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Zeneca ZD7114 acts as an antagonist at β_3-adrenoceptors in rat isolated ileum

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1 The relaxant effects of Zeneca ZD7114, BRL37344 (putative β_3-adrenoceptor agonists) and various phenylethylamine-based agonists were studied in isolated ileum of the rat where tone was increased with carbachol (0.5 μM). Agonist-induced relaxation was measured under equilibrium conditions with α-, β_1- and β_2-adrenoceptors inhibited.

2 Relaxant responses were obtained to isoprenaline, noradrenaline, and BRL37344, although, the efficacy of this latter agent was significantly lower than that of isoprenaline. Salbutamol caused weak relaxation (<20%) at high concentrations (10 μM) and ZD7114 was without significant relaxant effect even at high concentrations (10 μM).

3 Relaxant responses to isoprenaline and BRL37344 were weakly antagonized by high concentrations of (±)-propranolol (10 and 100 μM) yielding pK_B values of 5.7 with isoprenaline as the agonist and 5.5 with BRL37344 as the agonist.

4 The non-selective β-adrenoceptor antagonist, (±)-alprenolol (1–100 μM) caused competitive antagonism of the relaxant responses to isoprenaline (pA_2 value = 6.5). A similar pK_B value was obtained when BRL37344 was used as the agonist (6.4).

5 Relaxant effects of isoprenaline and BRL37344 were also antagonized by ZD7114 (1–100 μM) yielding pA_2 and pK_B values of 6.3 and 6.7 respectively.

6 The low potencies of (±)-propranolol and (±)-alprenolol as antagonists of the relaxant responses to isoprenaline and BRL37344 indicate that both the agonists and antagonists employed in the current study may interact with β_3-adrenoceptors in the rat isolated ileum. Contrary to the previous findings in guinea-pig ileum, where BRL37344 and ZD7114 were full agonists, in the current study, BRL37344 was a partial agonist and ZD7114 an antagonist at the β_3-adrenoceptor in rat ileum.

Keywords: Rat isolated ileum; β_3-adrenoceptor; Zeneca ZD7114; BRL37344

Introduction

Atypical or β_3-adrenoceptors have been identified in rat adipocytes (Arch *et al.*, 1984) and a variety of gastrointestinal smooth muscle preparations including guinea-pig and rat ileum (Bond & Clarke, 1987; 1988; van der Vliet *et al.*, 1990). Similar receptors have been implicated in the neuronal activation of the myenteric plexus of the guinea-pig (Taneja & Clarke, 1991). These sites can be differentiated from the classical β-adrenoceptors by the use of propranolol which exhibits low affinity for β_3-adrenoceptors. Moreover, agents such as BRL37344 and ZD7114 have been shown to have a high degree of selectivity for the β_3-adrenoceptor (Arch *et al.*, 1984; Holloway *et al.*, 1991a,b). These latter agents cause an inhibition of histamine-raised tone in guinea-pig ileum and are similar in potency and efficacy to the classical β-adrenoceptor agonist, isoprenaline (Growcott *et al.*, 1993). However, other workers have noted that BRL37344 may not always produce a full agonist response when compared to isoprenaline. For example, in human adipocytes, BRL37344 was found to be significantly less efficacious than isoprenaline in causing lipolysis whereas, in rat adipocytes, each agent possessed similar efficacy (Hollenga *et al.*, 1990). Further, in rat jejunum, where effects on resting tone were measured, BRL37344 produced a significantly lower maximum response when compared to isoprenaline (van der Vliet *et al.*, 1990). Although the authors gave no explanation for these differences in efficacy, similar observations have been previously noted with other β-adrenoceptor agonists such as, prenalterol (Kenakin & Beek, 1980). These authors addressed the issue by demonstrating that the intrinsic activity of prenalterol was determined, in part, by the receptor reserve of the tissue under study, i.e. in guinea-pig trachea, prenalterol was a full agonist, whereas in guinea-pig extensor digitorum, the agent possessed no agonist activity whatsoever and could act as an antagonist. Hence, receptor reserve could determine the final effect of a putative agonist – where reserve was high, full agonism would be observed, where the reserve was low, partial agonism or antagonism would be observed.

We have extended this argument to the current study in an attempt to establish the identity and function of the adrenoceptor present on the smooth muscle of rat isolated ileum. Using ZD7114, BRL37344 and a range of other agents, we have attempted to determine whether this site is different from that previously characterized in guinea-pig isolated ileum (Bond & Clarke, 1987; 1988; Growcott *et al.*, 1993).

A preliminary account of this work was presented at the meeting of the British Pharmacological Society, London, September, 1992.

Methods

Tissue preparation

Alderley Park rats (200–300 g) of either sex were killed by a blow to the head and cervical dislocation. The terminal 10 cm of ileum was removed and placed in Krebs solution. Segments (2–3 cm in length) were cut and the lumen washed through with Krebs solution to remove any traces of food. The segments were set up in 10 ml organ baths under a resting tension of 0.5 g in Krebs solution, maintained at 37°C and gassed with 95% O_2:5% CO_2. The composition (mM) of the Krebs solution was as follows: NaCl 118.9, $NaHCO_3$ 25.0, KCl 4.9, KH_2PO_4 1.2, glucose 11.1, $CaCl_2$ 2.5 and ascorbic acid 0.11. CGP20712A ((±)-1-[2-(3-carbamoyl-4-hydr-

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oxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluromethyl-2-imidazolyl)-phenoxy]-2-propanol), ICI 118551 (erythro-(±)-1-(7-methyindan-4-yloxy)-3-isopropylaminobutan-2-ol) and prazosin (all 0.1 μM) were present throughout the experiments to inhibit β_1-, β_2- and α_1-adrenoceptors respectively. Tissues were allowed to equilibrate for at least 30 min before any experimental procedures were begun.

Agonist activity

The relaxant effects of agonists were determined by measuring the inhibition of carbachol (0.5 μM)-induced tone by addition of the agonists. This concentration of carbachol was found to produce between 50–70% of the maximal achievable tone (data not shown). Adrenoceptor agonists were added cumulatively until a maximal relaxant effect was observed. Responses to agonists were then repeated 60 min later to check for any changes in tissue sensitivity.

Activity was expressed as an IC_{50} value, i.e. the concentration of agonist which produced a relaxant response that was 50% of the maximal effect.

Antagonist activity

Antagonist effects on the relaxant actions of the adrenoceptor agonists were assessed by using paired tissues, i.e. one tissue where the concentration-response curve to the agonist was constructed in the absence of antagonist and the other tissue where the same procedure was carried out in the presence of the antagonist. This procedure helped to assess the contribution of other factors such as tachyphylaxis. The equilibration time for the antagonists was 30 min.

Antagonist activity was determined by comparing the IC_{50} value obtained from the agonist-response curve in the absence of antagonist to that obtained in the presence of antagonist and calculated as a concentration-ratio. In some cases, a plot of log (agonist concentration-ratio −1) versus log [antagonist] was constructed. If the slope of the regression line was not significantly different from unity, then the antagonism was considered to be competitive (Arunlakshana & Schild, 1959). Where single concentrations of antagonist were used or where the antagonist effect was shown to be non-competitive, pK_B values were derived from the equation: pK_B = log(antagonist concentration ratio −1) −log[antagonist], according to the method of Furchgott (1972).

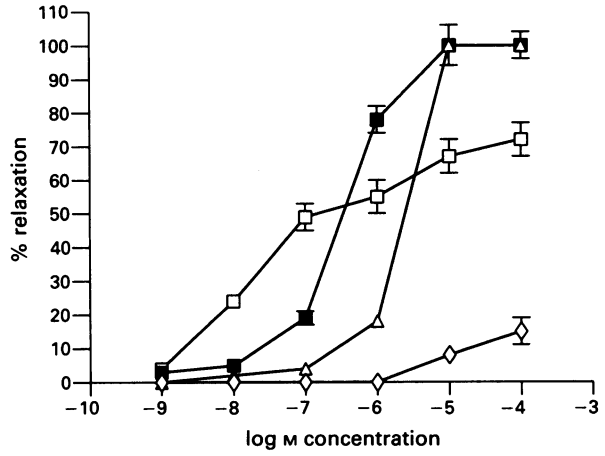

Figure 1 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves for isoprenaline (■, $n = 81$), BRL37344 (□, $n = 50$), noradrenaline (Δ, $n = 4$) and salbutamol (◇, $n = 4$) in the presence of CGP20712A, ICI 118551 and prazosin (all 0.1 μM). Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size. ZD7114 was without significant relaxant activity up to 10 μM.

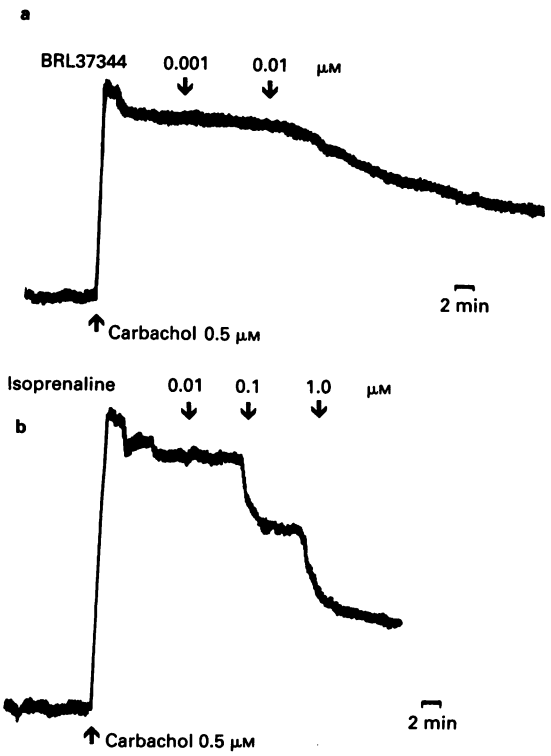

Figure 2 Typical traces showing the effects of (a) BRL37344 and (b) isoprenaline on carbachol (0.5 μM)-induced tone in the isolated ileum of the rat. Prazosin, CGP20712A and ICI 118551 (all 0.1 μM) were present throughout. Arrows indicate where agonists were added. Tone measurements were recorded isotonically.

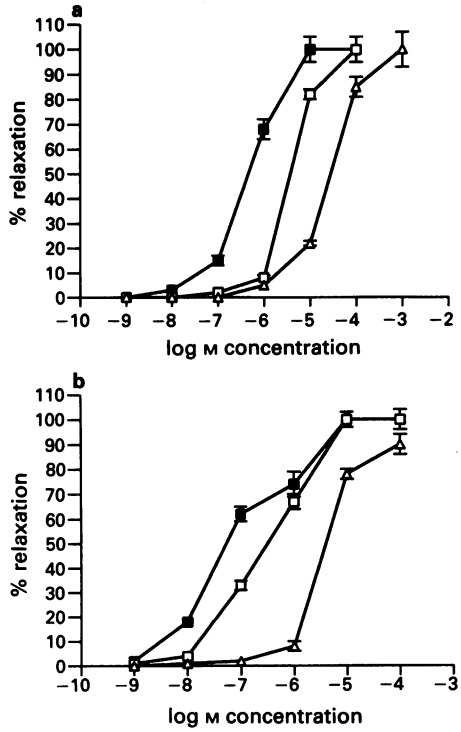

Figure 3 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 8$ and 9 respectively) and presence of propranolol 10 μM (□, $n = 4$) and 100 μM (Δ, $n = 4$) respectively. CGP 20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

Statistics

Results throughout are expressed as geometric mean values together with their respective 95% confidence limits. The numbers of rats used, n, given in parentheses. Slopes of the regression lines are given as 95% confidence limits. Statistical significance was determined using Student's t test where $P<0.05$ was considered to be significant.

Drugs used

The following drugs were used in the current study: (−)-isoprenaline hydrochloride (Sigma), (−)-noradrenaline bitartarate (Sigma), salbutamol hemisulphate (Sigma), carbachol chloride (Sigma) (±)-propranolol (Zeneca), (±)-alprenolol (Zeneca), CGP 20712A (CIBA-Geigy), ICI 118551 (Zeneca), ZD7114 ((S)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy]-N-(2-methoxyethyl)-phenoxyacetamide) and BRL37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylaminopropyl]phenoxy-acetate sesquijudrate) (both synthesized by B Rao, Research Department, Zeneca Pharmaceuticals, Alderley, Park Macclesfield). (±)-Alprenolol, ZD7114, BRL37344 were initially dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in distilled water. All other agents were dissolved in distilled water.

Results

Agonist activity

Isoprenaline, noradrenaline, and BRL37344 all caused graded relaxation of the tissues where tone had been raised with carbachol. Salbutamol produced a relaxation only at concentrations above 10 μM (i.e. between 10 and 20% of the isoprenaline maximum response) and ZD7114 was without any significant relaxant effect up to 10 μM. BRL37344 (EC_{50} 0.05 μM [0.004–0.9 μM], $n = 50$), was nine times more potent than isoprenaline (IC_{50} 0.45 μM [0.18–1.3 μM], $n = 81$) and 47 times more potent than noradrenaline (IC_{50} 2.3 μM [2.0–2.8 μM], $n = 4$). However, the maximum response obtained with BRL37344 (i.e. 71.4 ± 4.5%) was significantly ($P<0.05$) lower than that achieved with isoprenaline. Data are illustrated in Figure 1. The response profiles obtained with BRL 37344 and isoprenaline were quite different. BRL37344 produced a response that was slow in onset and continued to develop slowly, taking around 25 min to reach a plateau (Figure 2a). Isoprenaline, on the other hand produced a relatively rapid reduction in tone, responses reaching a plateau in about 5 min (Figure 2b).

It was noted that tachyphylaxis was present with the adrenoceptor agonists when concentration-response curves were repeated in the same tissues at hourly intervals (data not shown). Hence for antagonist studies (next section), paired tissue segments were used, allowing for one concentration curve per tissue.

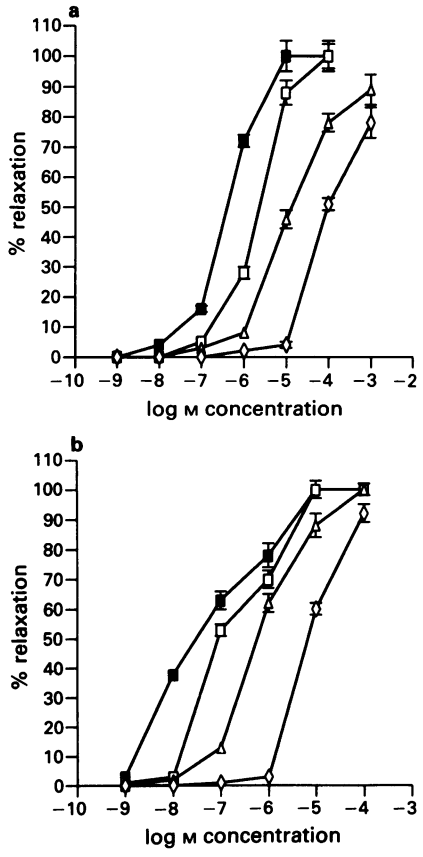

Figure 4 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 12$) and presence of alprenolol 1 μM (□, $n = 4$), 10 μM (△, $n = 4$) and 100 μM (◇, $n = 4$). CGP20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

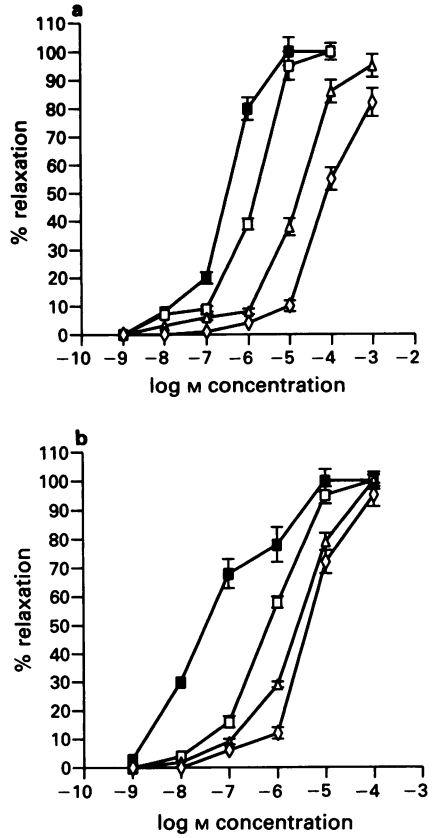

Figure 5 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 12$) and presence of ZD7114 1 μM (□, $n = 4$),10 μM (△, $n = 4$) and 100 μM (◇, $n = 4$). CGP20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

Antagonist activity

(±)-Propranolol (10 and 100 μM) weakly antagonized the relaxant reponses to both isoprenaline and BRL37344 (Figure 3a and b). pK_B values derived from these concentrations

of propranolol were 5.7 [5.3–6.4, $n=8$] and 5.5 [4.5–5.9, $n=9$] versus isoprenaline and BRL37344 respectively. These were not significantly different from each other. (±)-Alprenolol (1–100 μM) competitively antagonized the relaxant responses to isoprenaline (Figure 4a). The Arunlakshana-Schild plot of the data revealed a pA_2 of 6.5 [5.9–6.9, $n=12$, Figure 6a], the slope of the regression line not being significantly different from unity (0.9 [0.8–1.0]). When BRL 37344 was used as the agonist, its relaxant responses were also antagonized by (±)-alprenolol (1–100 μM, Figure 4b). However, the antagonist effect did not appear to be competitive as the slope of the Arunlakshana-Schild plot was significantly different from unity (0.5 [0.1–0.8], $P<0.05$). The pK_B value derived from these concentrations was 6.4 [5.8– 6.9, $n=12$], which was not significantly different from that obtained with (±)-alprenolol versus isoprenaline.

As ZD7114 was without significant agonist activity, it was tested as a potential antagonist of the relaxant responses to isoprenaline and BRL37344. When isoprenaline was used as the agonist, ZD7114 (1–100 μM) caused competitive antagonism of the relaxant responses (Figure 5a). An Arunlakshana-Schild plot of the data revealed a pA_2 value of 6.3 [5.9–6.8, $n=12$, Figure 6b), the slope of the regression line not being significantly different from unity (1.1 [0.8–1.2]). However, when BRL37344 was used as the agonist, the antagonist effect of ZD7114 (1–100 μM) did not appear to be competitive (Figure 5b) as the slope of the Arunlakshana-Schild plot was significantly different from unity (0.6 [0.5–0.6], $P<0.05$). The pK_B value derived from these concentrations of ZD7114 was 6.7 [5.9–7.4, $n=12$]. This value was not significantly different from that obtained with ZD7114 when isoprenaline was used as the agonist.

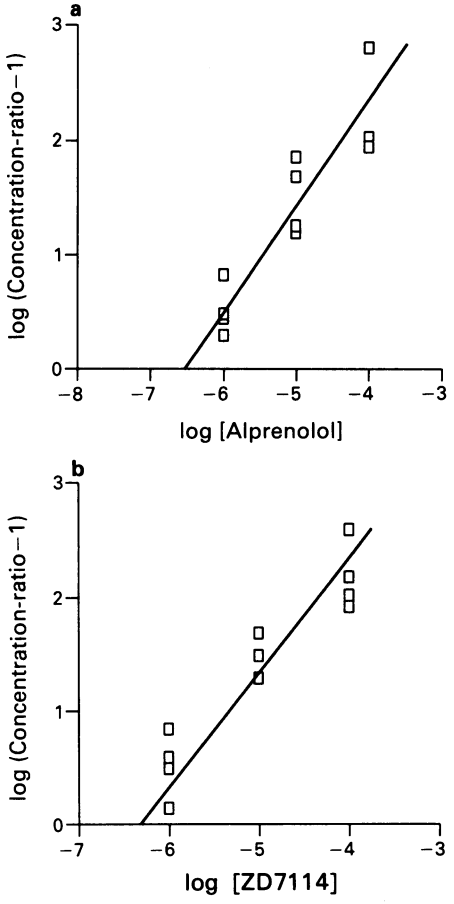

Figure 6 Arunlakshana-Schild plots of the data from (a) antagonism of isoprenaline by alprenolol, 1–100 μM (concentration-responses shown in Figure 4a), and (b) antagonism of isoprenaline by ZD7114, 1–100 μM (concentration-responses shown in Figure 5a).

Discussion

We have previously demonstrated that ZD7114 and BRL 37344 act at atypical or β_3-adrenoceptors in the guinea-pig (Growcott *et al.*, 1993). In this preparation, both agents possessed similar efficacy to isoprenaline, although both agents were less potent. In the current study, BRL37344 was the most potent of the agonists tested, although its efficacy was significantly lower than that of isoprenaline. ZD7114, on the other hand, was without any significant agonist activity. The reduced efficacy of BRL37344 compared to isoprenaline has been noted by other workers who have used the compound to evaluate potential β_3-adrenoceptor agonist activity in, for example, rat jejunum (van der Vliet *et al.*, 1990), human white adipocytes (Hollenga *et al.*, 1990) and colon (McLaughlin *et al.*, 1991). It was also interesting to note the difference in agonist profiles between isoprenaline and BRL 37344. Isoprenaline produced a fast relaxation whereas the response to BRL37344 was slow in onset and duration, the concentration responses covering some five log units. Although no direct reference to the speed of action of BRL 37344 was made by McLaughlin & MacDonald (1990), these workers did comment on the ability of this agent to produce profound tachyphylaxis in rat colon. Indeed, a close analogue of BRL37344, (BRL35135), has also been reported to produce tachyphylaxis in guinea-pig gastric fundus (Coleman *et al.*, 1987). The latter workers pointed out that neither isoprenaline nor salbutamol produced tachyphylaxis. The tendency for the BRL37344 compound to produce tachyphylaxis could possibly be explained by its slow onset and duration of action. Indeed, it has been suggested (Arch, 1989) that the bulky N-substituent of the BRL compounds could bind to accessory sites (possibly unrelated to the β-adrenoceptor). This might, in turn, mean that the rate at which these compounds access the β_3-adrenoceptor would be much slower than the classical ligands such as isoprenaline and noradrenaline. It was also noted that, with BRL37344 in the current studies, the concentration-responses appeared to be of a biphasic nature, suggesting that BRL37344 was interacting with more than one site. It seems unlikely that BRL 37344 would have interacted with more than one population of adrenoceptor as high concentrations of selective antagonists were employed in order to prevent this. Perhaps the postulated interaction with the accessory binding site (Arch, 1989) can again account for this or the compound may interact with some other mechanism which remains unknown. The answer to this question remains to be resolved.

The low efficacy of ZD7114 has also been noted in other parts of the rat gastrointestinal tract, i.e. distal colon (MacDonald & Lamont, 1992) and in human colon (Growcott, unpublished observations). Although speculation has been raised as to the homogeneity of atypical adrenoceptors (Bianchetti & Manara, 1990), it would seem inappropriate to apply that argument in the current study. It could be argued that differences in agonist efficacy result from differences in receptor coupling, i.e. in tissues where BRL37344 and ZD 7114 act as full agonists, receptor-coupling mechanisms are highly efficient, whereas in tissues where the efficacy of these agents was low, the efficiency of the receptor-coupling mechanisms was also low. If this were the case then it would be predicted that with an agonist possessing high intrinsic efficacy, such as isoprenaline, in a tissue with an efficient receptor coupling mechanism, e.g. guinea-pig ileum, this agent would be significantly more potent than in a tissue where receptor coupling efficiency was low (rat tissue, for example). Partial agonists would appear to lose efficacy as coupling efficiency declined (Kenakin, 1984). However, this argument cannot be the sole explanation for the observations made in the current study. Isoprenaline possessed full intrinsic efficacy in both guinea-pig (Growcott *et al.*, 1993) and rat ileum (present study). Indeed, there was no significant difference between the potency obtained with this agent on guinea-pig tissue when compared to that obtained on rat

tissue (IC_{50} values 0.50 and 0.45 μM respectively). However, both BRL37344 and ZD7114, compounds that possessed equal intrinsic activities when compared to isoprenaline in guinea-pig ileum, exhibited lower or no measurable intrinsic activities in rat ileum. Indeed, the latter compound exhibited antagonist activity in the rat ileum. An alternative explanation for these differences could be that guinea-pig and rat tissues contain differing levels of β_3-adrenoceptor reserves: in guinea-pig this is high, whereas in the rat, these levels are low. Hence, the efficacy of an agent at the β_3-adrenoceptor would be, in part, dependent upon the level of β_3-adrenoceptor reserve. Indeed, it appears that in the rat ileum, the β_3-adrenoceptor reserve is sufficiently low to allow ZD7114 to act as a competitive β_3-adrenoceptor antagonist (current study and MacDonald & Lamont, 1992) and for BRL37344 to act as a partial agonist. Indeed, in the absence of a suitable alkylating agent or irreversible antagonist to demonstrate receptor reserve and, hence alter the stimulus-response characteristics of tissues, it has been reported that functional antagonism of agonist responses can produce effects on the concentration-responses that resemble the effects observed with alkylating agents (Kenakin, 1984). Thus, functional antagonism can be achieved by addition of an agent, which through an effect at some other receptor, opposes the action of the primary stimulus. Using this technique in guinea-pig ileum, we have been able to demonstrate that ZD7114 can act as an antagonist of the responses to isoprenaline (Growcott *et al.*, 1992).

It is highly probable that the adrenoceptors involved in the responses to the agents used in the current study were of the atypical or β_3-subtype. The affinities of propranolol and alprenolol, as antagonists of the responses to both isoprenaline and BRL37344, in the current study, were comparable to those reported by other workers, i.e. pA_2 values of <7.0. These are significantly lower than would be expected for effects of these agents at classical β-adrenoceptors (i.e. pA_2 values in excess of 8.0). However, it is interesting to note that some workers find that responses to either isoprenaline and/or BRL37344 are completely insensitive to blockade with propranolol (Blue *et al.*, 1990), whereas others find that the actions of these two compounds can be blocked (McLaughlin & MacDonald, 1990). The affinity of propranolol in these latter studies, was however, lower than that obtained at classical adrenoceptors. In fact, the range of affinities expressed for propranolol at the atypical β-adrenoceptor is shared by another non-selective antagonist, alprenolol. The data obtained with this agent in the current study (i.e. pA_2 value of 6.5 versus isoprenaline) was significantly lower than would be expected for effects at classical β-adrenoceptors (i.e. pA_2 typically 8.6–9.0). This is in good agreement with other workers, e.g. Blue *et al.* (1990) using guinea-pig ileum (pA_2 value of 6.5 versus isoprenaline) but different from that obtained by van der Vliet *et al.* (1990) using rat jejunum (pA_2 value of 7.5 versus isoprenaline) and Bianchetti & Manara (1990) using rat colon (pA_2 value of 7.6 versus isoprenaline). A possible explanation for these apparent 'discrepancies' could be that in the studies where 'high' affinities have been obtained for propranolol or alprenolol a heterogeneous population of β-adrenoceptors exists and the affinities of these antagonists is an overestimate, including their effects at β_1-, β_2- and β_3-adrenoceptors. Interestingly, the pK_B value obtained for the antagonist effect of ZD7114 versus isoprenaline in the current study (6.33) was lower than that obtained for the same agent in the study of MacDonald & Lamont (1992), i.e. 7.3. In this latter study, it is not clear whether selective antagonists were present throughout the study to eliminate any possible contribution from classical adrenoceptors. Hence a direct comparison is not possible.

In the current study, the antagonist effects of propranolol, alprenolol and ZD7114 versus BRL37344 did not appear to be of a competitive nature. A possible explanation for this effect could be that because the responses elicited by BRL 37344 were composed of effects of the agent at more than one site (see above). This would lead to a non-linear Schild plot with an 'aggregate' slope less than unity (Kenakin, 1984). A further possibility could be that at the higher concentrations of BRL37344 'breakthrough' of the antagonists cocktail occurred and the resultant effect was due to an interaction with more than one type of adrenoceptor.

Whether the data generated in the animal tissues can be directly related to a human situation is, at present, difficult to answer. It would appear from comparison of the genomic sequences that there are only minor differences between the β_3-adrenoceptor in man, mouse and rat (Granneman *et al.*, 1992). Even so, there appear to be significant differences in the functional activity of recombinant rat and human β_3-adrenoceptors, when expressed in Chinese hamster ovary cells (Liggett, 1992). These differences could account, at least in part, for the results obtained in functional studies using isolated tissues which have implied marked discrepancies between human and animal β_3-adrenoceptors. However, differences in receptor density may also account for some of the inter-species differences seen in studies with isolated tissues. Only when agonists with high efficacy at the β_3-adrenoceptor are developed will some of these arguments be resolved.

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