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Modulation of histamine H_2 receptor signalling by G-protein-coupled receptor kinase 2 and 3

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1 To evaluate the role of G-protein-coupled receptor kinases (GRK) in the desensitization of the histamine H_2 receptor, the H_2 receptor was transiently cotransfected with GRK2, 3, 5 or 6 in COS-7 cells and the cyclic AMP levels in response to histamine were studied.

2 Coexpression of the H_2 receptor with GRK2 and 3 significantly decreased both the basal cyclic AMP levels and the cyclic AMP response to 100 μM histamine. Moreover, preincubation with 100 μM histamine desensitized the H_2 receptor response to $53 \pm 8\%$. Coexpression of GRK2 and 3 increased the H_2 receptor desensitization to $27 \pm 4\%$ and $24 \pm 4\%$ respectively. No effect on either cyclic AMP response or desensitization was found when GRK5, GRK6 or dominant negative mutants of GRK2 or 3 ($GRK2K^{220}R$ and $GRK3K^{220}R$) were coexpressed.

3 To study the role of the C-terminal tail in the GRK-mediated desensitization of the H_2 receptor, three truncations of C-tail were constructed: H_2T295, H_2T307 and H_2T341. H_2T307 and 341 H_2T341 expressed and responded normally to 100 μM histamine. The interaction of the H_2 receptor with GRK2 and 3 was also not altered upon truncation of the C-terminal tail.

4 These findings strongly suggest a role of GRK2 and 3 in the desensitization of the H_2 receptor. Furthermore, the finding that C-terminal truncations of the H_2 receptor did not abolish the effect of GRK2 and 3 suggests that the C-terminus is not involved in the GRK mediated desensitization of the histamine H_2 receptor.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinases; PKA, protein kinase A; PKC, protein kinase C

Introduction

The desensitization of G protein coupled receptor (GPCR) activity occurs within seconds following agonist stimulation and for most GPCRs is caused by phosphorylation of specific serine/threonine residues by two different classes of kinases. Many GPCRs are phosphorylated by second messenger-regulated kinases such as protein kinase A (PKA) and protein kinase C (PKC) or by specific G protein-coupled receptor kinases (GRKs). The latter specifically phosphorylates the agonist-occupied receptor promoting its interaction with β-arrestins (Ferguson *et al*., 1998; Lohse *et al*., 1996; Pitcher *et al*., 1998; Premont *et al*., 1995; Zhang *et al*., 1997). At low agonist concentrations, GPCR phosphorylation by the second-messenger-regulated kinases is thought to be responsible for heterologous GPCR desensitization, whereas GRK-mediated phosphorylation is considered to be involved in homologous GPCR desensitization (Ferguson *et al*., 1996; Inglese *et al*., 1993).

Currently three families of GRKs can be distinguished (Premont *et al*., 1995). GRK1 or rhodopsin kinase is localized in the cytosol and is only present in the eye. Farnesylation of its CAAX box in the C-terminal tail is required for membrane localization. GRK2 and GRK3, also called βARK1 and 2 respectively, are also localized in the cytosol but widely distributed in the body; their presence at the membrane is due to their ability to bind $\beta\gamma$ dimers which are released upon G-protein activation (Muller *et al*., 1997). Finally, the family of GRK4 (GRK4, 5 and 6) (Premont *et al*., 1996) is membrane-bound. GRK4 is very abundant in the testis, whereas GRK5 and 6 are ubiquitously expressed.

The extent to which the different GRKs contribute to desensitization *in vivo* remains controversial. In general, these proteins are not very GPCR specific; GRK2, for instance, desensitizes not only the α- and β-adrenergic receptors, but also the m_2-muscarinic, thyrotropin and substance P receptors (Debburman *et al*., 1995; Diviani *et al*., 1996; Iacovelli *et al*., 1996; Kwatra *et al*., 1993). GRK1, whose physiological substrate is rhodopsin (mainly for its specific location), is also able to phosphorylate the β_2-adrenergic receptors *in vitro* (Inglese *et al*., 1993). Furthermore GRK3 has been involved in the desensitization of one of the olfatory receptors (Dawson *et al*., 1993). The structural domains of GPCRs involved in the desensitization are mainly located in the carboxyl-terminal tail and the third intracellular loop (Inglese *et al*., 1993). Many receptors such as rhodopsin or the β_2-adrenergic receptor contain potential sites for GRK-mediated phosphorylation in their carboxyl-terminal tails (Palczewski *et al*., 1991; Premont *et al*., 1994). In contrast, receptors such as α_{2a}-adrenergic or m_2-muscarinic, with shorter carboxyl-terminal tails have phosphorylation targets in their third intracellular loop (Eason *et al*., 1995; Nakata *et al*., 1994).

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The histamine H_2 receptor is a member of the GPCR family which is coupled to both adenylyl cyclase and phospholipase C through G_s and G_q proteins respectively (Delvalle & Gantz, 1997; Hill *et al*., 1997; Leurs *et al*., 1995). As a result of this coupling, histamine initiates two biochemical pathways which result in an increased intracellular cyclic AMP or Ca^{2+} level (Fukushima *et al*., 1996; Kuhn *et al*., 1996; Wang *et al*., 1996). Moreover, receptor activation ultimately leads to receptor desensitization in spite of the continuous presence of the agonist (Smit *et al*., 1994). The desensitization of the histamine H_2 receptor has been previously investigated in several cellular systems expressing endogenously the H_2 receptor. In the human gastric cell line HGT-1, in clonal cytolytic T lymphocytes and in the human monocytic cell line U937, the endogenous histamine H_2 receptor rapidly desensitizes in response to histamine (Prost *et al*., 1984; Schreurs *et al*., 1984; Smit *et al*., 1994). However, little is known about the mechanisms which underlie histamine H_2 receptor desensitization or the receptor region involved. The human gastric carcinoma cell line MKN-45 possesses endogenous GRK2 and GRK6 and histamine H_2 receptors (Arima *et al*., 1991), which are homologously desensitized by histamine (Arima *et al*., 1993). In this cell line, treatment with a GRK2 or GRK6 antisense oligonucleotides specifically inhibits the mRNA expression of both GRKs, but only the GRK2 antisense oligonucleotide abolished the desensitization of the histamine H_2 receptor in response to histamine. These data suggest the involvement of GRK2 in the H_2 receptor desensitization (Nakata *et al*., 1996). The H_2 receptor domains involved in GRK interaction and thus receptor desensitization remains unknown. This receptor contains serine and threonine residues in the third intracellular loop and C-terminal tail which might serve as potential targets for serine/threonine kinases. Despite the relevance of the C-terminal tail of the histamine H_2 receptor in the agonist-induced H_2 receptor down-regulation (Fukushima *et al*., 1997; Smit *et al*., 1996b) previous results suggest that this region is not necessary for the receptor desensitization (Fukushima *et al*., 1997).

In this study we investigated in more detail the GRKs involved in the desensitization of the rat H_2 receptor by coexpressing the GPCR and GRKs in COS-7 cells. Moreover, we examined whether the C-terminal tail of the H_2 receptor contains structural domains involved in the interaction with GRKs. For this purpose, we used three receptor truncations H_2T295, H_2T307 and H_2T341 lacking 11, 10 and 3 serine/threonine residues respectively in the C-terminus, and studied their ability to undergo desensitization when coexpressed with or without GRK2 and GRK3.

Methods

Reagents and plasmids

Buffers, salts, bovine serum albumin (BSA), IBMX (1-methyl-3-isobutylxanthine), forskolin (FK), histamine dihydrochloride, protease inhibitors and cyclic AMP (cAMP) were from Sigma Chemical Company (U.S.A.). [2,8-^{3}H]-cyclic AMP (40 Ci mmol^{-1}) was obtained from Amersham. [^{125}I]-iodoaminopotentidine was synthesized in our laboratory as described previously (Leurs *et al*., 1994). Tissue culture media and cell culture reagents were obtained from GIBCO. pCMV containing cDNAs for bovine GRK2, 3 and its dominant negative mutants GRK2K^{220}R and GRK3K^{220}R as well as human GRK5 and GRK6 were generous gifts from Dr S. Cotecchia (Lausanne, Switzerland). The FLAG rat H_2 receptor cDNA was constructed by PCR as previously described (Smit *et al*., 1996b) and pharmacologically behaved as the wild type receptor (Smit *et al*., 1995). Truncated H_2 cDNAs at positions 295 (H_2T295), 307 (H_2T307), and 341 (H_2T341), were constructed by PCR using the rat H_2 receptor as a template (Smit *et al*., 1996b) and subcloned in pcDNA3 for expression in COS-7 cells.

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal calf serum, 2 mM glutamine, 100 units ml^{-1} of penicillin, 100 mg ml^{-1} of streptomycin at 37°C (95% O_2/5% CO_2) in a humidified incubator. Plasmid cDNAs were purified using Qiagen exchange chromatography and the transient transfection of cDNAs was carried out by DEAE-dextran. All the assays were performed 24 h after transfection.

Radioligand binding assays

Binding experiments were performed with 5–15 μg of cell homogenates in 0.4 ml of 50 mM Na_2/K phosphate buffer containing 0.2% gelatin and 0.3 nM [^{125}I]-iodoaminopotentidine ([^{125}I]-APT) (2000 Ci mmol^{-1}) for 90 min at 30°C. Nonspecific binding was determined in the presence of 1 μM tiotidine. Separation of bound and free radioactivity was carried out by vacuum filtration through GF/C filters treated with 0.3% polyethyleneimine. Data were analysed using the program Graphpad PRISM.

Cellular cyclic AMP assay

After the transfection, 6×10^5 cells were seeded in 12 well plates and allowed to grow for at least 24 h in DMEM/5% FCS. The day of the assay, the medium was removed and the cells were washed twice with serum-free DMEM containing 25 mM HEPES (pH 7.4 at 37°C) and 0.3 mM IBMX for 30 min at 37°C. Incubations with histamine (100 μM) was performed in the same serum-free medium at 37°C for 10 min. At the end of the incubation, the medium was removed and the cyclic AMP extracted with 0.2 ml of ice-cold 0.1 N HCl. The amount of cycle AMP in the neutralized homogenates was determined using a protein kinase A fraction from bovine adrenal glands as previously described (Smit *et al*., 1996b).

Western blot analysis of GRKs

1×10^6 COS-7 cells transfected with the cDNAs encoding GRK2, 3, 5, 6 and dominant negative mutants GRK2K^{220}R and GRK3K^{220}R were homogenized in 0.5 ml ice-cold RIPA buffer (PBS, 1% v v^{-1}) Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing 1 mM phenylmethyl sulphonylfluoride (PMSF) and 10 μg ml^{-1} benzamidine. The lysate was centrifuged at 15,000 × *g* for 15 min and the supernatant was subjected to SDS–PAGE (10%) and blotted onto nitrocellulose membranes.

The blots were blocked with TBS-T (10 mM Tris-HCl pH 7.5, 0.05% Tween-20) with 5% (w v^{-1}) non-fat milk powder for at least 30 min at room temperature. Incubations with 1 : 200–1 : 500 of specific rabbit IgG anti-GRK antibodies (Santa Cruz) were performed overnight at 4°C in TBS-T. After three washes with TBS-T, the membranes were incubated with a dilution of 1 : 4000 of a goat-anti-rabbit

horseradish peroxidase conjugated secondary antibody (BioRad) for 60 min at room temperature in TBS-T. Immunoreactive bands were identified using the ECL detection method (Amersham Pharmacia) and quantified by densitometry.

Statistics

Results were expressed as mean ± standard error (mean ± s.e.mean) of at least three independent experiments. Differences between means were analysed by Student's unpaired *t*-tests and were considered significant when $P<0.05$.

Results

Expression of the H_2 receptor and GRKs

COS-7 cells were transfected with the cDNAs encoding the rat histamine H_2 receptor and different GRKs (2, 3, 5, 6 and dominant negative mutants GRK2K^{220}R and GRK3K^{220}R) at a ratio of 5 : 1 (μg H_2 receptor cDNA vs μg GRK cDNA). The H_2 receptor expression was 0.75 ± 0.25 pmol mg^{-1} protein and was not affected by the coexpression of the various GRKs (data not shown).

The expression of bovine GRK2, GRK3, and human GRK5 and 6 was assessed by Western blots of cytosolic proteins using specific antiserums (Figure 1A). Using these antibodies we were able to detect in COS-7 cells endogenous expression of GRK2 and GRK6 (Figure 1A). Moreover, cotransfection of COS-7 with the cDNAs encoding the H_2 receptor and one of the GRKs, resulted in considerable overexpression of the respective GRK as demonstrated by immunoreactive bands at 78 (GRK2 and 3) and 70 kDa (GRK5 and 6). Densitometric analysis of the Western blots revealed that GRK2 and GRK6 were overexpressed 11 and 320 times respectively (Figure 1A). Due to the lack of endogenous expression of the other GRKs the fold overexpression could not be calculated. The expression levels of GRK2 and GRK3 increased progressively from 0.075 to 1.2 μg cDNA.10^6 transfected cells^{-1} (Figure 2B).

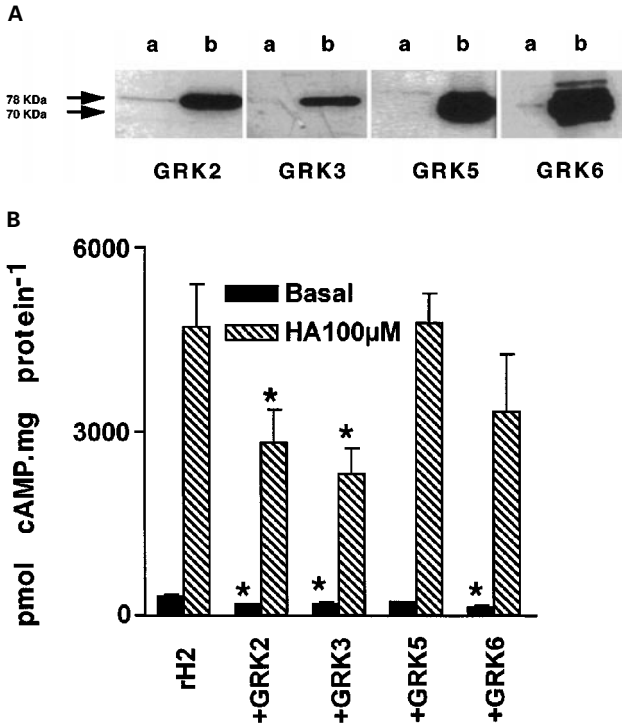

Figure 1 (A) Expression of GRKs in COS-7 cells. Western blot analysis of bovine GRK2 and 3 and human GRK5 and 6 were carried out after the loading of 10–20 μg of protein on a 10% SDS–PAGE gel. The GRK expression in cells transfected with pCMV bearing no insert (a) and different GRKs (b) was studied after 24 h of transfection. Proteins were transferred to nitrocellulose, incubated with anti-GRK antibodies and the bands were visualized by ECL. Similar results were obtained in three different experiments. (B) Effect of different GRKs on the cyclic AMP response of the H_2 receptor. COS-7 cells were transfected with the cDNA encoding the receptor alone or in combination with GRK cDNAs at a ratio of 5 : 1. Basal and the cyclic AMP response to 100 μM HA were studied after 24 h of transfection. Results are the mean ± s.e.mean of 10 experiments performed in triplicate. $^*P<0.05$.

Effect of GRKs on the cyclic AMP response of the H_2 receptor

Coexpression of GRK2 or GRK3 (1 μg cDNA 10^6 cells^{-1}) with the rat H_2 receptor, significantly decreased both the basal cyclic AMP level and the histamine (100 μM)-induced

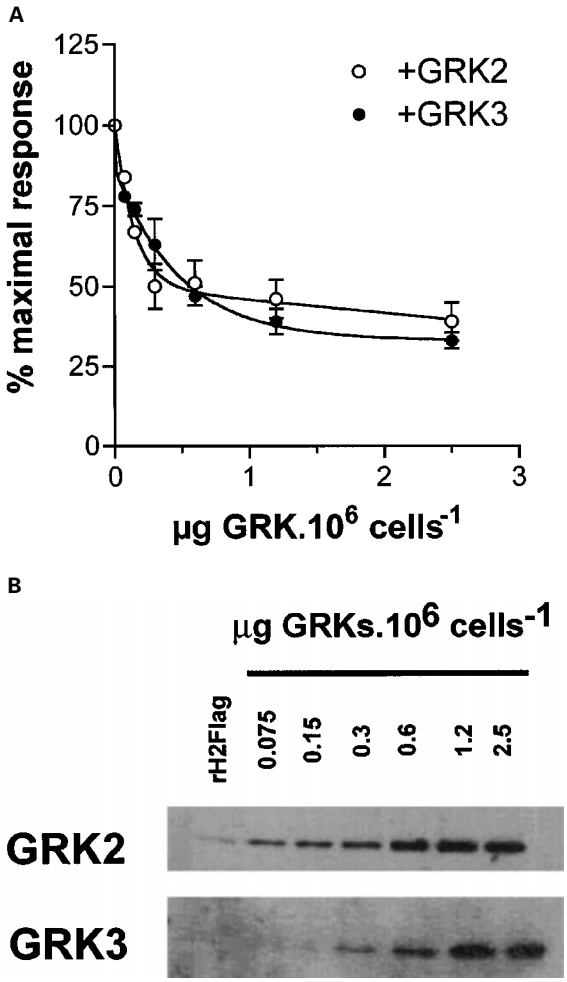

Figure 2 The effect of GRK2 and GRK3 on the cyclic AMP levels in response to 100 μM histamine. (A) COS-7 cells were transfected with 2.5 μg of the receptor alone or cotransfected with increasing amounts (from 0.075–2.5 μg) of the cDNAs coding for bovine GRK2 or GRK3. After 24 h, the cellular cyclic AMP response to 100 μM histamine was assessed. Results are expressed as percentage of response of COS-7 cells expressing only the receptor and are the mean ± s.e.mean of 10 experiments performed in triplicate. $^*P<0.05$. (B) Cell homogenates from the same transfections were resolved on a 10% SDS–PAGE as in Figure 1A. Similar results were obtained in three different experiments.

cyclic AMP response of the H_2 receptor (171 ± 20 and 2832 ± 519 pmol cyclic AMP mg^{-1} protein for GRK2; 180 ± 29 and 2313 ± 422 pmol cyclic AMP mg^{-1} protein for GRK3) as compared to COS-7 cells that only expressed the H_2 receptor (291 ± 38 and 4723 ± 682 pmol cyclic AMP mg^{-1} protein, $P<0.05$, $n=10$). (Figure 1B, Table 1). Coexpression of GRK5 or GRK6 did not affect significantly the histamine responsiveness, although GRK6 expression reduced the basal cyclic AMP (Figure 1B).

Coexpression of the H_2 receptor with increasing amounts of GRK2 and 3 caused a progressive decrease in the cyclic AMP response to histamine (10 min, 100 μM) with a half maximal inhibition at 0.2 μg of cDNA transfected.10^6 $cells^{-1}$ for both GRK2 and GRK3 (Figure 2A). Western blots of cytosolic proteins showed, indeed, a correlation between the inhibition of the cyclic AMP response and the increase in the expression of the GRKs (Figure 2B). Stimulation of COS-7 cells with increasing concentrations of histamine (0.1 nM–100 μM) for 10 min resulted in a dose-dependent increase of the cyclic AMP production with an EC_{50} value of 0.23 ± 0.07 μM ($n=5$). Coexpression of the H_2 receptor with GRK2 or GRK3 reduced the maximal cyclic AMP response to histamine to 46 and 65% respectively without affecting the EC_{50} (Figure 3, Table 2).

Moreover, a 10 min preincubation with 100 μM histamine, reduced the response of the H_2 receptor to a second histamine stimulation to 53 ± 8% (Figure 4, Table 1). The coexpression of GRK2 and 3 significantly decreased this response further to 27 ± 4% and 24 ± 4%, respectively ($P<0.05$, $n=10$) (Figure 4, Table 1). We found however no effect on the extent of histamine-induced desensitization when the GRK5 or 6 were coexpressed with the receptor (Figure 4, Table 1).

To investigate the specificity of the effect of GRK2 and GRK3, the dominant negative mutants $GRK2K^{220}R$ and $GRK2K^{220}R$ were cotransfected with the H_2 receptor. This

Table 2 Characteristics of the dose-response curve to histamine upon GRK2 and GRK3 coexpression in COS-7 cells

Expressed protein	*EC_{50} (μM)*	*Per cent maximal cyclic AMP response*	
rH_2	0.23 ± 0.07	100	$n=5$
$+GRK2$	0.15 ± 0.04	46 ± 6**	$n=5$
$+GRK3$	0.26 ± 0.05	65 ± 7**	$n=5$

The EC_{50} value and maximal cyclic AMP response were determined using nonlinear regression using the program Graphpad PRISM. Values shown are the means $\pm$ s.e.mean of five independent experiments. **Significant difference ($P<0.001$) compared with cells expressing the rat H_2 receptor.

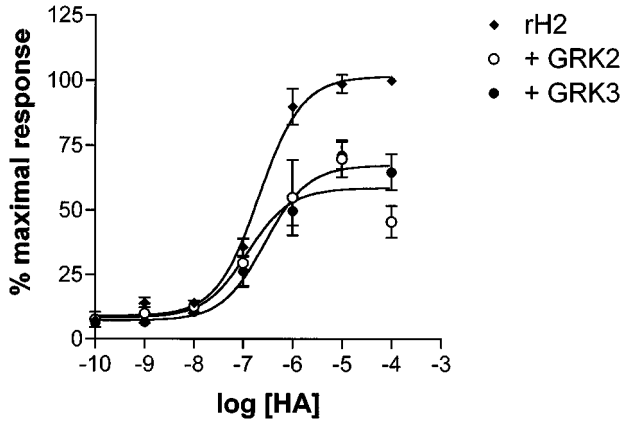

Figure 3 The effect of GRK2 and GRK3 on the histamine-induced cycle AMP production. COS-7 cells were transfected with 2.5 μg of the receptor alone or cotransfected with 0.5 μg of the cDNAs coding for bovine GRK2 or GRK3. After 24 h, the cellular cyclic AMP response to increasing concentrations of histamine was studied. Results are expressed as percentage of the maximal response obtained in COS-7 cells expressing only the H_2 receptor. Results are the mean $\pm$ s.e.mean of five experiments performed in triplicate. *$P<0.05$; **$P<0.001$.

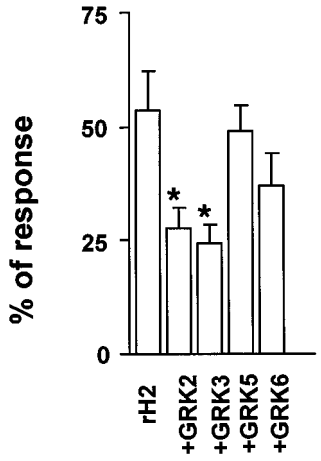

Figure 4 The effect of GRKs on the histamine-induced desensitization of the H_2 receptor. COS-7 cells were transfected with the cDNA encoding the receptor alone (rH_2) or in combination with GRK cDNAs (5 : 1). Cells were preincubated with 100 μM histamine for 10 min, washed and stimulated again with the same concentration of histamine. Results are expressed as percentage of response of COS-7 cells expressing only the H_2 receptor and preincubated with medium containing no histamine. Results are the mean $\pm$ s.e.mean of 10 transfections performed in triplicate. *$P<0.05$ compared to cells expressing only the H_2 receptor.

Table 1 Characteristics of the basal and histamine-induced cyclic AMP production and desentization in COS-7 cells co-expressing the rat H_2 receptor and different GRKs

	pmol *cyclic AMP.mg protein^{-1}*			
Expressed protein	*Basal*	*100 μM HA*	*Percent response after 100 μM HA preincubation*	
rH_2	291 ± 38	4723 ± 682	53 ± 8	$n=10$
$+GRK2$	171 ± 20*	2832 ± 519*	27 ± 4*	$n=10$
$+GRK3$	180 ± 29*	2313 ± 422*	24 ± 4*	$n=10$
$+GRK5$	208 ± 20	4760 ± 48	49 ± 6	$n=10$
$+GRK6$	127 ± 14*	3342 ± 902	37 ± 7	$n=10$
$+GRK2K^{220}R$	272 ± 69	5013 ± 988	46 ± 9	$n=10$
$+GRK3K^{220}R$	199 ± 31	3879 ± 968	51 ± 15	$n=10$

Results are the mean $\pm$ s.e.mean of 10 independent experiments. *Significant difference ($P<0.05$) compared with cells expressing only the rat H_2 receptor (rH_2).

resulted in a 7.4 fold overexpression of GRK2K^{220}R compared to endogenously expressed GRK2 (Figure 5). Due to lack of endogenous expression of GRK3 the overexpression of GRK2K^{220}R could not be quantified. The expression of these two mutants had no effect on the histamine response or desensitization when compared with COS-7 cells expressing the receptor alone (Figure 5, Table 1).

To demonstrate that the effects of GRKs are not due to an interference with signalling events downstream of the H_2 receptor, we measured cyclic AMP levels in response to forskolin in COS-7 cells co-transfected with the H_2 receptor and the GRKs. For a proper evaluation of the data one needs, however, to take into account that we previously showed that forskolin activation of adenylate cyclase is affected by constitutive GPCR activation of G_s proteins *via* e.g. the H_2 or TSH receptor (Alewijnse *et al*., 1997). In our present set of experiments we observed a similar effect. Increasing the H_2 receptor expression by transfecting increasing amounts of plasmid DNA increased both the basal and forskolin induced cyclic AMP levels due to the constitutive activity of the H_2 receptor (Figure 6). To check for potential non-specific effects of the GRK-overexpression the forskolin response was expressed as a percentage of the basal cyclic AMP levels. As can be seen in Figure 6 these values remain fairly constant upon overexpression of the GRKs. Based on these findings we conclude that the observed effect of the GRK2 and GRK3 is not downstream the H_2 receptor.

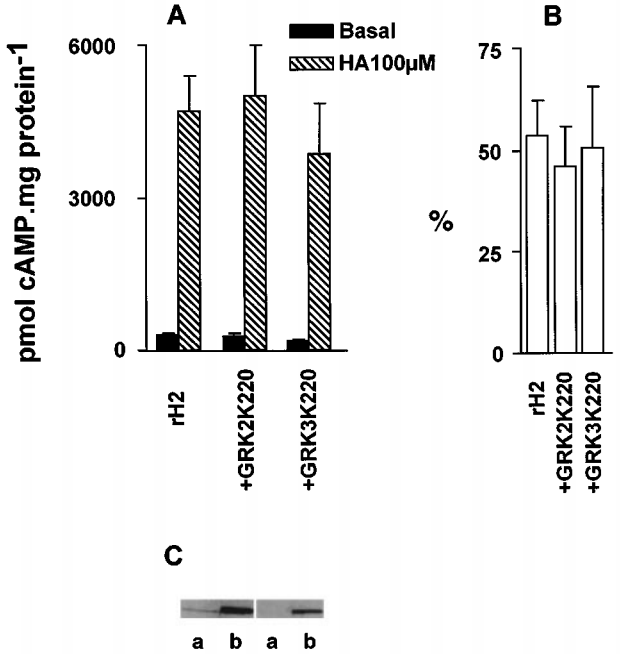

Figure 5 The effect of the dominant negative mutants GRK2K^{220}R and GRK3K^{220}R on the cyclic AMP response and desensitization of the H_2 receptor. COS-7 cells were transfected with the receptor alone or in combination with the dominant negative mutant GRK2K^{220}R or GRK3K^{220}R. (A) Basal and cyclic AMP levels in response to 100 μM histamine (HA) were studied after 24 h of transfection. (B) Cyclic AMP response after a desensitization with 100 μM histamine (HA) for 10 min. Results are the mean$\pm$s.e.mean of 10 experiments performed in triplicate. (A) Western blot from cell homogenates expressing the rat H_2 receptor alone (a) or in combination with the GRK mutants (b) were resolved after incubation with specific anti-GRK2 and GRK3 rabbit polyclonal antibodies as described in Methods.

Effect of C-terminal tail truncations on rat H_2 receptor desensitization

To study the role of the C-terminal tail in the GRK-mediated desensitization the wild type H_2 receptor and three mutant receptors with truncations at amino acid 295 (H_2T295), 307 (H_2T307) and 341 (H_2T341) (Figure 7) were expressed in COS-7 cells. The expression levels of the H_2T307 and H_2T341 mutants were similar to the wild type rat H_2 receptor (Table 3). However, the transient expression of the H_2T295 mutant did not result in detectable [^{125}I]-APT binding. This mutant receptor was therefore not used further in this study. COS-7 cells expressing the wild type H_2 receptor (0.6 ± 0.1 pmol mg^{-1} protein) or the H_2T341 or H_2T307 receptor all responded with an increase in cyclic AMP production after histamine stimulation. No difference in either the EC_{50} value or the maximal response was observed between the wild type of both receptor mutants (Figure 8,

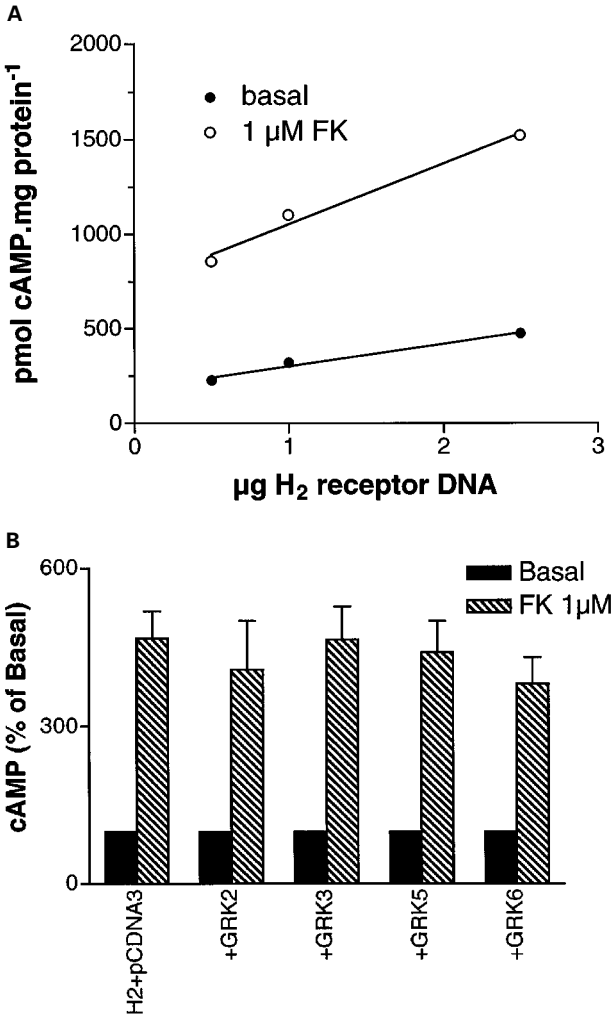

Figure 6 The effects of the GRK2 and GRK3 are not due to modulation of signal transduction pathways downstream of the H_2 receptor. COS-7 cells were transfected with the receptor alone (A) or in combination with GRK2, GRK3, GRK5 or GRK6 (B). (A) The H_2 receptor was expressed at various receptor densities by varying the amount of transfected cDNA. Basal and cyclic AMP levels in response to 1 μM forskolin (FK) were studied after 24 h of transfection. (B) Effects of co-expression of the H_2 receptor with the various GRKs on the basal and forskolin-stimulated (FK) cyclic AMP levels. The forskolin response was expressed as percentage of the basal cyclic AMP levels. Results are the mean$\pm$s.e.mean of three experiments.

Table 3). Moreover, a 10 min preincubation with 100 μM histamine is able to desensitize the responsiveness of the wild type rat H_2, H_2T307 and H_2T341 receptors to a second histamine stimulation to the same extent (Table 3).

The coexpression of GRK2 or GRK3 with either the wild type or the mutant H_2 receptors significantly decreased the histamine (100 μM)-induced cyclic AMP response of the respective receptor (Figure 9). For both H_2T307 and

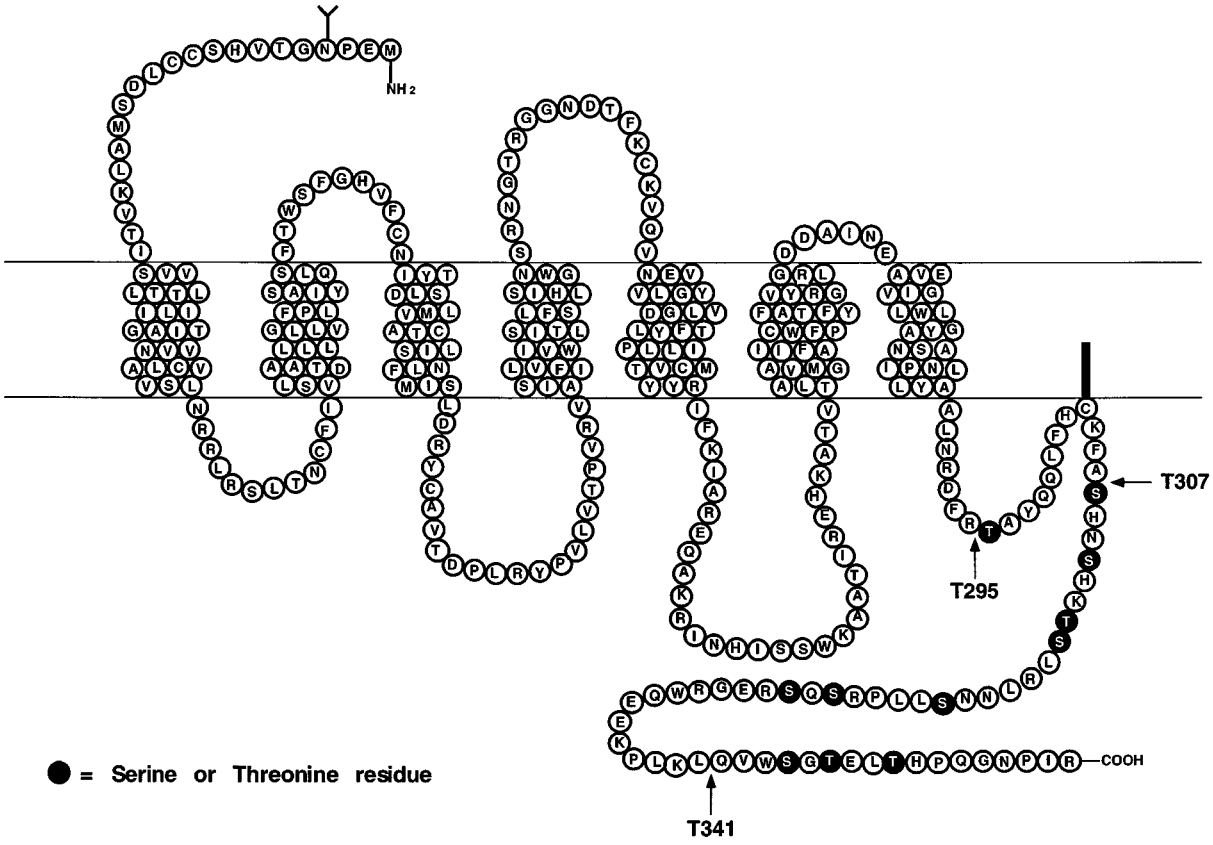

Figure 7 A schematic representation of the truncations in the C-terminal tail of the rat histamine H_2 receptor. The receptor was truncated at positions 341 (H_2T341), 307 (H_2T307) and 295 (H_2T295).

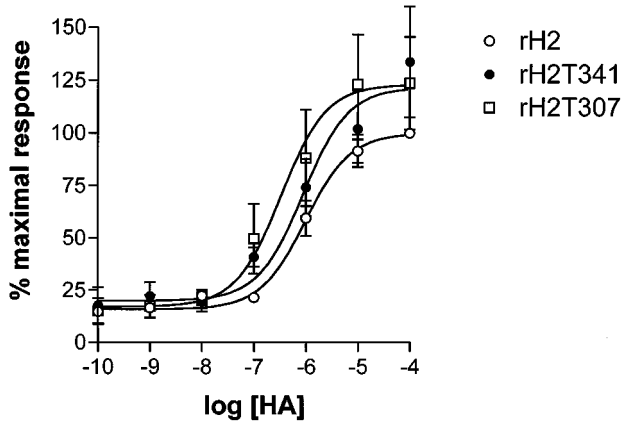

Figure 8 The effect of the truncation of the C-terminal tail of the rat H_2 receptor on the dose-response curve of histamine. COS-7 cells were transfected with 2.5 μg of the wild type H_2 receptor or the rH_2T341 and $rH_2$307 receptor mutants. The cellular cyclic AMP response to increased concentrations of histamine was studied 24 h after the transfection. Results are expressed as percentage of the maximal response obtained in COS-7 cells expressing the wild type receptor and are the mean$\pm$s.e.mean of four different experiments performed in triplicate. The curve was obtained by fitting a sigmoidal curve through the mean values using Graphpad PRISM. From these fits EC_{50} values of 0.9$\pm$0.3 μM; 0.9$\pm$0.4 μM and 0.3$\pm$0.3 μM for the wild type, rH_2T341 and rH_2T307 H_2 receptor mutants was obtained.

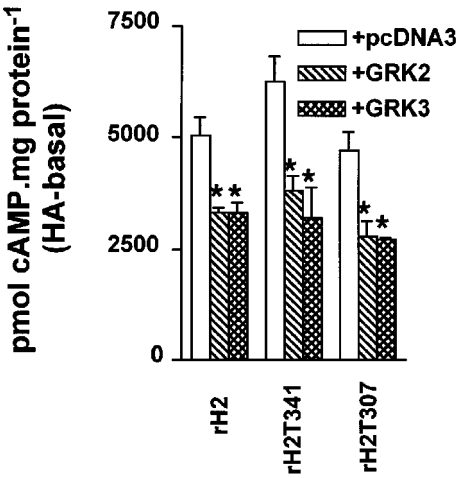

Figure 9 The effect of GRK2 and GRK3 on the response to histamine of C-terminal truncated receptors. The wild type H_2 receptor and the two receptor truncations rH_2T341 and $rH_2$307 were transfected alone or with GRK2 or GRK3 in COS-7 cells. AFter 24 h the cyclic AMP response to 100 μM of histamine was studied. Results are expressed as the difference between the maximal cyclic AMP response and the basal levels obtained in each case. Results are the mean$\pm$s.e.mean of four experiments performed in triplicate. *$P<0.05$.

Table 3 Characteristics of the histamine-induced cyclic AMP production and desensitization in COS-7 cells, expressing the rH_2, rH_2T341 and rH_2T307 receptors

Expressed protein	B_{max} pmol/mg prot	EC_{50} (μM)	pmol *cyclic AMP*·mgn protein^{-1} *Basal*	100 μM *HA*	*Per cent of desensitization*	
rH_2	0.58±0.1	0.83±0.3	361±57	5418±407	57±6	$n=4$
rH_2 T341	0.64±0.2	0.89±0.4	402±63	5931±804	62±8	$n=4$
rH_2 T307	0.51±0.1	0.98±0.4	300±65	5024±340	57±5	$n=4$

All values are the means±s.e.mean of four independent experiments. The receptor desensitization is expressed as percentage of the maximal response obtained in COS-7 cells preincubated with medium containing no histamine.

H_2T341 truncations a similar decrease in the response to histamine was found as for the wild type receptor (Figure 9).

Discussion

Previous papers have demonstrated rapid homologous desensitization of the H_2 receptor in several cell lines (Arima *et al*., 1993; Fukushima *et al*., 1994; Smit *et al*., 1994). However, the mechanistic basis of this desensitization has so far not been unraveled. In this study we investigated the interaction of different GRKs with the rat histamine H_2 receptor by measuring the cyclic AMP levels in COS-7 cells coexpressing the receptor with different GRKs. After GRK2 or GRK3 coexpression, both basal and maximal histamine-induced responses of the H_2 receptor were reduced when compared to COS-7 cells expressing only the rat H_2 receptor. GRK2 or GRK3 overexpression did not affect the potency of histamine, but only affected the maximal hisamine responsiveness. This effect of GRK overexpression is specific as overexpression of GRK5, GRK6 or the kinase deficient ($K^{220}R$) mutants of GRK2 and 3 did not affect these parameters.

While much is known about the modulation of the agonist responses, factors modulating the basal activity of GPCRs remain poorly understood. This is of special relevance in the case of spontaneously active receptors; receptors which in the absence of their agonist are able to signal downstream and that have been implicated in some important human disorders (Spiegel, 1996). The mechanisms underlying spontaneous GPCR activity are not completely understood (Leurs *et al*., 1998). Spontaneous activity is very often the result of a mutation in the GPCR (Spiegel, 1996) but, as we e.g. previously reported for the H_2 receptor (Alewijnse *et al*., 1998; Smit *et al*., 1996a), can also be observed for wild type GPCRs. Some spontaneous active receptors are constitutively phosphorylated by GRKs (Mhaouty-Kodja *et al*., 1999; Pei *et al*., 1994). These receptors, by mimicking the agonist occupied conformation, act as targets for GRKs even in the absence of the agonist. Our finding of lower basal cyclic AMP levels when the receptor is coexpressed with GRK2, 3 and 6 suggests a possible role of these kinases in the modulation of the spontaneous activity of the histamine H_2 receptor as well. Further studies will be necessary to confirm this hypothesis.

In our experimental system a preincubation of the H_2 receptor with histamine decreases the response to a second agonist exposure by approximately 50%. Overexpression of GRK2 and GRK3, but not GRK5 or GRK6 further increased the percentage of receptor desensitization, showing that these receptor kinases can desensitize the H_2 receptor. We detected by Western blot analysis a weak endogenous expression of GRK2 and GRK6, but failed to detect any endogenous GRK3 or GRK5. Since the dominant negative mutants $GRK2K^{220}R$ and $GRK3K^{220}R$ do not abolish the desensitization GRK2 and 3 are apparently not responsible for the observed H_2 receptor desensitization in COS-7 cells. This could indicate that the desensitization is mediated by endogenously expressed GRK6, but the lack of effect after overexpression of GRK6 argues against such a role. The observed H_2 receptor desensitization is therefore probably caused by PKA activation. Previously, we and others have shown already a role for PKA in the modulation of H_2 receptor function (Fukushima *et al*., 1996; Smit *et al*., 1996b). One should however keep in mind that $GRK2K^{220}R$ and $GRK3K^{220}R$ have previously been reported as dominant negative mutants of receptor phosphorylation triggered by GRK2 and GRK3, although receptor phosphorylation was not completely abolished by the GRK mutants (Diviani *et al*., 1996; Freedman *et al*., 1995). Moreover, a dominant negative effect of the two mutants on GPCR desensitization has, as far as we know, not been reported. An actual role of the endogenously expressed GRK2 in the H_2 receptor desensitization in COS-7 cells can, therefore, also not completely be excluded.

The domains of the H_2 receptor involved in the interaction of GRK2 and GRK3 are unknown. As most GPCRs, the H_2 receptor has potential phosphorylation sites in the carboxyl-terminal tail and the third intracellular loop (Gantz *et al*., 1991; Ruat *et al*., 1991). In general, the functional role of the C-terminus varies among receptors and in the histamine H_2 receptor this tail has been suggested to be involved in agonist-induced down-regulation but not in homologous desensitization (Fukushima *et al*., 1997; Smit *et al*., 1996b). We therefore investigated whether the C-terminus of the rat H_2 receptor is necessary for the interaction with GRK2 or 3. To this end, we truncated the tail at different positions to generate receptors lacking 3, 10 or 11 serine/threonine residues (H_2T341, H_2T307 and H_2T295 respectively). Although the transient expression of the H_2T295 mutant did not show detectable binding, previous papers have found mRNA for this particular truncation in both stably transfected CHO cells and transiently transfected HEK-293 cells (Smit *et al*., 1996b). When the C-terminal tail was removed in an epitope-tagged canine H_2 receptor, the truncated protein was detected by immunofluorescence in COS-7 cells but no functional receptor activity was observed, (Fukushima *et al*., 1997) suggesting an important role of the N-terminal portion of the C-terminus in the targeting of the receptor to the membrane.

When H_2T341 and H_2T307 were expressed in COS-7 cells, they responded to histamine with similar EC_{50} and maximal responses as the wild type rat H_2 receptor, as was previously observed in CHO cells (Smit *et al*., 1996b). Moreover, the mutant receptors were able to desensitize normally after a

pretreatment with histamine. GRK2 and GRK3 again further increase the desensitization of both truncated receptors. This effect was comparable to the wild type receptor, suggesting that the serine/threonine sites present in the C-terminus of the histamine H_2 receptor are not necessary for the action of GRK2 and 3.

In conclusion, the results of this study demonstrate for the first time that GRK2 and GRK3 can desensitize the rat H_2 receptor. Despite the role of the C-terminus in the internalization and down-regulation of the histamine H_2 receptor, this tail is not required for the interaction with GRK2 or 3, nor for the desensitization in response to histamine. Further research is needed in order to define the receptor region involved in the GRK-mediated desensitization. In this respect serine/threonine residues in the third intracellular loop of this receptor might be good candidates as potential targets for GRK interaction.

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