International Congress Series 1249 (2003) 131–138

Modulation of constitutive GPCR activity: a way of life?

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Received 16 April 2003; accepted 16 April 2003

Abstract

G-protein coupled receptors (GPCRs) are highly versatile signalling modules and, as such, one of the most important drug targets in many therapeutic areas. In recent years, the view on GPCR activation has been firmly challenged and it is now clear that GPCRs do not always need external ligands to modulate intracellular signal transduction. The histamine H_3 receptor shows a high level of constitutive activity both in vitro and in vivo. Constitutive H_3 receptor activity might be modulated by biological (receptor isoforms) and chemical means (H_3 receptor ligands). Furthermore, we recently found that constitutive activity seems to be a general characteristic of viral GPCRs, suggesting that the concept of constitutive GPCR activity is successfully exploited by a variety of pathogenic herpes viruses (e.g. HCMV and KSHV). As for most GPCRs, also for constitutively active viral GPCRs (endogenous) and synthetic ligands, can be found that shut down agonist-independent signalling, thereby acting as potential new anti-viral therapeutics.

Keywords: GPCR; Constitutive activity; Inverse agonism; Histamine receptor; Viral GPCR; ORF74; US28

1. Introduction

The concept of constitutive activity of G protein-coupled receptors (GPCRs) and the development of ligands, modulating this activity (agonist or inverse agonists) are currently attracting lots of interest. For many, clinically used GPCR antagonists inverse agonism has

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0531-5131/03
doi:10.1016/S0531-5131(03)00608-3

been discovered as a new mechanistic basis for the therapeutic action. It is only within the last decade that it became apparent that GPCRs display constitutive activity in the absence of agonist stimulation [1]. The occurrence of GPCR activity in the absence of agonist stimulation was first reported for the wild-type δ-opioid receptor in NG108-15 [2], and has now been described for a variety of (mutant) GPCRs [1].

2. Constitutive activity of histaminergic receptors

Currently, four histaminergic receptor subtypes have been identified [3]. Research in various laboratories revealed that these wild type receptors display considerable levels of constitutive activity, which had substantial consequences for the classification of histaminergic antagonists [4–8]. Initially, we observed that the H_2 receptor stably expressed in CHO cells displays constitutive activity at physiological receptor levels [4]. Interestingly, therapeutically important H_2 antagonists (cimetidine, famotidine and ranitidine), previously thought to act as competitive antagonists, actually act as inverse agonists. The first described H_2 antagonist burimamide was identified as a ligand with very low or no intrinsic activity [4], thereby behaving like a neutral antagonist. As expected, the neutral antagonist burimamide is able to block the effects of both an agonist (histamine) and an inverse agonist (cimetidine) on the cAMP levels in CHO cells expressing the H_2 receptor [9].

Recently, we have shown that also the human histamine H_1-receptor constitutively activates both PLC and NF-κB upon over-expression in COS-7 cells [5]. Importantly, all therapeutically important H_1 antagonists act as full inverse agonists at the H_1 receptor. In addition, we and other groups have shown that the recently cloned H_3 receptor also displays an unprecedented high level of constitutive activity [6,7]. Finally, also the recently discovered histamine H_4 receptor shows a considerable level of constitutive activity [8]. As such, all four histamine receptor subtypes show a high level of constitutive activity and actually do not need various reported techniques to measure inverse agonistic efficacies. It seems as if the histamine receptors, with the H_3 receptor as an outstanding example, show a high degree of conformational flexibility, which allows this class of GPCRs to adopt active conformation(s) quite easily.

2.1. Constitutive activity of histamine receptors depends on the cellular context

Agonists are known to have different intrinsic activities in different cell systems. The degree of constitutive GPCR activity and the concomitant inverse agonistic behaviour of GPCR ligands are also highly dependent upon the cellular context. One of the main determinants for constitutive GPCR signalling is the expression level of the respective GPCR, which has been shown for the H_1, H_2 and H_3 receptors. Transient expression of the histamine H_1 receptor in COS-7 cells leads to an expression-dependent increase in NF-κB activity as measured using a luciferase NF-κB reporter-gene assay [5]. For the H_3 receptor, we have recently cloned three new receptor isoforms, which are generated by alternative splicing of the last intron, resulting in proteins with only six transmembrane domains. These new isoforms do not bind H_3 receptor radioligands, do not signal in response to H_3 agonists, but are able to reduce membrane expression and signalling of the H_3 receptor

isoforms (unpublished data Leurs et al.). As such, these new isoforms can be seen as endogenous regulators of H_3 receptor signalling. It will be interesting to see if the expression levels of these isoforms are highly regulated under various pathophysiological conditions. Constitutive H_1 receptor activity can also be controlled by G protein expression levels as shown by the higher constitutive activity upon increase of $G\alpha_{11}$ expression (Bakker et al., unpublished observations). As a result, the signal to noise ratio for the measurement of inverse agonism by the H_1 antagonist mepyramine is improved with higher expression levels of $G\alpha_{11}$. Similarly, overexpression of $G\alpha_q$ results in constitutive signaling of the muscarinic m_1, m_3 and m_5 receptors, which could be inhibited by muscarinic antagonists [10].

Finally, constitutive GPCR signalling is also effected by over-expression of effector enzymes. Expression of adenylate cyclase isoform 2 in NG108-15 cells increases the basal signalling of a co-expressed β_2 receptor and consequently improves the read-out for detection of inverse agonism by β_2 receptor antagonists [11]. In addition, co-expression of RGS (regulator of G protein signaling) proteins has recently also been shown to lead to an increase in basal receptor signalling [12]. These data further demonstrate the importance of the cellular context for the detection of constitutive GPCR signalling and inverse agonism.

3. Viral GPCRs: constitutive signalling as their way of life?

Viruses pirate and modify key regulatory cellular molecules to elude the immune system, to promote virus dissemination or to modulate homeostasis of the cell. Cytomegalovirus (CMV), human herpesvirus types 6 and 7 (HHV-6 and-7) and Kaposi's sarcoma-associated herpesvirus (KSHV) all contain one or more DNA sequences encoding proteins with homology to cellular GPCRs. The function of virus-encoded GPCRs (vGPCRs) is currently not clear and little is known about the signaling pathways activated by these pirated receptors [13]. The vGPCRs show highest homology to the family of chemokine receptors. Chemokines constitute a large family of small proteins of 70–80 amino acids long and are involved in the control and regulation of the immune system during homeostasis and various pathological processes (e.g. inflammation, angiogenesis, oncogenesis). Chemokine receptors have recently attracted considerable attention because some members act as crucial cellular entry factors for the HIV virus [14]. Moreover, other members of this large family are considered as interesting drug targets for, e.g. rheumatoid arthritis, multiple sclerosis, atherosclerosis, psoriasis or T-cell lymphomas [14]. In view of the important role of chemokine receptors in particular, and GPCRs in general, it is obvious that vGPCRs are interesting targets for potentially innovative antiviral drug development.

3.1. KSHV-encoded ORF74, a constitutively active GPCR acting as oncogene

KSHV is a member of the γ-herpesvirus family and is associated with the pathogenesis of, Kaposi's sarcoma (KS), the most common malignancy in HIV-infected individuals. KS is a highly angiogenic multicentric tumor associated with abundant vasculature and spindle cell proliferation. The KSHV-encoded receptor ORF74 signals in a constitutively active manner to proliferative signaling pathways [15]. NIH-3T3 cells transfected with

ORF74 cause tumors when injected in nude mice [16] and transonic mice, expressing ORF74 within hematopoietic cells develop angioproliferative lesions that morphologically resemble KS lesions [17]. These data imply that ORF74 acts as a viral oncogene. ORF74 shows highest homology to the chemokine receptor CXCR2 (IL-8 receptor) but binds both CC and CXC chemokines, in contrast to CXCR2 and other chemokine receptors [15]. Remarkably, ORF74 constitutively initiates cellular transformation, tumorigenicity and induces a switch to an angiogenic phenotype in an agonist-independent manner. ORF74 has been shown by our laboratory and others to constitutively activate a variety of signal transduction cascades including activation of phosholipase C, various kinase pathways, RhoA and transcription factors. ORF74 appears to signal to a large diversity of G-proteins, allowing it to couple to a broad range of proliferative and anti-apoptotic signaling pathways in transfected cells (see for review Ref. [18]).

3.2. Constitutive activity of HCMV-encoded receptors

Cytomegalovirus (CMV) is a highly species-specific virus belonging to the beta herpesvirus subfamily. Species homologs have been identified in various mammalian hosts, including man (HCMV), rat (RCMV) and mouse (MCMV). HCMV is endemic in all human populations, with a seroprevalence ranging from 50% to 80%. Infection of immunocompetent hosts is usually asymptomatic, seldom it can cause a febrile illness (mononucleosis). In contrast, primary infection or reactivation of the virus in immunocompromised hosts can cause severe and even fatal disease. Damage is frequently observed in the liver, the brain and the retina of these individuals. Moreover, infection with CMV has been suggested to play a role in vascular disease processes such as vascular allograft rejection, restenosis and atherosclerosis (for review, see Ref. [18]). Little is known about the mechanisms underlying the pathogenicity of this virus. An interesting hypothesis suggests that expression of virally encoded genes may modify the phenotype of the infected cell. Interestingly, HCMV encodes four putative GPCRs, namely US27, US28, UL33 and UL78. Two of these GPCRs, UL33 and UL78, have RCMV and MCMV counterparts, whereas US27 and US28 are encoded exclusively by HCMV.

Recently, we have shown that US28 constitutively signals through a $G\alpha_q$ pathway leading to activation of PLC when transiently expressed in COS-7 cells [19]. In addition, US28 can constitutively activate NF-κB, a transcription factor that plays an important role in inflammatory events, such as atherosclerosis. Interestingly, CC-chemokines, such as RANTES and MCP-1, do not modulate the basal signal of this receptor, therefore behaving as neutral antagonists [19]. On the other hand, the soluble domain of the CX3C-chemokine fractalkine inhibits approximately 35% of US28-mediated response, and is therefore classified as a partial inverse agonist. By this means, fractalkine is suggested to play a role in CMV-entry into the target cell. Future investigations should further substantiate this.

Besides US28, the RCMV-encoded chemokine receptor R33 was also shown to constitutively activate multiple signaling pathways, including phospholipase C and CRE-, SRE- and NF-κB-mediated transcription while its cellular homologue CCR3 does not [20]. Recent findings in our laboratory (Casarosa et al., unpublished observations) and Waldhoer et al. [21] confirmed the constitutive signaling for the related MCMV encoded m33 and HCMV-encoded UL33.

In contrast to their cellular partners, these virus-encoded GPCRs apparently display high levels of constitutive activity. Although the role of these vGPCRs is not clear yet, it is believed that this phenomenon is an important feature of viral pathogenesis and viruses might exploit this GPCR property to modulate the homeostasis of infected cells via their encoded GPCR(s).

3.3. Identification of a non-peptidergic inverse agonist at US28

So far, all ligands reported to bind US28 belong to the family of chemokines and no small non-peptidergic ligands have been reported yet. Using phospholipase C activation as screening approach, a small non-peptidergic molecule, VUF2274, has been identified acting as inverse agonist at HCMV-encoded chemokine receptor US28 when expressed in COS-7 cells [22]. To our knowledge, this represents the first example of a non-peptidergic inverse agonist for US28 and in general for virus-encoded GPCRs.

Constitutive activity displayed by other vGPCRS, ORF74 and R33 and the histamine H_1 receptor, also a G_q-coupled receptor, is not affected by VUF2274 confirming that the inhibition at US28 is not due to a non-specific blockade of a downstream component in the phospholipase C signaling cascade or modulation of G protein function.

The ability of VUF2274 to act as inverse agonist at US28 was also tested in HCMV-infected human foreskin fibroblasts (HFF). This represents a more physiological system, since US28 expression is regulated by the virus and not by transfection. In collaboration with Mertens et al., we observed that HCMV infection of HFF is accompanied by a consistent increase in the intracellular levels of inositol phosphates [22]. This increase in PLC activity is not observed in cells infected with the deletion virus in which US28 is deleted (Δ28). As expected, the CX3C-chemokine fractalkine could partially inhibit the US28-mediated InsP production, while not affecting the signalling in Δ28- or mock-infected cells. These data are in line with previous results in COS-7 cells [19] and confirm the direct role played by US28 in PLC activation after infection with HCMV. Importantly, VUF2274 dose-dependently inhibited US28-mediated signalling in HCMV-infected fibroblasts [22]. These results, in line with data obtained in COS-7 cells, confirm that VUF2274 is acting as inverse agonist at US28 in HCMV-infected HFFs.

4. Final perspectives

The new concept of constitutive GPCR activity has received considerable attention in recent years. It is clear that constitutive activity of GPCRs highly depends on the 'cellular context' and, thus, will be tissue specific and differ among species and individuals. Interestingly, virus-encoded GPCRs display a high level of constitutive activity. This property might be a general characteristic of vGPCRs and viruses might exploit this GPCR property to modulate the homeostasis of infected cells via their encoded GPCR(s). As identified for the CMV-encoded receptor US28, the development of vGPCR-selective inverse agonists is essential for the elucidation of their role in viral pathogenesis and will serve as putative leads for innovative antiviral drug design.

References

[1] R.A. de Ligt, A.P. Kourounakis, A.P. IJzerman, Inverse agonism at G protein-coupled receptors: (patho)-physiological relevance and implications for drug discovery, Br. J. Pharmacol. 130 (1) (2000) 1–12.
[2] T. Costa, A. Herz, Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 7321–7325.
[3] L.B. Hough, Genomics meets histamine receptors: new subtypes, new receptors, Mol. Pharmacol. 59 (3) (2001) 415–419.
[4] M.J. Smit, R. Leurs, A.E. Alewijnse, J. Blauw, G.P.V. Amerongen, Y. Vandevrede, E. Roovers, H. Timmerman, Inverse agonism of histamine H_2 antagonists accounts for upregulation of spontaneously active histamine H_2 receptors, Proc. Natl. Acad. Sci. U. S. A. 93 (13) (1996) 6802–6807.
[5] R.A. Bakker, S.B. Schoonus, M.J. Smit, H. Timmerman, R. Leurs, Histamine H(1)-receptor activation of nuclear factor-kappa B: roles for G beta gamma-and G alpha (q/11)-subunits in constitutive and agonist-mediated signaling, Mol. Pharmacol. 60 (5) (2001) 1133–1142.
[6] K. Wieland, G. Bongers, Y. Yamamoto, T. Hashimoto, A. Yamatodani, W.M. Menge, H. Timmerman, T.W. Lovenberg, R. Leurs, Constitutive activity of histamine h(3) receptors stably expressed in SK-N-MC cells: display of agonism and inverse agonism by H(3) antagonists, J. Pharmacol. Exp. Ther. 299 (3) (2001) 908–914.
[7] S. Morisset, A. Rouleau, X. Ligneau, F. Gbahou, J. Tardivel-Lacombe, H. Stark, W. Schunack, C.R. Ganellin, J.C. Schwartz, J.M. Arrang, High constitutive activity of native H3 receptors regulates histamine neurons in brain, Nature 408 (6814) (2000) 860–864.
[8] K.L. Morse, J. Behan, T.M. Laz, R.E. West Jr., S.A. Greenfeder, J.C. Anthes, S. Umland, Y. Wan, R.W. Hipkin, W. Gonsiorek, N. Shin, E.L. Gustafson, X. Qiao, S. Wang, J.A. Hedrick, J. Greene, M. Bayne, F.J. Monsma Jr., Cloning and characterization of a novel human histamine receptor, J. Pharmacol. Exp. Ther. 296 (3) (2001) 1058–1066.
[9] A.E. Alewijnse, M.J. Smit, H. Hoffmann, D. Verzijl, H. Timmerman, R. Leurs, Constitutive activity and structural instability of the human H_2 receptor, J. Neurochem. 71 (1998) 799–807.
[10] E.S. Burstein, T.A. Spalding, H. Brauner-Osborne, M.R. Brann, Constitutive activation of muscarinic receptors by the G-protein Gq, FEBS Lett. 363 (3) (1995) 261–263.
[11] P.A. Stevens, G. Milligan, Efficacy of inverse agonists in cells overexpressing a constitutively active beta2-adrenoceptor and type II adenylyl cyclase, Br. J. Pharmacol. 123 (2) (1998) 335–343.
[12] P.J. Welsby, E. Kellett, G. Wilkinson, G. Milligan, Enhanced detection of receptor constitutive activity in the presence of regulators of G protein signaling: applications to the detection and analysis of inverse agonists and low-efficacy partial agonists, Mol. Pharmacol. 61 (5) (2002) 1211–1221.
[13] C. Vink, M.J. Smit, R. Leurs, C.A. Bruggeman, The role of cytomegalovirus-encoded homologs of G protein-coupled receptors and chemokines in manipulation of and evasion from the immune system, J. Clin. Virol. 23 (1–2) (2001) 43–55.
[14] P.M. Murphy, M. Baggiolini, I.F. Charo, C.A. Hebert, R. Horuk, K. Matsushima, L.H. Miller, J.J. Oppenheim, C.A. Power, International union of pharmacology, XXII. Nomenclature for chemokine receptors, Pharmacol. Rev. 52 (1) (2000) 145–176.
[15] L. Arvanitakis, E. Geras-Raaka, A. Varma, M.C. Gershengorn, E. Cesarman, Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation, Nature 385 (6614) (1997) 347–350.
[16] C. Bais, B. Santomasso, O. Coso, L. Arvanitakis, E.G. Raaka, J.S. Gutkind, A.S. Asch, E. Cesarman, M.C. Gershengorn, E.A. Mesri, M.C. Gerhengorn, G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator, Nature 391 (6662) (1998) 86–89.
[17] T.Y. Yang, S.C. Chen, M.W. Leach, D. Manfra, B. Homey, M. Wiekowski, L. Sullivan, C.H. Jenh, S.K. Narula, S.W. Chensue, S.A. Lira, Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma, J. Exp. Med. 191 (3) (2000) 445–454.
[18] M.J. Smit, C. Vink, D. Verzijl, P. Casarosa, C.A. Bruggeman, R. Leurs, Virally encoded G protein-coupled receptors: targets for potentially innovative anti-viral drug development, Current Drug Targets (2002) (in press).

[19] P. Casarosa, R.A. Bakker, D. Verzijl, M. Navis, H. Timmerman, R. Leurs, M.J. Smit, Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28, J. Biol. Chem. 276 (2) (2001) 1133–1137.
[20] Y.K. Gruijthuijsen, P. Casarosa, S.J. Kaptein, J.L. Broers, R. Leurs, C.A. Bruggeman, M.J. Smit, C. Vink, The rat cytomegalovirus R33-encoded G protein-coupled receptor signals in a constitutive fashion, J. Virol. 76 (3) (2002) 1328–1338.
[21] M. Waldhoer, T.N. Kledal, H. Farrell, T.W. Schwartz, Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities, J. Virol. 76 (16) (2002) 8161–8168.
[22] P. Casarosa, W.M. Menge, R. Minisini, C. Otto, J. van Heteren, A. Jongejan, H. Timmerman, B. Moepps, F. Kirchhoff, Th. Mertens, M.J. Smit, R. Leurs, Identification of the first non-peptidergic inverse agonist for a constitutively active viral-encoded G-protein coupled receptor, J. Biol. Chem. 278 (7) (2003) 5172–5178.

Discussion 11

P. Strange

The results on the interaction between 5-HT_{1B} and H_1 receptors were very interesting. Would you like to speculate on the mechanisms behind that?

R. Leurs

It's not exclusively H_1-5-HT_{1B}. It's a more general G_q/G_i coupling. In our view this really has to do with crosstalk at signalling level. At the moment we are studying whether $\beta\gamma$ that comes from one will be available for the G_q-coupled receptors. Because if you co-express the H_1 receptor with different combinations of $\beta\gamma$, you see that for this type of signalling there is preference for certain combinations but not for others. And this could be a way to influence signalling through the H_1 receptor if you have G_i-coupled receptors activated.

G. Milligan

I wasn't terribly sure with the histamine H_3 C-terminal splice variants, whether you feel it's actually stopping the expression of the traditional one, or actually retaining it intracellularly, a bit like the D3 NF construct.

R. Leurs

I think at the moment we can't really distinguish. They probably keep them inside. There should be a way to tackle that, and at the moment we haven't found the experimental tools to do it. But what we would like to do is to try and see if you could liberate them again so they can go up again. If you co-express it with other types of proteins and other types of receptors, you don't modulate the expression of the H_{3A} receptor. So it's not a competition at the level of protein production or such.

G. Milligan

Maybe we can discuss it later, but we've developed a kind of take on the ion channel ER-trapping approach for GPCRs to look for which ones interact together that might be useful for this.

R. Adan

Are the histamine 3D, E and F isoforms expressed in vivo, as well?

R. Leurs

I haven't shown the data, but we've done in situ hybridisation, and they are expressed in the brain, and in fact you have a distinct pattern of expression. Depending on the brain area you will have co-localization of the A and the D isoforms, to some extent, whereas in other brain areas you hardly have expression of the D, whereas you have expression of one

of the functional isoforms. Things will get interesting if this might change pathophysiological conditions. We've heard that, the Homer protein is suddenly up-regulated upon epileptic seizures. So this is a new area that's developing. On the other hand within the H_3 receptor field, this whole isoform stuff is really getting complicated, because in the human gene splicing is similar but still you have different isoforms. So you have the complexity like we have for the A3, for the rat H3, but in fact the real isoforms, their structural characteristics are different from the rat situation.

T. Schwartz

One thing that I just want to mention is that there's an NPY receptor, I think it's called Y6, which at least in man lacks TM7 that might be doing something similarly. I'm not aware that people have yet done co-transfections with that. Some of the experiments you have done in virally encoded receptors are really very interesting. Because that's sort of getting close to real life. What you do in your measurements, you're doing it sort of days after the infection?

R. Leurs

The measurements were done 15 hours after infection. Of course, kinetic experiments would be interesting; your hypothesis that the viral particle contains US28 molecules and that these receptor molecules will be introduced in the membrane early after infection is interesting. I fully support your idea to do similar experiments early after virus infection. But we haven't done it.

T. Schwartz

In in vitro ways we're doing the experiments, it may not be that important, because several of these genes are dispensable when you grow them in culture, whereas they are clearly important when you do it in vivo. That's true for a lot of the animal virus ones. It may be very difficult to do the right experiments. The antagonist you got there is apparently a CCR1 antagonist?

R. Leurs

It's a CCR1 antagonist as well, and of course its affinity is much better on CCR1 receptors than on US28. However, we've identified also other molecules having similar structural components where CCR1 activity is down. We have for example some H_1 receptor ligands that show also micromolar affinity for US28. There are ways to play with those type of ligands, to sort out the proper pharmacology.

T. Schwartz

From your slide it looks like you have identified this glutamic acid in TM7 as interacting amio acid with the small molecule. Have you shown that by mutagenesis?

R. Leurs

Yes, we've been mutating this residue to either alanine or glutamine. This glutamic acid residue is a highly conserved residue within the chemokine receptor family. For the CCR2 receptor this residue has been shown to interact with structurally similar small molecules. Mutation of this residue in US28 does not affect chemokine binding, but the affinity for the inverse agonist drops 30-fold.