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# Evidence against adenosine analogues being agonists at the growth hormone secretagogue receptor

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**Abstract**

Adenosine and adenosine analogues have been reported to act as agonists or partial agonists at the growth hormone secretagogue receptor 1a (GHSR1a). We have re-examined this question. A concentration-dependent increase in intracellular calcium concentration ($[Ca^{2+}]_i$) was observed in GHSR1a transfected HEK 293-EBNA cells stimulated with adenosine (EC50: 0.2 μM) or 2-chloroadenosine (EC50: 1.1 μM) but also in untransfected HEK 293-EBNA cells stimulated with 2-chloroadenosine (EC50: 0.67 μM) or 5′-*N*-ethylcarboxamidoadenosine (NECA) (EC50: 0.045 μM). These findings support endogenous expression of adenosine receptors, presumably $A_{2B}$ receptors in HEK 293-EBNA cells. In GHSR1a transfected CHO cells, lacking adenosine receptors, the GHSR1a agonist hGhrelin (EC50: 2.4 nM) increased $[Ca^{2+}]_i$, but no effects of adenosine, 2-chloroadenosine or NECA were detected. An inverse agonist of GHSR1a, [D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11] substance P, reduced hGhrelin effects but adenosine, 2-chloroadenosine or 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) did not. NECA increased the $[Ca^{2+}]_i$ in co-transfected (GHSR1a and $A_{2B}$ receptor) CHO cells (EC50: 0.053 μM), but no additive or synergistic effects on $[Ca^{2+}]_i$ or cAMP formation were observed after stimulation with NECA in the absence or in the presence of hGhrelin. In binding studies on GHSR1a transfected CHO cell membranes, [$^{125}$I]-hGhrelin binding could be displaced by the GHSR1a agonist MK-0677 (IC50: 0.34 nM), hGhrelin (IC50: 1.5 nM), and the substance P analogue (IC50: 0.64 μM) but not by adenosine or 2-chloroadenosine. We conclude that adenosine and analogues do not act as agonists or partial agonists at the GHSR1a and that cross-talk between the GHSR1a and $A_{2B}$ receptors is limited.


*Keywords:* GHSR1a; Adenosine $A_{2B}$ receptor; Ghrelin; CHO cells; HEK 293 cells

## 1. Introduction

The growth hormone secretagogue receptor 1a (GHSR1a) is a G protein-coupled seven transmembrane receptor [1] that is abundantly expressed not only in hypothalamus and pituitary, but also found in other areas of the brain [1,2]. Ghrelin, a 28 amino acid peptide hormone predominantly synthesized by endocrine cells in the stomach [3–5], was found to be a ligand at the GHSR1a [3].

Ghrelin, acting at GHSR1a, mediates both important endocrine and non-endocrine effects in the body; for reviews see [6,7]. For example, ghrelin is believed to control food intake and energy homeostasis. Ghrelin is released from endocrine cells in the stomach mucosa to provide a sensation of hunger that triggers meal initiation in humans [8].

For these reasons, and given the epidemic proportions of obesity-related diseases, GHSR1a is an interesting potential drug target, and the recent reports that the endogenous nucleoside adenosine is an agonist or partial agonist at the receptor are intriguing [9–11]. We have therefore re-examined this question and also studied whether there are any interactions between the adenosine $A_{2B}$ receptor [12] and the GHSR1a. We examined different cell types to explore if presence of GHSR1a invariably renders a cell responsive to adenosine analogues. We also examined if adenosine analogues could displace ghrelin from the receptor, and if adenosine could behave as a partial agonist to actually reduce responses to ghrelin.

Our results do not support the notion that adenosine or adenosine analogues are always agonists or partial agonists

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at GHS receptors and we also found only a very limited communication between the GHSR1a and $A_{2B}$ receptors in signaling. Hence, adenosine analogues are unlikely to become useful drugs for the GHSR1a target.

## 2. Materials and methods

### 2.1. Materials

5′-*N*-Ethylcarboxamidoadenosine (NECA), 2-chloroadenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), adenosine, substance P analogue ([D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11] substance P), probenicid, bovine serum albumin (BSA), aprotinin, leupeptin and pepstatin were purchased from Sigma (St Louis, MO). MK-0677 was from Biovitrum (Stockholm, Sweden). Human ghrelin (hGhrelin) was from Neosystems (Strasbourg, France) or Bachem (Bubendorf, Switzerland). Hygromycin B was from Roche Diagnostics (Mannheim, Germany). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). [His[$^{125}$I]]-Ghrelin (human) 25 μCi (925 kBq) was obtained from Perkin-Elmer (Boston, MA). Homogeneous time-resolved fluorescence (HTRF) assay reagents were from CIS bio international (Bagnols-sur-Ceze, France). Wheat germ agglutinin (WGA) Pvt. SPA scintillation beads (RPNQ 0001) were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Protein Kits and Micro BCA™ Protein Assay Reagent Kit were purchased from Pierce (Rockford, IL). Cell culture medium and additions were all from Gibco-Life Technologies (Täby, Sweden).

### 2.2. Cell culture

Human embryonic kidney 293 cells that constitutively express the Epstein–Barr virus nuclear antigen 1 (EBNA1) protein (HEK 293-EBNA cells), transiently transfected with the human GHSR1a EBNA plasmid construct PBV1051 (Biovitrum, Sweden) were cultured in Dulbecco's modified Eagle's medium (DMEM), glutamax, high glucose 4500 mg/l, 10% fetal bovine serum (FBS) and 250 μg/ml geneticin (G-418). Ordinary HEK 293-EBNA cells were cultured under the same conditions but without the selective antibiotic G-418.

Chinese hamster ovary (CHO) cells stably transfected with the human GHSR1a construct (Euroscreen, Belgium) were cultured in Ham's F12 medium, 10% FBS and 400 μg/ml G-418. Untransfected CHO cells were maintained under the same conditions but without G-418. The CHO cells stably transfected with the $A_{2B}$ receptor [13] were cultured in DMEM/F12 (1:1), 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 200 μg/ml G-418. The cells were grown adherent in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. Cells were sub-cultured two or three times weekly at a ratio between 1:3 and 1:10.

### 2.3. Transient transfections

For the transient transfection of the GHSR1a in the HEK 293-EBNA cells, 25,000 cells were seeded in 96-well poly-D-lysine coated plates (Biocoat™, Becton and Dickinson, Heidelberg, Germany) and the medium was replaced with G-418-free medium after 24 h. The cells were transfected by using the Lipofectamine 2000 kit according to the manufacturer's instructions. Briefly, 0.2 μg GHSR1a plasmid was diluted in 25 μl Opti-MEM (without serum) and 0.6 μl Lipofectamine 2000 was mixed gently with 25 μl Opti-MEM and incubated for 5 min at room temperature. The diluted GHSR1a plasmid was then gently mixed with the Lipofectamine 2000 solution followed by 20 min incubation at room temperature. Fifty microliters of GHSR1a plasmid–Lipofectamine 2000 complex was then added to each well in the 96-well plates. The growth medium was replaced with medium containing G-418 6 h after transfection. CHO cells stably transfected with the GHSR1a were transiently transfected with the $A_{2B}$ receptor in the same way as described above for the HEK 293-EBNA cells.

For the transient transfection of the GHSR1a in the CHO cells stably transfected with the $A_{2B}$ receptor, 1,750,000 cells were seeded out and grown overnight in 10 cm Petri dishes in medium without antibiotics. The CHO cells were then transfected with the GHSR1a plasmid. Twenty-four micrograms of GHSR1a plasmid was diluted in 1.5 ml Opti-MEM. At the same time, 60 μl Lipofectamine 2000 was diluted in 1.5 ml Opti-MEM and incubated for 5 min at room temperature. The diluted Lipofectamine 2000 was gently mixed with the diluted GHSR1a plasmid and after 20 min incubation at room temperature, added to the cells in the Petri dishes. The cells were resuspended after 24 h and 150,000 cells per well were seeded out in 24-well plates. The assays were performed 48 h after transfection.

### 2.4. Intracellular calcium concentration measurements

Approximately 30,000 cells per well were seeded out in 0.2 ml assay medium in 96-well poly-D-lysine coated plates and were then pre-incubated at 5% $CO_2$, 37 °C overnight. The plates were washed with preheated loading buffer (Hank's Balanced Salt Solution; HBSS with 15 mM HEPES containing 2.5 mM probenicid), pH 7.4. Then 100 μl Fluo-4 AM solution (4 μM) was added to each well followed by a 1 h incubation at 37 °C. The cells were then washed twice with the loading buffer and 100 μl buffer was added per well. After a 5 min equilibration period, $[Ca^{2+}]_i$ was measured in a Fluoroscan Ascent Spectrofluorometer (Labsystems, Hants, UK) with the plate chamber at 37 °C. The excitation wavelength was set to 488 nm and the emission wavelength to 538 nm. Background was subtracted. The fluorescence response was calculated in activity base (IDBS), an EXCEL-based result calculation program.

### 2.5. Membrane preparation

When the CHO cells had reached 90% confluence, they were rinsed twice with cold PBS ($Ca^{2+}$ and $Mg^{2+}$ free) and then suspended in cold buffer A containing 15 mM Tris–HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, pH 7.5 by using a rubber window scraper. After centrifugation at 1500 × *g* for 3 min at 4 °C the cells were resuspended in buffer A and homogenized using a Polytron homogenizer (4 × 30 s) at 4 °C. The homogenate was centrifuged at 40,000 × *g* for 25 min at 4 °C. The pellets were washed once with buffer A by mixing and centrifuged again. The pellets were suspended in buffer B containing 7.5 mM Tris–HCl, 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 25 mM sucrose, pH 7.5 and gently homogenized several times with a glass homogenizer. The membrane preparation was frozen in aliquots at −70 °C until used. The protein concentration was determined with a protein assay kit from Pierce.

### 2.6. Membrane GHSR1a binding

Scintillation proximity assay (SPA, Amersham Pharmacia Biotech) was used for GHS receptor binding. The wheat germ agglutinin beads were reconstructed with reaction buffer containing 50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4 to 40 mg/ml as a stock suspension. The beads and the membrane preparation were pre-incubated for 30 min at room temperature with gentle shaking. The bead suspension was centrifuged at 3400 rpm for 2 min and the beads were resuspended with binding buffer containing 25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% BSA, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin, pH 7.4. The radiolabeled [$^{125}$I]-hGhrelin was diluted with cold hGhrelin in the ratio of 1:3. The binding experiments were performed using 96-well microtiter plates. The total volume in each well was 200 μl, containing 0.25 mg beads, 4 μg membrane protein and 0.8 nM [$^{125}$I]-hGhrelin. The plate was gently shaken for 30 min and incubated overnight. The samples were counted using Microbeta counter (Wallac Trilux 1450 Microbeta counter, Turku, Finland) and the data were analyzed in the computer program GraphPad Prism (San Diego, CA).

### 2.7. cAMP assay

The homogeneous time-resolved fluorescence assay (CIS Bio International, France) was used for measuring the cAMP levels in the HEK 293-EBNA cells and in the CHO cells stably transfected with the GHSR1a with or without transiently transfected $A_{2B}$ plasmid. In the HTRF assay, the cells were seeded out in 96-well poly-D-lysine coated plates. After 24 h the cells were washed twice with serum-free medium and 100 μl of test substance solution was dispensed to each well in the cell plates. After 30 min of incubation at room temperature, 100 μl of conjugate and lysis buffer were added and after another 90 min of incubation, 30 μl of suspension was transferred to each well in 96-half-well readout plates (Costar, Cambridge, MA) with addition of 15 μl MAb anti-cAMP conjugated to europium cryptate, the donor, and 15 μl cAMP-XL665 conjugate, the acceptor. Samples without cAMP-XL665 were used as negative controls. The plates were incubated for 2.5 h and then read out with a spectrofluorometer (Wallac 1420 Victor$^2$V, Wallac Oy/Perkin-Elmer, Boston, MA) by using 665 nm and 620 nm. The fluorescence ratio of 665 nm/620 nm signals was calculated.

The cAMP levels in the CHO cells stably transfected with the $A_{2B}$ receptor with or without the transiently transfected GHSR1a plasmid were determined by using a protein binding assay. Briefly, 48 h after transfection with the GHSR1a plasmid, the CHO cells were washed twice with DMEM, 20 mM HEPES, pH 7.4. After a 10 min pre-incubation, the cells were stimulated with different concentrations (0.1–30 μM, higher concentrations were not used as they can cause effects not related to receptors) of NECA with or without presence of hGhrelin (0.01 or 0.1 μM) for 15 min at 37 °C. The reaction was stopped by addition of perchloric acid (final concentration of 0.4 M) and incubation on ice. After lysis and neutralization with KOH, the cAMP content in the supernatant was determined using the competitive [$^3$H]-cAMP-protein binding assay [14].

### 2.8. Statistical analysis

The dose–response curves were analyzed with GraphPAD Prism software (GraphPAD, San Diego, CA). The curves were calculated by using non-linear regression. Data are expressed as geometric mean with 95% confidence limits in parentheses. In each experiment the highest measured value was set to 100% and other values (after subtraction of blank) were related to it.

## 3. Results

### 3.1. Intracellular calcium concentration measurements

We first wanted to confirm the reported finding (see Section 1) that HEK 293 cells transfected with GHSR1a respond to adenosine or adenosine derivatives. We used HEK 293-EBNA cells transiently transfected with GHSR1a. Since GHSR1a signals via $G_{q/11}$ to increase the intracellular calcium concentration ($[Ca^{2+}]_i$) this was the response measured. As shown in Fig. 1A, adenosine and 2-chloroadenosine were indeed able to cause a concentration-dependent increase in $[Ca^{2+}]_i$ in HEK 293-EBNA cells transiently transfected with the GHSR1a. In fact, adenosine and the analogue were as efficacious as hGhrelin, but potency was different. Adenosine had an

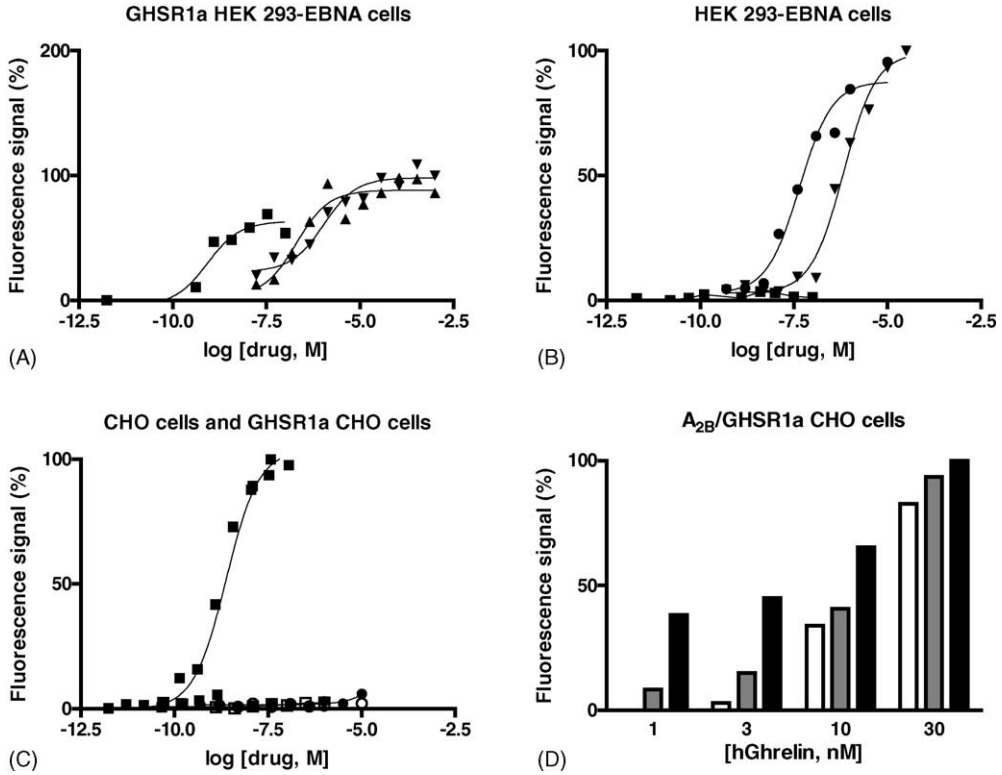


Fig. 1. Intracellular calcium concentration measurements; Panel A, GHSR1a transfected HEK 293-EBNA cells stimulated with increasing concentrations of hGhrelin (■), adenosine (▲) and 2-chloroadenosine (▼). Panel B, untransfected HEK 293-EBNA cells stimulated with NECA (●), 2-chloroadenosine (▼) and hGhrelin (■). Panel C, GHSR1a transfected CHO cells stimulated with hGhrelin (■) and NECA (●) and untransfected CHO cells stimulated with hGhrelin (□) and NECA (○). Panel D, co-transfected CHO cells (stably transfected with the GHSR1a and transiently transfected with the $A_{2B}$ receptor) stimulated with increasing concentrations of hGhrelin (white bars), in combination with 1 μM NECA (grey bars) and in combination with 3 μM NECA (black bars). There was no measurable effect when the cells were stimulated with 1 nM hGhrelin alone. All experiments were preformed in triplicate, except the experiment showed in panel B (single samples). The graphs show one representative experiment, but all experiments were performed at least three times.

EC50 value of 0.20 (0.015–2.6) μM (means and 95% confidence intervals) and 2-chloroadenosine 1.1 (0.17–6.9) μM. hGhrelin was about 1000 times more potent (EC50 value 0.80 (0.25–2.7) nM). The EC50 values reported in previous studies have been 2 μM for adenosine and 600 nM for NECA in HEK 293 cells stably expressing both the pig GHS receptor and the bioluminescence reporter gene aequorin [9] and 50 nM for adenosine in baby hamster kidney (BHK) cells transfected with the human GHS receptor [10]. The differences in EC50 values between our results and previous performed studies may, for example, be due to the use of different cell types.

We next examined if adenosine analogues were also active in HEK 293-EBNA cells not transfected with GHSR1a and we found that this was indeed the case (Fig. 1B). We could not directly compare the efficacy of adenosine analogues in transfected and untransfected cells, but the potency of 2-chloroadenosine was very similar (EC50 value 0.67 (0.44–1.02) μM). Another stable adenosine analogue, NECA, was approximately 10 times more potent (EC50 value 0.045 (0.021–0.097) μM). In these cells hGhrelin was not able to induce a rise in $[Ca^{2+}]_i$. Thus, our results indicate that HEK 293-EBNA cells possess a considerable number of endogenous $A_2$ receptors, presumably $A_{2B}$ receptors, irrespective of whether GHS receptors are present or not.

In HEK 293 cells that do not constitutively express the Epstein–Barr virus nuclear antigen 1 protein (EBNA1) and that are transfected with the GHSR1a, adenosine was also active, albeit possibly with a somewhat lower potency (EC50 value 2.5 μM, data not shown).

We therefore switched to CHO cells, which have no endogenous expression of $A_{2B}$ receptors [13,17]. Indeed, adenosine analogues did not raise $[Ca^{2+}]_i$ in these cells. There was no calcium signal in response to hGhrelin in untransfected CHO cells, but hGhrelin raised the $[Ca^{2+}]_i$ in CHO cells transfected with the GHSR1a. The EC50 value was 2.4 (1.5–3.8) nM. NECA was not able to induce an intracellular calcium response in either untransfected CHO cells or in CHO cells transfected with the GHSR1a (Fig. 1C), and adenosine and 2-chloroadenosine could not induce a response in CHO cells transfected with the GHSR1a (data not shown). This was not due to the inability of CHO cells to respond to adenosine analogues, since NECA elicited a rise in $[Ca^{2+}]_i$ in CHO cells transfected with both the GHS and the $A_{2B}$ receptors (Fig. 1D).

One characteristic of a partial agonist is that it should act as an antagonist of a full agonist. We therefore tested if that

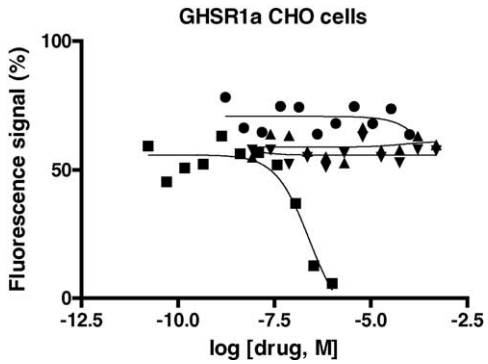


Fig. 2. Adenosine, 2-chloroadenosine and DPCPX did not antagonize hGhrelin signaling, but the substance P analogue did. The figure shows the calcium response when CHO cells transfected with GHSR1a were stimulated with increasing concentrations of adenosine (▲), 2-chloroadenosine (▼), DPCPX (●) and the substance P analogue (■), all in the presence of 4.8 nM hGhrelin. Stimulation with hGhrelin (0.1 μM) alone represents the maximal fluorescence response (100%). The experiment was preformed in triplicate. The graph shows one representative experiment, but the experiment was performed three times.

is indeed the case in CHO cells transfected with the GHSR1a. However, as seen in Fig. 2, adenosine did not influence the response to hGhrelin. The stable adenosine analogue 2-chloroadenosine and the adenosine $A_1$ receptor antagonist DPCPX were likewise ineffective. However, the substance P analogue, which has been reported to act as an inverse agonist of GHSR1a [18] did block the hGhrelin-induced response, showing that it can be blocked.

### 3.2. Radioligand binding assay

We next examined the binding to the GHSR1a by competitive binding experiments with the radioligand [$^{125}$I]-hGhrelin in membrane preparations from CHO cells expressing GHSR1a. In our experiments, the average $K_d$ (dissociation constant) value of hGhrelin was 0.3 nM, which is consistent with previous studies and the results with HEK 293-EBNA cells. As seen in Fig. 3 both the synthetic GHSR1a agonist MK-0677, and hGhrelin were able to displace [$^{125}$I]-hGhrelin binding with high potency. The IC50 values were 0.34 (0.32–0.35) nM and 1.5 (1.3–1.6) nM, respectively. The substance P analogue showed a rather low potency (IC50 value 0.64 (0.56–0.74) μM) in binding to the receptor, but the potency was similar to that seen in the functional assay (Fig. 2). However, neither 2-chloroadenosine nor NECA could influence the [$^{125}$I]-hGhrelin binding, indicating that there was no direct interaction between the adenosine analogues and the GHSR1a.

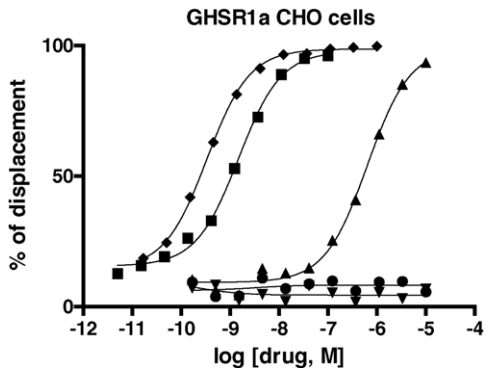


Fig. 3. Displacement of bound [$^{125}$I]-hGhrelin in membranes from GHSR1a transfected CHO cells by 2-chloroadenosine (▼), NECA (●), substance P analogue (▲), MK-0677 (◆) and hGhrelin (■). The experiment was preformed in triplicate. The graph shows one representative experiment, but the experiment was performed three times.

### 3.3. Interactions between hGhrelin and adenosine signaling

All the above data thus suggest that adenosine or adenosine analogues are not agonists or partial agonists at GHS receptors. It could, however, be that there are major interactions between the signaling pathways. Previous results have shown that signaling via $A_{2B}$ receptors can be very strongly affected by signals via other receptors [20]. We first studied if there was an additive or synergistic effect in calcium response when the co-transfected cells were stimulated with both NECA and hGhrelin, but we could not find any synergistic effect (Fig. 1D). The EC50 value of hGhrelin alone was 0.053 (0.013–0.22) μM; in combination with 1 μM NECA it was 0.081 (0.011–0.59) μM and in combination with 3 μM NECA 0.039 (0.0073–0.21) μM. An additive effect is however, seen at low doses (Fig. 1D). This is reasonable since both the $A_{2B}$ receptor and GHSR1a signal via $G_{q/11}$ [15,21,22]. The $A_{2B}$ receptor also signals via the $G_s$ subunit [23].

Finally, we investigated whether the presence or the absence of GHS receptor signaling affected signals via the $A_{2B}$ receptor. To study this we measured cAMP accumulation. NECA stimulated cAMP accumulation in a dose-dependent manner in transiently GHSR1a transfected HEK 293-EBNA cells (Fig. 4A). There was a slight increase in potency of NECA in the presence of hGhrelin (0.1 μM), even if the difference was not large or significant, Table 1.

The cAMP accumulation was also measured in CHO cells. NECA could not stimulate cAMP accumulation in cells not transfected with the $A_{2B}$ receptor, indicating that the response to NECA is entirely due to an $A_{2B}$ receptor stimulation (Fig. 4B). Both CHO cells stably transfected with the GHSR1a and transiently transfected with the $A_{2B}$ receptor and CHO cells stably transfected with the $A_{2B}$ and transiently transfected with the GHSR1a were used for the cAMP assay (Fig. 4B–D). The EC50 values are shown in Table 1. These results also show that there is no interaction between the $A_{2B}$ receptor and the GHSR1a, since it makes a very little difference in cAMP accumulation if NECA is added in the absence or in the presence of different concentrations of hGhrelin (0.01 or 0.1 μM). Fig. 4C and D show a very slight

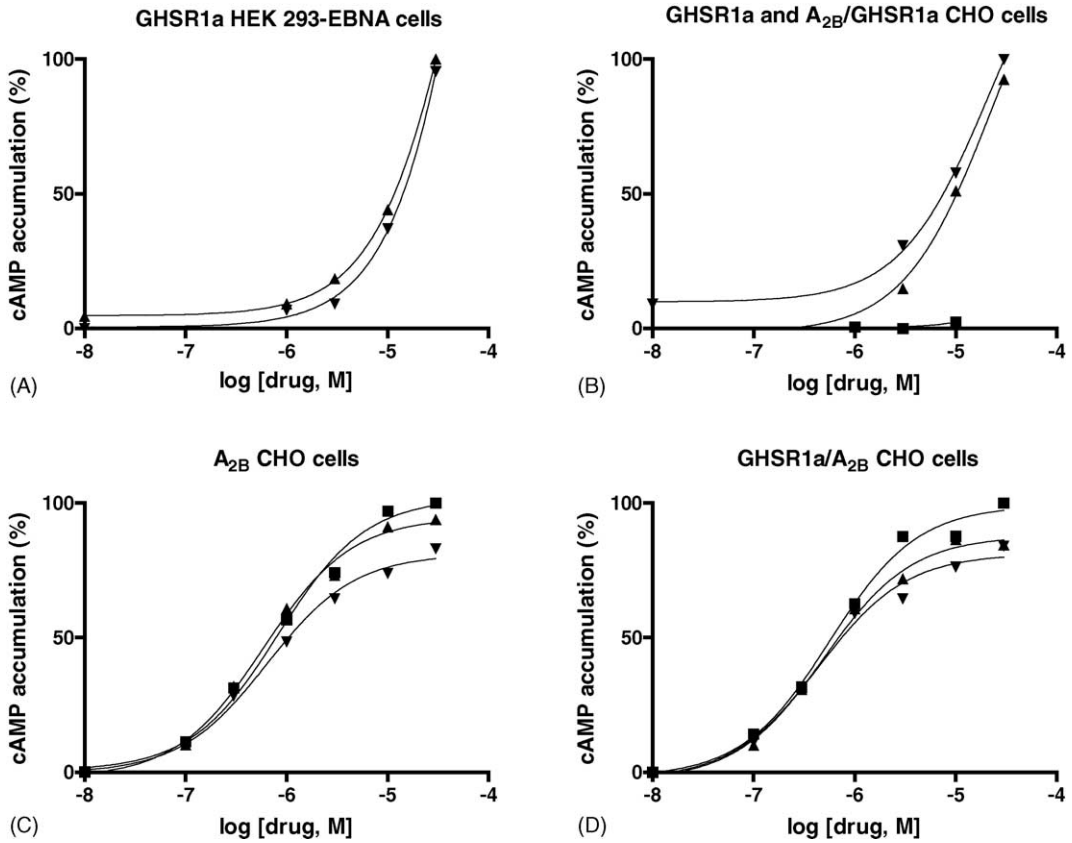


Fig. 4. Effects on cAMP accumulation. Panel A, GHSR1a transfected HEK 293-EBNA cells stimulated with NECA (▼) and NECA in presence of 0.1 μM hGhrelin (▲). Panel B, co-transfected CHO cells (stably transfected with GHSR1a and transiently transfected with the $A_{2B}$ receptor) stimulated with NECA (▼) and NECA in presence of 0.1 μM hGhrelin (▲) and in CHO cells stably transfected with GHSR1a stimulated with NECA (■). Panel C, CHO cells stably transfected with the $A_{2B}$ receptor stimulated with NECA (■), NECA in the presence of hGhrelin 0.01 μM (▲) and NECA in the presence of hGhrelin 0.1 μM (▼). Panel D, co-transfected CHO cells (stably transfected with the $A_{2B}$ receptor and transiently transfected with GHSR1a) stimulated with NECA (■), NECA in the presence of hGhrelin 0.01 μM (▲) and NECA in the presence of hGhrelin 0.1 μM (▼). The graphs show one representative experiment, but all experiments were performed at least three times.

tendency that addition of hGhrelin decreases the EC50 value (see also Table 1). However, this tendency is almost equally large when GHS receptors are present as when they are not. Furthermore, the EC50 value for NECA was somewhat lower in cells transfected with GHSR1a. All this could indicate some very minor interaction, but it is not so large that it would offer any therapeutic opportunities.

Table 1
Potency of NECA alone or in combination with 0.01 or 0.1 μM hGhrelin in HEK 293-EBNA cells, CHO cells stably transfected with GHSR1a and transiently transfected with the $A_{2B}$ receptor, CHO cells stably transfected with the $A_{2B}$ receptor and CHO cells stably transfected with the $A_{2B}$ receptor and transiently transfected with GHSR1a

| Treatment | EC50 value (μM) | 95% confidence interval |
|---|---|---|
| HEK 293-EBNA cells | | |
| NECA | 140.1 | 68.4–286.9 |
| NECA + hGhrelin 0.1 μM | 70.3 | 64.4–76.7 |
| $A_{2B}$ receptor (transiently) and GHSR1a (stably) transfected CHO cells | | |
| NECA | 21.2 | 18.0–25.0 |
| NECA + hGhrelin 0.1 μM | 22.7 | 17.6–29.2 |
| $A_{2B}$ receptor (stably) transfected CHO cells | | |
| NECA | 0.82 | 0.64–1.1 |
| NECA + hGhrelin 0.01 μM | 0.59 | 0.43–0.82 |
| NECA + hGhrelin 0.1 μM | 0.64 | 0.53–0.78 |
| GHSR1a (transiently) and $A_{2B}$ receptor (stably) transfected CHO cells | | |
| NECA | 0.56 | 0.43–0.74 |
| NECA + hGhrelin 0.01 μM | 0.49 | 0.35–0.69 |
| NECA + hGhrelin 0.1 μM | 0.44 | 0.30–0.64 |

## 4. Discussion

Our results strongly suggest that adenosine and analogues do not act as agonists or partial agonists at the GHSR1a. Although we could confirm that a HEK 293 cell line transfected with GHSR1a did respond also to adenosine, we also showed that this not was true in CHO cells (at least not in the CHO cell line that we used). Therefore we conclude that endogenous adenosine receptors, presumably $A_{2B}$ receptors appear to be present in our HEK 293-EBNA cells as well as in the HEK 293 cells used in previous studies [9–11].

Indeed, it has been shown that HEK 293 cells have an endogenous expression of the $A_{2B}$ receptor and that these receptors can trigger $[Ca^{2+}]_i$ mobilization when stimulated with NECA [15]. Similarly, BHK cells express both adenosine $A_1$ and $A_{2B}$ receptors [16]. Nevertheless previous studies [9–11], using HEK 293 and BHK cells, have reported that the calcium response mediated by adenosine and adenosine analogues was very low (albeit demonstrable) in the cells not transfected with the GHS receptor. However, our untransfected HEK 293-EBNA cells gave a strong response even when the cells were not transfected with the GHSR1a. This observation of a strong calcium response in our HEK 293-EBNA cells not transfected with GHSR1a could perhaps indicate that any transfected genes in HEK 293 and BHK cells may increase the normal expression of the $A_{2B}$ receptor in those cells, since HEK 293-EBNA cells are transfected, but with EBNA1, but other explanations are also possible.

The potency and efficacy of adenosine analogues at $A_{2B}$ receptors appears to be a function of not only the absolute level of receptor expression, but also other factors. For example, NECA was some 20 times more potent in CHO cells stably transfected with $A_{2B}$ receptors, when the receptor is presumably well integrated with the signaling machinery of the cell than in cells transiently transfected with the receptors.

In our experiments on the GHSR1a transfected CHO cells we first showed that adenosine and analogues are not able to stimulate to a rise $[Ca^{2+}]_i$. Second, we demonstrated that the calcium effect mediated by hGhrelin could not be antagonized by adenosine or analogues, despite the fact that such antagonism is a typical feature of a partial agonist. Third, we also showed that adenosine and analogues were not able to bind to the GHSR1a. These radioligand binding experiments were performed because the previous studies cited above concluded that adenosine (and analogues) bind to GHSR1a. In support of this conclusion Tullin et al. found that $[^3H]$-adenosine bound to BHK cells only if they were transfected with the GHS receptor. It should be noted, however, that $[^3H]$-adenosine is not recommended for use in adenosine receptor binding studies since it has low affinity and is metabolically unstable, being a substrate for many enzymes also present in membrane preparations [19]. In our binding studies we showed that 2-chloroadenosine and NECA are unable to displace the $[^{125}I]$-hGhrelin binding in membrane preparations from GHSR1a transfected CHO cells. Our results are in agreement with Smith et al. who reported that adenosine could not displace radiolabeled MK-0677 from GHSR1a [9]. However, these authors suggested that this is due to the substances occupying different sites on the GHS receptor. We do not favor this possibility, however, since the GHSR1a has a very short extracellular domain and drugs that bind between the helical domains of the receptor should interact when they are as bulky as the present ones. Instead we favor the parsimonious explanation that the adenosine analogues do not bind to GHSR1a. This also agrees with all our other data.

Thus, several lines of evidence indicate that adenosine is not a typical partial agonist at GHSR1a. There might be other interesting interactions between the two receptors depending on cross-talk between signaling cascades. Indeed, there is evidence that such interaction occurs between receptor coupled to $A_{2B}$ receptors, and receptors that mediate increases in intracellular calcium (e.g. [20]). However, we found that adenosine analogues caused at most a minor change in ghrelin responses, and conversely, ghrelin caused, if anything, a minor alteration in $A_{2B}$ signaling.

In conclusion, the present results strongly suggest that adenosine and adenosine analogues do not act as universal agonists or partial agonists at the GHSR1a and that cross-talk in the GHSR1 and $A_{2B}$ receptor signaling pathways is limited. Therefore, according to these results, it will not be a useful strategy to use adenosine related substances when targeting GHSR1a, e.g. for treatment of obesity-related diseases.

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