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# Expression of $\mu$-, $\delta$- and $\kappa$-opioid receptors in baculovirus-infected insect cells

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**Abstract**

The $\mu$-, $\delta$- and $\kappa$-opioid receptors have been expressed in Sf9 and 'High Five' insect cells using the baculovirus expression system. In both cell lines highest receptor levels (pmol/mg membrane protein) were observed 48 h after infection. Concomitant exposure to the narcotic antagonist naloxone (1 $\mu$M) enhanced the production of each receptor type. However, 'High Five' cells differed from Sf9 cells in a 2–3-fold higher receptor density in the cell membrane and were therefore employed for receptor characterization. In membranes of 'High Five' cells opioid receptor levels ranged from $1.0 \pm 0.2$ pmol/mg protein for the $\kappa$-opioid receptor, $1.7 \pm 0.2$ pmol/mg for the $\delta$-opioid receptor to $2.1 \pm 0.5$ pmol/mg for the $\mu$-opioid receptor. The $\mu$-, $\delta$- and $\kappa$-opioid receptor agonists [D-Ala$^2$, *N*-methyl-Phe$^4$-Gly-ol$^5$]enkephalin ([$^3$H]DAMGO), [D-Pen$^2$,D-Pen$^5$]enkephalin ([$^3$H]DPDPE) and (5$\alpha$,7$\alpha$,8$\beta$)-(+)-*N*-methyl-*N*-(7-(1-pyrrolidinyl-1-oxaspiro(4,5)dec-8-yl) benzeneacetamide ([$^3$H]U69,563) bound to the opioid receptors with $K_d$ values of $3.4 \pm 0.3$ nM, $4.5 \pm 0.1$ nM and $1.2 \pm 0.3$ nM, respectively, resembling those reported for opioid receptors expressed in mammalian cells. Testing the functionality of the receptors in 'High Five' cells, we found that high affinity agonist binding was strongly reduced in the presence of GTP$\gamma$S/sodium, indicating their coupling to G proteins. Furthermore, activation of the three receptor types inhibited forskolin-stimulated cAMP formation. The results presented here suggest that the 'High Five' cell/baculovirus system provides a convenient method for high level expression of functionally intact opioid receptors as judged by receptor binding studies, their G-protein coupling and inhibition of adenylyl cyclase.

*Keywords:* Opioid receptor; Baculovirus; 'High Five'; Sf9; Insect cell

## 1. Introduction

Opioid receptors are G-protein-coupled and have been pharmacologically classified into three distinct types, designated as $\mu$, $\delta$ and $\kappa$ (for review, see Loh and Smith, 1990). In contrast to the numerous studies investigating the pharmacological and signal transduction properties of opioid receptors (for review, see Knapp et al., 1995; Reisine and Bell, 1993), analysis of posttranslational mechanisms involved in the regulation of receptor activity has been limited since in neuronal tissues and cell lines receptor densities are too low. Thus, there is a need for a cell system expressing opioid receptors at high levels.

The recent cloning of cDNAs for the $\mu$-, $\delta$- and $\kappa$-opioid receptors (Chen et al., 1993; Yasuda et al., 1993; Kieffer et al., 1992; Evans et al., 1992) provides the basis for the expression of opioid receptors in heterologous cells. Mammalian cells have already been used for the expression of recombinant opioid receptors (Raynor et al., 1993; Prather et al., 1995; Chakrabarti et al., 1995; Tsu et al., 1995). An alternative may be provided by the baculovirus-insect cell system (Miller, 1988). Among the major advantages of this expression system are the convenient and reproducible overexpression of recombinant proteins combined with the protein modification, processing and transport system of higher eukaryotic cells (Miller, 1988). Since other members of inhibitory G-protein-coupled receptors such as the human 5-HT$_1$ receptors (Parker et al., 1994) or the human $\alpha_2$-C4 adrenoceptor (Oker-Blom et al., 1993) have been already successfully expressed in the baculovirus/Sf9 insect cell system, it seemed likely that this cell system may also be utilized to express opioid receptors in high levels.

In the present study we therefore investigated the expression of the $\mu$-, $\delta$- and $\kappa$-opioid receptor both in Sf9 and 'High Five' (Wickham, 1992) cells employing recombinant baculoviruses. The receptors expressed in 'High Five' cells were further characterized by their binding chracteristics, and their coupling to G proteins as well as

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their capacity to inhibit adenylyl cyclase. Preliminary results of this work have been presented (Obermeier et al., 1996).

## 2. Material and methods

### 2.1. Materials

[D-Ala$^2$, *N*-methyl-Phe$^4$-Gly-ol$^5$]enkephalin (DAMGO), [D-Pen$^2$,D-Pen$^5$]enkephalin (DPDPE) and [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr] (CTOP) were purchased from Bachem Biochemica (Heidelberg, Germany). (5$\alpha$,7$\alpha$,8$\beta$)-(+)-*N*-methyl-*N*-(7-(1-pyrrolidinyl-1-oxaspiro(4,5)dec-8-yl) benzeneacetamide (U69,593) and 17-cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2′,3′-indolomorphinan hydrochloride (naltrindole hydrochloride) were obtained from RBI (Natick, MA, USA). [$^3$H]DAMGO (58 Ci/mmol), [$^3$H]U69,563 (56 Ci/mmol) and adenosine 3′,5′-cyclic phosphoric acid, 2′-*O*-succinyl [$^{125}$I]iodotyrosinemethyl ([$^{125}$I]cyclic AMP) were from Amersham (Braunschweig, Germany). [$^3$H]DPDPE (32 Ci/mmol) was obtained from DuPont/NEN (Dreieich, Germany). *Spodoptera frugiperda* Sf9 cells and 'High Five' cells from *Trichoplusia ni* were kindly provided by P. Nasheuer, Department of Biochemistry, University of Munich (Munich, Germany). Cell culture reagents were from Gibco/BRL (Karlsruhe, Germany) and from PAN Systems (Aidenbach, Germany). All other chemicals were obtained from Sigma (Munich, Germany). The cDNAs for the mouse $\delta$- and $\kappa$-opioid receptors were kindly provided by G. Bell, University of Chicago, the cDNA for the rat $\mu$-opioid receptor was a kind gift of L. Yu, Indiana University School of Medicine. The BacPAK baculovirus expression system for the construction of recombinant baculoviruses was purchased from Clontech (Palo Alto, CA, USA).

### 2.2. Construction of recombinant baculoviruses

The 1.2 kb *Stu*I/*Sac*I mouse $\mu$-opioid receptor cDNA fragment was isolated from plasmid pRC/CMV (Chen et al., 1993) and inserted into the corresponding sites in the polylinker of the baculovirus transfer plasmid BacPAK8 (Clontech). The 1.2 kb *Pst*I fragment of the mouse $\kappa$-opioid receptor cDNA in the pCMV-6b expression vector (Yasuda et al., 1993) was cloned into the *Pst*I site of the baculovirus transfer plasmid BacPAK8. The 1.2 kb *Eco*RI/*Sac*I fragment coding for the mouse $\delta$-opioid receptor in the pCMV-6c expression vector (Yasuda et al., 1993) was cloned into the *Eco*RI/*Sac*I site of the baculovirus transfer plasmid BacPAK9 (Clontech). These vectors containing the $\mu$-, $\delta$- and $\kappa$-opioid receptor cDNA were co-transfected into Sf9 cells with a baculovirus DNA (BacPAK6; Clontech) containing a lethal deletion. Single recombinant baculoviruses were cloned by plaque assay according to Summers and Smith (1987), and propagated by three rounds of infection as described previously (Söhlemann et al., 1993).

### 2.3. Cell culture

Sf9 and 'High Five' cells were grown in monolayer culture in TC100 medium (Gibco/BRL, Karlsruhe, Germany) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum, glutamine (0.6 g/l), penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml) at 27°C. For the production of recombinant opioid receptors, Sf9 and 'High Five' cells, respectively, were plated in 150 cm$^2$ T flasks at $2 \times 10^7$ cells/flask and infected with single recombinant baculovirus encoding the $\mu$-, $\delta$- and $\kappa$-opioid receptor cDNA, respectively, at a multiplicity of infection of 3–5. Unless indicated otherwise, cells were harvested 48 h after infection.

### 2.4. Preparation of cell membranes

Cells were centrifuged at $200 \times g$, washed three times with ice cold phosphate buffered saline and resuspended in 3 ml of buffer A (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine). After homogenization by Polytron the suspension was passed five times through a 27 gauge needle. The homogenate was then centrifuged at $80 \times g$ to pellet unbroken cells and nuclei and the supernatant was centrifuged at $50\,000 \times g$ for 30 min. The resulting pellet was resuspended in buffer A at a protein concentration of 2–5 mg/ml and used immediately for radioligand binding assays. Protein content was determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as standard.

### 2.5. Radioligand binding assay

Radioligand displacement experiments were performed with minor modifications as described previously (Yu et al., 1986). In brief, membranes (100 $\mu$g protein/tube) were incubated in Tris buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$) with either 1 nM of [$^3$H]DAMGO ($\mu$-receptor specific agonist), [$^3$H]DPDPE ($\delta$-receptor specific agonist) or [$^3$H]U69,563 ($\kappa$-receptor specific agonist), respectively, in the presence of increasing concentrations of the corresponding unlabeled agonist (total volume 0.2 ml). Nonspecific binding was determined in the presence of 10 $\mu$M unlabeled agonist and accounted for less than 10% of total binding. Binding reactions were conducted to equilibrium (30 min; 27°C) and terminated by adding 1 ml of ice cold Tris buffer, followed by rapid filtration over Whatman GF/B glass-fiber filters presoaked in 0.1% (w/v) polyethylenimine. Filters were washed three times with 2 ml of ice cold buffer and submitted for liquid scintillation counting. In some experiments 100 $\mu$M guanosine 5′-[$\gamma$-thio]tri-

phosphate (GTPγS) and 100 mM sodium chloride was included into the reaction mixtures in order to define the fraction of G-protein-coupled receptors. All assays were performed in duplicate and binding parameters were determined by nonlinear regression analysis using the LIGAND program (Munson and Rodbard, 1980).

### 2.6. Measurement of intracellular cAMP

The ability of various opioid receptor agonists to inhibit forskolin-stimulated cAMP accumulation was assessed in intact 'High Five' cells 48 h after infection. Cells were seeded into 96 well plates (40 000 cells per well) and incubated in serum free TC100 medium at 27°C for 30 min in the presence of 100 μM isobutylmethylxanthine. Subsequently, serum free medium containing either no drugs (basal activity), forskolin (final concentration 30 μM) or forskolin plus various concentrations of opioid receptor agonists were added to the cells. Upon incubation (20 min, 27°C) cells were lysed in 0.1 N HCl and the amount of cAMP was determined by radioimmunoassay as described previously (Frandsen and Krishna, 1977). In some experiments, agonist ($10^{-6}$ M) mediated inhibition of forskolin-stimulated cAMP accumulation was measured in the presence of opioid receptor antagonists ($10^{-5}$ M) to determine the specificity of inhibition. Antagonists used were CTOP for the μ-opioid receptor, naltrindole for the δ-opioid receptor and naloxone for the κ-opioid receptor. All assays were done in duplicates.

## 3. Results

### 3.1. Kinetics of opioid receptor expression in Sf9 and 'High Five' cells

In Sf9 and 'High Five' cells infected with recombinant baculoviruses encoding the μ-, δ- and κ-opioid receptor cDNA, expression of each receptor type was detected by ligand-binding experiments using [$^3$H]DAMGO, [$^3$H]DPDPE and [$^3$H]U69,563, respectively, as receptor specific agonists. All three opioid receptors displayed very similar expression kinetics. As demonstrated exemplary for the μ-opioid receptor (Fig. 1), both in Sf9 and 'High Five' cells, receptor density expressed per mg membrane protein increased progressively and reached a maximum at 48 h post infectionem. Noninfected cells failed to display opioid binding sites. Membrane preparations of infected 'High Five' cells contained approximately 2–3-fold higher receptor levels as compared to infected Sf9 cells and were, thus, used for receptor characterization. Concomitant exposure of infected Sf9 and 'High Five' cells to the narcotic antagonist naloxone (1 μM) enhanced the expression of each opioid receptor type tested, at 48 and 72 h after infection.

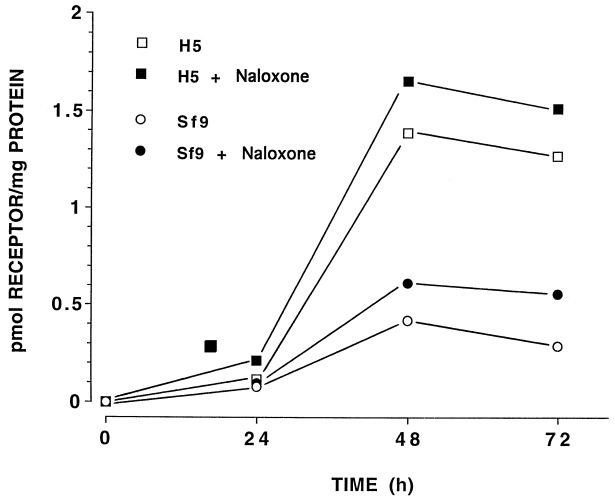


Fig. 1. Time-course of the expression of the μ-opioid receptor in Sf9 and 'High Five' cells. Cells were infected with recombinant baculoviruses encoding μ-opioid receptor cDNA in the presence (filled symbols) or absence (open symbols) of naloxone (1 μM). At the time points indicated, receptor levels in the membrane fraction were determined by radioligand binding using the receptor-specific agonist [$^3$H]DAMGO. The results are from a single experiment performed in duplicate. The experiment was repeated twice with similar results.

### 3.2. Agonist binding of opioid receptors expressed in 'High Five' cells

To characterize the binding properties of opioid receptors expressed in 'High Five' cells, membranes were prepared 48 h after infection and homologous displacement

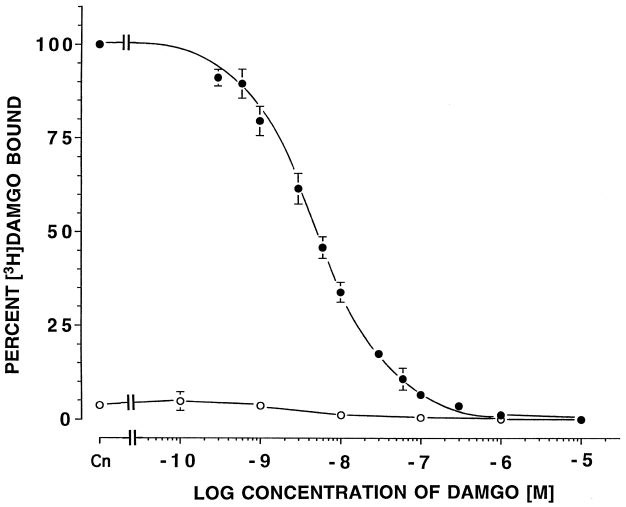


Fig. 2. Ligand binding of the μ-opioid receptor expressed in 'High Five' cells. Cell membranes were prepared 48 hours after infection and displacement of [$^3$H]DAMGO by increasing concentrations of unlabeled DAMGO was determined. Experiments were performed in the absence (●) or presence (○) of 100 μM GTPγS and 100 mM NaCl to define the fraction of G protein-coupled receptors. Control specific binding in the absence and presence of GTPγS and NaCl was 0.50 ± 0.07 pmol/mg protein (= 100% control) and 17.0 ± 0.3 fmol/mg protein (= 3.7% of the 100% control), respectively. Results are mean ± S.D. of three independent experiments performed in duplicate.

experiments were performed, using DAMGO, DPDPE and U69,563 as selective agonists for the $\mu$-, $\delta$- and $\kappa$-opioid receptor, respectively. Competition of unlabeled DAMGO with [$^3$H]DAMGO was concentration dependent (Fig. 2). Since agonist ligands are selective for the high affinity state of a receptor (Molinoff et al., 1981), the binding data was fitted to a single class of binding sites with an apparent $K_d$ of $3.4 \pm 0.3$ nM and a receptor density ($B_{max}$) of $2.1 \pm 0.5$ pmol/mg protein (Table 1). The $K_d$ values for the binding of DPDPE and U69,563 to the $\delta$- and $\kappa$-opioid receptor, respectively (displacement curves not shown) were calculated to be $4.5 \pm 0.1$ nM and $1.2 \pm 0.3$ nM with a $B_{max}$ of $1.7 \pm 0.2$ and $1.0 \pm 0.2$ pmol/mg protein, respectively (Table 1).

### 3.3. Functional studies of opioid receptors expressed in 'High Five' cells

Coupling of opioid receptors to G proteins is a prerequisite for their functional activity. To investigate the association of the expressed opioid receptors with G proteins, we tested the ability of the nonhydrolyzable GTP analogue GTP$\gamma$S (100 $\mu$M) in combination with NaCl (100 mM) to diminish binding of radiolabeled agonists to the receptors. As presented in Fig. 2, inclusion of GTP$\gamma$S and NaCl in the binding assays decreased specific labeling of the $\mu$-opioid receptor by $96.3 \pm 1.6$%. For the $\delta$- and $\kappa$-opioid receptor, decreases of $92.7 \pm 1.6$% and $79.9 \pm 5.7$%, respectively, were determined. These data suggest coupling of all three receptors to a G protein.

To determine whether the opioid receptors were also functional, we studied their ability to inhibit adenylyl cyclase. When 'High Five' cells expressing the $\mu$-, $\delta$- and $\kappa$-opioid receptor, respectively, were exposed to 30 $\mu$M forskolin they responded with an about 10-fold increase in cAMP production (basal: 30 pmol cAMP per $10^6$ cells and 20 min). DAMGO, DPDPE and U69,563, respectively, inhibited cAMP production in a dose-dependent manner, indicating functional activity of all three receptors (Fig. 3). With the highest concentration used ($10^{-5}$ M) reduction of cAMP accumulation by DAMGO, DPDPE and U69,563 was $28.4 \pm 1.6$%, $27.2 \pm 2.8$% and $39.0 \pm 3.9$%, respectively. The inhibition of cAMP production by 1 $\mu$M DAMGO, DPDPE and U69,563 was antagonized by a 10-fold excess of CTOP (a $\mu$-opioid receptor selective antagonist (Corbett et al., 1993)), naltrindole (a $\delta$-opioid receptor selective antagonist (Porthogese et al., 1988)) and naloxone (a nonspecific opioid receptor antagonist), respectively (data not shown).

Table 1
Binding parameters of $\mu$-, $\delta$- and $\kappa$-opioid receptors expressed in 'High Five' cells

| Receptor | Ligand | $K_d$ (nM) | $B_{max}$ (pmol/mg) |
|---|---|---|---|
| $\mu$ | DAMGO | $3.4 \pm 0.3$ | $2.1 \pm 0.5$ |
| $\delta$ | DPDPE | $4.5 \pm 0.1$ | $1.7 \pm 0.2$ |
| $\kappa$ | U69,563 | $1.2 \pm 0.3$ | $1.0 \pm 0.2$ |

$K_d$ and $B_{max}$ values were determined by nonlinear regression analysis of homologous displacement data using the LIGAND program. Values are mean $\pm$ S.D. of three independent experiments performed in duplicate.

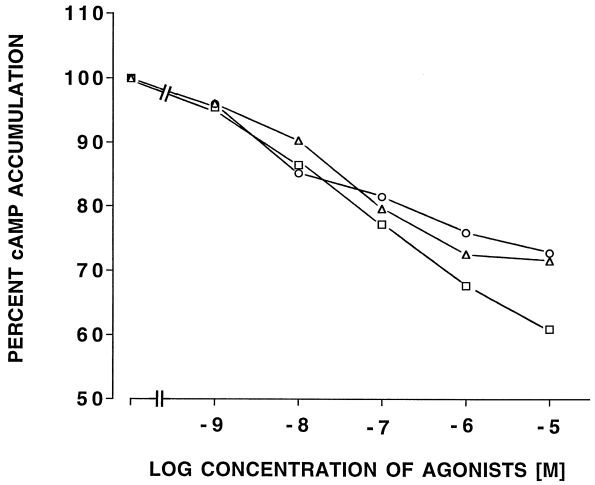


Fig. 3. Agonist-dependent inhibition of cAMP accumulation in 'High Five' cells expressing the $\mu$- ($\triangle$), $\delta$- ($\bigcirc$) and $\kappa$-opioid receptor ($\square$). Cells were stimulated with forskolin (30 $\mu$M) in the presence of increasing concentrations of DAMGO, DPDPE or U69,563, respectively, and cAMP production was determined. Results are mean of four independent experiments performed in duplicate. Error bars have been omitted for clarity (S.D. was less than 10%).

## 4. Discussion

The present study shows that the $\mu$-, $\delta$- and $\kappa$-opioid receptor can be expressed in Sf9 and 'High Five' cells using the baculovirus expression system. In both cell lines, receptor densities in the membrane fraction were highest 48 h after infection, which is typical for genes under the control of the polyhedrin promotor (Miller, 1988). Furthermore, the narcotic antagonist naloxone increased the amount of each opioid receptor in the membrane fraction, resembling findings described for mammalian cells (Schulz et al., 1979). Sf9 cells differed from 'High Five' cells in their expression capacity. In the latter, levels of all three opioid receptor types were about 2–3-fold higher, suggesting that 'High Five' cells are the more appropriate insect cell line for the expression of these receptors.

The production of opioid receptors with high affinity agonist binding in 'High Five' cells routinely yielded 1–2 pmol receptor/mg membrane protein which is approximately 5–10-fold higher as compared to endogenous opioid receptor levels in neuronal cells (Law et al., 1985; Cote et al., 1993; Kazmi and Mishra, 1987). Similar results have been reported for the heterologous expression of opioid receptors in mammalian cells, where levels generally ranged between 1–3 pmol receptor/mg membrane

protein (Raynor et al., 1993; Chakrabarti et al., 1995; Pei et al., 1995; Li et al., 1993; Kong et al., 1994). In parallel to the expression of other G-protein-coupled receptors (Parker et al., 1994; Oker-Blom et al., 1993) the baculovirus/insect cell expression system therefore provides an alternative to the production of opioid receptors in mammalian cells, as high level expression is convenient and highly reproducible.

To determine whether agonist binding properties of the opioid receptors expressed in 'High Five' insect cells correspond with those of receptors expressed in mammalian cells, homologous displacement experiments were performed. In membranes of 'High Five' cells, the $\mu$-, $\delta$- and $\kappa$-opioid receptor specific agonists DAMGO, DPDPE and U69,563, respectively, recognized a high affinity state of each opioid receptor, which probably represents receptor/G protein complexes as GTP$\gamma$S and sodium ions almost completely blocked agonist binding (DeLean et al., 1980; Limbird, 1988). The measured $K_d$ values (DAMGO: 3.4 nM; DPDPE: 4.5 nM; U69,563: 1.2 nM) are in good agreement with $K_d$ values reported for opioid receptors expressed in mammalian cells, ranging from 1.0–2.0 nM for DAMGO (Raynor et al., 1993; Kazmi and Mishra, 1987), 1.2–2.7 nM for DPDPE (Cote et al., 1993; Limbird, 1988) and 0.6–1.5 nM for U69,563 (Raynor et al., 1993; Meng et al., 1993). Furthermore, the $\delta$-opioid receptor endogenously expressed in mammalian NG108-15 cells (Garzon et al., 1995) and the recombinant $\delta$-opioid receptor expressed 'High Five' cells, displayed the same binding affinity for DPDPE (data not shown). These results clearly indicate that binding of specific agonists to opioid receptors expressed in 'High Five' cells does not differ from receptors expressed in mammalian cells.

The fact that the expressed opioid receptors were coupled to G proteins suggested already that they were functional. This notion was further tested by investigating their ability to inhibit the enzyme adenylyl cyclase. In mammalian cells, opioid receptors couple to inhibitory G proteins of the $G_{i/o}$ family and inhibit the enzyme adenylyl cyclase (Sharma et al., 1977). In 'High Five' cells expressing the $\mu$-, $\delta$- and $\kappa$-opioid receptor, respectively, opioids also inhibited adenylyl cyclase as demonstrated by a dose-dependent reduction of forskolin-stimulated cAMP production. Furthermore, inhibition of cAMP production was antagonized by opioid receptor antagonists, indicating functionality of receptors with respect to their signalling properties. Despite the high affinity binding of agonists to the expressed opioid receptors, the subsequent inhibition of forskolin-stimulated adenylyl cyclase did not exceed 30% for the $\mu$- and $\delta$-opioid receptor and 40% for the $\kappa$-opioid receptor, respectively. Similar results have been reported for other inhibitory G-protein-coupled receptors expressed in Sf9 cells such as several human 5-$HT_1$ receptor subtypes (Parker et al., 1994) or the human $\alpha_2$-C4 adrenoceptor (Oker-Blom et al., 1993), where cAMP accumulation was reduced by 30–50%. These results differ from mammalian cells, for which inhibition up to 90% within the same concentration range has been reported (Kong et al., 1994; Arden et al., 1995). Furthermore, the dose-response curves for the inhibition of cAMP accumulation in 'High Five' cells are rather shallow as compared to mammalian cells. In the latter it has been shown, that G-protein-mediated inhibition of adenylyl cyclase is strongly dependent on the type of the activated enzyme. Type I adenylyl cyclase for example is largely unresponsive to inhibition by $G_{i\alpha}$ (Taussig et al., 1993). Therefore, it seems possible, that the G proteins coupled to the opioid receptors expressed in 'High Five' cells and the type of adenylyl cyclase activated by forskolin in these cells may only be partially compatible. The prerequisite for a more detailed explanation would be the knowledge of the exact G protein and adenylyl cyclase composition in 'High Five' cells, which however is unknown to date.

In conclusion, our data indicate that the baculovirus/'High Five' insect cell expression system provides a useful model for the expression of functional $\mu$-, $\delta$- and $\kappa$-opioid receptors in high levels.

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