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Reduction of G Protein-Coupled Receptor Kinase 2 Expression in U-937 Cells Attenuates H2 Histamine Receptor Desensitization and Induces Cell Maturation

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ABSTRACT

Histamine and H2 agonists transiently induce an important cAMP response in promonocytic U-937 cells but fail to induce monocytic differentiation because of a rapid receptor desensitization mediated by G protein-coupled receptor kinases (GRKs). The aims of the present study were to investigate the participation of GRK2 in the desensitization mechanism of the H2 receptor in U-937 cells by reducing GRK2 levels through antisense technology and to evaluate the differentiating capacity of cells expressing lower GRK2 level, stimulated by H2 agonists. By stable U-937 cell transfection with a GRK2-antisense cDNA, we obtained D5 and A2 cell clones exhibiting a reduction in GRK2 expression and an H3 clone with no significant difference in GRK2 expression from control cells. The cAMP response induced by the H2 agonist in D5 and A2 but not in H3 cells was higher than in U-937 and persisted for a longer period of time, although the number of H2 receptors in D5 and A2 cells was lower than in U-937. Furthermore, D5 and A2 cells treated with H2 agonist showed patterns of c-Fos and CD88 expression consistent with monocytic differentiated cells. Overall, these results indicate a direct correlation between the expression of GRK2 and the desensitization of natively expressed H2 receptors in U-937 cells, suggesting that GRK2 plays a major role in the regulation of these receptors' response. In turn, desensitization process is a key component of H2 receptor signaling, determining the differentiation capability of promonocytic cells.

Histamine is a biogenic amine widely distributed throughout the body that works as a chemical messenger to exert numerous functions in central and peripheral tissues, including the induction of normal promyelocytic differentiation (Tasaka et al., 1994). These effects are mediated through three pharmacologically distinct subtypes of receptors, the H1, H2, and H3 receptors (Hill, 1990; Leurs et al., 1995; Hill et al., 1997). Recently, the H4 receptor was cloned and preliminarily characterized (Zhu et al., 2001). Molecular biology studies indicate that the histamine receptor belongs to the large multigene family of G protein-coupled receptors (GPCRs). Structurally, these receptors are characterized by seven transmembrane α-helices and functionally by their ability to transmit signals to effector molecules via G proteins (Dohlman et al., 1991). GPCRs play key physiological roles and their dysfunction results in several health disorders. The H2 receptor (H2r) causes cAMP accumulation through Gs protein activation in gastric cells, cardiac tissues, and other cell types, including smooth muscle cells and immune cells (Hill, 1990).

For a large number of GPCRs, rapid desensitization seems to involve receptor phosphorylation. G protein-coupled receptor kinases (GRKs) are responsible for homologous desensitization, whereas the second messenger-dependent kinases, protein kinases A (PKA) and C, could be involved in homologous and heterologous desensitization (Freedman and Lefkowitz, 1996; Post et al., 1996; Moffett et al., 2001). GRK-mediated phosphorylation of serine/threonine residues in the carboxyl tail and/or intracellular loops of GPCRs increases the receptor affinity to arrestin-type proteins, and this binding prevents any further coupling between the receptor and

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ABBREVIATIONS: GPCR, G protein-coupled receptor; H2r, histamine H2 receptor; GRK, G protein-coupled receptor kinase; PKA, protein kinase A; dbcAMP, dibutyryl cAMP; AC, adenylyl cyclase; IBMX, 3-isobutyl-1-methylxanthine; AM, acetoxymethyl ester; BSA, bovine serum albumin; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl; HA, hemagglutinin; PBS, phosphate buffered-saline; RPMI, Roswell Park Memorial Institute; BSS, buffered saline solution; $[Ca^{2+}]i$, intracellular Ca^{2+}.

G proteins. The complex formed by the phosphorylated GPCR and arrestin targets the activated receptor to clathrin-coated pits for subsequent internalization (Pitcher et al., 1998). There are six members of the GRK family: GRK1 through GRK6. On the basis of sequence homology, these can be classified into three groups: GRK1 (also known as rhodopsin kinase), GRK2 and -3 (also called β-adrenergic receptor kinases 1 and 2), and, finally, GRK4, -5, and -6. The mechanisms by which GRK activity is regulated can be divided into three categories: subcellular localization, alterations in intrinsic kinase activity, and alterations in GRK expression levels. Cytosolic GRK2 and -3 are translocated to the membrane after receptor activation, in a process facilitated by the interaction with released G$\beta\gamma$ dimers (Palczewski, 1997; Penn et al., 2000). Although GRK2, -3, -5, and -6 subtypes are ubiquitous, GRK2 is particularly abundant in peripheral blood leukocytes and in myeloid and lymphoid cell lines (Chuang et al., 1992).

The promonocytic U-937 cell line, derived from a histiocytic lymphoma, is considered an appropriate model to study the mechanism of cell differentiation (Harris and Ralph, 1985). It has been postulated that terminally differentiated cells would progressively lose their malignant capacity. In this cell line, dibutyryl cAMP (dbcAMP) and forskolin [an adenylyl cyclase (AC) activator] induce monocyte maturation (Gavison et al., 1988; Brodsky et al., 1998), whereas histamine-increased cAMP levels via H2r fail to promote differentiation, probably because of the rapid desensitization of the response (Shayo et al., 1997; Lemos Legnazzi et al., 2000). This early desensitization proved to be homologous and to involve receptor phosphorylation by the GRK family. Later, other mechanisms, such as an increase in nucleotide-phosphodiesterase activity or the reduction in receptor number, become involved in the decreased cell response to prolonged H2 agonist stimulation (Lemos Legnazzi et al., 2000).

The molecular mechanisms involved in H2r uncoupling have been studied in different systems. In COS-7 transfected cells, GRK2 and GRK3 phosphorylation mediates H2r desensitization (Shayo et al., 2001).

The aim of the present study was to investigate the participation of GRK2 in H2r desensitization in U-937 cells and to elucidate whether the lack of cell differentiation after treatment with H2 agonist was caused by rapid H2r desensitization. Results demonstrate that the attenuation of GRK2 levels by antisense cDNA reduces the desensitization of the H2r in U-937 cells and allows the H2 agonist to induce markers of cell maturation.

Materials and Methods

Materials. Restriction enzymes and DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA). Cell culture medium, DEAE-dextran, 3-isobutyl-1-methylxanthine (IBMX), cAMP, db-cAMP, forskolin, isoproterenol, Fura 2 acetoxymethyl ester (AM), bovine serum albumin (BSA), C5a, Tween-20, protease inhibitors, and phosphatase inhibitors, were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum was obtained from Invitrogen (Carlsbad, CA). Amthamine and 6[2-(4-imidazolyl)ethylamino]-*N*-(4-trifluoromethylphenyl)heptanecarboxamide were from Tocris Cookson Inc. (Ballwin, MO). *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl (H-89) was obtained from Calbiochem (La Jolla, CA). [^{3}H]cAMP and [^{3}H]tiotidine were purchased from PerkinElmer Life Sciences (Boston, MA) and [^{32}P]orthophosphate was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other chemicals were of analytical grade.

Plasmid Constructions. To prepare the GRK2 antisense construct, pBluescript was used to isolate the *Eco*RI/*Nhe*I fragment containing the GRK2 bovine cDNA. The eukaryotic expression vector pCEFL-HA (Teramoto et al., 1996) was cut with restriction endonucleases *Nhe*I and *Eco*RI. The larger *Nhe*I/*Eco*RI fragment from the plasmid was isolated and ligated to the fragment containing the GRK2 bovine cDNA. The result was a plasmid containing the GRK2 cDNA in reverse orientation with respect to the pCEFL-HA promoter (pCEFL-HA-antiGRK2). The orientation was confirmed by digestion with *Kpn*I and *Eco*RI, giving fragments of 7.7 and 0.3 kilobase pairs. GRK2 and -3 cDNAs were subcloned into the pCEFL vector (pCEFL-GRK2 and -3). Plasmid purification was performed using reagents from QIAGEN (Valencia, CA) according to the manufacturer's instructions.

Cell Culture. U-937 and COS-7 cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal calf serum and 50 μg/ml gentamicin.

Transient Transfection of COS-7 Cells. Transient transfection was performed by the DEAE-dextran technique as reported previously (Shayo et al., 2001). For GRK Western blotting, COS-7 cells were plated in 35-mm dishes and transfected at 80% confluence using 1 μg of each indicated plasmid. For the immunoprecipitation assay, cells were plated in 100-mm dishes and transfected with 2 μg of each construct. The total amount of plasmids was equaled with vector alone. All assays were performed 48 h after transfection.

Stable Transfection of U-937 Cells. Cells were harvested by centrifugation from cultures in exponential growth phase, washed once in phosphate-buffered saline (PBS), and resuspended at 10^6 cells/ml in fresh RPMI medium on ice. pCEFL-HA-antiGRK2 (10 μg) linearized with *Sal*I or pCEFL-HA linearized with *Eco*RI was added to cell suspension (400 μl) and kept 5 min on ice. 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 nonselective medium overnight. Cells were then plated in a 96-well culture plate in RPMI medium containing 0.8 mg/ml G-418. After 2 to 3 weeks, the surviving clones were amplified.

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% bromphenol blue), and sonicated to shear DNA. Samples were boiled for 5 min, and aliquots were subjected to electrophoresis in 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The residual binding sites were blocked with 5% nonfat powdered milk in PBS containing 0.05% Tween-20, and membranes were incubated with 1 μg/ml of anti-GRK2, -3, or c-Fos rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), in PBS containing 0.05% Tween-20. All subsequent washes were performed with the same buffer. Reactivity was developed using an anti-rabbit polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, according to the manufacturer's instructions (Amersham Biosciences).

Phosphorylation Assays. Transfected cells were preincubated for 1 h at 37°C in phosphate-free Dulbecco's modified Eagle's medium and labeled 3 h with 100 μCi/ml of $^{32}P_i$ at 37°C in fresh medium. H2 agonist was applied as indicated in figure legends. The reaction was stopped by placing the cells at 4°C and washing twice with ice-cold PBS, followed by the addition of 1 ml/plate of immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 1% Nonidet P-40, 0.1% SDS, 0.2 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 10 μM leupeptin, and 5 μM pepstatin). Lysed cells were centrifuged for 20 min at 12,000*g* at 4°C. The epitope-tagged H2 histamine receptor was immunoprecipitated from the supernatant by a 1 h incubation with the specific anti-HA antibody (Babco, Richmond, CA) at 4°C. Immunocomplexes were recovered with the aid of Protein A/G-

Sepharose (Santa Cruz Biotechnology) and washed five times with ice-cold immunoprecipitation buffer. Complexes were then dissociated by heating to 65°C for 10 min in sample buffer and separated by 12% SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to AGFA Curix RP1 films (Agfa Gevaert, Leverkusen, Germany). ^{32}P-labeling was quantified with the use of the Scion Image software (Scion Corporation, Frederick, MD).

cAMP Assay. Cells were resuspended in Hanks' solution supplemented or not with 1 mM IBMX, at a density of 10^6 cells/ml, preincubated 3 min at 37°C, and exposed for different periods of time to different chemicals at the indicated concentrations. The reaction was stopped by ethanol addition and centrifuged 10 min at 3000*g*. The ethanol phase was then dried and resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% BSA.

For the desensitization assay, pretreatment of cells with amthamine was performed in RPMI 1640 medium at 37°C 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 h, in the absence of IBMX. Cells were then washed and resuspended in Hanks' solution containing 1 mM IBMX at a density of 10^6 cells/ml and exposed for 10 min to 10 μM H_2 agonists to determine whether AC could still generate cAMP.

cAMP content was determined by means of competition with [^{3}H]cAMP for PKA, as described previously (Davio et al., 1995b). cAMP production is expressed as picomoles of cAMP produced per 10^6 cells.

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, 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-fibers filters was performed, followed by three washes with 3 ml of ice-cold buffer. Experiments on intact cells were carried out at 4°C to avoid internalization 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).

Intracellular Ca^{2+} Measurements. Fura 2-AM was used as a fluorescent indicator. Cells from 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 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES, pH 7.5) in the presence of 2 μM Fura 2-AM, 10 mM glucose, and 0.1% BSA. Cells were incubated for 30 min at 37°C in an atmosphere of 5% CO_2, by which time Fura 2 was trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS without Fura 2-AM, and brought to a density of 2×10^6 cells/ml of BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan), equipped with the CA-261 accessory (to measure Ca^{2+} with continuous stirring), the thermostat adjusted to 37°C, and an injection chamber. Intracellular Ca^{2+} ([Ca^{2+}]i) levels were registered every second by exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and their ratio (F340/F380) were tracked. Different agents were injected (5 μl) into the chamber as a 100-fold concentrated solution without interrupting recording. 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. (1985).

Results

Functionality of GRK2 Antisense Construct. As an initial step, the GRK2 antisense cDNA, constructed as detailed under *Materials and Methods*, was assayed for its capability to specifically inhibit the production of GRK2 protein in COS-7 transfected cells, because GRK2 and GRK3 share 72.5% of their sequence. COS-7 cells were transiently cotransfected with GRK2 antisense and GRK2 or GRK3 sense expression vectors, alternatively. After 48 h, Western blotting indicated that the antisense construct reduced GRK2 protein levels but did not interfere with GRK3 levels (Fig. 1A). H2r phosphorylation after H2 agonist stimulus was examined to determine whether the observed reductions in GRK2 expression were sufficient to produce a significant reduction in GRK2 activity. Cells transfected with the tagged receptor (HA-H2r) and GRK2 antisense expression vector reduced both HA-H2r phosphorylation induced by endogenous GRKs and the enhanced phosphorylation obtained after GRK2 transfection (Fig. 1B).

Creation of U-937 Cell Lines Containing GRK2 Antisense. The antisense-GRK2 construction was used to stably transfect U-937 cell line. Three clones resistant to G-418 were obtained: D5, A2, and H3. As a control, U-937 cells were transfected with the vector alone, resulting in the D2 clone.

The D5 and A2 clones were identified as having reduced levels of GRK2 expression compared with U-937 or D2 cells. Densitometry studies estimated 50 and 45% reductions in GRK2 levels for D5 and A2, respectively, whereas no modifications were observed in H3 cells (Fig. 2). Finally, all clones proved to express identical GRK3 and GRK6 levels (data not shown).

cAMP Response in GRK2-Antisense Clones. The consequence of GRK2 level reduction on receptor-mediated cAMP production and accumulation induced by the H2 agonist was examined in D5 cells (cells with lower GRK2 levels). In amthamine dose-response experiments, D5 cells' maximal response was higher than in control cells (U-937 and D2).

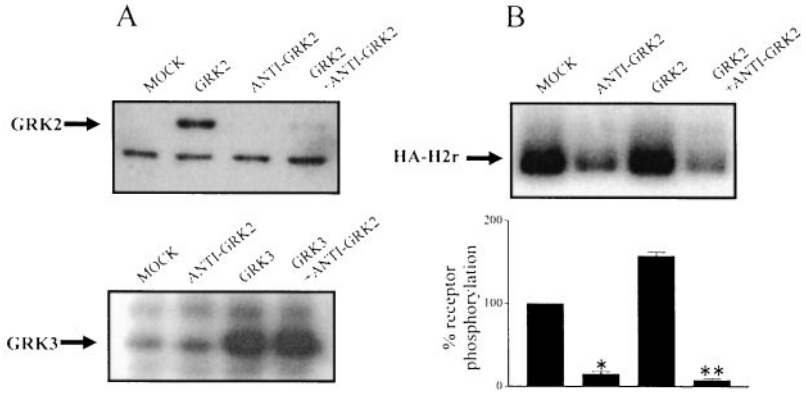

Fig. 1. Specific reductions of GRK2 levels and activity in COS-7 cells. A, COS-7 cells were transfected with pCEFL (MOCK), pCEFL-GRK2, -3, and GRK2 antisense (ANTI) as indicated 48 h after transfection. Cells were lysed, subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2 or GRK3. B, COS-7 cells were transfected with pCEFL-HA-H2r and pCEFL (MOCK), pCEFL-GRK2, and GRK2 antisense (ANTI), as indicated. After ^{32}P incorporation, cells were stimulated for 10 min with 10 μM amthamine. HA-H2r phosphorylation was determined as described under *Materials and Methods*. ^{32}P labeling was quantified using the Scion Image program. Data were calculated as the means ± S.E.M. of three experiments. *, $p <$ 0.01 compared with HA-H2r-transfected cells. **, $p < 0.001$ compared with HA-H2r and GRK2-transfected cells.

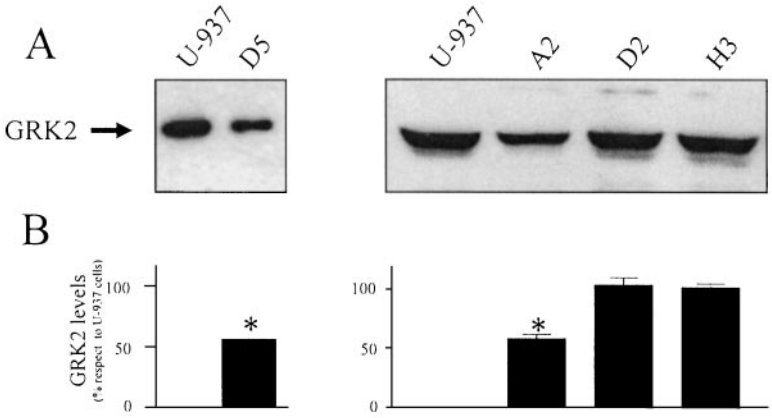

Fig. 2. Effect of GRK2 antisense expression on GRK2 levels. A, whole-cell lysates of the different clones were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against GRK2. B, densitometric analysis obtained with the Scion Image program. Data were calculated as the means ± S.E.M. of three experiments. *, $p < 0.01$ compared with U-937 cells.

Similar results were obtained for the A2 but not for the H3 clone, indicating that GRK2 reduction, not clonal variations, caused these increases. This higher response was attributable to an event upstream of AC activation, because in forskolin dose-response experiments, no differences were observed for D5 compared with U-937 cells (Fig. 3; Table 1).

For the evaluation of cAMP accumulation kinetics in D5 cells, D5 and control cells were treated with 10 μM amthamine for different periods of time in presence of IBMX. The cAMP response reached in each time by D5 cells was approximately 2-fold the U-937 levels. After a 60-min stimulus, when maximal response was reached, A2 cAMP levels were similar to those in D5 cells, whereas the H3 and D2 clones behaved as did U-937 (Fig. 4).

To corroborate the results mentioned above, a desensitization experiment, in which cells exposed to 10 μM amthamine for different periods of time were rechallenged with the same agent to determine the system capability to still generate cAMP, was performed. The U-937 desensitization curve was coincident with the one previously reported by our group (Lemos Legnazzi et al., 2000), showing a half-maximal desensitization time of 11.7 ± 1.6 min (mean ± S.E.M., $n = 4$). The reduction of GRK2 levels significantly modified the desensitization curve, because D5 cells showed a higher half-maximal desensitization time of 51.7 ± 5.3 min (mean ± S.E.M., $n = 3$), and similar results were obtained for A2 cells. D2 and H3 did not significantly modify the half-maximal desensitization time compared with U-937 (Fig. 5A; Table 2).

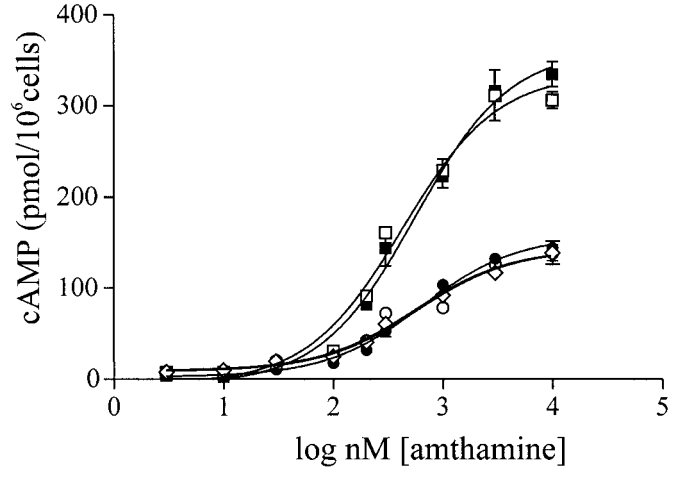

Fig. 3. Effect of GRK2 antisense expression on dose-response curves for cAMP production. U-937 (●), D2 (○), A2 (□), D5 (■), and H3 (◇) cells were incubated for 9 min with increasing concentrations of amthamine at 37°C in the presence of 1 mM IBMX, and cAMP levels determined. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

To elucidate whether the remaining H2r desensitization observed in D5 and A2 clones was caused by a heterologous component, such as H2r desensitization by PKA phosphorylation, desensitization assays in the presence of PKA inhibitor (H-89) were carried out. On the other hand, taking into account its known desensitization by PKA (Lohse et al., 1990), β_2 adrenergic receptor response was evaluated after H2 agonist pretreatment. The desensitization achieved by H2 agonist pretreatment was not prevented by H-89 in either U-937 or D5 cell lines. When desensitized cells were challenged with isoproterenol, the response evoked was similar to that for nonpretreated cells (Fig. 5B). Overall, these results indicate that a heterologous PKA-dependent component is not involved in the remaining H2r desensitization.

Because cAMP determinations in the above experiments were performed in the presence of IBMX (a phosphodiesterase inhibitor), and in an attempt to determine the cAMP levels resulting from the production-degradation balance, the time course of cAMP accumulation in the absence of IBMX was determined. Results indicated that D5 and A2 cells show higher cAMP levels with a slow response decay phase and superior residual response 2 h after stimulus than U-937, D2, or H3 cells (Fig. 6).

H2 Receptors in U-937 and GRK2-Antisense Clones. To further support the hypothesis that the differences in cAMP response were caused by the different levels of GRK2 and not by an increase in receptor numbers in D5 and A2 cells, [^{3}H]tiotidine binding experiments were carried out. Saturation analysis using intact cells revealed that D5 and A2 clones had a significantly lower receptor number than the other clones (U-937, D2, and H3), despite their increased cAMP response. In addition, there were no significant differences among K_d values (Fig. 7; Table 3).

Induction of Monocytic Maturation Markers in GRK2-Antisense Clones by H2 Agonist. In previous studies, we hypothesized that U-937 promonocytic cells were not capable of differentiating with H2 agonists because of the short H2r desensitization time (Shayo et al., 1997). Therefore, GRK2-antisense clones with a longer H2r desensitization time were analyzed for their capability to differentiate with amthamine. The sustained c-Fos expression in U-937 cells was associated with cell differentiation. In this way,

TABLE 1

cAMP response in GRK2-antisense clones

EC_{50} and maximal response (R_{max}) were calculated from the data in Fig. 3 using the equation for dose-response curves. The table shows the mean ± S.E.M.; the number of determinations (n) is in parentheses. The R_{max} of amthamine for the different clones with respect to U-937 were compared using an unpaired t test, and D5 and A2 clones were found to be significantly different.

	Amthamine		Forskolin	
	EC_{50}	R_{max}	EC_{50}	R_{max}
	nM	*pmol/10⁶ cells*	*mM*	*pmol/10⁶ cells*
U-937	465 ± 95 (5)	184 ± 35 (5)	161 ± 42 (3)	120 ± 27 (3)
D2	562 ± 97 (3)	143 ± 26 (3)	N.D.	N.D.
A2	426 ± 47 (3)	281 ± 67 (3)*	N.D.	N.D.
D5	472 ± 67 (3)	292 ± 38 (3)*	121 ± 37 (3)	116 ± 22 (3)
H3	584 ± 78 (3)	142 ± 27 (3)	N.D.	N.D.

N.D., not determined.
*, $p < 0.01$.

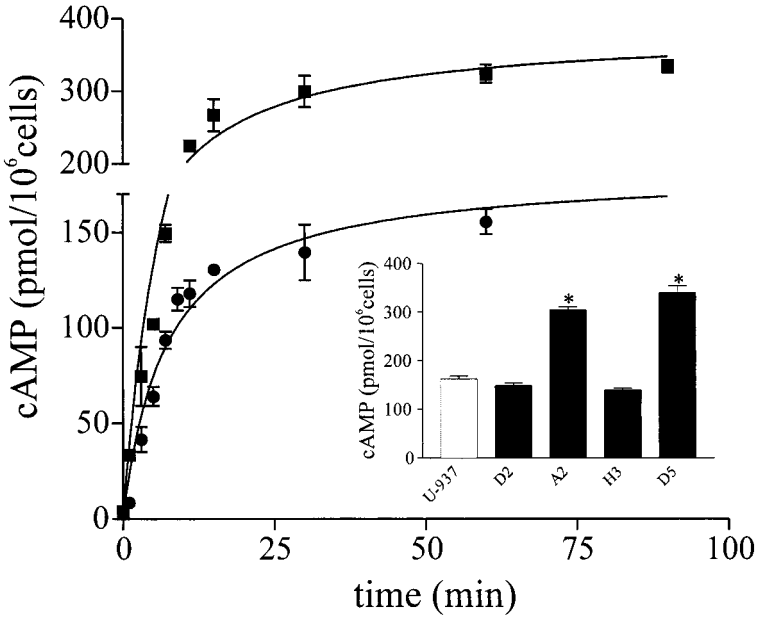

Fig. 4. Effect of GRK2 antisense expression on the time course of cAMP accumulation. U-937 (●) and D5 (■) cells were incubated for different periods of time with 10 μM amthamine at 37°C in the presence of 1 mM IBMX, and cAMP levels were determined. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Inset, D2, A2, and H3 cells were incubated for 60 min with 10 μM amthamine at 37°C in the presence of 1 mM IBMX, and cAMP levels were determined. Data were calculated as the means ± S.E.M. of three independent experiments. *, $p < 0.01$ compared with U-937 cells.

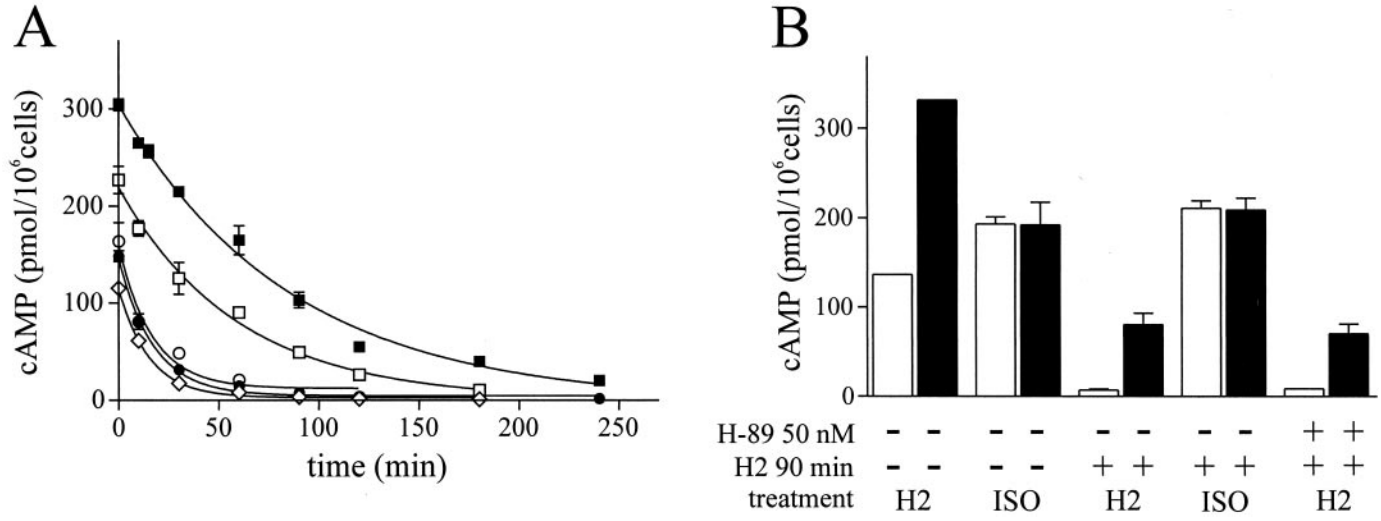

Fig. 5. Effect of GRK2 antisense expression on the desensitization kinetics. A, U-937 (●), D2 (○), A2 (□), D5 (■), and H3 (◇) cells were preincubated for different periods of time with 10 μM amthamine, washed, and restimulated with 10 μM amthamine in the presence of 1 mM IBMX. cAMP production was determined as described under *Materials and Methods*. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. B, U-937 (open bars) and D5 (closed bars) cells were pretreated 30 min or not with 50 nM H-89 and exposed 90 min or not to 10 μM amthamine. Cells were then washed and stimulated with 10 μM amthamine or 1 μM isoproterenol in the presence of 1 mM IBMX. Data were calculated as the means ± S.E.M. of three experiments.

TABLE 2

Desensitization of GRK2-antisense clones

The apparent rate of desensitization was calculated from the data in Fig. 5A using the equation for monoexponential decay. The table shows the mean ± S.E.M.; the number of determinations (n) is in parentheses. The apparent rates of desensitization were compared with U-937 using an unpaired t test, and the D5 and A2 clones were found to be significantly different.

Cell Clones	Half-Maximal Desensitization Time
	min
U-937	11.7 ± 1.6 (4)
D2	11.8 ± 1.5 (3)
A2	40.8 ± 5.3 (3)*
D5	51.7 ± 5.3 (3)*
H3	10.5 ± 1.2 (3)

*, $p < 0.01$.

U-937 treatment with amthamine produced a transient increase in c-Fos levels. However, in D5 cells, amthamine treatment induced a sustained pattern of c-Fos expression similar to that of dbcAMP-treated U-937 cells (Fig. 8).

On the other hand, C5a receptor (CD88) expression was assayed as a differentiation marker. The C5a receptor is a GPCR associated with Ca^{2+} release from intracellular stores (Burg et al., 1996). No response was observed in D5 or in control cells (U-937 and D2) when cells were stimulated with C5a without agonist pretreatment; cells stimulated with 6[2-(4-imidazolyl)ethylamino]-*N*-(4-trifluoromethylphenyl)heptanecarboxamide (H1 agonist), known to elevate $[Ca^{2+}]i$ levels, showed the typical spike, indicating that these cells were capable of evoking a Ca^{2+} response. After 2 days of 10 μM amthamine treatment, a $[Ca^{2+}]i$ spike was induced by C5a in D5 cells, whereas no response was observed in control cells. Similar results were obtained for A2, the other clone with low GRK2 levels. As a control-differentiating stimulus, cells were treated 2 days with dbcAMP; after stimulation with C5a, a $[Ca^{2+}]i$ spike was observed in all cell clones (Fig. 9).

Discussion

In a previous study, we showed that the human promonocytic cell line U-937 possesses histamine H2 receptors (Davio et al., 1995a). We also found that the H2 agonist induces homologous and GRK dependent desensitization of H2 receptors in U-937 cells (Shayo et al., 1997; Lemos Legnazzi et al., 2000).

Recently, we demonstrated that transient cotransfection of COS-7 cells with H2r and either GRK2 or GRK3, dampened the cAMP response after a subsequent H2 agonist treatment and involved H2r phosphorylation (Shayo et al., 2001). However, in the MKN-45 cell line, only GRK2 was described as responsible for the desensitization of H2r (Nakata et al., 1996). Because high levels of overexpressed proteins were reached in COS-7 cells for both receptors and GRKs, it is possible that, by mass action, even GRK subtypes with low affinity for the agonist-bound receptor may phosphorylate it in a heterologous system, although not under physiological conditions.

Antisense technology has been used to demonstrate the involvement of different GRKs in the desensitization of several receptors: GRK5 in the thyrotropin receptor (Nagayama et al., 1996), GRK2 in the A2 adenosine receptors (Willets et al., 1999), GRK2, -5, and -6 in the follicle-stimulating hormone receptor (Troispoux et al., 1999), GRK4 in the metabotropic glutamate receptor 1 (Sallesse et al., 2000), and GRK6 in the calcitonin gene-related peptide (Aiyar et al., 2000). In this study, using this technology, we attempted to evaluate the role of GRK2 in H2r desensitization in U-937 cells. For this purpose, using stable transfection of a GRK2 full-length cDNA antisense construct, we were able to specifically reduce protein levels of GRK2 in U-937 cells, indicated by a marked reduction in GRK2 but not GRK3 levels in D5 and A2 cells (stably-transfected U-937 cells with the GRK2 antisense construct). We described previously that U-937 cells express GRK2, -3, and -6 (Lemos Legnazzi et al., 2000). GRK6, which shares lower sequence homology with GRK2 rather than GRK3, showed no modified levels in D5 and A2 cells (data not shown). We also obtained an H3 clone, resistant to G-418, but with no reduction in GRK2 levels, probably because of the lack of GRK2 antisense cDNA expression.

This decrease in GRK2 expression correlates with an increase of cAMP levels in response to different doses of H2 agonist, and with the different kinetics of cAMP production compared with control cells (U-937 and D2).

In dose-response experiments, although the maximal re-

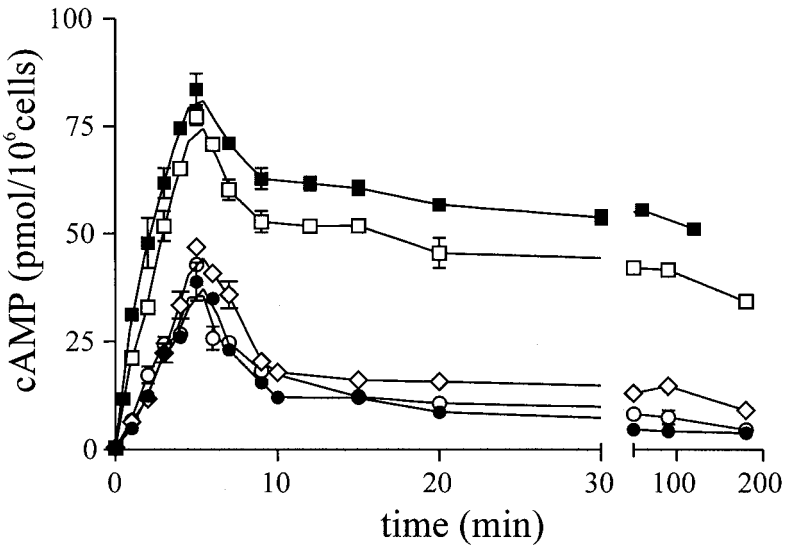

Fig. 6. Effect of GRK2 antisense on cAMP production in the absence of IBMX. U-937 (●), D2 (○), A2 (□), D5 (□), and H3 (◇) cells were incubated for different periods of time with 10 μM amthamine at 37°C, and cAMP levels were determined. Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

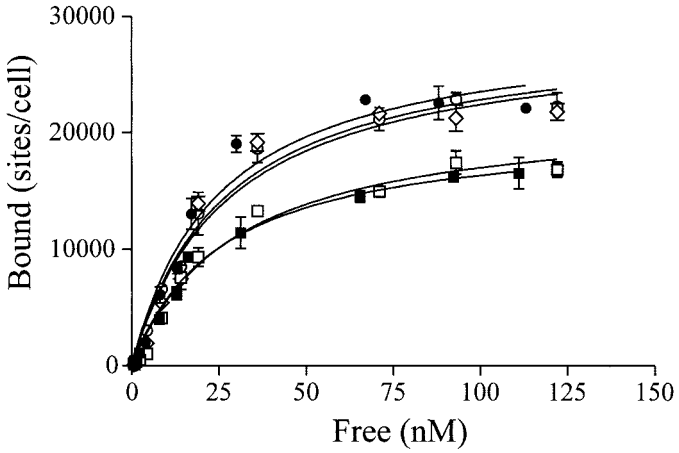

Fig. 7. [^{3}H]Tiotidine binding assay. Saturation assays for [^{3}H]tiotidine in U-937 (●), D2 (○), A2 (□), D5 (■), and H3 (◇) cells. Data were calculated as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments.

sponse was higher for D5 and A2 cells, the EC_{50} for amthamine was similar in all cell clones, indicating that the affinity of the agonist for the receptor was not modified. When stimulated with forskolin, the similar dose-response curves obtained for D5 and U-937 cells denoted that the modification observed in cAMP response in D5 cells was attributable to an event upstream of AC stimulation.

In time-course cAMP accumulation experiments and in the pretreatment assay, D5 and A2 cell desensitization seemed to be lower than in U-937, D2, or H3 cells, once again suggesting a role for GRK2 in this process. However, the desensitization was not completely blocked, possibly because of remaining GRK2 levels, other GRKs such as GRK3 [as observed in COS-7 cells (Shayo et al., 2001)], or to an alternative mechanism not involving GRKs. The presence of a component of heterologous desensitization involving PKA can be ruled out, because in desensitization experiments, the presence of a PKA inhibitor did not modify cAMP levels, and amthamine-pretreated cells did not modify isoproterenol response. The time-course cAMP accumulation experiments in the absence of IBMX gave rise to higher levels of cAMP in D5 and A2 cells as well as in assays in the presence of IBMX and in desensitization experiments. These results rule out the possibility of a compensatory mechanism, tending to maintain the original cell cAMP levels in the clones, such as an increase in phosphodiesterase activity in the initial step of the response. An induction of phosphodiesterase activity was observed in U-937 cells after 4 h of H2 agonist treatment, which was not involved in the mechanism of rapid desensitization (Lemos Legnazzi et al., 2000).

Even though we suppose that the reduction in GRK2 expression was responsible for a higher cAMP response after stimulation with amthamine, we could not discard the possibility that D5 and A2 cells possessed an increased receptor number. Surprisingly, D5 and A2 clones showed a significant decrease in membrane receptor number with respect to native cells or to transfected cells with no regulation of GRK2 levels. Our findings in GRK2 antisense-expressing cells suggest that GRKs not only regulate receptor desensitization but also play a role in the steady-state level of receptor expression. A recent study in Chinese hamster ovary cells demonstrates that the loss in GRK2-phosphorylating activity seemed to correlate with an increase in calcitonin receptor numbers (Horie and Insel, 2000), in contrast to the results observed for H2 receptors in U-937 cells. This evidence suggests that GRKs can regulate GPCRs and that its expression could be negatively or positively influenced by GRK activity depending on the receptor and/or the cell type. These results open an interesting question concerning the regulation between kinases involved in receptor phosphorylation and membrane receptor expression.

In a recent report, we showed that forskolin induces U-937 cell differentiation through a sustained rise in cAMP levels, whereas histamine or H2 agonists, which increase cAMP levels with a short desensitization half-time, failed to promote differentiation (Brodsky et al., 1998). Here, we evaluated the capacity of an H2 agonist to differentiate GRK2

TABLE 3

Binding of [^{3}H]tiotidine

K_d and maximal bound (B_{max}) were calculated using the equation for one binding site. The table shows the mean ± S.E.M.; the number of determination (n) is in parentheses. K_d and B_{max} values were compared with U-937 using an unpaired t test, and the D5 and A2 clones were found to be significantly different.

Cell Clones	K_d	B_{max}
	nM	*sites/cell*
U-937	26 ± 3 (6)	29,700 ± 1,600 (6)
D2	27 ± 4 (3)	28,960 ± 1,432 (3)
A2	29 ± 5 (3)	21,970 ± 1,143 (3)*
D5	26 ± 4 (3)	19,900 ± 900 (3)*
H3	28 ± 5 (3)	28,620 ± 1,829 (3)

*, $p < 0.01$.

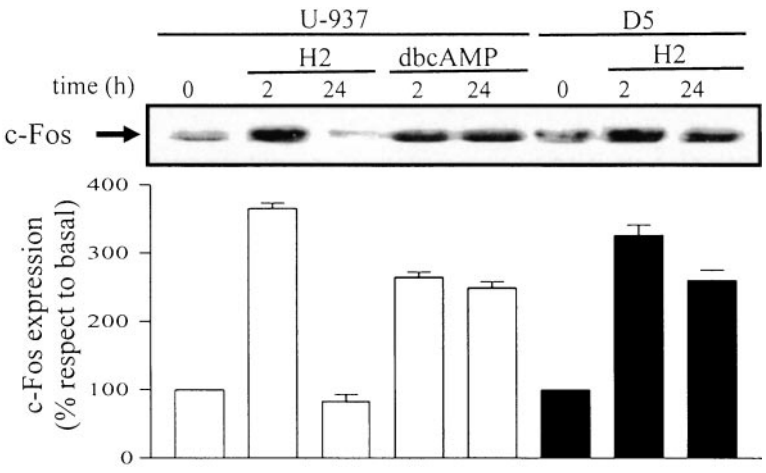

Fig. 8. c-Fos expression in U-937 and D5 cells. Top, cells were incubated with 10 μM amthamine or 0.4 mM dbcAMP for the indicated periods of time before harvesting, and lysed as described under *Materials and Methods*. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with polyclonal purified rabbit sera against c-Fos. Bottom, densitometric analysis obtained with the Scion Image program. Data were calculated as the means ± S.E.M. of three experiments.

antisense clones, which showed lower desensitization than U-937 cells.

In this study, the pattern of c-Fos expression and the induction of C5a receptor were evaluated as markers of cell maturation. Monocytic maturation in U-937 cells was associated with the sustained induction of c-fos gene expression by several differentiation agents including PMA (Liu and Wu, 1992) and dbcAMP (Shayo et al., 1997). When D5 cells were treated with H2 agonist, they showed a pattern of c-Fos expression similar to U-937 differentiated cells.

Concerning the chemoattractant C5a receptor, this receptor is up-regulated when cells are stimulated with differentiation agents (Burg et al., 1996). In accordance with this, H2 agonist-treated D5 and A2 cells, but not U-937 or D2 cells, induced cytosolic Ca^{2+} increases upon ligand activation, indicating the presence of the receptor in cells expressing GRK2 antisense cDNA. The stable induction of c-Fos levels and the expression of C5a receptors in the GRK2-attenuated cell lines treated with H2 agonists indicate induction of monocytic maturation.

Overall, reduction in GRK2 levels determined the higher and prolonged cAMP response mediated by H2r, because of lower receptor desensitization, which in turn allows amthamine-stimulated cell differentiation. These results establish an important correlation between duration and intensity of a signal and cellular response, showing that as a consequence of modulating the desensitization process, cells are able to switch from the proliferation to differentiation pathway, indicating that the desensitization mechanism is a key component of receptor signaling and not just the cause of the lack of response.

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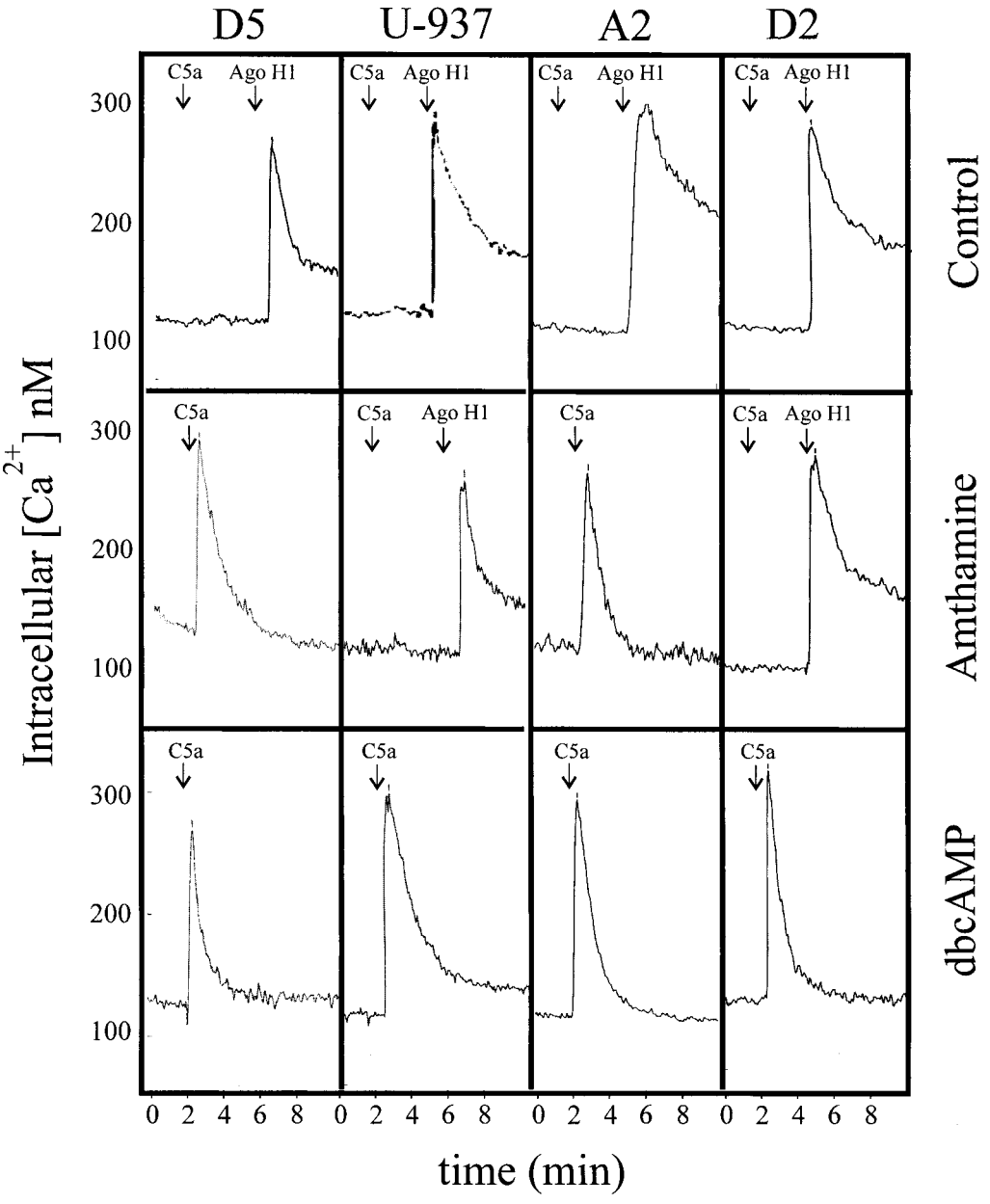

Fig. 9. Effect of C5a on $[Ca^{2+}]i$ in amthamine- or dbcAMP-treated cells. U-937, D2, A2, and D5 cells were cultured for 48 h in the presence of 10 μM amthamine or 0.4 mM dbcAMP. Control, nontreated cells. $[Ca^{2+}]i$ was determined as described under *Materials and Methods*. Arrows indicate the addition of 50 nM C5a or 10 μM H1 agonist. Similar results were obtained in at least three independent experiments.

to Dr. O. Coso (University of Buenos Aires, Buenos Aires, Argentina) for the expression vectors pCEFL and pCEFL-HA.

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