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Histamine H2 receptor overexpression induces U937 cell differentiation despite triggered mechanisms to attenuate cAMP signalling

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Abbreviations:
AC, adenylyl cyclase
ATRA, all trans retinoic acid
Ca^{2+}i, intracellular Ca^{2+}
dbcAMP, dibutyryl cAMP
Fura 2-AM, Fura 2 acetoxymethyl ester
GPCR, G protein-coupled receptor
GRK, G protein-coupled receptor kinase
G-418, geneticin
H2R, histamine H2 receptor

ABSTRACT

Knowing that cell-surface receptors that recognize and respond to extracellular stimuli are key components for the regular communication between individual cells required for the survival of any living organism, the aim of the present work was to investigate the effect of H2R overexpression on the U937 signal transduction pathway and its consequences on cell proliferation and differentiation. The overexpression of H2R led to an increase in cAMP basal levels, a leftward shift of agonist concentration–response curves, and similar maximal response to agonist treatment, suggesting that overexpressed H2Rs act as functional spare receptors. In this system cells triggered several mechanisms tending to restore cAMP basal levels to those of the naïve cells. H2R overexpression induced PDE activity stimulation and GRK2 overexpression. In spite of the onset of these regulatory mechanisms, H2 agonist and rolipram treatments induced the terminal differentiation of the H2R overexpressed clone, conversely to the naïve cells. Present findings show that stably H2R overexpression alters cAMP signalling as the result of not only the amounts of second messenger generated but also the activation or upregulation of various components of signalling cascade, leading to an adapted biologically unique system. This adaptation may represent an advantage or a disadvantage, depending on the biological system, but in any case, the existence of compensatory mechanisms should be considered when a clinical treatment is designed.

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HTMT dimaleate, 6[2-(4-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl)heptane-carboxamide
IBMX, isobutylmethylxanthine
PDE4, cyclic nucleotide phosphodiesterase 4
PGE2, prostaglandin E2
rhC5a, recombinant human C5a

1. Introduction

Cell-surface receptors recognizing and responding to extracellular stimuli are key components for the regular communication between individual cells required for the survival of any living organism. Most of transmembrane signal transduction in response to hormones and neurotransmitters is mediated by G protein-coupled receptors (GPCRs).

Histamine is a biogenic amine widely distributed throughout the body, which acts as a chemical messenger to exert numerous biological effects in central as well as peripheral tissues, including the induction of normal promyelocytic differentiation [1]. These effects are mediated through four pharmacologically distinct receptor subtypes, i.e. the H1, H2, H3 [2–4] and H4 [5] receptors. Molecular biology studies indicate that histamine receptors belong to the large multigene family of GPCRs. In particular, the H2 receptor (H2R) causes cAMP accumulation through Gs-protein activation, in various cell types including immune cells [2].

Innumerable reports show that GPCRs that activate the cAMP pathway exhibit distinct and often opposing effects on cell replication and differentiation in normal as well as malignant cells. Some of the mechanisms that may foster the opposite effects include signal compartmentalization, coupling to and activation of additional signalling pathways, and the cellular context in which the cAMP signal develops [6–8]. The final response of a biological system is highly dependent not only on the type but also on the amplitude (quantity) as well as on the time-course of the signal evoked [9–11].

Differentiation therapy is a novel and potentially less toxic form of cancer therapy that involves the use of several agents, alone or in combination, with the aim of modifying the state of differentiation and growth of cancer cells [12].

The promonocytic U937 cell line [13], can be differentiated in vitro to monocyte by agents that increase cAMP levels like forskolin [an adenylyl cyclase (AC) activator], prostaglandin E2 (PGE2) and dibutyryl cAMP (dbcAMP) [11,14,15]. The presence of H2R with its classical pharmacological profile and signalling properties has been described in this cell line [16]. However, cAMP stimulation via H2R fails to promote cell maturation [17]. We have reported that various mechanisms are involved in regulation of the time-course of H2-induced cAMP levels, including receptor desensitisation mediated by GRKs (mainly GRK2 and GRK3) [9] and cyclic nucleotide phosphodiesterase 4 (PDE4) activation [16]. These mechanisms account for the lack of H2-mediated cell differentiation. These processes tending to modulate quantitative characteristics of the cAMP response clearly influence cell behaviour [9].

Considering the impact of the regulation of cell signalling on cell behaviour, the aim of the present work was to investigate the effect of H2R overexpression on the U937 signal transduction pathway and its consequence on cell proliferation and differentiation.

Present findings showed that H2R overexpression triggered several mechanisms (namely PDE activity induction and GRK2 overexpression) tending to restore cAMP basal levels comparable to those of the naïve cells. In spite of the onset of these regulatory mechanisms, H2 agonist treatment induced the terminal differentiation of the H2R overexpressed clone. These findings provide new insights into the relevant role of receptor–effector stoichiometry regulation on cell behaviour, and further suggest that this regulation may be externally manipulated to achieve beneficial therapeutic effects in the future.

2. Materials and methods

2.1. Materials

Cell culture medium, antibiotics, isobutylmethylxanthine (IBMX), dibutyryl cAMP (dbcAMP), cAMP, ATP, forskolin, rolipram, 5′ nucleotidase (*Ophiophagus hannah* snake venom), Fura 2 acetoxymethyl ester (Fura 2-AM), bovine serum albumin (BSA), and recombinant human C5a (rhC5a) were obtained from Sigma Chemical Company (St. Louis, MO). Fetal calf serum was purchased from Natocor (Argentina). Amthamine, tiotidine and 6[2-(4-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl)heptane-carboxamide (HTMT dimaleate) were from Tocris Cookson Inc. (Ballwin, MO). [^{3}H]cAMP and [^{3}H]tiotidine were purchased from Perkin Elmer Life Sciences (Boston, MA). All other chemicals used were of analytical grade. Trifluoromethylphenyl histamine was a kindly gift of Dr. W. Schunack (Freie Universitat Berlin, Institut fur Pharmazie, Berlin, Germany).

2.2. Cell culture and transfection

U937 cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin.

For stable transfection, U937 cells were harvested by centrifugation from cultures in exponential growth phase, washed in phosphate-buffered saline (PBS), and resuspended at 2×10^7 cells/ml in fresh RPMI medium on ice. pCEFL-HA-H2R (10 μg), previously cloned [18], was linearized with SalI. Then it was added to cell suspension (400 μl) and kept on ice 5 min. Cells and DNA were then subjected to a pulse of 150 V at

a capacitance of 250 μF using a Gene Pulser (Bio-Rad, Hercules, CA). Cells were returned to ice for 5 min and incubated in a non-selective medium overnight. Cells were then plated in a 96-well culture plate in RPMI medium containing 0.8 mg/ml G-418. After 2–3 weeks, the surviving clones were amplified.

2.3. Radioligand binding assay

Triplicate assays were performed in polyethylene tubes in 50 mM Tris–HCl, pH 7.4. For saturation studies, increasing concentrations of [^{3}H]tiotidine, ranging from 0.4 to 240 nM, were incubated with 10^6 cells/tube, in the absence or presence of 1 μM tiotidine, in a total volume of 200 μl. After 40 min at 4 °C, incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris–HCl, pH 7.4, and rapid filtration under reduced pressure onto Whatman GF/B glass-fiber filters, followed by three washes with 3 ml ice-cold buffer. Experiments on intact cells were carried out at 4 °C to avoid internalisation of the ligand. Kinetic studies performed with 2 nM [^{3}H]tiotidine at 4 °C showed that equilibrium was reached after 30 min and persisted for 4 h (data not shown).

2.4. cAMP assays

For dose–response assays, cells were incubated 3 min in RPMI 1640 medium supplemented, or not, with 1 mM IBMX at 37 °C, followed by 9 or 5 min exposure to different concentrations of various agents.

For the desensitisation assays, pretreatment of cells with 10 μM amthamine was performed in RPMI 1640 medium at 37 °C in a 5% CO_2 humidified atmosphere for periods ranging from 1 min to 1 h, in the absence of IBMX. Cells at a density of 10^6 cells/ml were washed and resuspended in RPMI 1640 medium containing 1 mM IBMX, and exposed 9 min to 10 μM amthamine, to evaluate whether the system was able to generate cAMP.

For time-course cAMP accumulation studies, cells were resuspended in RPMI 1640 medium in the absence of IBMX, at a density of 10^6 cells/ml, and exposed different periods of time to 10 μM amthamine.

In all experiments, the reaction was stopped by ethanol addition followed by 5 min centrifugation at $2000 \times g$. The ethanol phase was then dried and resuspended in 50 mM Tris–HCl, pH 7.4, 0.1% BSA. Cyclic AMP content was determined by competition of [^{3}H]cAMP for PKA, as previously described [19].

2.5. Phosphodiesterase assay

Phosphodiesterase activity was assayed using a modified method based on that described by Thompson and Appleman [20]. Briefly, cells were resuspended in 0.3 ml PDE buffer (40 mM Tris–HCl, pH 8, 6 mM $MgCl_2$, 4 mM 2-mercaptoethanol) and disrupted by ultrasound followed by the addition or not of PDE inhibitors (1 mM IBMX and 10 μM rolipram). After 3 min reaction was initiated by the addition of a substrate mixture (0.1 ml) containing 1 μM cAMP and 500,000 dpm of [^{3}H]cAMP and incubated for 10 min at 30 °C. The reaction was stopped by boiling the mixture for 1 min. Then, 20 μl snake venom (5 mg/ml) was added and the mixture further incubated for 10 min at 30 °C. Reactions was stopped by the addition of 1 ml of 1:2 Dowex 1-X8, 50% methanol, followed by 15 min incubation at 4 °C and spun in clinical centrifuge. Radioactivity present in supernatants was measured by liquid scintillation.

2.6. Western blots

Cells were lysed in sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue), and sonicated to shear DNA. Samples were boiled 5 min, and aliquots were subjected to electrophoresis in 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% non-fat powdered milk in PBST (PBS containing 0.05% Tween-20), and membranes were incubated with 1 μg/ml of rabbit anti-GRK2 antibody or goat anti-actin antibody (Santa Cruz Biotechnology, CA), in PBST. All subsequent washes were performed with the same buffer. Reactivity was developed using an anti-rabbit or anti-goat polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham, Bukinghamshire, England). Image analyses were performed with the use of the Scion Image software (Scion Corporation, Frederick, MD).

2.7. Cell growth experiments

Cells (10^5 cells/ml) were seeded in 24-well plates and treated with different agents during 4 days. Cells were collected at the time indicated and the number of cells was determined using a cellular meter Coulter Z-1. Cell density in culture did not exceed 1.5×10^6 cells/ml.

2.8. Intracellular Ca^{2+} measurements

Fura 2-AM was used as a fluorescent indicator. An amount of 10^6 cells/ml was treated with amthamine or rolipram for 48 h. Cells of each experimental group were washed, resuspended and incubated in a buffered saline solution (BSS; 140 mM NaCl, 3.9 mM KCl, 0.7 mM KH_2PO_4, 0.5 mM $Na_2HPO_4{\cdot}12H_2O$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.5) in the presence of 2 μM Fura 2-AM. Cells were incubated for 30 min at 37 °C in an atmosphere of 5% CO_2, time by which Fura 2-AM was trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS, and brought to a density of 2×10^6 cells/ml in BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) provided with the CA-61 accessory and an injection chamber to measure Ca^{2+} with continuous stirring, with the thermostat adjusted to 37 °C. Intracellular Ca^{2+} ($[Ca^{2+}]i$) levels were registered every second by exposure to alternating 340-nm and 380-nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and their ratio (F_{340}/F_{380}) were tracked. Recombinant 50 nM hC5a, 10 μM HTMT dimaleate, 10 μM trifluoromethylphenyl histamine, or 100 μM ATP, were injected into the chamber without interrupting recording. HTMT dimaleate and trifluoromethylphenyl histamine, H1 histamine agonists, or ATP, were added as positive controls when no response was observed with rhC5a (data not shown). The preparation was calibrated by determining maximal fluorescence induced by 0.1% Triton X-100,

and minimal fluorescence in the presence of 6 mM EGTA (pH 8.3). $[Ca^{2+}]i$ was calculated according to Grynkiewicz et al. [21].

2.9. Chemotaxis assay

The "in vitro" locomotion of U937 cells was assayed using the micropore filter technique (Transwell 3521, Costar Corp., Cambridge, MA). Briefly, 10^5 control or 3 days treated cells, were seeded onto the top compartment of the chemotactic chambers in 0.1 ml of RPMI 1640 and placed in a 24-well tissue culture plate. A polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μm separates the top and bottom compartments. The bottom compartment was filled with 0.6 ml of medium with or without 5 nM rhC5a. Chambers were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. Migrated cells were collected and counted using a cellular meter Coulter Z-1.

2.10. Statistical analysis

Binding data and sigmoidal dose–response fittings were done using GraphPad Prism 3.00 for Windows, GraphPad Software (San Diego, CA). One-way ANOVA with Dunnett's post test was performed using GraphPad InStat version 3.01, GraphPad Software (San Diego, CA). Specific binding was calculated by subtraction of non-specific binding from total binding.

3. Results

3.1. Generation of a cell line stably overexpressing H2 receptors

To examine the effects of H2R overexpression on the U937 signal transduction pathway and its consequences on cell differentiation, we established U937 derived cell lines stably overexpressing the human H2R. By [^{3}H]tiotidine binding analysis a clone (U937-H2R), exhibiting seven-fold H2R levels as compared to U937 cells was selected (Fig. 1 and inset). In accordance with previous results, [^{3}H]tiotidine binding fitted better to a two-binding-site model, corresponding to different H2R populations [22]. It is worth noting that neither the affinity constants nor the proportions of each site were affected by H2R overexpression (U937 Kd_{High} = 2.2 ± 0.8 nM, $Bmax_{High}$ = 2550 ± 200 sites/cell, Kd_{Low} = 20 ± 3 nM, $Bmax_{Low}$ = 22,930 ± 2500 sites/cell; U927-H2R Kd_{High} = 2.1 ± 1.1 nM, $Bmax_{High}$ = 19,200 ± 1800 sites/cell, Kd_{Low} = 27 ± 10 nM, nM, $Bmax_{Low}$ = 168,800 ± 15,000 sites/cell).

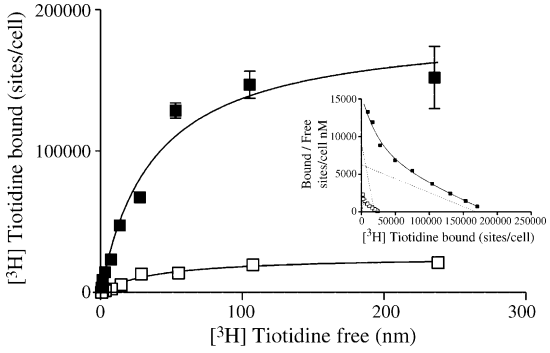

Fig. 1 – [^{3}H]Tiotidine binding assay in U937 and U937-H2R cells. Saturation assays for [^{3}H]tiotidine in U937 (□) or U937-H2R (■) cells. Data are the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Kd and Bmax values were calculated using the equation for two binding sites. Values are the mean ± S.E.M. (n = 3). U937: Kd_{High} = 2.2 ± 0.8 nM, $Bmax_{High}$ = 2550 ± 200 sites/cell, Kd_{Low} = 20 ± 3 nM, $Bmax_{Low}$ = 22,930 ± 2500 sites/cell; U927-H2R: Kd_{High} = 2.1 ± 1.1 nM, $Bmax_{High}$ = 19,200 ± 1800 sites/cell, Kd_{Low} = 27 ± 10 nM, $Bmax_{Low}$ = 168,800 ± 15,000 sites/cell. *Inset*: Scatchard representation of the saturation binding experiment.

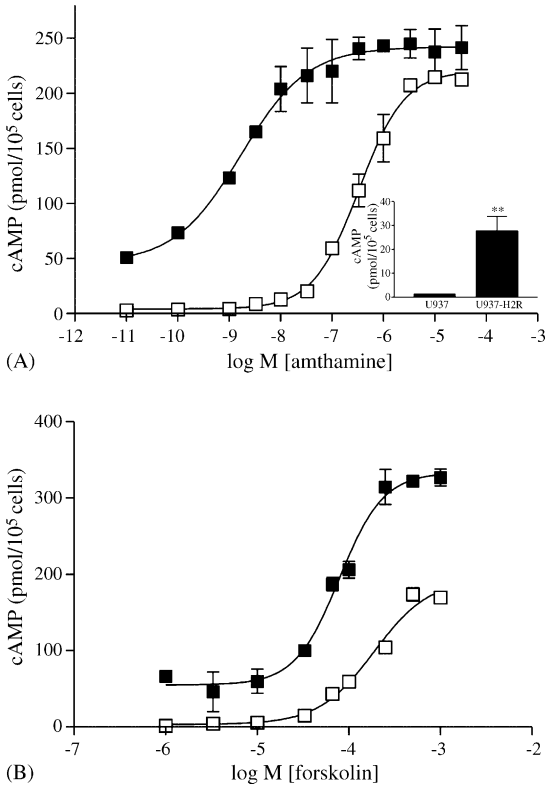

Fig. 2 – Concentration–response curves to cAMP production. (A) U937 (□) and U937-H2R (■) cells were exposed for 9 min to increasing concentrations of amthamine at 37 °C in the presence of 1 mM IBMX, and cAMP levels determined. Data are means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. *Inset*: Basal cAMP levels in U937 and U937-H2R. Data are means ± S.E.M. (n = 3). $^{**}p < 0.01$ vs. U937 cells. (B) U937 (□) and U937-H2R (■) cells were incubated for 9 min with increasing concentrations of forskolin at 37 °C in the presence of 1 mM IBMX, and cAMP levels determined. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

3.2. cAMP response in U937-H2R cells

The effect of H2R overexpression on cAMP production and accumulation induced by a selective H2 agonist (amthamine)

was examined in U937-H2R cells. Dose–response curves to amthamine in the presence of IBMX (PDE inhibitor) showed that the maximal response achieved in U937 and U937-H2R cells was similar. However, an increase in basal levels (1.21 ± 0.60 pmol versus 27.55 ± 3.51 pmol for U937 and U937-H2R, respectively) and a left shift in EC50 were observed in U937-H2R (pEC50 6.48 ± 0.05 versus 8.77 ± 0.18 for U937 and U937-H2R, respectively) as a consequence of receptor over-expression (Fig. 2A and inset). This sensitisation of the response could be attributed to a higher number of receptors coupled to G-protein. If so, forskolin-induced cAMP response should be higher due to a potentiation resulting from a cooperative effect between the binding of Gαs subunit and forskolin to AC [23]. As depicted in Fig. 2B, increasing concentration of forskolin evoked higher levels of cAMP in U937-H2R than in the naïve cell line. This result strengthens the conclusion that there are a higher number of active receptors (i.e. activating G proteins) in the cell line over-expressing H2R.

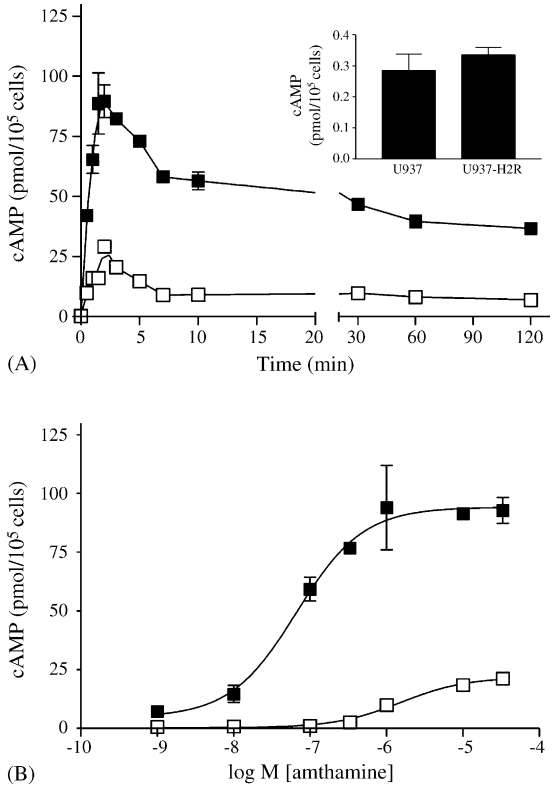

Fig. 3 – cAMP levels in the absence of IBMX. (A) Time course of cAMP levels on U937 (□) and U937-H2R (■). Cells were incubated for different periods of time with 10 μM amthamine at 37 °C, and cAMP levels were determined. (B) Concentration response curves on U937 (□) and U937-H2R (■). Cells were exposed for 5 min (the time to reach the maximal accumulation of cAMP) to increasing concentrations of amthamine at 37 °C, and cAMP levels determined. Data are mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. *Inset*: Basal cAMP levels in U937 and U937-H2R. Data are means ± S.E.M. ($n = 3$).

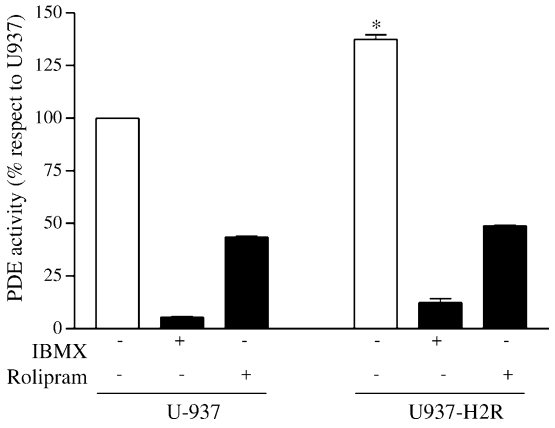

Fig. 4 – PDE activity in U937 and U937-H2R cells. The activity of PDE was assessed as described in Section 2 in the presence or in the absence of IBMX or rolipram. Data are means ± S.E.M. ($n = 3$). $^{*}p < 0.05$ vs. U937 basal activity.

In an attempt to determine cAMP levels resulting from the intracellular production–degradation balance, cAMP dose–response and time course assays were carried out in the absence of IBMX. In kinetic experiments, U937-H2R cells showed higher cAMP levels, with a higher residual response after 2 h stimulus than U937 cells (Fig. 3A). On the other hand, as expected, higher EC50s were found in dose–response experiments performed without IBMX at the time that cAMP reaches its maximum levels (5 min). A comparable left shift in pEC50 to that obtained in the presence of IBMX was observed (pEC50 5.82 ± 0.11 versus 7.18 ± 0.15 for U937 and U937-H2R, respectively) but the maximal responses were lower and significantly different (21.8 ± 1.1 pmol/10^6 cells versus 94.2 ± 3.9 pmol/10^6 cells for U937 and U937-H2R, respectively) (Fig. 3B). However, basal levels resulted similar in both cell lines supporting the existence of compensatory mechanisms attempting to restore cAMP levels to those of the naïve cells (Fig. 3A inset). Hence, we aimed to identify the mechanisms underlying this regulatory process.

3.3. *Phosphodiesterase activity*

Previous studies in U937 cells showed that agents that elevate cAMP regulate PDE4 activity. Thus, H2 agonist treatment results in augmented PDE activity after 2 h [16]. Total PDE activity, was evaluated in cell lysates of U937 and U937-H2R cells. U937-H2R cells exhibit an increase in basal PDE activity compared to U937 cells that was inhibited by IBMX, a general PDE inhibitor, as well as by rolipram, a specific PDE4 family inhibitor. These findings support that PDE4 accounts for the increase in total PDE activity (Fig. 4).

3.4. *GRK2 levels in H2R overexpressing cells*

Since GRK2 is another component involved in the cAMP response in U937 cells, GRK2 levels in U937-H2R cells were evaluated. In Western blot assays, the U937-H2R clone showed a 40% increase in GRK2 levels compared with those of U937 cells (Fig. 5).

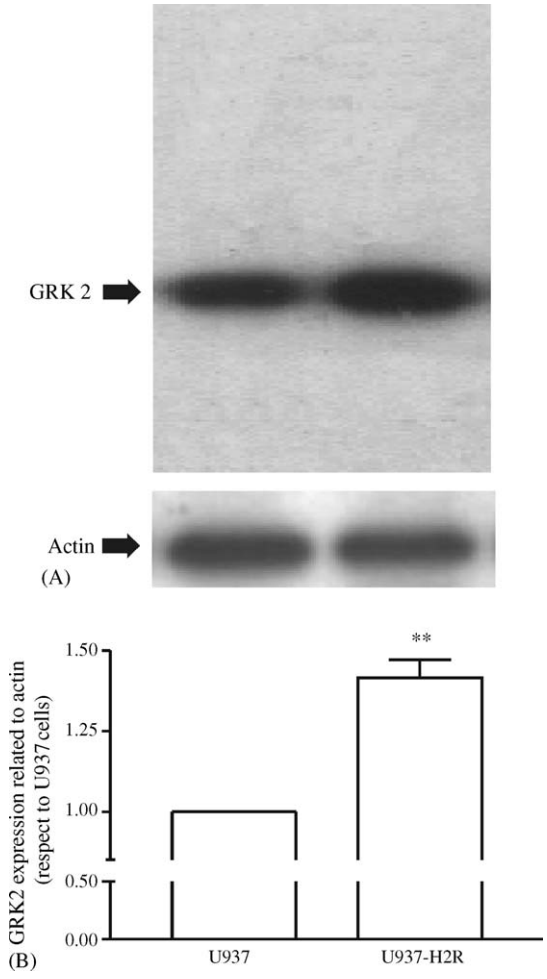

Fig. 5 – GRK2 expression in U937 and U937-H2R cells. (A) Whole-cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2 or goat sera against Actin as indicated in the methodology section. (B) Densitometric analysis obtained with the Scion Image program relative to actin intensity. Data are means ± S.E.M. ($n = 3$). $^{**}p < 0.01$ with respect to U937 cells.

3.5. *Desensitisation of U937-H2R cells*

We have previously reported that a reduction in GRK2 levels by cDNA antisense construct determines a higher and prolonged cAMP response mediated by H2R, due to lower receptor desensitisation, allowing H2 agonist-stimulated cell differentiation [9]. Therefore, it is likely to assume that an increase in the expression of this kinase may shorten the receptor response. In an attempt to evaluate this hypothesis, U937 and U937-H2R cells were exposed to 10 μM amthamine (maximal response) for different periods of time in the absence of IBMX, washed and re-stimulated to determine whether these cells were able to generate cAMP. U937 desensitisation curve was coincident with the curve previously reported by our group [16] showing a half-maximal desensitisation time of 10 ± 2 min (mean ± S.E.M.). However, in the H2R overexpression system the desensitisation curve

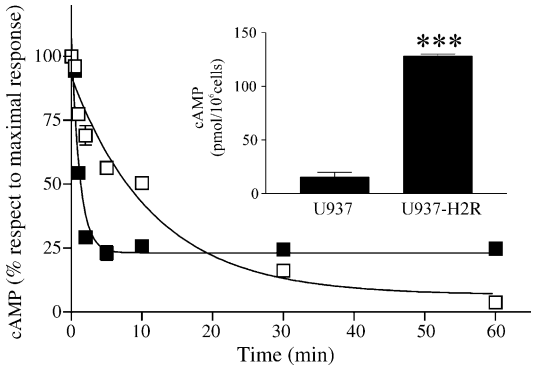

Fig. 6 – Effect of H2R overexpression on the desensitization kinetics. U937 (□) and U937-H2R (■) cells were preincubated for different periods of time with 10 μM amthamine at 37 °C, washed, and restimulated with 10 μM amthamine in the presence of 1 mM IBMX. cAMP production was determined as described in Section 2. Data are mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. *Inset*: Residual cAMP response. Cells were preincubated with 10 μM amthamine at 37 °C for 60 min, washed, and restimulated with 10 μM amthamine in the presence of 1 mM IBMX. $^{***}p < 0.01$ vs. U937 cells.

was modified (Fig. 6). U937-H2R cells showed a shorter half-maximal desensitisation time of 0.8 ± 0.1 min (mean ± S.E.M.). Nevertheless, a higher residual response significantly different from that of U937 cells was maintained up to 1 h (Fig. 6, inset).

3.6. *Amthamine and rolipram effects on U937-H2R cell proliferation and differentiation*

Up to now, the results obtained in U937 cells overexpressing H2R reveal the existence of different mechanisms tending to restore cAMP levels to those of naïve cells, such as an induction of PDE activity and an increase in GRK2 levels. Despite these mechanisms, cAMP production induced by H2 agonist in the absence of IBMX resulted in higher responses in U937-H2R than in control cells. Considering the ability of U937 cells to be differentiated by a sustained cAMP stimulus [11], the effect of amthamine and rolipram on U937-H2R cell proliferation and differentiation was analysed. Cell proliferation was evaluated in both cell lines treated for 4 days with 10 μM amthamine, 33 μM rolipram or 400 μM dbcAMP (positive control). In contrast to dbcAMP, neither amthamine nor rolipram were able to inhibit U937 cell division as it was previously reported [11]. On the other hand, treatment with amthamine and rolipram significantly inhibited U937-H2R cell proliferation (Fig. 7).

To evaluate U937-H2R cells ability to be differentiated in the presence of amthamine and rolipram, CD88 (C5a receptor) expression was assessed in order to evaluate terminal maturation. The C5a receptor is a GPCR associated with Ca^{2+} release from intracellular stores and it is also involved in chemotactic responses [24,25]. Thus, we evaluated its expression by the measurement of rhC5a induced

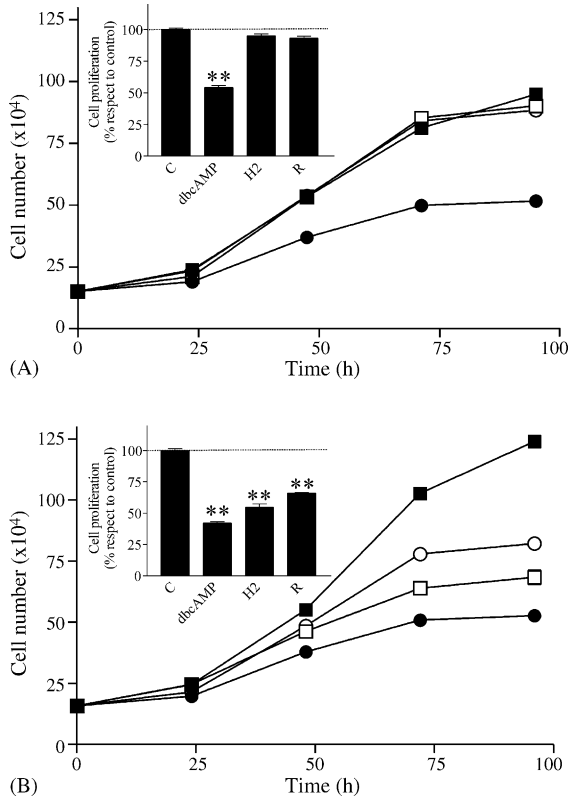

Fig. 7 – Effect of amthamine and rolipram on U937 and U937-H2R cell proliferation. U937 (A) or U937-H2R (B) cells were seeded in 24-well plates and treated with 10 μM amthamine (□), 33 μM rolipram (○), or 400 μM dbcAMP (●) up to 96 h. Control group (■) corresponds to non-treated cells. Data are mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. *Inset*: U937 and U937-H2R proliferation after 96 h of exposure to 10 μM amthamine (H2), 33 μM rolipram (R) or 400 μM dbcAMP. Control group (C) corresponds to non-treated cells. Data was expressed as a percent of the respective control. Control group proliferation considered 100% is shown as a dotted line. Data are means ± S.E.M. ($n = 3$). $^{**}p < 0.01$ vs. control group.

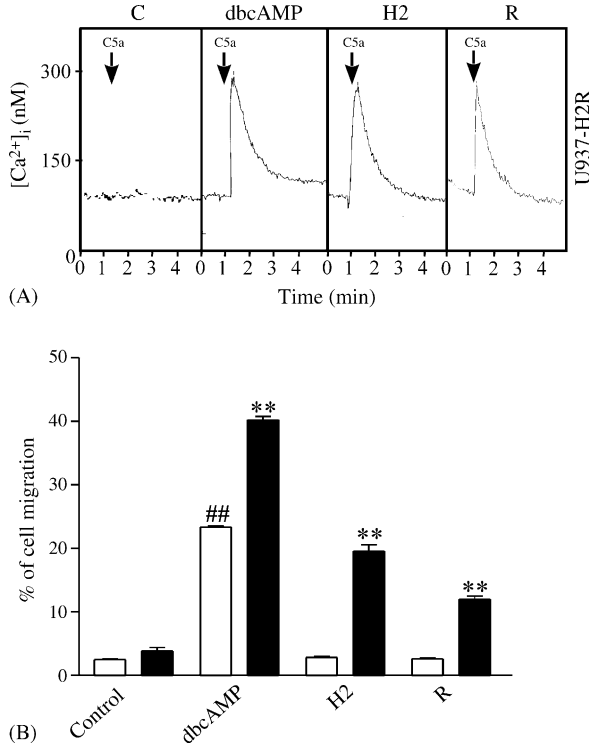

Fig. 8 – (A) Effect of rhC5a on $[Ca^{2+}]i$ in amthamine- or rolipram-treated U937-H2R cells. Cells were cultured for 48 h in presence of 10 μM amthamine (H2), 33 μM rolipram (R) or 400 μM dbcAMP. Control (C) represents non-treated cells. $[Ca^{2+}]i$ was determined as described under Section 2. Arrows indicate the addition of 50 nM rhC5a. Similar results were obtained in at least three independent experiments. (B) rhC5a chemotactic responses of amthamine- and rolipram-treated U937 (open bars) and U937-H2R cells (filled bars). Cells were incubated with 10 μM amthamine (H2), 33 μM rolipram (R) or 400 μM dbcAMP for 3 days and exposed to 5 nM rhC5a. Control represents non-treated cells. Chemotactic assay was performed as detailed in Section 2. Results are expressed as means ± S.E.M. ($n = 3$). $^{\#\#}p < 0.01$ vs. U937 control group. $^{**}p < 0.01$ vs. U937-H2R control group.

$[Ca^{2+}]i$ release and chemotaxis. As previously reported, no Ca^{2+} response was observed in U937 cells without treatment or pretreated for 2 days with 10 μM amthamine or 33 μM rolipram [11]. On the other hand, the U937-H2R cells treated with 10 μM amthamine and 33 μM rolipram for 2 days displayed a $[Ca^{2+}]i$ spike induced by rhC5a, similar to that evoked in dbcAMP treated cells (Fig. 8A). Moreover, consistently with C5a induced Ca^{2+} response, the chemotaxis of U937 cells treated with amthamine or rolipram did not significantly differ from control cells (without treatment) whereas the rhC5a induced migration of U937-H2R cells pretreated with amthamine and rolipram was similar to that of dbcAMP treated cells (Fig. 8B). These findings support the ability of H2 agonists to differentiate U937-H2R cells, highlighting the significance of receptor–effector stoichiometry on cell behaviour.

4. Discussion

The major finding of the present work is the relevance of the stoichiometry of the components of a signalling pathway in the biological response of a system.

To characterize the role of changing receptor levels in cellular proliferation and differentiation the H2R endogenous level in U937 cell line was manipulated and an overexpressed clone generated.

When the H2R were stably overexpressed (approximately seven-folds), [^{3}H]tiotidine was still able to recognize two distinct populations of the receptor, and neither the affinity constants nor the proportions of each site were affected by overexpression (Fig. 1). Since [^{3}H]tiotidine recognizes with different affinities the G-protein coupled and uncoupled states of the receptor [22], it can be inferred that both receptor species were proportionally

augmented, implying that a higher number of receptors in a G-protein coupled state existed.

The increased expression of the H2R led to an increase in basal cAMP levels, a leftward shift of agonist concentration–response curves, and a similar maximal response to agonist treatment (Fig. 2A). This increase in basal levels agrees with the notion of constitutive activity, a general phenomenon for GPCRs, described for the H2 receptor by Alewijnse et al. [26] that led to the reclassification of most H2 antagonists into inverse agonists. Similar results were obtained for β1 adrenergic receptor in transgenic mice and other GPCRs [27]. The augmentation in basal cAMP levels and in agonist potency suggests that overexpressed H2R act as functional spare receptors, and the slight difference in the agonist maximal response can be predicted if a limiting expression of any component in the signalling pathway different from the receptor (namely G-protein or effector AC) exists.

The sensitisation of the H2R response and the higher response to forskolin in the overexpression system (Fig. 2) can be attributed to the higher amount of the population of receptors coupled to G-protein observed in the binding studies.

Furthermore, cAMP production–degradation balance in the absence of IBMX was altered in the U937-H2R cell line. When stimulated with a specific H2 agonist, the overexpression system showed higher cAMP levels but the kinetic profile was similar in both cell lines (Fig. 3). As could be expected, dose–response experiments carried out without IBMX showed a right shift in EC50 and a diminish in maximal responses compared to the experiment performed in the presence of IBMX. It is worth noting that the maximal response for amthamine in U937 cells resulted significantly lower than the obtained in U937-RH2, indicating that the cAMP producing system is not saturated in this experimental setup. On the other hand, there was no difference in basal cAMP levels between the two cell lines (Fig. 3, inset), differing from the basal levels observed in the presence of IBMX (Fig. 2A, inset). This apparent discrepancy between the two experimental conditions suggests an increase in PDE activity responsible for the negligible difference in basal cAMP levels observed without IBMX. Previous studies in U937 cells have shown that agents that elevate cAMP content increase the activity of PDE4 [16,28]. PDE4 is the predominant cAMP-hydrolyzing isoenzyme class found in most immune and inflammatory cells [29]. In fact, in the U937-H2R cells we observed a 37% increase in total PDE activity, that was entirely due to an increase in the PDE4 family (Fig. 4). Although the mechanism governing such activation has not been addressed in the present study, it has been reported that a sustained stimulus may regulate PDE4 activity as a feedback loop to maintain cAMP homeostasis by means of various, mostly cAMP dependent, mechanisms [30]. However, in this case, the receptor overexpression does not lead to an increase in basal cAMP levels, suggesting the onset of other mechanisms. In fact, it seems more likely that a non-cAMP-dependent mechanism may lead to an increase in PDE activity. It is well known that GPCRs activate the ERK pathway [31], and that ERK mediated phosphorylation of short PDE4 variants results in the activation of these enzymes [32,33]. Therefore, it can be speculated that this activation may result from increase in the G-protein coupled receptor subpopulation. In this way, PDE would participate in U937-H2R cell adaptation to the receptor number elevation.

Another component involved in H2R signalling in U937 cells is GRK2. This kinase is responsible of H2R cell homologous desensitisation in U937 cells [9]. In the last few years it has became evident that GRKs, and GRK2 in particular, are proteins not only tightly regulated at the level of kinase activity or subcellular localization, but also at their expression level [34]. A relationship between altered GPCRs signalling and changes in the expression of GRKs has been clearly established by several experimental approaches. Cotransfection experiments in COS-7 cells using H2R and different GRKs demonstrated that GRK2 and GRK3 were the most potent in augmenting desensitisation mediated by H2R phosphorylation. Furthermore, binding experiments showed that GRK2 overexpression decreased the number of H2Rs in cell membrane, supporting a basal regulation of the H2R by GRK2. In addition, we have previously reported that the inhibition of GRK2 by cDNA antisense in U937 modifies the desensitisation kinetic of H2R and determines changes in cell behaviour [9].

In the present study, cells overexpressing H2Rs, exhibited increased GRK2 levels (Fig. 5). The mechanisms controlling GRK2 levels may result from an imbalance between transcriptional and post-transcriptional mechanisms (protein stability). This increment in GRK2 expression resulted in a shorter desensitisation time for the H2R overexpressed clone (Fig. 6). Nevertheless, the residual response after maximal desensitisation occurred proved to be higher in U937-H2R compared to the untransfected cells. In spite of the complexity of a cell system, these results are consistent with a simple model where GRK2 catalyses the desensitisation reaction by phosphorylating its substrate (i.e. H2R) to render a species of the receptor unable to signal (i.e. phosphorylated H2R). Hence, if the enzyme is upregulated and the substrate is overexpressed, the equilibrium would be reached faster and with higher levels of both substrate and product.

The regulation of GRK2 levels by agonists has been extensively studied in different systems [35–38]. However, in this case, kinase expression regulation is not due to an increment in basal second messenger levels but to an increase in receptor number. In fact, the increase in PDE4 activity and the upregulation of GRK2 expression are most likely the result of other mechanisms triggered by the receptor overexpression. Nevertheless, it appears that sustained agonist stimulation and receptor overexpression ultimately have similar effects, namely the triggering of mechanisms tending to regulate signalling activity.

It is worth noting that results obtained in time course, dose–response, and desensitisation experiments suggest that the mechanisms triggered as a consequence of receptor overexpression are able to reduce cAMP basal levels but are not able to manage cAMP levels in stimulated conditions. The experimental setup without IBMX reflects better what is occurring in the cell, and the conclusions drawn from these experiments are more representative of physiological conditions.

In a recent work, we established a correlation between the time-course of cAMP signalling induced by different agents (H2 agonist, prostaglandin E2 and forskolin) and evoked cellular responses such as proliferation and differentiation in U937 cells. Agents that elevate cAMP in a sustained manner

are able to differentiate U937 cells [11]. Consistently, related to U937 cells, the higher and sustained increase of cAMP levels in U937-H2R caused by amthamine (Figs. 3 and 6) induced the differentiation and hampered the proliferation of the over-expression clone.

Compared to the naïve cells, the surrogate system behaves in a distinctive way, since it is able to be differentiated by H2 agonist and rolipram pretreatments, highlighting the importance of the relative amounts of the components involved in a signal transduction pathway on the behaviour of a biological system. Remarkably, the cell system triggers several mechanisms tending to restore cAMP levels to those of the naïve cells (i.e. increases in GRK2 expression and PDE increased activity). The system appears to counterbalance the genetically introduced alteration.

Both observations may have practical consequences, considering the interest that the manipulation of protein could have on a potential therapy.

Concerning cancer therapies, most chemotherapeutic agents currently used display significant toxicity. A potential therapeutic alternative to treat this disease may include agents that induce cell differentiation, based on the hypothesis that many neoplastic cell types exhibit reversible defects in the course of differentiation. Differentiation induction as a therapeutic strategy has the greatest impact on hematopoietic malignancies, most notably on leukemia. This line of reasoning has culminated in the successful introduction of all trans retinoic acid (ATRA) as a primary treatment in patients with acute promyelocytic leukaemia [12]. Despite the success of ATRA and chemotherapy in the treatment of leukemia, a fair percentage (30–40%) of patients develops resistance to the ATRA treatment. Recently, arsenic trioxide (As2O3), has proven to be an effective drug in the treatment of patients with a refractory disease after ATRA or chemotherapy or both [39]. Remarkably, some reports evidence the synergic effect of ATRA or As2O3 with agents that mimic or modulate cAMP levels supporting a crosstalk between cAMP and ATRA or As2O3 signalling pathways [40,41]. This crosstalk is particularly attractive in terms of the development of novel pharmacological combinations to be used in the differentiation treatment of leukemia. In fact, various agents that modulate the cAMP pathway are already clinically available for the treatment of several malignancies [42,43].

Although most current strategies are restricted to ligand activation or blockade of receptors, human gene therapy including both overexpression or antisense approaches, may allow to manipulate GPCR signalling in a novel way. Present findings evidence how stably H2R overexpression alters cAMP signalling not only regarding the amount of second messenger generated but also the activation or upregulation of other components of the pathway, leading to a biologically adapted unique system. Indeed, it may represent an advantage or a disadvantage depending on the biological system. However, these mechanisms should be considered when a clinical treatment is designed.

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