli to trigger degranulation.

MATERIALS AND METHODS

Chemicals

A23187, charcoal, cytochalasin B, dextran (molecular mass 500,000 Da), fMLP, HEPES, Triton X-100, vitamin B_{12} and vitamin B_{12}-deficient BSA were purchased from Sigma Chemie (Deisenhofen, F.R.G.). Ficoll paque was obtained from Pharmacia LKB (Freiburg, F.R.G.), PBS tablets either from Flow Laboratories (Meckenheim, F.R.G.) or Unipath (Wesel, F.R.G.), and LTB_4 and 5-HETE from Paesel + Lorei (Frankfurt, F.R.G.). [^{57}Co]-Cyanocobalamin (intermediate sp. act. 556 kBq/μg) was purchased from Amersham Buchler (Braunschweig, F.R.G.). All other chemicals utilized were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.).

The inhibitors used were synthesized at the Chemistry Department of Bayer AG (Wuppertal, F.R.G.).

Isolation of human PMNL

The isolation of human PMNL (anticoagulated with sodium citrate) via dextran sedimentation, centrifugation on ficoll paque and hypotonic lysis of red blood cells were performed essentially as described previously [11]. Cells were stored in PBS/HEPES (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, 10 mmol/L HEPES, pH 7.4) at 4°.

Degranulation experiments

Incubation conditions. All assays were performed in duplicate in PBS/HEPES supplemented with 1 mmol/L Ca^{2+}/Mg^{2+} at 30°. Aliquots (1 mL) of the cell suspension (1.5–2 × 10^7 cells/mL) were (unless otherwise indicated) preincubated with cytochalasin B (2.5 μg/mL) in the absence or presence of the compounds for 5 min and then stimulated by the addition of either A23187 (2 μmol/L, final concentration) or fMLP (1 μmol/L, final concentration) for a further 5 min. Thereafter, the cells were spun down by centrifugation for 15 sec at 15,000 *g* in an Eppendorf centrifuge, and the supernatant removed to measure the activity/content of the marker proteins (see below). Stock solutions from all compounds used were prepared in DMSO and from LTB_4 and 5-HETE in ethanol so that the final concentration of each solvent in the assays never exceeded 0.5% (v/v).

Marker proteins. The activities of LDH (No. DG1340-UV) and β-glucuronidase (No. 325) were determined using commercially available kits from Sigma Chemie. The tests were performed according to the manufacturer's instructions with some minor modifications. For measurement of LDH activity (at 30°) as a marker for cytosol 0.02 mL aliquots of the samples were mixed with 1 mL of the working reagent and the decrease of absorbance continuously recorded at 340 nm for approx. 8 min. LDH activity was calculated by the mean absorbance change per min. To measure β-glucuronidase activity 0.2 mL aliquots of the samples were mixed with 0.6 mL of acetate buffer solution and 0.2 mL of phenolphthalein glucuronic acid solution and incubated for 1 hr at 56°. After addition of 5 mL AMP (2-amino-2-methyl-1-propanol) buffer absorbance was read at 546 nm. Results are expressed as a percentage of the LDH- or β-glucuronidase-activity, respectively, obtained in a cell suspension treated with Triton X-100 (0.1%, final concentration) which served as 100% control.

For the determination of the amount of vitamin B_{12}-binding protein, 0.4 mL aliquots of the samples were mixed with 0.6 mL PBS containing 60–70 ng vitamin B_{12}, of which 20–30 ng were [^{57}Co]-cyanocobalamin. This mixture was allowed to stand for approx. 30 min at room temperature before addition of 1 mL PBS containing 2.5–5% charcoal and 0.5% vitamin B_{12}-deficient BSA. After centrifugation for 15 min at 800 *g*, 1.5 mL aliquots of the supernatants were counted for radioactivity using a Multi-Crystal-Counter (LB 2104, Berthold, Wildbad, F.R.G.). Results are expressed as percentage of a 100% control as mentioned above. Under these conditions the calibration curve was linear from 0 to 50% of control.

Inhibition of BAY X1005 binding

The inhibition of BAY X1005 binding by the determined compounds was performed essentially as described previously [3] except that the final concentration of [^{14}C]BAY X1005 was 2 μmol/L. K_d values for the binding of the compounds to the low-

affinity binding site were calculated indirectly from the IC_{50} values for inhibition of BAY X1005 binding and the K_d value of 3.5 μmol/L determined earlier for BAY X1005 [3] according to:

$$K_d\,(X) = IC_{50}(X) \times K_d\,(\text{BAY X1005})/IC_{50}(\text{BAY X1005}).$$

Arachidonic acid metabolism

Aliquots (0.85 mL) of the cell suspension (1.5×10^7 cells/mL in PBS/HEPES supplemented with 1 mmol/L Ca^{2+}/Mg^{2+}) were preincubated for 5 min at 30° and then stimulated by addition of A23187 for an additional 5 min. Both the extraction and reverse phase HPLC analysis of 5-LOX metabolites have been described in detail elsewhere [3]. The determination of IC_{50} values for the inhibition of LTB_4 synthesis in A23187-stimulated human PMNL have been performed as described previously [2].

Statistics

Results are expressed as mean ± SD (standard deviation) of the number of experiments (N) indicated. IC_{50} values were determined graphically or by computerized linear regression analysis of log-concentrations (mol/L) versus inhibition (%). Statistical analysis of the mean values of two data sets was performed according to the Student's paired *t*-test.

RESULTS

It is known that cytochalasin B via unknown mechanisms substantially enhances the release of granule constituents upon stimulation of human PMNL, an effect which is reflected in the data shown in Fig. 1. In the presence of 2.5 μg/mL cytochalasin B and through the use of either 2 μmol/L A23187 or 1 μmol/L fMLP as a stimulus, a sufficiently high release of both β-glucuronidase (as a marker for specific granules) and vitamin B_{12}-binding protein (as a marker for azurophil granules) into the supernatant of the cells was obtained to enable inhibition studies to be performed.

In initial experiments the influence of BAY X1005 on granule release in comparison to MK-886 and A-64077 was investigated. The results summarized in Fig. 2 show that, in contrast to MK-886, neither BAY X1005 nor A-64077 had a significant effect on fMLP-stimulated granule release. Similarly, A23187-stimulated release of the vitamin B_{12}-binding protein was only marginally affected by the latter two compounds. However, A23187-stimulated release of β-glucuronidase was almost totally inhibited by all three inhibitors. The inhibition of granule release was not due to unspecific (toxic) effects since none of the compounds affected the viability of the cells, measured by the release of cytosolic LDH into the supernatant of these cells, at the highest concentration (10 μmol/L) tested (data not shown).

Because of the significant effect of the compounds tested so far on A23187-stimulated β-glucuronidase release, we extended our studies on this parameter by testing additional compounds. Indeed, all tested compounds were able to inhibit A23187-stimulated β-glucuronidase release and the IC_{50} values obtained are summarized in Table 1. According to our working hypothesis the potency of the compounds to bind to the BAY X1005 low-affinity binding site was investigated. For this purpose, the cells were incubated in the presence of 2 μmol/L [^{14}C]BAY X1005. At this concentration ≥80% specifically bound BAY X1005 can be calculated to be bound to its low-affinity binding site using the binding constants determined earlier [3]. From the IC_{50} values

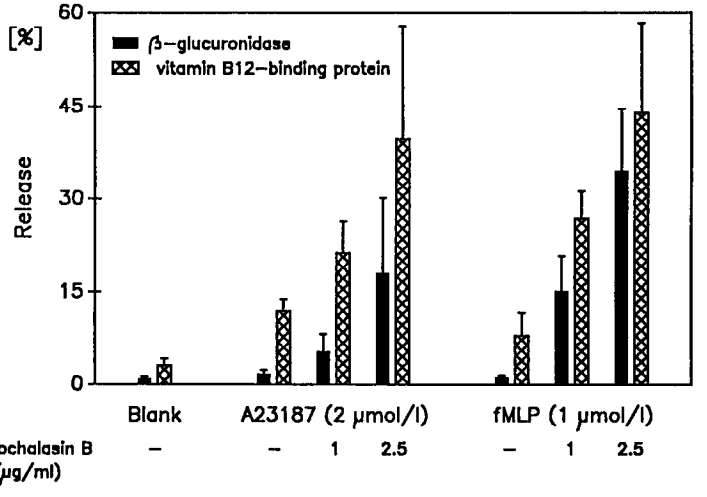

Fig. 1. Stimulation of azurophil (β-glucuronidase) and specific (vitamin B_{12}-binding protein) granule release by A23187 and fMLP in human PMNL. Human PMNL ($1.5–2 \times 10^7$ cells/mL) were preincubated in the absence or presence of the indicated concentrations of cytochalasin B for 5 min at 30°. After stimulation of the cells with A23187 (2 μmol/L) or fMLP (1 μmol/L) for an additional 5 min, the cells were spun down, and the supernatants removed in order to measure β-glucuronidase activity and vitamin B_{12}-binding protein content. Results are given as means ± SD from 5 to 11 independent experiments.

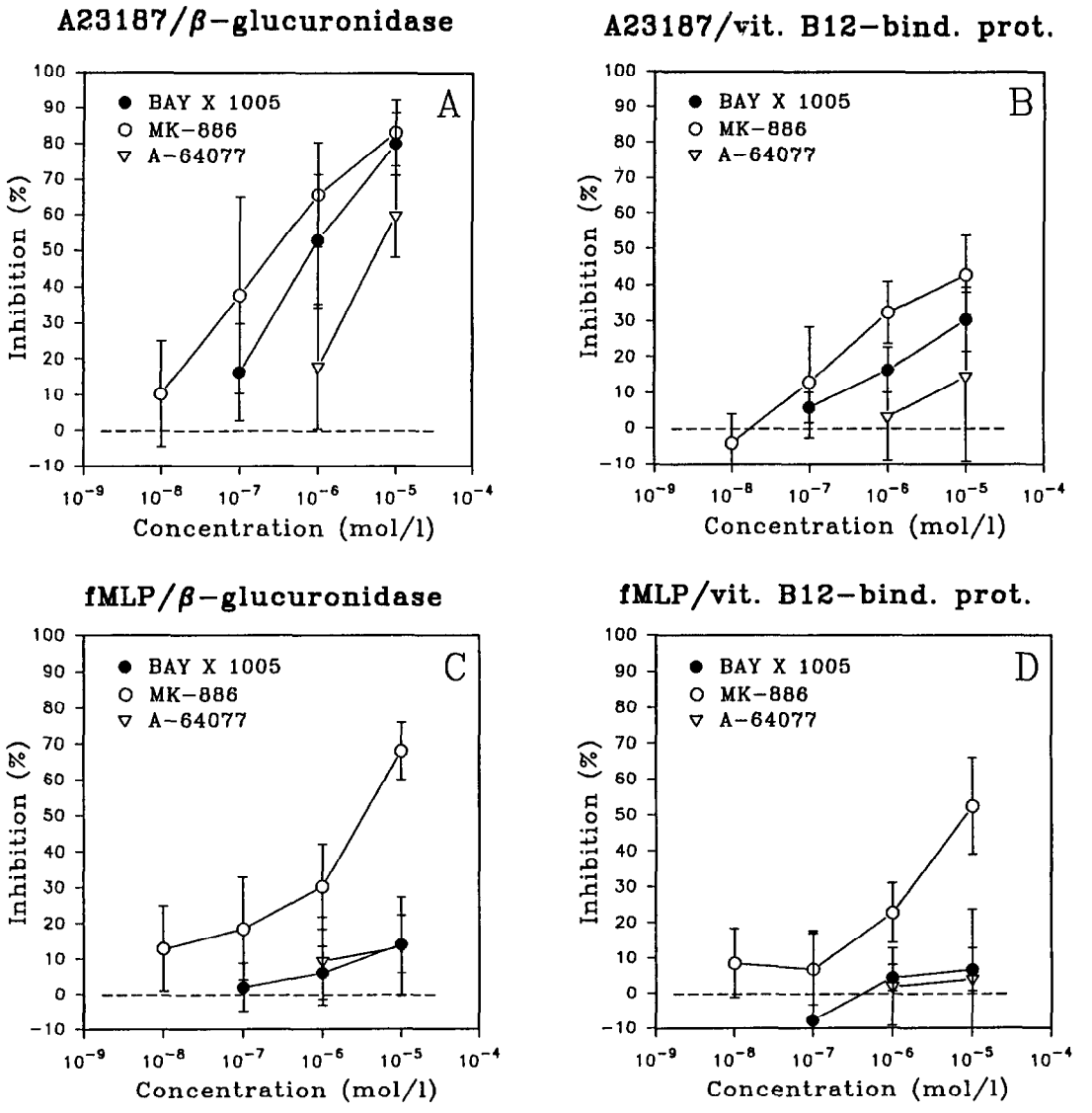

Fig. 2. Influence of BAY X1005, MK-886 and A-64077 on granule release in human PMNL. Human PMNL ($1.5–2 \times 10^7$ cells/mL) were preincubated in the presence of cytochalasin B (2.5 μg/mL) and the indicated concentrations of the compounds for 5 min at 30°. After stimulation of the assays with 2 μmol/L A23187 (A and B) or 1 μmol/L fMLP (C and D) for an additional 5 min, the cells were spun down and the supernatants removed in order to measure β-glucuronidase activity (A and C) or vitamin B_{12}-binding protein content (B and D). Results are given as means ± SD from four to eight independent experiments.

obtained for inhibition of BAY X1005 binding, K_d values were calculated as described in Materials and Methods. As shown in Table 1, a causal relationship between the binding of the compounds to the BAY X1005 low-affinity binding site and the inhibition of A23187-stimulated β-glucuronidase was possible in light of the results obtained for the four quinoline derivatives and MK-886. However, the direct 5-LOX inhibitors A-64077 and AA-861 also inhibited β-glucuronidase release. Since these two compounds did not interfere with BAY X1005 binding to the low-affinity binding site, the above-mentioned relationship appears to be unlikely.

However, the IC_{50} values of A-64077 and AA-861 for inhibition of β-glucuronidase release were quite similar to the IC_{50} values for inhibition of LTB_4 synthesis as a parameter for 5-LOX activity (Table 1). This raised the possibility that a link between these two parameters may exist; indeed, a high correlation ($r = 0.969$, $N = 7$) was obtained upon plotting corresponding IC_{50} values for all compounds investigated (Fig. 3).

It should be noted that, in contrast to the degranulation experiments, the influence of the compounds on either leukotriene synthesis or BAY X1005 binding were investigated in the absence of cytochalasin B. We have found, however, that cytochalasin B neither significantly influences BAY

Table 1. Comparison of the IC_{50} values for inhibition of A23187-stimulated β-glucuronidase release and LTB_4 synthesis, and the calculated K_d values for the binding to the BAY X1005 low-affinity binding site for various compounds in human PMNL

Compound	β-Glucuronidase release IC_{50} [μmol/L] (N)	BAY X 1005 low-affinity binding site K_d [μmol/L] (N)	LTB_4 synthesis IC_{50} [μmol/L] (N)
BAY X 1005	1.0 (5)	3.5 (3)	0.22 (7)
MK-886	0.33 (7)	0.63 (3)	0.09 (5)
A-64077	6.0 (4)	≥100* (3)	3.0 (5)
AA-861	0.94 (4)	≥10* (3)	0.24 (4)
1	1.0 (4)	NT	0.6 (5)
2	3.7 (4)	5.4 (3)	2.2 (3)
3	4.1 (4)	14.1 (3)	1.8 (3)

NT not tested; N, number of experiments; *Not soluble at higher concentrations.

The experimental conditions assessing the inhibition of A23187-stimulated β-glucuronidase release were identical to those described in the legend to Fig. 2. The inhibition of LTB_4 synthesis was determined under similar conditions but without cytochalasin B; LTB_4 was quantified by reverse phase HPLC analysis. The K_d values of the compounds for binding to the BAY X1005 low-affinity binding site were calculated from the inhibition of BAY X1005 binding (2 μmol/L) in PMNL suspensions (2×10^7 cells/mL) without cytochalasin B.

The enumerated compounds are: (1) *N*-(2-methylphenylsulphonyl)-*N'*-cyclopentyl-*N'*-4-(2-quinolylmethoxy) phenylurea; (2) *N*-methylsulphonyl-*N'*-cyclohexylmethyl-*N'*-4-(2-quinolylmethoxy)phenylurea; (3) 2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentylidenyl-acetic acid.

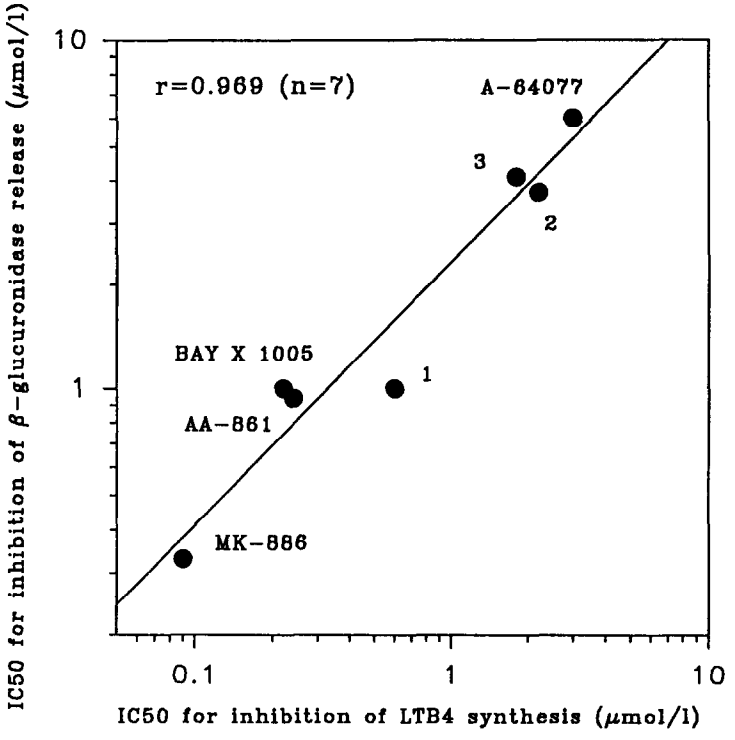

Fig. 3. Correlation between inhibition of A23187-stimulated LTB_4 synthesis and β-glucuronidase release by various compounds in human PMNL. Data are taken from Table 1.

X1005 binding nor A23187-stimulated leukotriene synthesis or the inhibition leukotriene synthesis by BAY X1005 (data not shown). These results suggest that cytochalasin B does not influence the comparison of the compound's effects with respect to the three parameters investigated.

To elucidate further the relationship between inhibition of LTB_4 synthesis and β-glucuronidase release, respectively, we sought to determine which 5-LOX-derived arachidonic acid metabolite(s) might be able to trigger azurophil granule release. Upon A23187 stimulation of human PMNL, LTB_4 and

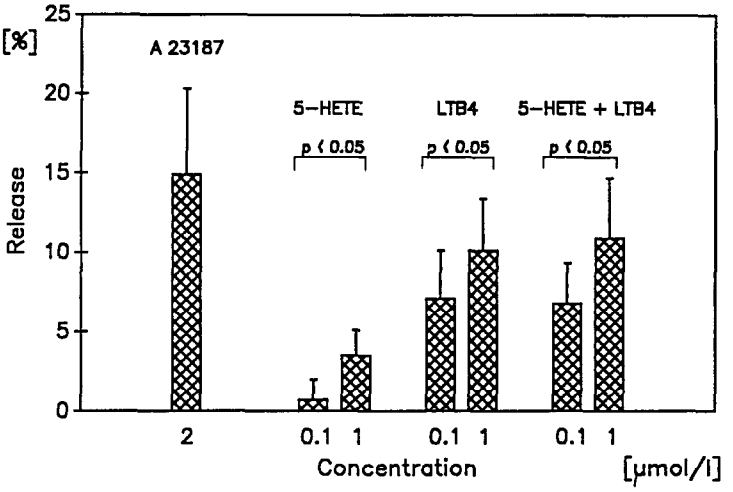

Fig. 4. Stimulation of β-glucuronidase release by A23187, 5-HETE or LTB_4 in human PMNL. Human PMNL ($1.5–2 \times 10^7$ cells/mL) were preincubated for 5 min at 30° in the presence of 2.5 µg/mL cytochalasin B. After stimulation of the cells for an additional 5 min with the indicated concentrations of A23187, 5-HETE, LTB_4 or a combination of 5-HETE plus LTB_4, the cells were spun down and the supernatants removed in order to measure β-glucuronidase activity. Results are given as means ± SD from four independent experiments. Statistical analysis was performed according to the paired *t*-test.

5-HETE are known to be the main biologically active 5-LOX metabolites, and Fig. 4 shows that LTB_4, but not 5-HETE, was able to stimulate β-glucuronidase release nearly to the same extent as A23187. 5-HETE-induced β-glucuronidase release was significantly lower ($P < 0.05$) than when induced by LTB_4. The concentrations of LTB_4 and 5-HETE chosen for these experiments were within the range of the amounts of these metabolites detected under conditions similar to those used for measuring β-glucuronidase release upon A23187 (2 µmol/L) stimulation (Table 2).

Furthermore, LTB_4 concentration-dependently enhanced A23187-stimulated β-glucuronidase release (Fig. 5) but only at A23187 concentrations (≥0.25 µmol/L) at which no significant amounts of 5-LOX metabolites were synthesized (Table 2).

Finally, the inhibition of A23187-stimulated β-glucuronidase release by both BAY X1005 and A-64077 could be totally reversed by addition of LTB_4 (Fig. 6).

DISCUSSION

This study was undertaken in an attempt to clarify the question of whether the putative target protein corresponding to the BAY X1005 low-affinity binding site [3] might be involved in the regulation of granule release in human PMNL.

Taking the receptor-agonist fMLP as a stimulus, neither BAY X1005 nor A-64077 up to a concentration of 10 µmol/L had a marked influence on the release of either β-glucuronidase or vitamin B_{12}-binding protein, respectively (Fig. 2C/D). The fact that in earlier experiments a K_d value of 3.5 µmol/L was determined for the binding of BAY

Table 2. A23187-stimulated formation of 5-LOX metabolites in human PMNL

	A23187 (2 µmol/L)	A23187 (0.5 µmol/L)	A23187 (0.25 µmol/L)
LTB_4	0.431 ± 0.080	0.279 ± 0.111	0.009 ± 0.016
5-HETE	1.381 ± 0.100	0.793 ± 0.291	ND
C_{20}-oxidized LTB_4	0.373 ± 0.068	0.217 ± 0.066	0.006 ± 0.006
6-*trans*-LTB_4 isomers	0.260 ± 0.022	0.112 ± 0.078	ND
Total 5-LOX metabolites	2.445 ± 0.175	1.401 ± 0.543	0.015 ± 0.022

Aliquots (0.85 mL) of human PMNL (1.5×10^7 cells/mL) were preincubated for 5 min at 30° before stimulation with the indicated concentrations of A23187 for an additional 5 min. After solid phase extraction, 5-LOX metabolites were analysed by reverse phase HPLC analysis. Values were calculated as µmol/L metabolites corresponding to a cell number of 1.5×10^7 cells/mL in the assays.

Results are given as means ± SD from three independent experiments. ND, not detectable.

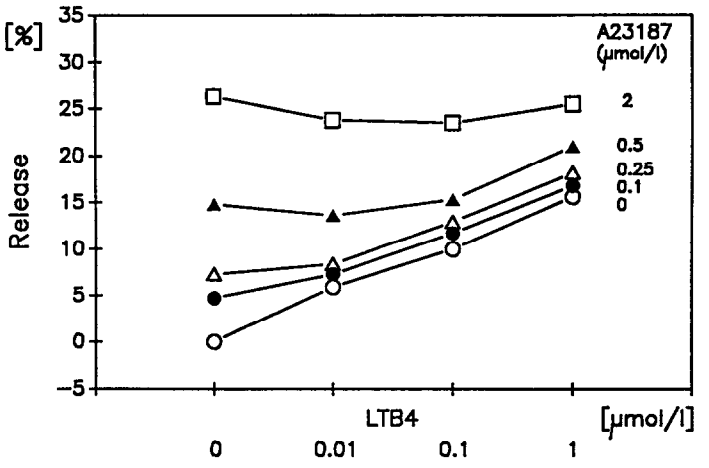

Fig. 5. Influence of LTB_4 on the A23187-stimulated β-glucuronidase release in human PMNL. Human PMNL ($1.5–2 \times 10^7$ cells/mL) were preincubated for 5 min at 30° in the presence of 2.5 μg/mL cytochalasin B. After stimulation of the cells with the indicated concentrations of LTB_4 and/or A23187 for an additional 5 min, the cells were spun down, and the supernatants removed in order to measure β-glucuronidase activity. Results are given as means from three independent experiments.

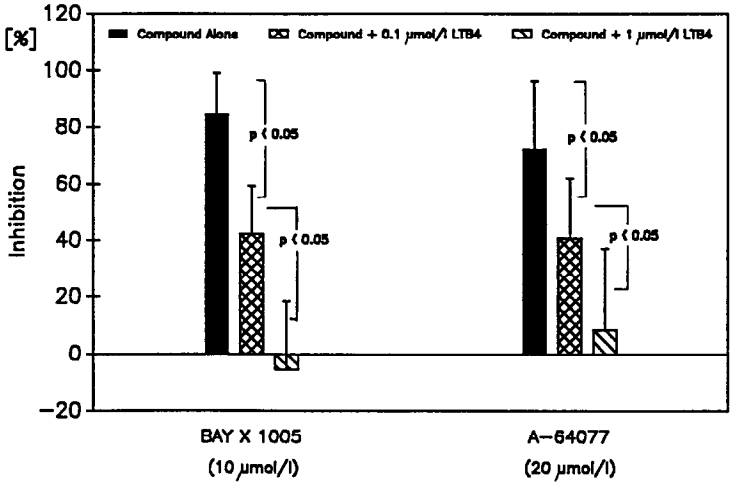

Fig. 6. Inhibition of A23187-stimulated β-glucuronidase release by BAY X1005 and A-64077 in human PMNL and its reversion by LTB_4. Human PMNL ($1.5–2 \times 10^7$ cells/mL) were preincubated for 5 min at 30° in the presence of 2.5 μg/mL cytochalasin B and the indicated concentrations of either BAY X1005 or A-64077. After stimulation of the cells for an additional 5 min with either A23187 (2 μmol/L) alone or in combination with 0.1 or 1 μmol/l LTB_4, respectively, the cells were spun down, and the supernatants removed to measure β-glucuronidase activity. Results are given as means ± SD from four independent experiments. Statistical analysis was performed according to the paired *t*-test.

X1005 to the low-affinity binding site [3] argues against an involvement of the putative target protein in the regulation of granule release at least under conditions of receptor–agonist stimulation. The reason why MK-886 at concentrations ≥1 μmol/L substantially inhibited the release of both azurophil and specific granules (Fig. 2C/D) has not been further investigated, but may be explained by the fact that MK-886 has been demonstrated to inhibit cAMP-phosphodiesterase enzymes *in vitro* [12]. Therefore, it is probable that MK-886 leads to elevated cAMP levels, known to inhibit receptor–agonist triggered events in human PMNL [13].

The use of specific inhibitors of leukotriene synthesis confirms the conclusion of Ozaki *et al.* [14] that the inhibitory effects of rather unspecific lipoxygenase inhibitors (e.g. nordihydroguaiaretic acid) on PMNL function cannot generally be attributed to inhibition of the 5-LOX pathway. This is also in agreement with the fact that soluble receptor–agonists such as fMLP are very weak stimuli for leukotriene formation in human PMNL [15] compared to A23187 (see below).

In contrast to the experiments discussed so far using fMLP to trigger granule release, A23187-stimulated β-glucuronidase release was almost totally

inhibited by all the compounds tested (Fig. 2A, Table 1). Yet, no obvious relationship between the IC_{50} values for inhibition of β-glucuronidase release and inhibition of BAY X1005 binding at the low-affinity binding site was found (Table 1). However, there is 4-fold evidence to suggest that the inhibition of A23187-stimulated β-glucuronidase release is largely due to the inhibition of endogenously synthesized LTB_4. Firstly, the IC_{50} values for inhibition of β-glucuronidase release correlated well ($r = 0.969$) with the IC_{50} values obtained for inhibition of LTB_4 synthesis (Fig. 3) independent of the mode of action of the compounds (direct 5-LOX inhibition as in the case of A-64077 and AA-861, and indirect 5-LOX inhibition mediated via binding to FLAP as in the case of MK-886, BAY X1005 and three other quinoline derivatives). Secondly, LTB_4 was found to stimulate β-glucuronidase release to virtually the same extent as A23187 (Fig. 4). Thirdly, LTB_4 substantially enhanced A23187-stimulated β-glucuronidase release (Fig. 5), but only at A23187 concentrations (≥0.25 μmol/L) which by themselves are not sufficient to trigger LTB_4 formation (Table 2). Fourthly, the inhibition of A23187-stimulated β-glucuronidase release by BAY X1005 or A-64077 could be totally reversed by the addition of LTB_4 (Fig. 6) which is in agreement with the fact that receptor–agonist-stimulated β-glucuronidase release (see fMLP) is largely insensitive to these compounds (Fig. 2C).

Since A23187-stimulated release of vitamin B_{12}-binding protein was much less affected by the compounds tested (Fig. 2B), one has to conclude that in contrast to azurophil granules the release of specific granules is less dependent on additional signal(s) provided by receptor activation beyond the Ca^{2+} signal provided by the Ca^{2+} ionophore A23187. Furthermore, studies using Ca^{2+} ionophores to link the extent of granule secretion to intracellular Ca^{2+} levels [16, 17] may lead to false interpretations since the experiments described in this paper clearly indicate that the stimulation of certain cell responses by Ca^{2+} ionophores may not always be the result of a direct Ca^{2+}-triggered event. Rather, the possibility should be considered that the stimulation of cells by Ca^{2+} ionophores is due to the intrinsic synthesis of mediators (e.g. LTB_4 as shown in this paper) acting in an autocrine fashion as secondary stimuli of the cells.

Taken together, the results obtained in this study did not corroborate the hypothesis that the putative target protein in the granule fraction of human PMNL corresponding to the BAY X1005 low-affinity binding site plays a crucial role in the regulation of lysosomal granule release. The importance of this binding site for any physiological response therefore awaits explanation. However, the results suggest that A23187-stimulated release of azurophil granules is largely mediated by the endogenous formation of the secondary mediator LTB_4 and compound effects can be explained by LTB_4 synthesis inhibition.

Acknowledgements—The authors thank Dr Fugmann (Bayer AG, Wuppertal, F.R.G.) for the synthesis of A-64077 and Dr Pleiss (Bayer AG, Wuppertal, F.R.G.) for the synthesis of [^{14}C]BAY X1005.

REFERENCES

1. Lewis RA, Austen KF and Soberman RJ, Leukotrienes and other products of the 5-lipoxygenase pathway—Biochemistry and relation to pathobiology in human diseases. *N Engl J Med* **323**: 645–655, 1990.
2. Fruchtmann R, Mohrs K-H, Hatzelmann A, Raddatz S, Fugmann B, Junge B, Müller-Peddinghaus R and Horstmann H, *In vitro* pharmacology of BAY X1005, a new inhibitor of leukotriene synthesis. *Agents Actions* **38**: 188–195, 1993.
3. Hatzelmann A, Fruchtmann R, Mohrs K-H, Raddatz S and Müller-Peddinghaus R, Mode of action of the new selective leukotriene synthesis inhibitor BAY X1005 ((*R*)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid) and structurally related compounds. *Biochem Pharmacol* **45**: 101–111, 1993.
4. Dixon RAF, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, Gillard JW and Miller DK, Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* **343**: 282–284, 1990.
5. Gillard J, Ford-Hutchinson AW, Chan C, Charleson S, Denis D, Foster A, Fortin R, Leger S, McFarlane CS, Morton H, Piechuta H, Riendeau D, Rouzer CA, Rockach J, Young R, MacIntyre DE, Peterson L, Bach T, Eiermann G, Hopple S, Humes J, Hupe L, Luell S, Metzger J, Meurer R, Miller DK, Opas E and Pacholok S, L-663,536 (MK-886) (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio -5- isopropylindol-2-yl]-2,2-dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. *Can J Physiol Pharmacol* **67**: 456–464, 1989.
6. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB and Brooks DW, 5-Lipoxygenase inhibitory activity of zileuton. *J Pharmacol Exp Ther* **256**: 929–937, 1991.
7. Yoshimoto T, Yokoyama C, Ochi K, Yamamoto S, Maki Y, Ashida Y, Terao S and Shiraishi M, 2,3,5-Trimethyl - 6 - (12 - hydroxy - 5, 10 - dodecadiynyl)-1,4 - benzoquinone (AA861), a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. *Biochim Biophys Acta* **713**: 470–473, 1982.
8. Johnson KJ, Varani J and Smolen JE, Neutrophil activation and function in health and disease. In: *Immunology Series* (Ed. Coffey RG), Vol. 57, pp. 1–46. Marcel Dekker, Inc., New York, 1992.
9. Parker CW, Neutrophil mechanisms. *Am Rev Respir Dis* **143**: S59–S60, 1991.
10. Borregaard N, The human neutrophil-function and dysfunction. *Eur J Haematol* **41**: 401–413, 1988.
11. Hatzelmann A and Ullrich V, Regulation of 5-lipoxygenase activity by the glutathione status in human polymorphonuclear leukocytes. *Eur J Biochem* **169**: 175–184, 1987.
12. Schmidt BH, Fruchtmann R, Hegemann L and Hatzelmann A, Allosteric inhibition of calmodulin-dependent cyclic nucleotide phosphodiesterase in vitro by the leukotriene synthesis inhibitor MK-886. *Naunyn-Schmiedeberg's Arch Pharmacol* **345** (Suppl. 2): 88, 1992.
13. Snyderman R, Smith CD and Verghese MW, Model for leukocyte regulation by chemoattractant receptors: roles of guanine nucleotide regulatory protein and polyphosphoinositide metabolism. *J Leukoc Biol* **40**: 785–800, 1986.
14. Ozaki Y, Ohashi T and Niwa Y, A comparative study on the effects of inhibitors of the lipoxygenase pathway on neutrophil function. *Biochem Pharmacol* **35**: 3481–3488, 1986.
15. Hatzelmann A, Haurand M and Ullrich V, Involvement of calcium in the thimerosal-stimulated formation of

leukotrienes by fMLP in human polymorphonuclear leukocytes. *Biochem Pharmacol* **39**: 559–567, 1990.
16. Lew PD, Monod A, Waldvogel FA, Dewald B, Baggiolini M and Pozzan T, Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. *J Cell Biol* **102**: 2197–2204, 1986.
17. Sengelov H, Kjeldsen L and Borregaard N, Control of exocytosis in early neutrophil activation. *J Immunol* **150**: 1535–1543, 1993.