Aryl propanolamines: comparison of activity at human β_3 receptors, rat β_3 receptors and rat atrial receptors mediating tachycardia

*¹Marlene L. Cohen, ¹William Bloomquist, ¹Aidas Kriauciunas, ¹Anthony Shuker & ¹David Calligaro

¹Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Drop Code 0520, Indianapolis, Indiana, 46285 U.S.A.

1 The *in vitro* activity of four aryl propanolamines was compared to two prototypic β_3 receptor agonists, CGP 12177 and CL316243 at the human β_3 receptor, the rat β_3 receptor in the stomach fundus and receptors mediating atrial tachycardia.

2 L-739,574 was the most potent (EC_{50} = 9 nM) and selective agonist at the human β_3 receptor with high maximal response (74% of the maximal response to isoproterenol).

3 A phenol-biaryl ether analogue possessed modest affinity for the human β_3 receptor (EC_{50} = 246 nM), but was highly efficacious with a maximal response 82% of the maximal response to isoproterenol. The other derivatives were intermediate in potency with low maximal responses.

4 These agonists at the human β_3 receptor did not activate the rat β_3 receptor in the rat stomach fundus. In fact, the aryl propanolamines (10^{-6} M) inhibited CL316243-induced activation of the rat β_3 receptor. Thus, agonist activity at the human β_3 receptor translated into antagonist activity at the rat β_3 receptor.

5 L739,574 and the phenol biaryl ether increased heart rate *via* β_1 receptors.

6 Although CGP12177 produced atrial tachycardia, neither the indole sulphonamide nor biphenyl biaryl ether did, although both had high affinity for the human β_3 receptor. Thus, the atrial tachycardic receptor was not identical to the human β_3 receptor.

7 These studies (a) characterized four aryl propanolamines with high affinity at the human β_3 receptor, (b) found that they were antagonists at the rat β_3 receptor, an observation with profound implications for *in vivo* rat data, and (c) established that the rodent atrial non- β_1 , β_2 or β_3 tachycardic receptor was also unrelated to the human β_3 receptor.

Keywords: Rat atria tachycardia; rat stomach fundus; propanolamines; human β_3 receptor; 'atypical' β receptor

Abbreviations: Beta, β ; beats per minute, BPM; cyclic adenosine monophosphate, cyclic AMP; Chinese hamster ovary, CHO; [S-4-(3-((1-*t*-butyl)amino)-2-hydroxypropoxy)-1,3-dihydro-2H-benzimidazol-2-one], CGP12177; Disodium [(*R,R*)-5-(2-((2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl)-1,3-benzodioxole-2,2-dicarboxylate], CL1316243; S-N-(4-(2-((2-hydroxy-3-(4-hydroxyphenyloxy)-propyl)amino)ethyl)phenyl) benzenesulfonamide, L-739,574

Introduction

Although activation of β_1 receptors is known to increase heart rate, evidence has been accumulating to suggest that some β_3 receptor agonists including the 'nonconventional partial agonist' CGP12177 may produce a chronotropic effect in rat atria that is mediated by a non β_1 , β_2 or β_3 rodent receptor (Kaumann, 1996; Malinkowski & Schicker, 1996; Kaumann & Molenaar, 1996). In those studies, CGP12177, which has high affinity for the human β_3 receptor increased rat atrial rate whereas CL316243, a ligand with high affinity for the rat, but not human β_3 receptor, did not increase heart rate. The fact that marked differences have been reported between the pharmacology of the rat and human β_3 receptor prompted the present study to identify pharmacological tools to use to examine whether the tachycardic receptor was identical to the human β_3 receptor.

For this reason, four aryl propanolamines (Figure 1) with high affinity for the human β_3 receptor (Dow, 1997) have been synthesized to determine whether all agonists with high affinity for the human β_3 receptor will increase heart rate. Before dissecting the effect of these aryl propanolamines on atrial rate, we characterized their activity at the human β_3 receptor using

the stably expressed human β_3 receptor and at β_1 , β_2 and rat β_3 receptors. Lastly, we determined the effect of these β_3 receptor agonists on atrial rate in an effort to understand whether activation of the human β_3 receptor was linked to atrial tachycardia.

Methods

Adenylyl cyclase activation at cloned human β_3 receptor The two exon human β_3 adrenergic receptor (Granneman *et al.*, 1993) was subcloned into the BCI restriction site using a phd expression vector (Grinnell *et al.*, 1987) before transfection into the DXB-11, Chinese hamster ovary (CHO) cell line by calcium phosphate precipitation methodology. The stably transfected cells were grown to 95% confluency in 95% Dulbecco's modified Eagles Medium (DMEM), 5% foetal bovine serum, 0.01% proline. Media was removed and the cells were washed with phosphate buffered (pH 7.4) saline (without magnesium and calcium). Cells were then lifted using an enzyme free cell dissociation solution (Specialty Media, Lavallete, NJ, U.S.A.) and pelleted by centrifugation. The cells were resuspended and added (15,000 per-well) to a 96-well plate. Cells were incubated at 37°C with receptor selective compounds for 20 min in buffer (Hank's balanced salt solution, 10 mM HEPES, 0.1%

* Author for correspondence.

Figure 1 Chemical structures of aryl propanolamines.

BSA, 1 mM L-ascorbic acid, 0.2% dimethyl sulphoxide, 1 mM 3-isobutyl-1-methylxanthine, pH 7.4). After halting the incubation with quench buffer (50 mM Na Acetate, 0.25% Triton X-100, pH 5.8), the cyclic AMP level was quantified by scintillation proximity assay (SPA) using a modification of the commercially available cyclic AMP kit (Amersham, Arlington Heights, IL, U.S.A.) with rabbit anti-cyclic AMP antibody (ICN Biomedicals, Aurora, OH, U.S.A.).

Sigmoidal dose response curves, from the whole cell receptor coupled cyclic AMP assay were fit to a four parameter logistic equation using non linear regression: $y = (a - d) / (1 + (\text{Dose}/c)^b) + d$ where a and d are responses at zero and maximal dose, b is the slope factor and c is the EC_{50} as previously described (DeLean *et al.*, 1978). EC_{50} was assessed as the concentration producing 50% of the maximum response to each agonist. The % maximal response was assessed relative to each agonist by the maximal response to isoproterenol by dividing the maximal response to isoproterenol $\times 100$.

Beta1 and beta2 receptor binding

[125]iodocyanopindolol binding to either human β_1 - or β_2 -adrenergic receptors was performed using transfected Sf9 cells (NEN Life Science Products, Inc., Boston MA, U.S.A.) according to the methods of Bylund & Snyder (1976). Nonspecific binding was determined in the presence of 1 μ M propranolol. Compounds were dissolved in dimethyl sulphoxide (DMSO), with final DMSO concentrations of less than 1%. Affinity constants (K_i) were determined using nonlinear least-squares regression software, Graphpad Inplot Inc., San Diego, CA, U.S.A. (Munso & Rodbard, 1980) and were calculated from IC_{50} values according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973). Affinities (K_i) were determined from eleven point concentration curves and were the means $\pm$ s.e. mean of at least three experiments.

In vitro rat stomach fundus relaxation

Male Sprague-Dawley rats (250–300 g; Harlan Sprague Dawley, Indianapolis, U.S.A.) were killed by cervical dislocation. The stomach fundus was removed and dissected free of connective tissue and two longitudinal preparations were obtained from each animal. The tissues were tied with thread at each end, the lower end being tied to a stationary metal hook and the upper end to a force transducer.

Tissues were mounted in organ baths containing 10 ml of modified Krebs' solution of the following composition (mM): NaCl 118.2, KCl 4.6, $CaCl_2 \cdot 2H_2O$ 1.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 10.0 and $NaHCO_3$ 24.8. Tissue bath solutions were maintained at 37°C and aerated with 95% O_2 -5% CO_2 . Propranolol (3×10^{-7} M) was added to the tissue baths to block β_1 and β_2 receptors without marked inhibition of β_3 receptors (Cohen *et al.*, 1995a). Tissues were placed under optimal resting force of 4 g and were allowed to equilibrate for 1 h before exposure to drugs. Isometric contractions were recorded as changes in grams of force on a Beckman Dynograph.

For relaxation studies, tissues were contracted with carbamylcholine (10^{-6} M) at an EC_{90} concentration based on previous studies (Secrest *et al.*, 1991). Once the carbamylcholine-induced contraction reached steady state, β receptor agonists were added cumulatively. Relaxation was calculated as a percentage of the initial carbamylcholine-induced force. EC_{50} values are the concentrations of β receptor agonist necessary to produce half-maximal relaxation of carbamylcholine-contracted tissues.

For studies with antagonists, tissues were preincubated with vehicle or antagonist for at least 45 min. Cumulative concentration responses to β receptor agonists were determined in the presence of antagonist or vehicle. Antagonist equilibrium dissociation constants (K_B) were determined for each concentration of antagonist according to the following equation:

$$K_B = \frac{[B]}{[DR - 1]}$$

where [B] is the concentration of the antagonist and dose ratio (DR) is the EC_{50} value of the agonist in the presence of the antagonist divided by the control EC_{50} value. These results are then expressed as the negative logarithm of the K_B (i.e., $-\log K_B$).

Rat atrial tachycardia

Male rats (150–300 g.) (Harlan Sprague Dawley, Indianapolis, Indiana, U.S.A.) were killed by cervical dislocation. Hearts were removed and the left and right atrium were dissected and mounted with thread in tissue baths containing 10 mls of modified Krebs' solution (see above). An initial optimum resting force of 1 g was applied to the atria (Cohen et al., 1982). Tissues were allowed to equilibrate approximately 30 min with vigorous oxygenation before exposure to drugs.

Agonists were added cumulatively once the spontaneous atrial rate reached a steady state from the previous addition. Agonist addition was continued until no further increase in atrial rate occurred or until a concentration of 10^{-4} M was reached. The increase in beats min^{-1} (b.p.m.) was measured for each concentration of agonist by means of a Beckmann Cardiometer.

Antagonists were evaluated by preincubation with tissue for at least 45 min. Cumulative concentration responses to the β receptor agonists were determined in the presence of antagonist or vehicle. Antagonist equilibrium dissociation constants were determined as indicated above.

Chemicals

All compounds used were reagent grade and prepared in saline, DMSO or propyleneglycol. CGP 12177A [S-4-(3-((1-*t*-butyl)amino)-2-hydroxy-propoxy)-1,3-dihydro-2H-benzimidazol-2-one], CL-316243 disodium [(*R,R*)-5-(2-((3-chlorophenyl)-2-hydroxyethyl)amino)propyl)-1,3-benzodioxole-2,2-dicarboxylate], indole sulphonamide [S-N-(4-(2-((2-hydroxy-3-(4'-indolyloxy)-propyl)amino)ethyl) phenyl)benzenesulphonamide], phenol-biaryl-ether [Ethyl-S-4-(4-(2-methyl-2-((2-hydroxy-3-(4-hydroxyphenyloxy)-propyl)amino)propyl) phenyloxy)-benzoate] and biphenyl-biaryl ether [S-4-(4-(2-methyl-2-((2-hydroxy-3-((2-phenyl)phenyloxy)-propyl)amino)propyl)phenyloxy) benzamide] were synthesized to 99% purity as determined by elemental analysis, HPLC and/or [^{13}C] spectroscopy at the Lilly Research Laboratories.

Results

Cloned human β_3 receptor agonist activity

Consistent with previous information, CL316243, a β_3 receptor agonist optimized for affinity at the rat β_3 receptor produced negligible activation of cyclic AMP formation in CHO cells stably expressing the human β_3 receptor (Table 1). In contrast, isoproterenol was a potent agonist (EC_{50} = 16 nM) with the greatest maximal activation of adenylyl cyclase of the agents studied. CGP12177 was a weak agonist at the human β_3 receptor (42% maximal response relative to isoproterenol).

All four aryl propanolamines were agonists at the human β_3 receptor as measured by their ability to increase cyclic AMP. Each of these β_3 receptor agonists was more potent than CGP12177. However, the four aryl propanolamines displayed marked diversity in agonist potency and efficacy at the human β_3 receptor (Table 1). Interestingly, both phenol bearing compounds (L-739,574 and the phenol-biaryl ether) possessed marked efficacy with maximal responses approximately 80% of the maximal response to isoproterenol at the human β_3 receptor, with L-739,574 considerably more potent than even isoproterenol. Compounds with an indole or biphenyl function were of intermediate potency. Nevertheless, although marked potency differences existed, these data document that all four

Figure 2 Effect of aryl propanolamines to relax carbamylcholine (10^{-6} M)-contracted rat stomach fundus in the presence of propranolol (3×10^{-7} M) relative to vehicle. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

Table 1 Human β receptor affinities activity of aryl propanolamines in CHO cells

Compound	β_1 K_i^a (nM $\pm$ s.e.)	β_2 K_i^a (nM $\pm$ s.e.)	EC_{50}^b (nM $\pm$ s.e.)	β_3 Maximal response ^c (percent $\pm$ s.e.)
Isoproterenol	—	—	16 $\pm$ 1	100
CGP12177	—	—	1375 $\pm$ 374	42 $\pm$ 5
CL316243	—	—	> 3160	14 $\pm$ 3
L-739,574	190 $\pm$ 60	360 $\pm$ 240	9 $\pm$ 0.5	74 $\pm$ 2
Indole sulfonamide	0.9 $\pm$ 0.2	0.6 $\pm$ 0.1	59 $\pm$ 11	55 $\pm$ 3
Biphenyl-biaryl ether	6.3 $\pm$ 2.9	2.9 $\pm$ 1.4	32 $\pm$ 4	16 $\pm$ 1
Phenol-biaryl ether	140 $\pm$ 10	17 $\pm$ 4	246 $\pm$ 46	82 $\pm$ 8

^aAffinities determined with radioligand binding as detailed in our Methods. ^b EC_{50} was the concentration producing 50% maximum response to each agonist in increasing cyclic AMP as detailed in Methods. ^cMaximal response was determined as the maximum increase in cyclic AMP relative to the maximal response to isoproterenol.

aryl propanolamines were relatively potent agonists at the human β_3 receptor.

Cloned human β_1 and β_2 receptor affinities

All four aryl propanolamines also interacted with β_1 and β_2 receptors, again with marked diversity in affinities. With the exception of L-739,574, affinities of these agents occurred in concentrations similar to or lower than concentrations that activated the β_3 receptor (Table 1). However, L-739,574 possessed only low affinity at β_1 and β_2 receptors and therefore showed high selectivity for β_3 receptors. Because of these potential β_1 and β_2 receptor interactions, subsequent studies in isolated stomach fundus and rat atria were conducted in the presence of β_1 and β_2 receptor blockade with propranolol (3×10^{-7} M), a concentration established to block β_1 and β_2 receptors (Cohen *et al.*, 1995a).

Rat stomach fundus β_3 receptor agonist activity

To examine the effect of these β_3 receptor agonists at the rat β_3 receptor, we utilized the rat stomach fundus, a preparation known to possess the rat β_3 receptor which mediates relaxation in this tissue (Cohen *et al.*, 1995a). CL316243 was a potent relaxant agonist in the rat stomach fundus ($EC_{50} = 3 \times 10^{-8}$ M) in contrast to CGP12177 (10^{-8} – 10^{-4} M) which did not relax the rat stomach fundus (less than 10% of the maximal possible relaxation which was similar to the relaxation produced by vehicle) (Figure 2).

Activity of the aryl propanolamines at the rat β_3 receptor in the stomach fundus did not parallel their activity at the human β_3 receptor. Like CGP12177, the biaryl ether derivatives did not markedly relax the rat stomach fundus in concentrations less than 10^{-5} M (Figure 2). In contrast, the sulfonamide derivatives relaxed the rat stomach fundus (in concentrations $>10^{-6}$ M) although the potency of these compounds was approximately 100 fold lower than CL316243. Cyanopindolol a known antagonist at the 'atypical' β receptor (McLaughlin & MacDonald, 1990; Blue *et al.*, 1989) was examined for its ability to inhibit the relaxation produced by these sulfonamide derivatives in the rat stomach fundus. Although cyanopindolol potently inhibited the relaxation produced by CL316243 in the rat stomach fundus [$-\log K_B = 7.62 \pm 0.20(3)$], cyanopindolol (10^{-6} M) had no effect on the relaxation produced by either the phenol or indole sulfonamide derivatives (Figure 3). Thus, the sulfonamide derivatives relaxed the rat stomach fundus by a mechanism independent of β_3 receptors.

Since these aryl propanolamine derivatives did not possess agonist activity at the rat β_3 receptor, we asked whether these agents had affinity as antagonists of the rat β_3 receptor. For these experiments, one sulphonamide and one biaryl ether derivative were examined, and both agents (10^{-6} M) dextrally shifted the relaxant concentration response curve produced by CL316243 (Figure 4). Thus, although not agonists at the rat β_3 receptor, these compounds possessed antagonist affinity for the rat β_3 receptor with the negative logarithm of the antagonist dissociation constant of $6.55 \pm 0.22(3)$ and $7.05 \pm 0.05(3)$ for the biphenyl biaryl ether and indole sulphonamide, respectively.

Figure 3 Effect of cyanopindolol (10^{-7} M) to inhibit relaxation of carbamylcholine (10^{-6} M) contracted rat stomach fundus strips by CL316243 (Top) L-739,574 (middle) and indole sulphonamide (bottom). Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

Figure 4 Effect of biphenyl-biaryl ether and indole sulfonamide (10^{-6} M) to inhibit the relaxation of carbamylcholine-induced contraction in rat stomach fundus by CL316243. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

Rat atrial tachycardic β receptors

Having characterized these aryl propanolamine derivatives as agonists at the human β_3 receptor and antagonists at the rat β_3 receptor, we determined the effect of these agents at β receptors mediating atrial tachycardia. Interestingly, of the β_3 receptor

Figure 5 Effect of aryl propanolamines to increase rat atrial rate. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

agonists studied, both phenol substituted derivatives modestly increased rate in rat atria although the potencies (EC_{50} values) were less than those observed with CGP12177 (Figure 5). Neither the indole nor the biphenyl-biaryl ether derivatives increased heart rate although both agents were agonists at the human β_3 receptor. CL316243 also did not increase rat atrial rate, although it was a potent agonist at the rat β_3 receptor.

Propranolol (3×10^{-7} M), in a concentration that inhibited β_1 and β_2 but not β_3 receptors (Cohen et al., 1995a) antagonized the tachycardia induced by isoproterenol and completely abolished the tachycardia produced by the phenol substituted propanolamine derivatives (Figure 6). In contrast, the tachycardia produced by CGP12177 was unaffected by propranolol (3×10^{-7} M). These studies suggest that the tachycardia produced by the aryl propanolamine derivatives, but not by CGP12177 resulted from β_1 receptor activation.

Discussion

Aryl propanolamines are known to have high affinity at β adrenergic receptors, an effect that has been most extensively studied with regard to β_1 and β_2 receptors. However, more recently with the cloning of the rat and human β_3 receptors, efforts have been directed toward identifying high affinity agonists at the human β_3 receptor. The localization of this receptor to brown adipose tissue and the gastrointestinal

Figure 6 Effect of propranolol to inhibit the increase in rat atrial rate produced by isoproterenol, CGP12177, L-739,574, and phenol-biaryl ether. Points are mean values and vertical bars represent the standard error of the mean for the number of tissues indicated in parenthesis.

tract has raised the possibility that β_3 receptor agonists might be effective as anti-obesity and anti-spasmodic agents. However, one concern with regard to such agents is beta receptor activation in the heart leading to tachycardia, an undesirable property in the clinic. Recent studies have suggested that tachycardia may not only result from β_1 receptor activation, but also *via* an atypical beta receptor found in rat (Kaumann & Molenaar, 1996) and human (Kaumann, 1996) atria.

Efforts to identify high affinity β_3 receptor agonists resulted in the synthesis of L-739,574 (Dow, 1997) and three additional aryl propanolamine derivatives. The first objective of the present study was to characterize the interactions of these aryl propanolamines at the various beta receptors to establish their utility as tools to further probe β -receptor interactions in atria and other tissues. These compounds possessed relatively high affinity as human β_3 receptor agonists with marked diversity in their potency and agonist intrinsic activity at the human β_3 receptor (Table 1). Furthermore, of these agents, L-739,574 was highly selective as a β_3 agonist possessing considerably lower affinity at β_1 and β_2 receptors (see Table 1).

With regard to interactions with rat β_3 receptors, although CL316243 was a potent agonist at the rat β_3 receptor in the stomach fundus (Cohen *et al.*, 1995a; Figure 2), none of the aryl propanolamines activated the rat β_3 receptor in the stomach fundus. Furthermore, even though L-739,574 was a potent agonist at the human β_3 receptor, it only weakly relaxed the rat stomach fundus and did so by a non-beta adrenergic and possibly non-selective mechanism since cyanopindolol which inhibits β_1 , β_2 and β_3 receptors did not block the relaxation. Although not agonists at the rat β_3 receptor, both the indole sulfphonamide and the biphenyl-biaryl ether were antagonists at the rat β_3 receptor. This example of compounds interacting as an agonist in one species (human) and an antagonist in another species (rat) underscores the importance of studying drug-receptor interactions in each species rather than extrapolating information from one species to another. Furthermore, these data documenting opposing effects of some β_3 receptor agonists at human and rat β_3 receptors suggest that *in vivo* data obtained in rats with agonists possessing high affinity at human β_3 receptors may reflect antagonist rather than agonist activity.

With regard to atrial tachycardia, our studies confirm previous data documenting that CGP12177 produced atrial

tachycardia whereas CL316243 did not (Cohen *et al.*, 1995b; Kaumann 1996; Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996). Of the aryl propanolamines with high agonist activity at the human β_3 receptor, only the phenol substituted compounds produced a modest increase in atrial rate, an increase that was totally abolished by propranolol (3×10^{-7} M) in a concentration known to inhibit β_1 and β_2 receptors (Cohen *et al.*, 1995a). In contrast, as previously reported (Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996), CGP12177-induced tachycardia was not blocked by the same concentration of propranolol. The inability of propranolol to block the tachycardia produced by CGP12177 is consistent with the suggestion (Kaumann & Molenaar, 1996; Malinkowski & Schlicker, 1996) that tachycardia induced by CGP12177 results from activation of a non- β_1 , β_2 , or β_3 receptor. Most importantly, the fact that these more potent agonists at the human β_3 receptor did not produce tachycardia *via* the non- β_1 , β_2 or β_3 receptor suggest that this tachycardic receptor is unrelated to and independent from the human β_3 receptor.

In summary, the present studies characterize the actions of four aryl propanolamines with high affinity for the human β_3 receptor. Although these compounds had high affinity at the human β_3 receptor, marked diversity occurred in the functional activation of the human β_3 receptor receptor with L-739574 as the most potent, selective, and efficacious agonist. Furthermore, in contrast to other β_3 receptor agonists, these aryl propanolamines were antagonists rather than agonists at the rat β_3 receptor, an observation with important implications to *in vivo* effects observed in rats. Lastly, the atrial tachycardic receptor, although previously established to be a non β_1 , β_2 or β_3 receptor (based on ligand affinities at rat β_3 receptors) has now clearly been shown to also be unrelated to the human β_3 receptor. These data support previous conclusions suggesting the presence of a fourth beta receptor associated with atrial tachycardia.

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