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Expression in animal cells of the 5-HT_{1A} receptor by a vaccinia virus vector system

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The co-infection or infection–transfection variants of the T7 RNA polymerase/vaccinia vector system were used to express 5-HT_{1A}Rs in COS-7, BSC-40 and GH_3 cells, with co-infection giving ca. 3-fold higher level than infection–transfection. Binding affinities were similar to those of the endogenous 5-HT_{1A}R, with highest affinities for 5-HT and 8-OH-DPAT. Functional properties were demonstrated by assays of agonist-stimulated GTPase activity and its inhibition by pertussin toxin. Immunoblot assays showed expression of the unglycosylated and glycosylated receptor protein in the membrane and, surprisingly, in the cytosolic fractions.

5-HT_{1A} receptor; Vaccinia virus vector; Receptor affinity; GTPase; COS-7, BSC-40 cells; GH_3 cells

1. INTRODUCTION

The vaccinia virus (VV) vector system offers unique advantages for transient expression of cDNAs in mammalian cels, including a broad host range and high level expression [1]. It is effective for expressing integral membrane proteins, including ion channels [2] and 7-helix receptors [3]. The serotonin 1A receptor (5-HT_{1A}R), a member of the 7-helix, G-protein-activating receptor family [4,5], is implicated in many pharmacological and behavioral actions including the control of cardiovascular functions, pain perception, anxiety and depression (for reviews, see [6]). These various functions are probably related to the coupling of the 5-HT_{1A}R to different effectors: adenylyl cyclase, phospholipase C and K^+ channels [7–9]. For these reasons, we have studied the use of the vaccinia system for expressing the 5-HT_{1A}R in a variety of cell types and have characterized the intracellular distribution of the expressed membrane protein.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Cells were grown in a humidified environment of 5% CO_2 at 37°C in the following media supplemented with penicillin G (100 U/ml) and streptomycin (100 μg/ml): COS-7 cells in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (FBS) and 2 mM L-glutamine; BSC-40 cells in minimum essential medium with 5% FBS and 2 mM L-glutamine; GH_3 cells in Ham's F-10 medium with 2.5% FBS and 15% horse serum.

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2.2. *Expression of the 5-HT_{1A}R by coinfection and infection–transfection*

Recombinant plasmid (pTM1-5HT1AR) and a recombinant vaccinia virus (VV:5HT1AR) carrying the 5-HT_{1A}R cDNA were constructed as reported previously [3]. Co-infection was performed by infecting cells with VV:5HT1AR and the helper VV (vTF7-3), each at multiplicity of infection (MOI) of 3–5. For infection–transfection, 6×10^6 cells were first infected with vTF7-3 at MOI of 3–5, and transfected with a mixture containing 30 μg. Lipofectin reagent (Bethesda Research Laboratories) and 10 μg pTM1-5HT1AR in 2 ml serum-free media for 12 h.

2.3. *Preparation of membrane for binding and GTPase assays*

Cells were lysed over ice with hypotonic buffer (5 mM Tris-HCl, 5 mM EDTA, 100 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, pH 7.4) and centrifuged at $1,000 \times g$ for 10 min to remove cell debris. The supernatant was centrifuged at $35,000 \times g$ for 20 min. The pellet was washed once in the lysis buffer and recentrifuged. Protein concentrations were determined according to Lowry et al. [10] using bovine serum albumin as the standard.

2.4. *Receptor binding and GTPase assays*

Saturation and competitive binding studies were performed with [^{3}H]serotonin (23.2–29.8 Ci/mmol) and analyzed with modification as described [11]. Incubation was carried out in a volume of 0.5 ml (50 mM Tris-HCl, 1 mM $MnCl_2$, pH 8.2) at room temperature for 1 h. GTPase activity was determined by the method of Cassel and Selinger [12]. The agonist-stimulated low-K_m GTPase activity was calculated by subtracting the high-K_m GTPase activity (determined in the presence of 50 μM unlabeled GTP) from the total GTPase activity.

2.5. *Immunoblots*

Cytosolic and membrane fractions were prepared from COS-7 cells [13]. Proteins were separated on a 0.1% SDS, 12.5% polyacrylamide gel and transferred onto a BA 85 nitrocellulose (Schleicher and Schuell) or Hybond N nylon filter (Amersham Corp.). Proteins were detected with a purified antibody directed against the predicted amino acids 242–267 of the 5-HT_{1A}R [4] by the colorimetric method using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate

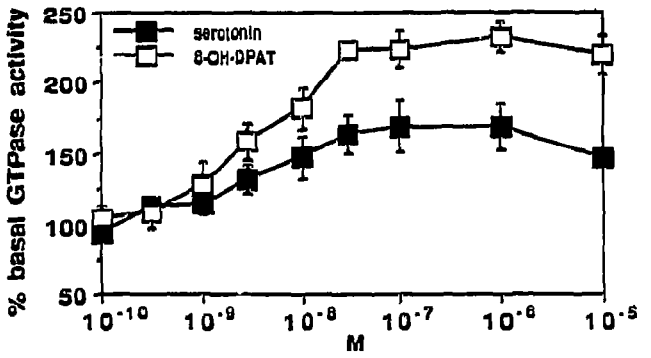

Fig. 1. Stimulation of low-K_m GTPase activity by serotonin and 8-OH-DPAT in COS-7 cells co-infected with VV:5HT1AR and vTF7-3. The high-K_m (non-specific) GTPase activity and basal low-K_m (agonist-sensitive) GTPase activity were 7.52 ± 1.62 nmol/mg protein/min and 30.44 ± 5.61 pmol/mg protein/min. Data are expressed as percent of basal low K_m GTPase activity in each assay. Results are mean ± S.E.M. from at least three separate coinfection experiments.

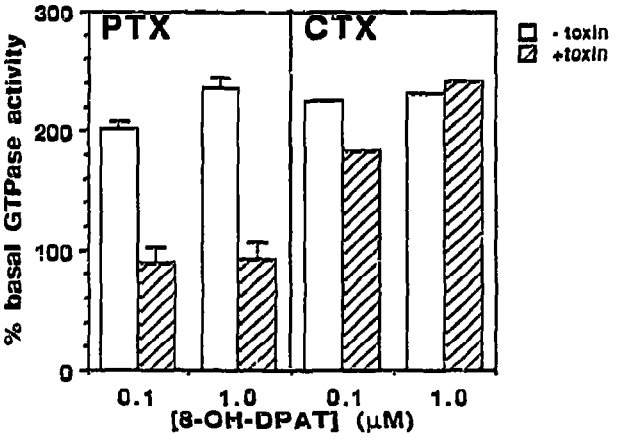

Fig. 2. Effects of toxin pretreatment on the stimulation of GTPase activity. Pertussis toxin (PTX) and cholera toxin (CTX) were pre-activated by incubating with 40 mM dithiothreitol, and 2 mM ATP for 20 min at 37°C. ADP-ribosylation of membranes from co-infected COS-7 cells was performed in buffer containing 100 mM Tris-HCl (pH 7.5), 20 μg/ml pertussis toxin or 50 μg/ml cholera toxin, 10 mM NAD, 1mM ATP, 6 mM DTT and 1 mM EDTA for 30 min at 37°C. The reaction was terminated by the addition of 1 ml ice-cold buffer (25 mM Tris–HCl, 0.1 mM EDTA). Membranes were washed two times in 10 volumes of the same buffer to remove the toxins and used immediately for GTPase assays. Control (no toxin treatment) and toxin experiments were performed simultaneously using the same membranes from each coinfection experiment. Data are expressed as the percent of the respective basal low-K_m GTPase activity (in the absence of 8-OH-DPAT). Results were derived from three separate coinfections for PTX pretreatment and two for CTX pretreatment.

(Promega) or the chemiluminescence method using Lumi-Phos 530 (Boehringer Mannheim).

2.6. *Materials*

Cell culture media were purchased from Irvine Scientific. Drugs were obtained from the following sources: serotonin HCl, dopamine HCl, norepinephrine HCl, epinephrine and pindolol (Sigma); 8-OH-DPAT HBr (8-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin), mianserin HCl and quipazine dimaleate (Research Biochemical Inc.); pertussis toxin and cholera toxin (List Biological Laboratories); [^{3}H]serotonin and [γ-^{32}P]GTP (Du-Pont New England Nuclear).

3. RESULTS AND DISCUSSION

3.1. *Expression level and pharmacology*

When 5-HT$_{1A}$Rs were expressed with either the co-infection or infection–transfection variants of the T7 RNA polymerase/vaccinia vector system, expression peaked at about 24 h postinfection and decreased after 2–3 days (data not shown). Therefore, membranes were prepared 24 h postinfection throughout the study. In the transfection–infection protocol, cells were infected with vTF7-3, which expresses the T7 RNA polymerase, and transfected with pTM1-5HT$_{1A}$R, carrying the receptor cDNA, driven by an T7 RNA polymerase promoter, with the 5′-untranslated region of the encephalomyocarditis virus to enhance cap-independent translation [14]. For the co-infection protocol, the 5-HT$_{1A}$R expression cassette of pTM1-5HT1AR was inserted into VV by homologous recombination to give VV:5-HT1AR, and cells were co-infected with vTF7-3 and VV:5-HT1AR. Expression levels of the 5-HT$_{1A}$Rs in BSC-40 and COS-7 cells (approximately 3 pmol/mg protein or 3×10^5 receptors/cell) were higher than that in GH$_3$ cells (Table I). The latter two cell types were chosen for study because they display signal transduction pathways that may couple to the expressed 5-HT$_{1A}$R: namely inhibition of adenylate cyclase in COS-7 cells [7] and potassium channel coupling in GH$_3$ cells [9]. Such levels are comparable to those of other 7-helix receptors expressed in various expression systems [7,15]. When compared in COS-7 cells, the coinfection protocol was about 3-fold as efficient as the infection–transfection protocol. This is probably because virus infection is more efficient than DNA transfection and because of intracellular replication by virus. Competitive binding

Table I

Expression of the 5-HT$_{1A}$R 24 h after coinfection or infection–transfection

	B_{max} (pmol/mg protein)	K_d (nM)
Co-infection[a]		
Monkey kidney COS-7 cells	2.88 ± 0.56	1.89 ± 0.28
Monkey kidney BSC-40 cells	2.57 ± 0.61	1.92 ± 0.24
Rat pituitary tumor GH$_3$ cells	0.75 ± 0.12*	1.92 ± 0.36
Infection–transfection in COS-7 cells[b]	0.82 ± 0.04*	1.79 ± 0.32

Data are expressed as mean ± S.E.M., from at least three independent experiments. No specific binding was detected in cells that were uninfected or infected with the wild type VV.

[a] COS-7 cells were co-infected with VV:5-HT$_{1A}$R and vTF7-3.

[b] COS-7 cells were infected with TF7-3 and transfected with pTM1-5HT1AR.

* $P < 0.05$, compared to coinfection in COS-7 cells, Student's t-test

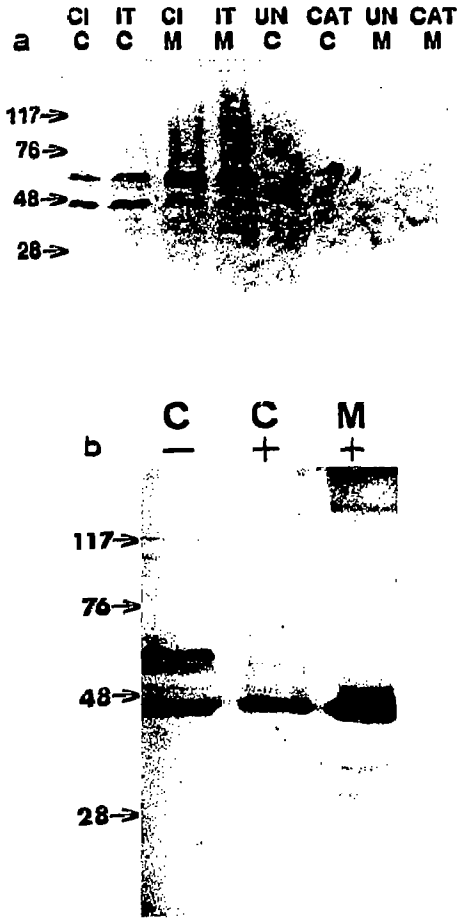

Fig. 3. Assessment of the 5-HT_{1A} receptor protein in the cytosolic (C) and membrane (M) fractions of COS-7 cells after coinfection (CI) or infection–transfection (IT). (a) Control cells were either uninfected (UN, lanes 5 and 7) or coinfected with vTF7-3 and vT7EMCAT, a virus expressing the CAT gene with the same flanking sequences as VV:5HT1AR (CAT, lanes 6 and 8). Ten (CI) or 40 μg protein (IT, UN, CAT) was loaded onto each lane, and analyzed by 12.5% SDS-PAGE. Proteins were immunoblotted and detected with the chemiluminescence method. (b) Cytosolic and membrane fractions from coinfected cells (+, lanes 2 and 3) were treated with 0.5 U of endoglycosidase F/glycopeptidase F (Boehringer Mannheim) for 20 h at 37°C in 20 mM potassium phosphate buffer, 10 mM EDTA, 0.2% SDS, 1% Nonidet P-40, 1% β-mercaptoethanol, pH 7.4. Lane 1 shows the result of untreated cytosolic fraction (−) from coinfected cells. Proteins were analyzed as in (a) and detected with the colorimetric method. Numbers at the left in (a) and (b) represent the positions of size standards in units of kDa.

assays showed a rank order of potency of 8-OH- DPAT ~5-HT>RU-24,969 > pindolol > mianserin > quipazine, and micromolar affinities for other biogenic amines, dopamine, epinephrine and norepinephrine (Table II). Therefore, the expressed receptor showed a 5-HT1A-type pharmacology [16].

3.2. *GTPase activity in coinfected COS-7 cells*

A functional coupling of the expressed receptor to G protein is indicated by its ability to stimulate GTPase activity (Fig. 1). The EC_{50} values for 5-HT and 8-OH-DPAT are estimated as 2.4 nM and 3.2 nM, respectively, which are close to the affinities found in the binding asays (Table I and II). The increase in GTPase activity in response to 8-OH-DPAT was abolished by pretreatment with pertussis toxin, but cholera toxin had no effect (Fig. 2). Thus, like the endogenous 5-HT_{1A}R [16], the expressed 5-HT_{1A}R in COS-7 cells acts primarily through a pertussis toxin-sensitive, cholera toxin-insensitive G protein.

3.3. *Intracellular distribution*

The observed values of B_{max} achieved for the 5-HT_{1A}R in the vaccinia/T7 hybrid expression system, are comparable to those achieved in other efficient gene transfer systems [7,15]. However, much higher expression levels of ca. 10% of total protein were achieved for expression of the cytoplasmic protein, chloramphenicol acetyltransferase (CAT), using the same VV system [17]. This discrepancy has prompted us to examine whether additional 5-HT_{1A}R protein is found in the cytoplasm in infected COS-7 cells. Relative levels of expression in the membrane and cytosolic fractions were compared by immunoblots (Fig. 3a). For each of the membrane fraction lanes, there was a strong band at 61 kDa, a weak band at 57 kDa, and a very weak band at 46 kDa. Similar bands were observed in the cytosolic fraction, but there was a much greater amount of immunoreactive protein at the 46-kDa band than the other bands. From its translated cDNA sequence, the calculated molecular weight of the 5-HT_{1A}R protein is 46 kDa and it contains 3 arginine residues as potential sites for *N*-linked glycosylation. To test whether the higher M_r components are glycosylated forms of the 46-kDa band, the fractions were treated with endoglycosidase F/glycopeptidase F which cleaves glycoproteins at the asparagine to *N*,*N*′-diacetylchitobiose bond, leaving a completely deglycosylated protein. After treatment, only the 46-kDa component remained (Fig. 3b). We

Table II

Affinity of the expressed 5-HT_{1A}R

Competitive ligand	K_i (nM)
Serotonin	1.13 ± 0.45
8-OH-DPAT	0.81 ± 0.12
RU-24969	4.74 ± 0.44
Pindolol	17.87 ± 2.04
Mianserin	518 ± 64
Quipazine	> 1,000
Dopamine	> 1,000
Norepinephrine	>10,000
Epinephrine	> 1,000

Assays were performed with 1 nM [^{3}H]serotonin and different competitive ligands. K_i values were calculated using the K_d value of co-infected COS-7 cells in Table I. The K_i values are expressed as mean ± S.E.M. from at least three separate coinfection experiments, except for quipazine, dopamine, and norepinephrine, the values of which were determined from two experiments.

therefore conclude that the 57- and 61-kDa forms are glycosylated. The protein in the cytosolic fraction is mostly not glycosylated, whereas most of the protein in the membrane fraction as prepared here is glycosylated. A quantitative comparison of the amounts of 5-HT_{1A}R in the membrane and cytosolic fraction was not attempted; this estimate would require the assumption that the glycosylated and nonglycosylated forms are equally reactive to the antibody as well as a knowledge of the fraction of total cell protein in the membrane fraction. Assuming the latter quantity is probably in the order of 5–10% and immunoreactivities are not grossly different between these two forms, the amount of 5-HT_{1A}R protein in the cytosol fraction is severalfold greater than in the membrane fraction.

The T7 RNA polymerase/vaccinia vector system has the unique advantage of a very broad host range, including primary cells. We have demonstrated that the system is effective for high level expression of a functional 7-helix G-protein-coupled receptor in several cell types. The unexpected finding is that a high level of immunoreactive protein was also found in the cytosolic fraction. While the expression levels achieved are adequate for pharmacological and functional studies, they are insufficient for structural studies. To achieve this latter goal, it will be necessary to overcome some unknown limiting factors on the expression of integral membrane proteins that do not apply to cytosolic proteins.

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