

# Adenosine does not bind to the growth hormone secretagogue receptor type-1a (GHS-R1a)

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## Abstract

Ghrelin regulates GH secretion and energy homeostasis through the GH secretagogue receptor type-1a (GHS-R1a). This G-protein coupled receptor shows the peculiarity to transduce information provided not just by ghrelin as well as by adenosine through a supposed binding site different from the characterized ghrelin-binding pocket. Indeed, adenosine triggers intracellular calcium rise through a distinct signaling pathway to the one described for ghrelin, although it fails to stimulate GH secretion. Despite multiple active conformations of GHS-R1a, suggested as an explanation for a ligand-dependent activation of the downstream signaling, the concept of adenosine as agonist for GHS-R1a has been re-evaluated. The results revealed that calcium rise of both ghrelin and adenosine appears to be mediated by receptors that did not show the same sensitivity to protein kinase C (PKC) activity in GHS-R1a-transfected HEK 293 cells (HEK-GHS-R1a cells). The binding analyses showed the

same number of adenosine-binding sites in both HEK 293 ( $B_{\max} = 2.01 \pm 0.15$  fmol/cell) and HEK-GHS-R1a cells ( $B_{\max} = 1.90 \pm 0.11$  fmol/cell). This binding was unaltered by different GHS-R1a antagonists. Western blot analysis showed a similar endogenous expression of endogenous adenosine receptor type-2b and -3 in both cell lines. The  $K_d$  values for adenosine were  $1.78 \mu\text{M}$  in HEK 293 cells and  $6.30 \mu\text{M}$  in HEK-GHS-R1a cells, pointing to a modification of agonist affinity induced by overexpression of the GHS-R1a. Additionally, adenosine failed to induce the GHS-R1a endocytosis, although it attenuates the ghrelin-induced GHS-R1a endocytosis. In conclusion, adenosine is not an agonist of the GHS-R1a and its action is mediated by the endogenous adenosine receptor type-2b and -3, which is able to partially use the intracellular signaling machinery of HEK-GHS-R1a cells.

*Journal of Endocrinology* (2006) **191**, 147–157

## Introduction

The ghrelin system is implicated in a variety of physiological events including growth hormone (GH) release, energy homeostasis, reproduction, sleep regulation, cardiovascular actions, corticotrope secretion, stimulation of lactotrope, and influence on gastroenteropancreatic functions (Korbonits *et al.* 2004, van der Lely *et al.* 2004). The ghrelin action on growth and energy homeostasis is mediated by the growth hormone secretagogue receptor type-1a (GHS-R1a), a G-protein coupled receptor, also called the ghrelin receptor (Shuto *et al.* 2002, Lall *et al.* 2004, Sun *et al.* 2004, Wettschureck *et al.* 2005). This receptor transduces information provided by ghrelin and by the group of synthetic growth hormone secretagogues (GHSs) not related structurally to the ghrelin. This property is explained by the existence of a common binding domain demonstrated by molecular modeling and site-directed mutagenesis studies developed with GHS peptide and non-peptide

agonists (Feighner *et al.* 1998). This binding site might determine that a conserved structure of the agonists recognizes a complementary conserved-binding pocket, which directs the variable part of the ligand and interacts with specific agonist-associated regions determining an overlapping in the agonist-binding site (Bondensgard *et al.* 2004). In addition, GHS-R1a appears to have other alternative ligands. This was revealed by studies carried out with adenosine (Smith *et al.* 2000, Tullin *et al.* 2000) and also with cortistatin (Deghenghi *et al.* 2001). Indeed, administration of adenosine to cells not expressing the GHS-R1a is devoid of action but when it is administered to cells expressing this receptor, adenosine triggers intracellular calcium rise, although it fails to stimulate growth hormone secretion (Tullin *et al.* 2000). Concerning intracellular calcium mobilization, a signaling pathway was proposed involving adenylate cyclase (AC)/protein kinase A (PKA)/IP<sub>3</sub>-receptors, different from those activated by ghrelin (Carreira *et al.* 2004). Based on such results, it appeared that the GHS-R1a was able to activate

different intracellular second messenger systems that appear to regulate separate agonist-dependent mechanisms for desensitization and internalization of this receptor. However, the fact that adenosine did not show cross-competition with ghrelin in binding to GHS-R1a (Camina *et al.* 2004, Carreira *et al.* 2004), a property described for cortistatin (Deghenghi *et al.* 2001), remained as discordant data, considering that it is a requisite to define alternative ligands for a specific receptor. It is important to note that the concept of adenosine as an agonist of GHS-R1a emerged from functional studies developed on cells over-expressing GHS-R1a. To date, there is growing evidence that high receptor expression levels, which are sometimes obtained in some cellular models, lead to unexpected interactions with other receptors that change the receptor properties and, inevitably, their functions. For this reason, we formulated the alternative hypothesis that adenosine does not bind to the GHS-R1a and that previous results could be fully explained by a cross-talk between two completely separated receptorial systems, the adenosine and the ghrelin systems. This is an important point taking into account, that the GHS-R1a appears to play a significant physiological role in the regulation of growth hormone secretion and, in particular, in the regulation of food intake and obesity. This converts GHS-R1a into a therapeutic target for the rational design of novel receptor ligands involved, in particular, in the regulation of the energy homeostasis. In this work, the binding characteristic and intracellular signaling activation of adenosine in cells expressing, or not, high numbers of ghrelin receptors have been addressed to assess the interplay between adenosine and ghrelin.

## Materials and Methods

### Materials

Human ghrelin was provided by Global Peptide (Fort Collins, CO, USA). Adenosine, D-Lys<sup>3</sup>-GHRP-6 and [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,D-Leu<sup>11</sup>]-substance P, phorbol 12-myristate 13-acetate (PMA), and cycloheximide were obtained from Sigma. <sup>125</sup>I-ghrelin, <sup>3</sup>H-adenosine, and the ECL detection kit was from Amersham Pharmacia Biotech. Polyallylamine hydrochloride was provided by Sigma. Glass slides were provided by Nalge Nunc (Nalge Europe Ltd, Hereford, UK). Fura-2 pentaacetoxymethylester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, USA). Anti-adenosine A3-R (H-80) rabbit polyclonal and anti-adenosine A2b-R (R-20) goat polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ghrelin analog BIM-28163 was a generous gift from Dr Michael Culler (IPSEN, Milford, MA, USA).

### HEK 293 cell line cultures

The HEK 293 cell line that stably expresses the human ghrelin receptor 1a (HEK-GHS-R1a) was a generous gift from Dr Roy Smith. The HEK-GHS-R1a cell line was

cultured in 100 mm dishes in high-glucose Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal calf serum, glutamine, and penicillin–streptomycin solution plus 500 µg/ml geneticin G-418 to 70–80% confluence for 3 days (Camina *et al.* 2004). The non-transfected HEK 293 cells were seeded in 100 mm dishes and cultured to 80% confluence for 3 days in DMEM supplemented with 10% (v/v) fetal calf serum. Media were supplemented with penicillin G (100 U/ml) and streptomycin sulfate (100 µg/ml). Cells were grown under a humidified atmosphere of 95% air, 5% CO<sub>2</sub> at 37 °C.

### CHO cell line cultures

The plasmid construction of the C-terminal tagging GHS-R1a with enhanced green fluorescent protein (EGFP), the transfection characteristics on CHO-K1 cells and selection of clones expressing the GHS-R1a-EGFP have been described previously (Camina *et al.* 2004). The CHO-K1 cell line that stably expresses the GHS-R1a-EGFP (CHO-GHS-R1a) was cultured in 100 mm dishes in Ham's F12 medium supplemented with 7.5% (v/v) fetal calf serum, 1 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C.

### Calcium measurements

Intracellular calcium measurements were performed in cell suspensions using the fluorescent calcium probe fura-2 as described previously (Camina *et al.* 2004).

### Whole cell-binding assay

Confluent monolayer cells (70–80%) were resuspended in binding buffer (containing, DMEM (pH 7.4) plus 1% (w/v) BSA), centrifuged at 500 *g* for 5 min at room temperature, washed twice and resuspended in proportion 1 × 100 mm plate/1.5 ml (500 000 cells/aliquot) in binding buffer supplemented with the radioligand (<sup>3</sup>H-adenosine, 2 µCi/ml; [<sup>125</sup>I]-ghrelin, 100 000 c.p.m./aliquot) in the absence or presence of unlabeled competitor (adenosine, 1 µM; ghrelin, 1 µM) for 2 h at 4 °C. After incubation, media were removed and the pellet was washed twice with binding buffer at 4 °C. Cell surface radioligand was determined by incubating the cells in 0.5 ml ice-cold acid buffer (containing 0.5 M NaCl; 0.2 M acetic acid, pH 2.0) for 10 min at 4 °C. Finally, the cells were centrifuged and the supernatants were counted in a β or a γ counter. For β counter, supernatants were mixed with scintillation liquid.

### Saturation analyses

Confluent monolayer cells (about 70–80%) were resuspended in binding buffer, centrifuged at 500 *g* for 3 min at room temperature, washed twice and then resuspended

( $5 \times 10^5$  cells/aliquot) in binding buffer supplemented with increasing concentrations of [ $^3\text{H}$ ]-adenosine (from 1 to 10  $\mu\text{M}$ ) in the absence or presence of unlabeled competitor (100 times [ $^3\text{H}$ ]-adenosine concentration used in each point) for 2 h at 4 °C. The media containing labeled adenosine was removed and the cells were washed twice with ice-cold binding buffer and the cell-surface bound adenosine was measured after treatment with acid buffer in a  $\beta$  counter. Non-linear curve-fitting procedures (GraphPad Prism, version 4.0, GraphPad Software, Inc, San Diego, CA, USA) were used to estimate the concentration of adenosine-binding sites ( $B_{\text{max}}$ ) and to calculate the  $K_d$ .

Non-specific binding, determined as radioactivity bound to cells in the presence of unlabeled competitor, represented about  $5.6 \pm 1.6$  and  $6.5 \pm 0.3\%$  for adenosine in HEK 293 and in HEK-GHS-R1a respectively.

#### Internalization assay and confocal microscopy

Trypsinized GHS-R1a-EGFP cells were diluted to obtain  $10^5$  cells/ml in Ham's F12 medium, seeded (200  $\mu\text{l}$ /well) on polyallylamine hydrochloride-coated (Sigma; 0.1 mg/ml for 30 min) 16-well glass (Lab-Tek Chamber Slides, Nalge Nunc), and cultured overnight in a humidified atmosphere of 95% air, 5%  $\text{CO}_2$  at 37 °C. In all experiments, 90 min before the beginning of the experiment, the medium was supplemented with cycloheximide (Sigma) to obtain a final concentration of 90  $\mu\text{M}$  in order to prevent *de novo* protein synthesis. Cells were pre-incubated for 30 min at 4 °C in ice-cold Earle's buffer (containing 25 mM HEPES; 140 mM NaCl; 5 mM KCl; 1.8 mM  $\text{CaCl}_2$ ; 3.6 mM  $\text{MgCl}_2$ , pH 7.4; complemented with 0.2% (w/v) BSA; 0.01% (w/v) glucose; 0.09 mM cycloheximide; 0.8 mM phenanthroline) in the presence or absence of different stimuli. Internalization was promoted by placing the cells at 37 °C for 20 or 60 min. Thereafter, the cells were rinsed three times with ice-cold Earle's buffer and subsequently fixed for 10 min with 4% paraformaldehyde dissolved in PBS. The cells were rinsed again in cold Earle's buffer, mounted using Vectashield (Vector Laboratories; Compiègne, France) and coverslipped for confocal microscopic examination. The cells were examined with a Leica TCS SP 2 (Leica Microsystems, Heidelberg, Germany) confocal laser scanning microscope mounted on a Leica DM IRBE inverted microscope equipped with an argon/krypton laser. EGFP fluorescence was detected with 100% excitation at 488 nm, using an RSP 500 (dichroic) mirror and the spectrophotometer set to acquire emission between 530 and 560 nm. Optical sections ( $1024 \times 1024$ ) of individual cells were taken at the equatorial level (level of the nucleus), using a  $63 \times 1.32$  NA oil-immersion objective.

#### Western blot analysis

The cells were incubated for 10 min with the lysis buffer (containing 150 mM NaCl, 1 mM EDTA, 50 mM

Tris-HCl, pH 7.4; 1% (v/v) NP-40, 0.25% (v/v) Na-deoxycholate, 1 mM supplemented with phenylmethylsulphonyl fluoride, 0.15 mM aprotinin, 2.16 mM leupeptin, 1.6 mM pepstatin, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF) at 4 °C. The solubilized cell lysates were pre-cleared by centrifuging at 14 000  $g$  for 10 min. The protein concentration was evaluated by the Bradford method. The same amount (30  $\mu\text{g}$ ) of protein of each sample was separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with goat polyclonal antibodies directed against the adenosine receptor type-2b (A2b-R) and rabbit polyclonal antibodies directed against adenosine receptor type-3 (A3-R). The primary antibodies were detected with corresponding peroxidase-conjugated IgG antibody. The ECL substrate was used to reveal bands, according to the manufacturer's instructions.

#### siRNA and cell transfection

Adenosine A2b-R and A3-R siRNAs were purchased from Santa Cruz Biotechnology. Three different strands for each siRNA were used in order to target different parts of the same mRNA to improve the knockdown efficiency. The siRNA sequences targeting adenosine receptors, as the manufacturer indicated, were the following. For A3-R: 5'-GCAUCA-CAAUCCACUUCUA-3', 5'-CAUGCCAACUCCAU-GAUGA-3', and 5'-CUAGUUGACUUACUGACAA-3'. For A2b-R: 5'-CACCAACAACUGCACAGAA-3', 5'-CUACCACGUAUCUAGCUAA-3', and 5'-CAGCUU-GAAUGGAUUCUAA-3'. A non-silencing RNA duplex was used as a control (Santa Cruz Biotechnology). 40–50% confluent cells in 100 mm plates, seeded 24 h before transfection, were transfected with siRNA using siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's modified instructions. In brief, 106.9  $\mu\text{l}$  transfection reagent were added to 792  $\mu\text{l}$  serum-free medium, while RNA mixtures containing 0.72 nmol siRNA and 792  $\mu\text{l}$  serum-free medium were prepared. After 5 min at room temperature, both solutions were combined and allowed to stand for 20 min at room temperature. The transfection mixture was added to cells containing 5 ml fresh serum-free medium. After the cells were incubated for 7 h at 37 °C, the medium was replaced with normal medium (serum containing). All assays were performed 48 h after siRNA transfection. A2b-R and A3-R expressions were determined by radioligand binding assays with [ $^3\text{H}$ ]-adenosine, as described above.

#### Data analysis

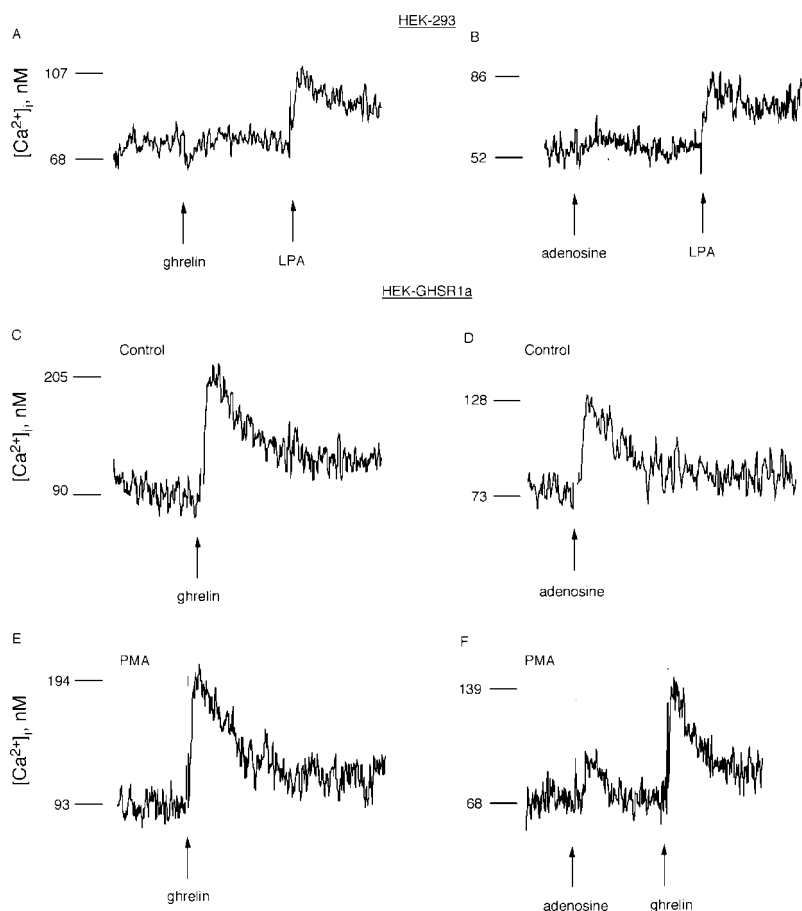
The results were expressed as the mean  $\pm$  S.E.M. Differences between means were evaluated by one-way ANOVA ( $*P < 0.05$ ).

## Results

### Desensitization of the ghrelin and adenosine responses by protein kinase C

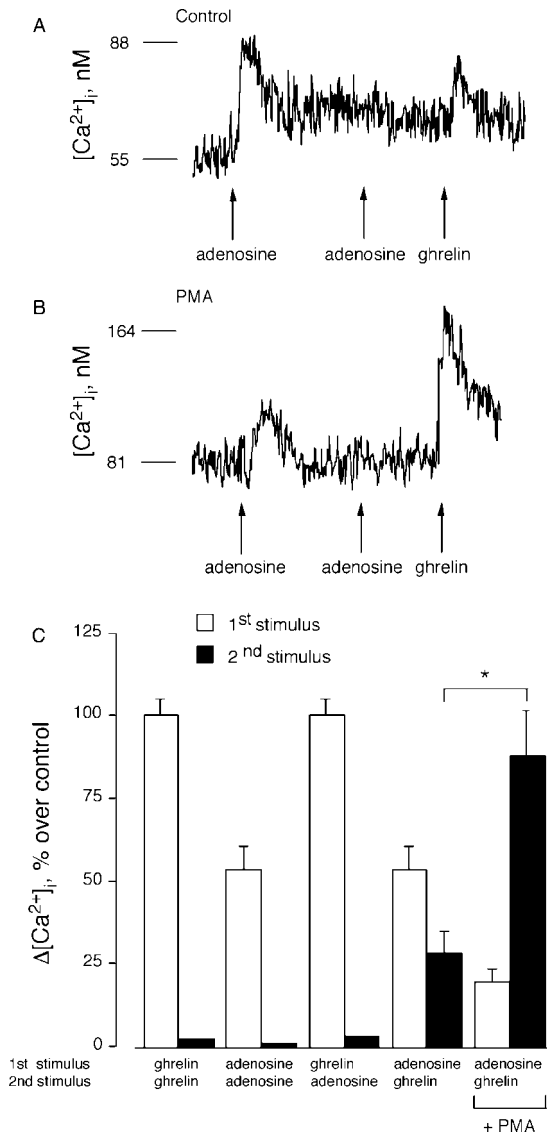
To evaluate if adenosine is a true ligand of the GHS-R1a, we monitored the intracellular calcium rise upon receptor activation. Neither ghrelin (100 nM) nor adenosine (100  $\mu$ M) had a significant effect on intracellular calcium mobilization on untransfected HEK 293 cells (Fig. 1A and B), which were fully responsive to lysophosphatidic acid (1  $\mu$ g/ml) used as an internal control of cell viability. In contrast, the addition of adenosine (100  $\mu$ M) to HEK 293 cells that stably express the GHS-R1a (HEK-GHS-R1a cells), induced a biphasic increase of  $[Ca^{2+}]_i$  with no detectable lag period (Fig. 1D), and ghrelin (100 nM) activated a more powerful response showing similar dynamics (Fig. 1C). The ghrelin-induced  $Ca^{2+}$  mobilization

is activated by phosphatidylinositol-specific phospholipase C, an enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate generating inositol 1,4,5-triphosphate and diacylglycerol. The formed diacylglycerol triggers the activation of PKC which, in turn, closes PKC-regulated receptors, such as L- $\alpha$ -lysophosphatidic acid receptor(s), with no effect on the GHS-R1a (Camina *et al.* 2004). In fact, the activation of PKC activity by means of the phorbol ester PMA (1  $\mu$ M, 5 min) did not alter the ghrelin-induced  $Ca^{2+}$  transient (Fig. 1E). However, the accurate administration of PMA (1  $\mu$ M, 5 min) caused a significant blockade of the adenosine-induced  $Ca^{2+}$  transient (Fig. 1F). These results were surprising since it was previously assumed that adenosine action is mediated through the GHS-R1a, then PKC activation should not modify the adenosine-induced calcium signal. In addition, PMA-activated PKC (1  $\mu$ M, 5 min) caused a non-significant reduction in [ $^3H$ ]-adenosine binding to the HEK-GHS-R1a cells (data not shown).

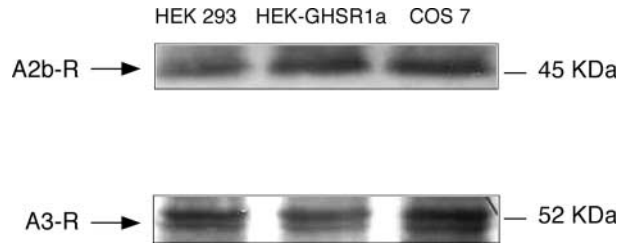


**Figure 1** Effects of (A) ghrelin (100 nM) and (B) adenosine (100  $\mu$ M) on intracellular calcium mobilization in untransfected HEK-293 cells. Effects of (C) ghrelin (100 nM) and (D) adenosine (100  $\mu$ M) on intracellular calcium mobilization in HEK-293 cells overexpressing the GHS-R1a. Pre-incubation in the presence of PMA (1  $\mu$ M, 5 min) inhibited adenosine–calcium signal (F) with no effect on ghrelin calcium transient (E,F). Results correspond to a representative experiment repeated six times with comparable results.

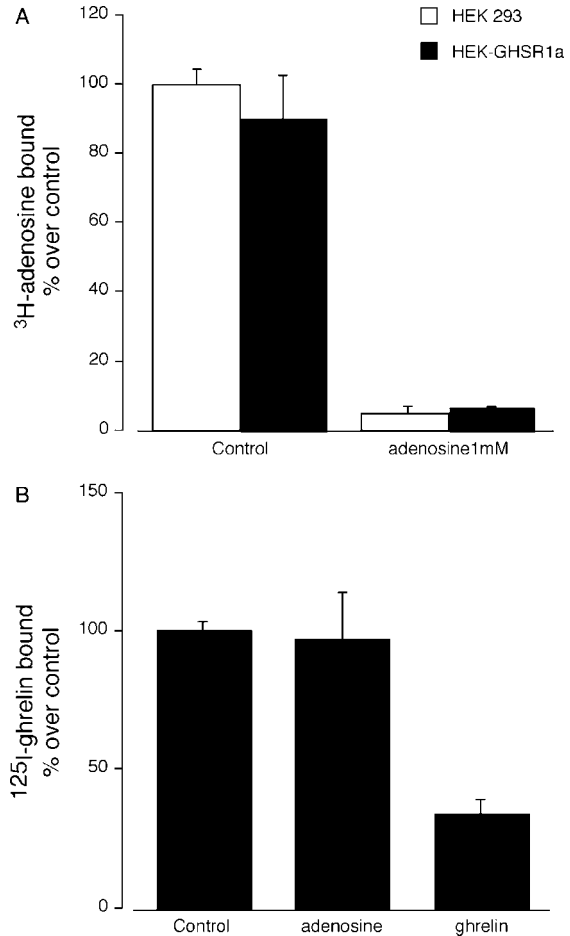
To gain further insight into the mechanism of action of adenosine, the role of PKC in the cross-talk between the adenosine and the ghrelin signaling pathways was assessed. As Fig. 2A shows, repeated administration of a saturating dose



**Figure 2** (A) The repeated administration of adenosine (100  $\mu$ M, two times) caused homologous desensitization and heterologous desensitization of ghrelin-induced calcium mobilization. (B) However, activation of protein kinase C (PKC) by treatment with PMA (1  $\mu$ M) inhibited the adenosine-induced homologous desensitization which allows recovery of the ghrelin calcium response. (C) Bar graph shows the ghrelin-induced homologous and heterologous desensitization to adenosine, the adenosine-induced homologous desensitization, the partial reduction of the ghrelin calcium response and the effect of PKC activation on the adenosine-induced cross-desensitization on the ghrelin calcium signal (100 nM; \* $P < 0.05$ ). Results (mean  $\pm$  S.E.M. of three independent quadruplicate experiments) are expressed as the percentage of maximum  $Ca^{2+}$  response.



**Figure 3** Western blot analysis of A2b-R and A3-R in HEK 293 and HEK-GHSR1a cells. The apparent molecular weight was 45 kDa for A2b-R and 52 kDa for A3-R. COS 7 cell line was taken as positive control.



**Figure 4** Competition analysis of [<sup>3</sup>H]-adenosine and [<sup>125</sup>I]-ghrelin in HEK 293 and HEK-GHSR1a cells. Binding was measured at the cell surface as described in Materials and Methods in HEK 293 (open bars) and HEK-GHSR1a (solid bars) cells. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabeled competitor). (A) The amount of cell-surface bound [<sup>3</sup>H]-adenosine was similar in both cellular systems. This binding was shown to be specific as bound radioligand was completely displaced by unlabeled adenosine (1 mM). (B) Binding of [<sup>125</sup>I]-ghrelin was not affected by unlabeled adenosine (1 mM) while unlabeled ghrelin (1  $\mu$ M) showed a complete displacement of surface bound [<sup>125</sup>I]-ghrelin. Data (mean  $\pm$  S.E.M.) are from three independent triplicate experiments.

of adenosine (100  $\mu\text{M}$ ) induced a homologous desensitization of its transmembrane signaling system and, in addition, reduced 48% the ghrelin-induced calcium response ( $P < 0.05$ ;  $n = 12$ ; Fig. 2C). However, when PKC was pre-activated by means of PMA (1  $\mu\text{M}$ , 5 min), adenosine was unable to cross-desensitize the ghrelin receptor (Fig. 2B and C). This supports the hypothesis that adenosine-induced calcium response is mediated by a receptor with different properties from the GHS-R1a.

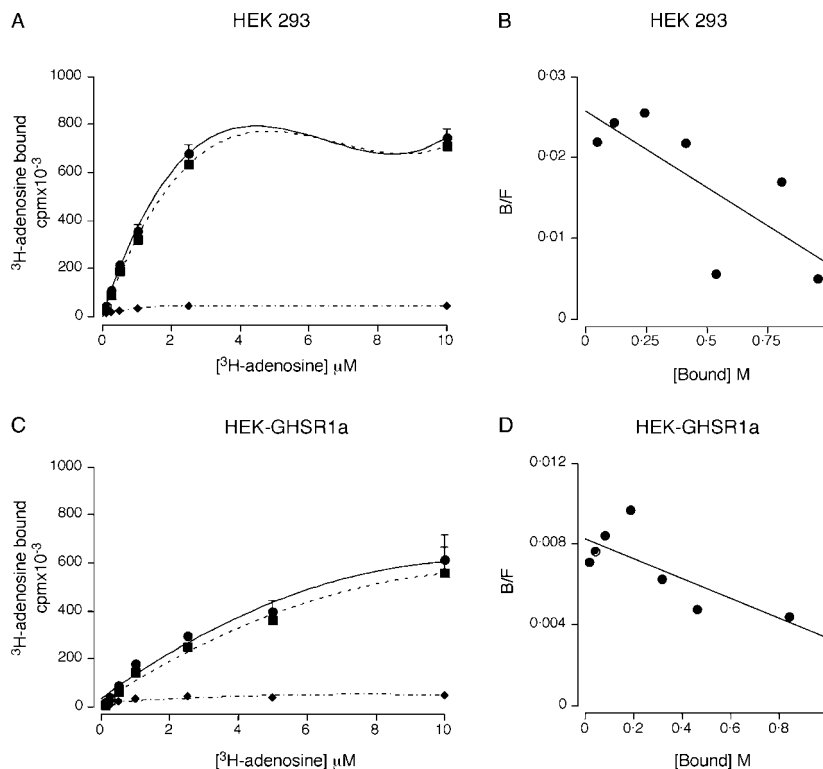
### Endogenous adenosine receptors

Suspecting that HEK 293 cells may be endowed with adenosine receptors which become activated after transfection of the GHS-R1a, the presence of adenosine receptors on HEK cells were evaluated. As Fig. 3 shows, adenosine receptor type-2b (A2b-R) was detected in both HEK 293 and HEK-GHS-R1a whole cell lysates by western blot analysis. Furthermore, a lower expression of adenosine receptor type-3 (A3-R) was also detected (Fig. 3). Despite the fact

that several studies had previously suggested the existence of endogenous A2b-R on HEK-293 cells (Cooper *et al.* 1997, Gao *et al.* 1999, Linden *et al.* 1999), the density of this receptor appeared to be too low to contribute to calcium activity of adenosine in these cells. To our knowledge, there is no data about the expression of A3-R on HEK-293 cells. The COS 7 cell line was taken as a positive control for these assays (Peters *et al.* 1998). No negative control is presented basically due to the wide distribution of both receptors (Feoktistov & Biaggioni 1997, Ralevic & Burnstock 1998).

### Radioligand binding studies

The above functional data suggest that the adenosine-activated signaling pathway was not mediated by GHS-R1a. Binding experiments with the radioligand [ $^3\text{H}$ ]-adenosine showed that the specific cell-surface bound [ $^3\text{H}$ ]-adenosine was comparable in HEK 293 and HEK-GHS-R1a cells (Fig. 4A). Competitive binding assays showed that adenosine did not displace bound [ $^{125}\text{I}$ ]-ghrelin in HEK-GHS-R1a cells



**Figure 5** Saturation curves of specific binding of [ $^3\text{H}$ ]-adenosine. Cells were incubated with increasing concentrations of [ $^3\text{H}$ ]-adenosine. Specific binding was determined in the presence of unlabeled adenosine (1 mM). (A) Representative saturation curve obtained in HEK 293 cells. (B) Scatchard analysis reveals a single class of limited capacity characterized by a  $K_d = 1.78 \pm 0.35 \mu\text{M}$  and  $B_{\text{max}} = 2.01 \pm 0.15 \text{ fmol/cell}$  in HEK 293 cells. (C) Representative saturation curve obtained in HEK-GHS-R1a cells. (D) Scatchard analysis showed a single class of limited capacity binding site characterized by a  $K_d = 6.30 \pm 0.75 \mu\text{M}$  and  $B_{\text{max}} = 1.90 \pm 0.11 \text{ fmol/cell}$  in HEK-GHS-R1a cells. The results are represented as means  $\pm$  S.E.M. of three independent experiments.  $\bullet$ , total binding;  $\blacksquare$ , specific binding;  $\blacklozenge$ , non-specific binding.

(Fig. 4B), a result that might be indicative of different binding sites in the same receptor or, by contrast, different receptors for both ligands. However, saturation-binding experiments at HEK-GHS-R1a cells with [<sup>3</sup>H]-adenosine exhibited a single-binding site with a  $K_d$  value of  $6.30 \pm 0.75 \mu\text{M}$  and a binding capacity ( $B_{\text{max}}$ ) of  $1.90 \pm 0.11 \text{ fmol/cell}$  (Fig. 5C and D). In HEK 293 cells, [<sup>3</sup>H]-adenosine saturation experiments revealed a single affinity site with a  $K_d$  value of  $1.78 \pm 0.35 \mu\text{M}$  and a binding capacity ( $B_{\text{max}}$ ) of  $2.01 \pm 0.15 \text{ fmol/cell}$  (Fig. 5A and B). For the saturation experiments in HEK-GHS-R1a cells, a two-site model did not give an improved fit over a one-site model (data not shown), a fact that further reinforces the suggestion that adenosine was not acting through the GHS-R1a.

A series of GHS-R1a antagonists, such as D-Lys<sup>3</sup> GHRP-6 (100  $\mu\text{M}$ ; Halem *et al.* 2004), the substance P analogue [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,D-Leu<sup>11</sup>]-substance P (10  $\mu\text{M}$ ; Carreira *et al.* 2004), and BIM 28163 (10  $\mu\text{M}$ ; Halem *et al.* 2004), were tested for their ability to compete for binding against [<sup>3</sup>H]-adenosine in HEK-GHS-R1a cells. None of the antagonists tested was able to compete for [<sup>3</sup>H]-adenosine binding, which was solely displaced by unlabeled adenosine (Fig. 6A). In contrast, D-Lys<sup>3</sup> GHRP-6 and substance P analogue competed for [<sup>125</sup>I]-ghrelin binding in HEK-GHS-R1a cells (Fig. 6B).

#### Assessment of internalization of GHS-R1a by confocal microscopy

In the resting CHO cells that stably express the human GHS-R1a fused to EGFP (CHO-GHS-R1a cells), fluorescence was predominantly confined to plasma membrane (control, Fig. 7A). After exposure to ghrelin (100 nM) for 20 min, the fluorescence disappeared from the plasma membrane to be redistributed to a population of intracellular fluorescent vesicles spread throughout the cytoplasm (Fig. 7A). On the contrary, no redistribution of the fluorescent labeling was observed in cells incubated with adenosine (100  $\mu\text{M}$ ) for 20 min (Fig. 7A). Large fluorescent vesicles reappeared partially at the cell surface after 60 min of incubation with ghrelin, while fluorescence was kept confined to the plasma membrane in cells incubated with adenosine during the same period (Fig. 7B). The lack of effect of adenosine on the GHS-R1a endocytosis supports the hypothesis that there is no direct action of adenosine on GHS-R1a. In addition, a slight reduction of the ghrelin-activated endocytosis (100 nM, 20 min) was observed after pre-incubating the cells with adenosine (100  $\mu\text{M}$ , 20 min; Fig. 8), supporting a cross-talk between both receptors.

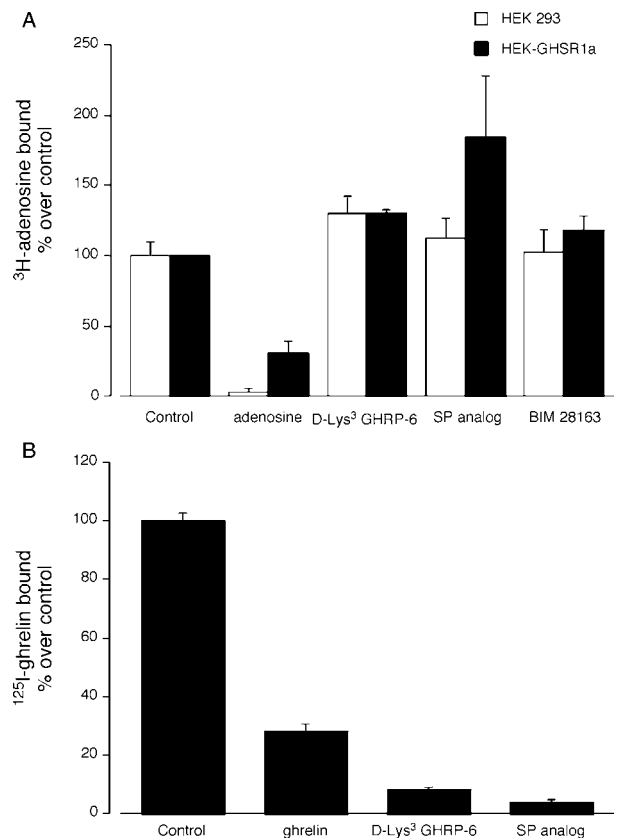
#### Effects of A2b-R and A3-R siRNAs

To test the role of endogenous adenosine receptors, we analyzed the adenosine-induced intracellular calcium rise after depleting cellular levels of A2b-R or A3-R by transfecting siRNA specifically directed against each isoform in HEK-GHS-R1a cells. In the presence of a non-targeting

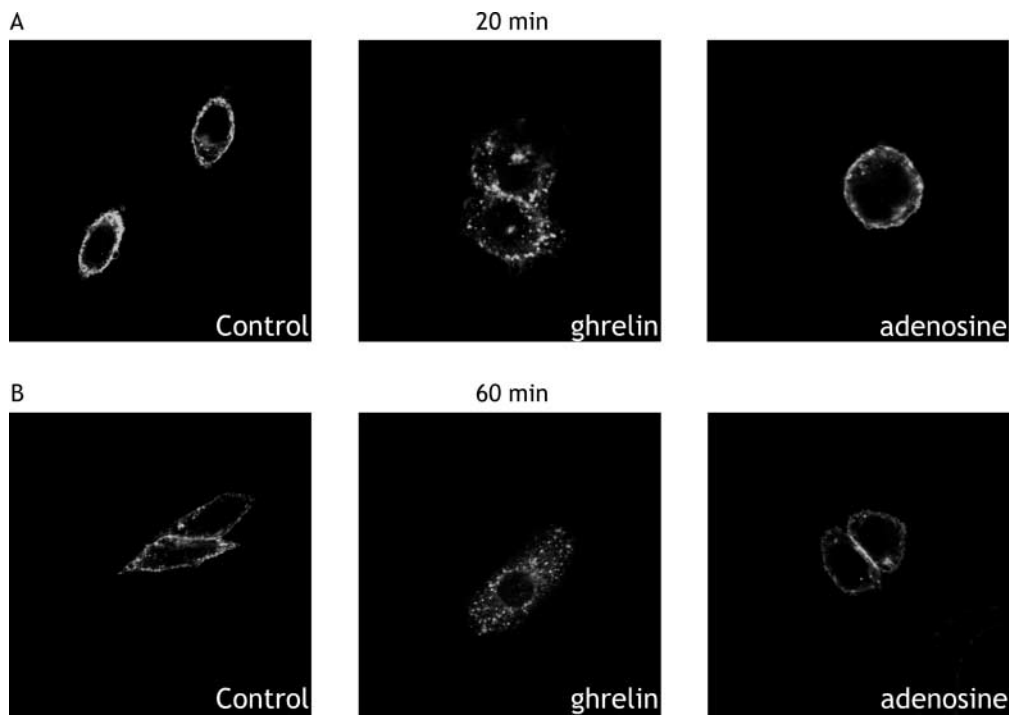
control siRNA, the cell-surface bound [<sup>3</sup>H]-adenosine was comparable to that observed without any siRNA transfection (Fig. 9A). However, both A2b-R and A3-R siRNAs effectively reduce the cell-surface bound [<sup>3</sup>H]-adenosine ( $46.03 \pm 5.71$  and  $44.47 \pm 2.73\%$  respectively). In the presence of a non-targeting control siRNA, adenosine (100  $\mu\text{M}$ ) stimulated intracellular calcium mobilization on HEK-GHS-R1a cells (Fig. 9B), which was identical to that observed with any siRNA transfection (data not shown). In contrast, both A2b-R and A3-R siRNAs individually reduced the adenosine-induced calcium peak by  $46.36 \pm 12.77$  and  $51.21 \pm 14.56\%$  respectively, suggesting that adenosine activity is in fact adenosine receptor dependent.

## Discussion

In this work, our findings suggest that adenosine does not bind to the GHS-R1a, contrary to previous reports (Smith



**Figure 6** Effect of GHS-R1a antagonists on [<sup>3</sup>H]-adenosine binding in HEK 293 (open bars) and HEK-GHS-R1a (solid bars) cells. (A) The tested compounds, D-Lys<sup>3</sup> GHRP-6 (100  $\mu\text{M}$ ), substance P analogue [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,D-Leu<sup>11</sup>]-substance P (SP analog; 10  $\mu\text{M}$ ) and BIM 28163 (10  $\mu\text{M}$ ), did not show any effect on surface bound [<sup>3</sup>H]-adenosine, while unlabeled adenosine (1  $\mu\text{M}$ ) displaced [<sup>3</sup>H]-adenosine. (B) D-Lys<sup>3</sup> GHRP-6 and substance P analogue completely displaced bound [<sup>125</sup>I]-ghrelin in HEK-GHS-R1a cells. Data (mean  $\pm$  S.E.M.) are from three independent triplicate experiments.

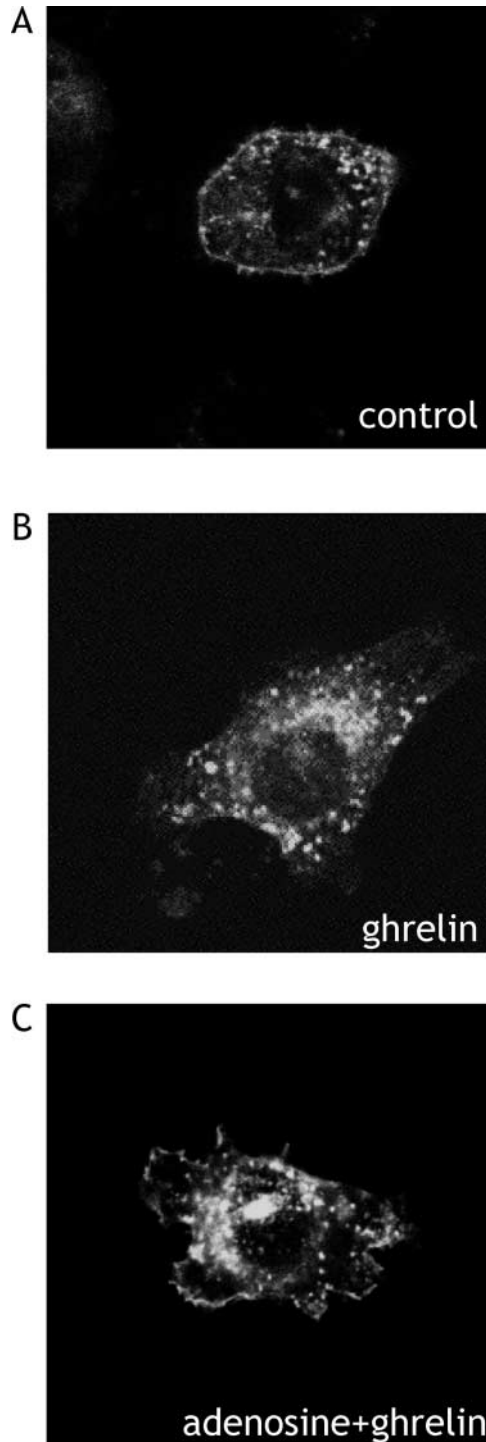


**Figure 7** Internalization of the GHS-R1a in CHO cells. The localization of the GHS-R-EGFP expressed in stably transfected CHO cells was visualized by confocal microscopy in cells incubated for (A) 20 min and (B) 60 min at 37 °C in the absence (control) or presence of 100 nM ghrelin and 100  $\mu$ M adenosine. The images are representative of four independent experiments.

*et al.* 2000, Tullin *et al.* 2000). Despite the fact that this receptor had been considered as an example of how a single receptor may activate two distinct intracellular signaling pathways depending on the agonist (Carreira *et al.* 2004), the data presented so far provide sufficient evidence to discard this concept. Initially, the intracellular calcium rise induced by adenosine in HEK-GHS-R1a cells appeared to be consistent with an action mediated by the GHS-R1a as no significant calcium rise was detected in HEK 293 cells and this effect was susceptible to inhibition by the GHS-R1a antagonist [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,D-Leu<sup>11</sup>]-substance P (Carreira *et al.* 2004); an action also detected in GHS-R1a-transfected BHK cells, the other cell line used to postulate adenosine as a ligand of the GHS-R1a (Tullin *et al.* 2000). Additionally, the low density of the endogenous adenosine receptor type-2b (A2b-R) in HEK-GHS-R1a cells and the lack of InPs generation supported this hypothesis (Linden *et al.* 1999, Smith *et al.* 2000, Carreira *et al.* 2004). In this way, adenosine seemed to activate a signaling cascade that involved the consecutive activation of AC and PKA through CTX-sensitive G-protein coupled to the GHS-R1a, a finding that described the GHS-R1a as a multifaceted receptor (Carreira *et al.* 2004). However, a closer analysis revealed some differences between the receptor that mediates the adenosine action and the ghrelin one. First, both systems appear to be under different regulation by PKC. Phorbol ester PMA did

not cause a suppression of the ghrelin-induced calcium response. On the contrary, adenosine failed to induce a calcium rise after PMA treatment, despite the clear ghrelin-induced calcium response. Furthermore, PMA pretreatment inhibited the adenosine-dependent homologous desensitization, and consequently adenosine failed to attenuate the calcium response associated with ghrelin. Despite the fact that this regulatory mechanism can operate at many levels within the cell, receptor or downstream signaling, the fact that ghrelin response was recovered after a complete suppression of adenosine response is consistent with signaling pathways regulated by distinct receptors. Second, and quite remarkably, binding experiments revealed a similar binding capacity for adenosine in HEK 293 and HEK-GHS-R1a cell lines. This saturable binding indicates a single-binding site in both cell lines, a result that fits well with an equivalent expression of adenosine receptor types-2b and -3 detected by western blot analysis in both cell lines. Despite the fact that A2b-R is also endogenously expressed in BHK cells (Mittal *et al.* 1999), adenosine showed a binding capacity apparently higher in GHS-R1a-transfected BHK cells than in untransfected cells (Tullin *et al.* 2000). However, these binding studies should be carried out with higher adenosine concentrations as weak calcium responses were observed when this concentration was increased in untransfected BHK cells (Tullin *et al.* 2000). Even in HEK cells, there are some notable differences in the pattern

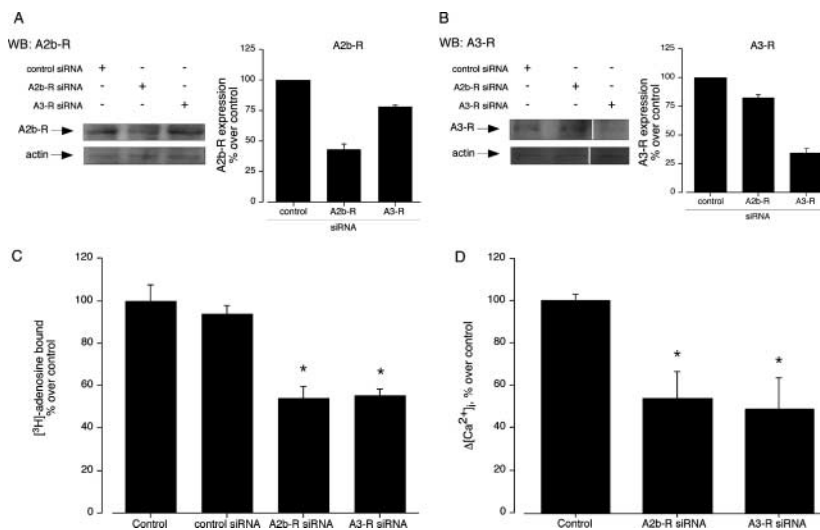




**Figure 8** Effect of adenosine on ghrelin-induced GHS-R1a endocytosis in CHO cells. The localization of the GHS-R1a-EGFP expressed in CHO cells was visualized by confocal microscopy in cells stimulated with ghrelin (100 nM), in the absence (B) or presence of adenosine (1 mM, 20 min pretreatment; C) for 20 min at 37 °C. The images are representative of four independent experiments.

of adenosine binding at different tested concentrations. Such differences tend to disappear at high concentrations. Taken together, our findings demonstrate that adenosine is not a ligand of the GHS-R1a, and its action is mediated by the A2b-R and A3-R in the HEK cell line. In fact, this conclusion is clearly endorsed by the reduction in adenosine functionality after depleting cellular levels of these receptors by transfecting siRNA specially directed against each isoform. This conclusion fits well with a recently published study in which evidence against adenosine analogues being partial agonists of GHS-R1a are shown (Johansson *et al.* 2005).

Interestingly, the activated adenosine receptor that is 'silent' in HEK 293 cells, was able to couple an intracellular signaling machinery by the presence of the GHS-R1a. This suggests an interaction between both the receptors that change the adenosine receptor properties, and consequently, its functionality. This fact could be explained by two alternative routes: either compartmentalization of signaling proteins within membrane microdomains as a consequence of overexpression of the GHS-R1a, implying that the efficiency of signal transduction is dictated by the transducer elements within microdomains (Maudsley *et al.* 2005), or modification of the receptor properties, including agonist affinity, potency, and efficacy, could be modified as a result of heterodimerization (Devi 2001, Rios *et al.* 2001, Terrillon & Bouvier 2004). The reduction in adenosine affinity in transfected cells suggests that the latter possibility is more plausible, as a higher  $K_d$  was detected. In addition, cross-talk between heterodimeric receptor pairs can modify positively or negatively the response to agonists resulting in either enhanced-G-protein activation or cross-inhibition (Ferre *et al.* 1998, Jordan & Devi 1999), even changes in G-protein-coupling specificity (George *et al.* 2000). In this particular case, the presence of GHS-R1a in HEK 293 cells appears to be essential for the qualitative coupling of A2b-R and A3-R to  $G_s$ -signaling pathway, as cAMP levels were elevated in response to adenosine in HEK-GHS-R1a cells, whereas no increase was determined in HEK 293 cells (Carreira *et al.* 2004). Furthermore, adenosine produces a signal attenuation of ghrelin. Decrease in the efficacy of inositol formation by ghrelin has been described in response to a combination of ghrelin and adenosine (Carreira *et al.* 2004). Similarly, adenosine treatment caused a partial blockade of the ghrelin-promoted internalization of the GHS-R1a, although it failed to induce GHS-R1a endocytosis, an action that seems to vary the GHS-R1a desensitization and trafficking, thus modulating the extent of receptor signaling. It thus appears that, in our experimental system, the overexpression of GHS-R1a modifies the efficacy of G-protein-coupling activities for A2b-R/A3-R with the consequent modulation of the GHS-R1a response. Interestingly, despite the fact that the adenosine-induced calcium rise was susceptible to inhibition by the GHS-R1a antagonist [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,D-Leu<sup>11</sup>]-substance P (Carreira *et al.* 2004), the adenosine binding was unaltered by this antagonist. Although this



**Figure 9** Effects of siRNA-suppressed A2b-R and A3-R expressions on the adenosine-induced intracellular calcium mobilization. HEK-GHS-R1a cells were transfected with the indicated siRNAs and 3 days after transfection, the efficacy of the treatments were determined by western blot ((A) for A2b-R and (B) for A3-R) and binding of [<sup>3</sup>H]-adenosine at the cell surface (C), and then the effect of adenosine (100 μM) on intracellular calcium mobilization was evaluated (D). Each data point represents the mean ± S.E.M. from three independent experiments (western blot).

discrepancy cannot be definitively explained at present, it is possible that this antagonist modifies the interactions between GHS-R1a and A2b-R/A3-R. Indeed, this agonist has been proven to prevent the GHS-R1a from constitutive endocytosis, with no apparent activation of GHS-R1a-associated signaling cascade (Holst *et al.* 2003, 2004). Even if the formation of heterodimers could have a crucial function in signal transduction, cross-talk regulation connecting the individual signaling pathways cannot be excluded.

At present, cells with high expression of a given receptor by transfection have been the preferred models for the study of receptor-associated signaling mechanisms. However, these models can lead to erroneous conclusions when used to determine the existence of ‘alternative’ ligands, a consequence of artifacts introduced by the high density of receptors. In this particular case, the data obtained in the present study strongly suggest that the expression of the GHS-R1a in HEK 293 cells modifies a number of A2b-R and A3-R properties, including adenosine affinity, G-protein-coupling efficacy, and adenosine-activated signaling potency. Thus, the fact that a practically non-operative receptor such as the adenosine receptor can become a receptor able to ‘use’ or to ‘modulate’ the ghrelin receptor, suggests that the interaction between both receptors is a mechanism that helps to aggregate the signal transduction machinery facilitating the modulation of signaling. It is clear that further studies examining the biochemical properties of the possible ghrelin-adenosine receptor dimers are necessary to understand their physiological significance.

## Acknowledgements

The expert technical assistance of Mary Lage and Sihara Pérez-Romero are greatly acknowledged. We wish to thank Dr Roy Smith (Baylor College of Medicine, Houston, USA) for the HEK-GHS-R1a cell line, and Dr Michael Culler (IPSEN, Milford, MA, USA) for the analog BIM-28163. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. This work was supported by grants from the FIS, the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Red de Grupos RGTO (G03/028), Red de Centros RCMN (C03/08), and Secretaría Xeral de Investigación e Desenvolvemento (PGIDIT02BTF91801, PGIDIT05BTF20802PR), Xunta de Galicia. JP. Camiña is a recipient of a Research Contract from the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, in the Research Area of the Complejo Hospitalario Universitario de Santiago.

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Received in final form 2 June 2006

Accepted 19 June 2006

Made available online as an Accepted Preprint  
17 July 2006