Pergamon

Biochemical Pharmacology, Vol. 49, No. 1, pp. 81–89, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00440-4

KAPPA OPIOID RECEPTORS EXPRESSED ON THREE RELATED THYMOMA CELL LINES

DIFFERENCES IN RECEPTOR–EFFECTOR COUPLING

DIANE M. P. LAWRENCE, DAVID B. JOSEPH and JEAN M. BIDLACK*

Department of Pharmacology, The University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

(*Received* 8 *February* 1994; *accepted* 16 *August* 1994)

Abstract—The mouse thymoma R1.1 cell line was shown previously to express a single high-affinity κ_1 opioid receptor that is negatively coupled through a pertussis toxin-sensitive G-protein to adenylyl cyclase. This study compared opioid receptor binding and inhibition of adenylyl cyclase activity in three unique derivatives of the R1.1 cell line. Membranes from the R1.G1 and R1E/TL8x.1.G1.OUA^r.1 (R1EGO) cell lines bound both [^{3}H]U69,593 and [^{3}H](−)-bremazocine with similar affinities compared with R1.1 membranes, whereas membranes from the R1E/TL8x.1 (R1E) cell line did not posses any opioid binding sites, detected by radioreceptor binding. The B_{max} values for [^{3}H]U69,593 and [^{3}H]-(−)-bremazocine binding to R1.G1 and R1EGO cell membranes were, respectively, 3- and 6-fold greater than those obtained with the parent R1.1 cell line. GTP and its nonhydrolyzable analog, Gpp(NH)p, inhibited [^{3}H]U69,593 binding to all three cell lines. Stimulation of low-K_m GTPase activity by the κ-selective agonist (−)U50,488 was greatest in R1.G1 membranes, followed by R1EGO and R1.1. The maximal inhibition of forskolin-stimulated adenylyl cyclase activity by (−)U50,488 was 66 ± 2% in R1.G1 and 49 ± 2% in R1EGO, compared with 37 ± 1% in R1.1 membranes. Whereas maximal inhibition of adenylyl cyclase activity did not correlate with receptor number among cell lines, the inhibition of cyclic AMP production did correlate with stimulation of low-K_m GTPase activity. The R1.1 cell line and its derivatives, R1.G1 and R1EGO, express a similar type of κ opioid receptor, which exhibits differences in coupling to G-proteins and to adenylyl cyclase among cell lines. These cell lines provide an excellent model system for studying the regulation of opioid receptor–adenylyl cyclase coupling efficiency.

Key words: kappa opioid receptor; lymphoma; lymphocyte; adenylyl cyclase; G-protein; GTP; GTPase; R1.1 thymoma cell line

Opioid alkaloids and peptides alter several immune parameters, including lymphocyte number and proliferation, antibody production, macrophage function, and natural killer cell activity. Whereas some opioid effects may be mediated by the central nervous system, there is substantial evidence for direct opioid effects on cells of the immune system. For example, direct immunomodulatory effects of κ opioid agonists include increased superoxide production in human polymorphonuclear leukocytes by less than 1 pM dynorphin A-(1–13); this effect is inhibited by naloxone pretreatment [1]. Foster and Moore [2] reported that dynorphin peptides enhance target cell lysis by activated murine macrophages in culture. In mouse splenocytes, T cell-dependent antibody production is inhibited by *in vitro* exposure of the isolated splenocytes to μ- and κ-selective opioid agonists [3] and by *in vivo* exposure to morphine [4]. The morphine-induced suppression is restored by the addition of adherent cells or macrophage-derived cytokines [4]. These studies provide evidence for direct opioid effects on the immune system, and thus some leukocytes may express opioid receptors.

The search for opioid binding to leukocytes has been difficult, however. The presence of opioid binding sites has been reported on human and murine leukocytes [5–7], as well as on some cell lines [8, 9]. However, in contrast to brain opioid receptors, these sites do not exhibit all of the classical opioid binding characteristics, such as stereoselectivity and high affinity for both alkaloids and peptides [10]. In addition, agonist-induced functional responses have not been correlated with these binding sites. It seems likely that only a small population of leukocytes actually express opioid receptors, and possibly only under certain conditions. If this is the case, binding studies with mixed cell populations may not show a detectable signal-to-noise ratio, even when receptors are present.

While studying more homogeneous cell populations, we recently found that the R1.1 mouse thymoma cell line expresses a high-affinity, stereoselective κ opioid receptor [11, 12]. Based on its opioid binding profile, the opioid receptor expressed on the R1.1 cell line belongs to the κ_1 subtype [11], using nomenclature defined previously [13, 14]. This finding was confirmed recently by Pasternak and colleagues [15]. Opioid receptor agonist binding to R1.1 cell membranes inhibits adenylyl cyclase activity

* Corresponding author: Jean M. Bidlack, Ph.D., Department of Pharmacology, University of Rochester, School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. Tel. (716) 275-5600; FAX (716) 244-9283.

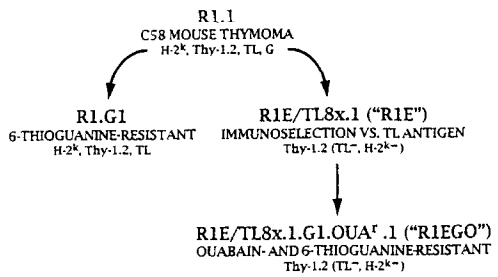


Fig. 1. Derivatives of the mouse R1.1 thymoma cell line.

by up to 40% [16]; this inhibition is blocked by the κ-selective antagonist nor-BNI* [17] and by culturing the cells with pertussis toxin [16]. These findings provided evidence of a lymphocytic κ opioid receptor with a binding profile and second messenger function apparently identical to brain κ opioid receptors. In addition, the presence of a single κ opioid receptor without μ and δ opioid receptors makes the R1.1 cell line an interesting model for biochemical and pharmacological studies of the κ opioid receptor.

The R1.1 cell line was derived from a spontaneous thymoma in a C58 mouse [18]. The experiments presented here tested whether three derivatives of the R1.1 thymoma cell line, shown in Fig. 1, also expressed opioid receptors. R1.1 cells express the major histocompatibility complex antigen H-2^k, the T cell marker Thy-1.2, the thymus leukemia antigen expressed by immature thymocytes, the Gross leukemia virus antigen [19], and the lymphocyte antigen Ly-23 [20]. They are also sensitive to cortisol and phytohemagglutinin [18] and to dexamethasone [21], characteristic of immature thymocytes. In addition to the κ opioid receptor, R1.1 cells also express insulin-like growth factor receptors that stimulate tyrosine kinase activity [22], as well as high-affinity β_2-adrenergic receptors that also bind reovirus type 3 [23, 24]. R1.G1 cells, the derivatives of R1.1 cells that are resistant to 6-thioguanine, express the thymus leukemia antigen, Thy-1.2 and H-2^k at levels consistent with the parent cells. The R1E cell line was developed from R1.1 cells by several cycles of *in vitro* immunoselection using complement and antibodies against the thymus leukemia antigen [19]. The R1E cell line expresses Thy-1.2 and the Gross leukemia virus antigen, but neither the thymus leukemia antigen, H-2^k [19, 25], β_2-microglobulin [26, 27], nor Ly-23 [20]. The third cell line, R1EGO, is a derivative of R1E that is resistant to 6-thioguanine and ouabain. Similar to the R1E cell line, the R1EGO cell line lacks the thymus leukemia antigen and H-2^k. Two of the three derivatives of R1.1 cells were found to express κ opioid receptors, and their binding properties and functional response of inhibiting adenylyl cyclase activity are reported here.

MATERIALS AND METHODS

Materials. The murine lymphoma cell lines R1.1 (TIB 42), R1.G1 (TIB 44), R1E (TIB 43) and R1EGO (TIB 45), shown in Fig. 1, were obtained from the American Type Culture Collection (Rockville, MD). For cell culturing, RPMI 1640 medium and iron-supplemented bovine calf serum were purchased from GIBCO Laboratories (Grand Island, NY) and Hyclone Laboratories (Logan, UT), respectively. [^{3}H]U69,593 (57 Ci/mmol) was purchased from Amersham (Arlington Heights, IL), and [^{3}H](−)-bremazocine (25.7 Ci/mmol), [γ-^{32}P]-GTP (30 Ci/mmol), [α-^{32}P]ATP (800 Ci/mmol) and [^{3}H]cyclic AMP (31.4 Ci/mmol) were obtained from DuPont/New England Nuclear (Wilmington, DE). U50,488 and nor-BNI were acquired from Research Biochemicals International (Natick, MA). (−)U50,488 was a gift from Drs. B. R. de Costa and K. C. Rice (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). DAMGO was purchased from Bachem (Torrance, CA), and ICI 174,864 was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY). Norit A decolorizing carbon was obtained from J. T. Baker Inc. (Phillipsburg, NJ). Nucleotides and all other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). Glass fiber filter sheets (No. 32) were purchased from Schleicher & Schuell, Inc. (Keene, NH). Ecoscint A and Ecolite (+) scintillation fluids were purchased from National Diagnostics (Atlanta, GA) and ICN Radiochemicals (Covina, CA), respectively.

Preparation of cell membranes. Lymphoma cell lines were cultured with RPMI 1640 medium, buffered with 12.5 mM HEPES, pH 7.2, and containing 300 μg/mL l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, 60 μM 2-ethanolamine, and 10% iron-supplemented bovine calf serum, in 5% CO_2 at 37°. For binding experiments, cells were harvested and centrifuged at 200 *g* for 15 min at 4°. The cells were resuspended in one-twentieth the original volume of 50 mM Tris–HCl, pH 7.5, homogenized with a Brinkmann Polytron homogenizer, setting 4 for 15 sec, and centrifuged at 39,000 *g* for 20 min at 4°. Membranes were resuspended in forty times the pellet volume of 50 mM Tris–HCl, pH 7.5, homogenized and centrifuged again at 39,000 *g* for 20 min at 4°. Membranes were then homogenized in ten times the pellet volume of 50 mM Tris–HCl, pH 7.5, for a final protein concentration of 6–12 mg/mL, as determined by the method of Bradford [28] using bovine serum albumin as a standard.

When membranes were prepared for low-K_m GTPase assays, the cells were initially centrifuged at 200 *g* for 15 min at 4°, then resuspended in 40 mL of 5 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM DTT, 0.32 mM sucrose (buffer A). Cells were

* Abbreviations: DAMGO, [D-Ala^2, (Me)Phe^4, Gly(ol)^5]enkephalin; DTT, dithiothreitol; pCl-DPDPE, pCl-[D-Pen^2,D-Pen^5]enkephalin; Gpp(NH)p, guanylyl-5′-imidodiphosphate; ICI 174,864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib is α-aminoisobutyric acid; nor-BNI, nor-binaltorphimine; R1E, R1E/TL8x.1; R1EGO, R1E/TL8x.1.G1.OUA^r.1; U50,488, (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]benzeneacetamidemethane-sulfonatehydrate; and U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide.

homogenized with 20 strokes of a glass tissue grinder, and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was collected and the pellet was resuspended in buffer A by vortexing, followed by centrifugation at 1000 *g* for 10 min at 4°. Both supernatants were combined and centrifuged at 22,500 *g* for 20 min at 4°. The pellet was resuspended in 40 mL of 5 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM DTT (buffer B) and centrifuged at 22,500 *g* for 30 min at 4°. Membranes were resuspended in buffer B, using 10 strokes of a glass tissue grinder, at a final protein concentration of 1.5 to 3.0 mg/mL.

For adenylyl cyclase experiments, after the initial centrifugation at 200 *g*, cells were resuspended in sucrose buffer (0.32 M sucrose, 40 mM HEPES, 2 mM EGTA, pH 7.6) and centrifuged again at 200 *g* for 15 min at 4°. Cells were again resuspended in sucrose buffer, homogenized with five strokes of a glass tissue grinder, and centrifuged at 22,000 *g* for 20 min at 4°, followed by resuspension in sucrose buffer for a final protein concentration of 10–15 mg/mL.

[^{3}H](−)-Bremazocine and [^{3}H]U69,593 binding to lymphoma cell membranes. [^{3}H](−)-Bremazocine binding to each cell line was measured as previously described [12]. Briefly, in a final volume of 4 mL of Tris–HCl, pH 7.5, 0.3 mg of membrane protein was incubated with 2.4 to 140 pM [^{3}H](−)-bremazocine for 60 min at 25°. To measure nonspecific binding, 1 μM naloxone was included. Membranes were filtered through glass fiber filters, washed three times with 4 mL of cold 50 mM Tris–HCl, pH 7.5, and counted in 2 mL of Ecoscint A scintillation fluid.

Experiments measuring [^{3}H]U69,593 binding to the cell lines were performed as previously described [11]. In these experiments, 0.3 mg of membrane protein was incubated with 0.05 to 1.34 nM [^{3}H]-U69,593 in a final volume of 1 mL of 50 mM Tris–HCl, pH 7.5. Nonspecific binding was measured by including 1 μM naloxone. The filters were pre-soaked in 0.25% polyethylenimine for at least 60 min before filtration.

Receptor specificity was addressed through competition studies with the κ-selective agonist U50,488 and κ-selective dynorphin peptides, the μ-selective peptide DAMGO, and the δ-selective peptide ICI 174,864. Membranes from each cell line were incubated with 0.4 nM [^{3}H]U69,593 and 12 concentrations of the competing ligand in a final volume of 1 mL of 50 mM Tris–HCl, pH 7.5, at 25° for 60 min before filtering.

To determine whether guanine nucleotides affected binding, membranes were incubated with 1 nM [^{3}H]-U69,593 and 12 concentrations of GTP or Gpp(NH)p under the same incubation conditions as described for competition studies. In addition, 100 μM concentrations of the nucleotides GDP, GMP, CTP, ATP, TTP and UTP were tested for their effects on the binding of 1 nM [^{3}H]U69,593 to each cell line.

Assay for low-K_m GTPase activity. [γ-^{32}P]GTP hydrolysis was measured using methods as previously described [29, 30], with some modification. Membranes, 10 μg protein/sample, were incubated with 8 concentrations of (−)U50,488 in a 100-μL reaction mixture containing 41 mM Tris–HCl, pH 7.5, 0.2 mM EGTA, 0.2 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM ATP, 1 mM adenylyl-5′-imidodiphosphate, 10 mM phosphocreatine, 5 U creatine phosphokinase, and 0.5 μM [γ-^{32}P]GTP (0.5 to 2.5 × 10^5 cpm/sample). Nonspecific ("high" K_m) GTP hydrolysis was measured in the presence of 50 μM GTP. The reaction mixture was equilibrated in a 37° water bath before the addition of membranes. After incubation of samples for 10 min at 37°, the reaction was terminated by the addition of 100 μL of ice-cold 40 mM H_3PO_4 followed by the immediate transfer to ice. Following the addition of 750 μL of ice-cold 5% Norit A decolorizing carbon suspension in 20 mM H_3PO_4, the samples were centrifuged at 11,700 *g* for 10 min at 4°. Radioactivity was measured in 650 μL aliquots of supernatant mixed with 2 mL of Ecoscint A scintillation fluid. Low-K_m GTPase activity was determined to be linear with time up to 15 min. Nonspecific GTPase activity accounted for 25–40% of total ^{32}P released by either R1.1 or R1EGO cell membranes, and 10–20% of total ^{32}P released by R1.G1 cell membranes. In the absence of membranes, less than 1% of total added radioactivity was released.

Assay for adenylyl cyclase activity. Methods for the quantification of cyclic AMP production in cell membranes as a measure of adenylyl cyclase activity were described previously [16]. Membranes, 175 μg protein/sample, prepared in sucrose buffer as described above, were incubated with 10 concentrations of (−)U50,488 in a final volume of 100 μL of 40 mM HEPES, pH 7.6, containing 60 nM forskolin, 0.1 mM [α-^{32}P]ATP (1.5 μCi/sample), 10 U creatine phosphokinase, 20 mM phosphocreatine, 1 mM cyclic AMP, 1 mM 1,10-*o*-phenanthroline, 60 μM isobutylmethylxanthine, 3 mM $MgCl_2$, 100 mM NaCl, and 0.1 mM GTP. After incubation for 15 min at 32°, followed by termination with 150 μL of 1 M $HClO_4$, the samples were centrifuged at 16,000 *g* for 4 min. The supernatants were washed over Dowex 50 ion-exchange columns and neutral alumina to collect the [^{32}P]cyclic AMP. The percentage of recovery for each sample was determined by including [^{3}H]cyclic AMP (10,000 cpm/sample) as a tracer. Eluents were counted by liquid scintillation spectrophotometry in 10 mL of Ecolite (+) scintillation fluid.

Data analysis. Saturation binding data were analyzed by nonlinear regression analysis with the LIGAND program [31]. The IC_{50} values for binding studies were determined using the least squares fit to a logarithm-probit analysis. K_i values were calculated using the formula $K_i = IC_{50}/(1 + S)$ where S = (concentration of radioligand)/(K_d of radioligand) [32]. Percent stimulation of low-K_m GTPase activity was calculated as follows: [specific GTPase activity in the presence of agonist/specific GTPase activity in the absence of agonist (basal) × 100%] − 100. The IC_{50} values for adenylyl cyclase assays were determined as the agonist concentration at which log $[I_{obs}/(I_{max} - I_{obs})] = 0$, where I_{obs} is the inhibition observed at a given agonist concentration, and I_{max} is the maximum inhibition observed that was not significantly different from that at the next higher and next lower concentrations tested. Statistical comparisons were made using Student's *t*-test for independent groups.

Table 1. [^{3}H]U69,593 and [^{3}H](−)-bremazocine binding to membranes from R1.1 and derivative cell lines

Cell line	[^{3}H]U69,593		[^{3}H](−)-Bremazocine	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
R1.1	0.28 ± 0.02	66 ± 5	0.015 ± 0.001	62 ± 8
R1.G1	0.35 ± 0.03	193 ± 33*	0.017 ± 0.002	187 ± 33*
R1E	—	—	—	—
R1EGO	0.49 ± 0.03*†	372 ± 16*†	0.035 ± 0.007*	436 ± 64*†

To determine K_d and B_{max} values for [^{3}H]U69,593 binding, 0.3 mg of membrane protein from each cell line, in a final volume of 1 mL of 50 mM Tris–HCl pH 7.5, was incubated with 0.05 to 1.3 nM [^{3}H]-U69,593 for 60 min at 25°. K_d and B_{max} values for [^{3}H](−)-bremazocine binding were determined by incubating 0.3 mg of membrane protein with 2.4 to 140 pM [^{3}H](−)-bremazocine in a final volume of 4 mL of 50 mM Tris–HCl, pH 7.5, for 60 min at 25°. Data are the mean values ± SEM from at least three experiments performed in triplicate.

* Significantly different from the R1.1 value ($P < 0.05$).

† Significantly different from the R1.G1 value ($P < 0.05$).

RESULTS

[^{3}H]U69,593 and [^{3}H](−)-bremazocine binding to lymphoma cell membranes. To characterize the opioid binding sites on the cell lines derived from the mouse thymoma R1.1, the κ-selective agonist [^{3}H]U69,593 and the nonselective opioid ligand [^{3}H]-(−)-bremazocine were used in binding studies. Membranes from R1.G1 and R1EGO cells bound both [^{3}H](−)-bremazocine and [^{3}H]U69,593 in a specific and saturable manner, with similar affinities compared to the parent R1.1 cell line. Table 1 shows the K_d and B_{max} values obtained by Scatchard analysis of the binding of both radioligands to R1.1, R1.G1, R1E and R1EGO thymoma cell lines. Results from experiments with R1.1 cell membranes were consistent with our previously reported findings [11, 12]. With either radioligand, the K_d values obtained with the R1.G1 cell line were the same as with R1.1 membranes, whereas the B_{max} values with R1.G1 membranes were 3-fold higher. Membranes from R1E cells showed no specific binding of [^{3}H]-(−)-bremazocine or [^{3}H]U69,593 (see Table 1) or [^{3}H]naloxone, or [^{3}H]DAMGO at concentrations up to 5 nM (data not shown). However, the R1E derivative, R1EGO, did show evidence of κ opioid binding. The K_d values for [^{3}H](−)-bremazocine and [^{3}H]U69,593 binding to R1EGO membranes were similar to those found with the R1.1 membranes, but the B_{max} values were six times greater than the binding capacity of the R1.1 cell line.

Analysis of [^{3}H](−)-bremazocine and [^{3}H]U69,593 saturation binding data resulted in linear best-fit models, and Hill coefficients for [^{3}H](−)-bremazocine and [^{3}H]U69,593 binding to R1.1, R1.G1 and R1EGO were all close to 1.0 (range of 0.965 to 1.027). None of the cell lines showed any specific binding of either the μ-selective peptide [^{3}H]DAMGO or the δ-selective peptide [^{3}H]pCl-DPDPE at a final concentration of 5 nM. The specific binding of 5 nM [^{3}H]naloxone, a nonselective opioid antagonist, was inhibited completely by an excess of the κ-selective compound U50,488 (data not shown). In addition, the B_{max} values obtained for each cell line with [^{3}H]U69,593 were not significantly different from those obtained with the relatively nonselective opioid [^{3}H](−)-bremazocine ($P > 0.05$). These findings provide evidence for the expression of a single κ opioid binding site on R1.1, R1.G1 and R1EGO cell lines.

Competition studies using U50,488, DAMGO and ICI 174,864, as well as the dynorphin peptides, confirmed the κ-opioid receptor specificity of [^{3}H]-U69,593 binding to R1.1, R1.G1 and R1EGO cell membranes. Table 2 shows the K_i values obtained for the two derivative cell lines, R1.G1 and R1EGO, compared with those reported previously for R1.1 membranes [11]. U50,488 and the dynorphin peptides were the most potent in competing with [^{3}H]U69,593 for binding to all three cell lines. Competition by the μ-selective peptide DAMGO was 1000-fold less potent than the κ-selective opioids, with K_i values greater than 200 nM. The δ-selective antagonist ICI 174,864 was ineffective at concentrations up to 30 μM. Thus, the high affinity of U50,488 and dynorphin peptide binding to the derivative cell lines R1.G1 and R1EGO is consistent with the κ_1 binding site expressed on the R1.1 cell line [11, 12].

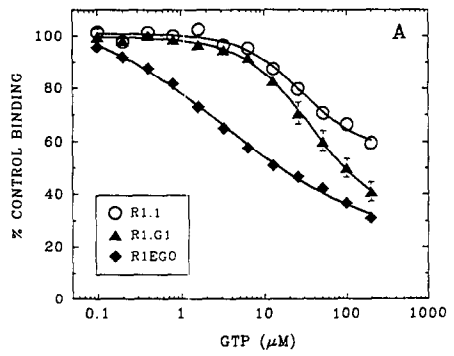
Guanine nucleotide inhibition of [^{3}H]U69,593 binding. To determine whether the binding sites on the R1.G1 and R1EGO cell lines were coupled to GTP-binding regulatory proteins, we tested whether GTP, at concentrations of 0.1 to 200 μM, inhibited [^{3}H]U69,593 binding to R1.G1 and R1EGO cell membranes. As shown in Fig. 2A, GTP diminished the binding of 1 nM [^{3}H]U69,593 to membranes from both derivatives, as well as the R1.1 cell line. The concentration-dependent effect did not reach a plateau in the concentration range tested. However, GTP was most potent in inhibiting binding to the R1EGO membranes, with inhibition observed with less than 1 μM GTP. At a concentration of 6 μM, GTP inhibited [^{3}H]U69,593 binding to R1EGO, R1.G1, and R1.1 membranes by 42 ± 2, 8 ± 1, and 5 ± 1%, respectively. The magnitude of inhibition

Table 2. K_i values for the inhibition of [^{3}H]U69,593 binding to R1.1, R1.G1 and R1EGO cell membranes by opioids

Opioid	K_i (nM)		
	R1.1*	R1.G1	R1EGO
U50,488	0.35 ± 0.13	0.30 ± 0.03	0.44 ± 0.03
Dynorphin A-(1–13)	1.25 ± 0.15	0.39 ± 0.03	0.28 ± 0.02
Dynorphin A-(1–17)	0.72 ± 0.04	1.23 ± 0.24	0.45 ± 0.02
Dynorphin B	0.23 ± 0.08	0.99 ± 0.06	0.44 ± 0.04
α-Neo-endorphin	0.23 ± 0.08	0.15 ± 0.003	0.24 ± 0.02
Dynorphin A-(2–17)	>300	>300	>300
DAMGO	214 ± 4	280 ± 7	320 ± 18
ICI 174,864	>3,000	>10,000	>10,000

Membranes from each cell line were incubated for 60 min at 25° with 0.4 nM [^{3}H]-U69,593 and 12 concentrations of the opioids, in a final volume of 1 mL of 50 mM Tris–HCl pH 7.5, as described in Materials and Methods. The IC_{50} values were determined from linear regression analysis, and converted to K_i values using the following K_d values for [^{3}H]U69,593 binding: 0.20 nM for R1.1 (see Ref. 11), 0.35 nM for R1.G1, and 0.49 nM for R1EGO, as reported in Table 1. Data are the means ± SEM from at least three experiments performed in triplicate.

* Data for R1.1 cell membranes were reported previously [11].



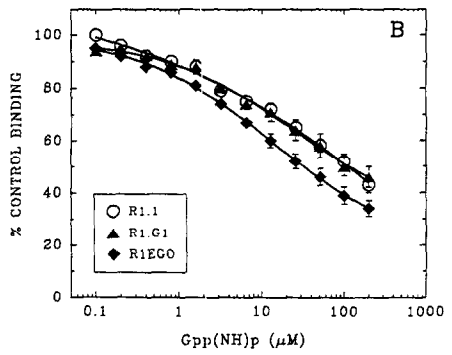


Fig. 2. GTP and Gpp(NH)p inhibition of [^{3}H]U69,593 binding. In a final volume of 1 mL of 50 mM Tris–HCl pH 7.5, 0.4 mg of membrane protein from R1.1, R1.G1, or R1EGO cell lines was incubated with 1 nM [^{3}H]U69,593 and 0–200 μM GTP (A) or Gpp(NH)p (B) for 60 min at 25°. Data represent the mean percent of control specific binding ± SEM from at least three experiments performed in triplicate.

Table 3. Nucleotide inhibition of the binding of 1 nM [^{3}H]-U69,593 to R1.1, R1.G1 and R1EGO cell membranes

Nucleotide (100 μM)	% Inhibition		
	R1.1	R1.G1	R1EGO
GTP	34 ± 1	50 ± 3	63 ± 2
GDP	39 ± 1	38 ± 3	64 ± 2
GMP	5 ± 1	5 ± 2	6 ± 0.8
CTP	10 ± 1	8 ± 1	9 ± 2
ATP	7 ± 2	8 ± 1	8 ± 2
UTP	15 ± 1	12 ± 2	16 ± 1
TTP	13 ± 0.6	11 ± 2	14 ± 1

Membranes were incubated for 60 min at 25° with 1 nM [^{3}H]U69,593 and a 100 μM concentration of each nucleotide, in a final volume of 1 mL of 50 mM Tris–HCl, pH 7.5, as described in Materials and Methods. Data are means ± SEM from at least three experiments performed in triplicate.

of [^{3}H]U69,593 binding with 200 μM GTP was not significantly different between R1.G1 and R1EGO membranes, 59 ± 3 and 69 ± 2%, respectively ($P > 0.05$), but the inhibition levels with both cell lines were significantly greater than with R1.1 membranes, 41 ± 1% ($P < 0.01$). The specificity of the GTP response was also tested by comparing the effects of 100 μM concentrations of the nucleotides GDP, GMP, CTP, ATP, TTP, and UTP with that of GTP. As shown in Table 3, only GDP inhibited [^{3}H]U69,593 binding to the same degree as GTP for R1.1, R1.G1 and R1EGO membranes.

A possible explanation for the greater potency of GTP in R1EGO membranes is that the rate of GTP hydrolysis is markedly slower in R1EGO membranes relative to R1.1 and R1.G1 membranes. To explore this possibility, we measured the inhibition of [^{3}H]-

U69,593 binding by 0.1 to 200 μM of the non-hydrolyzable guanine nucleotide Gpp(NH)p (Fig. 2B). In contrast to the marked differences in GTP potency among these cell lines, the potency of Gpp(NH)p was similar in all three cell lines. As observed with GTP, the concentration-dependent inhibition of binding did not reach a plateau. The magnitude of inhibition observed with 200 μM Gpp(NH)p was not significantly different among cell lines.

Low-K_m GTPase activity. The similarity in the Gpp(NH)p inhibition of agonist binding to R1.1, R1.G1, and R1EGO membranes suggests that differences in the rate of GTP hydrolysis account for the observed differences in GTP potency. To address this issue directly, the hydrolysis of 0.5 μM [γ-^{32}P]GTP was measured in membranes from each cell line. The values for basal low-K_m GTPase activity in R1.1, R1.G1, and R1EGO membranes were 19 ± 2, 41 ± 2, and 22 ± 1 pmol/mg protein/min, respectively. The value for basal activity in R1.G1 membranes was significantly greater than the values for R1.1 and R1EGO membranes ($P < 0.01$). If different rates of GTP hydrolysis were responsible for differences in GTP potency, then GTP hydrolysis in R1EGO membranes should have been much slower compared with that in R1.1 and R1.G1 membranes. Although the value for basal low-K_m GTPase activity in R1EGO membranes was nearly 50% lower than that of R1.G1 membranes, the values for basal activity were not significantly different between R1EGO and R1.1 membranes. Therefore, it is unlikely that the greater potency of GTP for inhibiting [^{3}H]U69,593 binding to R1EGO membranes is due to a slower rate of low-K_m GTPase activity in this cell line. To determine if differences in high-K_mGTPase activity were responsible for the differences in GTP potency among cell lines, we measured the hydrolysis of 50 μM [γ-^{32}P]GTP. The values for GTPase activity in R1.1, R1.G1, and R1EGO membranes were 971 ± 34, 723 ± 53, and 881 ± 42 pmol/mg protein/min, respectively. Again, the results indicate that the greater potency of GTP for inhibition of agonist binding to R1EGO membranes is not due to a slower rate of GTP hydrolysis in this cell line.

To further characterize the coupling of κ opioid receptors to G-proteins on each cell line, agonist-stimulated low-K_m GTPase activity was investigated (Fig. 3). The κ-selective agonist (−)U50,488 produced a concentration-dependent activation of low-K_m GTPase activity in each cell line. The values for stimulation of low-K_m GTPase activity by 30 μM (−)U50,488 in R1.1, R1.G1, and R1EGO membranes were 21 ± 3, 53 ± 5, and 32 ± 3%, respectively.

Inhibition of adenylyl cyclase activity. (−)U50,488 was tested for its effect on forskolin-stimulated adenylyl cyclase activity in membranes from the R1.G1 and R1EGO cell lines. As shown in Fig. 4, (−)U50,488 inhibited cyclic AMP production in R1.G1 and R1EGO membranes with similar potency but to a greater maximal level than was observed with R1.1 membranes. The inhibition was greatest in R1.G1 membranes, with a maximal inhibition (I_{max}) value of 66 ± 2%, and an IC_{50} value of

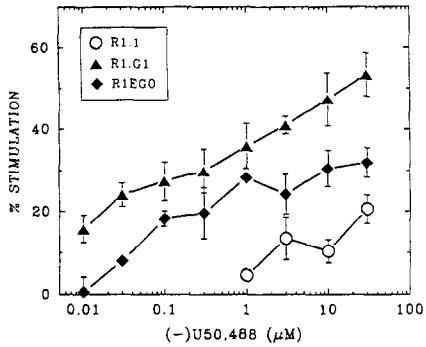


Fig. 3. (−)U50,488 stimulation of low-K_m GTPase activity. Membranes from R1.1, R1.G1 and R1EGO cells were incubated with 0.5 μM [γ-^{32}P]GTP in a final volume of 100 μL of 41 mM Tris–HCl pH 7.5, and assayed for low-K_m GTPase activity as described in Materials and Methods. Data represent the mean percent stimulation over basal activity ± SEM from three experiments performed in triplicate. Basal low-K_m GTPase activity in R1.1, R1.G1, and R1EGO cell membranes was 19 ± 2, 41 ± 2, and 22 ± 1 pmol/mg protein/min, respectively.

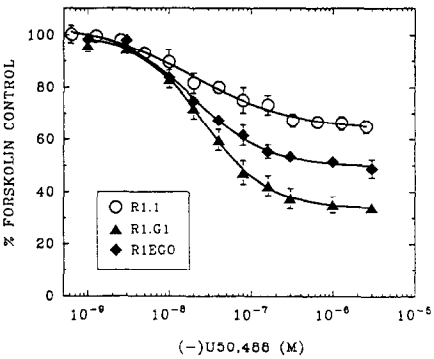


Fig. 4. (−)U50,488 inhibition of adenylyl cyclase activity. Membranes from R1.G1 and R1EGO cells were incubated with 0.1 mM [α-^{32}P]ATP in a final volume of 100 μL of 40 mM HEPES, pH 7.6, and assayed for cyclic AMP production, as described in Materials and Methods. For comparison, results with R1.1 membranes, as reported previously [16], are also shown. Data represent the mean percent of forskolin-stimulated control cyclic AMP production ± SEM from three experiments performed in triplicate. Forskolin-stimulated control cyclic AMP production in R1.G1 membranes was 3.3 ± 0.5 pmol/min/mg protein, which was 267 ± 16% of basal activity; in R1EGO membranes, forskolin-stimulated control activity was 1.8 ± 0.8 pmol/min/mg protein, 227 ± 5% of basal activity.

27 ± 3 nM. With R1EGO membranes, the I_{max} value was 49 ± 2%, and the IC_{50} value was 21 ± 1 nM. For comparison, the maximal (−)U50,488-mediated inhibition of cyclic AMP levels in R1.1 membranes was 37 ± 1% and the IC_{50} value was 51 ± 14 nM, as previously reported [16]. The I_{max} values for each cell line were significantly different from each other ($P < 0.01$), whereas the IC_{50} values were not

significantly different among cell lines ($P > 0.05$). For each cell line, the inhibitory effect of (−)U50,488 was blocked completely by an equimolar concentration of the κ-selective antagonist nor-BNI, which alone had no effect on cyclic AMP production (data not shown).

DISCUSSION

In this report, we have shown that two derivatives of the R1.1 cell line, R1.G1 and R1EGO, express a κ opioid receptor similar to that described previously in the parent cell line [11, 12]. Another derivative, R1E, did not show any specific opioid binding. As observed in the parent cell line, Scatchard analysis of saturation binding data, obtained with membranes from the R1.G1 and the R1EGO cell lines, yielded a linear fit and Hill coefficients near unity for both [^{3}H](−)-bremazocine and [^{3}H]U69,593. In addition, the number of sites bound by [^{3}H](−)-bremazocine on each cell line was not significantly different from those bound by [^{3}H]-U69,593. These observations, together with the absence of [^{3}H]pCl-DPDPE and [^{3}H]DAMGO binding, suggest the presence of a single population of κ opioid receptors on all three thymoma cell lines. The K_d values measured with either [^{3}H]U69,593 or [^{3}H](−)-bremazocine were similar among cell lines, but the maximal opioid binding capacity in R1.G1 and R1EGO membranes was 3- and 6-fold higher, respectively, than the parent R1.1 cell line.

Competition experiments showed that, as with R1.1 cell membranes, the κ-selective opioid agonists U50,488 and α-neo-endorphin were the most potent inhibitors of [^{3}H]U69,593 binding to R1.G1 and R1EGO membranes, with similar K_i values compared with R1.1 cells. High affinity for U50,488 indicates that the binding sites are of the κ_1-opioid receptor subtype, as was determined with the R1.1 cell line [11], using nomenclature described elsewhere [13, 14]. The dynorphin peptides, including dynorphin A-(1–17), dynorphin B and α-neo-endorphin, also potently inhibited [^{3}H]U69,593 binding to R1.G1 and R1EGO membranes. The high-affinity binding by both U50,488 and α-neo-endorphin in all three cell lines is consistent with the presence of a κ_{1b} binding site, as described by Clark *et al.* [14]. The μ-selective peptide DAMGO showed a 1000-fold lower affinity than U50,488 with each cell line; DAMGO binding to R1.1 membranes was shown to be due to crossover to the κ opioid site [11]. These results indicate that the R1.G1 and R1EGO cell lines each express a single κ_1 opioid binding site putatively identical to that expressed on R1.1 cells, but at a higher density.

Guanine nucleotide sensitivity of [^{3}H]U69,593 binding to R1.G1 and R1EGO membranes was demonstrated, suggesting that the binding sites are coupled to G-proteins. The potency of GTP inhibition of binding was similar in R1.1 and R1.G1 membranes, but a greater maximal inhibition was observed in the R1.G1 membranes. With R1EGO membranes, GTP was more potent, in that inhibition was observed at lower concentrations compared with the other cell lines. However, the inhibition curve with R1EGO membranes was more shallow than with R1.G1 and R1.1 membranes, and the inhibition of binding by 200 μM GTP did not differ significantly between R1.G1 and R1EGO membranes. Thus, the differences in the potency and magnitude of GTP inhibition of agonist binding to these cell lines do not directly correlate with differences in receptor number.

In contrast to GTP inhibition of [^{3}H]U69,593 binding, inhibition by Gpp(NH)p was similar in all three cell lines. It is unclear why GTP and Gpp(NH)p exhibit different profiles for the inhibition of agonist binding to each cell line. The basal rate of GTP hydrolysis measured in each cell line, in the presence of either 0.5 or 50 μM [γ-^{32}P]GTP, appears to exclude the possibility that the relatively high GTP potency in R1EGO membranes is due to a slower rate of GTP hydrolysis.

Stimulation of low-K_m GTPase activity in each cell line by the κ-selective agonist (−)U50,488 revealed that the magnitude of activation did not correlate with either the receptor density or the GTP sensitivity of agonist binding. This is clearly evident when the agonist-stimulated low-K_m GTPase activity in R1EGO membranes is compared with that of the other two cell lines. Although R1EGO membranes contained twice the number of κ opioid receptors as R1.G1 membranes, the maximal stimulation of low-K_m GTPase activity in R1EGO membranes was 40% lower than that of R1.G1 membranes. The sensitivity of agonist binding to GTP did not correlate with the magnitude of agonist-stimulated low-K_m GTPase activity, since GTP was more potent for inhibiting [^{3}H]U69,593 binding to R1EGO membranes than for inhibiting binding to R1.G1 membranes. The comparison of (−)U50,488-stimulated low-K_m GTPase activity in R1EGO and R1.1 membranes reveals another interesting result. Although the density of κ opioid receptors in R1EGO membranes was 6-fold greater than that of R1.1 membranes, the maximal stimulation of low-K_m GTPase activity in R1EGO membranes was only 55% greater than that of R1.1 membranes. If the coupling of κ opioid receptors to G-proteins was similar in each cell line, then the magnitude of stimulation of low-K_m GTPase activity in these cell lines would have correlated with receptor density. Therefore, receptor–G-protein coupling cannot be identical or even similar among these cell lines.

Another interesting observation from the GTPase studies was that the R1.G1 cell line exhibited the greatest level of both basal and agonist-stimulated low-K_m GTPase activity. The higher level of basal low-K_m GTPase activity in R1.G1 membranes suggests that this cell line expresses a greater number of G-proteins than either the R1.1 or R1EGO cell lines. This condition would be more conducive to receptor–G-protein coupling, and would explain the robust agonist-stimulated low-K_m GTPase activity observed in R1.G1 membranes.

Agonist-induced decreases in cyclic AMP production were detected in both R1.G1 and R1EGO membranes, showing that the functional response of these derivative cell line receptors, inhibition of adenylyl cyclase activity, is similar to the response seen in R1.1 membranes [16]. Both derivatives showed greater inhibition of adenylyl cyclase activity

than the parent cell line, with the greatest inhibition observed in R1.G1 membranes. The magnitude of cyclic AMP inhibition observed with each cell line correlated with neither the receptor density nor the GTP sensitivity of binding. For example, whereas R1EGO membranes contained twice the number of receptors than R1.G1 membranes, and showed greater agonist binding sensitivity to GTP, the magnitude of maximal inhibition of adenylyl cyclase activity was less in R1EGO membranes than in R1.G1 membranes. While studying δ opioid receptors on the neuroblastoma N18TG2 and neuroblastoma × glioma hybrid NG108-15 cell lines, Law and colleagues [33] found a difference in the magnitude of the inhibition of adenylyl cyclase activity that was not explained by a difference in receptor number. Similarly, Konkoy and Childers [34] recently reported that across several regions of guinea pig brain, the density of κ opioid receptors did not always correlate with the magnitude of inhibition of adenylyl cyclase activity. However, the magnitude of agonist inhibition of adenylyl cyclase activity in the R1.1 and its derivative cell lines did correlate with the magnitude of agonist stimulation of low-K_m GTPase activity (R1.G1 > R1EGO > R1.1). Thus, the relatively weak agonist response observed for the inhibition of cyclic AMP production in R1EGO membranes could be related to the low level of agonist-stimulated low-K_m GTPase activity in this cell line.

This is the first report of a series of cell lines expressing the same opioid receptor subtype with such intriguing relationships observed between receptor number, GTP inhibition of binding, stimulation of low-K_m GTPase activity, and maximal inhibition of adenylyl cyclase activity. The differences between the R1.1, R1.G1 and R1EGO cell lines appear to be related to differences in receptor–G-protein coupling. Also interesting is the finding that the R1E cell line, derived from the R1.1 cell line, does not express opioid receptors, whereas the R1E derivative, R1EGO, expresses high levels of κ opioid receptors. These mouse thymoma cell lines provide an excellent opportunity for further study of the cellular mechanisms of neurohormonal immunomodulation, as well as basic function and regulation of the κ_1 opioid receptor and its coupling to second messengers.

Acknowledgements—We thank LeeAnn Higgins, Timothy Tramontana and Lalitha D. Saripalli for excellent technical assistance and Drs. B. R. de Costa and K. C. Rice for their gift of the (−)-isomer of U50,488. This work was supported by US Public Health Service Grants DA04355 and DA07232 from the National Institute on Drug Abuse.

REFERENCES

1. Sharp BM, Keane WF, Suh HJ, Gekker G, Tsukayama D and Peterson PK, Opioid peptides rapidly stimulate superoxide production by human polymorphonuclear leukocytes and macrophages. *Endocrinology* **117**: 793–795, 1985.
2. Foster JS and Moore RN, Dynorphin and related opioid peptides enhance tumoricidal activity mediated by murine peritoneal macrophages. *J Leuko Biol* **42**: 171–174, 1987.
3. Taub DD, Eisenstein TK, Geller EB, Adler MW and Rogers TJ, Immunomodulatory activity of μ- and κ-selective opioid agonists. *Proc Natl Acad Sci USA* **88**: 360–364, 1991.
4. Bussiere JL, Adler MW, Rogers TJ and Eisenstein TK, Cytokine reversal of morphine-induced suppression of the antibody response. *J Pharmacol Exp Ther* **264**: 591–597, 1993.
5. Madden JJ, Donahoe RM, Zwemer-Collins J, Shafer DA and Falek A, Binding of naloxone to human T lymphocytes. *Biochem Pharmacol* **36**: 4103–4109, 1987.
6. Ovadia H, Nitsan P and Abramsky O, Characterization of opiate binding sites on membranes of rat lymphocytes. *J Neuroimmunol* **21**: 93–102, 1989.
7. Roy S, Ge B-L, Ramakrishnan S, Lee NM and Loh HH, [^{3}H]Morphine binding is enhanced by IL-1-stimulated thymocyte proliferation. *FEBS Lett* **287**: 93–96, 1991.
8. Fiorica E and Spector S, Opioid binding site in EL-4 thymoma cell line. *Life Sci* **42**: 199–206, 1988.
9. Carr DJJ, DeCosta BR, Kim C-H, Jacobson AE, Guarcello V, Rice KC and Blalock JE, Opioid receptors on cells of the immune system: Evidence for δ- and κ-classes. *J Endocrinol* **122**: 161–168, 1989.
10. Sibinga NES and Goldstein A, Opioid peptides and opioid receptors in cells of the immune system. *Annu Rev Immunol* **6**: 219–249, 1988.
11. Bidlack JM, Saripalli LD and Lawrence DMP, κ-Opioid binding sites on a murine lymphoma cell line. *Eur J Pharmacol* **227**: 257–265, 1992.
12. Lawrence DMP and Bidlack JM, Kappa opioid binding sites on the R1.1 murine lymphoma cell line: Sensitivity to cations and guanine nucleotides. *J Neuroimmunol* **41**: 223–230, 1992.
13. Zukin RS, Eghbali M, Olive D, Unterwald EM and Tempel A, Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for κ_1 and κ_2 opioid receptors. *Proc Natl Acad Sci USA* **85**: 4061–4065, 1988.
14. Clark JA, Liu L, Price M, Hersh B, Edelson M and Pasternak GW, Kappa opiate receptor multiplicity: Evidence for two U50,488-sensitive κ_1 subtypes and a novel κ_3 subtype. *J Pharmacol Exp Ther* **251**: 461–468, 1989.
15. Brown GP, Standifer KM and Pasternak GW, Biochemical characterization of κ_1 opiate binding sites in R1.1 thymoma cells. *Soc Neurosci Abstr* **19**: 1154, 1993.
16. Lawrence DMP and Bidlack JM, The kappa opioid receptor expressed on the mouse R1.1 thymoma cell line is coupled to adenylyl cyclase through a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein. *J Pharmacol Exp Ther* **266**: 1678–1683, 1993.
17. Portoghese PS, Lipkowski AW and Takemori AE, Binaltorphimine and nor-binaltorphimine, potent and selective κ-opioid receptor antagonists. *Life Sci* **40**: 1287–1292, 1987.
18. Ralph P, Retention of lymphocyte characteristics by myelomas and θ^+-lymphomas: Sensitivity to cortisol and phytohemagglutinin. *J Immunol* **110**: 1470–1475, 1973.
19. Hyman R and Stallings V, Characterization of a TL$^-$ variant of a homozygous TL$^+$ mouse lymphoma. *Immunogenetics* **3**: 75–84, 1976.
20. Gasser DL, Ziebur J and Matsumoto K, A lymphocyte antigen encoded by a locus closely linked to H-3 on the second chromosome of the mouse. *Proc Natl Acad Sci USA* **80**: 6620–6623, 1983.
21. Szekely JG, Lobreau AU, Einspenner M and Raaphorst GP, The use of flow cytometry to measure glucocorticoid-induced killing of lymphoid cell lines. *Basic Appl Histochem* **31**: 153–164, 1987.
22. Verland S and Gammeltoft S, Functional receptors for

insulin-like growth factors I and II in rat thymocytes and mouse thymoma cells. *Mol Cell Endocrinol* **67**: 207–216, 1989.

23. Co MS, Gaulton GN, Tominaga A, Homcy CJ, Fields BN and Greene MI, Structural similarities between the mammalian β-adrenergic and reovirus type 3 receptors. *Proc Natl Acad Sci USA* **82**: 5315–5318, 1985.
24. Liu J, Co MS and Greene MI, Reovirus type 3 and [^{125}I]iodocyanopindolol bind to distinct domains on the beta-adrenergic like receptor. *Immunol Res* **7**: 232–238, 1988.
25. Hyman R and Stallings V, Analysis of hybrids between an H-2$^+$, TL$^-$ lymphoma and an H-2$^+$, TL$^+$ lymphoma and its H-2$^-$, TL$^-$ variant subline. *Immunogenetics* **4**: 171–181, 1977.
26. Hyman R and Trowbridge I, Analysis of lymphocyte surface antigen expression by the use of variant cell lines. *Cold Spring Harbor Symp Quant Biol* **41**: 407–415, 1976.
27. Parnes JR and Seidman JG, Structure of wild-type and mutant mouse β_2-microglobulin genes. *Cell* **29**: 661–669, 1982.
28. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
29. Selley DE and Bidlack JM, Effects of β-endorphin on *mu* and *delta* opioid receptor-coupled G-protein activity: Low K_m GTPase studies. *J Pharmacol Exp Ther* **263**: 99–104, 1992.
30. Vachon L, Costa T and Herz A, GTPase and adenylate cyclase desensitize at different rates in NG108-15 cells. *Mol Pharmacol* **31**: 159–168, 1987.
31. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
32. Cheng Y-c and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
33. Law PY, Koehler JE and Loh HH, Comparison of opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 and neuroblastoma × glioma NG108-15 hybrid cell lines. *Mol Pharmacol* **21**: 483–491, 1982.
34. Konkoy CS and Childers SR, Relationship between kappa$_1$ opioid receptor binding and inhibition of adenylyl cyclase in guinea pig brain membranes. *Biochem Pharmacol* **45**: 207–216, 1993.