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Original article

Differential functional effects of two 5-HT$_4$ receptor isoforms in adult cardiomyocytes

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Abstract

Serotonin 5-HT$_4$ receptors are present in human atrial myocytes and have been proposed to contribute to the generation of atrial fibrillation. However, 5-HT$_4$ receptors have so far been only found in human and pig atria and are absent from the heart of small laboratory animals, such as rat, guinea pig, rabbit and frog, which limits the experimental settings for studying their functional properties. In this study, we developed an adenovirus expression system to examine the properties of two human 5-HT$_4$ receptor splice variants, h5-HT$_{4(b)}$ and h5-HT$_{4(d)}$, expressed in adult cardiomyocytes devoid of native 5-HT$_4$ receptors. When expressed in the HL-1 murine cell line of atrial origin, both receptors caused specific binding of the 5-HT$_4$ selective antagonist GR113808 and activated adenylyl cyclase in the presence of serotonin (5-HT, 1 μM). When expressed in freshly isolated adult rat ventricular cardiomyocytes, a stimulation of the L-type Ca^{2+} current ($I_{Ca,L}$) by 5-HT (100 nM) was revealed. Both effects were blocked by GR113808. In HL-1 cells, the h5-HT$_{4(d)}$ receptor was found to be more efficiently coupled to adenylyl cyclase than the h5-HT$_{4(b)}$. Pertussis toxin treatment (250 ng/ml for 5 h) potentiated the stimulatory effect of 5-HT on $I_{Ca,L}$ in rat myocytes expressing the h5-HT$_{4(b)}$ but not the h5-HT$_{4(d)}$ receptor, indicating a likely coupling of the (b) isoform to both Gs and Gi/o proteins. Adenoviral expression of h5-HT$_4$ receptor isoforms in adult cardiac myocytes provides a valuable means for the exploration of the receptor signaling cascades in normal and pathological situations.

Keywords: Serotonin; 5-HT$_4$ receptor; Adenovirus; L-type Ca^{2+} current; Cyclic AMP; Rat cardiomyocytes; HL-1 cell line

1. Introduction

The serotonin 5-HT$_4$ receptor, a member of the G protein-coupled receptor family, activates adenylyl cyclase and mediates many cellular functions, both in the central nervous system and at the periphery [1]. In the heart, 5-HT$_4$ receptors exert strong positive chronotropic, inotropic and lusitropic effects in human and pig atria. These effects are associated with an increase in intracellular cAMP, leading to activation of the hyperpolarization-activated pacemaker current I_f [2] and the L-type Ca^{2+} current ($I_{Ca,L}$) in human atrial myocytes [3,4]. Although the effect of 5-HT$_4$ receptor activation on I_f and $I_{Ca,L}$ resemble the effects of a β-adrenergic agonist such as isoprenaline, 5-HT$_4$ receptors unlike β-adrenergic receptors exert their effects exclusively on the atrial tissue and, until recently, had been assumed to have no functional effects in ventricles [5,6]; but see [7]. The restricted location of 5-HT$_4$ receptors in the atrium, together with its stimulatory effects on $I_{Ca,L}$ and I_f, makes it reasonable to assume that 5-HT$_4$ receptors may participate in the control of cardiac rhythmic and/or arrhythmic activity [8,9]. In support of this hypoth-

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esis, a 5-HT_4 receptor antagonist was shown to inhibit atrial fibrillation induced by rapid pacing in juvenile pigs [10].

Although only one gene for the 5-HT_4 receptor has been found in human, eight C-terminal isoforms (a–g, i, n) and one internal splice variant (hb) have been currently identified [4,11–16]. These human 5-HT_4 receptor (h5-HT_4) isoforms display distinct patterns of tissue distribution [11,17]. For instance, the h5-$HT_{4(b)}$ variant is the most abundant isoform in human brain and heart [17], while the h5-$HT_{4(d)}$ receptor splice variant is only present in the gastro-intestinal (GI) tract [11]. All h5-HT_4 receptor isoforms share the same primary binding site for 5-HT_4 ligands and so their pharmacology is identical in binding studies [1]. However, striking differences have been reported in their signaling properties. For instance, in CHO cells, the h5-$HT_{4(e)}$ receptor isoform is less potent than the h5-$HT_{4(d)}$ isoform to increase cAMP accumulation in response to 5-HT_4 agonists [14,18]. In HEK 293 cells, the h5-$HT_{4(b)}$ receptor isoform is able to activate both to Gi/o and Gs proteins whereas the h5-$HT_{4(a)}$ receptor is only coupled to Gs proteins [19]. Furthermore, a given 5-HT_4 receptor isoform may have different signaling properties depending on the cellular environment in which it is expressed (e.g. h5-$HT_{4(g)}$ receptor isoform expressed in CHO vs. C6-glial cell lines [14]). These observations come from in vitro studies using different cell lines and to date there is no information on the functional effects of h5-HT_4 receptor isoforms in cardiac myocytes.

Thus, the functional differences between receptor variants when expressed in cell lines emphasize the importance to study the properties of native 5-HT_4 receptors in their natural environment. However, a major drawback in studying native cardiac 5-HT_4 receptors is that these receptors have so far been only found in human and pig atria and are absent from the heart of small laboratory animals, such as rat, guinea pig, rabbit and frog [3,20]. In the rat, only the messenger RNA coding for 5-HT_4 receptors has been detected in the atria, with no evidence for the corresponding protein [21,22]. For these reasons we decided to set up an expression system of h5-HT_4 receptor splice variants in adult cardiomyocytes. As cardiac myocytes are difficult to transform with plasmids, we developed two bicistronic adenovirus encoding two distinct proteins, the enhanced green fluorescent protein (eGFP) and either the h5-$HT_{4(b)}$ or h5-$HT_{4(d)}$ receptor isoform. In this study, we report the expression of these two adenovirus in two cardiac preparations, the mouse cardiac cell line HL-1 [23] and adult rat ventricular cardiomyocytes. Human 5-HT_4 receptor expression caused specific binding of a 5-HT_4 selective ligand, as well as serotonin (5-HT) induced cAMP production and stimulation of $I_{Ca,L}$. In contrast to previous studies showing a constitutive activity of h5-HT_4 receptor isoforms in different cell lines, we did not find any intrinsic activity of h5-$HT_{4(b)}$ or h5-$HT_{4(d)}$ receptor isoforms on cAMP production and $I_{Ca,L}$ in cardiac myocytes. In addition, we demonstrate that the h5-$HT_{4(b)}$ receptor retains its capacity to activate pertussis toxin-sensitive G proteins when expressed in adult rat cardiomyocytes.

2. Materials and methods

The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off J* n°L358, 18 December 1986), the local (CREEA Ile-de-France Sud) ethics committee guidelines and the French decree n°87/748 of October 19, 1987 (*J Off République Française*, 20 October 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (no. 04226, April 12, 1991).

2.1. Adenovirus

Type 5 adenovirus were generated using the AdEasy vector system as described previously [24]. Inserts for the human 5-$HT_{4(b)}$ (AC: Y12505) and 5-$HT_{4(d)}$ (AC: Y12507) [11] receptors were obtained locally. Bicistronic adenovirus bearing either the h5-$HT_{4(b)}$ receptor or the h5-$HT_{4(d)}$ receptor under the control of a cytomegalovirus (CMV) promoter, and eGFP under a second CMV promotor were constructed and amplified. The viruses were cesium chloride-purified, viral DNA was extracted from an aliquot, optical density was determined and used to calculate the concentration of virus [24]. The titer of biologically active virus (bav) was determined by titrations in cardiac myocytes and was defined as the number of virus that was just sufficient to transfect 100% of a given number of cardiac myocytes. It generally amounted to 1/30 of total virus number. All further infections were done at a certain multiplicity of infection (MOI) based on this definition, e.g. 10×10^6 bav/1×10^6 cells = MOI 10.

2.2. Expression in HL–1 cells and measure of cAMP

HL-1 cells were obtained from Dr. W.C. Claycomb, Louisiana State University Medical Center, New Orleans, LA [23]. They were grown in "Claycomb medium" (JRH Biosciences, Andover, UK) according to the manufacturer's instructions. For the infection, confluent cells were plated at 1/2 dilution in 24 wells culture plate. After 24 h, the adenovirus was applied to the cells in the culture medium at the appropriate concentration. After another 24 h the adenovirus and noradrenaline were removed from the medium and foetal bovine serum (FBS) in the medium was replaced with dialyzed FBS to minimize exposure of the cells to serotonin (5-HT). Finally, 24 h later the cells were stimulated with 5-HT in the presence of 5 mM theophyllin (a non-selective phosphodiesterase inhibitor) and 10 μM pargylin (a monoamine oxydase inhibitor). The cells were lyzed in 15% ice-cold perchloric acid, neutralized by KOH 2 N and cAMP in the lysate was measured by a commercial radioimmunoassay kit (Immunotech, Marseille, France). Expression of eGFP was monitored by measuring the fluorescence in a microplate reader (FL600, Bio Tek, Winooski, VT, USA) through the bottom of the wells with the excitation wavelength set at 485 ± 20 nm and the detection at 530 ± 25 nm.

2.3. Binding experiments

Cultured HL-1 cells were infected by the viruses as described above. Forty-eight hours after the infection the cells were detached in PBS with a scraper and broken down. Membranes were rinsed twice in PBS by centrifugation (15 min at 10,000 rpm) and re-suspended in binding buffer (hepes 50 mM, pH 7.4). Crude membranes were incubated with 0.2 nM [^{3}H]GR113808 (Amersham) at 25 °C for 30 min in binding buffer. Non-specific binding was measured in the presence of 10 μM of non-radioactive GR113808. The incubation was stopped by rapid filtration on Whatman GF/B filters pre-soaked in polyethylenimine 0.1% in a semi-automatic filtering machine (Brandel, Gaithersburg, MD, USA). Filters were washed with ice-cold 50 mM Tris–HCl, pH 7.4 and counted by liquid scintillation.

2.4. Infection of rat ventricular cardiomyocytes

Adult rat ventricular cells were isolated as described earlier [25], plated in laminin coated plastic Petri dishes and covered with Dulbecco's modified Eagles medium (DMEM) containing 1.2 mM $CaCl_2$, 20 mM hepes, 1 × penicillin/streptomycin and 2.5% FBS, pH adjusted to 7.6 with NaOH. Infection of rat cardiomyocytes was performed essentially as described by [26] for mouse cells. One hour after plating, the myocytes were infected with the adenovirus diluted in the same medium without FBS. The concentration of virus was adjusted for the number of cells alive after resuspension in the culture medium. After 24–48 h incubation with the adenovirus at 37 °C in 5%CO_2, the cardiomyocytes where used for recordings.

2.5. Electrophysiological recordings

For recording, cardiomyocyte-containing Petri dishes were placed on the stage of an inverted microscope equipped with fluorescence detection (excitation: 490 nm; detection: 535 nm). Cells were transferred into the recording solution composed of (mM): hepes 10, glucose 5, NaCl 107, Na-pyruvate 5, $NaHCO_3$ 4, NaH_2PO_4 0.8, CsCl 20, $CaCl_2$ 1.8, $MgCl_2$ 1.8, pH 7.4 with NaOH. L-type Ca^{2+} currents ($I_{Ca,L}$) were recorded from eGFP positive cells in the whole-cell configuration of the patch-clamp technique with the help of a computer-controlled patch-clamp amplifier (Visual-Patch 500, Biologic, Claix, France). Pulse generation and data acquisition were performed with the Visual-Patch 500 V 1.31B software (Biologic). Pipettes were made of soft glass (Drummond, Broomall, PA, USA) and had a resistance of around 1 MΩ when filled with the following pipette solution (mM): hepes 10, CsCl 118, $CaCl_2$ 0.062 (pCa 8.5), $MgCl_2$ 4, EGTA 5, Na_2CP 5, Na_2ATP 3, Na_2GTP 0.416, pH 7.3 with CsOH. Drugs were perfused by gravity. The holding potential was kept at –50 mV and the current was elicited by 400 ms duration depolarization to 0 mV every 8 s. Occasionally a holding potential of –80 mV was used and the depolarization to 0 mV was preceded by a 50 ms prepulse to –50 mV. When indicated, pertussis toxin (PTX) was added to the incubation medium of the cardiomyocytes at a concentration of 250 ng/ml for at least 5 h prior to the experiment. All the experiments were done at room temperature (21–27 °C), and the temperature did not vary by more than 1 °C in a given experiment.

2.6. Drugs

GR113808 ([1-[2-(methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate) was a gift from Glaxo Research Group (Ware, Hertfordshire, UK). [3H]-GR113808 was purchased from Amersham (Orsay, France). Serotonin (5-hydroxytryptamine or 5-HT) was from Aldrich (L'Isle d'Abeau Chesnes, France). L858051 and pertussis toxin (PTX) were from Calbiochem-Merck (Darmstadt, Germany).

2.7. Data analysis

Currents were sampled at a frequency of 20 kHz (filtered at 5 kHz) and were not compensated for capacitive and leak currents. On-line analysis of the recordings was made with the Biotools program (Biologic) and Origin (Microcal Software Inc., Northampton, MA, USA). For each membrane depolarization, $I_{Ca,L}$ amplitude was measured as the difference between peak inward current and the current at the end of the 400 ms pulse and plotted as a function of time. To account for rundown, the resulting curve was fitted to an exponential decay and the $I_{Ca,L}$ peak amplitude measured in presence of a drug (I_{drug}) was expressed relatively to the fitted, extrapolated basal $I_{Ca,L}$ amplitude (I_{basal}) at the same time point. In each cell, the effect (E) of a drug on $I_{Ca,L}$ was estimated as the percentage variation from basal and was calculated as: $E = 100\ (I_{drug} - I_{basal})/I_{basal}$. Likewise, for dose response determination, increasing doses of the drug were successively added to the bath and the amplitude of $I_{Ca,L}$ at each dose was expressed with reference to the non-stimulated basal current for that cell.

2.8. Statistics

Error bars are given as standard errors to the mean (S.E.M.). The number of cells is denoted by n. For statistical evaluation the paired and unpaired Student's t-test were used, and a difference was considered statistically significant when P was <0.05.

3. Results

3.1. Functional expression of h5-HT$_4$ receptors in the HL-1 cell line

HL-1 is a cell line derived from the atria of a transgenic mouse expressing the SV40 big T antigen under the control

of the ANF (atrial natriuretic factor) promoter [27]. HL-1 cells express several adult cardiac cell markers and receptors [23] and, when confluent, beat spontaneously. When HL-1 cells were infected with either the h5-$HT_{4(b)}$ or the h5-$HT_{4(d)}$ receptor adenovirus construct, a green fluorescence due to concomitant eGFP expression was detectable 24 h after infection, and continued to increase until 48 h. MOI values were usually kept below 300 because higher values induced progressive cell death. Fig. 1A shows that adenovirus infection of HL-1 cells with either the h5-$HT_{4(b)}$ or the h5-$HT_{4(d)}$ receptor adenovirus at a MOI of 100 revealed a significant and specific binding of [^{3}H]GR113808, a highly selective 5-HT_4 receptor antagonist [1], which was not observed in non-infected cells. This indicates the presence of h5-HT_4 receptors in a crude membrane fraction of HL-1 cells. To verify that these receptors were functionally coupled to adenylyl cyclase, the effect of a saturating concentration of 5-HT (1 μM) on intracellular cAMP production was examined in infected and non-infected HL-1 cells using radioimmunoassay. As shown in Fig. 1B, 5-HT had no effect on cAMP concentration in non-infected cells, whereas L858051, a hydrophilic forskolin analogue [28], increased cAMP approximately fourfold. However, in infected HL-1 cells, 5-HT increased cAMP >twofold and this stimulation was fully antagonized by the 5-HT_4 antagonist GR113808 (1 μM). Interestingly, a similar increase in cAMP was observed with the h5-$HT_{4(b)}$ receptor adenovirus at MOI 100 as with h5-$HT_{4(d)}$ isoform at a 10-fold lower virus amount (Fig. 1B), which might suggest a preferential coupling of the (d) isoform to adenylyl cyclase.

To examine this hypothesis, we systematically quantified the green fluorescence of the infected cells, considering that because our adenovirus constructs were bicistronic for eGFP and the 5-HT_4 receptor, the fluorescence should reflect the amount of receptor expressed. Fig. 2 shows that, as expected, the amount of cAMP produced in response to 5-HT (1 μM)

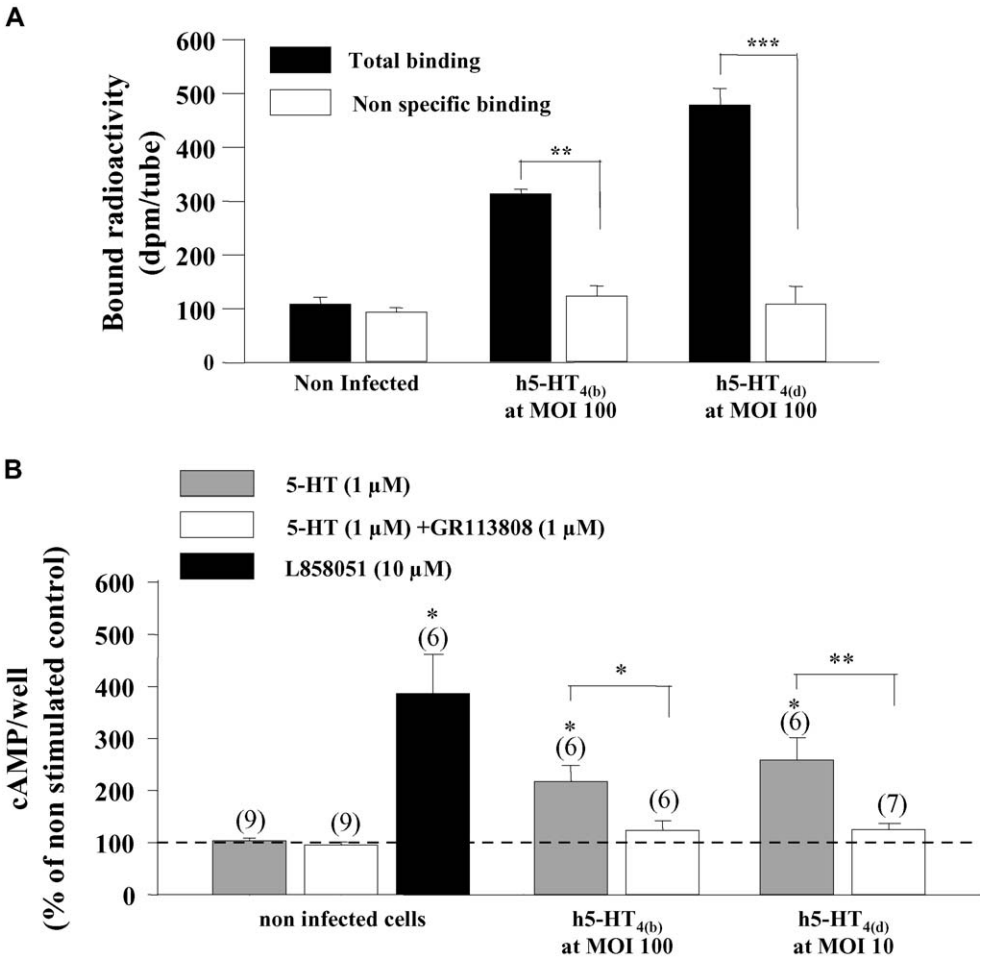

Fig. 1. Functional adenoviral expression of h5-HT_4 receptors in HL-1 cells. **A**, Binding of [^{3}H]GR113808 (0.2 nM) to crude membranes from non-infected HL-1 cells or from cells infected with the adenovirus expressing the h5-$HT_{4(b)}$ (MOI 100, 48 h) or h5-$HT_{4(d)}$ receptors (MOI 100, 48 h). Non-specific binding (open bars) measured in presence of 10 μM non-radioactive GR113808. Each bar indicates the mean ± S.E.M. of triplicates (**, $P < 0.01$; ***, $P < 0.001$). **B**, Effect of 5-HT (1 μM) alone (gray bars) or in the presence of the specific 5-HT_4 antagonist GR113808 (1 μM, open bars) on adenylyl cyclase activity in non-infected HL-1 cells or in cells infected with the adenovirus expressing the h5-$HT_{4(b)}$ (MOI 100, 48 h) or h5-$HT_{4(d)}$ receptors (MOI 100, 48 h). Control experiments with the hydrophilic forskolin analogue L858051 demonstrates a stimulatable adenylyl cyclase in non-infected cells (black bars). Results are expressed as percentage of the control in non-stimulated cells infected with the same construct at the same MOI. Each bar indicates the mean ± S.E.M. of the number of experiments indicated near the bar (*, $P < 0.05$; **, $P < 0.01$).

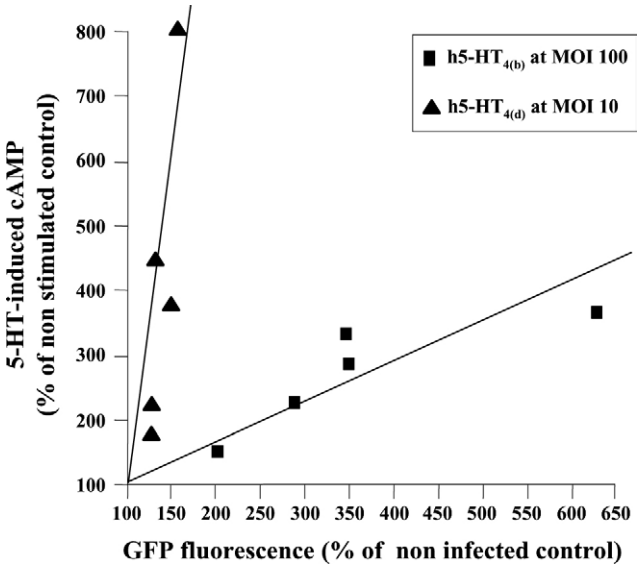

Fig. 2. The h5-$HT_{4(d)}$ receptor is more efficiently coupled to adenylyl cyclase than the h5-$HT_{4(b)}$ receptor. Correlation between the cAMP response to 5-HT (1 μM) and the eGFP fluorescence in HL-1 cells infected with the adenovirus encoding either the h5-$HT4_{(b)}$ (MOI = 100, 48 h, squares) or the h5-$HT4_{(d)}$ isoform (MOI 10, 48 h, triangles).

increased linearly as a function of the eGFP fluorescence for both the h5-$HT_{4(b)}$ and h5-$HT_{4(d)}$ receptor isoforms. However, the data differ for the two receptors, as the results indicate that a similar production of cAMP is observed for a > 10-fold lower expression of the h5-$HT_{4(d)}$ isoform as compared to the h5-$HT_{4(b)}$ isoform. Confocal imaging of HL-1 cells infected with the h5-$HT_{4(b)}$ isoform at different MOIs ranging from 30 to 300 indicated a linear relationship between the number of transfected cells and MOI (not shown). This eliminates the possibility that only a limited number of transfected cells contributed to the total fluorescence in cells transfected with the h5-$HT_{4(b)}$ isoform. Thus, these data confirm our hypothesis that the h5-$HT_{4(d)}$ receptor isoform is >10 times more efficiently coupled to its downstream effector than the h5-$HT_{4(b)}$ isoform.

3.2. Functional expression of h5-HT_4 receptors in adult rat ventricular myocytes

Freshly isolated adult ventricular cardiomyocytes were infected with either the h5-$HT_{4(b)}$ or the h5-$HT_{4(d)}$ receptor adenovirus construct. After 24 h infection, the proportion of bright fluorescent cells averaged 52 ± 2% (mean ± S.E.M., n = 16) of the total population of living cells, indicating that half the population of cells had expressed the 5-HT_4 receptor. Next, we examined whether these receptors were functional. Because native cardiac 5-HT_4 receptors in human atrial myocytes activate the L-type Ca^{2+} current ($I_{Ca,L}$) [3,4], we anticipated that $I_{Ca,L}$ in infected rat ventricular myocytes should also respond positively to 5-HT. Whole-cell $I_{Ca,L}$ was recorded in non-infected and infected fluorescent cells at 0 mV. Fig. 3 shows that the basal $I_{Ca,L}$ current density did not vary significantly between non-infected and infected cells, whether the (b) or (d) h5-HT_4 receptor isoform was expressed, and whether the cells were infected during 24 or 48 h and at two different MOIs. This indicates that constitutive activity of the h5-HT_4 receptors does not contribute to a significant increase in adenylyl cyclase and stimulation of $I_{Ca,L}$ in this system.

Next, we tested the effect of 5-HT on $I_{Ca,L}$ in infected cells recognized by their fluorescence. Fig. 4 shows an example of the response obtained in a rat cardiomyocyte expressing the h5-$HT_{4(d)}$ receptor isoform. 5-HT (100 nM) strongly stimu-

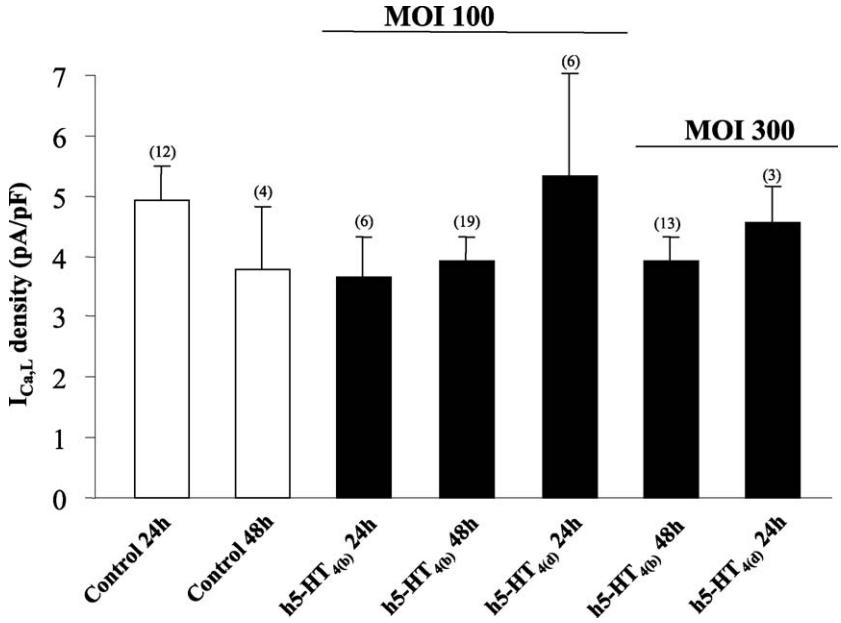

Fig. 3. $I_{Ca,L}$ density in rat ventricular myocytes infected by h5-$HT_{4(b)}$ or h5-$HT_{4(d)}$ constructs. $I_{Ca,L}$ density (pA/pF) was measured at a test potential of 0 mV, from a holding potential of –50 mV, 24 or 48 h after dissociation and plating. The myocytes were either not-infected (open bars) or infected with the adenovirus expressing the h5-$HT_{4(b)}$ or h5-$HT_{4(d)}$ receptors at the MOI and for the duration indicated. Each bar indicates the mean ± S.E.M. of the number of experiments indicated.

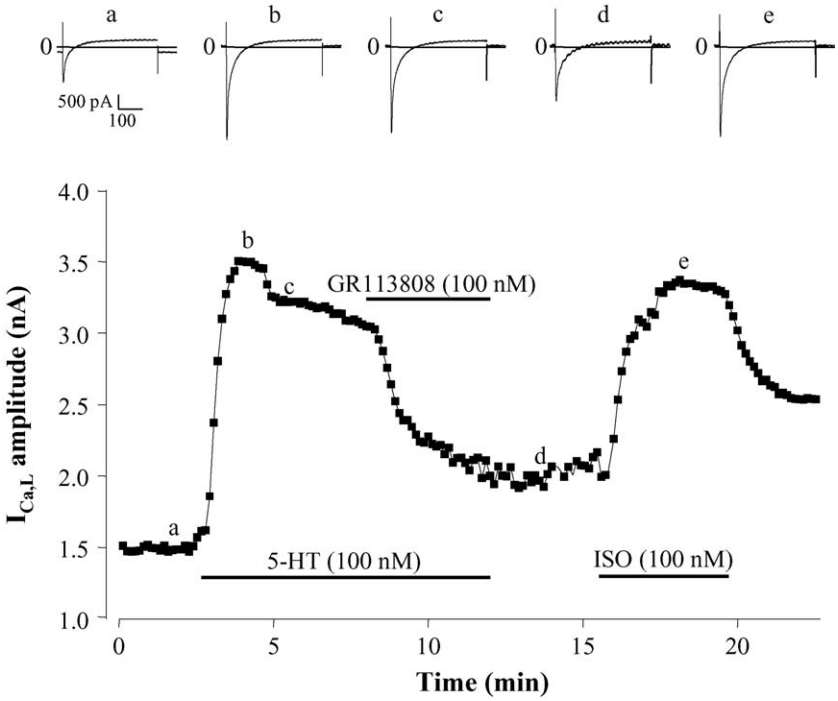

Fig. 4. 5-HT activates $I_{Ca,L}$ in infected adult rat ventricular myocytes. Time course of the effects of 5-HT and GR113808 on $I_{Ca,L}$ in a rat ventricular myocyte infected with the h5-$HT_{4(d)}$ adenovirus (24 h, MOI 300). Each point corresponds to a net $I_{Ca,L}$ amplitude measured every 8 s during a 400 ms test potential at 0 mV, from a –50 mV holding potential. The cell was first perfused with control solution and then exposed to 5-HT (100 nM) alone or in the presence of GR113808 (100 nM) during the periods indicated by the solid lines. After washout of the 5-HT_4 ligands, the cell was perfused with isoprenaline (ISO, 100 nM). The individual current traces on the top were obtained at time points indicated by the corresponding letters in the main graph.

lated $I_{Ca,L}$, and this response was fully antagonized by a 5-HT_4 receptor antagonist, GR113808 (100 nM). The stimulatory effect of 5-HT was similar to the effect of the β-adrenergic receptor agonist isoprenaline (ISO, 100 nM) tested on the same cell after washout of the 5-HT_4 receptor ligands. To verify that the $I_{Ca,L}$ response to 5-HT was due to adenovirus 5-HT_4 receptor expression, non-infected myocytes were also tested. Fig. 5 shows a typical experiment obtained in a non-fluorescent cell. When the cell was exposed to 100 nM 5-HT, $I_{Ca,L}$ did not change although the current responded positively to a subsequent challenge with ISO (10 nM). This confirms the absence of endogenous 5-HT_4 receptors in adult rat ventricular myocytes [3].

Fig. 6 summarizes our results on adult rat ventricular myocytes. At a MOI of 100, myocytes infected during 24 h by the h5-$HT_{4(b)}$ adenovirus construct did not respond to 5-HT while those infected by the h5-$HT_{4(d)}$ adenovirus responded to 5-HT by a 60–100% increase in $I_{Ca,L}$. Increasing threefold the amount of viral particles or a twofold longer infection at a MOI of 100 revealed a 5-HT response in myocytes infected by the h5-$HT_{4(b)}$ adenovirus. But a similar procedure with the h5-$HT_{4(d)}$ adenovirus doubled the response to 5-HT ($P < 0.01$) which then became similar to the response of $I_{Ca,L}$ in non-infected myocytes to a saturating concentration of ISO (100 nM) or L858051 (50 μM). The smaller response of $I_{Ca,L}$ to 5-HT in myocytes infected with the (b) vs. (d) isoform may again indicate a better coupling of the h5-$HT_{4(d)}$ isoform to its effector. As a negative control, 5-HT had no effect on $I_{Ca,L}$ in non-infected myocytes. In all cases, GR113808 (100 nM) fully antagonized the effect of 5-HT. Altogether, these data demonstrate the efficacy of our adenovirus constructs to express 5-HT_4 receptors with distinct functional properties in rat ventricular cardiomyocytes.

3.3. PTX sensitivity of the h5-$HT_{4(b)}$ response in adult rat ventricular myocytes

Since we gathered evidence suggesting a less efficient coupling in cardiac myocytes of the h5-$HT_{4(b)}$ as compared to the h5-$HT_{4(d)}$ receptor to both cAMP production and $I_{Ca,L}$, we questioned whether this could be due to a simultaneous coupling of the (b) isoform to both a stimulatory and an inhibitory G protein pathway. To examine this, HL-1 cells and adult rat ventricular myocytes infected with the h5-$HT_{4(b)}$ receptor were incubated (overnight for HL-1 cells and 5 h for rat cardiomyocytes) with PTX and the effect of 5-HT on cAMP (HL-1 cells) and $I_{Ca,L}$ (rat cardiomyocytes) was then re-evaluated. The effect of 5-HT on cAMP accumulation in HL-1 cells was not affected by PTX (not shown), but the degree of Gi/o inactivation by PTX has not been tested in this cellular system. In contrast, PTX treatment significantly increased the $I_{Ca,L}$ response to 5-HT in rat ventricular cardiomyocytes infected with the 5-$HT_{4(b)}$ adenovirus. Here, the efficacy of the PTX treatment was controlled by its ability to block the muscarinic inhibition of the 5-HT-stimulation of $I_{Ca,L}$ (not shown). Fig. 7 shows the effects of four increasing concentrations of 5-HT on $I_{Ca,L}$ in rat ventricular cardiomyocytes infected with the h5-$HT_{4(b)}$ receptor adenovirus construct, in the absence

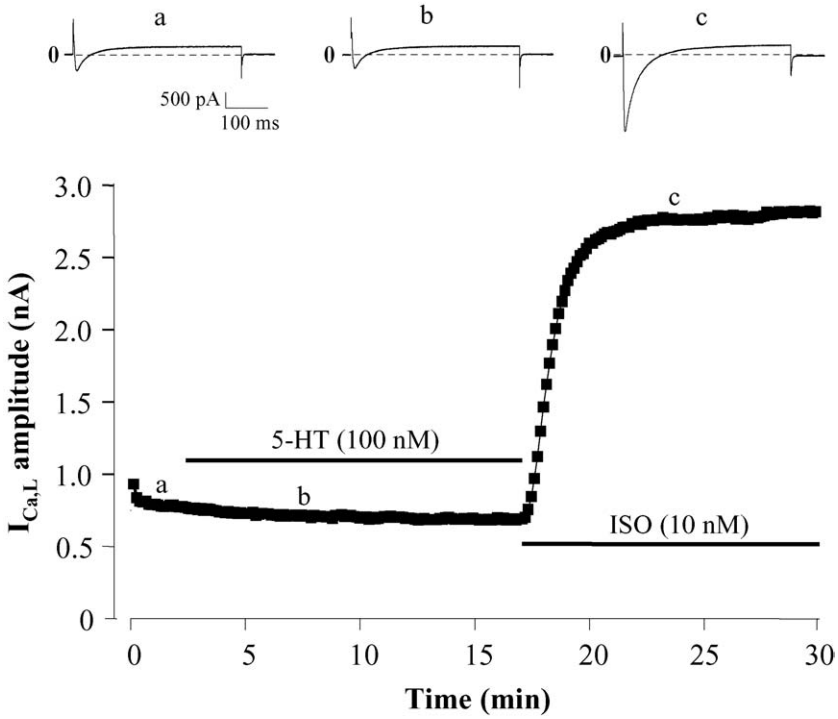

Fig. 5. 5-HT has no effect on $I_{Ca,L}$ in non-infected adult rat ventricular myocytes. Time course of the effects of 5-HT and isoprenaline (ISO) on $I_{Ca,L}$ in a non-infected rat ventricular myocyte, 24 h after isolation. Each point corresponds to a net $I_{Ca,L}$ amplitude measured every 8 s during a 400 ms test potential at 0 mV, from a –50 mV holding potential. The cell was first perfused with control solution and then exposed to 5-HT (100 nM) or ISO (10 nM) during the periods indicated by the solid lines. The individual current traces on the top were obtained at time points indicated by the corresponding letters in the main graph.

or presence of PTX. While PTX treatment had no effect on basal $I_{Ca,L}$ current density (inset in Fig. 7), it significantly increased the response of $I_{Ca,L}$ to 5-HT at every concentration tested. PTX treatment increased by >70% the maximal response to 5-HT, but the threshold response produced by 3 nM 5-HT was increased fourfold. This indicates that activation of Gi/o proteins may preclude a stimulatory response to 5-HT via the h5-$HT_{4(b)}$ isoform at small agonist concentrations. At variance with the h5-$HT_{4(b)}$ receptor isoform, the response of $I_{Ca,L}$ to 5-HT (100 nM) stimulation in rat cardi-

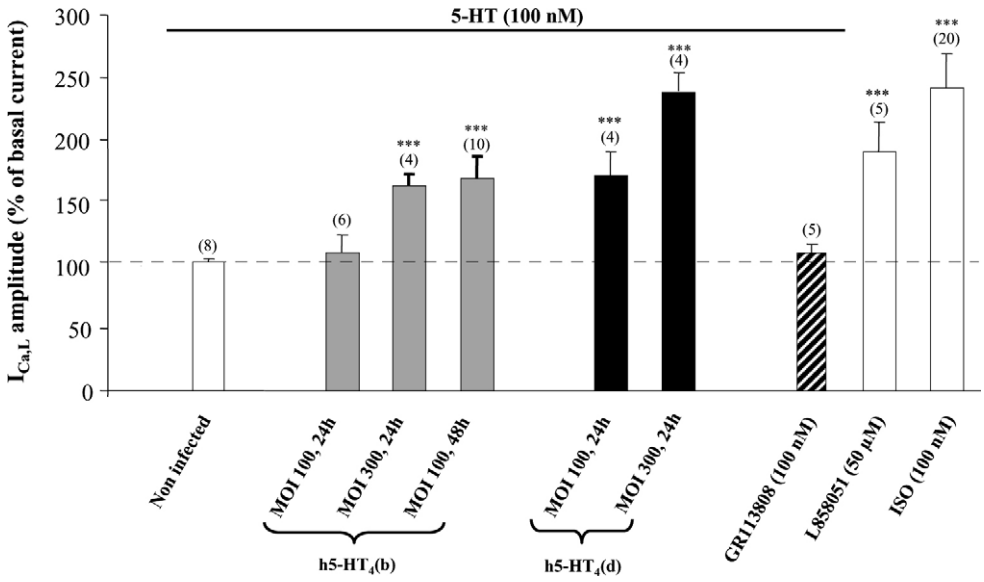

Fig. 6. Summary of the effects of 5-HT on $I_{Ca,L}$ in rat ventricular myocytes. 5-HT (100 nM) was tested on $I_{Ca,L}$ in non-infected rat ventricular myocytes (open bars) or in myocytes infected by the h5-$HT_{4(b)}$ (gray bars) or h5-$HT_{4(d)}$ receptor adenovirus construct (black bars) at MOI 100 or 300 and for 24 or 48 h as indicated. The hatched bar indicate the response to 5-HT in the presence of 100 nM GR113808 in a total of 5 cells expressing the (d) and one cell the (b) isoform. Since non-infected cells did not respond to 5-HT, isoprenaline (100 nM) or L858051 (50 µM) were tested in these cells (open bars on the right). Results are expressed in percentage of the basal, non-stimulated current. Each bar indicates the mean ± S.E.M. of the number of experiments indicated near the bar (***, $P < 0.001$ with respect to basal $I_{Ca,L}$ in the absence of drug).

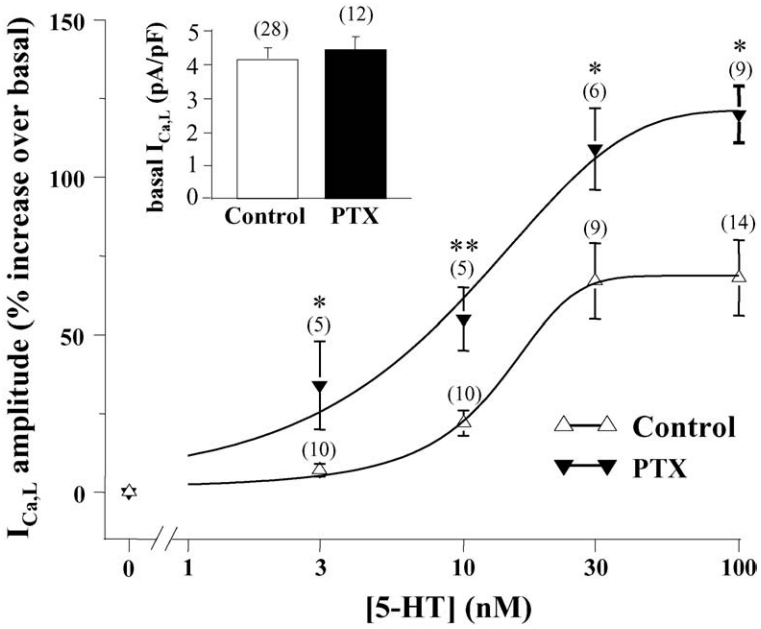

Fig. 7. Effect of PTX on $I_{Ca,L}$ response to 5-HT in rat ventricular myocytes. Adult rat ventricular myocytes were infected by the h5-$HT_{4(b)}$ adenovirus (MOI 300, 24 h or MOI 100, 48 h), and their $I_{Ca,L}$ response to 5-HT was tested in the absence of PTX (open triangles) or after >5 h incubation with 250 ng/ml Pertussis toxin (PTX, closed triangles). Results are expressed as percent increase over basal, non-stimulated current. Each point indicates the mean ± S.E.M. of the number of experiments indicated near the point (*, $P < 0.05$; **, $P < 0.01$ comparing control with PTX treated cells). The inset shows the lack of effect of PTX on basal $I_{Ca,L}$ amplitude.

omyocytes infected with the h5-$HT_{4(d)}$ receptor adenovirus construct (MOI 300, 24 h infection) was similar in the absence or presence of PTX (respectively, 238 ± 14%, $n = 3$, and 251 ± 29%, $n = 4$, of control amplitude).

4. Discussion

In this study, we developed two adenoviral constructs encoding h5-HT_4 receptors, and tested for their efficacy in two different cardiac preparations devoid of native 5-HT_4 receptors. One of the viruses encodes the (b) splice variant of the h5-HT_4 receptor, a variant normally expressed in the human atrium [17]. The other one encodes the (d) splice variant, a variant not usually expressed in heart but expressed solely in the GI tract [11].

When expressed in the HL-1 murine cell line of atrial origin, both receptors were found to activate adenylyl cyclase in the presence of 5-HT. When expressed in freshly isolated adult rat ventricular cardiomyocytes, a stimulation of the L-type Ca^{2+} current ($I_{Ca,L}$) by 5-HT was revealed. In HL-1 cells, the h5-$HT_{4(d)}$ receptor was found to be more efficiently coupled to adenylyl cyclase than the h5-$HT_{4(b)}$. Pertussis toxin (PTX) treatment potentiated the stimulatory effect of 5-HT on $I_{Ca,L}$ in rat myocytes expressing the h5-$HT_{4(b)}$ but not the h5-$HT_{4(d)}$ receptor, indicating a likely coupling of the (b) isoform to both Gs and Gi/o proteins.

Surprisingly, none of the adenovirally expressed h5-HT_4 receptors showed any constitutive activity on cAMP production or $I_{Ca,L}$ in cardiac myocytes. This contrasts with previous findings showing that recombinant mouse, rat and h5-HT_4 receptor isoforms displayed intrinsic activities in the absence of 5-HT_4 ligands when expressed in COS-7 and CHO cells at physiological densities [12,14,18]. The reasons for these discrepancies might be explained by the cellular environment in which specific proteins might interact with 5-HT_4 receptor intracellular C-terminal tails and therefore influence the capacity of the receptor to spontaneously isomerize from its inactive to its active form [12].

5-HT produced no effect on cAMP concentration in HL-1 cells and on $I_{Ca,L}$ in rat ventricular myocytes, suggesting the absence of functional endogenous 5-HT_4 receptors in these cellular models. This was consistent with the absence of mRNA for the 5-HT_4 receptor in HL-1 cells (not shown) and rat ventricular myocytes [21]. 5-HT had also no effect on $I_{Ca,L}$ or contractility in rat atrial tissue [3] although this tissue contains mRNA for the 5-HT_4 receptor [21] and 5-HT_4 agonists can increase the pressure-stimulated release of ANF in rat left atria [29]. A somewhat similar situation may apply to human and pig ventricle, where no contractile effect of 5-HT_4 agonists were initially reported [5,6,30,31], although mRNA for 5-HT_4 receptor appears to be present [7,32; but see 4,11]. In this case, the lack of contractile response may be due to an insufficient cAMP production, or to a highly compartmentalized signal due to cAMP phosphodiesterase activity [33,34]. Indeed, inhibition of phosphodiesterase activity by IBMX revealed an effect of 5-HT_4 agonists both in human and pig ventricular tissue [7]. Interestingly, it has been recently reported that 5-HT_4 receptors may be also coupled to the newly discovered cAMP-GEF/Epac, which belongs to a novel cAMP binding protein family having guanine nucleotide exchange factor (GEF) activity [35,36]. 5-HT_4 receptor-

induced Epac activation regulates exocytosis in a PKA independent manner in different cellular systems. Whether these 5-HT receptors are coupled or not to the cAMP-GEF, Epac in cardiac myocytes is still an open question.

The contribution of 5-HT_4 receptors to cardiac function may also differ between normal and pathological situations. With respect to its preferential expression in atrial tissue, and its coupling to two inward currents, I_f [2] and $I_{Ca,L}$ [3,4], the 5-HT_4 receptor has been proposed to participate in the generation of cardiac arrhythmias, particularly atrial fibrillation (AF) [8,9]. For instance, the 5-HT_4 receptor antagonist RS-100302 was shown to inhibit AF induced by rapid pacing in juvenile pigs [10] and another such antagonist, piboserod, is currently being evaluated in AF [37]. 5-HT_4 receptor antagonists may also be of potential interest in the treatment of heart failure, as both the mRNA for 5-HT_4 receptors and the contractile response to 5-HT_4 agonists in ventricle appear up-regulated in a rat model of heart failure [38] and in human failing hearts [7].

All the above studies emphasize the need for exploring in more detail the functional properties of 5-HT_4 receptors in cardiac myocytes. Ideally, this should be done in a cardiac cellular model naturally expressing the 5-HT_4 receptor. Unfortunately, since human and pig are the only animal species expressing functional cardiac 5-HT_4 receptors, and only in atrium, this sets obvious practical limitations in pursuing that ideal goal. For this reason, we developed adenoviral constructs to allow expression of 5-HT_4 receptors in adult cardiac myocytes isolated from laboratory animals devoid of functional cardiac 5-HT_4 receptors. This study clearly shows that two human 5-HT_4 receptors heterologously expressed in cardiac myocytes couple to cAMP generation and $I_{Ca,L}$ in a manner which is sensitive to the 5-HT_4 antagonist GR113808. Although the dominant human cardiac 5-HT_4 receptor appears to be the (b) isoform [17], other isoforms are also expressed [4,11,17,32]. Since the binding sites for agonists and antagonists are located in the common part to all 5-HT_4 receptor isoforms [39,40], no isoform-selective 5-HT_4 receptor ligand is available yet, making it impossible to study the contribution of a particular isoform to the overall effects of 5-HT on cardiac function. In this respect, adenoviral expression of a single 5-HT_4 receptor isoform provides a clear advantage. In this study, we found that the h5-$HT_{4(d)}$ isoform is more efficiently coupled to adenylyl cyclase in HL-1 and to $I_{Ca,L}$ in rat cardiomyocytes than the h5-$HT_{4(b)}$ isoform. Our PTX experiments in rat cardiomyocytes indicate that part of this difference may be due to the dual coupling of the (b) isoform to both Gs and Gi/o proteins, while the (d) isoform is only coupled to Gs. A similar difference was found between the (b) and (a) isoforms when expressed in HEK 203 cells [19]. This confirms an observation initially reported for the EP3 family of prostaglandin PGE_2 receptors [41] that different C-terminal extremities of G protein coupled receptors may confer different coupling to G proteins and second messenger systems. Interestingly, PTX treatment of native human atrial membranes enhanced the stimulatory effect of 5-HT on adenylyl cyclase activity [42], indicating that native cardiac 5-HT_4 receptors behave similarly to the (b) isoform expressed by adenovirus. As both 5-HT_4 receptor [7,38] and Gαi protein [43] expression is increased in heart failure, the participation of a dual coupling of the h5-$HT_{4(b)}$ receptor to Gs and Gi in this setting is not obvious. Adenoviral expression of h5-HT_4 receptor isoforms in cellular models of cardiac hypertrophy, or cardiac gene transfer in intact rats using a catheter-based technique [44] could provide possible means to address that question.

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