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Adenovirus-mediated expression of 5-HT_{1B} receptors in cardiac ventricle myocytes; coupling to inwardly rectifying K^+ channels

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Abstract

The 5-HT_{1B} receptor is expressed on nerve terminals where it inhibits neurotransmitter release. When expressed ectopically in fibroblasts, the 5-HT_{1B} receptor inhibits adenylyl cyclase. However, in the central nervous system, the effect of this receptor on neurotransmitter release appears to be cAMP-independent. We therefore investigated alternative effector systems that might be activated by the 5-HT_{1B} receptor. We constructed a recombinant adenovirus that allows expression of high levels of the 5-HT_{1B} receptor in a variety of cells. We chose cardiac ventricle myocytes because they express a muscarinic-gated, inwardly rectifying K^+ channel (i_{KACh}). In infected ventricle cells, both 5-HT and the muscarinic receptor agonist, carbachol, elicited a similar inwardly rectifying K^+ current. The currents elicited by these agonists were pertussis-toxin sensitive and were not additive. These results suggest a common signal transduction pathway for 5-HT_{1B} and muscarinic receptors in ventricle cells. © 1997 Elsevier Science B.V.

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1. Introduction

The neurotransmitter serotonin (5-HT) is involved in a wide range of behaviors and serotonergic drugs are used in the treatment of a number of neuropsychiatric disorders such as depression, anxiety, and eating disorders (Sleight et al., 1991). Underlying 5-HT's pleiotropy are at least 13 distinct mammalian receptors (for reviews, see Hoyer et al., 1994; Saudou and Hen, 1994). Except for the 5-HT_3 receptors, which are ligand-gated ion channels, all the other 5-HT receptors belong to the large family of G-protein coupled receptors. The 5-HT_1 receptors bind 5-HT with high affinity and are negatively coupled to adenylyl cyclase. The 5-HT_2 family includes receptors that stimulate phospholipase C. The 5-HT_4, 5-HT_6, and 5-HT_7 receptors stimulate adenylyl cyclase while the effectors of the 5-HT_5 receptors have not yet been characterized. To date, cloning studies have identified five unique G-protein coupled receptors belonging to the 5-HT_1 class: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}. Besides inhibiting adenylyl cyclase, these receptors can also interact with other effector systems. The 5-HT_{1A} receptor, for example, has been shown to stimulate phospholipase C (Fargin et al., 1989), to potentiate inwardly rectifying K^+ channels, and to attenuate voltage-activated calcium channels (Karschin et al., 1991; Penington et al., 1991; Okuhara and Beck, 1994).

Unlike the 5-HT_{1A} receptor, the 5-HT_{1B} receptor has not been extensively studied in terms of effector systems that operate in vivo. The 5-HT_{1B} receptor is found in kidney as well as in many brain structures such as the basal ganglia, cerebellum, and hippocampus (Saudou and Hen, 1994). This receptor is localized predominantly on axon terminals (Boschert et al., 1994) where it is believed to inhibit neurotransmitter release. For example, stimulation of 5-HT_{1B} receptors localized on serotonergic nerve terminals results in an inhibition of 5-HT release (Engel et al., 1986; Göthert et al., 1987). The 5-HT_{1B} receptor is also present on non-serotonergic terminals where it has been shown to inhibit the release of acetylcholine (Maura and Raiteri, 1986), noradrenaline (Molderings et al., 1987), and GABA (Johnson et al., 1992). However, the intracellular effector systems which mediate this inhibition of neurotransmitter release have not yet been identified. A

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coupling with adenylyl cyclase is unlikely since inhibition of 5-HT release resulting from the stimulation of 5-HT_{1B} autoreceptors located on serotoninergic terminals is not affected by cAMP (Ramdine et al., 1989). In addition, there is no evidence for coupling of the 5-HT_{1B} receptor to phospholipase C (Adham et al., 1992). Other possibilities include the modulation of ion channels such as K^+ or calcium channels. In order to study the possible coupling of the 5-HT_{1B} receptor to a K^+ channel, we expressed an epitope-tagged mouse 5-HT_{1B} receptor in cardiac ventricle myocytes. We chose these cells because they have been shown to express a G protein gated K^+ conductance with properties similar to the muscarinic K^+ current found in atrium (Koumi and Wasserstrom, 1994; Ito et al., 1995) and also because electrophysiological studies of the 5-HT_{1B} receptor in neurons is difficult due to the axon terminal localization of this receptor (Boschert et al., 1994). In order to express the 5-HT_{1B} receptor in ventricle cells, we constructed a replication-deficient adenovirus which expresses an N-terminal hemagglutinin-tagged version of the 5-HT_{1B} receptor. Electrophysiological experiments and a cAMP assay were used to study the intracellular effectors of the 5-HT_{1B} receptor. The results were compared with those obtained with the muscarinic receptor which is endogenously expressed in ventricle myocytes. We show here that the 5-HT_{1B} receptor activates an inwardly rectifying K^+ current, and inhibits adenylyl cyclase activity when it is expressed in ventricle myocytes via an adenovirus vector.

2. Materials and methods

2.1. Construction of a tagged 5-HT_{1B} receptor

In order to monitor the expression of the 5-HT_{1B} receptor in infected ventricle myocytes, we generated a tagged version of this receptor. The hemagglutinin epitope (YPYDVPDYA) which is recognized by the commercially available monoclonal antibody HA12CA5 (Boehringer) was linked to the extracellular amino-terminus of the mouse 5-HT_{1B} receptor (Maroteaux et al., 1992). Two complementary oligonucleotides were used that contain the sequence coding for the HA epitope as well as a Kozak sequence (CACC) and initiator methionine sequence. These oligonucleotides also contained *Hin*dIII and *Bam*HI sites which were used to subclone this fragment into the *Hin*dIII and *Bam*HI sites of the pSL1180 plasmid (Pharmacia). The 5-HT_{1B} receptor was fused to the C-terminus of the HA epitope as follows: the sequence encoding the 5-HT_{1B} receptor flanked with the restriction sites *Bam*HI and *Sal*I was engineered using polymerase chain reaction (PCR). The PCR products were digested by *Bam*HI and *Sal*I and subcloned into pSL1180-Tag to generate Tag-5-HT_{1B}-pSL1180. The final product was verified by double-stranded dideoxy DNA sequencing to ensure against unwanted PCR-induced mutations.

2.2. Recombinant adenovirus construction

A recombinant adenovirus expressing the Tag-5-HT_{1B} receptor was constructed. Most of the procedures were performed as described previously (Rosenfeld et al., 1992). In brief, we first engineered the plasmid pAd.CMV-Tag-1B as follows: The Tag-5-HT_{1B}-PSL1180 was cut by *Hin*dIII and *Sal*I digestion and was subcloned into the pAd.CMV (a gift from Dr. Falk-Paderson, Cornell University, New York, NY). This plasmid contains the first 454 nucleotides of adenovirus 5 including the 5′-ITR (inverted terminal repeat, which is necessary for the replication) and adjacent packaging sequences, the early promoter of the *Cyto megalo* virus, a β globin splice site, a polylinker, a polyadenylation signal from SV40, and finally, the sequence from 3329 to 6503 of adenovirus 5 encoding for polypeptide IX with its 3′ flanking region. The pAd-CMV-Tag-1B plasmid was linearized using the restriction enzyme *Xmn*I. The linearized plasmid was co-transfected in HEK 293 cells (ATCC No.: CRL 1573) together with the large *Cla*I DNA fragment of the d1324 mutant of type 5 adenovirus (Rosenfeld et al., 1992) which contains all the viral genome except 914 bp at the left end and is deleted for the E1 and E3 regions. Homologous recombination events between these two DNA molecules generated a replication-deficient adenovirus, Adeno-1B, which was carrying the Tag-5-HT_{1B} receptor cDNA (Fig. 1). The presence of this DNA was confirmed by PCR using specific oligonucleotides for the 5-HT_{1B} receptor. The Adeno-1B viruses were amplified by growth in HEK 293 cultured in a T175 flask. When complete cytopathic effect was observed, the cells were transferred to a 50 ml tube and then collected by centrifugation for 20 min at 2500 rpm. The pellet was resuspended in 5 ml of Dulbecco's modified Eagles's medium/2% fetal calf serum and the virus was released by three cycles of freezing and thawing. To eliminate cell remnants, the viral preparation was cen-

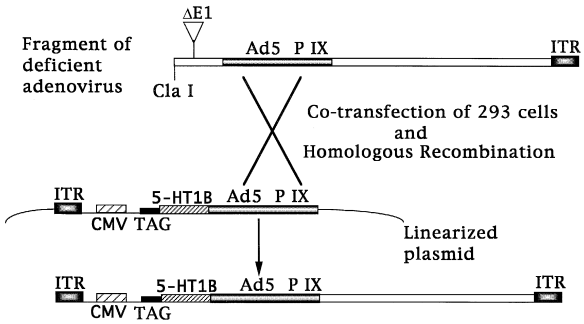

Fig. 1. Construction of a replication deficient recombinant adenovirus encoding the 5-HT_{1B} receptor. This cartoon summarizes the construction of the recombinant adenovirus containing the cDNA encoding the tagged 5-HT_{1B} receptor. See Section 2 for further explanation. ITR: inverted terminal repeat; CMV: early promoter of the Cyto megalo virus, Plasmid is pAd.CMV-Tag-1B.

trifuged at 3000 rpm. The supernatants were distributed into freezing vials and stored at −80°C. The titer of the Adeno-1B was determined using a plaque assay. This titer was estimated to be 10^{10} pfu/ml.

2.3. Isolation and culturing of neonatal rat ventricle myocytes

Protocols employed in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Columbia University.

A previously described trypsin dissociation procedure was employed to culture neonatal rat ventricular myocytes (Zhang et al., 1992). Briefly, hearts were removed from 1–2 day old animals, the ventricles were dissected free from the remainder of the heart, and minced into 1 mm^3 cubes. The tissue was dissociated in a series of digestions in a trypsin containing solution, then resuspended in culture medium (minimum essential medium + 10% fetal calf serum). Fibroblast proliferation was minimized by using a 60 min preplating at 37°C.

2.4. Immunolocalization of the Tag-5-HT_{1B} receptor

Ventricle cells were plated onto glass coverslips at a density of 500,000 cells/ml. 24–96 h later they were infected overnight with 2×10^8 pfu of Adeno-1B. 24 h later cells were fixed in 4% paraformaldehyde and 4% sucrose in PBS for 15 min at 37°C. Then the cells were rinsed three times in PBS and permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature. Nonspecific sites were saturated with 10% goat serum in PBS supplemented with 0.1% Triton for 1 h at 37°C. To perform immunostaining on non-permeabilized cells we used the same protocol as above except that triton was omitted. The cells were incubated with 10 ng of mouse monoclonal anti-hemagglutinin and rabbit polyclonal alpha-actinin antibodies in PBS containing 3% goat serum for 1 h at 37°C. Following three washes with PBS (10 min/wash), a 1/200 dilution of Cyanin 3 conjugated goat anti-mouse IgG and FITC conjugated anti-rabbit antibodies in PBS was added, and the cells were incubated for 1 h at room temperature. At the end of this incubation, the cells were washed five times with PBS (10 min/wash), mounted onto glass slides with Aqua-poly/mount (PolyScience) and examined with a Leitz fluorescence microscope.

2.5. Electrophysiological experiments

Primary cultures of neonatal rat ventricle myocytes were used in these experiments. In some experiments cells were incubated for 24 h prior to the experiment with pertussis toxin (List Biological Lab. CA) at a concentration of 100 ng/ml. On the morning of an experiment, a 4–6 d old monolayer culture was resuspended using a 2–4 min exposure to 0.2% trypsin, then replated at low density to provide isolated single cells for patch clamping. During the experiment the cells were placed in a recording chamber and superfused constantly through a fast perfusion system that allowed a complete change of the solution surrounding the cell in less than 3 s (DiFrancesco et al., 1986). The control solution used was a high K Tyrode (mM): NaCl 140, KCl 25, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (Hepes) 5, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5.5, pH 7.4. The intracellular solution used in the patch pipette was: aspartic acid 130, KOH 146, NaCl 10, Hepes 10, EGTA 5, $CaCl_2$ 2, ATP(Mg) 2, GTP 0.2, pH 7.2. Carbamylcholine chloride (carbachol) or serotonin (5-HT) were prepared fresh daily and used at a final concentration of 10 μM. Carbachol was purchased from Sigma Chemicals, St. Louis, MO; 5-HT from Merck. All the experiments were conducted at 33°C. To antagonize the response to 5-HT in cells infected with Adeno-1B, cells were exposed to 1 μM 5-HT, either alone or in the presence of 3 μM of the $5\text{-}HT_{1B}$ antagonist, methiothepin (RBI; Natick, MA). When the experiments were done with antagonist, the cells were first superfused with methiothepin for 30–60 s, then exposed to 5-HT in the continued presence of antagonist.

The tight seal, patch clamp technique in whole cell configuration was used to record ionic currents. The cells were generally clamped at −90 mV and a stable inward holding current was confirmed prior to testing the effect of the drugs. In order to obtain a larger current, the external K^+ concentration was raised to 25 mM, resulting in a theoretical reversal potential for K^+ of −43 mV. Pipettes were prepared from borosilicate glass capillaries (Sutter Instrument, CA) and fire polished using a microforge (Narishige Scientific Instrument Lab, Tokyo). When filled with the pipette solution, the pipette had a tip resistance of 2–4 MΩ. On-line capacitance correction was employed in some experiments. Series resistance compensation was not necessary given the small amplitude of the currents recorded. Voltage protocols were controlled via PC software (patch clamp 5.5) and the acquired data recorded both on a video cassette recorder (VCR) tape and digitized and stored in a PC. The traces stored in the PC were first low pass filtered at a corner frequency of 1 kHz. Sampling rate was set at 100 Hz. Data analysis was performed using patch clamp 6.0 and MicroCal Origin 3.5 or 4.0 software. When it is stated in the results that there was no response to a particular agonist, it indicates that any drug sensitive current was < 5 pA (our limit of resolution). Cell capacitance was routinely measured for all cells using a 10 mV hyperpolarizing step. The current trace obtained was integrated over time to determine the cell capacitance.

2.6. cAMP assay

Ventricle cells were seeded into 24 well plates at a density of 250,000 cells/well. After 2 d, cells were infected with 5×10^7 pfu of virus per well. 24 h later the medium was changed and cells were incubated for another 24 h. After this time the cells were washed once with PBS and incubated for 15 min at 37°C with 100 μM isobutyl-

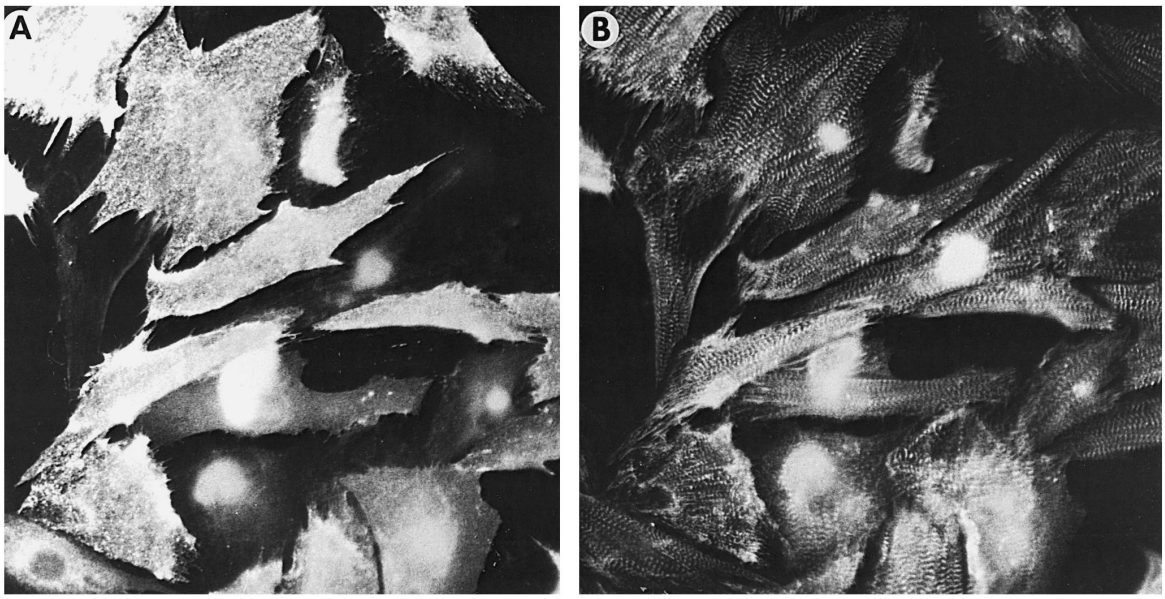

Fig. 2. Expression of the 5-HT_{1B} receptor in ventricle myocytes. Double-labeled immunofluorescence analysis was performed on ventricle myocyte cells which were grown on coverslips and infected as described in Section 2. Cells were stained with either anti-hemagglutinin (A) or anti-alpha-actinin antibodies (B), followed by fluorescently labeled secondary antibodies (Cy3 conjugated goat anti-mouse and fluorescein-labeled anti-rabbit).

methylxanthine and test agents in PBS. The reaction was stopped by aspiration of the medium, followed by the addition of 500 μl of ice cold ethanol. After 2 h at room temperature, the ethanol fraction was collected and lyophilized in a speed vac. The pellet was reconstituted and cAMP was quantified by radioimmunoassay (Immunotech radioimmunoassay kit 1117).

3. Results

3.1. Expression of a recombinant adenovirus encoding the 5-HT_{1B} receptor in ventricle myocytes

In order to determine the efficiency of infection of the 5-HT_{1B} receptor in ventricle myocytes in culture, we performed a double immunofluorescence labeling using both an anti-hemagglutinin antibody as well as an antibody against alpha-actinin which, when expressed in muscle cells, displays a characteristic striated pattern. Immunostaining of Adeno-1B infected cells with the anti-hemagglutinin antibody revealed that the 5-HT_{1B} receptor is highly expressed in these cells, with a pattern resembling that of cell surface staining. The same pattern and intensity of staining was obtained in non-permeabilized cells which further suggests that this receptor is predominately localized at the cell surface (data not shown). When stained with the antibody against alpha-actinin, more than 90% of the cells displayed a striated pattern, indicating that the primary culture was composed mostly of myocytes. These experiments also revealed that about 95% of the cells which were stained with the anti-alpha-actinin antibody were also stained with the anti-hemagglutinin antibody (Fig. 2). Adeno-1B is therefore very efficient at expressing the 5-HT_{1B} receptor in ventricle myocyte cells.

3.2. 5-HT increases an inward K^+ current in ventricle myocytes infected with Adeno-1B

To study the possibility of coupling of the 5-HT_{1B} receptor to a K^+ channel, we measured the current elicited

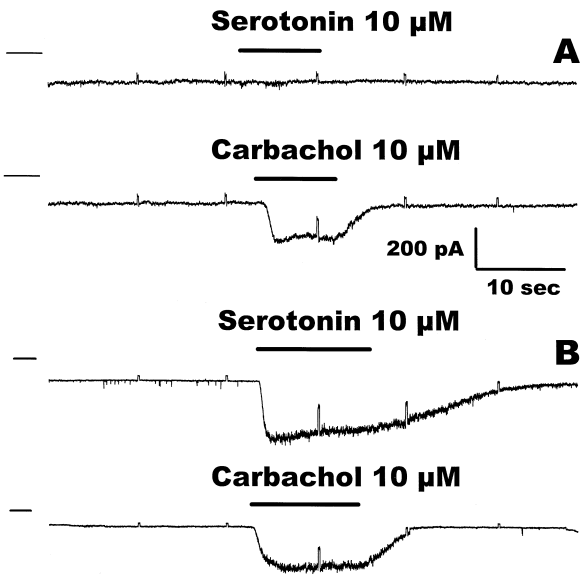

Fig. 3. After infection with Adeno-1B, ventricle myocytes respond to 5-HT. (A) Recordings from control cells show that an inward current is activated upon carbachol (CCh) perfusion (10 μM), while in the same conditions 5-HT (10 μM) does not elicit any effect. (B) Infected cells with Adeno-1B after 48 h respond to both agonists with an increase of net inward current. Pulses to test conductance were every 10 s. The bars on the upper left represent the zero current level.

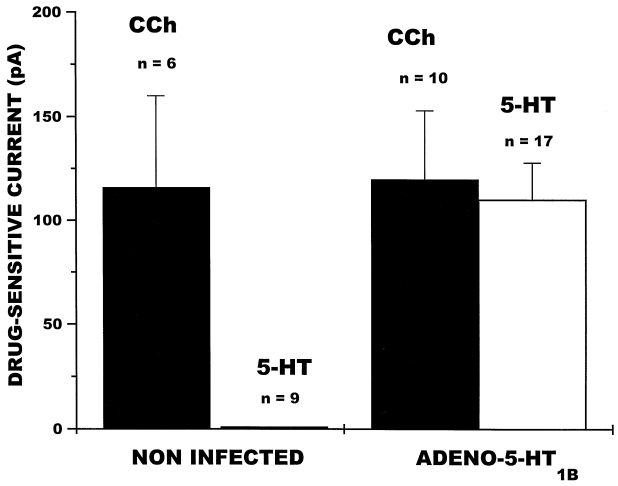

Fig. 4. Mean data of effect of 5-HT and carbachol on net current. Bar graph showing drug-sensitive currents for control cells (left) and infected cells (right). Carbachol (CCh) was equally effective in both preparations, but 5-HT was only effective in the infected cells.

by 5-HT or carbachol, an agonist of the muscarinic receptor in ventricle cells which were either non-infected or infected with Adeno-1B. Fig. 3A shows representative recordings obtained from control cells (i.e. non-infected) clamped at -90 mV. The effect of externally applied drugs on the holding current was determined. The upper panel illustrates that no effect was elicited from a 10 s exposure to 10 μM 5-HT (bar). Small (20 mV) depolarizing pulses were applied to some cells every 10 s to provide an index of membrane conductance. None of the non-infected cells tested elicited a detectable 5-HT response ($n = 9$), while all responded to carbachol ($n = 6$). The lack of effect of 5-HT on modifying the ionic fluxes across the membrane confirms that, even if present, 5-HT receptors are not functionally coupled to ligand gated ionic channels in these cells under our experimental conditions. When 10 μM carbachol was used as the perfusing drug (lower panel), after a relatively brief delay mainly due to drug delivery time, a clear inward current was observed. Upon drug wash-out, the current returned to the control level. Evaluation of membrane conductance in the presence or absence of carbachol using the small voltage pulses clearly demonstrates a reversible increase of conductance in the presence of this agonist. The mean increase in conductance was $87 \pm 33\%$ ($n = 3$).

Fig. 3B shows similar recordings obtained from Adeno-1B infected cells in the presence of either 5-HT or carbachol. Similar to the cell shown in Fig. 3A, carbachol perfusion elicited an inward deflection of the holding current which was reversible upon washout of the drug (lower panel). All 10 infected cells tested responded to carbachol. The presence of a carbachol effect indicates that the physiological receptor–effector coupling was not disrupted by viral infection. When 5-HT was used to perfuse the Adeno-1B infected cells (upper panel), a steady-state inward current was activated. All 17 infected cells tested responded to 5-HT. The effect of 5-HT, like that of carbachol, was reversible and was also associated with an increase in membrane conductance. 5-HT increased conductance $126 \pm 53\%$ ($n = 8$) and carbachol increased conductance $178 \pm 86\%$ ($n = 5$). These increases did not differ statistically from each other, or from that of carbachol in the non-infected cells (ANOVA, $p = 0.86$). The bar-graph shown in Fig. 4 summarizes the amplitude of the currents (mean $\pm$ SEM) recorded in control and Adeno-1B infected cells. Except for the lack of 5-HT induced current in non-infected cells, the other three groups have amplitudes that are not significantly different ($p = 0.97$). In order to show that the currents observed in Adeno-1B infected cells were not due to a non-specific effect of viral

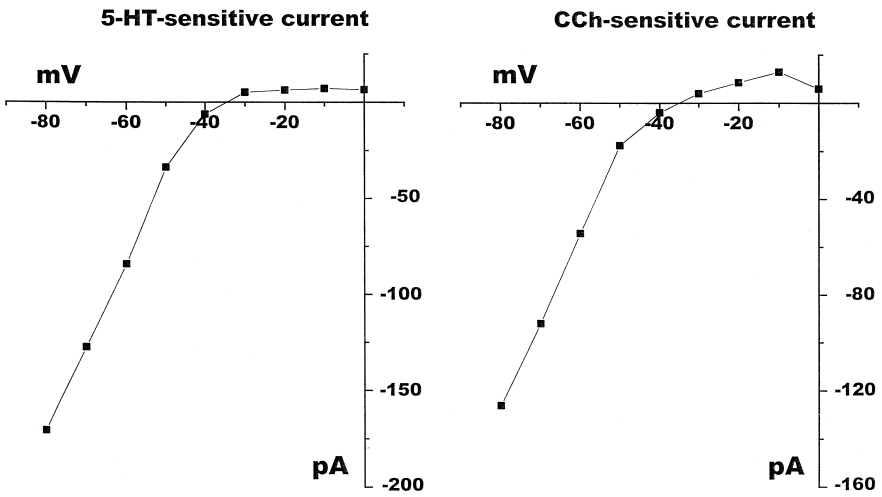

Fig. 5. The 5-HT and carbachol sensitive currents are inwardly rectifying. A series of voltage steps were imposed from a holding potential of -40 mV and the current at the end of a 500 ms test pulse was measured. The protocol was repeated prior to and during superfusion with 10 μM 5-HT (left) or carbachol (CCh, right) and the drug-sensitive difference current determined. 5-HT and carbachol were tested on different cells. A total of 5 cells were tested with each drug and representative I–V relations are shown.

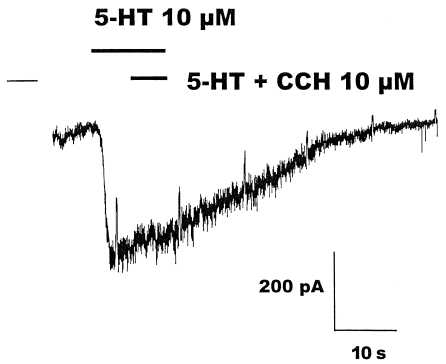

Fig. 6. Maximal responses to 5-HT and carbachol occlude each other. When a maximal current response was induced by 5-HT (10 μM), additional application of carbachol (CCh, 10 μM) in an infected ventricle cell produced no further response. The same type of experiment was repeated in 5 cells, with similar results, regardless of the order of agonist exposure.

infection, but were a direct consequence of the expression of the 5-HT_{1B} receptor, we measured the responses to carbachol and 5-HT in cells infected with a recombinant adenovirus expressing β-galactosidase (Adeno-βgal) under the control of the *Cyto megalo* virus early promoter. These infected cells exhibited a normal response to carbachol ($n = 4$) but no response in any cell ($n = 5$) to 5-HT (data not shown). We also antagonized the response to 5-HT (1 μM) with methiothepin (3 μM), an antagonist of the 5-HT_{1B} receptor, in cells infected with Adeno-1B. All 5 cells exposed to 5-HT (1 μM) in the absence of antagonist exhibited a 5-HT sensitive current (mean $\pm$ SEM = 131 $\pm$ 84 pA, $n = 5$). Of 5 cells from the identical culture preparation tested in the presence of methiothepin, only one exhibited a measurable 5-HT sensitive current, and it was less than 20 pA (mean $\pm$ SEM = 3.8 $\pm$ 3.8 pA, $n = 5$). The response of the two groups was statistically different ($p = 0.016$).

While these experiments demonstrate that the 5-HT_{1B} receptor can couple to an ionic channel, they do not prove that the cascade or channel activated by 5-HT and carbachol are the same. To confirm this, additional experiments were undertaken. First, we determined the current–voltage relation of the drug-sensitive current by imposing a series of voltage steps before and during exposure, and subtracted the two curves to generate the drug-sensitive current. Fig. 5 shows representative *I–V* relations for an infected cell treated with 5-HT (left) and another infected cell treated with carbachol (right). It is clear that activation of either the 5-HT_{1B} or the muscarinic receptors results in a similar inwardly rectifying current (Pfaffinger et al., 1985). The activation of an adenylyl cyclase inhibitory G-protein (G_i) is known to directly couple the muscarinic receptor K^+ current (Codina et al., 1987; Yatani et al., 1988). Therefore, we next tested for the presence of a G_i-dependent step in the cascade following the activation of the 5-HT_{1B} receptor. The cells were pretreated for 24 h with pertussis toxin, which is known to inactivate all the G_i/G_0 proteins in these cells (Steinberg et al., 1989). When cells were subsequently exposed to either 5-HT ($n = 5$) or carbachol ($n = 5$) no changes in membrane conductance were observed in any cells (data non shown). This indicates that the coupling between the 5-HT_{1B} receptor and the observed increase of membrane conductance, is mediated by a pertussis toxin-sensitive G protein. Another indication that 5-HT and carbachol might act via a common pathway would be if the effects of the two agonists were not additive. A cell was first exposed to 5-HT (10 μM) long enough to reach a steady state, then the perfusion was rapidly switched to a solution containing both carbachol and 5-HT (10 μM each). In this experiment carbachol elicited no additional effect (Fig. 6). In other experiments, no evidence of additivity was found, independent of which drug was applied first (data not shown). The desensitization of the response seen in Fig. 6 was present to a variable degree in individual cells. This is consistent with the reported effect of cell dialysis on this process (Shui et al., 1995).

3.3. 5-HT inhibits adenylyl cyclase activity in ventricle myocytes infected with Adeno-1B

Since 5-HT_{1B} receptors have been shown to inhibit adenylyl cyclase in fibroblasts (Voigt et al., 1991; Adham et al., 1992; Maroteaux et al., 1992), we investigated whether that coupling was also effective in myocytes. The concentration of cAMP detected in the assay using either infected or non-infected cells was 2.2 nM in baseline

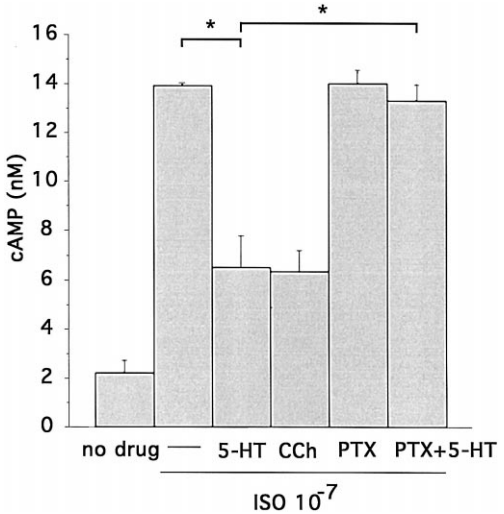

Fig. 7. 5-HT and carbachol induced decrease in cAMP levels in myocyte cells infected with Adeno-1B. In infected cells, the application of 10 μM carbachol (CCh) or serotonin inhibited the isoproterenol (100 nM) stimulated level of cAMP via a pertussis toxin-sensitive pathway. Pertussis toxin was applied at a concentration of 100 ng/ml 20 h before the addition of different drugs. Data are the mean of three independent experiments, with each determination performed in duplicate. The inhibitory effect of 5-HT and the blockade of 5-HT's effect by pertussis toxin were statistically significant ($^* p < 0.01$).

conditions and was stimulated up to 14 nM in the presence of 100 nM of isoproterenol, which is an agonist of the endogenously expressed β-adrenergic receptors. In non-infected cells, application of 10 μM of serotonin had no effect on either the basal level of cAMP or on the inhibition of isoproterenol stimulated cAMP levels (data not shown). In contrast, in cells infected with Adeno-1B, serotonin (10 μM) inhibited the isoproterenol-stimulated cAMP level from 14 nM to 5 nM (Fig. 7). Incubation of infected cells for 24 h in the presence of 100 ng/ml pertussis toxin blocked the effect of serotonin, indicating that the 5-HT_{1B} receptor inhibits adenylyl cyclase activity via a pertussis toxin-sensitive G protein. In both infected (Fig. 7) and non-infected cells (data not shown), carbachol inhibited the isoproterenol-stimulated cAMP accumulation from 14 nM to 6 nM. 5-HT_{1B} receptors therefore appear to be coupled as efficiently to adenylyl cyclase as do the endogenous muscarinic receptors.

4. Discussion

The presented data shows that the 5-HT_{1B} receptor, when expressed in ventricle cells via a recombinant adenovirus, can couple to an inwardly rectifying K^+ channel.

We compared the effects of carbachol on endogenous muscarinic receptors with the effects of serotonin on the 5-HT_{1B} receptor expressing cells. These data clearly demonstrate a reversible increase of membrane conductance in the presence of carbachol in non-infected cells and in the cells infected with Adeno-βgal. Since carbachol has been shown to activate muscarinic-gated, inwardly rectifying K^+ channels (i_{KACh}) in ventricle cells (Koumi and Wasserstrom, 1994; Ito et al., 1995), it is reasonable to assume that the carbachol-activated inward current observed in our experiments was due to the opening of the same channels. In non-infected cells or in those infected with Adeno-βgal, 5-HT had no effect, while, in cells infected with Adeno-1B, both 5-HT and carbachol activated a steady-state inward current. The similar *I–V* relations of the carbachol-sensitive and 5-HT-sensitive currents, and the non-additivity of the response suggest that 5-HT and carbachol elicit the opening of the same channels. Under the conditions used for the electrophysiological recordings, we were not able to observe any differences between the activation rates for the carbachol and 5-HT responses. However, we noticed an apparent slower reversal of the 5-HT effect (Fig. 3b) but did not analyze it since it would not be meaningful in the absence of data on relative receptor number and affinity. The coupling of the 5-HT_{1B} receptor to inwardly rectifying K^+ channels (i_{KACh}) is probably not the consequence of an overexpression of 5-HT_{1B} receptors in cardiac cells, because the same response was recorded in cells expressing low levels of the 5-HT_{1B} receptor as those expressing high levels, as measured by immunohistochemistry (see Fig. 2).

The activation of a similar inwardly rectifying K^+ current by 5-HT or carbachol, the non-additivity of the carbachol and 5-HT effects, the inhibition of adenylyl cyclase by both 5-HT and carbachol, and the suppression of the responses to these agonists by pertussis toxin, all indicate that the muscarinic and 5-HT_{1B} receptors share common effector pathways. In addition, since muscarinic receptors have been shown to couple directly to inwardly rectifying K^+ channels (i_{KACh}) (Codina et al., 1987; Yatani et al., 1988), it is likely that the 5-HT_{1B} receptor also couples directly to these channels via a pertussis toxin-sensitive G-protein. Ventricle cells express all three types of G_i isoforms, whereas little G_0 is found in these cells (Matesic et al., 1991). In addition, the muscarinic receptors in ventricle cells have been shown to be associated only with $G_{i\alpha 2}$ (Matesic et al., 1991). More experiments are needed to show whether the 5-HT_{1B} receptor is also preferentially associated with one of the $G_{i\alpha}$ isoforms present in ventricle cells.

It has been shown that activation of the inwardly rectifying K^+ channel proceeds through a direct coupling between the G protein beta–gamma subunits and the channel itself (Logothetis et al., 1987; Wickman et al., 1994). These observations suggest that the activation of any G-protein coupled receptor might activate these channels as a result of the production of free $\beta\gamma$ subunits. However, in atrial myocytes, the atrial, muscarinic gated, inwardly rectifying K^+ channel is activated by G_i coupled (muscarinic) but not by G_s coupled receptors (β-adrenergic receptors) (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985). This paradox might be explained by the recent observation that atrial G protein-activated K^+ channels can be directly inhibited by certain G_α subunits such as α_s, α_0, and α_{i1}, but not by α_{i2} and α_{i3} (Schreibmayer et al., 1996). These observations might suggest a preferential association of the 5-HT_{1B} receptor with α_{i2} or α_{i3}.

In neurons, the 5-HT_{1B} receptor is localized on axon terminals, where it inhibits neurotransmitter release (Boschert et al., 1994). The inhibition of neurotransmitter release by activation of this presynaptic receptor probably results from the modulation of effector systems found in nerve terminals. Adenylyl cyclase is not a likely candidate because stimulation of adenylyl cyclase in rat hippocampal slices had no effect on 5-HT_{1B}-mediated inhibition of [^{3}H]5-HT release (Ramdine et al., 1989). G-protein gated inwardly rectifying K^+ channels might be better candidates because of our present finding.

In summary, we have used ventricle cells to show that the 5-HT_{1B} receptor can couple to an inwardly rectifying K^+ channel in these cells. The immunostaining and electrophysiological experiments reveal that all ventricle cells exposed to the recombinant adenovirus express the 5-HT_{1B} receptor and responded to 5-HT. This expression persisted for at least 2 weeks after infection and no cytopathic effect was observed (data not shown). The adenovirus is therefore a very efficient system for expressing exogenous

genes in cardiac cells and as a consequence is very well suited for single cell studies.

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