International Congress Series 1249 (2003) 55–61

Inverse agonism at β_1-adrenergic receptors

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Received 16 April 2003; accepted 17 April 2003

Abstract

Constitutive activity and inverse agonism have been described for numerous G-protein-coupled receptors, including the β_2-adrenergic receptor. We have investigated whether these properties also exist for the β_1-adrenergic receptor. One line of experiments was done with cell lines transiently or stably expressing the human β_1-adrenergic receptor measuring intracellular cAMP or adenylyl cyclase activity. In a second set of experiments, we used transgenic mice with cardiac overexpression of the human β_1-adrenergic receptor and measured spontaneous beating frequency of isolated atria as a physiological read-out. Both sets of experiments revealed that the human β_1-adrenergic receptor displays constitutive activity, but this constitutive activity is considerably lower than that of the β_2-subtype. We then tested various β-adrenergic antagonists for their ability to modulate this constitutive activity. In the β_1-receptor-transgenic atria, the β_1-selective antagonists metoprolol and bisoprolol showed significant inverse agonistic activity, whereas carvedilol behaved as a neutral antagonist. In contrast, in cellular cAMP-assays, metoprolol, bisoprolol, propranolol and carvedilol all displayed similar inverse agonist activities. We conclude that also the human β_1-adrenergic receptor displays constitutive activity and that—depending on the read-out—β-adrenergic "antagonists" currently in clinical use may differ in their inverse agonistic activity at this receptor.

Keywords: β_1-Adrenergic receptor; Adenylyl cyclase; Constitutive activity; Inverse agonism; Cardiac frequency

1. Introduction

Constitutive activity of signalling proteins, i.e. the activity in the absence of an activating ligand or other agent, has been known for many years. It has been observed in receptors of various families, in G-proteins and other GTP-binding proteins as well as

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0531-5131/03 © 2003 Published by Elsevier Science B.V.
doi:10.1016/S0531-5131(03)00678-2

downstream signalling elements. Constitutive activity can be markedly increased by specific mutations, and such mutated signalling proteins often have great clinical importance. For example, various forms of cancer are associated with activating mutations in tyrosine kinase receptors or in GTP-binding proteins such as ras.

In the field of G-protein-coupled receptors, constitutive activity was first observed for opiate receptors, and this observation paved the way to the detection of compounds, which inhibited this constitutive activity [1]. These compounds with "negative intrinsic activity" have been given various names, but the term "inverse agonists" is now the most accepted. It was soon realized that the constitutive activity of G-protein-coupled receptors was due to spontaneous interactions with their G-proteins [2]. Since then, constitutive activity and inverse agonism have been described for a large number of G-protein-coupled receptors. Constitutive activity at G-protein-coupled receptors has gained clinical importance in two respects. First, many G-protein-coupled receptors can carry various activating mutations which can cause diseases; examples are activating mutations in the thyroid stimulating hormone (TSH) receptor which cause hyperthyroidism, and some activating receptor mutations are even tumorigenic [3]. Second, in the case of receptors with high constitutive activity, effective functional blockade of receptor activity requires the use of inverse agonists; for example, the anti-ulcer drugs acting at the H_2-histamine receptor such as cimetidine and ranitidine are in fact all inverse agonists at this receptor [4,5].

Among the β-adrenergic receptors, constitutive activity is well-documented for the β_2-subtype [6,7]. This can be seen, for example, from the fact that expression of these receptors in cell lines leads to an increase in intracellular cAMP in the absence of ligands. Transgenic expression of the β_2-adrenergic receptor in the mouse heart causes an increase in beating frequency [7]. In both cases, inverse agonists have been identified among the classical "antagonists"—for example propranolol or ICI 118551—which act as inverse agonists and block the effects caused by the unoccupied receptors. Much less is known about the β_1-subtype in this respect. Constitutively active mutants of this receptor have been generated by site-specific mutations [8] along the lines described earlier for other G-protein-coupled receptors [9], but there was very little evidence for constitutive activity in the native β_1-receptor. This lack of knowledge is surprising given that clinically blockade of β_1-adrenergic receptors is much more important than that of the β_2-subtype.

Activation of cardiac β_1-adrenergic receptors plays a central role in regulating the physiological responses of the heart to an increased demand [10]. Blockade of cardiac β_1-adrenergic receptors is a key strategy in reducing cardiac output and energy demand in a variety of diseases including most importantly hypertension and coronary heart disease. More recently, blockade of cardiac β_1-adrenergic receptors has also been demonstrated to be useful in treating heart failure [11]. This is presumably due to the fact that the increased stimulation of these receptors by increased sympathetic activity (which accompanies heart failure) results in structural and functional damage to the heart [12–14]. Given the importance of inverse agonism for therapy at other receptors, it was of interest to investigate the level of constitutive activity of the human β_1-adrenergic receptor, to measure whether inverse agonism occurs at this receptor and to determine whether the available β-adrenergic receptor antagonists differ in such inverse agonist properties.

We have done two types of study to investigate these questions: (a) studies with cell lines transiently or stably expressing the human β_1-adrenergic receptor measuring intra-

cellular cAMP or adenylyl cyclase activity, and (b) studies with transgenic mice with cardiac overexpression of the human β_1-adrenergic receptor measuring spontaneous beating frequency of isolated atria.

2. Constitutive activity of β_1-adrenergic receptors in transfected cell lines

In a first set of studies, we investigated cAMP-levels and adenylyl cyclase activities in cells transfected with the human β_1- or β_2-adrenergic receptors. In transiently transfected COS-7 cells, the accumulation of intracellular cAMP after inhibition of phosphodiesterases with isobutyl methyl xanthine (IBMX) was determined as a function of receptor expression [15]. For both receptor subtypes, an increase in basal cAMP levels was detected, which was dependent on the amount of cDNA transfected and on the resulting receptor levels. However, the increase of cAMP levels was about six-fold higher for the β_2- than for the β_1-subtype (Table 1). Per picomole of receptor expressed, the accumulation of cAMP increased 2.5-fold for the β_2-subtype and 0.4-fold for the β_1-subtype. These results showed that the constitutive activity of the β_1-adrenergic receptor is lower than that of the β_2-subtype, but that it is clearly detectable.

Similar results were obtained with CHO-cells stably expressing the different human β-adrenergic receptors (data not shown). Using adenylyl cyclase activity of membranes prepared from such cells, we could confirm that the β_1-subtype does indeed possess constitutive activity, even though this property is more pronounced for the β_2-subtype. In these experiments, the constitutive activity of the β_2-subtype was about four-fold higher than that of the β_1-subtype (data not shown).

3. Constitutive activity of β_1-adrenergic receptors in hearts from transgenic mice

Since β_1-adrenergic receptors appeared to possess constitutive activity in transfected cells, we went on to try to discover this property with a physiological read-out. Transgenic mice overexpressing the human β_1-adrenergic receptor [14] proved to be a suitable model for this purpose. In vivo, these mice have an increased cardiac frequency. An increased frequency was also seen in spontaneously beating isolated right atria from these mice (Table 2). In this experimental set-up, no catecholamines stimulate the receptors; this is evident from the fact that depletion of endogenous catecholamines by treating the animals with reserpine—a treatment that reduced cardiac catecholamine levels by more than

Table 1
Constitutive activity of β_1- and β_2-adrenergic receptors in transiently transfected COS-7 cells

Receptor subtype	cAMP-accumulation (% increase per pmol receptor)
β_1-Adrenergic receptor	42 ± 3
β_2-Adrenergic receptor	253 ± 18

COS-7 cells were transiently transfected with various amounts of cDNA for the receptors resulting in receptor levels between 0.03 and 2.6 pmol/mg membrane protein, and cAMP-accumulation was determined 20 min after the addition of the phosphodiesterase inhibitor IBMX (0.5 mM). Data are derived from Ref. [15].

Table 2
Constitutive activity of β_1-adrenergic receptors in isolated atria from transgenic mice overexpressing the receptors

Receptor subtype	Frequency (beats per minute)
Wild-type	$\approx 331 \pm 10$
β_1-Adrenergic receptor	$\approx 382 \pm 23$

Spontaneous frequencies were recorded in organ bath experiments with atria from non-transgenic mice, or transgenic mice overexpressing the human β_1-adrenergic receptor (2.7 ± 0.3 pmol/mg membrane protein). Data are derived from Ref. [15].

99.9%—did not affect the frequency of atria from wild-type mice nor the increased frequency in transgenic mice (data not shown).

The frequency of the β_1-adrenergic receptor-transgenic atria was increased by about 50 beats per minute compared to the wild-type atria. Although this represents an increase by only $\approx$ 15%, the effect was highly reproducible. It indicates that constitutive activity of the β_1-adrenergic receptor can be discovered using a physiological read-out, even though it is modest.

4. Inverse agonism at β_1-adrenergic receptors

With these two models to determine constitutive activity of the β_1-adrenergic receptor, we then investigated whether compounds usually classified as antagonists might in fact act as inverse agonists at these receptors. In these experiments, we studied four clinically used "antagonists", the β_1-selective compounds bisoprolol and metoprolol, the nonselective β_1/β_2-"antagonist" propranolol, the nonselective α- and β-receptor-"antagonist" carvedilol and the highly β_1-selective experimental compound CGP 20712A (Table 3).

In the β_1-receptor-transgenic mouse atria, the three β_1-selective compounds CGP20712A, bisoprolol and metoprolol all behaved as inverse agonists. Propranolol

Table 3
Inverse agonism at β_1- and β_2-adrenergic receptors

Compound	Inverse agonism	
	Transgenic atria (decrease in beats per minute) (% inhibition of transgene-induced frequency increase)	Cell membranes (decrease in %)
CGP 20712	42 ± 17 (82)*	25 ± 5*
Bisoprolol	23 ± 14 (45)*	33 ± 9*
Metoprolol	20 ± 5 (39)*	24 ± 11*
Propranolol	11 ± 11 $(20)^{NS}$	35 ± 6*
Carvedilol	$[-9 \pm 8^{NS}]$	28 ± 9*

Inverse agonist properties of compounds classically regarded as "β-adrenergic receptor antagonists" were assayed either in cell membranes from CHO-cells overexpressing the human β_1-adrenergic receptor or in isolated atria from transgenic mice as in Table 2. Data are derived from Ref. [15] for the isolated atria or are unpublished. Note that negative figures (in square brackets) mean that this compound acts as a partial agonist, not as an inverse agonist in this experimental model.
NS = not significant.
*$p < 0.05$.

caused only a small and statistically not significant decrease of the spontaneous beating rate of right transgenic atria. The nonselective β-adrenergic receptor antagonist carvedilol did not display inverse agonist activity at the β_1-adrenergic receptor, but rather caused a slight, statistically not significant, stimulation.

Slightly different results were obtained in assays measuring adenylyl cyclase activity in membranes from CHO-cells stably expressing β_1-adrenergic receptors. Here, all five compounds appeared to act as inverse agonists and reduced adenylyl cyclase activity by 25% to 35%. Thus, in contrast to the data obtained in the physiological model, there were no distinctions between the different compounds investigated.

5. Discussion

Our investigations of constitutive activity of the human β_1-adrenergic receptor had four major results: (1) The human β_1-adrenergic receptor displays constitutive activity. (2) The constitutive activity of the β_1-adrenergic receptor is considerably lower than that of the β_2-subtype. (3) Several β-adrenergic antagonists display inverse agonist activity at the β_1-adrenergic receptor. (4) The extent of inverse agonist activity depends on the choice of experimental model.

The clinical interest in these results is potential differences between various β-receptor "antagonists" in terms of their clinical efficacy. Recent large trials in heart failure have yielded different results for the compounds studied. Both for bisoprolol and for metoprolol, benefits in mortality from heart failure patients have been demonstrated in large clinical trials [16,17]. In contrast, xamoterol, a partial agonist at the β_1-adrenergic receptor, led to an increase in mortality [18]. These data suggest that β_1-mediated signalling in a diseased heart, even at reduced or very low levels, is generally detrimental. Carvedilol, which in addition to acting at β-adrenergic receptors is a potent α_1-adrenergic receptor antagonist and has significant antioxidative properties, is clinically useful in heart failure even though it was not an inverse β_1-adrenergic receptors agonist in the atrial model; however, it did display inverse agonist activity at β_1-adrenergic receptors expressed in CHO-cells.

We conclude that the human β_1-adrenergic receptor shows constitutive activity, and that several clinically used drugs display inverse agonist activity at this receptor. Future studies will be aimed at investigating the correlation between inverse agonist activity of classical "β_1-receptor blockers" and their therapeutic efficacy.

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Discussion 5

M. Lohse

I will ask you a question. We have just started doing experiments with so-called inverse agonists. For several receptors, there are no good inverse agonists. But in those cases that we have studied there are often very surprising responses. Most importantly, the kinetics of inverse agonist effects are often quite different from those produced by partial or full agonists. So my question goes to all of you, but in particular to Tommaso Costa, whether we can make any predictions about the expected kinetics of an inverse agonist response.

T. Costa

Do you think that what you are looking at is not the active G protein-bound form, but a sort of pre-active state? Is that what you see or is it both? Could you be looking at a slow search of the active conformation?

M. Lohse

We can't really distinguish. All we know is that so far we are unable to make the G protein alter either the kinetics or the extent of our direction. That could be a technical problem, because we are working with over-expressed receptors and there is not enough G protein around. We have tried reconstituting it with G proteins, but this is difficult to

achieve in a quantitative assay. So it may be either the R* without the G proteins, or it may be R* G.

T. Costa

Didn't you get those rate measurements also in membranes?

M. Lohse

No, for technical reasons we can't do that in membranes, we can do it only in intact cells. So the rate could be changed, depending on whether the G protein is there or not.

A. Newman-Tancredi

I was just wondering if this is a quantitative measure that you are deriving. Can you extrapolate from the extent of loss of response to number of receptors which are occupied by the agonists? Is that something you can do?

M. Lohse

Not really, I think. But we can measure with binding assays that we are occupying all receptors that are available. In a stable cell line they're really all at the cell surface and we can measure with radio ligand binding that they are all occupied within a very short period of time.

A. Newman-Tancredi

And do those kinetics correspond to the kinetics you're seeing for the loss of response?

M. Lohse

I don't know of a binding assay that you can do within the millisecond scale.

A. Newman-Tancredi

It looks like the response signal you're getting there is a lot more rapid than any association of drug, doesn't it?

M. Lohse

I don't think so. At least in the adrenergic field, we're dealing with low affinity binding that is in the micromolar range and I think if you can extrapolate that that is complete within a few seconds. I don't think the kinetics are wrong, and that there is a major difference between the binding kinetics and this response.

A. Newman-Tancredi

I would have thought that the kinetics are quicker than you're saying. If you have got a micromolar ligand, it's going to be on quicker than that.

M. Lohse

Yes, but I would be concerned if the response is there before the ligand has bound.