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Characterization of human 5-$HT_{4(d)}$ receptor desensitization in CHO cells

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1 Serotonin 5-HT_4 receptor isoforms differ in their C-terminal tail and yet little is known about their regulation. In this study, we investigated the desensitization of two human 5-HT_4 receptors stably expressed in CHO cells, with a special emphasis on the h5-$HT_{4(d)}$ isoform.

2 Exposure of h5-$HT_{4(d)}$ and h5-$HT_{4(e)}$ receptors to 1 μM 5-HT induced a rapid desensitization of the adenylyl cyclase response. The h5-$HT_{4(d)}$ receptor desensitized with a faster rate ($t_{1/2} < 5$ min) than the h5-$HT_{4(e)}$ receptor ($t_{1/2} = 15$ min) and after 10 min 5-HT treatment cAMP production was reduced by $\sim 70\%$.

3 5-HT-induced h5-$HT_{4(d)}$ receptor desensitization was mimicked by 8-Bromo-cAMP, a cAMP analogue, and was inhibited by [n-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide, 2HCl] (H-89), an inhibitor of cAMP-dependent protein kinase (PKA). Inhibitors of endocytosis (sucrose, 0.45 M and concanavaline A, 0.25 mg ml^{-1}) partially reversed the h5-$HT_{4(d)}$ receptor desensitization process.

4 Given the prominent role of PKA in agonist-induced desensitization, we mutated the four putative PKA phosphorylation sites present in the third intracellular loop (Ser242, Thr253, Thr255) and the C terminal tail (Ser338) of the h5-$HT_{4(d)}$ receptor. Surprisingly, mutated receptors in which either one or all four putative phosphorylation sites were substituted to alanine did not impair receptor desensitization suggesting that PKA might act on nonconsensus sites.

5 Altogether, our data demonstrate that the C-terminal tail of h5-HT_4 receptors may influence the rate of agonist-induced desensitization and we provide evidence for a major role of PKA in h5-$HT_{4(d)}$ receptor desensitization.

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Keywords: cyclic AMP; serotoninergic receptors; desensitization; protein kinase A; G-protein-coupled receptors; adenylyl cyclase; G protein-coupled receptor kinase

Abbreviations: 8-Bromo-cAMP, 8-bromoadenosine 3′, 5′-cyclic monophosphate; CHO, Chinese hamster ovary cells; Con A, concanavaline A; dFCS, dialyzed FCS; DMSO, dimethyl sulphoxide; FCS, foetal calf serum; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; h5-HT_4, human 5-HT_4 receptor; H-89, [n-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide, 2HCl]; PKA, cAMP-dependent protein kinase; PKC, calcium-dependent protein kinase

Introduction

Serotonin 5-HT4 receptors exert pleiotropic effects in vertebrates and are thought to be involved in a variety of central and peripheral disorders including cardiac arrhythmias, neurodegenerative disorders, irritable bowel syndrome, or gastroparesis (Kaumann, 1994; Reynolds *et al*., 1995; Hedge & Eglen, 1996; De Ponti & Tonini, 2001). They are members of the large family of G protein-coupled receptors (GPCRs) and molecular studies have revealed the existence of several splice variants which differ mainly in their C-terminal tail. To date, eight human 5-HT_4 receptor isoforms have been identified and are called h5-$HT_{4(a-g)}$ and h5-$HT_{4(n)}$ (Blondel *et al*., 1997; 1998; Claeysen *et al*., 1997; 1999; Van den Wyngaert *et al*., 1997; Bender *et al*., 2000; Mialet *et al*., 2000a; Vilaró *et al*., 2002). Most of the h5-HT_4 receptor splice variants are widely distributed in the central nervous system and in peripheral organs such as the atrium and the gastroinstestinal tract (Medhurst *et al*., 2001).

The pharmacological binding profile determined in competition studies with the specific 5-HT_4 receptor antagonist [^{3}H]-GR113808 revealed the same rank order of affinity of 5-HT_4 compounds for recombinant h5-HT_4 receptors transfected in mammalian cells (Blondel *et al*., 1998; Mialet *et al*., 2000a, b). Furthermore, all the h5-HT_4 variants stimulate adenylyl cyclase and raise intracellular cAMP levels (Blondel *et al*., 1998; Claeysen *et al*., 1999; Bender *et al*., 2000; Mialet *et al*., 2000a; Vilaró *et al*., 2002). However, the efficacy and the potency of a given 5-HT_4 ligand depend strongly on the variant considered. For instance, we have recently shown that the h5-$HT_{4(e)}$ receptor isoform was much less potent than the h5-$HT_{4(d)}$ receptor to increase cAMP synthesis in response to agonists indicating that the C-terminal tail of the 5-HT_4 receptor, which is much shorter in the (d) than in the (e) variant, may directly influence its functional properties (Mialet *et al*., 2000a, b). Further

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differences between different 5-HT$_4$ splice variants may extend to the type of G proteins activated by the receptor (Pindon *et al*., 2002).

An important cellular mechanism that modulates the functional activity of GPCRs is the agonist-induced desensitization which is defined as a regulatory phenomenon by which the intensity of a cellular response decreases over time despite the continuous presence of the agonist. In many cases, receptor phosphorylation on serine and threonine residues present in the carboxyl-terminal domain or intracellular loops of the receptor appears to play a major role in initiating the desensitization process which is reported to be homologous or heterologous (Bunemann *et al*., 1999; Seibold *et al*., 2000). Receptor phosphorylation can be achieved by G protein-coupled receptor kinases (GRKs) which recognize the agonist occupied form of the receptor in a process termed homologous desensitization (Pitcher *et al*., 1998; Bunemann & Hosey, 1999). Subsequently, this phosphorylation promotes β-arrestin binding which uncouples the receptor from G protein and potentially induces its internalization by endocytosis (Krupnick & Benovic, 1998). In heterologous desensitization, phosphorylation is mediated by non-specific cellular protein kinases such as cAMP-dependent protein kinase (PKA) and calcium-dependent protein kinase (PKC) (Lefkowitz, 1998).

It is well known that GPCR desensitization may limit the biological effects of therapeutic molecules. Therefore, given the potential therapeutic applications of 5-HT$_4$ receptor ligands for treatments of memory disorders, arrhythmia or the dysfunction of the alimentary tract, it is crucial to get insights into the activation and signalling mechanism of h5-HT$_4$ receptor isoforms. The molecular mechanisms involved in 5-HT$_4$ receptor desensitization have not been yet characterized and discordant results were reported in the different biological models used in the studies. For example, a rapid and homologous desensitization was observed in mouse colliculi neurons (Ansanay *et al*., 1992) and rat oesophagus (Rondé *et al*., 1995) while a lesser extent of desensitization was found in human atrium (Kaumann *et al*., 1990). These observations raise the question of the existence of h5-HT$_4$ receptor isoforms having distinct properties of desensitization. Thus, the present study was undertaken (1) to determine the desensitization profile of two human serotonin 5-HT$_4$ receptors isoforms: the h5-HT$_{4(d)}$ isoform which is normally expressed exclusively in the gastrointestinal tract (Blondel *et al*., 1998) and the h5-HT$_{4(e)}$ isoform largely expressed in the brain (Claeysen *et al*., 1999), and (2) to dissect the molecular signalling pathway involved in the h5-HT$_{4(d)}$ receptor isoform desensitization process.

Methods

Materials

5-HT, theophylline, sucrose, GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) and concanavaline A (Con A) were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). 8-Bromo-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) and the protein kinase A inhibitor, H-89 were from Calbiochem (France Biochem, Meudon, France). H-89 and GF 109203X were dissolved in ethanol 50% and dimethyl sulphoxide (DMSO), respectively.

Cell culture

Cell culture materials and reagents were obtained from Life Technologies (Cergy Pontoise, France). CHO, a Chinese hamster ovary cell line, was purchased from ATCC (Rockville, U.S.A). Stock cultures of CHO cells were grown at 37°C and 5% CO_2 in Ham-F12 medium supplemented with 10% foetal calf serum (FCS) or 5% dialyzed FCS (dFCS), 10 mM HEPES (pH 7.4) and antibiotics. CHO cells stably expressing 5-HT$_{4(d)}$ and 5-HT$_{4(e)}$ receptor isoforms have been previously described (Mialet *et al*., 2000a, b). To maintain stable expression of 5-HT$_{4(d)}$ and 5-HT$_{4(e)}$ receptors in the cell lines, cells were cultured in the presence of 1 mg ml^{-1} of neomycin.

Site-directed mutagenesis

The full coding region of the h5-HT$_{4(d)}$ receptor cDNA was subcloned in the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.) as previously described (Blondel *et al*., 1998). Mutations of PKA consensus sites in the h5-HT$_{4(d)}$ receptor isoform were introduced using Quick-Change Site-directed Mutagenesis kit (Stratagene, Montigny-le-Bretonneux, France). The primers used were: Ser242→Ala, 5′-GAGCAGGCCTCAG**GCG**GCAGACCA-GC-3′; Thr253→Ala, 5′-CATCGCATGAGG**GCA**GAGAC-CAAAGCAG-3′; Ser338→Ala, 5′-GCGCTACCGAAGAC-CT**GCC**ATTCTGGGCC-3′; Thr255→Ala, 5′-GCATGGA-CAGAG**GCC**AAAGCAGCCAAG-3′, Thr253→Ala, Thr 255→Ala, 5′-GCATGAGG**GCA**GAG**GCC**AAAGCAGC-CAAG-3′. The quadruple mutant was obtained from the Ser242/Ala single mutant by replacing one by one the three other amino acids. We generated successively a double mutant Ser242/Ala, Thr253/Ala, then a triple mutant Ser242/Ala, Thr253/Ala, Ser338/Ala and finally a quadruple mutant Ser242/Ala, Thr253/Ala, Ser338/Ala, Thr255/Ala. Mutants were analysed by restriction enzymes and the authenticity of each mutation was confirmed by DNA sequencing (Genome Express, Montreuil, France).

Cyclic AMP radioimmunoassay

Stably transfected CHO cells in 24-well plates, or transiently transfected CHO cells in 12-well plates were incubated 48 h before experimentation with Ham-F12 medium containing 5% dFCS to remove any trace of 5-HT in culture medium. For measurement of cAMP accumulation, cells were incubated 10 min in serum-free medium supplemented with 5 mM theophylline, 10 μM pargyline, 1 μM GR127935 (to block the activity of endogenous 5-HT$_{1B}$ receptors) and 1 μM 5-HT. The reaction was stopped by aspiration of the medium and addition of 50 μl ice-cold perchloric acid (20%). After a 30 min period, neutralization buffer was added (HEPES 40 μM, KOH 2N) and cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Immunotech, Marseille, France).

Desensitization experiments

For desensitization experiments, cells were preincubated for various periods of time with or without 1 μM 5-HT and in the presence of 1 μM GR127935 to block the activity of endogenous 5-HT$_{1B}$ receptors. Then, the cells were washed three times (2 min each) with serum-free medium. Upon this washing period, the cells were restimulated with 5-HT and cAMP determination was performed. The involvement of cAMP in the desensitization process was examined with the membrane permeant 8-Bromo-cAMP (100 μM). One inhibitor of PKA, H-89 (10 μM) and one of inhibitor of PKC, GF 109203X (10 μM) were used to test the role of PKA and PKC in 5-HT-induced receptor desensitization. At the concentration used in our study, these protein kinase inhibitors are known to be effective in CHO cells (Smeets *et al*., 1998; May *et al*., 1999; Robert *et al*., 2001). We investigated the effects of two inhibitors of internalization, hypertonic sucrose (0.45 M) and Con A (0.25 mg ml^{-1}) on the agonist-induced desensitization of the 5-HT$_{4(d)}$ receptor. Both agents employed at the above concentrations are known to inhibit agonist-induced internalization of other Gs-coupled receptors such as the A_{2A} adenosine receptor and the β2-adrenoreceptor (Pippig *et al*., 1995; Mundell & Kelly, 1998). All the drugs used in this study were added during the 1 h preincubation period.

Statistical analysis

One way ANOVA, followed by Newman-Keuls *post hoc* test was used to assess the differences between the experimental groups. Values of $P<0.05$ were considered significant.

Results

Time course analysis of 5-HT-induced cAMP production in CHO cells stably expressing h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors

Cyclic AMP responses to 5-HT were investigated in the CHO cell line stably transfected with similar levels of h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors (493$\pm$25 fmol mg^{-1} protein for the (d) variant and 347$\pm$7 fmol mg^{-1} protein for the (e) variant) (Mialet *et al*., 2000a, b) (Figure 1). The time-course of cAMP production and subsequent cAMP assays were examined in the presence of a non selective phosphodiesterase inhibitor, theophylline (5 mM). One μM 5-HT caused a time dependent increase of cAMP production in both clonal cell lines. Figure 1 shows that maximal level of cAMP production was reached with a faster rate for the h5-HT$_{4(d)}$ receptor (Figure 1A) than for the h5-HT$_{4(e)}$ receptor isoform (Figure 1B). Maximal effect of 1 μM 5-HT on cAMP accumulation was observed at 5 min for the h5-HT$_{4(d)}$ receptor and 15 min for the h5-HT$_{4(e)}$ receptor. In contrast, the absolute magnitude of cAMP production in CHO cells transfected with the (e) variant was substantially greater than that seen with the h5-HT$_{4(d)}$ receptor isoform. Indeed, 5-HT increased basal level of cAMP by 6 and 13 fold at the h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptor, respectively (Figure 1).

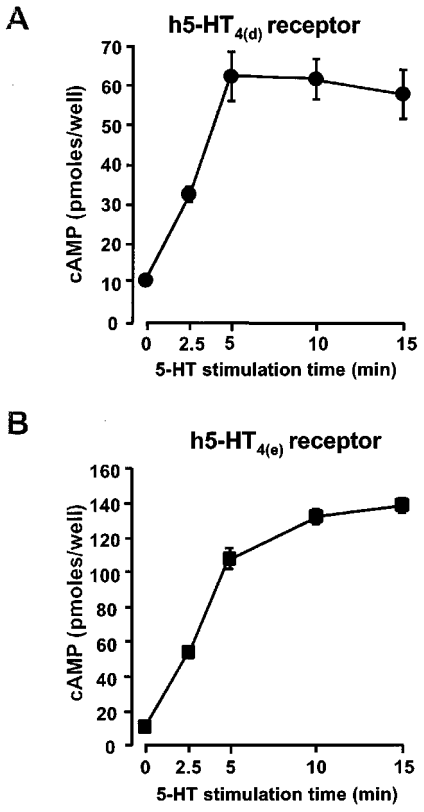

Figure 1 Time course analysis of cAMP production in CHO cells stably expressing h5-HT$_{4(d)}$ (A) and h5-HT$_{4(e)}$ receptors (B). CHO cells treated with 1 μM 5-HT were incubated at indicated times. The reaction was stopped and cAMP levels were then quantified as described in Methods and expressed in pmoles per well. cAMP basal levels were respectively 11 pmoles well^{-1} for the h5-HT$_{4(e)}$ receptor and 12 pmoles well^{-1} for the h5-HT$_{4(d)}$ receptor. Maximal cAMP levels were 63 pmoles well^{-1} at 5 min for the h5-HT$_{4(d)}$ receptor and 139 pmoles well^{-1} at 15 min for the h5-HT$_{4(e)}$ receptor. Results are means$\pm$s.e.mean. This experiment is representative of three independent experiments performed in triplicate.

Agonist-induced desensitization of h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors

When transfected CHO cells were exposed to 5-HT (1 μM) for extended periods of time and assayed for cAMP production, a time-dependent loss of coupling efficiency to its effector was observed both for the h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors (Figure 2). As shown in Figure 2, desensitization proceeded in two phases for both receptors, an initial rapid phase (between 0 and 15 min) and a secondary slower phase (15 min to 1 h). In the first phase, the rate of desensitization of the 5-HT$_{4(d)}$ receptor was substantially faster than that of the 5-HT$_{4(e)}$ receptor. Indeed, a 5 min exposure of the 5-HT$_{4(d)}$ receptor to 5-HT was sufficient to reduce by 50% the response of adenylyl cyclase to 1 μM 5-HT, while 15 min were necessary to produce a similar effect on the 5-HT$_{4(e)}$ receptor (Figure 2). The secondary phase was somewhat similar for the two 5-HT$_4$ receptors, and both responses to 5-HT reached a plateau after 1–2 h corresponding to approximately 20% of the control cAMP production. Since the h5-HT$_{4(d)}$ receptor isoform is only expressed in human (Blondel *et al*., 1998),

we thought it was particularly interesting to focus the rest of the study on this isoform.

Next, we investigated the recovery from desensitization of the h5-HT$_{4(d)}$ receptor. In the experiment illustrated in Figure 3, a 30 min exposure to 1 μM 5-HT was used to produce a strong desensitization of the h5-HT$_{4(d)}$ receptor-mediated cAMP response. Subsequently, the cells were exposed to a 5-HT free solution for increasing periods of time (from 5 to 120 min) and the capacity of the cells to resensitize to 5-HT was examined by measuring the cAMP response to a second challenge with 1 μM 5-HT. As shown in Figure 3, the cAMP response recovered only partially. Resensitization of the 5-HT$_{4(d)}$ receptor began 10 min after washout of 5-HT. This process was slow and only 70% of the adenylyl cyclase activity recovered after a 2 h resensitization period (Figure 3). We did not try any time points beyond 2 h but based on previous experiments performed on native 5-HT$_4$ receptors in mouse colliculi neurons, one can speculate that full resensitization of the 5-HT$_{4(d)}$ receptor requires two to three days (Ansanay *et al*., 1992).

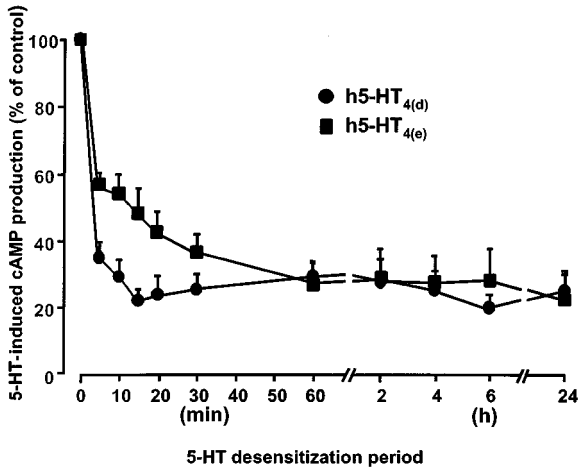

Figure 2 Time course of desensitization of 5-HT-mediated cAMP production in CHO cells expressing h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptor isoforms. Cells were preincubated with 5-HT (1 μM) in the presence of an antagonist of the 5-HT$_{1B}$, GR127935 (1 μM), during the desensitization period for the times indicated. 5-HT was then removed by three washes with serum-free medium and cells were analysed for 5-HT-induced cAMP production. For each desensitization period with 5-HT, cAMP stimulation (per cent over basal) was normalized to control stimulation (preincubated with vehicle only). Each point is the mean$\pm$s.e.mean of four independent experiments performed in duplicate.

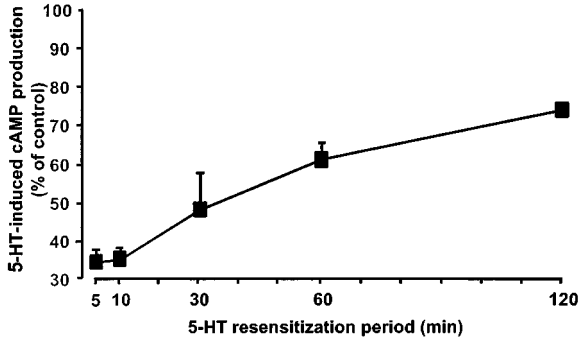

Figure 3 Time course of resensitization of the h5-HT$_{4(d)}$ receptor in CHO cells. Cells were preincubated for 30 min (desensitization period) with 5-HT (1 μM) in the presence of GR127935 (1 μM). Ligand was removed and cells were washed three times with serum-free medium. Recovery period was started by incubation in 5% dFCS supplemented-medium at 37°C during increasing periods (5 min to 2 h). After the recovery period, 5-HT-induced cAMP production was quantified (per cent over basal). Results were normalized to control stimulation (preincubated with vehicle only) and each point is the mean$\pm$s.e.mean of three independent experiments performed in duplicate.

Effects of protein kinase inhibitors on 5-HT-induced desensitization of the h5-HT$_{4(d)}$ receptor

To explore the involvement of PKA in the h5-HT$_{4(d)}$ receptor desensitization process, we tested the effects of the PKA inhibitor, H-89 (10 μM) on cAMP production. The drug was added during the 1 h preincubation period to 5-HT. Stock solution of H-89 (10^{-2}M) was prepared in ethanol 50% but a final concentration of 0.05% ethanol had no effect on the desensitization process (data not shown). As shown in Figure 4A, cAMP accumulation after 1 h preincubation with 1 μM 5-HT was only 39$\pm$7% of the control value (no preincubation with 5-HT). In

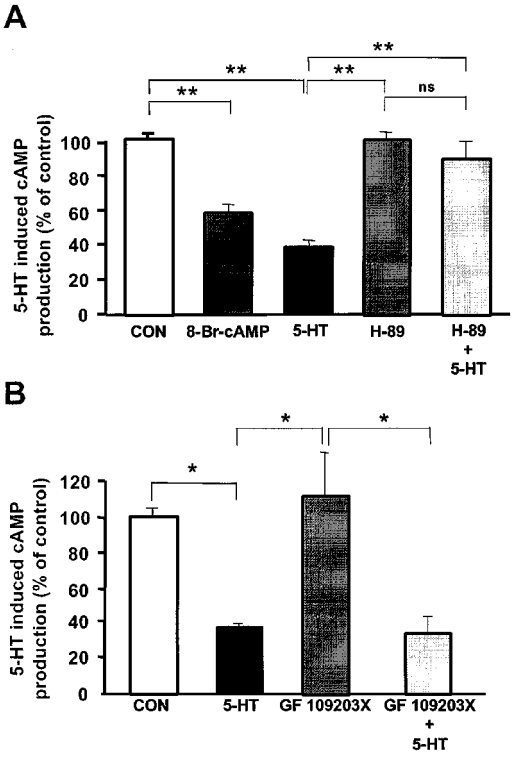

Figure 4 Effect of inhibitors or activators of second messenger kinases on h5-HT$_{4(d)}$ desensitization in CHO cells. CHO cells expressing the h5-HT$_{4(d)}$ receptor were preincubated for 60 min either in control solution (CON) or with the following drugs: (A) 8-Bromo-cAMP (100 μM), 5-HT (1 μM), H-89 (10 μM), or H-89+5-HT; (B) 5-HT (1 μM), GF 109203X (10 μM), or GF 109203X+5-HT. Cells were then washed three times with serum-free medium and 5-HT-induced cAMP production was quantified (per cent stimulation over basal). Results were normalized to control stimulation (preincubated with vehicle only) for each experiment, and were expressed as means$\pm$s.e.mean of four separate experiments performed in duplicate. $^{**}P<0.01$; $^{*}P<0.05$ versus indicated values; ns, non significant.

contrast, when H-89 was added during the preincubation period, cAMP production reached 89±14% of the control vehicle indicating that the desensitization process was almost completely inhibited (Figure 4A). No statistical difference was observed between H-89 treated cells and H-89+5-HT treated cells (Figure 4A). To examine whether activation of PKA was also sufficient to induce receptor desensitization, 5-HT was replaced by 8-Bromo-cAMP (10 μM), a membrane permeant cAMP analogue and PKA activator, during the 1 h preincubation period. As shown in Figure 4A, 8-Bromo-cAMP alone reduced by 42±5% the cAMP-response to 5-HT, indicating that activation of PKA is sufficient to desensitize the h5-HT$_{4(d)}$ receptor.

Next, we investigated the involvement of PKC in 5-HT$_{4(d)}$ receptor desensitization. As observed in Figure 4A, a 60 min exposure of the 5-HT$_{4(d)}$ receptor to 5-HT was sufficient to reduce by 60% the response of adenylyl cyclase to 1 μM 5-HT (Figure 4B). Unlike PKA, PKC did not seem to play a role in the desensitization process. Indeed, in the presence of GF 109203X (10 μM), a potent and selective PKC inhibitor (Toullec *et al*., 1991), 5-HT still desensitized the 5-HT$_{4(d)}$ receptor (Figure 4B). cAMP levels were similar to the vehicle control value (0.1% DMSO) when GF 109203X (10 μM) was incubated without 5-HT (Figure 4B). Altogether these results indicate that activation of PKA (but not PKC) is necessary to mediate agonist-dependent h5-HT$_{4(d)}$ receptor desensitization.

Inhibitors of internalization and h5-HT$_{(4d)}$ receptor function

Two main mechanisms are responsible for GPCR desensitization: uncoupling of the receptor from the G proteins and receptor internalization. To determine the relative contribution of the latter mechanism, we used two inhibitors of internalization, Con A and hypertonic sucrose. CHO cells expressing the 5-HT$_{4(d)}$ receptor were pretreated with 1 μM 5-HT in the presence of either Con A (0.25 mg ml^{-1}) or sucrose (0.45 M) and following this treatment 5-HT (1 μM)-induced cAMP accumulation was determined. As shown in Figure 5, both inhibitors of endocytosis significantly attenuated 5-HT-induced desensitization. No statistical difference was observed between 5-HT and sucrose and Con A treated cells and their respective control (sucrose and Con A applied alone) (Figure 5).

Mutation of consensus PKA phosphorylation sites on h5-HT$_{4(d)}$ receptor

Consensus sequences for PKA predict the presence of basic amino acids, particularly arginine, in the amino-terminus of the phospho-acceptor serine or threonine separated by one or two residues (Kennelly & Krebs, 1991). Examination of the cytoplasmic regions of the h5-HT$_{4(d)}$ receptor indicates the presence of four potential sequences for PKA-induced phosphorylation. Three residues (Ser242, Thr253 and Thr255) are found in the third intracellular loop of the h5-HT$_{4(d)}$ receptor and one residue (Ser338) is found in its carboxyl-terminal tail. Using site-directed mutagenesis, we modified these potential phosphorylation sites by changing serines and threonines to alanine as to preclude potential phosphorylation of these sites.

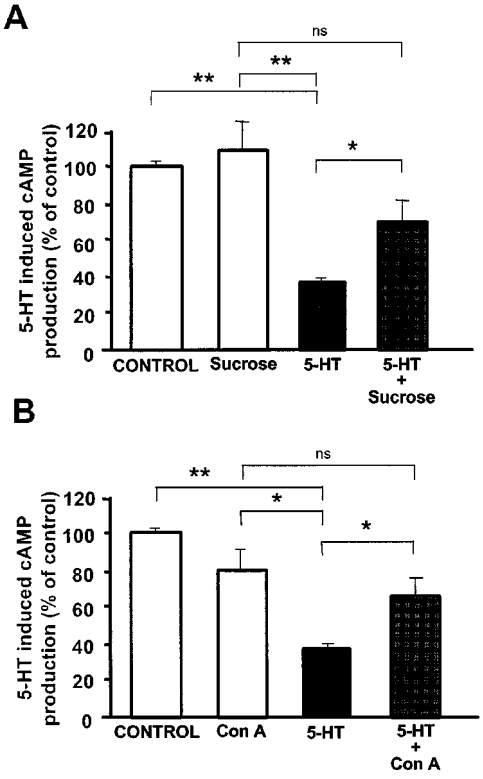

Figure 5 Effect of sucrose or Con A on 5-HT$_{4(d)}$ receptor desensitization in CHO cells. Cells were pretreated with 1 μM 5-HT, or sucrose (0.45 M) (A), or Con A (0.25 mg ml^{-1}) (B), or 1 μM 5-HT and sucrose (0.45 M) (A), or 1 μM 5-HT and Con A (0.25 mg ml^{-1}) (B) for 60 min, washed three times with serum-free medium, then the production of cAMP in response to 1 μM 5-HT was determined as described in Methods. Results were normalized to control stimulation (preincubated with vehicle only) for each experiment, and were expressed as means±s.e.mean of four (n=4) (A) or five (n=5) (B) separate experiments performed in duplicate. $^{**}P<0.01$; $^{*}P<0.05$ versus indicated values; ns, non significant.

In a first step, we constructed four mutants containing only a single amino acid substitution. Mutant receptors were transiently expressed in CHO cells to characterize their kinetics of desensitization (Figure 6A). All single mutant receptors were able to stimulate cAMP production in a similar manner as the wild type receptor (data not shown). When we tested the four mutant receptors for their ability to undergo functional agonist-induced desensitization, we observed the same kinetic and intensity of desensitization as for the wild-type receptor (Figure 6A). Therefore in a second step, another construct was created in which all the four potential phosphorylation sites were simultaneously replaced by alanines. This quadruple mutant receptor was as potent as the wild type receptor to stimulate cAMP production (data not shown). Most surprisingly, this quadruple mutated receptor had similar kinetics of desensitization as the wild type receptor (Figure 6B). These results indicate that Ser242, Thr253, Thr255 and Ser338, although present in consensus PKA phosphorylation sites, are not involved in the h5-HT$_{4(d)}$ receptor desensitization process.

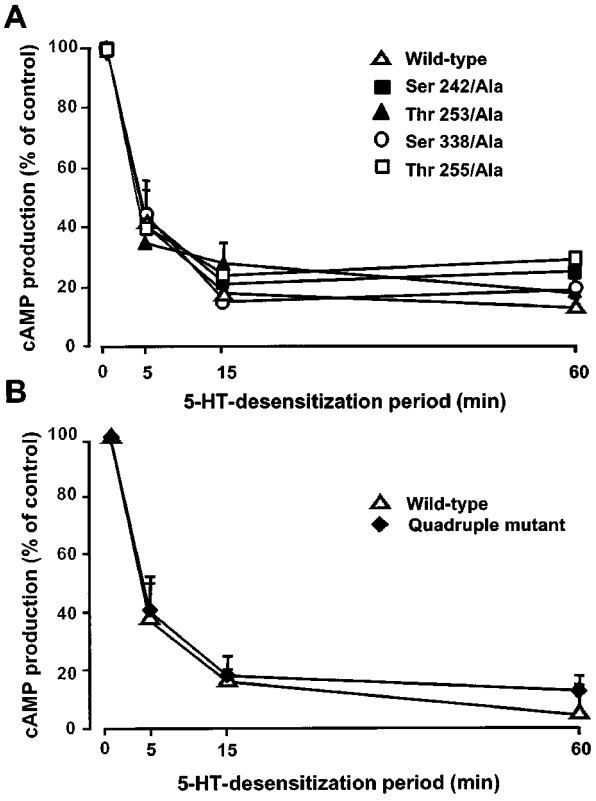

Figure 6 Desensitization profile of wild-type and mutant 5-HT$_{4(d)}$ receptors transiently expressed in CHO cells. Wild-type h5-HT$_{4(d)}$ receptor and mutant receptors in which either each of the four consensus PKA phosphorylation sites (A) or all four at once (B) were substituted to alanines were transiently expressed in CHO cells and 5-HT-induced desensitization of cAMP response was measured. Transfected cells were preincubated with 5-HT for 0 min, 5 min, 15 min or 1 h, then they were washed three times with serum-free medium and stimulated with 1 μM 5-HT to determine cAMP response. Results are expressed as the percentage of control stimulation (preincubated with vehicle only). Results are means $\pm$ s.e.mean of four independent experiments performed in triplicate.

Discussion

The 5-HT$_4$ receptor mediates important physiological effects of 5-HT, such as intestinal motility or memory facilitation, and the concept has emerged that 5-HT$_4$ agonists may be used as therapeutic drugs for the treatment of various disorders, such as the irritable bowel syndrome (De Ponti & Tonini, 2001) or neurodegenerative disorders (Marchetti *et al*., 2000; Robert *et al*., 2001). In that respect, it is conceivable that the therapeutic effects of 5-HT$_4$ receptor agonists might decrease over time if the portion of active receptors decreases due to desensitization. It is therefore important to delineate the molecular determinants involved in this process in order to increase the therapeutic utility of 5-HT$_4$ ligands.

Although earlier studies have addressed the mechanisms of desensitization of 5-HT$_4$ receptors in native tissues, such as mouse colliculi neurons (Ansanay *et al*., 1992) and rat oesophagus (Rondé *et al*., 1995), the intrinsic desensitization properties of distinct 5-HT$_4$ receptor isoforms expressed in heterologous systems have never been described. Here, we found that pretreatment of CHO cells with a saturating concentration of 5-HT desensitized both h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors in a time-dependent manner. Half-maximal desensitization were obtained at 5 min and 15 min agonist exposure for h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors, respectively. The higher sensitivity of the h5-HT$_{4(d)}$ receptor to the desensitization process cannot be attributed to a different level of expression of the two receptors since both receptors were stably expressed in CHO cells at a similar density (Mialet *et al*., 2000a, b). It is also difficult to explain the differences based on the primary structure of the receptors, because the carboxyl-terminal tail of the h5-HT$_{4(d)}$ receptor is shorter than that of the h5-HT$_{4(e)}$ receptor, thus precluding the existence of additional phosphorylation sites for protein kinases on the (d) variant. Interestingly, we showed in a previous study that h5-HT$_{4(d)}$ and h5-HT$_{4(e)}$ receptors possessed distinct coupling efficiencies and potencies in response to 5-HT$_4$ agonists (Mialet *et al*., 2000a, b). The h5-HT$_{4(d)}$ receptor was 5 fold more potent to activate cAMP production in response to 5-HT than the h5-HT$_{4(e)}$ receptor in CHO cells (EC_{50} value of 5-HT was 5.3 ± 0.6 nM and 25 ± 6 nM for the (d) and (e) variants, respectively) (Mialet *et al*., 2000a; b). Thus, the potent coupling of the (d) variant to its effector may increase PKA activity which could trigger the desensitization process. Accordingly, we found that inhibition of PKA reversed most of the agonist-mediated desensitization of the h5-HT$_{4(d)}$ receptor, while inhibition of PKC had no effect. This indicates that PKA plays a pivotal role in this process. Activation of PKA was not only necessary but also sufficient to desensitize the h5-HT$_{4(d)}$ receptor, since a direct, receptor-independent, activation of PKA mimicked the 5-HT-mediated desensitization of the h5-HT$_{4(d)}$ receptor. These results suggest that activation of PKA may participate in heterologous desensitization of the h5-HT$_{4(d)}$ receptor. One of the downstream mechanisms responsible for receptor desensitization must involve endocytosis and receptor sequestration, since inhibitors of endocytosis reversed by about 50% the agonist-mediated desensitization of the h5-HT$_{4(d)}$ receptor.

In our study, we found that 20% of the cAMP response was resistant to desensitization for both receptors. This could be explained by the lack and/or low abundance of specific proteins in CHO cells which may participate in the 5-HT$_4$ receptor desensitization process. Indeed, besides second messenger-activated protein kinases, GPCR desensitization may also involve the recruitment of additional kinases belonging to the family of G protein-coupled receptor kinases (GRK) which phosphorylate the agonist-occupied form of GPCRs leading to their functional uncoupling (for review, see Bunemann & Hosey, 1999). For instance, PKA-mediated phosphorylation of the β_2-adrenergic receptor favoured subsequent phosphorylation of the receptor by GRKs and thus the extent of the functional uncoupling and desensitization of the receptor (Moffett *et al*., 2001). Stable cell clones expressing different GRK sub-types will allow us to clearly show their involvement in h5-HT$_4$ receptor desensitization.

At first glance, the implication of PKA in the h5-HT$_{4(d)}$ receptor desensitization process is surprising because previous studies performed on native 5-HT$_4$ receptors in mouse colliculi neurons and rat oesophagus desensitization showed a mechanism strictly homologous involving phosphorylation of the 5-HT$_4$ receptor *via* the GRK pathway (Ansanay *et al*., 1992; Rondé *et al*., 1995). PKA was not found to be involved

in this regulation. Species differences could explain this discrepancy since sequence and number of splice variants are not strictly conserved among rat, mouse and human. For instance, the 5-HT$_{4(d)}$ receptor isoform is only expressed in human and has not been reported in rat and mouse (Blondel *et al*., 1998; Claeysen *et al*., 1999). Yet, 5-HT$_4$ receptor splice variants have a specific pattern of expression and distinct functional properties (Mialet *et al*., 2000a, b; Medhurst *et al*., 2001). Finally, we can speculate that cell specific differences in both GRK subtype and quantity may affect the signalling through a given 5-HT$_4$ receptor isoform expressed in that cell. Altogether these observations may confer to a given tissue a specific mechanism to finely regulate the desensitization process.

To further investigate the prominent role of PKA in agonist-induced desensitization, site-directed mutagenesis techniques were used to alter each of the putative PKA phosphorylation sites in the h5-HT$_{4(d)}$ protein followed by heterologous expression in CHO cells. Surprisingly, analysis of single mutated receptors, in which only one of the four putative phosphorylation sites was modified, did not reveal any effect on 5-HT-induced desensitization of the h5-HT$_{4(d)}$ receptor. Moreover, the quadruple mutants in which Ser242, Thr253, Thr255 and Ser338 were substituted to Ala failed to impair receptor desensitization. There are at least two possible interpretations of these data. Firstly, 5-HT-induced desensitization may occur through a pathway that does not involve direct phosphorylation of the h5-HT$_{4(d)}$ receptor on the mutated sites but rather on other potential phosphorylation sites present in the receptor. Such an observation has been reported for other PKA regulated proteins such as the L-type cardiac calcium channel which is phosphorylated on PKA non-consensus sites (Gerhardstein *et al*., 1999). Since the h5-HT$_{4(d)}$ receptor possesses several serine and threonine residues in the third intracellular loop and carboxyl-terminal tail, one can make the hypothesis that PKA may phosphorylate these non-consensus sites. Obviously, definitive proof that PKA directly phosphorylates the 5-HT$_{4(d)}$ receptor on its third intracellular loop and/or C-terminal tail must await the production of sufficient quantities of purified receptor for use in *in vitro* phosphorylation assays with purified PKA. Secondly, the h5-HT$_{4(d)}$ receptor may not necessarily be directly phosphorylated by PKA upon receptor activation, but another PKA protein substrate might be involved in receptor desensitization. One possible candidate for such a substrate is the adenylyl cyclase, which is acting downstream of the receptor. Indeed, among the great diversity of adenylyl cyclase isoforms in mammalian tissues, adenylyl cyclase type V and VI have been shown to be inhibited by PKA (Iwami *et al*., 1995; Chen *et al*., 1997). Such a negative feedback regulation might contribute to the h5-HT$_4$ receptor desensitization process. However, apart from brain and heart, adenylyl cyclase type V and VI are expressed at relatively low levels in most tissues (Defer *et al*., 2000) and given the large number of adenylyl cyclase isoforms, it is rather difficult to determine precisely which isoform is involved in the 5-HT$_4$ signalling cascade in CHO cells.

In summary, the data presented here suggest that the carboxyl domain of the h5-HT$_4$ receptor not only plays important roles in coupling efficacy but also in agonist-induced receptor desensitization. We have also demonstrated a major role for PKA in the desensitization of the 5-HT$_{4(d)}$ receptor.

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