The 5-HT_4 receptor antagonist ML10375 inhibits the constitutive activity of human 5-$HT_{4(c)}$ receptor

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Transient expression in COS-7 cells of the recombinant human 5-hydroxytryptamine (5-HT) h5-$HT_{4(c)}$ receptor isoform led to constitutive activity of the receptor. The 5-HT_4 receptor antagonist 2-(*cis*-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate (ML10375) at 1 μM completely abolished the 5-HT (1 μM)-mediated increase in adenylyl cyclase activity in COS-7 cells expressing the h5-$HT_{4(c)}$ receptor. Moreover, ML10375 also reduced basal cAMP levels in cells over-expressing the receptor, even in the absence of agonist. The inhibitory effect of ML10375 on basal adenylyl cyclase activity was not modified by pre-treatment of the cells with pertussis toxin, indicating that ML10375 acts through inactivation of spontaneously active h5-$HT_{4(c)}$ receptors rather than through a G_i/G_o regulatory pathway. We conclude that ML10375 acts as an inverse agonist on the h5-$HT_{4(c)}$ receptor.

Keywords: Human; serotonin 5-HT_4 receptors; inverse agonist; ML10375

Introduction In recent years, a number of studies have shown that some G-protein-coupled receptors (GPCR) can spontaneously oscillate between an active and inactive receptor conformation (Leff, 1995; Pauwels & Wurch, 1998). In heterologous expression systems, these receptors can attain the active conformation in the absence of agonist by spontaneous isomerization and display constitutive activity by interacting with the G protein (Lefkowitz *et al*., 1993). In native systems, GPCR can also exhibit constitutive activity either as intact proteins (Barker *et al*., 1994) or following point mutations that can have severe pathological consequences (Shenker *et al*., 1993; Schipani *et al*, 1995). Certain ligands, referred to as 'inverse agonists', appear capable of driving these receptors to the inactive state and reduce their constitutive activity (Leff, 1995; Pauwels & Wurch, 1998). While inverse agonists may provide powerful tools to investigate mechanisms controlling basal receptor activation in native cells, their ability to induce negative intrinsic activity as well as to decrease receptor density (Barker *et al*., 1994) make them also attractive therapeutic agents as compared to corresponding neutral antagonists (Milligan *et al*., 1995; Pauwels & Wurch, 1998). We have recently reported the functional characterization of four alternative splice variants of the human 5-HT_4 receptor that differ in their C-terminus sequences (Blondel *et al*., 1998). We have found that transient expression of one of the isoform, the h5-$HT_{4(c)}$ receptor, in COS-7 cells gave a constitutive activation of adenylyl cyclase leading to an increase in basal cAMP levels (Blondel *et al*., 1998). Using this recombinant expression system, we now report for the first time that a 5-HT_4 receptor antagonist, 2-(*cis*-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate (ML10375: Yang *et al*., 1997), can act as an inverse agonist of the human h5-$HT_{4(c)}$ receptor.

Methods *DNA transfection* The full coding region of the h5-$HT_{4(c)}$ cDNA was subcloned in the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.). Transfections were performed using the vector Polyethylenimine (PEI) as described (Boussif *et al*., 1995). Cells were transfected using a mixture of DNA and PEI at a ratio of 20 nmol PEI mg^{-1} DNA in 0.9% NaCl. COS-7 cells were seeded 1 day before transfection into 12-well plates at a density of 5×10^5 cells $well^{-1}$, incubated for 6 h with plasmid DNA (4 or 8 μg $well^{-1}$) and assayed 24 h after transfection. Cells transfected with the h5-$HT_{4(c)}$ cDNA construct were compared with mock-transfected cells that were only exposed to the crude pRC/CMV plasmid.

Membrane preparation and radioligand binding assays Membrane preparations and saturation analysis of ^{3}H-labelled GR113808 {[1-[2-(methylsulphonyl)amino] ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate} binding from cells expressing the h5-$HT_{4(c)}$ receptor were performed as described earlier (Blondel *et al*., 1998). When COS-7 cells were transfected with 4 μg or 8 μg of plasmid DNA $well^{-1}$, the density of receptors (B_{max}) was, respectively, 78 ± 6 and 5006 ± 226 fmol mg^{-1} protein, with similar K_d values for GR113808 (0.30 ± 0.08 and 0.65 ± 0.08 nM).

Measurement of cAMP formation For measurement of intracellular cAMP accumulation, transiently transfected COS-7 cells were incubated 24 h after transfection in Dulbecco's modified Eagle's medium containing 5 mM theophylline, 10 mM HEPES and 10 μM pargyline for 15 min at 37°C, 5% CO2. 5-HT (1 μM), ML10375 (1 μM) or forskolin (10 μM) were added and incubated for an additional 15 min at 37°C, 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 500 μl of ice-cold ethanol. After 1 h at room temperature, the ethanol fraction was collected and lyophilized. The pellet was reconstituted and cAMP was quantified using a radioimmunoassay (E.R.I.A, Diagnostics Pasteur radio-immunoassay kit 79830). Student's *t*-tests were performed using the QuickTTest software.

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Materials PEI (MW 800 kD) was from Fluka (L'Isle d'Abeau Chesnes, France). DMEM was obtained from Gibco-BRL (Life Technologies, Cergy Pontoise, France. Pertussis Toxin (PTX) was from Calbiochem. GR113808 was a generous gift from Glaxo Research Group (Ware, Hertfordshire, U.K.) and [^{3}H]-GR113808 was from Amersham (Arlington Heights, IL, U.S.A.). All other drugs were from Sigma (Ville d'Abeau Chesnes, France). ML10375 was synthesized as recently described (Yang *et al*., 1997). All drugs were dissolved in ionic aqueous solution

Results We have recently reported the functional characterization of four alternative splice variants of the human 5-HT$_4$ receptor that differ in their C-terminus sequences (Blondel *et al*., 1998). While all h5-HT$_4$ receptor subtypes expressed in COS-7 cells displayed a similar ability to couple to adenylyl cyclase when exposed to 5-HT, expression of the h5-HT$_{4(c)}$ isoform gave a constitutive activation of adenylyl cyclase that resulted in an increased basal cAMP level (Blondel *et al*., 1998). When COS-7 cells were transfected with 4 μg or 8 μg of plasmid DNA well^{-1} to obtain increasing levels of h5-HT$_{4(c)}$ receptor expression (see Methods), basal cAMP levels were increased by 59% and 235%, respectively (Figure 1a). 5-HT

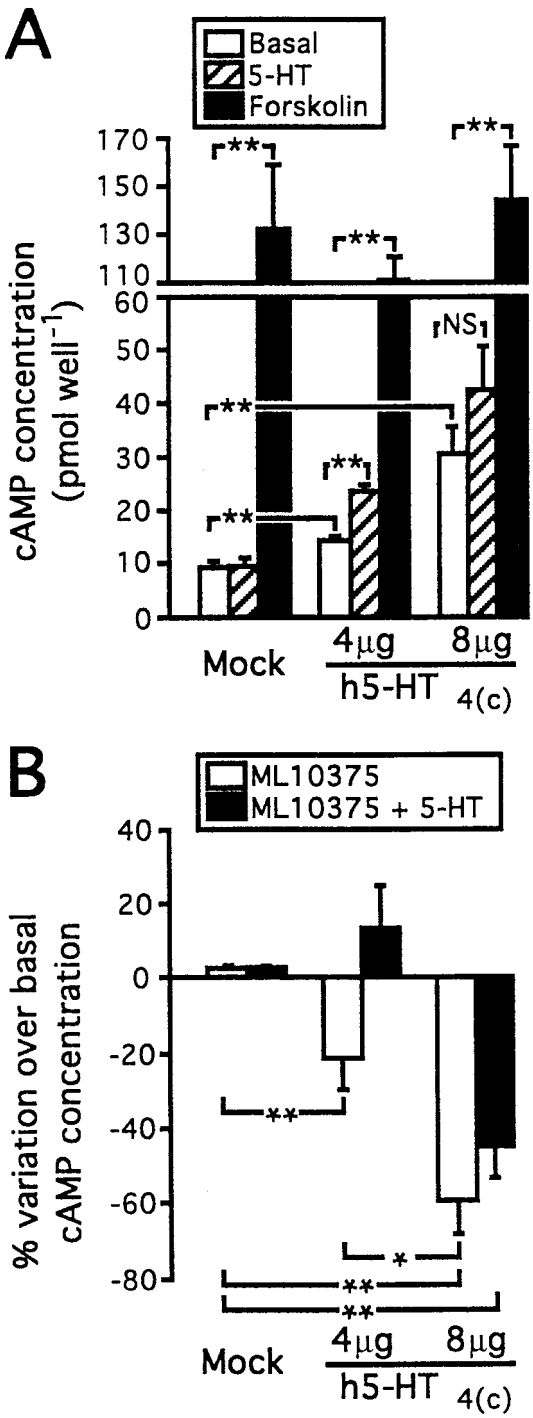

Figure 1 cAMP responses to 5-HT (1 μM), forskolin (10 μM) and the 5-HT$_4$ antagonist ML10375 (1 μM) using the h5-HT$_{4(c)}$ receptor transiently expressed in COS-7 cells or mock transfected cells as control. Cells were transfected using 4 or 8 μg well^{-1} of plasmid DNA. Effect of ML10375 on 5-HT-induced cAMP accumulation was assayed by addition of the drug during the 15 min pre-incubation period, followed by addition of 5-HT for 15 min. Values are means$\pm$s.e.mean. of six to eleven experiments. NS: non significant; *$P<0.05$ and **$P<0.01$ vs indicated values (*t*-test).

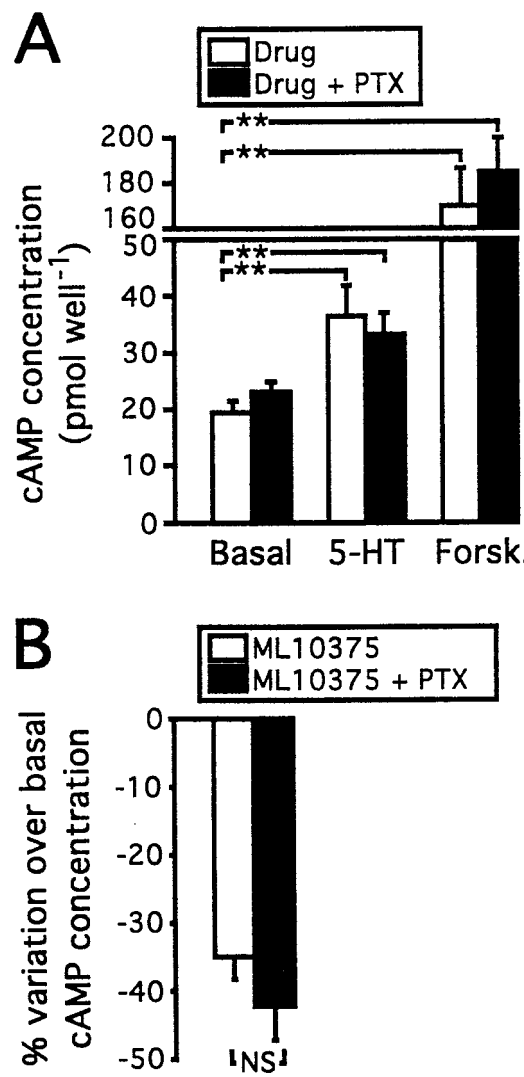

Figure 2 cAMP responses to 5-HT (1 μM), forskolin (10 μM) and the 5-HT$_4$ antagonist ML10375 (1 μM) using the h5-HT$_{4(c)}$ receptor transiently expressed in COS-7 cells. Cells were transfected using 6 μg well^{-1} of plasmid DNA. Responses were studied in the presence or absence of a 16 h preincubation with pertussis toxin (PTX, 100 ng ml^{-1}). Incubation conditions were the same as the one described in Figure 1. Values are means$\pm$s.e.mean. of six to eleven experiments. NS: non significant; **$P<0.01$ vs indicated values (*t*-test).

(1 μM) induced a 64% increase in basal cAMP levels in cells transfected with 4 μg of plasmid DNA, but did not increase significantly the basal level of cAMP in cells transfected with 8 μg of plasmid DNA (Figure 1a). This last result indicates that in cells overexpressing the h5-$HT_{4(c)}$ receptor isoform, the prevalence of spontaneously active receptor prevents further stimulatory effect of 5-HT on the adenylyl cyclase activity. Forskolin (10 μM) induced similar increases in cAMP concentrations in cells transfected by either 4 μg or 8 μg of plasmid DNA and in mock-transfected cells, indicating that the potential for maximal activation of adenylyl cyclase was not impaired in cells overexpressing the h5-$HT_{4(c)}$ receptor isoform. In COS-7 cells transfected using 4 μg and 8 μg of plasmid DNA, ML10375 (1 μM) decreased basal cAMP values by 24% and 62%, respectively (Figure 1b). In cells transfected with 4 μg of plasmid DNA, preincubation with ML10375 (1 μM) prevented a significant increase in basal cAMP after 5-HT (1 μM) stimulation. In cells transfected with 8 μg of plasmid DNA, preincubation with ML10375 (1 μM) induced a 47% decrease in basal cyclic level even in the presence of 5-HT (1 μM). To test if the effect of ML10375 on cAMP levels corresponded to an inactivation of the spontaneously active h5-$HT_{4(c)}$ receptors or to an inhibition of adenylyl cyclase activity through a G_i regulatory pathway, the effect of ML10375 was tested in COS-7 cells transfected with 6 μg of h5-$HT_{4(c)}$ plasmid DNA in the presence or absence of PTX (100 ng ml^{-1}). PTX treatment did not modify either the basal cAMP level or the 5-HT- and forskolin-induced cAMP stimulation in the transfected cells (Figure 2a). Moreover, PTX treatment did not modify significantly the ML10375-induced reduction in basal cAMP in transfected COS-7 cells (Figure 2b).

Discussion The 5-HT_4 antagonist ML10375 was shown recently to block competitively the 5-HT-induced relaxation of rat oesophageal muscles with a pA_2 of 8.6 (Yang *et al*., 1997) and to bind to cloned human 5-HT_4 receptor isoforms with K_i values in the range of 0.5–2.5 nM (Blondel *et al*., 1998). Here, we found that ML10375 also antagonized the 5-HT-induced cAMP response mediated by the h5-$HT_{4(c)}$ receptor isoform in transiently transfected COS-7 cells. However, ML10375 also reduced basal adenylyl cyclase activity in h5-$HT_{4(c)}$-transfected cells. Inhibitory effects of ML10375 on basal cAMP levels were observed in the range of 1 nM to 1 μM concentrations. Although it was difficult to derive an IC_{50} value from our experiments in COS-7 cells due to large differences in transfection efficacy between individual experiments, preliminary experiments in CHO cells stably expressing the h5-$HT_{4(c)}$-receptor showed that ML10375 reduced basal cAMP levels in a dose-dependent manner with an IC_{50} of 16 nM (data not shown), i.e. in the same range of concentrations at which it binds specifically to the receptor (Blondel *et al*., 1998). Inhibition of basal adenylyl cyclase was not due to a receptor activation of PTX-sensitive G proteins (G_i and G_o) but was rather mediated by an inverse agonistic effect of ML10375. This phenomenon represents the first of its kind in the 5-HT_4 receptor family. ML10375 will be a powerful tool in the future to investigate whether different levels of constitutive activity exist in native tissues, and can be related to preferential expression of a particular h5-HT_4 receptor subtype. Indeed, the existence of a tissue-dependent and/or isoform-dependent constitutive activity of the h5-HT_4 receptor may explain some of the differences observed in the pharmacological properties of 5-HT_4 agonists in different regions of the brain and among peripheral tissues (Blondel *et al*., 1997; 1998). Moreover, ML10375 inverse agonistic properties are likely to be of therapeutic relevance in the treatment of disorders involving 5-HT_4 receptors (Blondel *et al*.,1998), particularly if these disorders are directly related to constitutive h5-HT_4 receptor activation.

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