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Research Article

Functional expression of mammalian opioid receptors in insect cells and high-throughput screening platforms for receptor ligand mimetics

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Abstract. Lepidopteran cell lines have been engineered to constitutively express high levels of mouse δ opioid receptors either alone or in combination with human Gα16 protein. Biochemical and pharmacological studies demonstrate that these lines contain all the mediator G proteins and downstream effectors required for opioid receptor function, including phospholipase C, and that expression of exogenous Gα16 does not contribute significantly to increased receptor responses upon activation. The activation of the phospholipase C pathway in the transformed cells upon stimulation with known receptor ligands results in easily and quantitatively measurable increases in free intracellular calcium, which can be monitored by automated fluorescent methods, while the addition of specific antagonists blocks the agonist-induced responses. Therefore, the transformed lepidopteran cell lines can be used as sensitive high-throughput screening platforms for fast detection of δ opioid receptor ligand mimetics (agonists and antagonists) in collections of natural products and synthetic compounds.

Key words. Mammalian opioid receptor; delta opioid receptor; G-protein-coupled receptor; insect cell expression system; Bm5 cell; high throughput screening assay; ligand mimetic.

Introduction

In mammals, opioids affect a number of physiological functions ranging from hormone secretion, neurotransmitter release, feeding, respiratory depression, gastrointenstinal mobility and immunosuppression [1]. Opiate drugs mediate their analgesic, euphoriant and rewarding effects by activating opioid receptors [2, 3]. Pharmacological and molecular studies have demonstrated the existence of three opioid receptor subtypes, μ, δ and κ (MOR, DOR and KOR, respectively), which are members of the G-protein-coupled receptor (GPCR) superfamily that couple to Giα/Goα types of G proteins and inhibit adenylyl cyclase activity [4–6]. Beside this well-known response, opioid receptors have also been shown to regulate diverse second-messenger systems such as phospholipase Cβ (PLCβ) and mitogen-activated protein kinase (MAPK), as well as the function of a variety of ion channels [5–7].

The clinical use of opiate drugs is limited due to their tendency to cause tolerance and dependence after prolonged or repeated administration [2, 3]. The existence of such undesirable side effects of current opioid drugs has

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stimulated the search for new drugs that can act as potent painkillers without causing dependence or addiction. Several approaches are used to achieve this goal, ranging from in silico modeling of putative ligands to the ligand-binding region of opioid receptors to the screening of libraries of chemical compounds or collections of natural products using available in vitro or cell-based high-throughput screening (HTS) systems [8, 9].

Cultured insect cells occupy an important biotechnological niche, mainly by serving as hosts for baculovirus-vector-based expression of recombinant proteins [10, 11]. As an alternative to the baculovirus expression systems, where proteins are produced in batch mode, plasmid-based lepidopteran expression systems were also recently developed, in which transformed lepidopteran cell lines are engineered for continuous expression of high levels of recombinant proteins with pharmaceutical value [12, 13]. In comparison to mammalian expression systems, the lepidopteran-cell-based expression systems display several advantageous features for biotechnological applications. First, the cells can adapt easily to growth in spinner flasks and grow to very high densities, at temperatures ranging from 23 to 28 °C, in inexpensive media and without the need for CO_2 supplementation [14]. Second and most important, several cell lines are available that grow efficiently and allow protein production in serum-free media of defined chemical composition [15], which are considered safe for production of recombinant proteins, especially those destined for use in pharmaceutical applications due to the lack of potential harmful pathogens such as viruses, mycoplasmas and prions [11].

While lepidopteran cell lines are used routinely to produce proteins of biological and pharmaceutical value, their use as screening systems for bioactive products remains largely unexplored. Recently, a transformed silkmoth (*Bombyx mori*)-derived cell line (Bm5 cells) was generated that has incorporated an ecdysone (insect moulting hormone)-inducible reporter cassette in its genome and responds effectively to the application of ecdysone agonists by induction of green fluorescence in the transformed cells [16]. This transformed cell line was used successfully to screen for ecdysone agonists and antagonists in chemical libraries and collections of plant extracts. The above example illustrates that lepidopteran cell lines can be successfully engineered to function as screening systems for ligands of nuclear receptors such as the insect ecdysone receptor. On the other hand, little is known regarding the capacity of lepidopteran cell lines to function as detection systems for GPCR ligands.

The goal of the present study was to develop a novel HTS system that detects ligands for a mammalian DOR using lepidopteran cell lines. We succeeded in generating transformed silkmoth Bm5 cells that stably express the murine DOR, either alone or in combination with an auxiliary factor, the human Gα16 protein. Successful receptor expression was achieved as detected by high-affinity ligand binding and receptor-mediated activation of G proteins. The functionality of the heterologous system was also evaluated by measurements of PLC stimulation and intracellular Ca^{2+} mobilization upon agonist activation. Our data provide the basis for developing a lepidopteran-cell-based HTS system that can be used for the rapid detection of novel bioactive metabolites that interact with mammalian DORs.

Materials and methods

Expression plasmids

Restriction fragments containing the open reading frames of the mouse δ_1 opioid receptor (DOR) [4, 17] and the human Gα16 protein [18] (1.2-kb *Eco*RV/*Sac*I fragment for DOR, and 1.1-kb *Kpn*I/*Eco*RV fragment for human Gα16) were cloned in the *Sma*I site of the lepidopteran expression vector pEA [19] after trimming the restriction sites of *Sac*I and *Kpn*I by T4 DNA polymerase. The correct sense orientation of the inserts in the obtained expression plasmids, pEA-DOR and pEA-Gα16, was verified by sequencing. The expression plasmid pBmIE1 contains a 3.8-kb *Cla*I fragment from the BmNPV genome that encompasses the *ie*1 gene [20]. IE1 expression is achieved under the control of its own promoter [21].

Tissue culture, transfection, transformation and clonal selection.

Bm5 cells were routinely subcultured at 28 °C in IPL-41 insect medium [22] supplemented with 0.35 g/l $NaHCO_3$, 2.6 g/l tryptose phosphate, 9 g/l sucrose, 0.069 mg/l $ZnSO_4$. $7H_2O$, 7.59 mg/l $AlK(SO_4)$. $12H_2O$ and 10% fetal bovine serum (FBS). Transfections were performed as previously described [12] with slight modifications. Bm5 cells were seeded into six-well plates (35 mm diameter) at a density of 10^6 cells/well (2 ml/well) and transfected for 5 h with 0.25 ml of transfection solution containing 30 µg/ml lipofectin (Invitrogen) and 5.05 µg/ml of total plasmid DNA in basal IPL-41 medium. The plasmid DNA content in the transfection solutions consisted of the expression plasmid for the mouse DOR (4 µg/ml), or the expression plasmids for DOR and Gα16 (2 µg/ml each), combined with DNA of plasmid pBmIE1 (1 µg/ml), which expresses the baculovirus (*B. mori* nuclear polyhedrosis virus or BmNPV) transcriptional co-activator IE1 [21], and the antibiotic selection plasmid pBmA.PAC (0.05 µg/ml) conferring resistance to growth inhibition by puromycin [23]. The IE1 expression plasmid was included because of its capability to boost expression from the pEA-based expression vectors by up to two orders of magnitude [19]. After 5 h, the transfection medium was replaced with complete IPL-41 medium,

supplemented with 50 µg/ml gentamycin (2 ml/well). To obtain stably transformed cell lines, the culture medium was replaced at 48 h post-transfection with fresh medium supplemented with 15 µg/ml puromycin for selection of puromycin resistance. Heterogeneous populations of transformed cells were obtained and maintained by weekly subculturing in 25-cm^2 cell culture flasks in the presence of 15 µg/ml puromycin. Cell lines stably expressing the DOR alone were designated δ1, while cell lines stably expressing DOR and Gα16 were termed δ3G. To obtain semi-clonal lines from the δ3G line, small numbers (one to ten) of cells were seeded in individual wells of a 96-well plate. Amplified cultures were subsequently screened by [^{3}H]diprenorphine (NEN) binding to membrane protein extracts. The highest-expressing semi-clonal line (2 pmol/mg of the DOR), derived from seven individual cells and designated δ5G, was used for further experiments.

Cell membrane preparations

Cells were harvested, collected by centrifugation at 2000 g for 5 min and washed once with phosphate-buffered saline (PBS), pH 7.5. The cells were resuspended in ice-cold buffer A (10 mM Tris, pH 7.5, 0.1 mM EDTA) with approximately 2×10^6 cells/ml buffer, and lysed with 30 strokes of the pestle of a Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at 2000 g for 2 min. The supernatant was further centrifuged at 200,000 g for 20 min at 4 °C. The membrane pellet was resuspended in buffer A at a protein concentration of approximately 1.5 mg/ml, and stored in aliquots at –70 °C. Protein concentration was measured according to the method of Bradford [24]. Yields of approximately 220 µg of membrane protein per 10^6 Bm5 cells were typically obtained.

Binding experiments

Binding experiments with opioid agonists and antagonists were performed essentially as previously described [25]. Briefly, cells were harvested, washed with PBS and resuspended in buffer A. Whole-cell binding experiments were performed at 30 °C for 45 min in 10 mM $MgCl_2$, 100 mM NaCl and 50 mM Tris-HCl, pH 7.5 (buffer B, antagonist binding) or in 20 mM $MgCl_2$, 50 mM sucrose and 20 mM Tris-HCl, pH 7.5 (buffer C, agonist binding). Protein concentration in the different assays varied between 60–100 µg per reaction. For saturation binding experiments, [^{3}H]diprenorphine and [^{3}H]DADLE ([D-Ala^2, D-Leu^5]enkephalin; NEN) were used at concentrations of 0.5–12 nM and 0.3–17 nM, respectively. Non-specific binding was assessed in parallel assays in the presence of 10 µM of the non-selective opiate antagonist naloxone (Sigma). Reactions were terminated by rapid filtration through GF/B filters (Whatman) presoaked in 10 mM Tris-HCl, pH 7.5, followed by three 4-ml washes with the same ice-cold buffer, using a Brandel apparatus. Filters were counted in a Packard Tri-Carb 2100TR liquid scintillation analyzer. Data were analyzed and saturation parameters (K_d and B_{max}) were calculated using the RadLig 4.0 program.

[^{35}S]GTPγS binding studies

GTPγS binding of the δ3G cell membranes (15–20 µg) was performed in 100-µl reaction volumes containing 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 10 µM GDP, 0.2 mM ascorbate, 0.3–0.5 nM [^{35}S]GTPγS (NEN) and the ligand DSLET (1 nM –10 µM; Sigma). The samples were incubated for 2 h at 4 °C. The reaction was terminated by rapid filtration on presoaked GF/B filters (Whatman), followed by three washes with cold 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$ using a Brandel cell harvester. Bound radioactivity was quantified by counting the filters in scintillation cocktail. Specific binding was calculated by subtracting the amount of [^{35}S]GTPγS bound in the presence of 10 µM unlabeled GTPγS from total bound [^{35}S]GTPγS.

Western blot analysis

Membranes from Bm5 cells (δ1 and δ3G cell lines) were used for SDS polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes. The PVDF membranes were exposed overnight at 4 °C to rabbit or goat polyclonal antibodies raised against the various types of G proteins at 1:1000 dilutions. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) according to the manufacturer's instructions, using X-ray film (Fuji). Most of the antibodies used in these experiments were kindly provided by Prof. G. Milligan, University of Glasgow and were as follows: (i) SG3 antiserum (rabbit) recognizing Gαi1 and Gαi2 equally well, (ii) OC1 antiserum (rabbit) recognizing Gαo and (iii) CQ2 antiserum (rabbit) recognizing Gαq and Gα11 [26]. T20 antibodies (rabbit) recognizing Gβ and anti-Gα16 antibodies (goat) were purchased from Santa Cruz Biotechnology.

Measurements of inositol trisphosphate accumulation

Measurements of inositol (1,4,5) triphosphate [Ins(1,4,5)P_3] accumulation were performed by a modification of a previously established protocol [27]. Briefly, transformed Bm5 cells were incubated in 12-well plates (10^6 cells/ml per well plated in IPL41 medium containing 10% FBS, supplemented with 15 µg/ml puromycin and 50 µg/ml gentamycin) in the presence of myo-[^{3}H]inositol (NEN; 1 µCi/ml per well) for 24 h before assaying. Cells were challenged at 28 °C for 30 min with variable amounts (0.05–10 µM) of DSLET ([D-Ser^2, Leu^5]enkephalyl-Thr; Sigma) in HBSS buffer (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-

glucose, 21 mM HEPES, pH 7.4), supplemented with 10 mM LiCl. The reaction was stopped by removal of the drug and addition of 1 ml of stop solution (96% v/v methanol, 0.23% v/v HCl). Following addition of 0.7 ml of chloroform and spinning at 1000 g for 5 min, the aqueous phase (~0.7 ml) was removed, mixed with 2 ml of 5 µM *myo*-inositol (Sigma) and loaded onto ion exchange AG-501-X8 resin (Bio-rad). The columns were washed three times, each with 4 ml of 5 µM *myo*-inositol solution. The tritiated labeled product was eluted with 2 ml of 1 M ammonium formate. The levels of Ins(1,4,5)P_3 were determined by the ratio of the radioactivity bound in the aqueous and lipid phases.

Intracellular Ca^{2+} measurements

One to five hundred thousand δ1, δ3G or δ5G cells (depending on the type of assay used) were incubated for 30–45 min in 10 mM Hepes pH 7.4, 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$ containing 2.5 mM probenecid (ICN Biomedicals) (buffer D) and 5 µM Fluo3 (Molecular Probes). The cells were then washed three times with buffer D and incubated for 20–30 min in the same buffer. Fluorometric measurements after stimulation with various concentrations of the opioid agonists DSLET and DPDPE ([D-Pen^2, D-Pen^5]enkephalin; RBI) were performed either in a stirring cuvette (5×10^5 cells per measurement) using an LS50 fluorometer (Perkin Elmer) or in the wells of 96-well microtiter plates (1×10^5 cells per well) using a FluoSTAR Galaxy microplate fluorescence reader (BMG Laboratories) with filter sets for excitation at 488 nm and emission at 525 nm. Antagonist detection assays were carried out under the same conditions except that the cells were preincubated with the antagonist for 15 min prior to the addition of the agonist. For measurements of the sizes of the intracellular pools of Ca^{2+} in Bm5 and HEK293 cells, fluorescent readings were taken after addition of 60 µg/ml digitonin to numbers of cells equal to those used for the agonist activity measurements.

Results

Identification of G protein content of Bm5 cells

To identify the nature of the G proteins present in the silkmoth cells, we performed immunoblots of rat brain and Bm5 cell membrane proteins using a series of antisera recognizing the Gαi2, Gαo and Gαq proteins as well as the G$\beta\gamma$ subunit of the G protein complex [26]. As shown in figure 1, immunoblotting with the antiserum that specifically recognizes Gαo identified a single polypeptide of 39 kDa in Bm5 membranes, which co-migrated with a polypeptide of equivalent size in rat brain, a tissue known to express high levels of this protein. The antiserum that recognizes the Gαi1 and Gαi2 proteins identified a

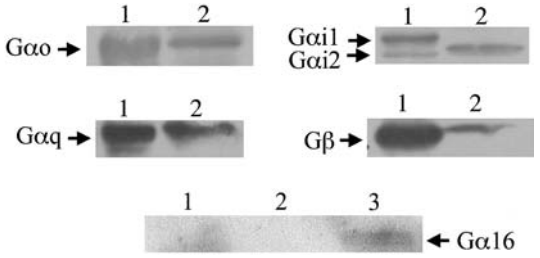

Figure 1. Immunological detection of G proteins in δ1 and δ3G cell lines. Upper panels: membranes from rat brain (40 µg) (lanes 1) and δ1 cells (lanes 2) were immunoblotted for Gαo using antiserum OC2, for Gαq/11 with the antiserum CQ2, for Gαi2 with the SG1 specific antiserum and for the Gβ subunit with the BN3 antiserum. Lower panel: expression of Gα16 in the δ3G cell line. Membrane proteins (50 µg) of untransfected Bm5 cells (lane 1), Bm5 cells transfected with vector alone (lane 2) and δ3G cells (lane 3) were resolved by electrophoresis and processed for immunodetection of Gα16 using an anti-Gα16-specific antibody (Santa Cruz).

41- and a 40-kDa polypeptide in rat brain membranes and only a single polypeptide in Bm5 cell membranes, which migrated with a mobility intermediate to Gαi1 and Gαi2. The presence of Gαq in Bm5 cell membranes was also demonstrated by immunoblotting with the antibody that recognizes this protein. Finally, the antiserum that recognizes the β subunit of G proteins identified a 35-kDa polypeptide in Bm5 cells, suggesting the presence of $\beta\gamma$ complexes in these cells. On the other hand, when similar immunoblotting studies were performed in δ3G cells, which were stably transfected with Gα16, the presence of a 37-kDa polypeptide corresponding to Gα16 was revealed, which was absent from untransfected Bm5 cells or cells transfected with the empty expression vector. These experiments suggest that Bm5 cells contain a full complement of G proteins and that the human Gα16 protein is also expressed in the δ3G line.

Expression of DOR in Bm5 cells

Bm5 cells were co-transfected with pEA.DOR (alone or in combination with pEA.Gα16), pBmIE1 and pBmA.PAC and cell lines surviving the antibiotic selection process were selected and examined for DOR expression by ligand-binding assays. Of the selected cell lines tested, the δ1 and δ3G lines (the latter co-transfected with Gα16), which displayed high [^{3}H]diprenorphine binding (fig. 2), were selected for further characterization.

As shown in figure 2, saturation analysis of specific [^{3}H]diprenorphine binding in whole cells indicated K_d values of 0.7 ± 0.3 and 0.5 ± 0.06 nM for the δ1 and δ3G cell lines, respectively, indicating high-affinity binding of the antagonist, with B_{max} values of 1400 ± 200 and 266 ± 24 fmol/mg, respectively (means ± SE, n=3 in each case). Similar binding experiments employing the DOR-specific agonist DADLE revealed K_d values of 0.3 ± 0.01 and 0.7 ± 0.1 nM and B_{max} values of 112 ± 11 and 75 ± 9

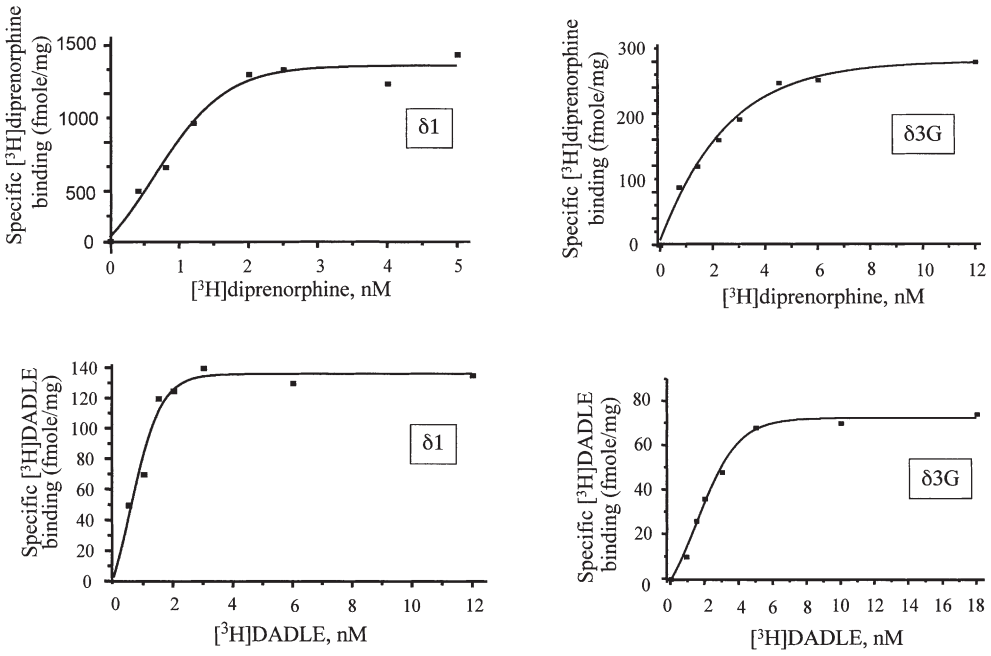

Figure 2. Binding parameters of DOR in the δ1 and δ3G lines. Whole cells from the δ1 and δG3 lines (50 µg protein equivalent) were incubated with varying concentrations of [^{3}H]diprenorphine and [^{3}H]DADLE for 45 min at 30 °C. Non-specific binding was assessed by the presence of 10 µM naloxone. The results are presented as mean values ± SE of three independent experiments.

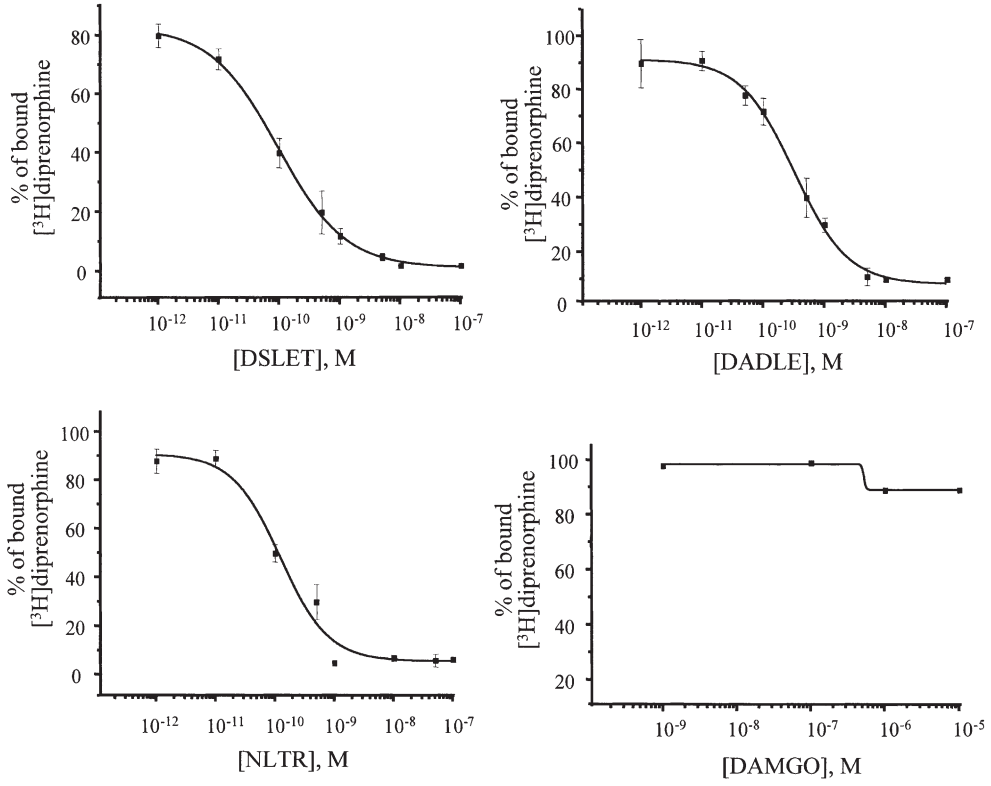

Figure 3. Competition of various opioid ligands for specific [^{3}H]diprenorphine binding. Membranes (50 µg protein equivalent) of the δ3G line were incubated with [^{3}H]diprenorphine (2 nM) and increasing concentrations of the agonists DSLET and DADLE, the DOR-selective antagonist naltrindole and the MOR-selective agonist DAMGO. Non-specific binding was measured in the presence of 10 µM naloxone. The results are presented as mean values ± SE of three independent experiments.

fmol/mg for the δ1 and δ3G cell lines, respectively (means ± SE, n=3 in each case). Binding assays performed with untransfected Bm5 cells did not show any ligand binding (data not shown).

The differential B_{max} values observed with the two ligands used in these experiments were expected, because the peptide agonist DADLE binds opioid receptors with lower affinity than the alkaloid antagonist diprenorphine. Thus, binding occurs only on receptors at a high-affinity state involving coupling with endogenous or over-expressed (in the case of δ3G) G protein complexes. Diprenorphine binding to the receptors, on the other hand, occurs independently of receptor affinity state. Moreover, DADLE binds only to cell surface receptors while diprenophine binds to both cell surface and endocytosed receptors.

Pharmacological characterization of the δ3G line

To define in more detail the pharmacological profile of the δ3G line, which co-expresses the Gα16 protein, we analyzed the ability of various DOR-specific ligands to displace specific [^{3}H]diprenorphine binding from the δ3G cell membranes in the presence of 10 µM naloxone. Figure 3 shows that 10 µM of each of the DOR agonists DSLET, DADLE and the DOR antagonist naltrindole completely displaced [^{3}H]diprenorphine binding. The IC_{50} for DSLET, DADLE and naltrindole was 0.09, 0.32, and 0.12 nM, respectively, suggesting the presence of high-affinity binding sites for DOR in the transformed cell line. In contrast, the MOR-selective agonist DAMGO was unable to displace specific [^{3}H]diprenorphine binding.

The mouse DOR mediates activation of G proteins in Bm5 cells

Preliminary work has shown that preincubation of membranes obtained from the δ3G line with the non-hydrolyable analog GppNHp, which uncouples the receptor from the G proteins, reduces the specific [^{3}H]DADLE and [^{3}H]diprenorphine binding displacement ability by opioid analogs (data not shown). This finding suggested that the expressed receptor couples functionally to the endogenous or over-expressed G proteins present in Bm5 cells. To provide further evidence for the DOR-mediated activation of G proteins, we have employed an assay that examines the DSLET concentration-dependent binding of [^{35}S]GTPγS to cell membranes. Because G protein activation involves stimulation of GDP/GTP exchange in the Gα subunit, G protein activation can be measured through the incorporation of the non-hydrolyzable GTP analog [^{35}S]GTPγS [28]. As shown in figure 4, DSLET stimulated specific [^{35}S]GTPγS binding to the membrane fraction of the δ3G cells in a concentration-dependent manner. The EC_{50} value for DSLET was 9.8 ± 0.9 nM, and the maximal agonist-induced increase in specific

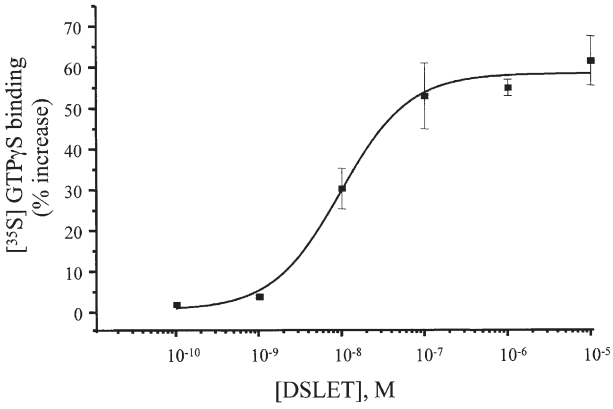

Figure 4. DSLET-stimulated [^{35}S]GTPγS binding to membranes of δ3G cells. The ability of increasing concentrations of DSLET to stimulate the rate of guanine nucleotide exchange in membranes of the δ3G cells was measured. Membranes (15 µg protein equivalent) were incubated for 60 min at 30 °C with 50 nCi [^{35}S]GTPγS in the presence of increasing concentrations of DSLET. Basal [^{35}S]GTPγS binding was 22 fmol/mg. Data are presented as mean values of DSLET-stimulated [^{35}S]GTPγS binding (% increase over basal levels) ± SE from three independent experiments.

[^{35}S]GTPγS binding (60% increase over the basal levels) was achieved at 1 µM DSLET. As expected, the DOR-specific antagonist naltrexone had no effect on [^{35}S]GTPγS binding (data not shown). Moreover, the effect of DSLET was specific for the DOR, as DSLET did not affect [^{35}S]GTPγS binding to the membranes of the parental Bm5 cells (data not shown).

Inositol trisphosphate accumulation

Opioid receptors, besides coupling to Gαi/Gαo proteins, have previously been demonstrated to also couple to other G proteins, which can stimulate PLCβ and mediate intracellular Ca^{2+} mobilization [29–31]. Given that Gαq proteins are expressed endogenously in Bm5 cells (fig. 1), coupling to the PLCβ pathway may also occur in these cells. To test this possibility, we measured the ability of the stably expressed DOR to stimulate Ins(1,4,5)P_3 formation in the δ1 and δ3G lines. Accumulation of Ins(1,4,5)P_3 in the presence of varying concentrations of DSLET occurred in an agonist dose-dependent manner for both the δ1 and δ3G cell lines (fig. 5), with an approximately twofold increase in Ins(1,4,5)P3 accumulation occurring in the δ3G line relative to δ1. This result suggests a possible contribution of the human Gα16 to receptor-mediated Ins(1,4,5)P_3 formation. Neither the MOR-specific agonist DAMGO nor the non-specific opioid antagonist naloxone at 10 µM was able to elicit any stimulation of the PLC (data not shown). Collectively, these observations suggest that the activation of PLC pathway can be indeed mediated through stimulation of the DOR via both the endogenous and the heterologous Gα proteins.

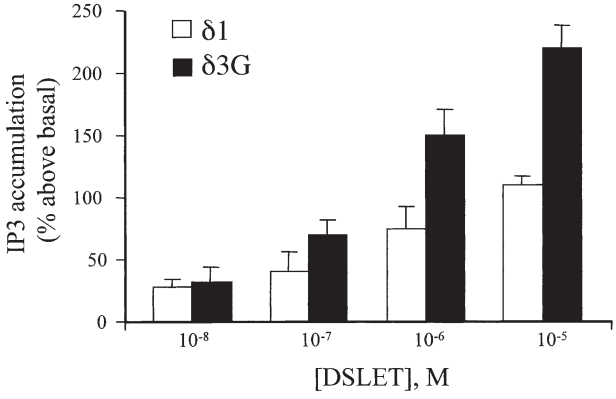

Figure 5. DSLET-mediated PLC activation in δ1 and δ3G cells. δ1 and δG3 cells were labelled with 1 µCi/ml *myo*-[^{3}H] inositol for 24 h before assaying for Ins(1,4,5)P3 formation in the absence or presence of 0.05–10 µM DSLET. Data represent the mean ± SE values of triplicate determinations of three independent experiments.

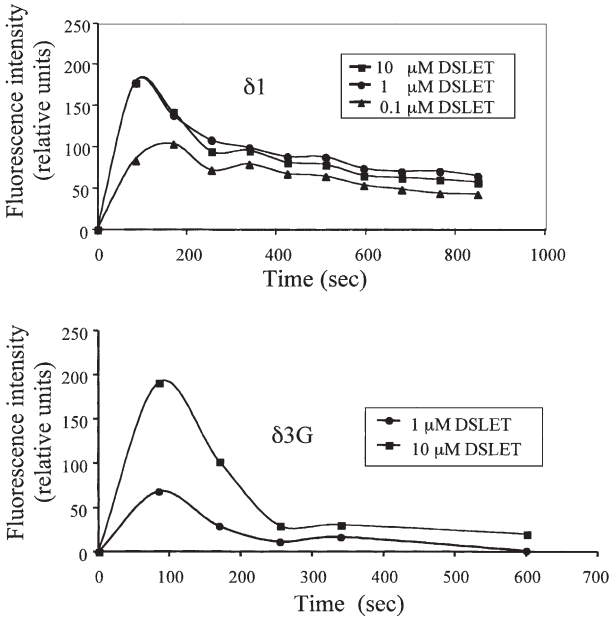

Figure 6. DSLET-mediated changes in intracellular Ca^{2+} release in the δ1 and δ3G lines. The increases in Ca^{2+} release were measured using the Ca^{2+}-binding dye, Fluo-3. The cells were treated with 0.1, 1 or 10 µM DSLET and fluorescent signals were determined at 85-s intervals using a FluoSTAR Galaxy apparatus. Averages of the results from three independent measurements are shown. Note that release of the entire pool of intracellularly stored Ca^{2+} effected by digitonin treatment of the cells produces a fluorescence reading of 230 units above background in this assay.

Measurements of intracellular Ca^{2+} release

Previous observations on SH-SY5Y neural and NG108-15 neuroblastoma cells have shown that activation of the opioid receptors can lead to increases in Ca^{2+} mobilization from intracellular stores [31, 32]. To capitalize on these observations toward the development of an easy and fast HTS protocol, we measured the ability of the DOR expressed in the δ1 or δ3G cells to mobilize intracellular Ca^{2+} in the presence of various concentrations of DSLET. Measurements of Ca^{2+} release using the fluorescent Ca^{2+}-sensitive dye Fluo-3 [33] were performed in 96-well microtiter plates seeded with confluent monolayers of the δ1 and δ3G lines using a microtiter plate fluorescence reader. As is evident from the examples shown in figure 6, concentrations of 0.1–1 µM of DSLET produced a significant elevation of Ca^{2+} release in the transformed Bm5 cell lines 60 s after the addition of the agonist, as demonstrated by the increase in fluorescence relative to that obtained from the parental Bm5 cells. Interestingly, although the maximal response for Ca^{2+} release was similar for the two cell lines (δ1 and δ3G), indicating the presence of sufficient amounts of endogenous Gαq/11 proteins to sustain a robust calcium response in both, the δ1 line showed easily measurable responses at a lower agonist concentration (0.1 µM) than the δ3G line and a maximal response at 1 µM DSLET. Given that the release of all stored Ca^{2+} of the Bm5 cells, achieved by digitonin treatment under equivalent assay conditions, results in a fluorescence response of 230 units above background (data not shown), the maximal responses obtained from the δ1 and δ3G lines upon receptor stimulation by high concentrations of the agonist (200 units of fluorescence) reflect nearly quantitative release of the stored Ca^{2+}. The higher sensitivity of the δ1 line relative to δ3G at low agonist concentrations in this assay may be due either to a better receptor coupling efficiency in the δ1 line or the higher quantity of functional receptor expressed by it (see table 1). Thus, despite the differences observed in the assays involving measurements of stimulation of Ins(1,4,5)P_3 formation, co-expression of Gα16 in the Bm5 cells does not appear to confer any advantages on the transformed cells with regard to the magnitude of the DOR functional response in the Ca^{2+} release assay. This may be due to the pool size of endogenous Gαq proteins, which may be sufficient to cause release of nearly all intracellular Ca^{2+} from its storage sites upon receptor activation.

Supporting evidence for the notion that the differences in the numbers of functional receptor molecules expressed by the two cell lines are responsible for the observed increased responsiveness of the δ1 line to lower agonist concentrations was obtained through the selection of a semi-clonal cell line, δ5G (originating from a small number of cells of the δ3G cell population), which expresses higher amounts of functional receptor molecules than the δ3G line. As shown in table 1, the δ5G line displays [^{3}H]diprenorphine and [^{3}H]DADLE binding with B_{max} values of 2037 and 210 fmol/mg membrane protein, respectively. In addition, as shown in figure 7 (inset), DOR-mediated stimulation of IP3 formation in the δ5G

Table 1. Overview of functional characteristics of the Bm5-derived cell lines δ1, δ3G and δ5G and the mammalian cell lines SH-SY5Y and δHEK293 with respect to specific [^{3}H]diprenorphine and [^{3}H]DADLE binding and activation of PLC and Ca^{2+} release by DSLET.

	δ1	**δ3G**	**δ5G**	**δHEK293**	**SH-SY5Y**
B_{max} (fmol/mg) [^{3}H]diprenorphine	1400±200	266±15	2037±98	2200±110	265±2
B_{max} (fmol/mg) [^{3}H]DADLE	112±11	75±9	210±15	490±18	51±11*
EC_{50} for IP3 formation (μM DSLET)	1.00±0.05	0.60±0.03	0.10±0.01	0.66±0.04	ND
EC_{50} for Ca^{2+} release (μM DSLET)	0.10±0.02	0.32±0.04	0.29±0.02	0.56±0.03	1.63±0.08

Although the EC_{50} for the calcium response in the δ1 line is lower than that for the δ5G line (100 versus 290 nM), the latter line gives a more robust response over a wider range of concentrations. Thus, a clear calcium response was observed at 10 nM for the δ5G line, in contrast to the δ1 line. ND, not determined. *B_{max} value determined by DSLET binding [33].

cells occurs in an agonist dose-dependent and saturable manner with an EC_{50} of 100 nM for DSLET (see also table 1).

To analyze in more detail the functionality of the δ5G line, quantitative assays for intracellular Ca^{2+} release following agonist stimulation were carried out in parallel with a neural cell line, SH-SY5Y, which expresses relatively low levels of functional DOR (B_{max} of 265 and 51 fmol/mg membrane protein for specific [^{3}H]diprenorphine and [^{3}H]DSLET binding, respectively [34]; table 1), and a line of transformed HEK293 cells, δHEK293, which stably expresses mouse DOR at high levels [35] (B_{max} of 2200 and 490 fmol/mg membrane protein for specific [^{3}H]diprenorphine and [^{3}H]DSLET binding, respectively; table 1). As exemplified by the data presented in figure 7, reliably measurable releases of Ca^{2+} from the intracellular stores of the δ5G (and δHEK293) cells were observed upon agonist stimulation at concentrations as low as 10 nM, while no responses could be obtained in the SH-SY5Y neural cell line at this agonist concentration. Furthermore, the maximal Ca^{2+} release responses obtained from the δ5G cells were significantly higher than those obtained from SH-SY5Y cells and similar to those obtained from the transformed HEK293 clonal line (calculated EC_{50} values of 0.29, 1.63 and 0.56 μM DSLET, respectively; table 1). One needs to stress that while the HEK293 clone uniformly expresses the mouse DOR, the δ5G cell line is semi-clonal, with cells expressing the receptor at high levels constituting approximately 15–20% of the total population (data not shown).

Finally, to demonstrate the flexibility of the specific expression vector-Bm5 cell system, we examined the responses obtained from it with another DOR-specific agonist [D-Pen^{2,5}]enkephalin (DPDPE) and the antagonist naloxone. As can be seen in figure 8A, addition of DPDPE in the δ5G cells caused easily measurable responses of Ca^{2+} release even at a concentration of 1 nM (calculated EC_{50} of 0.12 μM DPDPE for the Ca^{2+} release). On the other hand, preincubation of the cells with various concentrations of naloxone (fig. 8B) resulted in significant reduction in the Ca^{2+} release caused by the subsequent addition of 1 μM DPDPE and almost complete blockage of the release at a concentration equal to that of the agonist. The IC_{50} for naloxone was calculated at 0.37 μM.

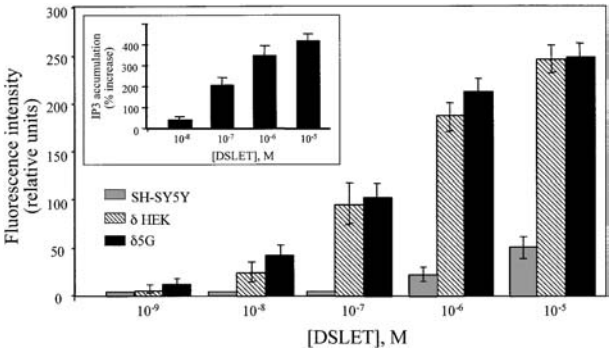

Figure 7. DSLET-mediated changes in intracellular Ca^{2+} release in the δ5G, SH-SY5Y and δHEK293 cell lines. Cells loaded with the calcium-binding dye Fluo-3 were treated with different concentrations of DSLET (1 nM – 10 μM) and increases in intracellular calcium were measured in a stirring cuvette of a LS50 Perkin-Elmer fluorometer. Results from three independent measurements are shown. The inset shows the DSLET-mediated activation of PLC in the δ5G line (% increase in IP3 accumulation).

Discussion

Opiate-based drugs are used extensively for the treatment of acute pain. However, such drugs have the disadvantages of loosing their effectiveness upon prolonged use and causing significant dependence-related problems. Because of this, new drugs are needed for acute-pain treatment, based on new ligands capable of activating opioid receptor function without triggering undesirable side effects. Prominent among the tools that can be employed to identify new opioid receptor-specific ligand mimetics (agonists or antagonists) representing leads for new pharmacological agents are cell-based HTS systems that may be used for the rapid examination of large chemical libraries or collections of plant extracts for the presence of such ligand mimetics. Consequently, a need exists for

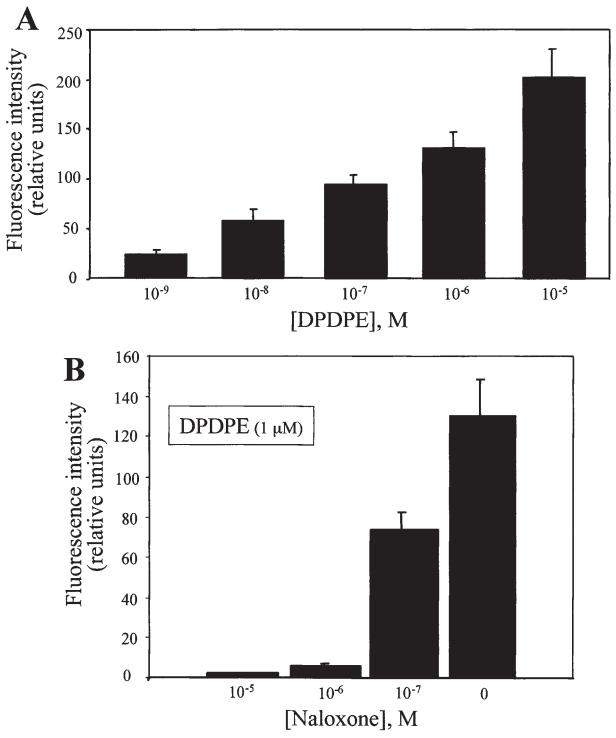

Figure 8. Calcium response assays for the DOR-selective agonist DPDPE and blocking of agonist-mediated activation by the opioid antagonist naloxone. (*A*): Cells of clone δ5G were treated with increasing concentrations of DPDPE (from 1 nM – 10 μM) and responses of intracellular calcium were determined. (*B*): For the antagonist measurements, cells were pre-treated for 15 min with different concentrations of naloxone (100 nM – 10 μM), and calcium responses were recorded upon subsequent challenge with 1 μM DPDPE.

the development of robust cell-based expression systems that can express efficiently functional opioid receptors in a background that contains the components required for proper functioning of this class of receptors (G proteins), as well as the downstream effectors of opioid receptor function. Such systems should be capable of recording reliably minute changes in host cell responses through the use of sensitive reporter molecules, and should also be amenable to miniaturization.

A new plasmid-based system for protein expression in lepidopteran cells has become available recently, which employs three distinct genetic elements – the silkmoth actin promoter [22], the BmNPV (baculovirus) *hr3* enhancer element [19] and the gene encoding the BmNPV IE1 co-activator [21] – to drive heterologous gene expression in silkmoth Bm5 cells and other lepidopteran cell lines [12, 23]. The modularity of this expression system allows an at-will manipulation of the levels of heterologous gene expression from moderate to very high. This feature makes the system particularly attractive for several applications in addition to the mere high-level expression of foreign genes [11, 14], particularly with respect to the functional expression of receptor molecules, where expression at a high level does not always translate into proper functional responses. Although this system has already been used to develop HTS systems for targets representing nuclear receptors [16], its usefulness for functional expression of GPCRs, in general, and opioid receptors, in particular, has not been reported.

In this report, we documented the suitability of both the specific expression system and the specific cell line to which this has been coupled for functional expression of a mammalian δ opioid receptor (mouse DOR) and a mammalian G protein (human Gα16) with the aim of developing of a HTS system allowing fast detection of molecules capable of acting as DOR-specific ligands. Our results demonstrate that when coupled to the specific expression system, silkmoth Bm5 cells can express high levels of murine DOR and can be used as a HTS platform for fast, efficient and reliable detection of DOR ligand mimetics based on the monitoring of fluorescence changes occurring in the expressing cells in response to changes in the activity of the receptor expressed in them. Furthermore, our results suggest that this newly developed system does not require the presence of exogenous Gα16 in order to achieve reliable Ca^{2+} release responses at low ligand concentrations.

Because Bm5 cells were not used previously as hosts for functional expression of GPCRs, our work entailed a biochemical analysis of the cells themselves in terms of their content of endogenous G proteins. In addition, we carried out a detailed pharmacological characterization of the coupling efficiency of the latter and a promiscuous mammalian G protein (human Gα16 introduced in the cells by co-transfection) to the heterologous opioid receptor. Finally, we examined the overall behavior of the receptor in terms of downstream effector activation represented by the endogenous PLC pathway, which stimulates Ins(1,4,5)P3 formation and an associated release of Ca^{2+} from its intracellular stores. The latter were monitored through the appearance of measurable fluorescence changes in cells preloaded with the Ca^{2+}-binding marker Fluo-3 [33].

Our analysis has established that Bm5 cells contain a full complement of homologs of the most prominent mammalian G proteins, which include Gαo, Gαi, Gαq/11 and Gβ. Moreover, the quantitative analysis of the murine DOR expressed in these cells, carried out by specific agonist and antagonist binding assays (table 1), revealed not only high levels of receptor accumulation in the cells but also high levels of membrane-bound receptor (amounting to 2 pmol/mg membrane protein or approximately 30,000 ligand-binding-competent receptor molecules per cell for the δ5G semi-clonal line).

The pharmacological profiles of the δ1, δ3G and δ5G lines (ligand-binding specificity, coupling to the endogenous and the heterologous G proteins and downstream signaling

to the PLC pathway) were found to be similar to those reported for opioid receptors normally expressed in mammalian cells [25, 36]. In particular, both the receptor-coupling efficiency, confirmed by measurements of agonist-induced binding of [^{35}S]GTPγS to the membranes of the transformed cells, and the alterations in agonist binding occurring in the presence of the non-hydrolyzable analog GppNHp suggest proper receptor coupling to the endogenous as well as the human Gα proteins. Furthermore, by monitoring the PLC effector pathway of DSLET-activated DOR, which results in PLC activation, Ins(1,4,5)P3 formation and release of Ca^{2+} from its intracellular storage sites [6, 31], easily measurable receptor activation was demonstrated for all cell lines at nanomolar concentrations of DSLET (100 nM for δ1 and 10 nM for δ5G; fig. 6, 7). Thus, our results demonstrate not only that the over-expressed murine DOR assumes a conformation appropriate for high-affinity binding of receptor-specific ligands in Bm5 cells, but also that all components of the Ca^{2+} release pathway, including Gαq, PLC, Ins(1,4,5)P_3 and Ca^{2+} stored in the endoplasmic reticulum, are present in the Bm5 cell line.

The initial comparisons in the responses of the δ1 and δ3G lines, which contained similar numbers of membrane-bound receptor, suggested that an improvement in the stimulation of Ins(1,4,5)P_3 formation could be obtained by the co-expression of the human Gα16 protein. This is in agreement with previous reports suggesting that the use of the promiscuous Gα16 protein frequently increases the responses of GPCRs toward the PLCβ pathway [37–39]. However, the results of the Ca^{2+} release assays have also shown very clearly that the presence of the exogenous Gα16 did not add to the robustness of the responses obtained upon agonist stimulation. Presumably this may happen because the level of endogenous Gαq proteins is sufficient to induce levels of Ins(1,4,5)P_3 accumulation capable of effecting the release of nearly all stored Ca^{2+} upon receptor activation.

A number of previous reports have demonstrated the efficacy of functional expression of mammalian opioid receptors in mammalian host cells [40–44]. Nevertheless, our side-by-side comparisons of the δ5G line to a transformed HEK293 cell line expressing high levels of the mouse DOR under cytomegalovirus promoter control [35] demonstrated directly the potential that the Bm5 system has for the detection of DOR ligand mimetics in HTS assays relative to comparable mammalian systems. While similar fluorescence induction values were obtained from the two systems in the Ca^{2+} release assays, the use of insect cells is advantageous because of the lower maintenance costs and the higher cell densities that can be achieved in culture [11, 14]. Furthermore, while the δHEK293 cells homogeneously express membrane-bound DOR [35], the δ5G line, which expresses 2 pmolDOR/mg membrane protein, represents a low-complexity semi-clonal line containing cells expressing the receptor at different levels. Therefore, a true clonal line derived from the existing δ5G line and expressing the receptor at uniformly high levels will probably represent an even more sensitive HTS system capable of reliably detecting ligand mimetics at concentrations much lower than the 10 nM range, which represents the most reliable lower detection limit of the currently available δ5G line (note, however, that easily measurable Ca^{2+} release responses in the δ5G cells can also be obtained even with 1 nM of DSLET and DPDPE; fig. 7, 8).

In terms of other insect systems developed for expression of functional opioid receptors, we should mention the earlier work on baculovirus-mediated expression in High-Five and Sf9 cell lines. For these systems, receptor yields ranging from 1 to 9 pmol/mg membrane protein, functional coupling to endogenous G proteins and down-regulation of cAMP signaling by agonists were reported [45–48]. Although the amounts of expression obtained with baculovirus systems were similar to and, in some cases, higher than the values obtained in our study, the baculovirus system cannot be used as a HTS system because of the batch-type nature of protein expression and the degradation of the cellular contents at the end of the infection cycle, when cell lysis occurs [11]. Demonstration of the functional properties of the opioid receptors in the baculovirus system can only be obtained during a narrow window of expression prior to the attainment of high expression levels and the initiation of cellular lysis (typically 24 h), i.e. when expression levels are sub-optimal [10].

More recently, insect cell lines constitutively expressing opioid receptors were also generated [49, 50]. In the first case, the IE2 promoter from the OpNPV baculovirus was used to drive expression of the human MOR in lepidopteran Sf9 cells. Although high-affinity binding sites (K_d of 0.037 nM for diprenorphine) could be detected, B_{max} values established by whole-cell [^{3}H]diprenorphine binding assays revealed a total of 11,000–15,000 receptor molecules per cell, while values for high-affinity membrane-bound receptors (DADLE-binding assays) were not examined [49]. In the second case, the *Drosophila* inducible metallothionein promoter was employed also to express the human MOR in *Drosophila* S2 cells. Levels of 20,000–30,000 receptors per cell were reported in this case, based on whole cell diprenorphine binding assays, but the levels of high-affinity membrane-bound receptors were also not determined [50]. Thus, the expression levels obtained from our expression system (2 pmol of specific [^{3}H]diprenorphine binding per milligram membrane protein for the δ5G cell line, which corresponds to approximately 30,000 ligand-binding-competent receptors per cell) are comparable to or higher than the values obtained from other insect cell systems employing different types of expression systems.

In conclusion, we have demonstrated that Bm5 cells represent suitable hosts for functional expression of opioid receptors (and, presumably, other GPCRs as well). Furthermore, we have shown that the combination of Bm5 cells and the specific expression system employed with them can be used as components of cell-based HTS systems for the fast, sensitive and reliable detection of molecules with mammalian DOR-specific ligand activities based on the monitoring of agonist-mediated changes in intracellular calcium release with fluorescent indicators. Thus, the cell-based system, which is composed of the Bm5 cells and the specific expression system, proves to be a powerful alternative both to other insect-cell-based screening systems developed earlier for different types of GPCRs [51–53] and to the mammalian systems that are available for the same purposes.

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