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Differential ion current activation by human 5-HT_{1A} receptors in *Xenopus* oocytes: Evidence for agonist-directed trafficking of receptor signalling

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Abstract

The subject of the present study was the functional and pharmacological characterization of human 5-HT_{1A} receptor regulation of ion channels in *Xenopus* oocytes. Activation of the heterologously expressed human 5-HT_{1A} receptor induced two distinct currents in *Xenopus* oocytes, consisting of a smooth inward current (I_{smooth}) and an oscillatory calcium-activated chloride current, $I_{Cl(Ca)}$. 5-HT_{1A} receptor coupling to both ionic responses as well as to co-expressed inward rectifier potassium (GIRK) channels was pharmacologically characterized using 5-HT_{1A} receptor agonists. The relative order of efficacy for activation of GIRK current was 5-HT ≈ F13714 ≈ L694,247 ≈ LY228,729 > flesinoxan ≈ (±)8-OH-DPAT. In contrast, flesinoxan and (±)8-OH-DPAT typically failed to activate $I_{Cl(Ca)}$. The other ligands behaved as full or partial agonists, exhibiting an efficacy rank order of 5-HT ≈ L694,247 > F13714 ≈ LY228,729. The pharmacological profile of I_{smooth} activation was completely distinct: flesinoxan and F13714 were inactive and rather exhibited an inhibition of this current. I_{smooth} was activated by the other agonists with an efficacy order of L694,247 > 5-HT ≈ LY228,729 > (±)8-OH-DPAT. Moreover, activation of I_{smooth} was not affected by application of pertussis toxin or the non-hydrolyzable GDP-analogue, guanosine-5′-*O*-(2-thio)-diphosphate (GDPβS), suggesting a GTP binding protein-independent pathway. Together, these results suggest the existence of distinct and agonist-specific signalling states of this receptor.

Keywords: GPCR; 5-HT_{1A} receptor; Agonist trafficking; Electrophysiology; *Xenopus* oocytes

1. Introduction

Serotonergic receptors have been classified into seven subfamilies. All receptor types with the exception of the 5-HT_3 receptor belong to the superfamily of G protein-coupled receptors (GPCRs), also called seven trans-membrane (7-TM) receptors. The GPCRs of the 5-HT_1 family are coupled to inhibitory G proteins of the $G_{i/o}$ class. Among these, the 5-HT_{1A} receptor has attracted particular interest as a major target for drug development, since multiple therapeutic indications for 5-HT_{1A} receptor ligands are possible. For example, drugs acting at 5-HT_{1A} receptors exhibit promising anxiolytic and antidepressant (Blier and Ward, 2003) as well as

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antipsychotic (Bantick et al., 2001) and analgesic (Bardin et al., 2003) properties.

A large number of signalling pathways coupled to the 5-HT_{1A} receptor has been described in assays based on heterologous expression of this receptor, suggesting a capacity to mediate a considerable diversity of cellular effects (reviewed in Raymond et al., 1999; Albert and Tiberi, 2001). The techniques most frequently employed to compare pharmacological properties of 5-HT_{1A} ligands in recombinant systems are measurements of adenylyl cyclase activity (Fowler et al., 1992; Pauwels et al., 1993; Varrault et al., 1992), radioligand binding experiments (Assié et al., 1999; Fargin et al., 1988; Varrault et al., 1992) or quantification of guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]-GTPγS) binding (Newman-Tancredi et al., 1997; Pauwels et al., 1997; Stanton and Beer, 1997). In addition, data based on 5-HT_{1A} receptor-mediated effects such as phospholipase C (PLC) activation (Boddeke et al., 1992; Pauwels and Colpaert, 2003), MAPK activation (Cussac et al., 2002) or extracellular acidification (Dunlop et al., 1998) have been reported.

In this context, a point of particular interest is the detection of differential signalling of 5-HT_{1A} ligands (Pauwels, 2000; Pauwels and Colpaert, 2003). Such diversity in ligand activity either detected in the same assay under different conditions or when comparing different assays, may rely on various factors, such as the properties of the signalling pathway and the experimental system, as well as the different levels of receptor expression (Kenakin, 1996). In addition, a differential action of agonists in different systems may be due to a phenomenon that is referred to as 'agonist trafficking of receptor signals' (Kenakin, 1995), i.e. a selective preference of certain ligands to activate a particular biochemical pathway when compared to another. Thus, differential pharmacological outcomes of 5-HT_{1A} receptor activation assays have been demonstrated depending on cellular system, on the second-messenger pathway under study or on the particular conditions of the assay (e.g. Hoyer and Boddeke, 1993; Newman-Tancredi et al., 1997, 2002; Pauwels and Colpaert, 2003; Pauwels et al., 1997), including certain indications of agonist-specific trafficking of receptor stimulus (reviewed in Newman-Tancredi, 2003).

Xenopus oocytes are a widely used and well-described heterologous expression system. They have proven very useful in various electrophysiological assays, including those dealing with GPCR coupling to membrane channels (Lee and Durieux, 1999). In this system, coupling of 5-HT_{1A} receptor has been reported for the inhibition and activation of adenylyl cyclase (Noh and Han, 1998; Uezono et al., 1993) as well as for the activation of inward rectifier potassium (GIRK) channels and PLC (Dascal et al., 1993; Ni et al., 1997).

However, extensive pharmacological studies of 5-HT_{1A} receptors in *Xenopus* oocytes have not been reported. In the present study, we performed recordings of three different ion currents in *Xenopus* oocytes as an alternative approach to characterize ligand activity at the 5-HT_{1A} receptor and their putative signalling diversity at different cellular responses.

Parts of the results presented here have been previously published in abstract form (Heusler et al., 2004).

2. Materials and methods

2.1. RNA preparation

Plasmids containing the coding sequence for the human 5-HT_{1A} receptor or the T149A h5-HT_{1A} receptor mutant (see Wurch et al., 2003 for mutant construction) were subcloned into the *Xenopus* high expression vector pGEMHE (Liman et al., 1992) and designated pGEMHE/h5HT_{1A} and pGEMHE/h5HT_{1A} T149A, respectively. Plasmids pSP/GIRK1 and pBScMXT/GIRK2 encoding the GIRK1 and GIRK2 potassium channel subunits were prepared as described (Ulens et al., 2000). Plasmids were linearised with *Nhe*I (pGEMHE/h5HT_{1A}, pGEMHE/h5HT_{1A} T149A), *Eco*RI (pSP/GIRK1) or *Sal*I (pBScMXT/GIRK2) and in vitro transcription of RNA was performed using the T7 (pGEMHE/h5HT_{1A}, pGEMHE/h5HT1A T149A), SP6 (pSP/GIRK1) or T3 (pBScMXT/GIRK2) mMessage mMachine transcription kit (Ambion, Austin, TX). RNA was purified using the RNeasy RNA cleanup kit (Qiagen, Courtaboeuf, France), quantified spectrometrically, diluted to the appropriate concentration in RNAse-free water and stored at −80 °C prior to use.

2.2. Xenopus laevis oocyte expression

Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the European Directive 86/609/EEC, and the protocols were carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research (protocol approved by the ethics committee under number 242). For isolation of oocytes, adult female *X. laevis* frogs were deeply anaesthetized by immersion in water containing 1 g/l Tricaine methanesulfonate (MS222) buffered with 0.7 g/l sodium bicarbonate. Fragments of ovarian lobes were surgically removed from an incision made in the abdomen. Oocytes were placed in Ca^{2+}-free ND96 solution (see below) and defolliculated by treatment with collagenase (2 mg/ml, Sigma type 1A) for 2 h. Defolliculated oocytes were placed in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH

7.5 with NaOH). Healthy-looking stage V/VI oocytes were selected and injected with a roughly 50 nl volume of RNA solution containing 1 ng of RNA encoding either the wt h5-HT_{1A} receptor or the T149A mutant h5-HT_{1A} receptor. For experiments based on GIRK current activation, oocytes were injected with a 50-nl volume of RNA solution containing the RNAs coding for the GIRK1 and GIRK2 channel subunits at a concentration of 10 pg RNA/oocyte each with or without addition of h5-HT_{1A} receptor RNA at 1 pg/oocyte (agonist experiments) or 10 pg/oocyte (PTX control experiments; no measurable 5-HT-induced currents were observed in oocytes injected with 10 pg h5-HT_{1A} receptor RNA alone, not shown). After injection, oocytes were kept at 17 °C in ND96 solution supplemented with 50 mg/l gentamicin.

2.3. Electrophysiological recordings

Whole-cell oocyte currents were recorded using the two-electrode voltage clamp technique and a Geneclamp 500 amplifier (Axon Instruments, Union City, CA). For recording, oocytes were placed in a recording chamber. If not stated otherwise, oocytes were continuously superfused (2.5–3.5 ml/min) with ND96 solution (see above for ionic composition), a standard oocyte recording solution (see e.g. Lee and Durieux, 1999) for all protocols applied. Microelectrodes filled with 3 M KCl had resistances of 0.3–0.8 MΩ. All experiments were performed at room temperature (20–23 °C). In standard experiments, oocytes were voltage-clamped at −90 mV in ND96 and ligand-induced currents were measured as deflexions in holding current. For quantification of I_{smooth}, current was measured in the steady-state phase after drug application, but before the appearance of $I_{Cl(Ca)}$, and baseline current level before drug application was subtracted. In experiments on ion channel blockers, I_{smooth} was activated by two consecutive activations by 5-HT at 1 μM, separated by a 5-min washout period. All putative blocking agents were applied during the second application of 5-HT and during a 1-min preincubation period. Steady-state current level in the presence of the blocking agents was defined as baseline for the second application of 5-HT (of particular importance in the case of effects of the agents on baseline currents; see Section 3). Calcium chelators were injected in this experimental series to avoid the induction of $I_{Cl(Ca)}$. Routinely, EGTA was used as calcium chelator, except in experiments involving the application of La^{3+} and Gd^{3+}. Here, large unspecific currents were observed in EGTA-loaded cells, probably due to direct interactions of the lanthanides with EGTA (see Caldwell et al., 1998). Therefore, BAPTA was injected in this experimental series to avoid these effects. For the examination of current/voltage behaviour of I_{smooth}, cells were clamped at −35 mV in ND96 and voltage ramps from −100 mV to +60 mV were continuously applied for 1 s every 4 s. The current activated by 5-HT was measured before the activation of $I_{Cl(Ca)}$ (easily detected as large current activation in the positive voltage range after several tens of seconds); for analysis of 5-HT induced current, last four ramps before the inset of $I_{Cl(Ca)}$ were averaged and currents before receptor activation were subtracted (average of four ramps before 5-HT application). GIRK currents were recorded following a well-established protocol for quantification of these currents in *Xenopus* oocytes, as described previously (see Ulens et al., 2000; compare also legend to Fig. 2). Briefly, basal as well as agonist-induced GIRK currents were measured in high-potassium solution (hK, containing 96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5 with KOH) at a holding potential of −70 mV. Superfusion of oocytes with hK enables K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials (see Ulens et al., 2000). 500 ms voltage pulses to +20 mV were applied every 20 s to verify the absence of $I_{Cl(Ca)}$. At the end of each experiment, $BaCl_2$ (1 mM, dissolved in hK) was applied to quantify the GIRK-independent currents in high-potassium solution, and agonist-evoked GIRK currents were quantified relative to basal GIRK currents according to the algorithm presented previously (Ulens et al., 2000). For evaluation of receptor-independent effects of agonists on GIRK currents, oocytes were injected with GIRK RNA alone, and agonists were applied as in experiments on receptor activation. GIRK current level after 30 s of agonist application was determined and differences to the usual GIRK current decline as measured in control oocytes of the same donor frog without drug application were evaluated. All drugs were tested in at least six independent experiments. For drugs exhibiting statistically significant differences to the control group, agonist efficacy values in the GIRK assay were corrected for the respective value. For quantification of agonist-evoked chloride currents ($I_{Cl(Ca)}$, see Section 3), a protocol similar to that described by Hartzell (1996) was performed. Oocytes were clamped at −35 mV in ND96 and repeated voltage-steps to +20 mV were applied for 1 s every 10 s. The application of this protocol offers two main advantages, especially for the detection of small currents: (a) $I_{Cl(Ca)}$ is amplified at depolarised potentials as a function of its current/voltage relationship (see e.g. Hartzell, 1996); (b) activation of $I_{Cl(Ca)}$ can be easily detected during a +20-mV voltage step because of its particular, slowly developing time course (Hartzell, 1996). To quantify $I_{Cl(Ca)}$, current was measured at the end of the +20 mV voltage step (averaged over the last 50 ms). To determine the efficacy of the agonists to activate $I_{Cl(Ca)}$, we chose the maximal $I_{Cl(Ca)}$ amplitude during a 4-min application of each drug at 10 μM (the respective

control current value of the last voltage step before the first appearance of $I_{Cl(Ca)}$ was subtracted). All experiments were performed with oocytes from at least two different donor frogs. Unless otherwise specified, only one drug application was performed for each single oocyte.

2.4. Data analysis

The pClamp 8 software (Axon Instruments) was used for data acquisition. Data are presented as means ± S.E.M. Statistical analysis of differences between groups was carried out using the Student's *t*-test, a probability of $p < 0.05$ was considered statistically significant. Isotherms were analysed by non-linear regression, using GraphPad Prism (GraphPad Software Inc., San Diego, CA) to yield EC_{50} values.

2.5. Drugs

Serotonin (5-HT) and (±)8-hydroxy-2-(di-*n*-propylamino)tetralin [(±)8-OH-DPAT] were obtained from Sigma-RBI (Saint Quentin Fallavier, France). (−)-4-(Dipropylamino)-1,3,4,5-tetrahydrobenz{c,d,}indole-6-carboxamide (LY228,729) was from Eli Lilly and Company (Indianapolis, IN, USA). 2-[5-[3-(4-Methylsulfonylamino) benzyl-1,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine (L694,247) was from Tocris (Bioblock, Illkirch, France). Flesinoxan, 3-chloro-4-fluorophenyl-(4-fluoro-4-{[(5-methyl-6-methylamino-pyridine-2-ylmethyl)-amino]-methyl}-piperidine-1-yl)-methanone fumaric acid salt (F13714) and *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide dihydrochloride (WAY100,635) were synthesised at the Centre de Recherche Pierre Fabre (Castres, France). Stock solutions of LY228,729 and L694,247 at 10^{-2} M were prepared in dimethyl sulfoxide, all other ligands were dissolved in water. Stock solutions of ligands were dissolved to the appropriate concentrations in recording buffer prior to use and applied by superfusion. The *Bordetella pertussis* toxin (PTX) A-protomer was purchased from Calbiochem (VWR, Fontenay Sous Bois, France). Tricaine was from Acros (Noisy Le Grand, France). Gentamicin, collagenase, amiloride, benzamil, $GdCl_3$, $LaCl_3$, niflumic acid (NFA), flufenamic acid (FFA), GDPβS, 1,2-bis(2-aminophenoxy)-ethane *N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Sigma–Aldrich (Saint Quentin Fallavier, France). Stock solution of the PTX A-protomer was 10 μg/100 μl in 50 mM TRIS, 500 μM ethylenediaminetetraacetic acid (EDTA), 0.2% CHAPS, pH 8.0 (as supplied by the furnisher). Prior to use, the PTX stock solution was mixed 1:1 with 100 mM dithiothreitol (DTT) and was incubated at 37 °C for 30 min. After incubation, the solution was further diluted 1:1 with 100 mM NAD^+. Oocytes were injected with 50 nl solution containing either 1.25 or 0.625 ng PTX A-protomer. A 200-mM stock solution of GDPβS was prepared in water and stored at −80 °C. Further dilution was done prior to injection. GDPβS was injected to yield a final intracellular concentration of 1 mM (assuming a roughly 1 μl intracellular oocyte volume). EGTA and BAPTA were injected in water to a final intracellular concentration of 200 μM.

3. Results

3.1. h5-HT_{1A} receptor-mediated currents in Xenopus oocytes

In oocytes injected with 1 ng of RNA coding for the human 5-HT_{1A} receptor, application of 5-HT at 1 μM or 10 μM typically induced a biphasic response (Fig. 1A). The first current component was a smooth inward current (I_{smooth}), starting within the first seconds after application of 5-HT. The amplitude of this current was usually between 20 nA and 150 nA at a holding potential of −90 mV, showing relatively constant values within the same batch of oocytes. After a delay of several tens of seconds, an oscillatory current response appeared superimposed upon the smooth current (Fig. 1A). Sharp single peaks followed by much smaller oscillations as well as sustained oscillations of similar size were observed. The oscillatory current response often lasted minutes after the cessation of 5-HT application or was followed by an elevated level of holding current. The peak size of the oscillatory current was between 50 nA and 2 μA, depending on the respective oocyte batch. The characteristics of the oscillatory current as well as typical inter-oocyte variations in response pattern and onset/offset kinetics (see e.g. Pin et al., 1992) suggest that 5-HT activated the well-described oocyte calcium-dependent chloride current ($I_{Cl(Ca)}$). This current is frequently used as indicator of GPCR activation in *Xenopus* oocytes (see e.g. Lee and Durieux, 1999), and 5-HT_{1A} receptor coupling to this current has been reported previously (Ni et al., 1997). In line with this, the oscillatory current component induced by 5-HT was abolished when oocytes were pre-injected with EGTA, which is known to suppress $I_{Cl(Ca)}$ by preventing its calcium-dependent activation, whereas I_{smooth} could still be evoked in the presence of EGTA (Fig. 1B). Suppressing $I_{Cl(Ca)}$ also revealed that I_{smooth} typically showed a constant steady-state level of activation in the presence of 5-HT, but that it deactivated soon after washout of the agonist. Further, both currents could be separated by their differential dependence on the holding potential. When the holding potential was changed to about −10 mV, I_{smooth} was reduced but its direction was still inward, while the

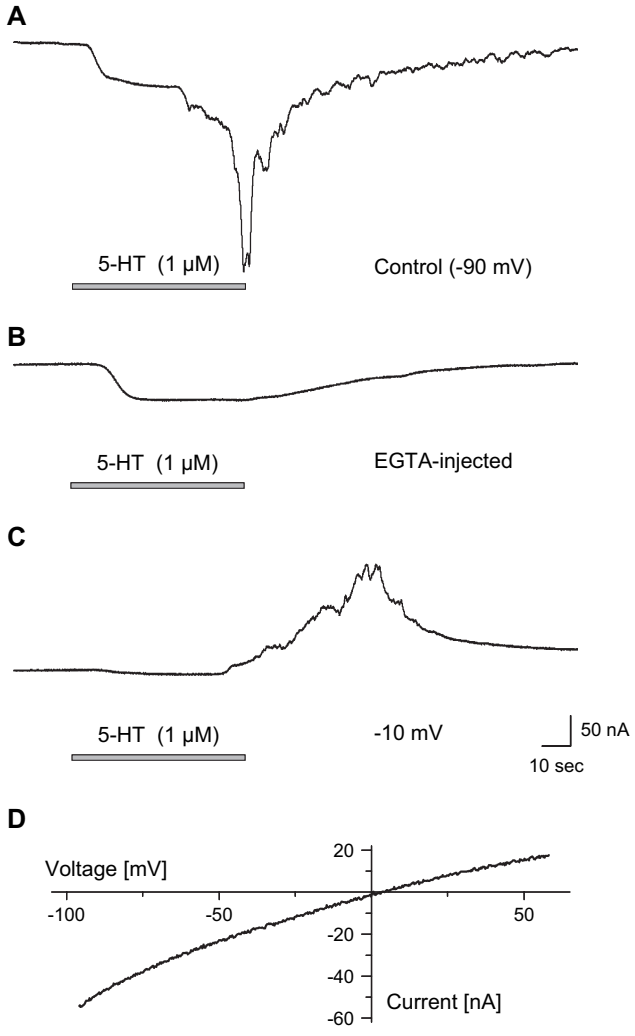

Fig. 1. Agonist-evoked currents in *Xenopus* oocytes expressing the human 5-HT_{1A} receptor. (A) Application of 5-HT (1 μM) typically evoked a biphasic current response in oocytes clamped at −90 mV in ND96 solution. (B) Injection of EGTA selectively abolished the oscillatory current. (C) In oocytes clamped at −10 mV, current direction was inversed for the oscillatory current but not for the smooth current. Oocytes were injected with 1 ng of h5-HT_{1A} receptor RNA. Calibration bars in (C) apply to current traces (A)–(C). All traces from different oocytes. Elevated levels in holding current in (A) and (C) after 5-HT washout are attributed to the typical slowly decreasing sustained component of $I_{Cl(Ca)}$ (compare e.g. Pin et al., 1992; Parekh et al., 1993). (D) Current–voltage relationship of I_{smooth} in ND96 solution. Repetitive voltage ramps were applied as outlined in Section 2. The current induced by 1 μM 5-HT before the activation of the oscillatory component is shown (basal current response subtracted, see Section 2).

direction of the oscillatory current typically was reversed to outward (Fig. 1C). The reversal potential of the oscillatory current was determined as about −15 mV (results not shown), consistent with previous results for chloride current reversal potential of *Xenopus* oocytes in ND96 (Centinaio et al., 1997; Fairman et al., 1998). Determination of I_{smooth} reversal potential was difficult to obtain due to prominent current drifts in oocytes constantly clamped to holding potentials more positive than −10 mV (no similar current shifts were observed in experiments conducted at negative holding potentials described below). In order to obtain an estimation of the reversal potential of this current, we applied voltage ramps from a holding potential of −35 mV. The smooth current activated by 5-HT (measured before the onset of $I_{Cl(Ca)}$, current in the absence of 5-HT subtracted) reversed at 4.9 ± 1.4 mV ($n = 28$). A slight inward rectification was observed (see example in Fig. 1D). The reversal potential of around 5 mV argues for a current response not strictly selective for a single ion species (Dascal, 1987).

Given the abundance of non-selective cation channels (NSCCs) in *Xenopus* oocytes (see Weber, 1999), we assumed that those channels might underlie I_{smooth}. To test for this possibility, we applied several typical blockers as have been used in other studies on NSCCs in *Xenopus* oocytes (compare e.g. Bielfeld-Ackermann et al., 1998; Weber, 1999). 5-HT-induced currents in the absence and the presence of agents were measured on the same oocyte, and the current amplitude in the presence of the agents was expressed as percentage of the current induced by 5-HT alone. To avoid the activation of $I_{Cl(Ca)}$, these experiments were performed on oocytes loaded with calcium chelators (EGTA or BAPTA, see Section 2). The results obtained with the different ion channel blockers are summarized in Table 1.

While two consecutive applications of 5-HT induced comparable currents in control experiments without ion channel blocker, most of the agents tested turned out to effectively block the smooth current evoked by activating the 5-HT_{1A} receptor. The current decrease induced by the anion channel blocker NFA was small and did not reach statistical significance, consistent with its relative low efficacy in blocking NSCCs (e.g. Chen et al., 1993). Thus, these results support the hypothesis of an NSCC carrying the smooth current response.

Table 1
Effects of ion channel blockers on I_{smooth}

Agent	Relative size of second response (%)	n	Significance
Control	99.2 ± 5.8	6	n. a.
FFA (100 μM)	53.7 ± 1.4	6	$p < 0.001$
NFA (100 μM)	86.9 ± 1.6	6	n.s.; ($p = 0.11$)
Gd^{3+} (100 μM)	59.2 ± 4.7	5	$p < 0.01$
La^{3+} (100 μM)	74.3 ± 2.0	5	$p < 0.01$
Amiloride (100 μM)	25.9 ± 2.7	6	$p < 0.001$
Benzamil (10 μM)	49.4 ± 5.5	8	$p < 0.001$

Current was evoked by two consecutive applications of 5-HT (1 μM) in oocytes clamped at −90 mV in ND96 solution. Current amplitude of the second response during application of the respective ion channel blocker is expressed as percentage of the first response (mean ± SEM). Statistical significance of effects with respect to the control group is indicated. Experiments were conducted on oocytes loaded with EGTA or BAPTA (Gd^{3+}, La^{3+}). See Section 2 for more detail.

Surprisingly, amiloride and its derivative benzamil induced increases in holding current at −90 mV when applied to oocytes containing the 5-HT_{1A} receptor (not shown). The analysis of this phenomenon will be subject of a separated study.

3.2. Current activation by the T149A receptor mutant

To further characterize h5-HT_{1A} receptor coupling, we expressed the T149A 5-HT_{1A} receptor mutant in oocytes. This mutant has been reported to lack activation of some, but not all, biochemical pathways coupled to the wild type 5-HT_{1A} receptor (Lembo et al., 1997). 5-HT induced no detectable $I_{Cl(Ca)}$ in oocytes expressing the T149A 5-HT_{1A} receptor mutant ($n = 27$) whereas I_{smooth} was consistently activated with higher amplitude as compared with oocytes expressing wild type h5-HT_{1A} receptors (on average 1.6 ± 0.1 fold of wild type effect in the same oocyte batch, $n = 27$; not shown).

3.3. Pharmacological activation profile of co-expressed GIRK channels

We next investigated the activation of the above described ion currents by other 5-HT_{1A} receptor agonists than 5-HT to possibly identify differences in their pharmacological activation profiles. First, the ability of each ligand to activate the h5-HT_{1A} receptor expressed in *Xenopus* oocytes was verified by stimulation of potassium channels of the GIRK type (Dascal, 1997). In these cells, 5-HT evoked a receptor-dependent increase in I_{GIRK} when applied during superfusion of oocytes with a high-potassium (hK) extracellular solution, and this receptor-dependent current was not observed in the presence of the selective 5-HT_{1A} receptor antagonist, WAY100,635 (not shown). Experiments revealed that all agonists tested (each at 10 μM) induced receptor-dependent increases in I_{GIRK}, with (±)8-OH-DPAT and flesinoxan behaving as partial agonists (Figs. 2 and 3 and Table 2). However, (±)8-OH-DPAT and F13714 modulated I_{GIRK} in oocytes only injected with GIRK channel RNA by decreasing the level of hK-induced basal I_{GIRK} by 12.9% and 13.0%, respectively. Therefore, quantitative estimations of efficacy of both ligands have been corrected by their direct effect on I_{GIRK} (correction already included in values given in Table 2 and Fig. 3A).

3.4. Pharmacological activation profile of $I_{Cl(Ca)}$

When activation of $I_{Cl(Ca)}$ was quantified using a current step protocol adopted from Hartzell (1996), the concentration–response curve of 5-HT ($pEC_{50} = 7.0$, Fig. 4) was in good agreement with that reported by Ni et al. (1997), as well as with results on 5-HT_{1A} receptor-mediated calcium liberation in other cell types (Boddeke et al., 1992; Pauwels and Colpaert, 2003). Further, current induction was prevented by application of the 5-HT_{1A} receptor antagonist, WAY100,635. L694,247 was of similar efficacy as 5-HT (Table 2 and Fig. 3B), whereas F13714 and LY228,729 behaved as partial agonists. In contrast, flesinoxan and (±)8-OH-DPAT only rarely activated $I_{Cl(Ca)}$ (detectable $I_{Cl(Ca)}$ in 4/10 and 1/10 oocytes, respectively; each of these currents smaller than 10% of the average current evoked by 5-HT; Table 2, Figs. 2 and 3B).

3.5. Pharmacological activation profile of I_{smooth}

When 5-HT_{1A} receptor agonists were evaluated on I_{smooth} activation, the current induced by L694,247 was much stronger than that of 5-HT (Table 2 and Fig. 3C). LY228,729 was as effective as 5-HT. I_{smooth} evoked by (±)8-OH-DPAT was quite small, but current activation was consistently observed in all oocytes tested ($n = 8$). All these stimulations were blocked by the selective 5-HT_{1A} receptor antagonist WAY100,635 (Fig. 5).

Further, WAY100,635 itself induced a certain decrease of holding current (−29.6 ± 1.9% of I_{smooth} induced by 5-HT, $n = 23$). A similar effect was observed for flesinoxan and F13714 (Table 2 and Figs. 2, 6 and 7). However, reduction of current levels induced by WAY100,635 and F13714 were completely surmountable by increasing concentration of 5-HT (Fig. 6), suggesting that these ligands acted via h5-HT_{1A} receptors. These results further revealed that WAY100,635 and F13714 behaved as competitive antagonists at h5-HT_{1A} receptor-mediated I_{smooth} activation (Fig. 6).

Concentration–response curves for modulation of I_{smooth} showed that 5-HT ($pEC_{50} = 6.6$) and L694,247 ($pEC_{50} = 6.8$) exhibited similar potency while LY228,729 ($pEC_{50} = 7.3$) was more potent (Fig. 7), in agreement with results on h5-HT_{1A} receptor-mediated [^{35}S]-GTPγS binding (Pauwels et al., 1997, and unpublished results). Interestingly, 5-HT seemed to be more potent in experiments on competitive receptor inhibition (Fig. 6), possibly due to the presence of intracellular EGTA in the respective experimental series.

3.6. Influence of pertussis toxin (PTX) and GDPβS on h5-HT_{1A} receptor-mediated currents

5-HT_{1A} receptors preferentially couple to GTP binding proteins of the $G_{i/o}$ family that are sensitive to treatment by PTX. The non-hydrolysable analogue of GDP, GDPβS, has a broader action by blocking the exchange of GDP by GTP on all G proteins. First, we examined the effects of PTX injection on the occurrence of the different currents (injection of 0.6–1.2 ng of PTX per oocyte, 5–24 h before recording; no differences as a function of variable incubation times). Vehicle-injected

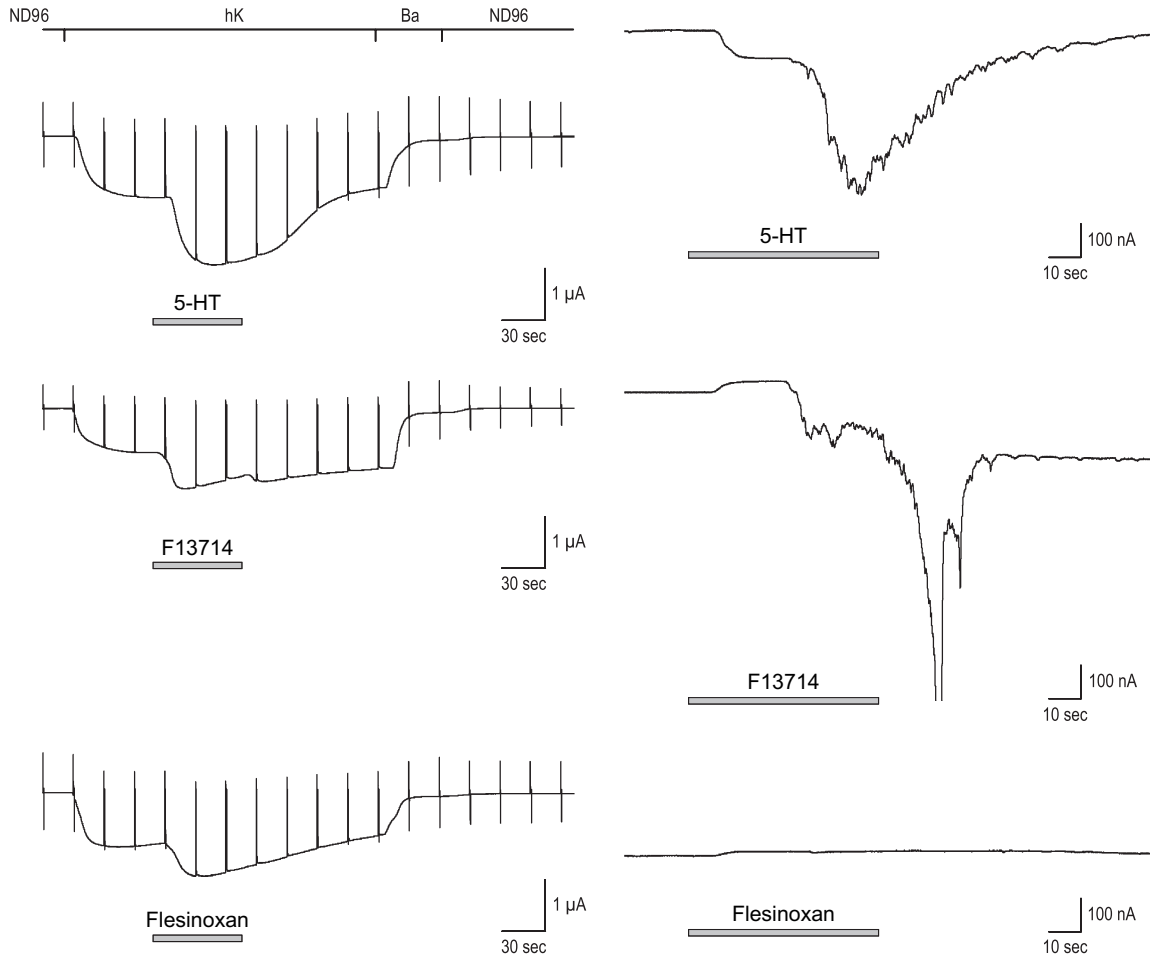

Fig. 2. Ion current activations by various 5-HT_{1A} receptor ligands. Left side: activation of I_{GIRK}. Oocytes coinjected with h5-HT_{1A} receptor RNA (1 pg/oocyte) and GIRK1/2 channel subunit RNAs (10 pg of each subunit/oocyte) were clamped at −70 mV, and repeated control pulses to +20 mV were applied. Changes in recording buffer (top) and application of agonists (bars) are indicated. At the beginning and at the end of each experiment, oocytes were kept in ND96 solution, containing 96 mM Na^+ and 2 mM K^+ (see Section 2). Basal I_{GIRK} was activated by high-potassium (hK) solution, containing 96 mM K^+. Ligands (5-HT, F13714 and flesinoxan, respectively, each at 10 µM, dissolved in hK) were applied during 1 min after 1 min in hK, as indicated by the bars. At the end of each experiment, $BaCl_2$ (1 mM in hK) was applied to quantify GIRK-independent effects of hK (Ba). Ligand-evoked GIRK currents were observed with all agonists tested. Please note the current increase after washout of F13714, indicating the receptor-independent current blockade induced by this compound. Right side: differential activation of endogenous oocyte ion currents $I_{Cl(Ca)}$ and I_{smooth}. Oocytes injected with h5-HT_{1A} receptor RNA (1 ng/oocyte) were clamped at −90 mV in ND96 solution and ligands were applied for 1 min as indicated by the bars (10 µM each). While 5-HT evoked a two-component current response consisting of I_{smooth} and $I_{Cl(Ca)}$, F13714 only activated $I_{Cl(Ca)}$, and flesinoxan failed to induce both current responses. Elevated levels in holding current after washout of agonists in upper traces are attributed to the typical slowly decreasing sustained component of $I_{Cl(Ca)}$ (compare e.g. Pin et al., 1992; Parekh et al., 1993).

cells were used as a control. PTX treatment reduced agonist-induced I_{GIRK} by more than 90% (not shown), whereas I_{smooth} was not affected (Fig. 8A). Mean amplitude of I_{smooth} in PTX-injected cells ($n = 9$) was 92.9 ± 4.2% versus control (100%, non-significant differences). The PTX-dependence of $I_{Cl(Ca)}$ could not be examined because of the presence of EDTA in the buffer of PTX solution that blocked $I_{Cl(Ca)}$ (see Fig. 1B and Fig. 8A, upper panel). Second, we examined the effect of GDPβS injection (final intracellular concentration of GDPβS was estimated at 1 mM). GDPβS injection did not lead to a decrease of I_{smooth}. Amplitude of this current induced by 5-HT in oocytes injected with GDPβS was 116.3 ± 9.3% when compared to current induced in non-injected oocytes ($n = 13$, non-significant differences). $I_{Cl(Ca)}$, on the other hand, was heavily affected by GDPβS injection (Fig. 8B). While $I_{Cl(Ca)}$ was consistently observed in all control oocytes ($n = 11$), only 2 of 13 GDPβS-injected oocytes showed this current. Taken together, these results showed that I_{GIRK} and $I_{Cl(Ca)}$ were induced via G protein-dependent pathways and suggested that I_{smooth} was not mediated by a G protein-dependent mechanism.

4. Discussion

The main findings of the study are: (1) the pharmacological characterisation of 5-HT_{1A} receptor

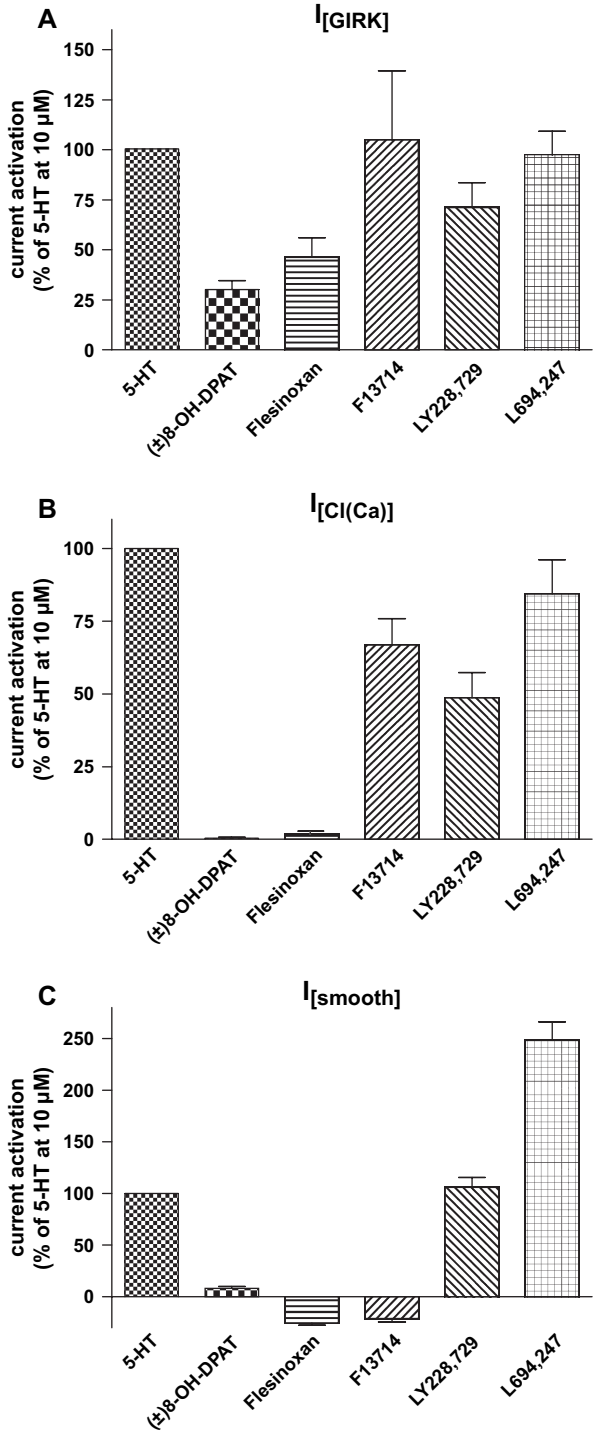

Fig. 3. Pharmacological activation profiles of h5-HT_{1A} receptor-mediated current responses. (A) I_{GIRK}. (B) $I_{Cl(Ca)}$. (C) I_{smooth}. See legends to Figs. 2 and 4 for experimental design. Currents were quantified as outlined in Section 2. All agonists were applied at 10 µM, dissolved in the respective recording buffer. All data normalized to the mean response induced by 10 µM 5-HT in independent oocytes from the same batch. Group size represented by each bar is 9–10 independent experiments in (A), 8–20 in (B), and 8–9 in C (see Table 2). Values represented in these histograms are given in Table 2. Efficacy values for (±)8-OH-DPAT and F13714 were corrected for receptor-independent effects in the GIRK assay (see text for details).

coupling to three different current responses in *Xenopus* oocytes, (2) the demonstration that activation of these currents by a series of 5-HT_{1A} ligands yields dramatically distinct activation profiles, and (3) the PTX and GDPβS insensitivity of 5-HT_{1A} receptor coupling to I_{smooth} suggests a G protein-independent phenomenon.

4.1. I_{GIRK}

The most "classical" assay of the experiments performed in our study is the evaluation of I_{GIRK} (Dascal, 1997). GIRK-mediated currents are a major target of 5-HT_{1A} receptors in neurons (Barnes and Sharp, 1999). In this case, the results of the ligand efficacies were in accordance with expectations based on other methods. It seems worth to mention in this context that the outcome of an assay based on liberation of G protein βγ subunits does not result in a significantly differential activation profile when compared to studies based on [^{35}S]-GTPγS binding (Pauwels et al., 1997; Pauwels and Colpaert, 2003) or on Gα activation (e.g. Pauwels et al., 1993), at least not for the compounds tested here. An unexpected finding of our experiments was the receptor-independent attenuation of I_{GIRK} by the 5-HT_{1A} receptor agonists (±)8-OH-DPAT and F13714. Various substances of different classes have been found to inhibit GIRK channels (see Kobayashi et al., 2004, for review), but this has not been reported for 5-HT_{1A} receptor agonists. Thus, (±)8-OH-DPAT and F13714 behave like certain opioid receptor agonists that activate I_{GIRK} via opioid receptors but inhibit the same currents in a receptor-independent manner (Ulens et al., 1999).

4.2. $I_{Cl(Ca)}$

In contrast to I_{GIRK} activation, coupling to endogenous oocyte ion currents was more diverse. In the case of the calcium-dependent chloride current, $I_{Cl(Ca)}$, the activation pattern essentially conserved the efficacy rank order observed with I_{GIRK} activation, but it showed a marked preference for efficacious agonists. This observation is in accordance with a previous study (Ni et al., 1997), reporting only weak (±)8-OH-DPAT-induced $I_{Cl(Ca)}$ activation via 5-HT_{1A} receptors expressed in *Xenopus* oocytes.

The efficacy values observed for the different agonists essentially reproduce our findings when exploring h5-HT_{1A} receptor-mediated calcium liberation in recombinant CHO-K1 cells (Pauwels and Colpaert, 2003). In particular, flesinoxan and (±)8-OH-DPAT induce weak responses in both assays, resulting in much lower efficacy values than obtained in [^{35}S]-GTPγS binding assays (Pauwels and Colpaert, 2003) or adenylyl cyclase experiments (Fowler et al., 1992; Pauwels et al., 1993).

Table 2
Relative efficacy values of 5-HT_{1A} receptor ligands at different current responses

Ligand	I_{GIRK}	$I_{Cl(Ca)}$	I_{smooth}
5-HT	100	100	100
(±)8-OH-DPAT	29.8 ± 4.4 ($n = 9$)[a]	0.4 ± 0.4 ($n = 10$)	8.2 ± 1.8 ($n = 8$)
Flesinoxan	46.4 ± 9.7 ($n = 10$)	1.9 ± 1.2 ($n = 10$)	−25.2 ± 2.3 ($n = 8$)
F13714	104.9 ± 34.6 ($n = 9$)[a]	66.8 ± 9.1 ($n = 17$)	−21.2 ± 3.2 ($n = 8$)
LY228,729	71.1 ± 12.5 ($n = 9$)	48.6 ± 8.7 ($n = 16$)	106.0 ± 9.6 ($n = 9$)
L694,247	97.2 ± 12.1 ($n = 9$)	84.4 ± 11.7 ($n = 17$)	248.1 ± 18.2 ($n = 8$)

Pharmacological activation profile of three different ion currents induced by h5-HT_{1A} receptor activation in *Xenopus* oocytes. Currents were quantified as outlined in Section 2. All agonists were applied at 10 μM, dissolved in the respective recording buffer. All values are in percentage, mean ± SEM, normalized to the mean response induced by 10 μM of 5-HT (100%) in independent oocytes from the same batch. See legends of Figs. 2–4 for further details.

[a] Values corrected for receptor-independent effects (see text for details).

The mechanism responsible for this inability of low efficacy agonists to stimulate $I_{Cl(Ca)}$ remains speculative. Differences in agonist efficacies and/or potencies at the 5-HT_{1A} receptor have been reported repeatedly when activation of the PLC pathway was compared to results obtained in other assays (see e.g. Hoyer and Boddeke, 1993). It has been argued that 5-HT_{1A} receptor coupling to the PLC pathway is relatively weak and that a lack of receptor reserve at this pathway might underlie the observed differences (Hoyer and Boddeke, 1993). However, potential activation of distinct receptor conformations by the respective agonists should also be considered (Hoyer and Boddeke, 1993; Pauwels and Colpaert, 2003).

4.3. I_{smooth}

The third current response observed with h5-HT_{1A} receptor activation in *Xenopus* oocytes was a smooth inward current (I_{smooth}) preceding $I_{Cl(Ca)}$. Both responses could be clearly distinguished from each other by functional and pharmacological examination. In addition, the T149A point-mutated h5-HT_{1A} receptor did not activate $I_{Cl(Ca)}$, but efficiently mediated I_{smooth}.

Of particular interest was the pharmacological activation profile of I_{smooth}, which differed strongly from both other h5-HT_{1A} receptor-mediated cellular responses. Most notably was the behaviour of F13714 when compared to 5-HT. This drug did not induce I_{smooth}, while it behaved as highly efficacious agonist in previous studies (Pauwels and Colpaert, 2003) and with respect to activation of I_{GIRK} and $I_{Cl(Ca)}$ in experiments presented here. Importantly, F13714 behaved as a competitive antagonist for I_{smooth}, supporting its highly distinct behaviour for different h5-HT_{1A} receptor-mediated effects. L694,247, in contrast, displayed higher relative efficacy than 5-HT at the I_{smooth} response.

The unusual pharmacological profile as well as its insensitivity to PTX and GDPβS (see below) raises the possibility that the activation of I_{smooth} was in fact not

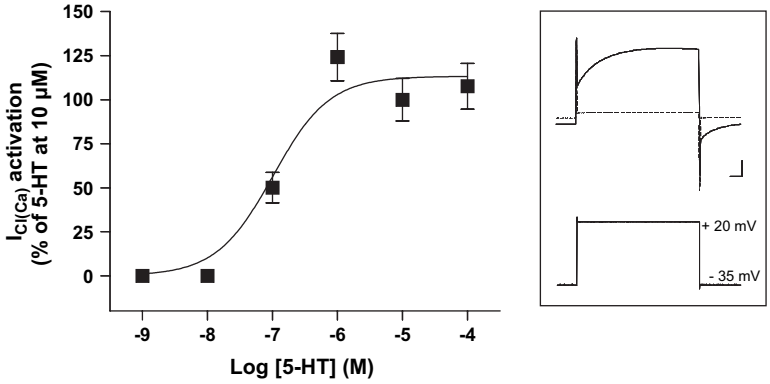

Fig. 4. h5-HT_{1A} receptor-mediated activation of $I_{Cl(Ca)}$. Concentration–response curve with 5-HT as agonist. Experiments performed on oocytes injected with 1 ng h5-HT_{1A} receptor RNA. Currents were quantified according to a protocol adapted from Hartzell (1996) as described in Section 2. Value of maximal $I_{Cl(Ca)}$ during a 4-min ligand application was taken as measure for ligand efficacy. Each data point represents mean values from 6 to 8 independent oocytes. Data normalized to the mean response induced by 10 μM 5-HT recorded in oocytes of the same experimental series. Representative examples of current traces immediately before onset of $I_{Cl(Ca)}$ (dashed line) and at maximal current activation (solid line) are given in the inset (upper panel, both traces in the continuing presence of 5-HT). The voltage protocol (1 s voltage step to +20 mV from a holding potential of −35 mV) is represented below (lower panel). Please note the slight shift in holding current at −35 mV before the voltage step (representing $I_{Cl(Ca)}$ at this potential) and the strong slowly activating $I_{Cl(Ca)}$ during the +20 mV voltage step. Time and current calibration bars represent 100 ms and 250 nA, respectively.

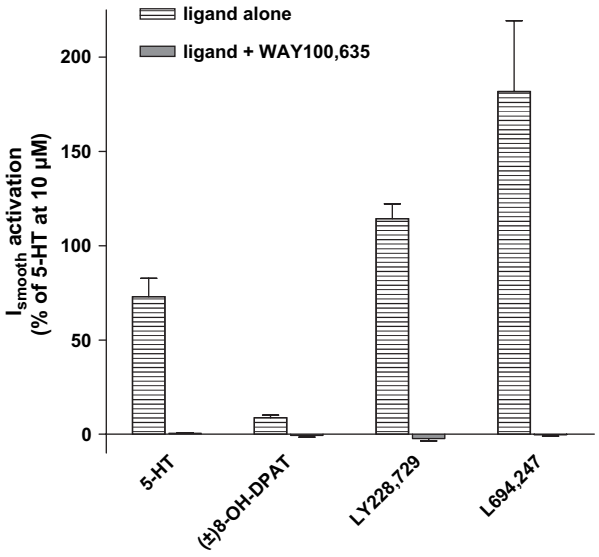

Fig. 5. Antagonism by WAY100,635 of ligand-induced I_{smooth} activation. Ligands were applied at a concentration of 1 µM in the absence or presence of WAY100,635 (10 µM) at separate oocytes and I_{smooth} amplitude was determined. In experiments involving WAY100,635, oocytes were pre-exposed to WAY100,635 for 60 s before agonist application, and the current level in the presence of WAY100,635 was defined as new baseline level. Each column represents mean responses obtained from 3 to 5 independent oocytes. Data were normalized to the mean response induced by 10 µM 5-HT in separate oocytes from the same batch.

mediated by the 5-HT_{1A} receptor. This assumption seems unlikely for three main reasons. First, there was no activation of currents by 5-HT_{1A} receptor ligands in oocytes not injected with h5-HT_{1A} receptor RNA. Second, responses to all agonists were blocked by the very selective 5-HT_{1A} receptor antagonist WAY100,635. This antagonist is routinely used for the identification of 5-HT_{1A} receptor-mediated responses and is the most selective 5-HT_{1A} receptor antagonist available to date (see Hoyer et al., 2002; Newman-Tancredi et al., 2001). Third, the selective 5-HT_{1A} receptor ligand, F13714, (Koek et al., 2001), induced a rightward shift in the dose/response curve of 5-HT, similar to that observed with WAY100,635. Thus, even if F13714 does not activate I_{smooth}, this selective ligand binds to the receptor and acts as competitive antagonist, further supporting that activation of 5-HT_{1A} receptors underlies the effects observed here.

A specific issue in the context of the activation profile of I_{smooth} is the decrease in holding current induced by WAY100,635, flesinoxan and F13714. It is not clear from the present data if this represents a 5-HT_{1A} receptor-mediated current response different from I_{smooth} (presumably masked by I_{smooth} when other agonists are applied) or if these ligands behave as inverse agonists for I_{smooth} induction. Indeed, inverse agonist properties of the antagonist WAY100,635 have been previously reported (Cosi and Koek, 2000), and might be related to the effects observed here.

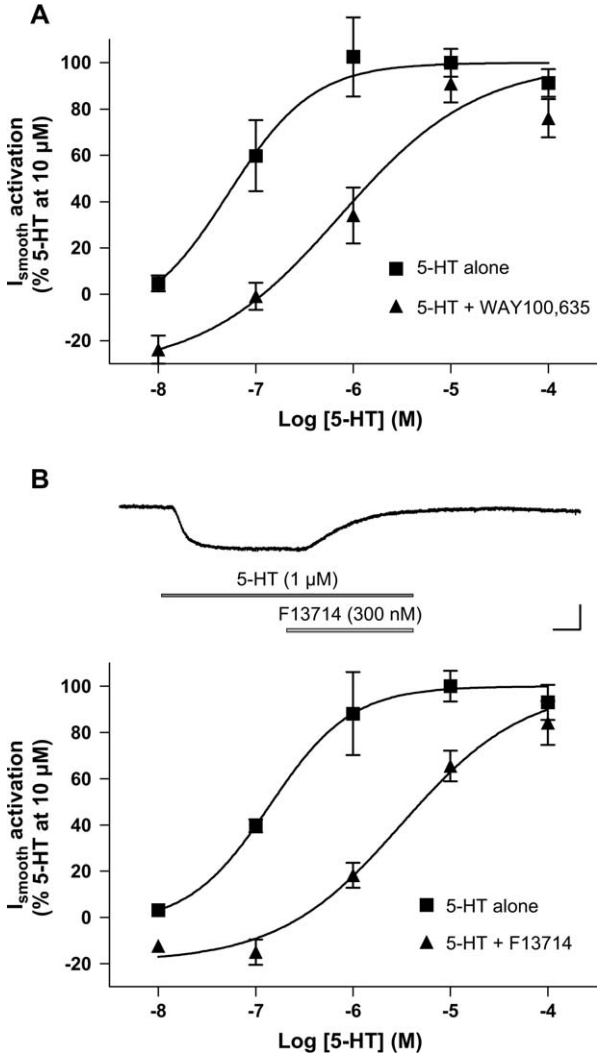

Fig. 6. Competitive antagonism of WAY100,635 (A) and F13714 (B) at I_{smooth} induction. Both compounds induce a rightward shift in the concentration–response curve of 5-HT. Oocytes were clamped at −90 mV in ND96, and I_{smooth} was first activated by 5-HT for 90 s followed by application of the antagonistic ligand for another 90 s at a fixed concentration (300 nM) in the continuing presence of 5-HT (see inset for an example). Amplitude of I_{smooth} was measured before and during the application of the antagonistic ligand in the same oocyte and concentration–response curves for both conditions were depicted. Please note the values below 0 at low concentrations of 5-HT, consistent with the effects of WAY100,635 and F13714 alone. All experiments were performed in EGTA-injected cells to avoid induction of $I_{Cl(Ca)}$. Data points represent mean values from 4 to 6 oocytes. Data normalized to the mean response induced by 10 µM 5-HT in the respective series of experiments. Inset: example of an experiment performed to determine the amount of I_{smooth} inhibition. Time and current calibration bars represent 20 s and 25 nA, respectively.

Although a detailed characterisation of I_{smooth} is beyond of the scope of the present study, its current-voltage behaviour and sensitivity to ion channel blockers indicate that a non-selective cation channel might underlie this current. This assumption seems likely in the light of the abundance of this ion channel type in *Xenopus* oocytes (Weber, 1999). Interestingly, a current

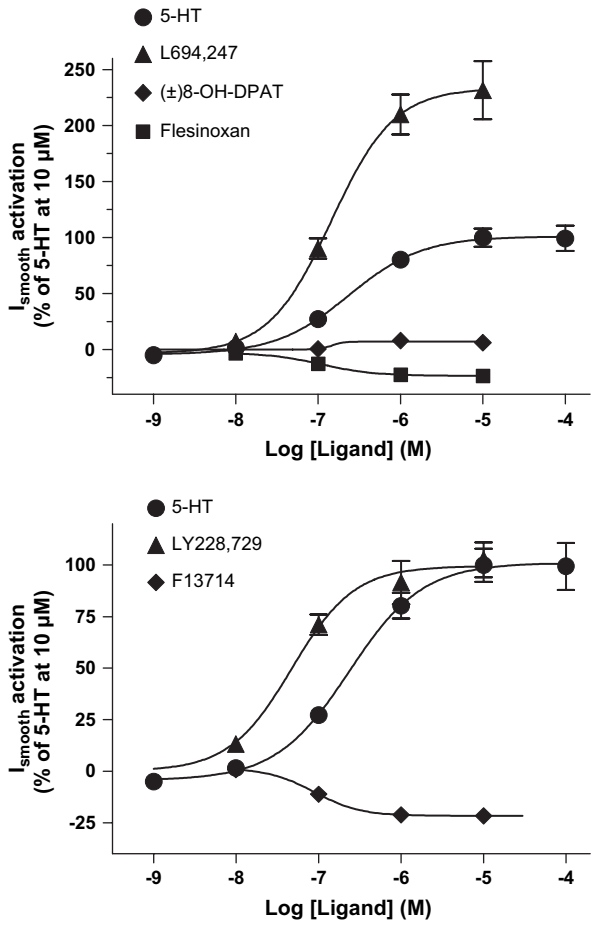

Fig. 7. Concentration–response curves for I_{smooth} activation. I_{smooth} was determined as described in Section 2. Each data point represents mean values from 4 to 6 independent oocytes. Data normalized to the mean response induced by 10 µM 5-HT in oocytes from the same batch.

activation pattern similar to that found here (smooth inward current and $I_{Cl(Ca)}$) was reported for activation of the m2 muscarinic acetylcholine receptor (Fukuda et al., 1987). Here, the smooth current was cation selective. However, further work will be necessary to characterise the nature of I_{smooth} activated by 5-HT_{1A} receptors in more detail.

4.4. Selective activation of h5-HT_{1A} receptor signalling

The activation profile of I_{smooth} differs significantly from those of the other currents activated via the same receptor. This cannot consistently be explained by a "strength-of-signal"-based mechanism, since we observed a different rank order in relative ligand efficacies. Therefore, our data strongly suggest that different active receptor states underlie the activation of diverse signalling pathways. Indications for pathway-selective agonism at the 5-HT_{1A} receptor have been presented previously (e.g. Gettys et al., 1994; Malmberg and Strange, 2000; Newman-Tancredi et al., 2002; Pauwels and Colpaert, 2003; reviewed in Newman-Tancredi, 2003). However, although Gettys et al. (1994) show a partially reversed efficacy rank order of 5-HT_{1A} receptor ligands for activation of different G proteins, highly distinct pharmacological profiles as reported herein have not yet been reported.

The notion that different h5-HT_{1A} receptor states mediate the activation of different current responses is further supported by the fact that the T149A point mutation abolished $I_{Cl(Ca)}$ activation, but left the induction of I_{smooth} intact or even enhanced it (possibly due to a lower rate of PKC-induced desensitisation, compare Wu et al., 2002). This affirms that different mechanisms of activation are possible at the level of the receptor itself. Our results are in line with reports showing that the T149A mutant is uncoupled from selected biochemical pathways, including the PLC pathway in different cell types (Lembo et al., 1997; Wurch et al., 2003).

4.5. Coupling of h5-HT_{1A} receptor in Xenopus oocytes

Another particular feature of I_{smooth} activation besides its unusual pharmacology is its obvious insensitivity to GDPβS and PTX. Injection of both agents did not affect this pathway, whereas they efficiently inhibited coupling to $I_{Cl(Ca)}$ and I_{GIRK}. Some caution should be retained, since difficulties with PTX applications in *Xenopus* oocytes have been reported (Blitzer et al., 1993), and coupling of the 5-HT_{1A} receptor to PTX-insensitive G proteins seems to be possible under certain circumstances (Malmberg and Strange, 2000; Wurch and Pauwels, 2003). In addition, it cannot be excluded that GDPβS acts differently on different G proteins depending on amount or type of the G protein involved. Indeed, higher concentrations of GDPβS than reported here could not be tested due to deleterious effects on oocytes (unpublished observation). However, the results with GDPβS and PTX at least argue in favour of the possibility that the h5-HT_{1A} receptor mediates I_{smooth} activation via a G protein-independent mechanism.

There are a growing number of reports indicating G protein-independent signalling by GPCRs, including regulation of ion channels (reviewed in Heuss and Gerber, 2000). Most notably, the 5-HT_{1A} receptor has been shown to inhibit T-type Ca^{2+}-channels in a fashion not affected by PTX or analogues of guanine nucleotides, including GDPβS (Sun and Dale, 1999). Assumptions on the possible mechanisms of G protein-independent signalling by 5-HT_{1A} receptors remain speculative; however, likely mechanisms of receptor-mediated ion channel regulation include G protein-independent signalling cascades (as shown for metabotropic glutamate

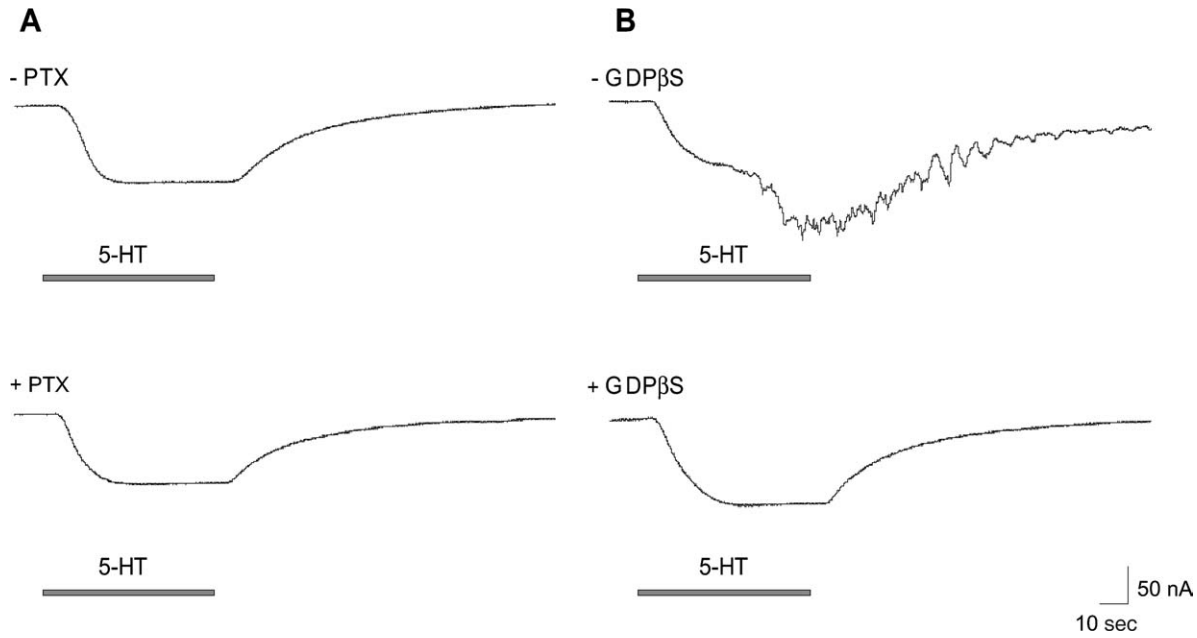

Fig 8. Effects of PTX and GDPβS on h5-HT_{1A} receptor-mediated currents. Control recordings are shown in the upper trace. Recordings from oocytes injected with PTX (A) or GDPβS (B), as described in Section 2, are shown in the lower trace. 5-HT (1 μM) was applied to oocytes from the same batch and currents were recorded at a holding potential of −90 mV in ND96 solution. Note the absence of $I_{Cl(Ca)}$ in oocytes injected with PTX and vehicle (A, see text for details) as well as in oocytes injected with GDPβS (B).

receptors, see Heuss et al., 1999; Benquet et al., 2002) as well as direct protein-protein interactions (shown for dopaminergic receptors, see Liu et al., 2000; Lee et al., 2002). Notwithstanding the lack of mechanistic insight, the assumption of G protein-independent signalling is attractive in the present context, as it seems rather plausible that the unusual pharmacological profile of 5-HT_{1A} receptor activation observed for I_{smooth} would correspond to an unusual mode of signal transduction. In line with this, two recent studies suggest agonist-selective activation of a G protein-independent pathway mediated *via* the β_2 adrenergic and the V2 vasopressin receptor activation (Azzi et al., 2003; Baker et al., 2003).

5. Conclusions

In summary, our study underlines the diversity of h5-HT_{1A} receptor signalling by examination of coupling to three distinct ion currents in *Xenopus* oocytes. h5-HT_{1A} receptor-mediated induction of $I_{Cl(Ca)}$ was selectively obtained with efficacious agonists, whereas an unprecedented ligand activation pattern was obtained for activation of a smooth inward current. Moreover, this latter current was not affected by agents that uncouple G protein-dependent signalling or by a point mutation in the receptor protein that abolished $I_{Cl(Ca)}$ induction. Together, these results suggest the existence of multiple functionally distinct active states of the human 5-HT_{1A} receptor, revealing agonist-directed signalling of this receptor in *Xenopus* oocytes.

Acknowledgments

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