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The cysteinyl-leukotriene receptor antagonist BAY u9773 is a competitive antagonist of leukotriene C_4 in the guinea-pig ileum

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Abstract

Two main classes of receptors exist for leukotrienes C_4, D_4 and E_4, collectively named cysteinyl-leukotrienes (CysLTs). The $CysLT_1$ receptor is blocked by currently available leukotriene antagonists, and the $CysLT_2$ receptor is defined by the absence of selective antagonists. The contractile response to leukotriene C_4 in guinea-pig ileum longitudinal muscle is resistant to $CysLT_1$ receptor antagonists. However, the leukotriene E_4 analogue BAY u9773 (6(*R*)-(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid) has recently been reported to inhibit $CysLT_2$ responses. Therefore BAY u9773 was evaluated for antagonism of the effect of leukotriene C_4 in the guinea-pig ileum longitudinal muscle. We found that BAY u9773 (0.3–10 μM) did not contract the preparation, but produced a concentration-dependent rightward shift in the concentration-response relation for leukotriene C_4. Schild plot analysis yielded a slope which was not significantly different from unity and a pA_2 value of 6.1. The inhibition of leukotriene C_4 by BAY u9773 was not altered by antagonism of $CysLT_1$ receptors by ICI 198,615 {[1-[[2-methoxy-4-[[(phenylsulfonyl)amino]carbonyl]-phenyl]methyl]-1H-indazol-6-yl]carbamic acid cyclopentyl ester} (100 nM). The $CysLT_1$ receptor agonist, leukotriene E_4 (1 μM), contracted the preparation but did not inhibit the contraction induced by leukotriene C_4. Taken together, the antagonism exerted by BAY u9773 appeared unrelated to actions on $CysLT_1$ receptors. In conclusion, BAY u9773 was a useful selective competitive antagonist of leukotriene C_4, and the findings support the classification of the receptors for leukotriene C_4 in the guinea-pig ileum as $CysLT_2$.

Keywords: Cysteinyl-leukotriene receptor antagonist; Guinea pig; Ileum; BAY u9773

1. Introduction

Activation of mast cells and other inflammatory cells leads to production of cysteinyl-leukotrienes (leukotrienes C_4, D_4 and E_4) from arachidonic acid by the 5-lipoxygenase pathway (Samuelsson et al., 1987). The cysteinyl-leukotrienes (CysLTs) are potent spasmogens and make up the biological entity previously described as SRS-A (slow reacting substance of anaphylaxis) (Samuelsson et al., 1987). There is new strong evidence in support of suggestions that pulmonary generation of cysteinyl-leukotrienes constitutes a final common event for the many different factors causing airway obstruction in asthmatics (Dahlén and Dahlén, 1995). Moreover, drugs which inhibit the formation or action of cysteinyl-leukotrienes have been found to exert potent anti-asthmatic effects. Such antileukotriene drugs are being introduced as a new therapy in asthma (Chung, 1995).

Receptor antagonists for cysteinyl-leukotrienes constitute one main class of antileukotrienes. Following the lead provided by the acetophenon compound FPL 55712 (Augstein et al., 1973), a great number of antagonists for cysteinyl-leukotrienes have been developed. The currently developed antagonists, termed $CysLT_1$ receptor antagonists (Coleman et al., 1995), generally inhibit the action of leukotriene D_4 and leukotriene E_4 on the guinea-pig trachea or ileum (Krell et al., 1983; Snyder and Krell, 1984; Gardiner et al., 1990). On the other hand, the effects of leukotriene C_4 and leukotriene D_4 on human pulmonary veins (Labat et al., 1992) or ferret spleen (Snyder and Krell, 1986; Gardiner et al., 1993), and the contractile effects of leukotriene C_4 on guinea-pig trachea (Snyder and Krell, 1984) have been found resistant to $CysLT_1$ receptor antagonists. The receptors for cysteinyl-leuko-

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trienes which are resistant to currently available $CysLT_1$ receptor antagonists have been designated $CysLT_2$ (Coleman et al., 1995). At present, however, there is no selective $CysLT_2$ receptor antagonist, making it possible that these receptors are a heterogeneous group. There are observations supporting the latter possibility (Tudhope et al., 1994).

Gardiner and colleagues recently reported that the leukotriene E_4 analogue, BAY u9773, had a broader antagonistic activity than other antagonists of cysteinyl-leukotrienes (Tudhope et al., 1994). In addition to functional and binding data supporting $CysLT_1$ antagonism, BAY u9773 was able to antagonise effects of leukotriene C_4 or leukotriene D_4 at purported $CysLT_2$-receptors in preparations such as guinea-pig trachea, ferret spleen and sheep bronchus. The antagonism by BAY u9773 was generally competitive, with pA_2 values ranging between 6.8 and 7.7. Consistent with such findings, BAY u9773 was also able to inhibit the effects of leukotriene C_4 and leukotriene D_4 on human pulmonary veins (Labat et al., 1992). In the latter preparation, however, the antagonistic properties of BAY u9773 were shared to some extent by leukotriene E_4, and both compounds had significant contractile activity.

The effect of leukotriene C_4 in the guinea-pig ileum is not antagonised by current $CysLT_1$ receptor antagonists (Gardiner et al., 1990). The aim of the study was to investigate whether the effect of leukotriene C_4 in the guinea-pig ileum could be described as a $CysLT_2$ response, according to the current classification, by investigating if BAY u9773 could inhibit the response to leukotriene C_4 in the guinea-pig ileum. Furthermore, the similarity in activity between BAY u9773 and leukotriene E_4 on human pulmonary veins led to the hypothesis that BAY u9773 is a partial agonist for certain cysteinyl-leukotriene responses. Therefore, the study included evaluation of whether or not BAY u9773 and leukotriene E_4 had similar effects on spontaneous tone and on the contraction induced by leukotriene C_4 in this preparation.

2. Materials and methods

2.1. Materials

Synthetic leukotrienes and BAY u9773 (6(*R*)-(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid) were from Cascade Biochemical (Reading, UK), ICI 198,615 {[1-[[2-methoxy-4-[[(phenylsulfonyl)amino]carbonyl]-phenyl]methyl]-1H-indazol-6-yl]-carbamic acid cyclopentyl ester} from Zeneca Pharmaceuticals (Macclesfield, UK), U-46619 (9,11-dideoxy-9α, 11a-methanoepoxy-Prostaglandin $F_{2\alpha}$) from Cayman Chemical (Ann Arbor, MI, USA). Histamine, *S*-hexyl-glutathione (*S*-hexyl GSH), L-cysteine and other standard reagents were all from Sigma (St. Louis, MO, USA).

2.2. Preparation

Guinea pigs (250–400 g body weight) of either sex were killed by a sharp blow to the head and bled. The terminal 30–40 cm of ileum was removed and put into Tyrode's solution (composition, mM: NaCl: 149.2; KCl: 2.7; $NaHCO_3$: 11.9; $CaCl_2$: 1.8 $MgCl_2$: 0.5; NaH_2PO_4: 0.4 and glucose: 5.5). Longitudinal muscle strips were prepared as described (Rang, 1964; Kosterlitz et al., 1970). Four longitudinal muscle strips were tied together with a cotton thread and cut into preparations of approximately 10 mm length. Six preparations were obtained from each animal.

2.3. Experimental protocol

The preparations were placed in 5 ml tissue baths containing Tyrode's solution gassed with 6.5% CO_2 in O_2 and kept at 37°C. Contractile responses were recorded on a Grass Model 7 Polygraph or on a Riken Denshi Speedex recorder SP-K4 via isometric Grass (Quincy, MA) FT03C or Experimetria (Budapest) FSG-01 force-displacement transducers. The resting tension of the preparation was kept at 5 mN throughout the experiment. The preparations were allowed to equilibrate for at least an hour before agonists were added. Unless otherwise stated, *S*-hexyl GSH (100 μM) and L-cysteine (5 mM) were added to Tyrode's solution after the equilibration period, in order to inhibit the conversion of leukotriene C_4 and leukotriene D_4, respectively (Örning and Hammarström, 1980; Sok et al., 1980).

The experiment started with non-cumulative addition of histamine (the final bath concentrations were 0.3 and 1.0 μM). Histamine was left in contact with the preparation until the peak contraction had been obtained (approximately 30 s), after which the bath fluid was changed twice. The two concentrations were added three to four times each in sequential order. If the response of the preparation to this first cycle of histamine challenges was more than 1 mN for the lower concentration and 2 mN for the higher concentration, the preparation was included in the study.

The experiment then continued with the administration of leukotriene C_4 (10 nM), the response to which was followed for 15 min or longer. After repeated changes of bath fluid and, if required, adjustment of baseline tension, a second identical cycle of histamine challenges was performed. In the experiments where the effects of purported antagonists on the concentration-response relation for leukotriene C_4 were evaluated, antagonist or solvent was added at the end of the second histamine cycle. Ten minutes later, a cumulative challenge with leukotriene C_4 (1–1000 nM) was performed. The concentration of leukotriene C_4 was normally increased every ten minutes, but if the contraction had reached its plateau earlier, the next concentration was given before the contraction started to fade. At least two out of six preparations from each

animal were used as controls. In some experiments, the effects of drugs were studied on the effect of cumulative challenge with histamine (0.1–10 μM), given at the same time of the experiment as when leukotriene C_4 was studied. The time interval between dose-increments was 2 min or less for histamine.

In some experiments, the influence of drugs or the conversion inhibitors was evaluated with respect to effects on the contractile response to single doses of leukotrienes or histamine, given when the second cycle of histamine challenges had been completed.

2.4. Data analysis

The responses to agonists were expressed as percentages of the contraction induced by a supramaximal concentration of histamine (100 μM) at the end of the experiment. For Schild plot analysis concentration-response curves were obtained in the absence and presence of different concentrations of antagonist (Arunlakshana and Schild, 1959). Concentration ratios (Jenkinson et al., 1995) were calculated at the EC_{25} level (25% of the maximal histamine response), which corresponds to the half-maximal value of the response produced by cysteinyl-leukotrienes in this particular experimental protocol, the leukotriene C_4 maximum being about 50% of the histamine maximum. The 95% confidence interval for the pA_2 value was calculated.

Statistical hypotheses were tested with Student's two-tailed *t*-test for unpaired or paired samples, and a *P* value less than 0.05 was considered to indicate a significant difference. Statistical evaluation was done with the PC program Sigma Stat for Windows version 1.0 (Jandel Scientific Software, San Rafael, CA, USA).

3. Results

3.1. Influence of the conversion inhibitors on the preparation

When the conversion inhibitors (see Section 2) *S*-hexyl GSH and L-cysteine were added to the preparations after the first cycle of histamine responses, there was frequently a contraction, amounting to less than 10% of the maximum (8.6 ± 7%, mean ± SD, $n = 33$). The contraction was however short-lasting and the preparations relaxed spontaneously to regain baseline tension within 5 min.

Repeated challenge with single concentrations of leukotriene C_4, leukotriene D_4 or histamine evoked responses of equal magnitude before and after addition of the conversion inhibitors (Table 1).

Prolonged (more than 5 h) exposure to the conversion inhibitors however appeared to cause a small but significant depression of tissue reactivity. Thus the maximum response to histamine was lower ($P < 0.05$) in preparations receiving the conversion inhibitors (14.3 ± 9.0 mN tension increase, mean ± S.D., $n = 95$) than in the controls (22.6 ± 11 mN, $n = 52$). Therefore, for the evaluation of drug effects on the response to leukotrienes, an experimental design with experiments of relatively short duration and cumulative challenge with leukotriene C_4 was selected (Fig. 1).

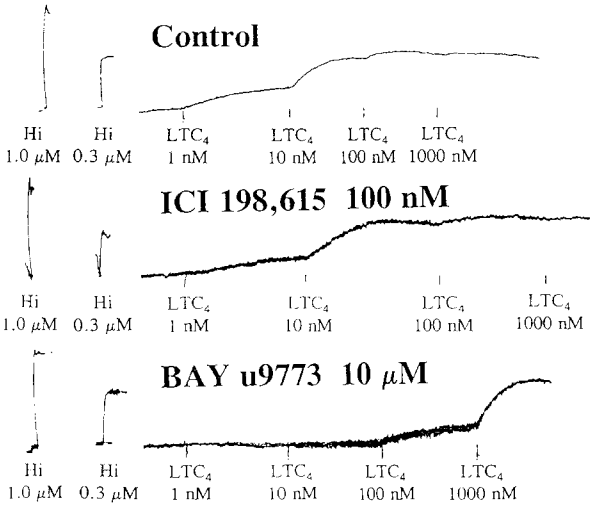

Fig. 1. Tracings from organ bath experiments with guinea-pig ileum longitudinal muscle. Cumulative challenge with leukotriene C_4 (LTC_4 1–1000 nM) in controls (top), after exposure to the conventional $CysLT_1$ receptor antagonist, ICI 198,615 (100 nM, middle), and the novel antagonist, BAY u9773 (10 μM, bottom). The contractile responses to single concentrations of histamine are indicated as reference. All experiments were conducted in the presence of the conversion inhibitors, *S*-hexyl GSH (100 μM) and L-cysteine (5 mM).

3.2. Effect of ICI 198,615 on leukotriene C_4 and leukotriene D_4

ICI 198,615 (100 nM) did not inhibit the leukotriene C_4 response in the presence of conversion inhibitors (Fig. 1), whereas the effect of leukotriene D_4 was effectively antagonised (not shown). A tenfold higher concentration of ICI 198,615 ($n = 6$) was also ineffective against leukotriene C_4 (not shown). These findings are consistent with previous observations (Gardiner et al., 1990).

Table 1
Responses to leukotriene C_4, leukotriene D_4 and histamine (means ± S.D. (n) [a]) before and after the administration of the conversion inhibitors, ShGSH and L-cysteine

	LTC_4 (10 nM)	LTD_4 (10 nM)	Hi (0.3 μM)	Hi (1.0 μM)
Before	18 ± 8 (4)	11 ± 7(4)	32 ± 22 (4)	77 ± 42 (4)
After	20 ± 6 (4) ns	11 ± 5 (4) ns	28 ± 12 (4) ns	61 ± 16 (4) ns

[a] For histamine n is the number of preparations, with means calculated for 3–6 observations on each tissue before and after the manipulation.

3.3. Effects of BAY u9773

3.3.1. Influence on the spontaneous tone of the preparation and on histamine responsiveness

BAY u9773 did not contract the preparation. The changes in tone during the pre-treatment period were insignificant and within the variations observed in preparations given solvent (Fig. 2a). The responsiveness of the preparations to histamine was not significantly affected by BAY u9773 (Fig. 2b).

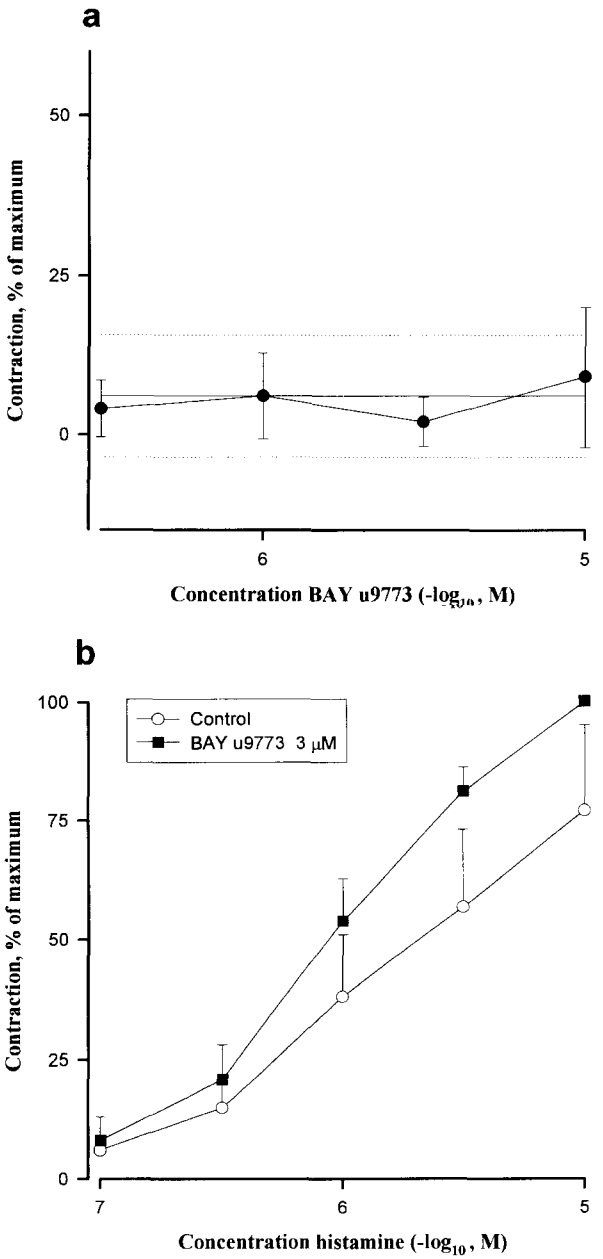

Fig. 2. Basal tone in guinea-pig ileum longitudinal muscle recorded during the ten minutes preceding challenge with leukotriene C_4 (a). BAY u9773 (filled circles) did not affect the basal tone. The solid line shows the mean spontaneous increase in tone for controls, with standard deviation indicated by dotted lines. Concentration–response curve for histamine in guinea-pig ileum longitudinal muscle (b). The contractile response to histamine (open circles) was not affected by BAY u9773 (3 μM, filled squares). Error bars were omitted when within symbol size.

Table 2
The effect of BAY u9773 on the maximum histamine response

	n	Mean of maximum (mN)	S.D.
Controls	18	7.6	4.6
BAY u9773 0.3 μM	6	14	9.0
BAY u9773 1.0 μM	6	7.2	2.3
BAY u9773 3.0 μM	6	12	8.3
BAY u9773 10 μM	7	8.6	3.8

3.3.2. Effect on maximal contraction

The maximum responses to histamine in the different preparations were not related to the concentration of BAY u9773 administered in the experiment (Table 2). Likewise, if all preparations given BAY u9773 (in different doses) were compared with the control group, there was no significant difference between maximal responses in the two groups (7.6 ± 4.6 mN; $n = 18$ versus 10 ± 6.6 mN; $n = 25$; $P > 0.05$)

3.3.3. Effect of BAY u9773 on leukotriene C_4

BAY u9773 was evaluated over the concentration range 0.3–10 μM against cumulative challenge with leukotriene C_4 (1–1000 nM). BAY u9773 was found to produce a concentration-dependent rightward shift in the concentration-response-relation for leukotriene C_4 (Fig. 3a). With 0.3 μM of BAY u9773, no significant inhibition was observed, but with the concentration increased to 1.0 μM, there was a significant inhibition surmounted at a leukotriene C_4 concentration of 100 nM. With BAY u9773 at concentrations of 3 and 10 μM, the inhibition was surmounted at a leukotriene C_4 concentration of 1 μM. Schild plot analysis yielded a slope which was not significantly different from unity ($r^2 = 0.99$) and the pA_2 value was 6.1 (95% C.I.: 5.9–6.3) (Fig. 3b).

3.3.4. Effect of ICI 198,615 on the antagonism of leukotriene C_4 by BAY u9773

Pre-treatment with the $CysLT_1$ receptor antagonist, ICI 198,615 (100 nM), did not affect the inhibition of the leukotriene C_4 response by BAY u9773 (10 μM) (Table 3).

Table 3
The influence of the $CysLT_1$ receptor antagonist, ICI 198,615, on the antagonism of the leukotriene C_4-response by BAY u9773, expressed as percent of maximum (mean ± S.D.)

	LTC_4 10 nM	LTC_4 100 nM	LTC_4 1000 nM
BAY u9773 10 μM	3.3 ± 1.2	14 ± 2.5	35 ± 8.5
ICI 198,615 100 nM + BAY u9773 10 mM	2.0 ± 1.0 ns	9.7 ± 2.1 ns	25 ± 5.5 ns

3.4. Effect of leukotriene E_4 on leukotriene C_4

Leukotriene E_4 itself caused a concentration-related contraction of the preparation. The responses to the various concentrations of leukotriene E_4, expressed as % of the maximum response to histamine, were 10 ± 7.7%, 14 ± 7.2% and 31 ± 11% for 30, 100 and 1000 nM respectively. The response to leukotriene C_4 was increased progressively in preparations precontracted with leukotriene E_4, suggesting an additive effect (Fig. 4).

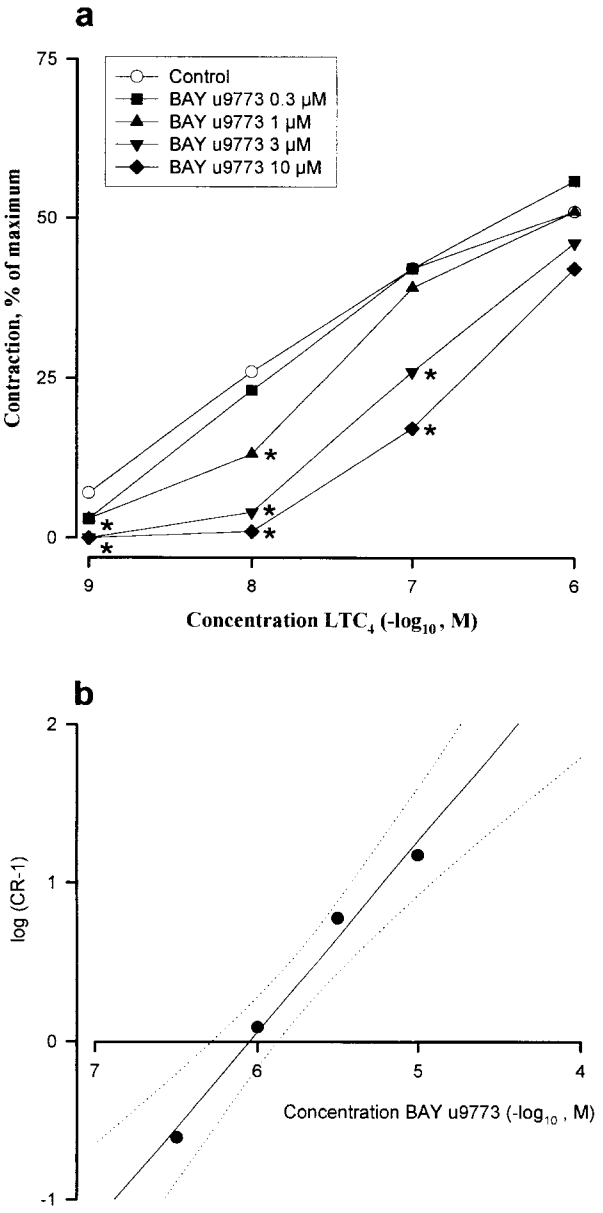

Fig. 3. (a) The concentration-response curve for leukotriene C_4 in guinea-pig ileum longitudinal muscle was inhibited in a concentration-dependent fashion by BAY u9773 (0.3 μM, filled squares; 1 μM, filled triangles pointing upwards; 3 μM, filled triangles pointing downwards; 10 μM, filled diamonds). Error bars were omitted for clarity. Stars indicate a significant difference from controls ($P < 0.05$) at the indicated concentration. (b) The linear Schild plot yielded a slope not significantly different from unity ($r^2 = 0.99$) and a pA_2 value of 6.1 (95% C.I. 5.9–6.3).

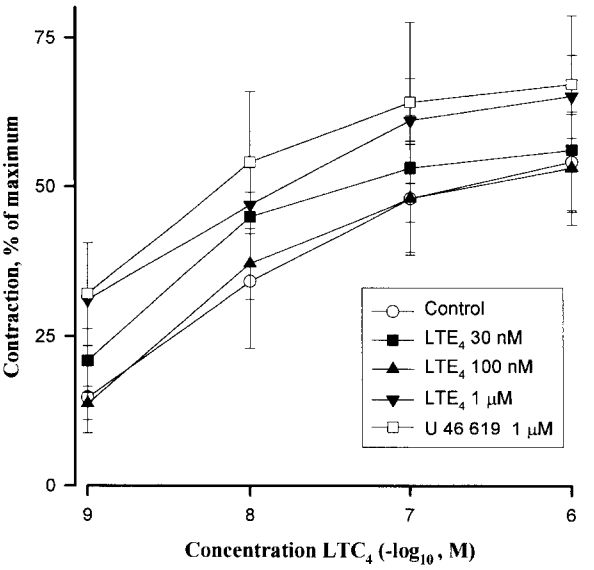

Fig. 4. Concentration–response curves for leukotriene C_4 in guinea-pig ileum longitudinal muscle after pre-treatment with leukotriene E_4 (30 nM, filled squares; 100 nM, filled triangles pointing upwards; 1 μM, filled triangles pointing downwards) or U-46619 (1 μM, open squares).

3.5. U-46619

Since leukotriene E_4 produced a precontraction, the influence of precontraction with another agent on the response to cumulative challenge with leukotriene C_4 was evaluated. The thromboxane (TP) receptor agonist, U-46619, was used, and the concentration (1 μM) was selected so as to produce a precontraction comparable to that obtained with the highest dose of leukotriene E_4 (Fig. 4). The response to leukotriene C_4 was increased by U-46619 in an additive fashion, similar to when the preparation was precontracted with leukotriene E_4 (Fig. 4).

4. Discussion

We found that BAY u9773 competitively inhibited the contractile response to leukotriene C_4. The effect was selective for the contraction induced by leukotriene C_4, and there was no depression of the reactivity to subthreshold or supramaximal concentrations of histamine. The pA_2 value for the antagonism of leukotriene C_4 by BAY u9773 was 6.1 (5.9–6.3) in our study. In other tissues, the pA_2 value for BAY u9773 has been reported to be somewhat higher (6.8–7.7) (Tudhope et al., 1994). We have not investigated the effect of BAY u9773 in tissues other than the guinea-pig ileum, and it remains to establish whether significantly different values are observed when different tissues are studied with identical methods. Nevertheless, the findings of a linear Schild plot for BAY u9773 against

leukotriene C_4 strongly supports the notion that BAY u9773 is a competitive antagonist at $CysLT_2$ receptors in the guinea-pig ileum.

The structural similarities between leukotriene E_4 and BAY u9773 raise the possibility that BAY u9773 activates the $CysLT_1$-receptor. It has been reported that leukotriene E_4 (Gardiner et al., 1990) or a $CysLT_1$-agonist, MDL 28,753 (Gieske et al., 1990) may inhibit the response to leukotriene C_4. Thus, activation of the $CysLT_1$-receptor could theoretically lead to inhibition of the leukotriene C_4 response, perhaps, at least for leukotriene E_4, by partial agonism. However, the $CysLT_1$ receptor antagonist, ICI 198,615, did not affect the inhibition of the contractile response to leukotriene C_4 caused by BAY u9773. This indicates that the mechanism of inhibition was not related to agonism at the $CysLT_1$ receptor. This conclusion is also supported by the fact that BAY u9773 is devoid of spasmogenic activity at the concentrations where competitive antagonism of leukotriene C_4 was demonstrated.

We found no evidence that leukotriene E_4 (1 μM) inhibited the effect of leukotriene C_4, whereas other investigators had reported that leukotriene E_4 inhibits the response to leukotriene C_4 in the guinea-pig ileum (Gardiner et al., 1990). There are several factors which make it difficult to compare their findings with ours. For example, they used a tenfold higher concentration of leukotriene E_4 (10 μM), and determined the concentration–response curve with leukotriene C_4 at a later time-point (30 min), when the initial, almost maximal, response to leukotriene E_4 had faded. Furthermore, whole segments of ileum were used, as opposed to the longitudinal muscle in the present study, and isotonic contractions were followed in the presence of indomethacin. In addition, the effect observed with leukotriene E_4 against leukotriene C_4 was relatively marginal compared to the shift in the leukotriene D_4 concentration–response curve obtained in the same study. Prolonged exposure to leukotriene E_4 may cause desensitisation of $CysLT_2$-receptors. However, our present findings show that, when BAY u9773 and leukotriene E_4 were compared under identical conditions (10 min preincubation), their effects on the contractile response to leukotriene C_4 were opposite. Leukotriene E_4, as well as the thromboxane receptor agonist, U-46619, produced a contraction which was added to the effect of leukotriene C_4. On the other hand, BAY u9773 caused concentration-dependent antagonism of leukotriene C_4. The conclusion that agonism of $CysLT_1$ receptors is unrelated to the mechanism of the antagonism exerted by BAY u9773 is further supported by a previous observation. Namely, the $CysLT_1$ receptor agonist MDL 28,753 inhibited leukotriene C_4-induced contractions in the ileum only in the presence of a $CysLT_1$ receptor antagonist (Gieske et al., 1990). However, the mode of action of MDL 28,753 was not further investigated.

The conversion inhibitors, *S*-hexyl GSH and L-cysteine, were used in order to inhibit the metabolism of leukotriene C_4 and leukotriene D_4, respectively. In previous studies, the gamma-glutamyl transferase inhibitor, L-serine borate, has been found to exert unspecific contractile effects (Gardiner et al., 1990). The inhibitors selected for this study caused a small and transient contraction only on the first addition to the preparations. There was no acute effect on the responses to histamine, leukotriene C_4 or leukotriene D_4, indicating that there was no major general change in reactivity of the smooth muscle. With time (hours) however there was a small but significant depression of the maximum response to histamine at the end of the experiments. Therefore, a short experimental protocol was chosen for the present study of BAY u9773. Nevertheless, compared with previous protocols using L-cysteine and L-serine borate, the conversion inhibitors we now used represent an improved strategy with fewer unspecific effects. Finally, in this context, since the response to leukotriene C_4 was not significantly altered by the use of conversion inhibitors, we conclude that the spasmogenic properties of leukotriene C_4 in this preparation under the present experimental conditions, were not caused by its metabolites to any great extent. The same conclusion has been reached by other investigators who used older types of conversion inhibitors and similar, if not identical, experimental conditions (Krilis et al., 1983; Gardiner et al., 1990; Gieske et al., 1990).

In conclusion, to the best of our knowledge, BAY u9773 is to date the only compound which has been shown to provide competitive antagonism of leukotriene C_4 in the guinea-pig ileum in the absence of $CysLT_1$ receptor antagonists. The inhibition by BAY u9773 was unlikely to be explainable in terms of partial agonism at $CysLT_1$ or $CysLT_2$ receptors. As proposed earlier, (Tudhope et al., 1994), the compound, BAY u9773, should be a useful tool for further investigations of receptor subtypes for cysteinyl-leukotrienes. Although currently available $CysLT_1$ receptor antagonists are effective in the symptomatic treatment of asthma (Chung, 1995), many effects of cysteinyl-leukotrienes in man are quite resistant to this class of antagonists, for example the wheal and flare response to leukotriene D_4 in human skin (Dahlén et al., 1994) and the effects of leukotriene C_4 and leukotriene D_4 on pulmonary vessels (Labat et al., 1992). Therefore, further development of this field requires intensified delineation of the nature of different leukotriene receptors. The contractile effect of leukotriene C_4 in the guinea-pig ileum in the presence of conversion inhibitors appears to be a suitable functional assay for further exploration of $CysLT_2$ receptors.

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