Biochemical Pharmacology, Vol. 60, pp. 159–166, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00295-1

Rapid Desensitization and Slow Recovery of the Cyclic AMP Response Mediated by Histamine H_2 Receptors in the U937 Cell Line

Bibiana Lemos Legnazzi, Carina Shayo,† Federico Monczor,*‡ Maria E. Martin,* Natalia Fernandez,† Andres Brodsky,§ Alberto Baldi† and Carlos Davio*‡‖*

*Radioisotope Laboratory, School of Pharmacy and Biochemistry, and §Hematology Division and Immunogenetic Service,"José de San Martín" Clinical Hospital, School of Medicine, University of Buenos Aires, Buenos Aires; †Institute of Biology and Experimental Medicine, National Research Council of Argentina, Buenos Aires; and ‡National Research Council of Argentina, Buenos Aires, Argentina

ABSTRACT. The present study focused on the desensitization process of the H_2 receptor in U937 cells and the recovery of the cyclic AMP (cAMP) response. Treatment of U937 leukemic cells with the H_2 histamine receptor agonists (±)-N^1-[3-(3,4-difluorophenyl)-3-(pyridin-2-yl)propyl]-N^2-[3-(1H-imidazol-4-yl)propyl]guanidine (BU-E-75) and amthamine produced a rapid desensitization characterized by decreased cAMP production ($T_{1/2}$ = 20 min). Pretreatment with 10 μM BU-E-75 did not induce modifications in the responses to prostaglandin E_2, isoproterenol, or forskolin. H_2 receptor desensitization was not affected by protein kinase A and C inhibitors, but was reduced drastically by Zn^{2+} and heparin, known to act as inhibitors of G protein-coupled receptor kinases. Recovery studies of the cAMP response showed that cAMP levels reached 50% of the initial values within 5 hr. Furthermore, desensitization produced an important decrease in the basal level of this cyclic nucleotide. The minimal value was observed 12 hr later, and corresponded to approximately 1.3% of the initial basal level (7.5 vs 0.1 pmol/10^6 cells). This result could be explained by an increase in phosphodiesterase activity following 10 μM BU-E-75 treatment. When cells were exposed for 2 hr to an H_2 agonist, binding assays showed no modification in the number of H_2 receptors; internalization began just after 8 hr. Although the initial desensitization seems to involve G protein-coupled receptor kinases, results indicate that additional mechanisms of regulation were triggered by the H_2 agonists. BIOCHEM PHARMACOL **60**;2:159–166, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. homologous desensitization; G protein-coupled receptor kinases; phosphodiesterases; [^{3}H]tiotidine binding; cell differentiation

Based on the differences in the pharmacological profiles of several drugs, three histamine receptors (H_1, H_2, and H_3) can be distinguished [1]. Molecular biological studies indicate that the H_2 histamine receptor belongs to the large multigene family of G protein¶-coupled receptors [2]. Structurally, these receptors are characterized by seven transmembrane α-helices, and functionally, by their ability to transmit signals to effector molecules via G proteins [3]. It is generally accepted that the H_2 receptor is coupled to the adenylyl cyclase system, through direct interaction with a G_s protein family [4].

Cellular responses to agonists of G protein-coupled receptors are attenuated rapidly. Mechanisms of signal attenuation include ligand removal from the extracellular fluid, desensitization of receptor function (uncoupling), endocytosis, and down-regulation [5]. An important component of desensitization, which occurs within seconds to minutes of receptor activation, is the uncoupling of the activated receptors from their G protein by receptor phosphorylation. GRKs, and the second messenger-dependent kinases PKA and PKC, are responsible for homologous and heterologous desensitization, respectively [6]. There are six members of the GRK family, known as GRK1 through GRK6. On the basis of sequence homology, these can be classified into three groups: (a) GRK1 (also known as rhodopsin kinase), (b) GRK2 and 3 (also called β-adrenergic receptor kinases 1 and 2, or βARK1 and 2, respectively), and (c) GRK4, 5, and 6. Cytosolic GRK2 and 3 are translocated to the membrane upon receptor activation, in a process facilitated by interaction with the Gβγ dimers released [7]. Although the GRK2, 3, 5, and 6 subtypes are ubiquitous, GRK2 is particularly abundant in peripheral

‖ Corresponding author: Carlos Davio, Ph.D., Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 PB, (1113) Buenos Aires, Argentina. Tel. (54) 114-964-8277; FAX (54) 114-508-3645. E-mail: cardavio@huemul.ffyb.uba.ar

¶ *Abbreviations:* G proteins, GTP-binding regulatory proteins; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; GRKs, G protein-coupled receptor kinases; cAMP, cyclic AMP; cGMP, cyclic GMP; db-cAMP, dibutyryl-cAMP; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; FBS, fetal bovine serum; and AC, adenylyl cyclase.

Received 21 June 1999; accepted 10 November 1999.

blood leukocytes, as well as in myeloid and lymphoid cell lines [8]. Agonist-induced receptor endocytosis contributes to desensitization by depleting the cell surface of high-affinity receptors, whereas recycling of internalized receptors mediates the resensitization of cellular responses [9]. Receptor down-regulation is a form of desensitization that occurs during continuous, long-term exposure of cells to receptor agonists. Down-regulation is characterized by the depletion of the cellular content due to alterations in the rate of receptor degradation and synthesis [10]. These regulatory events are important, because they govern the ability of cells to respond to agonists. This desensitization mechanism may have serious consequences during various physiological and pathological processes [11].

Recent studies have reported that the H_2 receptor-mediated cAMP response is desensitized rapidly in various cell types [12, 13]. However, in contrast to β-adrenergic receptors [5], the mechanism of H_2 receptor desensitization is still not well understood.

In the human promonocytic cell line U937, an H_2 histamine receptor was described that increases cAMP production directly, with a classical pharmacological profile [13]. Recently, we found concentration-dependent regulation of c-*fos* expression with histamine concentrations corresponding to those that increase cAMP production via H_2 receptors [14]. In addition, a histamine H_1 receptor capable of inducing a concentration-dependent increase in phosphoinositide turnover was characterized in these cells [15].

In the U937 cell line, sustained PKA stimulation, such as that induced by db-cAMP and forskolin, results in a differentiation process, though histamine and H_2 agonists are unable to trigger it [14, 16]. These experimental results strongly suggest that this lack of response may be due to a rapid H_2 receptor-mediated desensitization of cAMP production.

The present study focused on the desensitization process of the H_2 receptor in U937 cells, and on the recovery of the cAMP response. The participation of GRKs in the desensitization process and the slow recovery of the cAMP response, and its relationship to the induction of PDE activity and the kinetics of receptor internalization, are shown.

MATERIALS AND METHODS

Chemicals

Histamine dihydrochloride, forskolin, PGE_2, IBMX, isoproterenol, cAMP, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7), famotidine, and mepyramine were obtained from the Sigma Chemical Co. Amthamine, tiotidine, and *N*-(2-aminoethyl)-5-isoquinolinesulphonamide (H9) were purchased from Tocris Cookson Inc. 2′-*O*-Anthraniloyl cAMP was purchased from GIBCO BRL. Dimaprit, (±)-N^1-[3-(4-fluorophenyl)-3-(pyridin-2-yl)propyl]-N^2-[3-(1H-imidazol-4-yl)propyl]guanidine (arpromidine), and (±)-N^1-[3-(3,4-difluorophenyl)-3-(pyridin-2-yl)-propyl]-N^2-[3-(1H-imidazol-4-yl)propyl]guanidine (BU-E-75) were provided by Dr. W. Schunack of the Frëie Universität, and Dr. A. Buschauer of the Regensburg Universität. [^{3}H]cAMP and [^{3}H]tiotidine were purchased from New England Nuclear. All other chemicals were of analytical grade.

Cell Culture

U937 cells, obtained from the American Type Culture Collection, were cultured at 37° in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS. Cells were passed routinely every 3 days and seeded at a density of 2.5×10^5 cells/mL. Cell viability was monitored in every assay by trypan blue exclusion.

cAMP Assay

Cells were resuspended in Hanks' solution supplemented with 0.8 mM IBMX, at a density of 10^6 cells/mL, preincubated for 3 min at 37°, and exposed for 9 min to different chemicals at the indicated concentrations. The reaction was stopped by a 3-min centrifugation at 3000 *g*; the resulting pellet was resuspended in ethanol for cAMP extraction and further centrifuged for 10 min at 3000 *g*. Then the ethanol phase was dried and resuspended in 50 mM Tris–HCl, pH 7.4. cAMP content was determined by means of competition with [^{3}H]cAMP for PKA, as previously described [17].

Desensitization Experiments

Pretreatment of cells with BU-E-75 or amthamine was performed in RPMI 1640 medium, supplemented with 10% FBS, at 37° in a 5% CO_2 humidified atmosphere. Cells were exposed to 10 μM H_2 agonists (maximal response) for periods ranging from 1 min to 3 hr, in the absence of IBMX. Then cells were washed and resuspended in Hanks' solution containing 0.8 mM IBMX, at a density of 10^6 cells/mL, and exposed for 9 min to 10 μM H_2 agonists, 1 μM isoproterenol, 75 μM forskolin, or 1 μM PGE_2, to determine whether the AC could still generate cAMP.

Cells were pretreated with 20 μM H7 or H9, PKA and PKC inhibitors, or 200 μM Zn^{2+} (a GRK inhibitor) [18] for 20 min at 37°, following which the desensitization assays were carried out in the presence of inhibitors. Low M_r (≅3000) heparin was allowed to enter the cells by preincubating the cultures overnight in the presence of heparin together with 5 μg/mL of lipofectamine (GIBCO BRL), before desensitization. cAMP production is expressed as picomoles of cAMP produced per 10^6 cells, or represented as the percentage of stimulation relative to basal levels.

Assay of Phosphodiesterase Activity

Several fluorescent analogues of cAMP and cGMP have been used as substrates of PDE to determine activity [19]. For this study, the fluorescent cAMP analogue 2′-*O*-anthraniloyl cAMP was used to characterize U937 cell PDE activity.

The U937 cells were resuspended in PDE buffer (10 mM HEPES, 90 mM KCl, 0.7 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.0), and disrupted with ultrasound. The reaction mixture was prepared in a quartz cuvette with 1 mL of cellular lysate as a source of PDE, and 1 mL of 8 μM 2′-*O*-anthraniloyl cAMP solution (2x) in PDE buffer. This suspension was mixed thoroughly, and the change in fluorescence emission at 430 nm (330 nm excitation) was recorded. A calibration curve was prepared with substrate standards covering the 1–8 μM range, and the fluorescence values were converted to femtomoles of substrate degraded per minute per 10^6 cells, at 37°.

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 were incubated with 10^6 cells/tube (or purified membrane fraction corresponding to this number of cells), in the absence or presence of 1 μM tiotidine, in a total volume of 200 μL. After 40 min at 4°, 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 was performed, followed by three 3-mL washes with ice-cold buffer. Experiments on intact cells were carried out at 4° to avoid internalization of the ligand. Kinetic studies showed that equilibrium was reached after 30 min and persisted for 4 hr (data not shown). A purified membrane fraction was obtained by disrupting cells by sonication in 50 mM Tris–HCl, pH 7.4, and centrifuging for 15 min at 8500 *g*. The supernatant was centrifuged further for 40 min at 30,000 *g*, and the resulting pellet was resuspended in 50 mM Tris–HCl, pH 7.4. For desensitization assays, cells were exposed to 10 μM BU-E-75 and washed carefully with Hanks' solution before being included in the binding assay.

Western Blot Analysis

Cells (3×10^6) were lysed in 300 μL of 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. Then samples were boiled for 5 min, and 20-μL aliquots were electrophoresed in 12% SDS–polyacrylamide gels and transferred to nitrocellulose paper. The residual binding sites were blocked with 5% nonfat powdered milk in PBST (PBS containing 0.05% Tween-20), and membranes were incubated with 1 μg/mL of anti-GRK (2, 3, 5, and 6) rabbit sera (Santa Cruz Biotechnology) in PBST. All subsequent washes were performed with PBST. Reactivity was developed using an anti-rabbit polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham Life Science).

Data Analysis

Results are expressed as the means ± SEM of at least three independent experiments, and statistical significance was analyzed by one-way ANOVA. Experimental data for saturation and inhibition studies were analyzed using the nonlinear fitting method of steepest descent to reach the lowest sum of squares supplied by PRISM (Graph Pad Software, Inc.). To facilitate comparisons, IC_{50} values were converted to K_i values using the Cheng and Prusoff equation [20].

TABLE 1. Induction of cAMP production in U937 cells by H_2 agonists

Agonist	EC_{50} (μM)	Maximal response (pmol/10^6 cells)
Histamine	2.7 ± 0.3	120 ± 3
Dimaprit	0.25 ± 0.04	70 ± 11
Arpromidine	0.31 ± 0.03	65 ± 7
Amthamine	1.00 ± 0.30	69 ± 7
BU-E-75	0.27 ± 0.04	60 ± 4

Data are the means ± SEM, obtained in four independent experiments performed on different preparations of U937 cells.

RESULTS

cAMP Production by H_2 Agonists in U937 Cells

The ability of U937 cells to produce cAMP when stimulated with histamine or dimaprit was similar to that reported by other authors [13]. Similar results were obtained with other H_2 agonists such as BU-E-75, arpromidine [21], and amthamine (Table 1).

Histamine H_2 Receptor Desensitization

H_2 receptor desensitization assays were performed with specific H_2 agonists to avoid interactions between the signaling pathways of both receptor subtypes, as previously described [22].

After pretreatment ranging from 1 min to 3 hr with a given H_2 agonist, a decrease in cAMP production was observed when the cells were re-challenged with the same agonist (Fig. 1A). BU-E-75 and amthamine no longer induced a response when cells were pretreated for 2 hr. Half-maximal desensitization was observed at 20 ± 3 min (mean ± SEM, N = 4) for BU-E-75, and 14 ± 3 min (mean ± SEM, N = 3) for amthamine. When cells were incubated with both 10 μM BU-E-75 and 10 μM famotidine (a specific H_2 antagonist), desensitization was prevented completely (Fig. 1B). Finally, this effect was not observed with 10 μM mepyramine, a specific H_1 antagonist (Fig. 1B).

To establish whether the observed desensitization was confined to the histamine H_2 receptor, we examined the effects of H_2 agonist pretreatment on the responses to PGE_2, isoproterenol (a β-adrenergic agonist), and forskolin (a direct activator of AC). When U937 cells were stimulated with PGE_2, isoproterenol, or forskolin, a concentration-dependent increase in cAMP production with EC_{50} values of 40 ± 3 nM (mean ± SEM, N = 5), 20 ± 3 nM (means ± SEM, N = 4), and 19 ± 2 μM (mean ± range, N = 2), respectively, was detected. In the pretreated cells, concentration–response curves showed similar results (data not shown), although no changes were observed in the

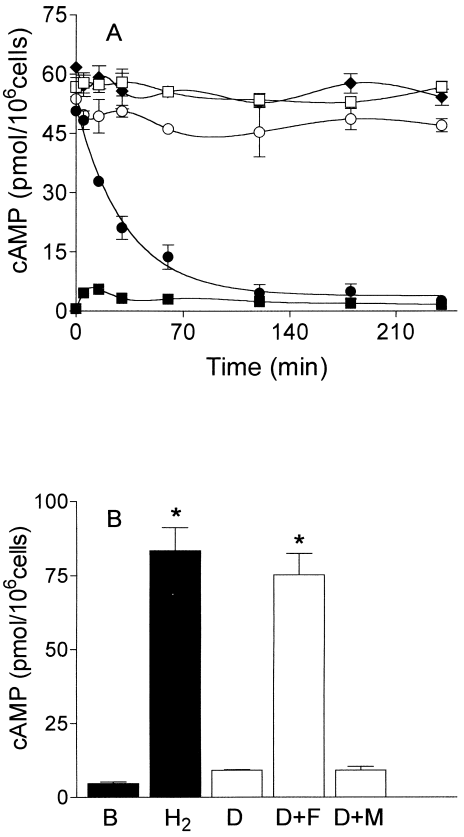

FIG. 1. H_2 agonist-induced desensitization of the histamine H_2 receptor. (A) U937 cells were preincubated for different periods of time with 10 μM BU-E-75, washed, incubated for 3 min in Hanks' medium supplemented with 0.8 mM IBMX, and finally stimulated with 10 μM BU-E-75 (●), 1 μM PGE_2 (○), 1 μM isoproterenol (♦), or 75 μM forskolin (□). Basal cAMP levels were evaluated at each experimental time (■). cAMP production was determined as described in Materials and Methods. Data are expressed as pmol/10^6 cells, and were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments. (B) U937 cells were preincubated with 10 μM BU-E-75 (D); 10 μM BU-E-75 and 10 μM famotidine (D+F); or 10 μM BU-E-75 and 10 μM mepyramine (D+M) for 2 hr, washed, incubated for 3 min in Hanks' medium supplemented with 0.8 mM IBMX, and stimulated with 10 μM BU-E-75. cAMP production was determined as described in Materials and Methods. Filled bars represent control cells that were not exposed to the desensitization stimulus: (B) basal levels, (H_2) stimulation with 10 μM BU-E-75. Data were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments. Key: (*) $P < 0.001$, by ANOVA for multigroup comparison followed by Bonferroni's test.

desensitization assays following 1 μM PGE_2, 1 μM isoproterenol, or 75 μM forskolin stimulus (Fig. 1A).

To determine whether protein kinases are involved in the desensitization of the H_2 receptor in U937 cells, experiments were performed in the presence of H7 or H9 as inhibitors of PKA and PKC, and Zn^{2+} and heparin as inhibitors of GRKs.

Results indicated that 20 μM H7 and H9 had no effect on desensitization ($T_{1/2}$ = 18 ± 4 min, mean ± range, N = 2, and 14 ± 4 min, mean ± SEM, N = 3, respectively) (Fig. 2). No modifications were observed either in cAMP basal levels or in the PGE_2-mediated response (data not shown). Pretreatment of the cells for 30 min with 200 μM Zn^{2+} completely blocked the desensitization of the AC response (Fig. 2). Because Zn^{2+} affected the viability of the cells, amthamine treatment was limited to 60 min in these experiments. At the concentration used, and for incubation times not exceeding 60 min, Zn^{2+} had no effect on amthamine stimulation in naive cells (data not shown). Low M_r (≅3000) heparin was allowed to enter the cells by preincubating the cultures overnight in the presence of 1 or 5 μM heparin together with 5 μg/mL of lipofectamine, before desensitization. Heparin or lipofectamine alone did not alter the effect of amthamine on the cAMP response (data not shown), but in the presence of heparin and lipofectamine, desensitization of the H_2 receptor was reduced markedly. cAMP accumulation was still 30 ± 5 or 15 ± 3% of the control value in the presence of 5 or 1 μM heparin, respectively, compared to 2 ± 1% for cells desensitized in the absence of heparin (Fig. 2, inset). Viability of cells was reduced drastically at concentrations higher than 10 μM (data not shown). To identify the GRKs expressed in these cells, extracts were used in immunoblots. The expression of GRK2, 3, and 6 was not modified by treatment with the H_2 agonist (Fig. 3), whereas GRK5 showed no positive band (data not shown).

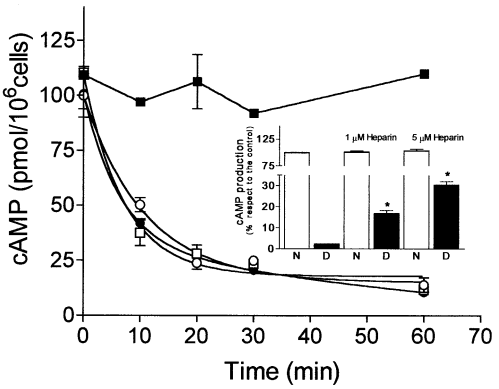

FIG. 2. Effect of H7 or H9 as inhibitors of PKA and PKC, and Zn^{2+} or heparin as inhibitors of GRKs on desensitization of the H_2 receptor. U937 cells were pretreated with 20 μM H7 (●), 20 μM H9 (○), 200 μM Zn^{2+} (■), or not pretreated (□), and exposed for different periods of time to 10 μM amthamine. Cells were then washed, incubated for 3 min in Hanks' medium supplemented with 0.8 mM IBMX, and then stimulated with 10 μM amthamine. cAMP production was determined as described in Materials and Methods. Data are expressed as pmol/10^6 cells, and were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments. (Inset) Effect of heparin on desensitization of the H_2 receptor. cAMP levels were determined as described in Materials and Methods in naive (N) and 1-hr-desensitized (D) cells, before or after treatment with 1 or 5 μM heparin. Data are expressed as means ± SD (N = 3, each determination performed in duplicate). Key: (*) $P < 0.01$, by ANOVA test for multigroup comparison followed by Bonferroni's test.

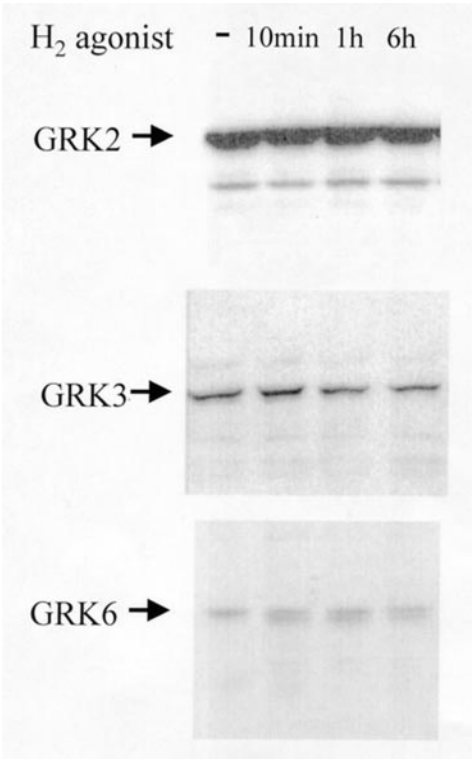

FIG. 3. Expression of GRKs in U937 cells. U937 cells were incubated with 10 μM amthamine for the indicated periods of time before harvesting, and lysed as described in Materials and Methods. Samples were electrophoresed on 12% SDS–polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with polyclonal purified rabbit sera against GRK2, GRK3, and GRK6.

Recovery of the cAMP Response Following BU-E-75 Desensitization

To determine the recovery of the cAMP response following pretreatment with BU-E-75, cells were pretreated with 10 μM BU-E-75 for 2 hr, the period required for total desensitization. Cells then were washed, and basal and re-stimulated levels of cAMP were evaluated. cAMP levels from cells receiving no pretreatment represent control values.

A slow recovery of the BU-E-75-induced intracellular cAMP accumulation could be observed in pretreated cells. When the desensitizing stimulus was removed, 50% of the initial response was recovered within 5 hr, while 100% recovery was achieved after 15 hr (Fig. 4).

Results showed that pretreatment for 2 hr induced modifications in the basal levels of cAMP with respect to control cells. Minimal values were observed 12 hr later, and corresponded to approximately 1.3% of the initial basal level (7.5 vs 0.1 pmol/10^6 cells) (inset Fig. 4). These results can be explained by an increase in PDE activity following 10 μM BU-E-75 treatment.

Phosphodiesterase Activity

Total PDE activity in U937 cells was determined in a mixture of cytosolic and membrane fractions, using 8 μM anthraniloyl cAMP. Two hours after treatment with 10 μM

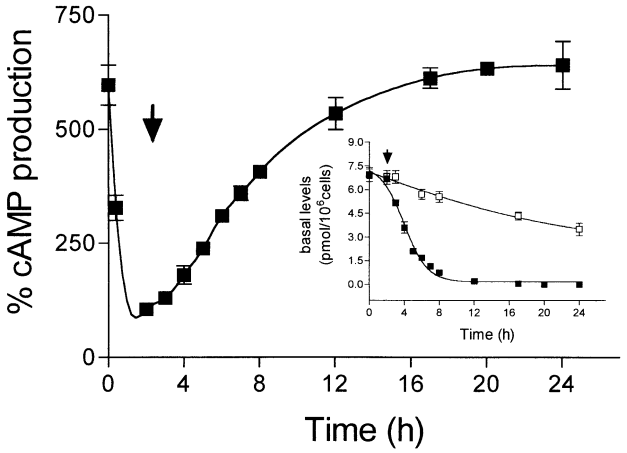

FIG. 4. Recovery from BU-E-75 desensitization. Cells were treated with 10 μM BU-E-75 for 2 hr, washed (↓), and the capacity of H_2 receptors to generate an increase of intracellular cAMP at different times was evaluated. Results are expressed as percent of stimulation; basal levels are considered as 100% at the different times. Data were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments. (Inset) Cells were pretreated with BU-E-75 for 2 hr (■) or not (□), washed (↓), and basal cAMP levels at different periods of time were evaluated by incubating cells for 12 min in Hanks' solution, supplemented with 0.8 mM IBMX. Data were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments.

BU-E-75, a 2-fold increase in PDE activity, compared with untreated cells (2.25 ± 0.09 fmol/min per 10^6 cells, mean ± SEM, N = 3 vs 1.33 ± 0.16 fmol/min per 10^6 cells, mean ± SEM, N = 3) was observed (Fig. 5). Prolonged treatment resulted in a marked increase in PDE activity, reaching a maximum within 24 hr (7.18 ± 1.30 fmol/min per 10^6 cells, mean ± SEM, N = 3) (Fig. 5).

[^{3}H]Tiotidine Binding Assay

To evaluate whether receptor internalization takes place during the desensitization process, [^{3}H]tiotidine binding

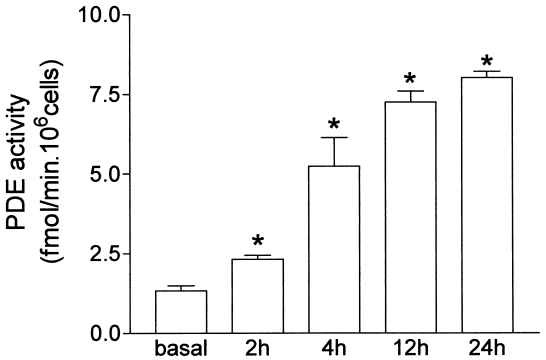

FIG. 5. PDE activity in U937 cells. Cells were incubated for different periods of time with 10 μM BU-E-75, and the activity of PDE was evaluated as described in Materials and Methods. Basal values correspond to untreated cells. Data were calculated as the means ± SEM of three independent experiments. Key: (*) $P < 0.05$ with respect to basal activity.

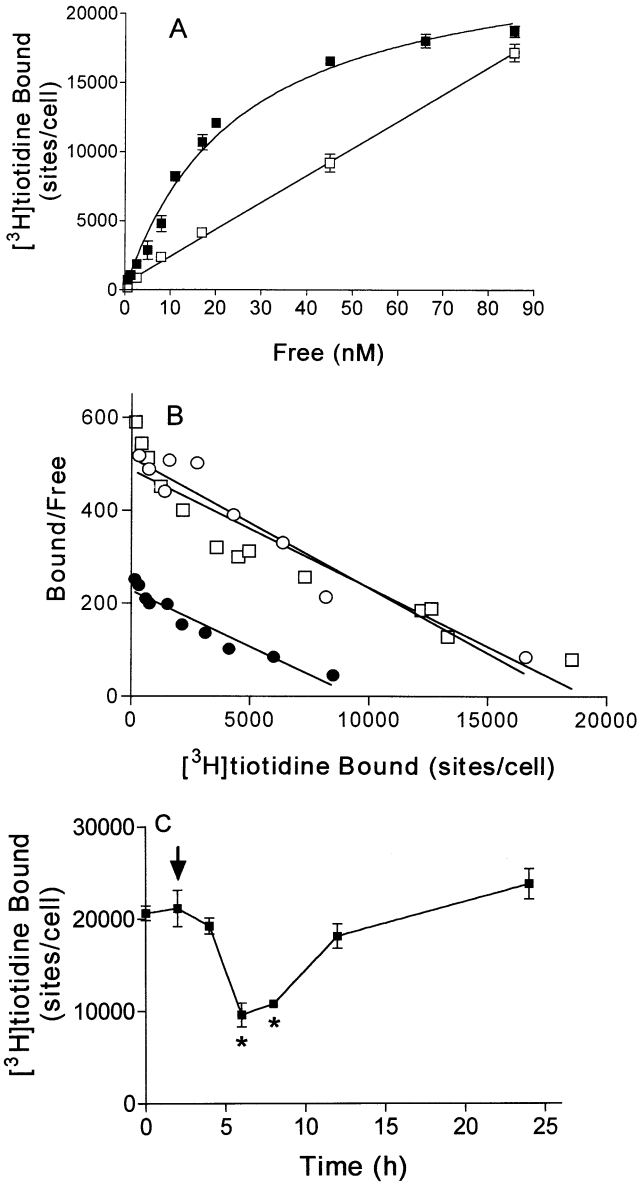

FIG. 6. [^{3}H]Tiotidine binding to U937 cells. (A) Saturation assay for [^{3}H]tiotidine in U937 cells: (■) specific binding and (□) nonspecific binding to intact cells. Data were calculated as the means ± SEM of assay triplicates. Similar results were obtained in five independent experiments. (B) Scatchard plot of a saturation assay with [^{3}H]tiotidine on intact cells, incubated with 10 μM BU-E-75 for 2 hr (○), after 6 hr of total desensitization (●), or without treatment (□). Data were calculated as the means ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments. (C) Cells were treated with 10 μM BU-E-75 for 2 hr, washed (↓), and total binding at different times was evaluated. Data represent the Q total from a saturation assay with [^{3}H]tiotidine on intact cells, calculated as the means ± SEM of three independent experiments. Key: (*) $P < 0.01$ with respect to control.

experiments were carried out. Saturation analysis using intact cells revealed a [^{3}H]tiotidine binding site (Q = 20,500 ± 1,800 sites/cell, K_d = 20 ± 3 nM, mean ± SEM, N = 5) (Fig. 6A). Similar results were obtained using the purified membrane fraction (data not shown). To confirm that [^{3}H]tiotidine specifically labeled the H_2 receptor,

TABLE 2. Inhibition constants (K_i) for [^{3}H]tiotidine binding

Compound	IC_{50} (nM)	K_i (nM)
Tiotidine	74.5 ± 20	37 ± 17
Famotidine	317 ± 60	156 ± 34
BU-E-75	319 ± 20	160 ± 19
Mepyramine	ND	ND

The IC_{50} Values were calculated from a nonlinear regression program by the method of Cheng and Prusoff [20]. Results are expressed as the means ± SEM, N = 3–4. $K_i = IC_{50}/1 + [T]Kd^{-1}$, where IC_{50} is the concentration of the agent required for 50% inhibition of specific [^{3}H]tiotidine binding, [T] is the concentration of [^{3}H]tiotidine in the assay (20 nM), and K_d is the dissociation constant for [^{3}H]tiotidine, considered as 20 nM. ND, < 20% inhibition at 5×10^{-5} M.

inhibition of [^{3}H]tiotidine binding to U937 cells by several H_2 agonists and antagonists was evaluated (Table 2). The maximal inhibition of [^{3}H]tiotidine binding by each compound did not differ significantly from that obtained in the presence of 1 μM tiotidine, i.e. there was no displacement of the nonspecific component that routinely accounted for 50–55% of the total binding. Binding experiments were performed using intact cells pretreated for 2 hr with 10 μM BU-E-75, at which time complete loss of cAMP responsiveness occurred. Results showed that the number of sites remained unmodified (Q = 21,800 ± 2,114 sites/cell, mean ± SEM, N = 3) (Fig. 6B). Cells were then washed to remove the desensitizing stimulus, and the number of sites were evaluated for different periods of time. Similar results were observed after 2 hr of total desensitization (Fig. 6C). At this time, the number of sites decreased, reaching a minimum value at 4 hr (Q = 9446 ± 514 sites/cell; K_d = 22 ± 8 nM, mean ± SEM, N = 3) (Fig. 6, B and C), and a total recovery of binding sites was achieved after 10 hr (Fig. 6C).

DISCUSSION

The U937 cell line is derived from the pleural fluid of a patient with diffuse histiocytic lymphoma [23]. The presence of H_2 receptors in this cell line has been described, showing a classic pharmacological profile similar to the one described for the guinea pig right atrium [13]. For this reason, these cells represent an interesting model for studying the signal transduction pathway for H_2 receptors. Recent studies indicate a rapid desensitization of the H_2 receptor by 100 μM histamine [13].

In this study, using 10 μM BU-E-75 and amthamine, we demonstrated a specific desensitization with a $T_{1/2}$ of approximately 20 min, similar to the one described for histamine [13]. This process was blocked by 10 μM famotidine, whereas mepyramine did not modify desensitization, indicating that the cross-talk between the histamine H_1 and H_2 receptor [15] is not involved in the mechanism of desensitization.

Desensitization proved to be homologous, since pretreatment did not induce modifications in the responses to PGE_2, isoproterenol, or forskolin. Pretreatment of cells with H7 and H9 did not modify H_2 receptor desensitization

kinetics, indicating that neither PKA nor PKC is involved in this process. Desensitization of the histamine H_2 receptor has been noted in the human leukemia cell line HL-60, and the human gastric adenocarcinoma cell line MKN-45. Rapid homologous and heterologous desensitizations have been observed in HL-60 cells. In the case of the MKN-45 cell line, cAMP-independent homologous desensitization (via GRK2) could be detected, but heterologous desensitization was absent [24, 25]. For other receptors such as the β_2-adrenergic receptor, both PKA-dependent and PKA-independent mechanisms (GRKs) have been shown to contribute to agonist-induced desensitization, as well as in the sequestration of the receptors from the cell surface [26]. However, there are several reports of G_s-coupled receptors in which no PKA-mediated desensitization was observed [11]. In U937 cells, we recently reported heterologous desensitization of H_2 receptors via PKA, following forskolin stimulation [16].

Zn^{2+} has been shown to inhibit phosphorylation of the β_2-adrenergic receptor by βARK, and completely blocks the time-dependent desensitization and phosphorylation of the δ-opioid receptors in SK-N-BE cells [18]. In the present study, we detected an inhibition of the desensitization process in the presence of Zn^{2+}. Furthermore, a pronounced inhibition of desensitization in the presence of 5 μM heparin was also observed, a result that strongly suggests that a member of the GRK family is involved in this event. Immunoblots showed the expression of three members of this family in U937 cells (GRK2, 3, and 6), indicating that some GRKs could be involved in H_2 receptor desensitization.

The decrease in the basal levels of cAMP after 2 hr of treatment is a consequence of the induction of PDE activity. Previous studies with U937 cells have shown that the activity of cyclic nucleotide phosphodiesterase 4 (PDE4) is increased by agents that elevate cAMP content [27]. PDE4 is the predominant cAMP-hydrolyzing isoenzyme class found in most, if not all, immune and inflammatory cells [28].

The present data show that up to 2 hr, the time in which receptor desensitization is nearly complete, internalization does not take place. Similar results have been described for H_2 receptors in HL-60 cells [24], and in the gastric carcinoma cell line HGT-1 [29]. Moreover, desensitization studies of histamine H_2 receptor expressed in the HEK-293 cell line [30] and in CHO cells [12] indicate a rapid receptor desensitization with subsequent internalization, followed by a rapid recovery of the response.

Binding experiments showed that receptor internalization began after 2 hr, when total desensitization took place. After removal of the desensitizing stimulus, the number of sites reached a minimum at approximately 4 hr, and total recovery of the binding sites was achieved after 10 hr. H_2 receptor internalization and recycling to the membrane correlates with the recovery of the cAMP response discussed previously.

It has been postulated that the internalization of seven transmembrane receptors is necessary for the dephosphorylation and recycling of the receptor to the membrane to allow recovery of the response [11]. In addition, it has been reported that in β-adrenergic receptors prevention of sequestration abolishes the recovery of the biological response [11].

Histamine receptors have been classified according to their pharmacological properties [31]. During the last few years, study of the molecular biology of G protein-coupled receptors has led to a dramatic increase of receptor subtypes [11, 32], and in many cases has allowed the explanation of different regulatory mechanisms.

cAMP levels are implicated in growth regulation of normal and malignant cells [33, 34]. This second messenger is also involved in apoptotic cell death in lymphoid [35] and myeloid cells [36–38]. In HL-60 cells, histamine, acting through H_2 receptors, produces an increase in cAMP intracellular levels that induces cell maturation. In this system, the reported $T_{1/2}$ of desensitization is 60 min [39].

We recently have found that histamine is not capable of inhibiting U937 cell proliferation, despite the marked but transient increase in cAMP levels, whereas important inhibition was observed with db-cAMP and forskolin [14, 16]. Accordingly, we have shown that forskolin and db-cAMP, but not H_2 agonists, induced cells to acquire the ability to produce superoxide anion, to express cell surface maturation markers, and to increase chemotaxis, indicating cellular differentiation [14, 16]. These results suggest that the binding of agonists to H_2 histamine receptors triggers an early specific response characterized by an increase in cAMP levels, but the rapid desensitization ($T_{1/2}$ = 20 min) makes this response insufficient to induce differentiation of U937 cells [14, 16].

The histamine receptor has an important role in many physiological and pathological processes. A better understanding of the receptor signal transduction regulation might be of importance in therapeutic manipulation.

This study was supported by grants from the Antorchas Foundation (A-13218/1–000026), the University of Buenos Aires (JB10), and CONICET-FONCYT, Argentina (PICT-1598; 4406).

References

1. Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankeley NP, Young JM, Schunack W, Levi R and Haas HL, International Union of Pharmacology. XIII: Classification of histamine receptors. *Pharmacol Rev* **49:** 253–278, 1997.
2. Gantz I, Schaffer M, DelValle J, Logsdon C, Campbell V, Uhler M and Yamada T, Molecular cloning of a gene encoding the histamine H_2 receptor. *Proc Natl Acad Sci USA* **88:** 429–433, 1991.
3. Dohlman HG, Thorner J, Caron MG and Lefkowitz RJ, Model systems for the study of seven-transmembrane segment-receptors. *Annu Rev Biochem* **60:** 653–688, 1991.
4. Kühn B, Schmid A, Harteneck C, Gudermann T and Schultz G, G proteins of the G_q family couple the H_2 histamine receptor to phospholipase C. *Mol Endocrinol* **10:** 1697–1707, 1996.
5. Grady EF, Böhm KS and Bunnett NW, Turning of the signal:

Mechanisms that attenuate signaling by G protein-coupled receptors. *Am J Physiol* **273:** G586–G601, 1997.
6. Freedman NJ and Lefkowitz RJ, Desensitization of G protein-coupled receptors. *Recent Prog Horm Res* **51:** 319–353, 1996.
7. Palczewski K, GTP-binding-protein-coupled receptor kinases—two mechanistic models. *Eur J Biochem* **248:** 261–269, 1997.
8. Chuang TT, Sallese M, Ambrosini G, Parruti G and De Blasi A, High expression of β-adrenergic receptor kinase in human peripheral blood leukocytes. Isoproterenol and platelet activating factor can induce kinase translocation. *J Biol Chem* **267:** 6886–6892, 1992.
9. Trowbridge IS, Collawn JF and Hopkins RC, Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu Rev Cell Biol* **9:** 129–161, 1993.
10. Hadcock JR and Malbon CC, Agonist regulation of gene expression of adrenergic receptors and G proteins. *J Neurochem* **60:** 1–9, 1993.
11. Lohse M, Molecular mechanisms of membrane receptor desensitization. *Biochim Biophys Acta* **1179:** 171–188, 1993.
12. Fukushima Y, Oka Y, Katagiri H, Saitoh T, Asano T, Ishihara H, Matsuhashi N, Kodama T, Yazaki Y and Sugano K, Desensitization of canine H2 receptor expressed in Chinese hamster ovary cells. *Biochem Biophys Res Commun* **190:** 1149–1155, 1993.
13. Smit M, Leurs R, Shukrula S, Bast A and Timmerman H, Rapid desensitization of the histamine H_2 receptor on the human monocytic cell line U937. *Eur J Pharmacol* **288:** 17–25, 1994.
14. Shayo C, Davio C, Brodsky A, Mladovan AG, Lemos Legnazzi B, Rivera E and Baldi A, Histamine modulates the expression of c-*fos* through cyclic AMP production via the H_2 receptor in the human promonocytic cell line U937. *Mol Pharmacol* **51:** 983–990, 1997.
15. Davio C, Baldi A, Shayo C, Brodsky A, Cricco G, Bergoc R and Rivera E, H_1 and H_2 histamine receptors in histiocytic lymphoma cell line U-937. *Inflamm Res* **44**(Suppl 1)**:** S72–S73, 1995.
16. Brodsky A, Davio C, Shayo C, Lemos Legnazzi B, Barbosa M, Lardo M, Morelli A, Baldi A, Sanchez Avalos JC and Rivera E, Forskolin induces U937 cell line differentiation as a result of a sustained cAMP elevation. *Eur J Pharmacol* **350:** 121–127, 1998.
17. Davio CA, Cricco GP, Bergoc RM and Rivera ES, H_1 and H_2 histamine receptors in *N*-nitroso-*N*-methylurea (NMU)-induced carcinomas with atypical coupling to signal transducers. *Biochem Pharmacol* **50:** 91–96, 1995.
18. Hasbi A, Polastron J, Allouche S, Stanasila L, Massotte D and Jauzac P, Desensitization of the δ-opioid receptor correlates with its phosphorylation in SK-N-BE cells: Involvement of a G protein-coupled receptor kinase. *J Neurochem* **70:** 2129–2138, 1998.
19. Kincaid RL and Manganiello VC, Assay of cyclic nucleotide phosphodiesterase using radiolabeled and fluorescent substrates. *Methods Enzymol* **159:** 457–470, 1988.
20. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
21. Felix SB, Buschauer A and Baumann G, Therapeutic value of H_2 receptor stimulation in congestive heart failure. Hemodynamic effects of BU-E-76, BU-E-75 and arpromidine (BU-E-50) in comparison to impromidine. *Agents Actions Suppl* **33:** 257–269, 1991.
22. Schwartz JC, Arrang JM, Garbarg M, Pollard H and Ruat M, Histaminergic transmission in the mammalian brain. *Physiol Rev* **71:** 1–51, 1991.
23. Sundstrom C and Nilsson K, Establishment and characterization of human histiocytic lymphoma cell line U937. *Int J Cancer* **17:** 565–572, 1976.
24. Sawutz D, Kalinyak K, Whitsett J and Johnson C, Histamine H_2 receptor desensitization in HL-60 human promyelocytic leukemia cells. *J Pharmacol Exp Ther* **231:** 1–6, 1984.
25. Nakata H, Kinoshita Y, Kishi K, Fukuda H, Kawanami C, Matsushima Y, Asahara M, Okada A, Maekawa T and Chiba T, Involvement of β-adrenergic receptor kinase-1 in homologous desensitization of histamine H_2 receptors in human gastric carcinoma cell line MKN-45. *Digestion* **57:** 406–410, 1996.
26. Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ, Multiple pathways of rapid β_2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* **265:** 3202–3209, 1990.
27. Torphy TJ, Zhou H-L, Foley JJ, Sarau HM, Manning CD and Barnette MS, Salbutamol up-regulates PDE4 activity and induces a heterologous desensitization of U937 cells to prostaglandin E_2. Implications for the therapeutic use of β-adrenoceptor agonists. *J Biol Chem* **270:** 23598–23604, 1995.
28. Giembycz MA, Phosphodiesterase 4 and tolerance to β_2-adrenoreceptor agonists in asthma. *Trends Pharmacol Sci* **17:** 331–336, 1996.
29. Enami S and Gespach C, Desensitization by histamine of H_2 receptor activity in HGT-1 human cancerous gastric cells. *Agents Actions* **18:** 130–136, 1986.
30. Smit MJ, Timmerman H, Alewijnse AE, Punin M, van den Nieuwenhof I, Blauw J, van Minnen J and Leurs R, Visualization of agonist-induced internalization of histamine H_2 receptors. *Biochem Biophys Res Commun* **214:** 1138–1145, 1995.
31. Leurs R, Smit MJ and Timmerman H, Molecular pharmacological aspects of histamine receptors. *Pharmacol Ther* **66:** 413–463, 1995.
32. Saxena RP, Serotonin receptors: Subtypes, functional responses and therapeutic relevance. *Pharmacol Ther* **66:** 339–368, 1995.
33. Cho-Chung YS, Clair T, Tortora G and Yokozaki H, Role of site-selective cyclic AMP analogs in the control and reversal of malignancy. *Pharmacol Ther* **50:** 1–33, 1991.
34. Hartwell L, Cell cycle. cAMPing out. *Nature* **371:** 286, 1994.
35. McConkey DJ, Orrenius S and Jondal M, Agents that elevate cyclic AMP stimulate DNA fragmentation in thymocytes. *J Immunol* **145:** 1227–1230, 1990.
36. Duprez E, Gjertsen BT, Bernard O, Lanotte M and Døskeland SO, Antiapoptotic effect of heterozygously expressed mutant RI (Ala^{336}→Asp) subunit of cAMP kinase I in a rat leukemia cell line. *J Biol Chem* **268:** 8332–8340, 1993.
37. Tasaka K, Doi M, Nakaya N and Mio M, Reinforcement effect of histamine on the differentiation of murine myeloblasts and promyelocytes: Externalization of granulocyte colony-stimulating factor receptors induced by histamine. *Mol Pharmacol* **45:** 837–845, 1994.
38. Vintermyr O, Gjertsen BT, Lanotte M and Døskeland SO, Microinjected catalytic subunit of cAMP-dependent protein kinase induces apoptosis in myeloid leukemia (IPC-81) cells. *Exp Cell Res* **206:** 157–161, 1993.
39. Seifert R, Höer A, Schwaner I and Buschauer A, Histamine increases cytosolic Ca^{2+} in HL-60 promyelocytes predominantly via H_2 receptors with an unique agonist/antagonist profile and induces functional differentiation. *Mol Pharmacol* **42:** 235–241, 1992.