Functional characterisation of the *Drosophila* 5-HT$_{dro1}$ and 5-HT$_{dro2B}$ serotonin receptors in insect cells: activation of a $G_{\alpha s}$-like protein by 5-HT$_{dro1}$ but lack of coupling to inhibitory G-proteins by 5-HT$_{dro2B}$

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Received 27 December 1995

Abstract Insect cells are routinely used for the production of receptor proteins. Expression of the *Drosophila* 5-HT$_{dro1}$ serotonin receptor resulted in positive coupling of the receptor to adenylyl cyclase via the $G_{\alpha s}$ G-protein subtype. The *Drosophila* 5-HT$_{dro2B}$ receptor stimulated the metabolism of inositol phospholipid via a pertussis toxin-insensitive G-protein, but exhibited no detectable inhibition of adenylyl cyclase. Immunoblot analysis of the endogenous G-proteins revealed that Sf9 cells lack the G-protein subtypes $G_{\alpha i1-3}$ and $G_{\alpha o}$, but express the subtypes $G_{\alpha s}$ and $G_{\alpha q}$.

Key words: *Drosophila* 5-HT$_{dro1}$ receptor; *Drosophila* 5-HT$_{dro2B}$ receptor; Baculovirus expression; Insect cell (Sf9); G-protein

1. Introduction

The baculovirus expression system which utilizes insect cell lines derived from *Spodoptera frugiperda* (Sf9 or Sf21 cells) [1] has proved to be a popular system for the characterization of receptors and ion channels [2]. However, it is unclear whether Sf9 cells synthesize a full complement of G-proteins to enable the use of this cell line for detailed studies on receptor-G-protein interaction. The available reports [3,4] appear to highlight some inconsistencies in the class of G-protein subtypes that may be present in Sf9 cells.

We chose to express two *Drosophila* serotonin receptors, the 5-HT$_{dro1}$ [5] and 5-HT$_{dro2B}$ [6], in Sf9 cells. Previous studies had assigned the 5-HT$_{dro1}$ to the family of serotonin receptors that activate adenylyl cyclase (AC), while the 5-HT$_{dro2B}$ receptor appears to belong to the family of 5-HT receptors that are coupled negatively to AC. Therefore, the functional coupling of the 5-HT$_{dro1}$ and 5-HT$_{dro2B}$ receptors via different classes of endogenous G-proteins in Sf9 cells could be investigated. In addition, the dominant classes of G-protein subtypes present in these cells have been identified by immunoblotting. The data presented here suggest that the 5-HT$_{dro1}$ receptor positively couples to AC via the $G_{\alpha s}$ subtype, whilst the 5-HT$_{dro2B}$ receptor stimulated the production of inositol triphosphate via subtype $G_{\alpha q}$. We were unable to detect receptor-mediated inhibition of AC following expression of the 5-HT$_{dro2B}$ receptor in insect cells.

2. Materials and methods

2.1. Production of recombinant baculoviruses

Standard molecular biology techniques were used to produce the recombinant baculovirus transfer vectors pAc5HT$_{dro1}$ and pAc5HT$_{dro2B}$. All procedures for cell culture, production of recombinant viruses by co-transfection, identification and amplification of recombinant viruses were as detailed [7]. The recombinant viruses Ac5HT$_{dro1}$ and Ac5HT$_{dro2B}$ were produced by co-transfection of Sf21 cells with linearized BacPAK6 DNA [8] and either pAc5HT$_{dro1}$ or pAc5HT$_{dro2B}$, using Lipofectin (Gibco Life Technologies).

2.2. 5-[^{3}H]HT binding assays

For ligand binding analysis, virus-infected Sf9 cells were harvested at 48 h post infection and used to prepare membranes as described [9]. Aliquots of the prepared membranes (50–80 μg protein) were incubated with various concentrations of 5-[^{3}H]HT ranging from 0.75 nM to 3 μM in a final assay volume of 0.25 ml. Specific binding was defined using 1 mM 5-HT for all experiments. All experiments were carried out in duplicate and the data were analysed using computer fitting program LIGAND [10].

2.3. [^{3}H]Inositol labelling experiments and cAMP assays

Cells infected with the Ac5HT$_{dro2B}$ virus were transferred to *myo*-inositol-free TC100 medium, plus 5% fetal calf serum. At 22 h post-infection, 2 μCi *myo*-[^{3}H]inositol/1 × 10^6 cells was added in the presence or absence of pertussis toxin (2.5 μg/ml). The cells were pelleted at 36 h post-infection, washed three times in assay buffer; phosphate-buffered saline (PBS), 1.25 mM $CaCl_2$ and 1 mg/ml amino acid-free bovine serum albumin (BSA). All subsequent procedures were carried out as described [11]. Stimulation of cAMP was achieved by incubating Sf9 cells in the presence of 5-HT according to the procedure described [12]. Levels of cAMP in the samples were quantified using a radioimmunoassay kit (Immunotech) according to the supplier's instructions.

2.4. Immunoblot analysis

Membrane preparations from Sf9 cells were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting [13]. The membranes were blocked for 1 h at room temperature in PBS/3% skimmed milk, washed 3 times in PBS, and then incubated in primary antibody for 1 h at room temperature. The primary antibodies AS/7, EC/2, RM/1, SW/1 and GC/2 were purchased from Dupont NEN and used at a 1:1000 dilution in PBS. The antibodies IM1, OC1 and ON1 were kindly provided by Professor G. Milligan (University of Glasgow). Antisera IM1 and OC1 were used at a 1:2000 dilution and OC1 at a 1:800 dilution. The blots were washed and incubated for 1 h at room temperature in secondary antibody conjugated to peroxidase, diluted at 1:1000. The blots were then washed, and antibody complexes detected using an ECL kit (Amersham International).

3. Results and discussion

Two recombinant baculoviruses, containing the *Drosophila* 5-HT$_{dro1}$ and 5-HT$_{dro2B}$ receptor genes under the control of

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SSDI S0014-5793(96)00078-6

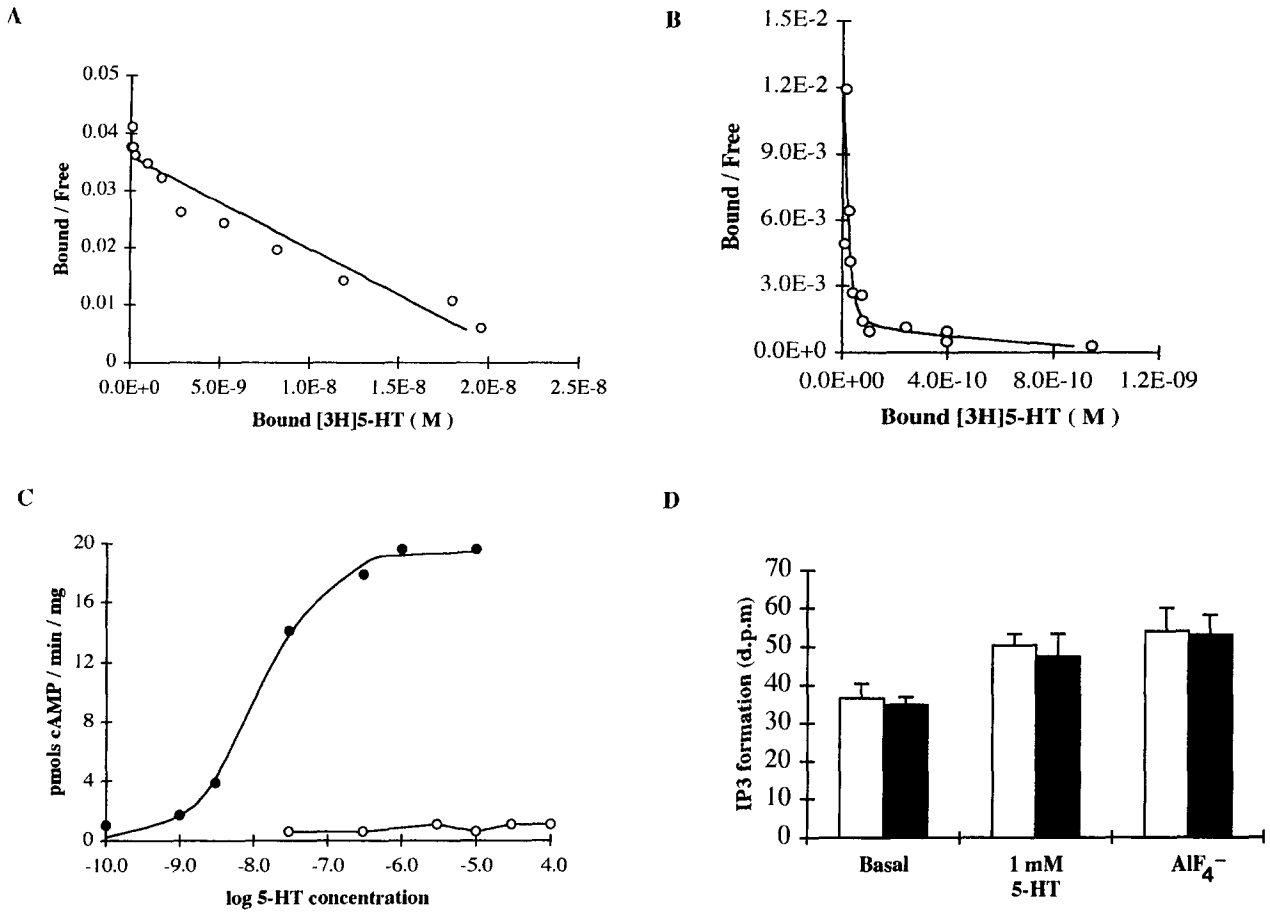

Fig. 1. (A,B) Scatchard plots of 5-[^{3}H]HT binding to the 5-HT_{dro1} (A) and 5-HT_{dro2B} (B) receptors, following expression in Sf9 cells. (C) 5-HT induced stimulation of cAMP in Sf9 cells infected with the recombinant baculovirus $Ac5HT_{dro1}$ harvested at 30 h post-infection (solid circles). Open circles represent Sf9 cells infected with the modified baculovirus AcMNPV-*lacZ*. (D) Effect of pertussis toxin on the accumulation of inositol triphosphates in Sf9 cells infected with the recombinant virus $Ac5HT_{dro2B}$. Solid bars indicate cells incubated for 18 h in the presence of 2.5 μg/ml pertussis toxin prior to assay. Fluoroaluminate (AlF_4^-) was obtained by combining 20 mM NaF with 10 μM $AlCl_3$.

the highly expressed polyhedrin gene promoter were constructed. Crude membranes prepared from $Ac5HT_{dro1}$-and $Ac5HT_{dro2B}$-infected Sf9 cells at 48 h post-infection, were found to bind 5-[^{3}H]HT in a specific and saturable manner (Fig. 1A,B). Analysis of the saturation data for the 5-HT_{dro1} receptor showed the existence of a single binding site with a K_d of 0.64 (±0.04) μM and a receptor density (B_{max}) of 61 (±1.8) pmol/mg of membrane protein (Fig. 1A). To characterise further the 5-HT_{dro1} protein, the ability of this receptor to increase cAMP levels in Sf9 cells was investigated. In the presence of 5-HT, $Ac5HT_{dro1}$ infected Sf9 cells showed a dose-dependent increase in cAMP (Fig. 1C). The concentration of 5-HT required for half-maximal stimulation (EC_{50}) was 12.9 (±0.8) nM. Control experiments performed using a modified *Autographa californica* nucleopolyhedrosis virus (AcMNPV), AcMNPV-*lacZ* [7], did not result in detectable increases in cAMP levels above basal (Fig. 1C).

Analysis of the saturation data obtained for the $Ac5HT_{dro2B}$ receptor revealed the expression of two classes of receptor binding site for the radiolabeled agonist 5-[^{3}H]HT, (K_{d1} = 3.8(±0.5) nM; B_{max} = 0.057(±0.010) pmol/mg protein and K_{d2} = 1.7 μM; B_{max} = 2.03 pmol/mg of protein) (Fig. 1B). The density of 5-HT_{dro2B} receptor expression was considerably lower than that achieved with the 5-HT_{dro1} receptor. However, the existence of a high-affinity ligand binding site suggested coupling of the 5-HT_{dro2B} receptor with an endogenous G-protein in Sf9 cells.

Previous studies using a stably-transformed mouse cell line (NIH3T3) expressing the 5-HT_{dro2B} receptor failed to detect 5-[^{3}H]HT binding, but clearly demonstrated the ability of this receptor not only to induce a decrease in cAMP levels stimulated by forskolin, but also to increase levels of intracellular inositol triphosphates (IP_3) [6]. We were unable to detect a 5-HT induced decrease in forskolin-stimulated cAMP levels in Sf9 cells expressing the 5-HT_{dro2B} receptor. However, incubation of $Ac5HT_{dro2B}$-infected Sf9 cells with 1 mM 5-HT resulted in a significant increase ($P < 0.01$) in intracellular IP_3 levels (Fig. 1D). This stimulation was comparable to that evoked by the positive control (ALF_4^-) suggesting that 1 mM 5-HT induces near-maximal activation of phospholipase C (PLC) (Fig. 1D). Pertussis toxin at 2.5 μg/ml had no effect on the 5-HT-induced IP_3 increase. The pertussis-toxin insensitive G-proteins ($G_{\alpha q}$ and $G_{\alpha 11}$) have been implicated in the activation of PLC [14]. Therefore, our data suggested that stimulation of the IP_3 transduction pathway in insect cells might involve the $G_{\alpha q}$ family of G-proteins.

The preferential coupling of the 5-HT_{dro2B} receptor to IP_3 production, was further investigated by characterisation of the G_α/G_β subunit profile in Sf9 cell membranes using antibodies raised to mammalian G-proteins. Immunoblotting with the

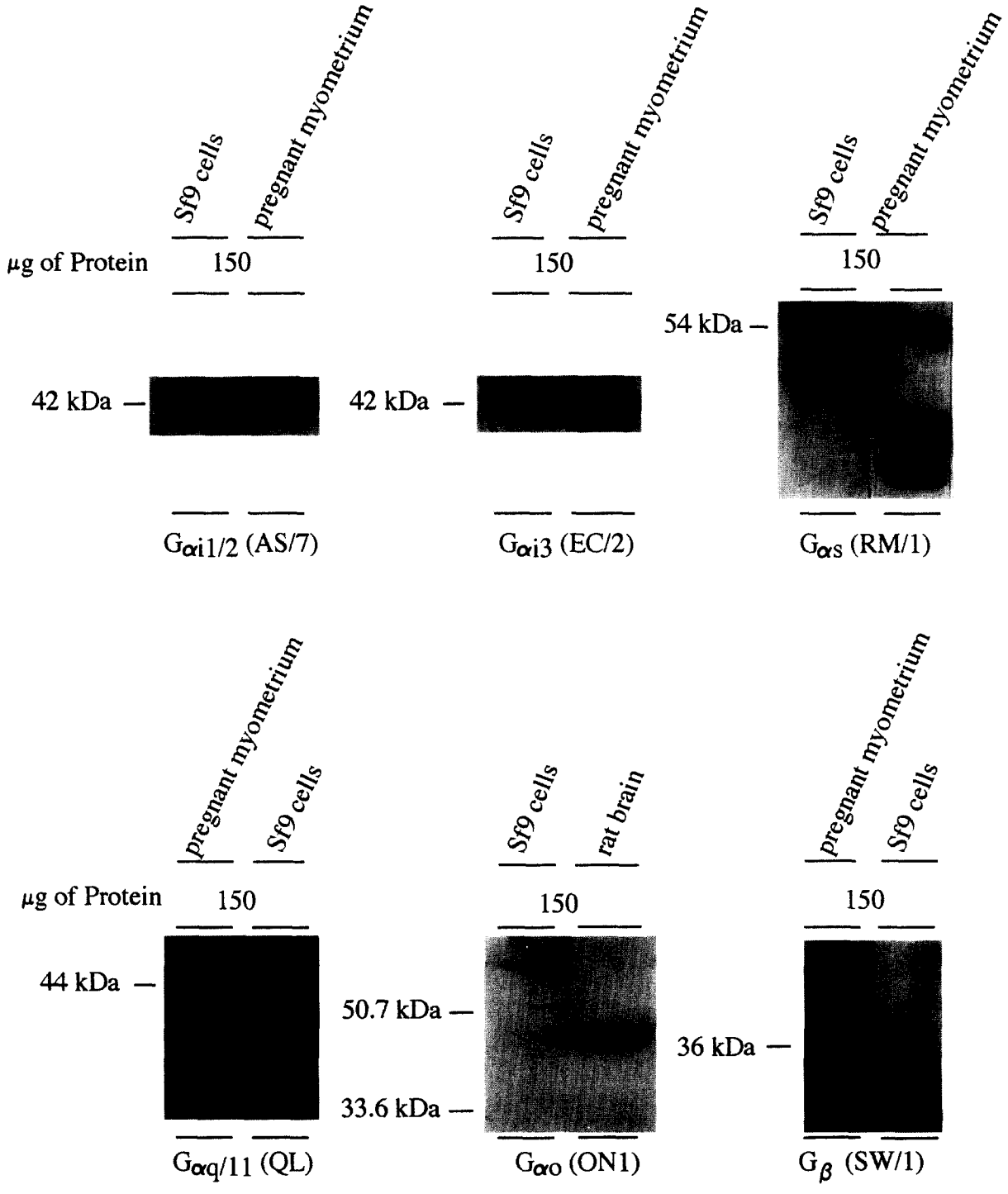

Fig. 2. Detection of G-proteins in Sf9 cell membranes by immunoblotting. Immunoblots were probed with antibodies raised against $G_{\alpha i1/2}$ (AS/7); $G_{\alpha i3}$ (EC/2); $G_{\alpha s}$ (RM/1); $G_{\alpha q/11}$ (QL); $G_{\alpha o}$ (ON1) and G_{β} (SW/1). Membranes obtained from human pregnant myometrium provided positive controls for all immunoblots except for the detection of $G_{\alpha o}$, where a membrane preparation from rat brain was used.

$G_{\alpha s}$ specific antibody (RM/1) detected a 50 kDa protein in agreement with previous results [3] (Fig. 2). Sf9 membranes probed with the G_{β}-specific antibody (SW/1) resulted in the detection of a protein that co-migrated with the 36 kDa G_{β} subunit found in the positive control (Fig. 2). However, the antibodies AS/7 ($G_{\alpha 1/2}$) or EC/2 ($G_{\alpha i3}$) failed to detect inhibitory G-proteins in Sf9 membranes in agreement with [3] and [4], respectively. Similarly, using the $G_{\alpha o}$-specific antibodies GC/2, IM1, OCI and ON1 we were unable to detect the $G_{\alpha o}$ subtype in these cells. The inability to detect $G_{\alpha o}$-like proteins in Sf9 membranes differed from previous reports [4] and [15], which demonstrated the existence of the $G_{\alpha o}$ subtype in insect cells. In contrast, with the anti-$G_{\alpha q/11}$ antibody (QL), we detected a 42 kDa protein in Sf9 cell membranes (Fig. 2). This protein probably represented $G_{\alpha q}$ as the apparent molecular mass of 42 kDa was similar to that observed for mammalian $G_{\alpha q}$ [13]. The detection of a $G_{\alpha q}$-like protein, together with the insensitivity to pertussis toxin of the observed 5-

HT_{dro2B} receptor-mediated IP_3 production, suggested that this receptor coupled to an endogenous $G_{\alpha q}$ protein in Sf9 cells.

In conclusion, the data we have obtained from both functional assays for receptor activity and immunoblotting studies using a panel of anti-G-protein antibodies suggest that Sf9 cells express the adenylyl cyclase stimulatory G-protein ($G_{\alpha s}$) and at least one member of the $G_{\alpha q}$ family of G-proteins. However, Sf9 cells appear to lack three subtypes of inhibitory G-protein ($G_{\alpha i1-3}$), which would suggest that these cells would be unsuitable for the functional analysis of receptors that preferentially couple to these G-proteins. The apparent inability to detect any $G_{\alpha o}$ subunits in this study indicates that variability in the levels of G_{α}-subunits may occur, and in this respect our results highlight the need to individually assess the functional characteristics exhibited by each G-protein coupled receptor following expression in insect cells.

Acknowledgements: We thank Sylvain Phaneuf for help with the inositol labelling experiments. This work was funded by the UK BBSRC.

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