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An Immortalized Myocyte Cell Line, HL-1, Expresses a Functional δ-Opioid Receptor

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C. L. Neilan, E. Kenyon, M. A. Kovach, K. Bowden, W. C. Claycomb, J. R. Traynor and S. F. Bolling. An Immortalized Myocyte Cell Line, HL-1, Expresses a Functional δ-Opioid Receptor. *Journal of Molecular and Cellular Cardiology* (2000) **32**, 2187–2193. The present study characterizes opioid receptors in an immortalized myocyte cell line, HL-1. Displacement of [^{3}H]bremazocine by selective ligands for the mu (μ), delta (δ), and kappa (κ) receptors revealed that only the δ-selective ligands could fully displace specific [^{3}H]bremazocine binding, indicating the presence of only the δ-receptor in these cells. Saturation binding studies with the δ-antagonist naltrindole afforded a B_{max} of 32 fmols/mg protein and a K_D value for [^{3}H]naltrindole of 0.46 nM. The binding affinities of various δ ligands for the receptor in HL-1 cell membranes obtained from competition binding assays were similar to those obtained using membranes from a neuroblastoma × glioma cell line, NG108-15. Finally, various δ-agonists were found to stimulate the binding of [^{35}S]GTPγS, confirming coupling of the cardiac δ-receptor to G-protein. DADLE (D-Ala-D-Leu-enkephalin) was found to be the most efficacious in this assay, stimulating the binding of [^{35}S]GTPγS to 27% above basal level. The above results indicate that the HL-1 cell line contains a functionally coupled δ-opioid receptor and therefore provides an *in vitro* model by which to study the direct effects of opioids on cardiac opioid receptors.

Key Words: Cardiac myocytes; δ-opioid receptor; Radioligand binding; [^{35}S]GTPγS binding.

Introduction

Opiates and opioid peptides are known to exert significant cardiovascular effects including regulation of blood pressure and heart rate, via an action on the central nervous system.[1] In addition it has been shown that opioids exert a direct effect on the heart, and indeed there are several lines of evidence to suggest that cardiac opioid receptors and their ligands play a role in cardioprotection against prolonged ischemic insult and subsequent reperfusion.[2–4] The exact type(s) of opioid receptors involved in mediating cardioprotection is not known, but there is substantial evidence to suggest that both delta (δ) and kappa (κ) receptors play an important role.[4–6]

The presence of δ- and κ-opioid receptor types in rat cardiomyocyte membrane preparations has been demonstrated by a number of groups.[7–9] As yet no evidence has been found to support the existence of the mu (μ) opioid receptor in cardiac myocytes.[9,10] In addition, the only binding data available has been carried out in either rat or guinea pig cardiac membranes. Several functional studies have shown that upon activation of cardiac opioid receptors there is an activation of protein kinase C,[11,12] stimulation of phospholipase C,[13,14] increased inositol 1,4,5-triphosphate (IP_3), increased in-

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tracellular calcium and changes in pH.[15] In the following study the pharmacological characterization of opioid receptors in an immortalized mouse atrial cardiac myocyte cell line is presented. The study provides evidence for the presence of a functional δ-opioid receptor and thus provides a model for the susequent investigation of the direct effects of δ-opioids on cardiac myocytes.

Materials and Methods

Cell culture and membrane preparation

HL-1 cardiac myocytes[16] were cultured under a 5% CO_2 atmosphere in Ex-Cell 320 medium supplemented with 10% fetal bovine serum, 50 μg/ml endothelial cell growth supplement, 10 μg/ml insulin, 1 μM retinoic acid, 1 $\times$ non-essential amino acids and 100 μM norepinephrine to stimulate contractions. Culture flasks were precoated with 2 μg/cm fibronectin/0.02% gelatin solution. NG108-15 neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2% HAT supplement. Once cells had reached confluency they were harvested in HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid], 20 mM pH 7.4)-buffered saline containing 1 mM EDTA (NG108-15 cells), or trypsin-EDTA (HL-1 cells), then dispersed by agitation and collected by centrifugation at 500 $\times$ **g**. The cell pellet was suspended in 50 mM Tris-HCl buffer pH 7.4, and homogenized with a tissue tearor (Biospec Products, Bartlesville, OK, USA). The resultant homogenate was centrifuged for 15 min at approximately 40 000 $\times$ **g** at 4°C and the pellet collected, resuspended and recentrifuged. The final pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4; separated into 0.5 ml aliquots (0.75–1.0 mg protein) and frozen at -80°C. Protein concentration for cell membrane preparations was determined by the method of Lowry *et al.*,[17] using a bovine serum albumin standard.

Saturation binding assays

HL-1 cell membranes (300–500 μg protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4 for 1 h with varying concentration of [^{3}H]-naltrindole in the presence of either water (control) or 10 μM naloxone to determine total specific binding. The reaction was terminated by filtering the samples through glass fiber filters (Schleicher and Schuell #32, Keene, NH, USA) mounted in a Brandel 24-well harvester. The filters were subsequently washed three times with ice-cold Tris-HCl, pH 7.4 and radioactivity determined by scintillation counting after addition of 3 ml of Ultima Gold liquid scintillation fluid. Binding capacities (B_{max}) and equilibrium dissociation constants (K_D) were calculated from non-linear regression using GraphPad Prism, San Diego, CA, USA.

Competition binding assays

HL-1 cell membranes (300–500 μg protein), or NG108-15 cell membranes (100–150 μg protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4 for 1 h with radioligand and varying concentrations of unlabeled ligand to give a final volume of 1 ml (NG108-15 cells) or 500 μl (HL-1 cells). Non-specific binding was defined with 10 μM naloxone. The reaction was terminated by filtration as above, and filters were subjected to liquid scintillation counting. K_i values were determined using GraphPad Prism, using K_D values of 0.20 nM for [^{3}H]diprenorphine and 0.46 nM for [^{3}H]naltrindole as determined by saturation assay. For the determination of type(s) of opioid receptors present [^{3}H]bremazocine was used at a concentration of 1.0 nM.

[^{35}S]GTPγS binding assays

Agonist stimulation of [^{35}S]GTPγS binding was measured as described by Traynor and Nahorski.[18] Cell membranes (300–500 μg protein) were incubated for 1 h at 30°C in GTPγS binding buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). [^{35}S]GTPγS (guanosine-5′-O-(3-thio)-triphosphate) (50 pM), and GDP (guanosine 5′-diphosphate) (either 30 or 100 μM) were added to give a final volume of 500 μl. The reaction was terminated by rapid filtration as above except that samples were washed with GTPγS binding buffer, pH 7.4, and radioactivity retained on filters analysed by scintillation counting (see above). Basal binding was determined in the absence of unlabeled ligand.

Drugs

[^{3}H]diprenorphine (58 Ci/mmol), [^{3}H]naltrindole (33 Ci/mmol), [^{3}H]bremazocine (26.5 Ci/mmol) and

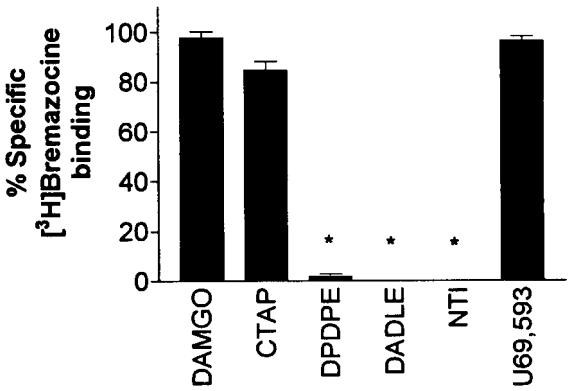

Figure 1 The displacement of specific [^{3}H]bremazocine binding by selective μ- (DAMGO, 100 nM, CTAP, 300 nM), δ- (DPDPE, DADLE, naltrindole, all 1 μM), and κ- (U69, 593,1 μM) opioid ligands. Values represent mean $\pm$ S.E.M. for three experiments performed in duplicate. *P<0.05, Student's t-test.

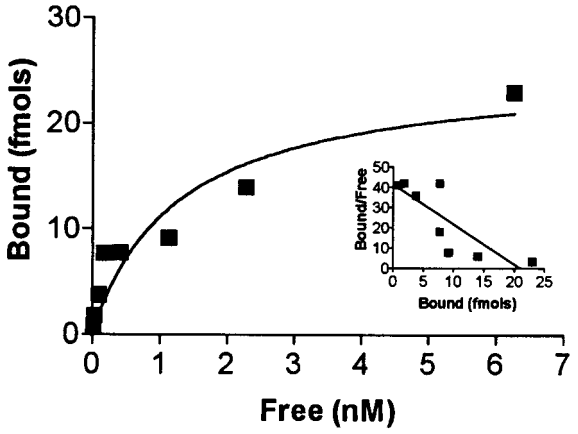

Figure 2 Representative curve of saturation binding of [^{3}H]naltrindole to receptors in HL-1 cell membranes. Shown in the inset is the corresponding Scatchard plot.

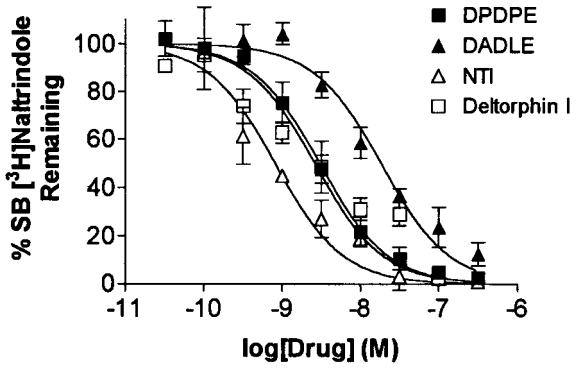

Figure 3 The displacement of specific [^{3}H]naltrindole binding by various δ-ligands. [^{3}H]Naltrindole was used at a concentration of 0.46 nM as determined by saturation binding. Values represent mean $\pm$ S.E.M. for three experiments performed in duplicate.

[^{35}S]GTPγS (1250 Ci/mmol) were purchased from DuPont NEN, Boston, MA, USA. The following drugs were generous gifts from the National Institute on Drug Abuse, Rockville, MD, USA: naloxone HCl, SNC80, deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), DADLE ([D-Ala^2, D-Leu^5]enkephalin) and DPDPE ([D-Pen^2, D-Pen^5]enkephalin). CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) and naltrindole were a kind gift from NIH (Bethesda, MD, USA). DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol) was purchased from Tocris Cookson, (Ballwin, MO, USA), DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) from Multiple Peptide Systems (San Diego, CA, USA), and U69,593 from Research Biochemicals International (Natick, MA, USA).

Results

The displacement of the non-selective opioid ligand [^{3}H]bremazocine by various unlabelled opioid ligands revealed that only δ-selective ligands, DPDPE, DADLE and naltrindole, all at a concentration of 1 μM, were able to fully displace specific [^{3}H]bremazocine binding from membranes prepared from HL-1 cells (Fig. 1). Neither the μ-selective ligand DAMGO nor the κ-selective ligand U69,593 at concentrations of 100 nM and 1 μM respectively, were able to displace any specifically bound radioligand. These concentrations are approximately 100- and 1000-fold higher than their affinities for the μ- and κ-receptor respectively. At a concentration of 300 nM the μ-selective antagonist CTAP was able to displace only 15% of specific [^{3}H]bremazocine binding.

Saturation binding of the selective δ-antagonist [^{3}H]naltrindole to membranes prepared from HL-1 cells afforded data best fit to a single site with a binding capacity (B_{max}) value of 32 fmols/mg of protein, and a dissociation constant (K_D) for [^{3}H]naltrindole of 0.46 $\pm$ 0.05 nM. A representative saturation binding curve is shown in Figure 2 with the Scatchard plot as the inset.

In order to determine if the receptor found in HL-1 cells was pharmacologically similar to the neuronal δ-opioid receptor, binding affinities of various δ-ligands were determined in HL-1 cells and compared to the values obtained from a neuroblastoma $\times$ glioma cell line, NG108-15, which endogenously expresses the mouse δ-receptor.[19] Figure 3 shows the competition binding

Table 1 K_i values for the inhibition of specific [^{3}H]diprenorphine binding (NG108-15 cell membranes) and [^{3}H]naltrindole binding (HL-1 cell membranes). [^{3}H]Diprenorphine was used at a concentration of 0.20 nM and [^{3}H]naltrindole at a concentration of 0.46 nM, as determined by K_D values obtained from saturation binding.

Ligand	NG-108-15	HL-1
DADLE	3.02 ± 1.30	12.21 ± 3.30
DPDPE	1.96 ± 0.76	1.96 ± 0.56
Deltorphin 1	2.84 ± 1.07	2.12 ± 0.86
Naltrindole	0.21 ± 0.03	0.52 ± 0.07

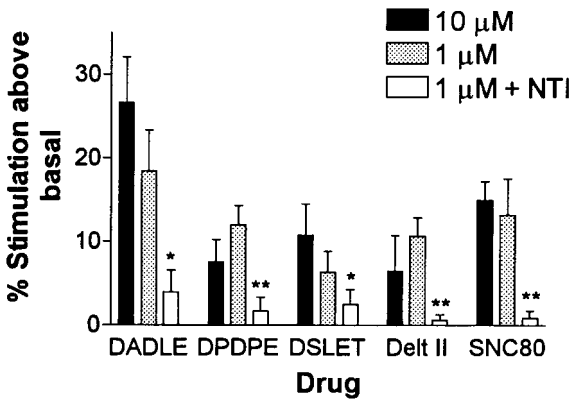

Figure 4 Stimulation of [^{35}S]GTPγS binding by the various δ-agonists at 10 and 1 μM concentrations, and inhibition of agonist stimulation (1 μM) by naltrindole (1 μM). Values represent mean ± S.E.M. for at least three experiments performed in duplicate. Significant differences were found between stimulation produced by 1 μM agonist and stimulation produced by 1 μM agonist in the presence of naltrindole. *P<0.05, **P<0.01, Student's t-test.

curves obtained for the displacement of [^{3}H]naltrindole by four δ-ligands, the peptide agonists DPDPE, DADLE, and deltorphin 1, and the non-peptide antagonist naltrindole in membranes prepared from HL-1 cells. All ligands afforded Hill coefficients of close to unity indicating recognition of a single binding site. The binding affinities (K_i) values determined for these competition curves are given in Table 1 and are compared with those obtained in NG108-15 cell membranes.

In order to determine if the receptor present in HL-1 cells was functionally coupled to G-proteins, the ability of various δ-opioid agonists to stimulate the binding of [^{35}S]GTPγS was assessed (Fig. 4). Optimum level of GDP for use in this assay using the HL-1 cell line was determined as being 30 μM (data not shown). The peptide agonist DADLE, at a concentration of 10 μM, was found to be the most efficacious in this assay, affording a stimulation of [^{35}S]GTPγS binding of 26.6 ± 5.4% above basal level of [^{35}S]GTPγS binding. The non-peptide agonist, SNC80 (10 μM), afforded a stimulation of 14.9 ± 2.2% above basal. The three other δ-peptides examined, DPDPE, DSLET, and deltorphin II afforded maximal stimulations of 12.0 ± 2.2, 9.9 ± 6.2, and 12.9 ± 2.7% respectively. Concurrent addition of the δ-antagonist naltrindole (1 μM) significantly blocked stimulation of [^{35}S]GTPγS binding by the agonists at 1 μM concentrations, confirming a δ-specific agonist effect. Significant differences between individual points were determined by Student's t-test. No significant differences were found between stimulation with 10 μM *v* 1 μM agonist, indicating that maximal effect of agonist was achieved at 1 μM.

Discussion

HL-1 cells, derived from a mouse atrial cardiomyocyte tumor, have been shown to retain the phenotypic characteristics of the adult cardiomyocyte, yet are able to proliferate and can be serially passaged.[16] The above study provides evidence for the presence of a functional δ-opioid receptor in these cells. This agrees with previous findings that opioid receptors exist in the myocardium; low levels of binding of opioids to cardiac muscle from guinea pig and rat hearts have been reported.[20,21] In more recent years several groups have reported binding of δ- and κ-opioids to crude membranes prepared from rat heart,[8,22,23] from purified rat cardiac sarcolemmal preparations[7] and from membranes prepared from rat cardiomyocyte cultures.[9]

Interestingly, in the HL-1 cell line, the standard κ-agonist U69,593, did not displace any specific [^{3}H]bremazocine binding. This is contrary to previous findings that this ligand does bind to cardiac membranes.[7,8,22] The difference may be attributable to species differences (rat *v* mouse) or indeed may be due to the fact that we are using an immortalized myocyte cell line. In addition, the standard μ-opioid peptides DAMGO and CTAP, used at doses selective for the μ-receptor, could not displace any specific [^{3}H]bremazocine binding. This is in agreement with previous findings that no appreciable μ-receptor binding can be identified in cardiac membranes.[7,8,24] The ability of only the δ-ligands to fully displace all [^{3}H]bremazocine binding argues strongly for the presence of the δ-receptor alone in the HL-1 cells.

The level of δ-opioid receptors is, however, low. Saturation binding using [^{3}H]naltrindole revealed specific, saturable binding to a receptor population

of 32 fmols/mg protein. A previous study by Ela *et al.*,[10] reported a B_{max} of only 12.9 fmols/mg protein for the δ-receptor in rat ventricular cardiomyocytes. Again the differences observed may be due to species differences, the use of different heart chambers, or, although more unlikely, the use of a different radioligand. Interestingly, studies on the relative opioid receptor number in the four chambers of the heart have shown that the largest population of opioid receptors is found in the right atrium.[8,24]

To further characterize the receptor found in the HL-1 cell line, the binding affinities of the δ-opioid peptides DPDPE, DADLE and deltorphin I, and the δ-non-peptide naltrindole were compared to those obtained using membranes prepared from NG108-15 cells. DPDPE and deltorphin I had similar K_i values for the δ-receptor in both cell lines. DADLE appeared to have somewhat lower affinity (12.2 nM) for the receptor in the HL-1 cells compared to a value of 3.0 nM for the receptor in NG108-15 cells. The antagonist naltrindole also had a slightly lower affinity for the receptor in HL-1 cells (0.52 nM compared to 0.21 nM in NG108-15 cells).

Although no cloning data is available, the existence of δ-receptor subtypes has been proposed,[25,26] based on *in vivo* antagonism studies using naltrindole and its analogues, including naltriben (NTB), a putative δ_2 antagonist, and benzylidenenaltrexone (BNTX), a putative δ_1 antagonist. DPDPE and DADLE are agonists at the putative δ_1 subtype and the peptides DSLET and deltorphin II are agonists at the putative δ_2 subtype. It has been suggested that the δ-receptor in NG108-15 cells is of the δ_2 subtype.[27,28] Given that the K_i value for DPDPE is similar in both cell lines, and that the affinity of DADLE is even lower for the receptor in HL-1 cells, it is tempting to propose that just the δ_2 subtype existed in this cell line. In addition, Hill coefficients were all close to unity indicating a homogenous receptor population in both cell lines.

One other possible explanation for the lower affinity of DADLE may be the increased presence of proteases in this cell line compared to in NG108-15 cells. No displacement of [^{3}H]naltrindole from receptor in HL-1 cells was obtained by the peptide [Leu^5]-enkephalin unless a protease inhibitor cocktail was present in the binding buffer, and the incubation temperature decreased to 4°C (data not shown). It may be that DADLE is similarly sensitive to proteases in HL-1 cells and therefore is partially degraded upon incubation with the membrane fraction.

The cell signalling events that occur upon agonist activation of opioid receptors have been well characterized, and indeed, there are many reports of direct effects of opioids on cardiomyocyte opioid receptors. Opioids increase inositol 1,4,5-tri-phosphate (IP_3) and elevate intracellular free calcium by increasing mobilization of calcium from intracellular stores;[15] they cause an activation of protein kinase C,[11,12] and a stimulation of phospholipase C.[13,14] Opioids mediate these signal transduction events *via* their interaction with G-proteins, and it has been shown that the effects of opioids on myocardial contraction are blocked by pertussis toxin,[29] indicating that the G_i/G_o family of G-proteins is involved in this process. Despite all of the reports on cell signalling mechanisms downstream of G-protein activation, the direct activation of G_i/G_o G-proteins in cardiac tissue has not yet been shown. In this study, we chose to use the [^{35}S]GTPγS assay in order to determine if δ-receptor agonists could directly stimulate the binding of [^{35}S]GTPγS thus demonstrating a functional coupling of the receptor to G_i/G_o. All agonists stimulated the binding of [^{35}S]GTPγS using GDP at a level of 30 μM. At 10 μM GDP no stimulation by the agonists was observed, and using 100 μM GDP stimulation dropped significantly (data not shown). Other studies have highlighted this dependence on GDP, for example stimulation of the binding of [^{35}S]GTPγS by μ-opioid agonists in SH-SY5Y cells,[18 by the] δ-receptor in NG108-15 cells,[28] and also by the muscarinic receptor in porcine cardiac membranes.[30] Stimulation by the agonist was blocked by the concurrent addition of 1 μM naltrindole, thus confirming a δ-specific response. Naltrindole alone did not produce any stimulation (data not shown). The rank order of efficacy between agonists was as follows: DADLE>SNC80>DPDPE>DSLET>deltorphin II. This corelates with previously reported data using NG108-15 cells,[28] C6 glioma cells[31] and COS[32] cells both transfected with the rat δ-receptor.

There has been considerable excitement in recent years following the discovery that opioids (in particular δ- and κ-ligands) may play an important role in cardioprotection against prolonged ischemic insult and subsequent reperfusion. It has been shown that isolated rabbit hearts pre-treated with DADLE exhibit improved functional recovery following cardioplegic ischemia and reperfusion.[4] Similar results were observed by Kevelaitis *et al.*,[33] who reported improved functional preservation of cold-stored rat hearts pre-treated with DADLE. Opioids also appear to mimic the effects of ischemic preconditioning.[2,3] The exact mechanism by which opioids afford cardioprotection is not known, although it is believed to be a result of an action on the K_{ATP} channel.[5,33,34] All of the above studies

have been carried out using either whole hearts or primary myocyte cultures. The results of the present study clearly show that the HL-1 cell line contains a functionally coupled δ-opioid receptor and thus provides a valid model with which to study the direct effects of opioids on cardiac δ-receptors whilst eliminating lengthy tissue preparation times and complications of mixed tissues.

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