Exploring prospects of β3-adrenoceptor agonists and inverse agonists for colon motility control

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Abstract

Inverse agonists are useful active ingredient of drugs clinically used to treat diseases mainly involving receptors endowed with non-endogenous agonist induced activity (constitutive or basal activity). SP-1e and SP-1g are the first two potent and highly selective β3-adrenoceptor inverse agonists [EC_{50}=181 nM (IA=-64%) and 136 nM (IA=-73%), respectively], which their peculiar activity seems due to the absolute configurations of the two stereogenic centres present in each molecule. Rat proximal colon motility measurements allowed their further pharmacological characterization and pA_2 values determination by Schild analysis (7.89 and 8.16, respectively). The purpose of our work is a further characterization of our novel β3-adrenoceptor agonists (SP-1a-d, SP-1f,1h) and inverse agonists (SP-1e and SP1g) on rat proximal colon motility and a confirmation of their inverse agonist nature in a more complex system like the functional test on rat proximal colon. Male Wistar rats segment of the proximal colon were placed in organ baths containing Krebs solution. Muscle tension was recorded isotonically. Cumulative β3-AR agonists doses experiments were performed for each test compound: isoprenaline, BRL37344, SP-1a-d, SP-1f and SP-1h were dissolved in Krebs. The EC_{50} values of each agonists and pA_2 of inverse agonists were determined. SP-1a-d, SP-1f and SP-1h in rat colon have a muscle relaxing effect thus confirming their partial agonist activity found in CHO-K1 cell line. SP-1e and SP-1g behaved as antagonists with pA_2 values of 7.89 and 8.16, respectively. In conclusion, experiments carried out by using isolated rat proximal colon allowed us to determine the pA_2 values of the two β3-AR inverse agonists and add knowledge on the behavior of a novel set of compounds and their possible value as agents useful whenever is necessary to also control the colon motility.

Introduction

The gastrointestinal (GI) tract produces large amounts of catecholamines, which contribute to the regulation of gastrointestinal motility, secretion, local blood flow, and immune responses.[1] It has been reported that both α- and β-adrenoceptors mediate noradrenaline-activated K^+ secretion in the distal colonic mucosa.[2] It is generally accepted that catecholamines can relax gastrointestinal smooth muscle by acting on post-junctional α- and β-adrenoceptors.

All three β-adrenoceptor subtypes are expressed in GI tract were they modulate also colon motility. In particular, β1-adrenoreceptors are expressed in neurons and nerve fibres in both the myenteric and submucosal plexus in several species. The β2-adrenoceptor is in rodent submucosal plexus neurons and nerve fibres, and in the mouse myenteric plexus. The β3-adrenoceptors are localized on myenteric cholinergic neurons in the human colon and in nerve fibres of the rat myenteric plexus and deep muscular plexus where they were in close opposition to interstitial cells of Cajal.[1]

β3-adrenoceptors (β3-AR) are widely distributed in the gastrointestinal tract of several species, including humans and rats. In particular, they are expressed on gut vascular and nonvascular smooth muscle, where they mediate relaxation and are probably involved in the control of blood flow. The β3-AR modulates colonic motility, in particular, elevated tone and spontaneous contractions of isolated human colon are reduced and inhibited by β3-AR agonists such as SR 58611A or CGP 12177A,[3-5] respectively (Figure 1).

In addition, activation of β3-AR by their agonists leads to an inhibition of cholinergic contractions and evokes somatostatine release, resulting in a decrease of intestinal motility and secretion, and inducing analgesia.[6]

β3-AR displays a different degree of stereoselectivity towards several known traditional β-AR ligands. In particular, β3-AR stereoselective dependency is lower for agonists such as isoprenaline and noradrenaline and is higher for antagonists (i.e. propranolol) than β1- and β2-AR.[7] We uncovered a set of new compounds that stereoselectively interact with β3-AR.[8]

Novel compounds were assayed by measuring cAMP levels in CHO-k1 cells expressing human cloned β3-AR to determine their β3-AR activity and as a result, novel potent and selective stereogenic β3-AR agonists were disclosed together with the discovery of the first two potent and selective β3-AR inverse agonists SP-1e and SP-1g was absolutely the most striking finding.[8,9]

Herein, we report a further characterization of such compounds by measuring their effect on ex vivo rat proximal colon. Colon motility modification is compromised in several diseases.[10] Hence, it is crucial to know how physiological colon tone and contraction can pharmacologiclly be recovered.
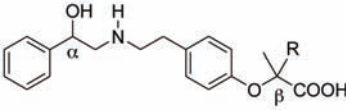
Materials and Methods

Statistics

The EC_{50} values were obtained from non-linear iterative curve fitting by GraphPad Prism® 3.0. One way-ANOVA analysis of variance was used to estimate the significance of difference. $P<0.05$ was considered statistically significant. The pA_2 and slope values were calculated according to the method described by Arunlakshana and Schild.[11]

Animals: experimental conditions

Male Wistar rats weighing 300-350 g were killed by decapitation. The first 2.5-3.0 cm segment of the proximal colon, starting from the ileo-caecal junction, was dissected and quickly washed in saline and then placed in 20 mL organ baths containing Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 11 mM glucose) completed with 1 μM phentolamine, 0.5 μM desmethylimipramine and 30

Table 1. Structures, absolute configurations and activity values of compounds SP-1-a–h measured in (CHO)-K1cell line and in *ex vivo* rat colon.

Compd	R	Abs. config.	EC$_{50}$ [a] (nM±SEM)[b] $h\beta_3$-AR in CHO[8] (IA%)[c]	EC$_{50}$ (M±SEM) [d] rat β_3-AR (IA%)[e]
SP-1a	CH$_3$	α-*rac*	4.9±0.2 (68)	6.02±1.2 (37)
SP-1b	CH$_3$	αR	3.9±2.1 (72)	0.20±0.03 (100)
SP-1c	CH$_3$	βS	3.4±0.8 (76)	3.67±0.4 (37)
SP-1d	H	α-*rac*, β-*rac*	3.8±0.7 (65)	4.98±0.9 (100)
SP-1f	H	αR, βS	2.7±0.7 (50)	0.73±0.03 (100)
SP-1h	H	αR, βR	235±37 (34)	0.08±0.02 (100)
	Isoprenaline		5.8±1.2 (100)	0.10±0.08 (100)
	BRL37344		1.1±0.3 (76)	0.042±0.005 (100)

[a]EC$_{50}$: substance concentration which produces a cAMP response equal to 50% of its maximal response; [b]SEM, standard error mean from at least three experiments (n≥3); [c]IA: Intrinsic Activity is the fitted maximal value of the concentration-response curve, expressed as a percent of the maximal response to *R*-(-)-isoproterenol (10^{-4} M). [d]EC$_{50}$: substance concentration evaluated in functional test in which colon relaxation is evaluated; [e]IA is the fitted maximal value of the concentration-response curve, expressed as a percent of the maximal relaxant effect obtained with *R*-(-)-isoproterenol (10^{-4} M).
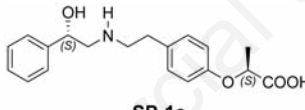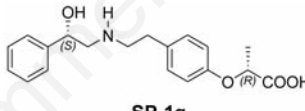
μM hydrocortisone (the latter two for preventing intraneuronal and extraneuronal uptake of catecholamines, respectively) at 37.0°C and bubbled with a 5% CO_2 and 95% O_2 gas. The bathing medium contained 1 μM betaxolol and 1 μM ICI118551 to block β_1 and β_2-AR, respectively. The strip was placed under a 1 g load, washed several times and allowed to develop stable spontaneous tone. Muscle tension was recorded isotonically using Fort 10 transducers original WPI, connected to a PowerLab 4/20 AD Instrument recorder.

After a three hours equilibration period, cumulative agonists doses experiments were performed for each test compound: isoprenaline, BRL37344, SP-1a-d, SP-1f and SP-1h were dissolved in Krebs and then cumulatively (10 nM, 50 nM, 100 nM, 0.5 μM, 1 μM, 3 μM, 5 μM, 10 μM and 20 μM) added with an interval of 40 seconds between each dose. The concentration of agonists to produce a half maximal contraction (EC$_{50}$) was determined with a non-linear curve fit program (GraphPad Prism® 3.0, GraphPad Software, Inc. San Diego, CA, USA) using the mean response of at least 3 separate trials. All animals were maintained in accordance with the guidelines for the care and use of laboratory animals of the Italian Ministry of Health.

Table 2. Structures, absolute configurations and activity values of compounds SP1-e and SP1-g measured in (CHO)-K1cell line and in ex vivo rat colon.

Compd	EC$_{50}$ [a] (nM±SEM)[b] h β_3-AR in CHO (IA%)[c]	pA$_2$[d] rat β_3-AR
SP-1e	181±19 nM (-64%)	7.89±1.5
SP-1g	136±20 nM (-73%)	8.16±0.8

[a]EC50: substance concentration which produces a cAMP response equal to 50% of its maximal response; [b]SEM, standard error mean from at least three experiments (n≥3); [c]IA, Intrinsic Activity is the fitted maximal value of the concentration-response curve, expressed as a percent of the maximal response to *R*-(-)-isoproterenol (10^{-4} M); [d]pA$_2$ value of inverse agonists determined by using *R*-(-)-isoproterenol.

Table 3. Possible clinical use of β_3-AR inverse agonists.

Metabolic (insulin resistance) syndrome	In upper-body obese subjects with signs of the metabolic syndrome, β_3-AR blockade might preferentially inhibit fatty acid release from visceral adipose tissue and improve some of the metabolic abnormalities associated with the high portal fatty acid flux.[12]
Cachexia	In cachectic cancer patients, the remarkable loss in adipose tissue (~30% of pre-illness stable weight at diagnosis) seems to be mediated by a lipid mobilizing factor (LMF). Reported evidences demonstrate that, at least in part, LMF produces this effect by interaction with β_3-AR.[21]
Heart failure	Selective β_3-AR agonists should serve in the early stage of heart failure, whereas highly selective antagonists/inverse agonists might be useful in the advanced stage of the disease.[8]

Results

SP-1a-h behaviour strongly depends on the absolute configuration of the stereocentre(s) present in the molecules (Tables 1 and 2). In particular, SP-1a-d, SP-1f and SP-1h in rat colon have a muscle relaxing effect thus confirming their partial agonist activity found in
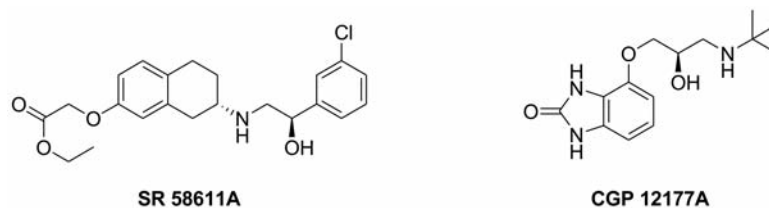

Figure 1. SR 58611A and CGP 12177A β_3-AR agonist chemical structures.
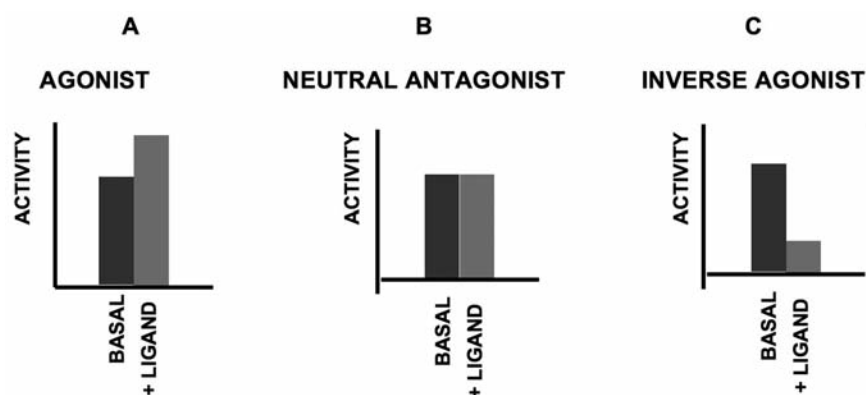
Figure 2. Inverse agonists are ligands that, by binding to a constitutively activated receptor, reduce or suppress its basal activity thus shifting a cell signaling cascade in the opposite direction to that produced by an agonist.
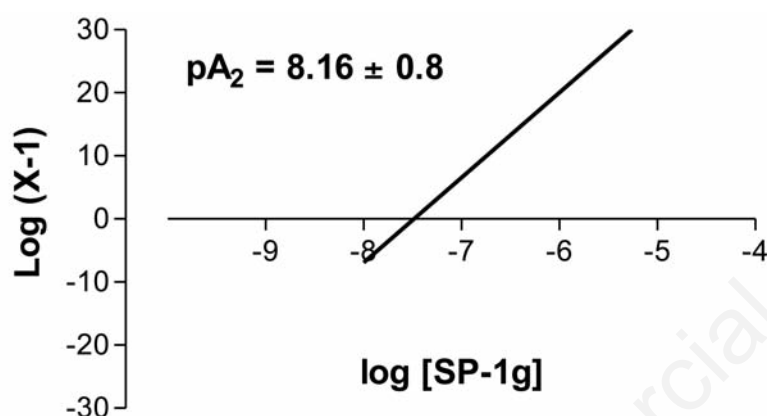

Figure 3. Schild plots of SP-1e and SP-1g in the rat proximal colon. All experiments are in triplicates and the pA2 values are expressed as the mean ± SEM.

CHO-K1 cell line.[8] The different EC_{50} and intrinsic activity values between CHO and rat colon are mainly due to the sequence difference of human and rat receptor, particularly located in the binding site.

SP-1b, in *ex vivo* experiments (Table 1), was found to be a full agonist (EC_{50}=0.20 μM and IA=100%) with respect to the corresponding racemic form SP-1a (EC_{50} = 6.02 μM) and its αS- enantiomer SP-1c (EC_{50}=3.67 μM) which instead behaved as partial agonists (IA=37%) thus confirming that the configuration at Cα is generally (R) in β_3-AR agonists.

Compounds with two stereocentres, such as SP-1d, SP-1f and SP-1h were found to be full agonists at rat colon and endowed with different potencies, 4.98 μM for the racemic form SP-1d, 0.73 μM for αR,βS-SP-1f and 0.08 μM for αR,βR-SP-1h.

The β_3-AR inverse agonists SP-1e (5 M) and SP1g (5 μM) were pre-incubated for 15 min and then the effect of the addition of isoprenaline was evaluated. Their potency was determined plotting the suitable results to perform the Schild analysis. The pA$_2$ values [−log (Antagonist)] were determined by a linear curve fit program (GraphPad Prism® 3.0) using the mean response of at least 3 separate experiments. (αS, βR)-SP-1g was found to be the most potent inverse agonist with an EC_{50}=136 nM and IA=-63%. Its epimer (αS, βS)-SP-1e had comparable potency and intrinsic activity (EC_{50}=181 nM, IA=-64%) (Table 2).

Discussion

The β_3-AR, like the other β-AR subtypes, is a seven-transmembrane domain (7TD) G-protein coupled receptor (GPCR). It is usually coupled to a Gs protein and its stimulation increases the production of cAMP.[12] On the other hand, in the human heart the signal of β_3-AR is transduced by the Gi-eNOS-NOcGMP pathway and produce negative inotropic effect.[13-14] A lot of β_3-AR agonists have been uncovered and extensively characterized.[15] Conversely, very little is known about β_3-AR inverse agonists.[16,17] Inverse agonism is not a new concept and it describes ligand behavior displaying negative efficacy.[18] In particular, for GPCRs, it has been widely assumed that inverse agonists suppress the agonist-independent activity (constitutive activity) of the receptor by stabilizing it in its inactive state (Figure 2).[19]

Novel findings suggest that some classical β-AR antagonists behave either as partial agonists, neutral antagonists, or inverse agonists in cell systems expressing the wild type or a constitutively activated mutant of the human β-AR. For example, selective β_1-AR antagonists with significant inverse agonistic activity, in rat myocardium, such as metoprolol,[20] have been proved to be safe in the treatment of heart failure. Very little is known about β_3-AR inverse agonists, but they could have different possible clinical applications (Table 3).[16-17,21]

Knowing the potential therapeutic applications of β_3-AR inverse agonists, it was crucial a further characterization of our two compounds and a confirmation of their inverse agonist nature in a more complex system like the functional test on rat proximal colon. SP-1e and SP-1g behaved as antagonists (in functional test in which colon motility is evaluated, it is not possible to discriminate antagonism from inverse agonism). Schild analysis allows to determine pA$_2$ values (Table 2, Figure 3).

Conclusions

In conclusion, experiments carried out by using isolated rat proximal colon allowed us to determine the pA$_2$ values of the two β_3-AR inverse agonists. The behavior of a novel set of compounds add knowledge on their possible value as agents useful whenever is necessary also to control the colon motility.

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