ELSEVIER

European Journal of Pharmacology 348 (1998) 85–93

ejp

Catecholaminergic CATH.a cells express predominantly δ-opioid receptors

Claudia Bouvier [1], Dorina Avram [1], Valerie J. Peterson, Barbara Hettinger, Ken Soderstrom, Thomas F. Murray [2], Mark Leid *

Laboratory of Molecular Pharmacology, College of Pharmacy, Oregon State University, Corvallis, OR 97331, USA

Received 20 November 1997; revised 9 February 1998; accepted 13 February 1998

Abstract

CATH.a cells are a catecholaminergic cell line of neuronal origin. The opioid receptor complement expressed by CATH.a cells was defined pharmacologically and by reverse transcription-polymerase chain reaction (RT-PCR). CATH.a cells were found to express mRNA encoding all three of the major subtypes of opioid receptors. The relative abundance of CATH.a cell opioid receptor transcripts was $\delta > \kappa > \mu$. Pharmacological and functional data were in agreement with the results of RT-PCR inasmuch as δ-opioid receptor was identified as the most abundant opioid receptor subtype expressed by CATH.a cells. In addition, at least one of the opioid signalling pathways, inhibition of adenylyl cyclase activity, was found to be operant in this cell line. CATH.a cells should be of general utility for the study of opioid receptor signalling mechanisms in the context of catecholaminergic neurons. © 1998 Elsevier Science B.V.

Keywords: CATH.a cell; Noradrenergic neuron; Opioid receptor; Cell culture

1. Introduction

Opiates exert their cellular effects via interaction with several receptor subtypes that were initially identified by pharmacological studies. Molecular cloning has confirmed the existence of at least three major subtypes of opioid receptors: the μ-, δ- and the κ-opioid receptors (Chen et al., 1993; Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993; Wang et al., 1993) as well as the opioid receptor-related protein ORL1/LC132 (Bunzow et al., 1994; Mollereau et al., 1994; Wang et al., 1994) for which an endogenous ligand has been identified (Butour et al., 1997; Meunier et al., 1995; Reinscheid et al., 1995; Tian et al., 1997). Although the ligand specificities and signalling mechanisms of individual opiate receptors can be studied in isolation through use of non-neuronal cells stably expressing a particular opioid receptor subtype, characterization of opiate action at the level of the neuron requires use of either brain tissue or neuronal cell lines.

The CATH.a cell line was developed from a brainstem tumor of a transgenic mouse expressing the SV40 T antigen driven by the tyrosine hydroxylase promoter (Suri et al., 1993). These immortalized cells are not of glial origin but rather are catecholaminergic in nature expressing dopamine β hydroxylase and tyrosine hydroxylase, and synthesizing dopamine and norepinephrine (Suri et al., 1993). The catecholaminergic phenotype of CATH.a cells suggests that these cells may be derived from either locus coeruleus or possibly other discrete catecholaminergic nuclei in brain (Suri et al., 1993). CATH.a cells also express several markers of differentiated neurons including neurofilament proteins (Suri et al., 1993) and voltage-sensitive potassium (Baraban et al., 1995), Na^+ and Ca^{2+} (Lazaroff et al., 1996) channels. Bundey et al. (1997) have demonstrated that CATH.a cells exhibit functional signalling responses to corticotrophin-releasing factor, vasoactive intestinal peptide, bradykinin, and muscarinic acetylcholine and α_2-adrenergic receptor agonists. CATH.a cells are somewhat heterogeneous in culture and do not display an overt neuronal morphology (Baraban et al., 1995; Qi et al., 1997; Suri et al., 1993). However, recent studies have

* Corresponding author. Tel.: +1-541-737-5809; fax: +1-541-737-3999; e-mail: leidm@ccmail.orst.edu

[1] The first two authors contributed equally to this work.

[2] Present address: Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602, USA.

demonstrated that serum and/or protein starvation of a particular subclone of CATH.a cells induces differentiation to CATH.a differentiated (CAD) cells (Qi et al., 1997). CAD cells exhibit a pronounced neuronal morphology and express a wider range of neuronal-specific markers than the parental CATH.a cell line.

Although CATH.a cells have been used widely as a neuronal cell line, a complete pharmacological characterization of the opioid receptor complement expressed by this cell line has not been reported. The present study was undertaken to elucidate the subtype(s) of opioid receptor expressed by CATH.a cells and to characterize the functional coupling of these receptors to signal transduction pathways.

2. Materials and methods

2.1. Materials

[^{3}H]Diprenorphine was purchased from Amersham (Arlington Heights, IL) and [^{3}H][D-Pen^2-D-Pen^5]enkephalin ([^{3}H]DPDPE), [^{3}H]adenine and [^{14}C]CAMP were purchased from DuPont NEN (Boston, MA). Bio-Rad AG-50W-X4 cation exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA). Neutral alumina, bestatin, captopril, forskolin and [D-Ala^2, *N*-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) were purchased from Sigma (St. Louis, MO), DPDPE was obtained from Bachem (Torrance, CA), and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl-benzeneacetamide methanesulfonate (U50488) and levorphanol were purchased from RBI (Natick, MA). 4-[(3-Butoxy-4-methoxyphenyl) methyl]-2-imidazolidinone (Ro20-1724) was a gift from Hoffman-LaRoche (Nutley, NJ). All other chemicals were purchased from Gibco-BRL (Grand Island, NY), Sigma or Bachem.

2.2. Tissue culture and preparation of membranes

Mouse CATH.a cells were grown and passaged essentially as described by Suri et al. (1993) on 100 mm plates except that the RPMI medium was supplemented with 2.5% fetal bovine serum (Hyclone) and 7.5% horse serum (Gibco). Under these cell culture conditions, no evidence of CATH.a cell differentiation was observed. Membranes derived from CATH.a cells were prepared as follows: cell monolayers were rinsed twice with 5 ml of ice-cold, 50 mM Tris–HCl pH 7.4 (Buffer A), detached with a rubber policeman, homogenized in a Dounce homogenizer and centrifuged at $37\,000 \times g$ for 10 min at 4°C. The pellets were washed twice, by resuspension and centrifugation as above, with 5 ml of Buffer A and then the washed and pelleted membranes were stored at −80°C until used. Chinese hamster ovary (CHO) cells stably expressing recombinant μ, κ, and δ-opioid receptors (CHOμ, CHOκ and CHOδ, respectively) were maintained in F12 medium containing 700 μg/ml G418 and supplemented with 10% fetal bovine serum as previously described (Bunzow et al., 1995). These cells were harvested and membranes prepared as described for CATH.a cells.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from CATH.a cells using Tri-Reagent (Molecular Research Center). Poly A+ mRNA was prepared from total RNA by double purification on oligo dT-Sepharose (Pharmacia) using standard techniques. First-strand cDNA synthesis was carried out using 1 μg of CATH.a cell poly A+ mRNA, 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco-BRL), and 100 ng of random hexameric primers (Promega). Reverse transcription reactions were performed at 37°C for 60 min and terminated by heating at 65°C for 15 min. Amplification of tyrosine hydroxylase was used as an internal control for reverse transcription and all polymerase chain reactions as follows: 5% of each randomly-primed, reverse transcription reaction was subjected to 5 rounds of PCR (denaturation at 94°C for 25 s, annealing at 45°C for 1 min, extension at 72°C for 50 s) using only tyrosine hydroxylase primers (see Table 1). Opioid receptor subtype-specific primers (Table 1) were introduced into the reactions and an additional 42 cycles were carried out as described above except that the annealing temperature was increased to 54°C. Twenty percent of each amplification reaction was subjected to electrophoresis on a 2% agarose gel and the amplified products were visualized by ethidium bromide staining. The gel was then blotted to a nylon membrane (ZetaProbe, Biorad) which was incubated in prehybridization solution (0.75 M NaCl, 0.075 M sodium citrate, 0.5% sodium dodecyl sulfate and Denhardt's solution without albumin) at 42°C for 4 h prior to probing overnight at 42°C with [^{32}P] end-labelled oligonucleotides specific for μ, κ and δ-opioid receptors (see Table 1). The blot was washed in 2× sodium chloride/sodium citrate (SSC; 300 mM NaCl, 30 mM sodium citrate) at 42°C and then subjected to autoradiography with intensifying screens for 4 to 24 h.

2.4. Radioligand receptor binding assays

Frozen membrane pellets were resuspended in 50 volumes of ice-cold Buffer A and used immediately in radioligand binding assays. The protein concentration was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard. Equilibrium saturation and competition experiments using [^{3}H]diprenorphine (39 Ci/mmol) were performed at 25°C for 90 min and typically employed 150–400 μg (CATH.a cells) or 80–100 μg (CHO cells expressing recombinant opioid

Table 1
PCR primers used for amplification of opioid receptor subtype and tyrosine hydroxylase cDNA from CATH.a cells

cDNA	5′ Primer (5′-3′)	Oligonucleotide probe (5′-3′)	3′ Primer (5′-3′)	Expected size of amplified PCR product (bp)
DOR	CTGGACTTCCGGACACCAGCC (nt 703–724)	TGGTATGCATGCTCCAGTTCCCCAG (nt 821–845)	ACGCAGATCTTGGTCACAGTG (nt 884–863)	181
KOR	TTGGACTTCCGAACACCTTTG (nt 684–704)	ATGTCATTGAATGCTCCTTGCAGT (nt 802–826)	ACACAGATCTTCATGAAGAGA (nt 873–852)	189
MOR	CTGGATTTCCGTACCCCCCGA (nt 743–764)	TAGATTGCACCCTCACTTTCTCTCA (nt 861–885)	ACACAGATTTTGAGCAGGTTC (nt 924–903)	181
TH	ACCTTTCCTTCCTTTATTG (nt 1501–1520)	–	AAATGCATAGGGTACCACC (nt 1773–1754)	272

receptors) of membrane protein. All radioligand binding experiments were carried out in 2.0 ml of Buffer A supplemented with 3 mM magnesium acetate, 30 μM captopril, 10 μM bestatin and 50 μM L-leucyl–L-leucine, and the reactions were terminated by rapid filtration over GF/B filters (which had been presoaked in 0.5% polyethylenimine to reduce non-specific binding) using a Brandel Cell Harvester. The filters were rapidly washed (3 × 4 ml) with ice-cold Buffer A, dried and eluted overnight in CytoScint (ICN) scintillation cocktail prior to counting using a Beckman LS6000SC scintillation counter. Non-specific binding was defined as that occurring in the presence of either 10 μM levorphanol or 10 μM DPDPE, both of which gave identical results. The [^{3}H]dprenorphine concentration used in all competition experiments ranged from 2 to 5 nM. Equilibrium saturation experiments using [^{3}H]DPDPE (32.4 Ci/mmol) were performed at 25°C for 90 min as described above except the magnesium acetate concentration used in the assay employing this agonist radioligand was 9 mM rather than 3 mM.

2.5. Adenylyl cyclase activity assays

Adenylyl cyclase assays were performed according to a modification of the method described by Salomon (1979). CATH.a cell monolayers were washed twice with serum-free RPMI medium (Gibco-BRL) and then incubated for 4 h in 1 ml of the same medium containing 1.2 μCi [^{3}H]adenine (26.9 Ci/mmol). This media was then aspirated and replaced with serum-free RPMI medium containing a phosphodiesterase inhibitor (50 μM Ro20-1724) and peptidase inhibitors (10 μM bestatin, 30 μM captopril, 50 μM L-leucyl–L-leucine). These cultures were incubated at 37°C for 30 min in the presence of 5 μM forskolin and increasing concentrations of DPDPE. Incubations were terminated by the addition of 300 μl of stop solution (2% sodium dodecyl sulfate [SDS], 1.3 mM cAMP), followed by addition of 750 μl water, and 100 μl concentrated perchloric acid. [^{14}C]cAMP (5000 cpm in 50 μl; 52.3 mCi/mmol) was added to each plate and used to normalize for cAMP recovery. After transferring the contents of culture dishes to 1.5 ml centrifuge tubes, 12 M KOH was added to neutralize the samples. The resulting precipitate was removed by centrifugation at 10 000 × g for 10 min and cAMP in the supernatant was isolated by sequential chromatography over AG-50W-X4 cation exchange resin and neutral alumina columns. The amount of [^{3}H]cAMP and [^{14}C]cAMP present in eluates from these columns was quantified simultaneously by two-channel liquid scintillatography using a Beckman LS 6000SC scintillation counter. Counts were corrected for crossover and recovery.

2.6. Data analysis

An iterative, curve-fitting routine (GraphPad Prism) was used in all data analyses.

3. Results

3.1. Opioid receptor subtype-specific RT-PCR

The results of RT-PCR analysis revealed that CATH.a cells express mRNA encoding all three of the major subtypes of opioid receptors (Fig. 1A and B). However, the relative abundance of the three opioid receptor transcripts varied dramatically. Delta opioid receptor mRNA was found to be the most abundant opioid receptor transcript expressed in CATH.a cells and the corresponding amplified fragment was detected by both ethidium bromide staining (Fig. 1A, lane 2) and by Southern blotting (Fig. 1B, lane 2). Although amplified fragments derived from either κ or μ transcripts were not detectable by ethidium bromide staining (Fig. 1A, lanes 4 and 6, respectively), Southern analysis of amplification reactions revealed the presence of both transcripts in CATH.a cells (Fig. 1B, lanes 4 and 6, respectively). From these studies, we conclude that the relative expression level of the three opioid receptor genes in CATH.a cells is: $\delta > \kappa > \mu$.

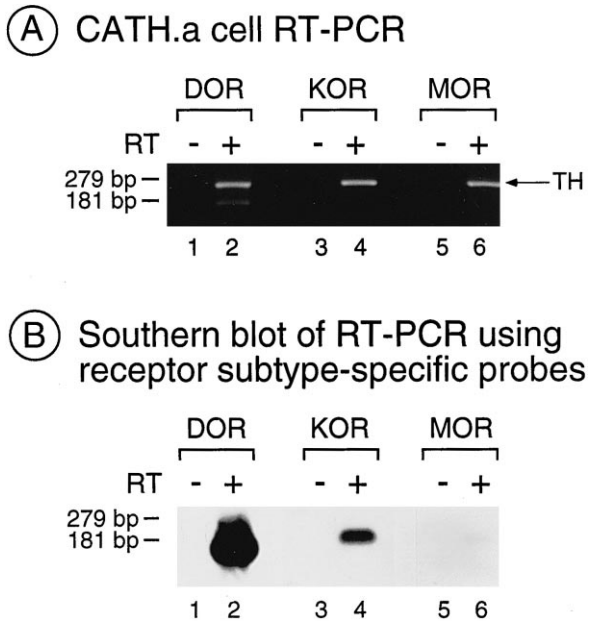

Fig. 1. RT-PCR analysis of CATH.a cell opioid receptor mRNA. (A) Ethidium bromide-stained gel representing RT-PCR analysis of CATH.a cell mRNA. Reactions were carried out in the absence (lanes 1, 3, and 5) and presence (lanes 2, 4, and 6) of reverse transcriptase (RT) and δ (DOR)-, κ (KOR)-, μ (MOR)-specific primers as indicated. The migration of two markers (279 bp and 181 bp) is indicated to the left and that of tyrosine hydroxylase (TH) is indicated to the right of the gel. Tyrosine hydroxylase, which is highly expressed in CATH.a cells, was used as an internal control for amplification reactions and gel loading. The expected sizes of amplified fragments from δ, κ, μ receptors are 181, 189 and 181 bp, respectively. (B) Southern analysis of the gel shown in A. These blots were individually probed with [^{32}P]-labeled, opioid receptor subtype-selective probes under stringency conditions which minimized cross-hybridization.

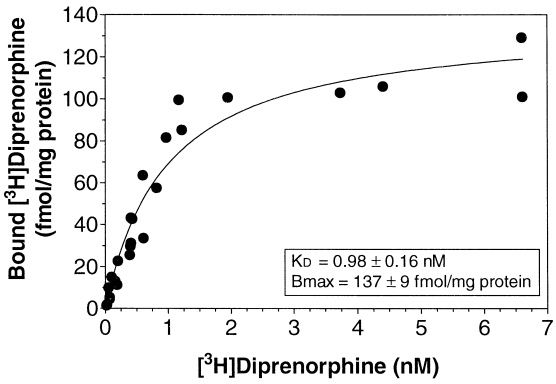

Fig. 2. Saturation isotherm of [^{3}H]diprenorphine binding to CATH.a cell membrane preparations. Data were pooled from four independent experiments performed in duplicate. Non-specific binding was defined in the presence of 10 μM levorphanol, however, use of 10 μM DPDPE to define non-specific binding yielded identical results. The theoretical curve shown was obtained by non-linear regression analysis which yielded the parameter estimates given in the figure.

3.2. Radioligand binding studies

Radioligand binding experiments were carried out to verify the existence, and to determine the subtype complement of CATH.a cell opioid receptors. A non-selective opioid receptor antagonist radioligand, [^{3}H]diprenorphine, was used for the initial radioligand binding studies to establish the presence and quantitate the density of opioid receptors in CATH.a cell membrane preparations. [^{3}H]Diprenorphine bound saturably and with high affinity (K_D = 0.98 ± 0.16 nM) to an apparently homogenous population of recognition sites in membrane preparations derived from CATH.a cells (B_{max} = 137 ± 9 fmol/mg of protein; Fig. 2). Definition of non-specific binding using either levorphanol or DPDPE (each at 10 μM) yielded identical parameter estimates for [^{3}H]diprenorphine saturation experiments. The affinity of [^{3}H]diprenorphine for recombinant μ-, κ- and δ-opioid receptors was determined in parallel saturation experiments to be 0.23, 0.31 and 0.62 nM, respectively (data not shown). Although not definitive, the affinity with which [^{3}H]diprenorphine labelled CATH.a membranes was most similar to that of recombinant δ-opioid receptor.

Toward the goal of pharmacological characterization of the CATH.a cell opioid receptor population, the ability of three prototypic, receptor subtype-selective compounds (δ-selective DPDPE, μ-selective DAMGO, and κ-selective U50488) to inhibit [^{3}H]diprenorphine binding to CATH.a cell membrane preparations was evaluated. Competition radioligand binding experiments were carried out in parallel using membranes derived from CHO cells stably expressing recombinant μ, K, and δ-opioid receptors to facilitate unambiguous classification of the opioid receptor subtype(s) expressed by CATH.a cells. Parameter estimates which describe these titration experiments are given in Table 2. The rank-order of potency with which subtype-selective compounds inhibited [^{3}H]diprenorphine binding in CATH.a membranes was as follows: DPDPE > > > DAMGO > U50488

DPDPE inhibition of [^{3}H]diprenorphine binding in CATH.a cell membrane preparations was nearly identical to that obtained using CHO cells stably expressing δ-opioid receptor (Table 2). Moreover, the weak potency with which DAMGO and U50488 displaced [^{3}H]diprenorphine from CATH.a cell membranes was consistent with the low affinity of these opioids as inhibitors of [^{3}H]diprenorphine binding to recombinant δ-opioid receptor (Table 2). These findings, together with the results of RT-PCR, suggest that the predominant opioid receptor expressed by CATH.a cells is of the δ subtype.

Titration of [^{3}H]diprenorphine binding by DAMGO, U50488 and DPDPE was biphasic when using membranes prepared from CHO-μ, -κ and -δ, respectively, consistent with the existence of high (G protein-coupled) and low (uncoupled) agonist affinity states of these recombinant receptors (Table 2). Titration of [^{3}H]diprenorphine binding to CATH.a cell membranes by DPDPE, but neither DAMGO nor U50488, was also biphasic suggestive of a heterologous δ-opioid receptor population in these cells (Table 2). The interconvertible nature of the two populations of CATH.a cell δ-opioid receptors was investigated by examining the influence of Gpp(NH)p on DPDPE

Table 2
Parameter estimates for inhibition of [^{3}H]diprenorphine binding by subtype-selective, opioid receptor agonists in membrane preparations derived from CATH.a cells and stably-transfected CHO cells expressing recombinant opioid receptor subtypes

	CHO-μ	CHO-K	CHO-δ	CATH.a
	K_I ± S.E. (nM)			
DAMGO	0.21 ± 0.02 (64%) 12.50 ± 9.90 (36%)	275 ± 23	70 ± 3	268 ± 113
U50488	109 ± 6.3	0.04 ± 0.01 (42%) 2.25 ± 0.07 (58%)	> 10 000	> 10 000
DPDPE	438 ± 17	> 10,000	0.46 ± 0.03 (94%) 36.7 ± 15 (6%)	0.48 ± 0.14 (31%) 3.12 ± 0.50 (69%)

Parameter estimates were obtained by fitting raw data with a logistic equation using an iterative curve-fitting routine (GraphPad). Two-site models that provided a statistically significant improvement in fit are indicated along with the corresponding fraction of receptor sites described by that affinity state (in parentheses). All data were obtained from a single, representative experiment performed in triplicate and replicated 3–5 times.

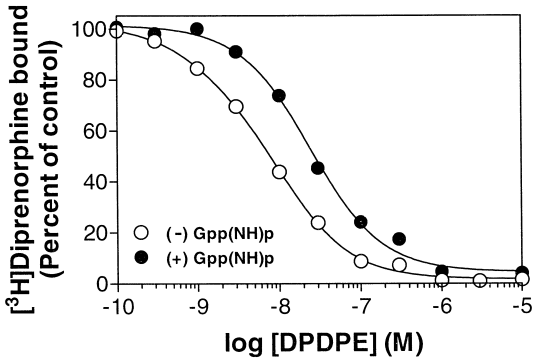

Fig. 3. Regulation of DPDPE displacement of [^{3}H]diprenorphine binding in CATH.a cell membranes by Gpp(NH)p. Data shown are from a representative experiment performed in triplicate and replicated twice. DPDPE titration of [^{3}H]diprenorphine was biphasic in the absence but monophasic in the presence of 300 μM Gpp(NH)p. The curves shown were obtained by non-linear regression and are based on parameter estimates given in Table 3.

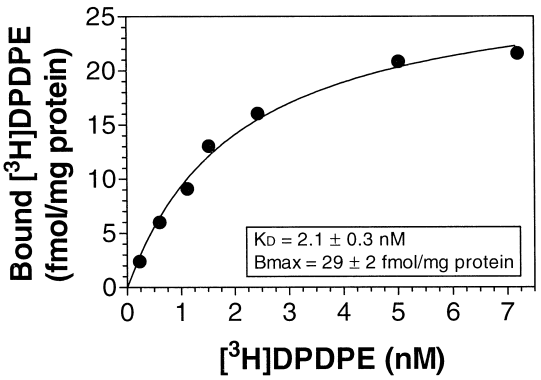

Fig. 4. Saturation isotherm of [^{3}H]DPDPE binding to CATH.a cell membrane preparations. The data presented are derived from a representative experiment performed in duplicate and replicated twice. Non-specific binding was defined in the presence of either unlabelled DPDPE or levorphanol (each at 10 μM) which gave identical parameter estimates for [^{3}H]DPDPE binding. The theoretical curve shown is based on the parameter estimates given.

titration of [^{3}H]diprenorphine binding. DPDPE titration curves were monophasic in the presence of Gpp(NH)p and corresponded to the low affinity component of curves generated in the absence of this non-hydrolyzable guanine nucleotide (Fig. 3, Table 3). These experiments suggest that the CATH.a cell δ-opioid receptor, like that in membrane preparations derived from other cell lines (Law et al., 1985), exists in two interconvertible agonist affinity states.

Toward the goal of providing direct evidence to support the hypothesis that CATH.a cells express predominantly δ-opioid receptor, radioligand binding experiments were carried out using the δ-opioid receptor-selective agonist radioligand [^{3}H]DPDPE (Fig. 4). Consonant with the labelling of a δ-opioid receptor, [^{3}H]DPDPE bound saturably and with high affinity to CATH.a cell membranes. The density of CATH.a cell δ-opioid receptor labelled by the agonist [^{3}H]DPDPE was approximately 5-fold lower than that labelled by [^{3}H]diprenorphine. The number of sites labelled by [^{3}H]DPDPE, however, corresponds to the high agonist affinity component of DPDPE titration of [^{3}H]diprenorphine (22–32% high affinity, 68–78% low affinity, see Fig. 3 and Tables 2 and 3). Therefore, these findings are consistent with data derived from RT-PCR and competition radioligand binding experiments described above in that δ-opioid receptor appears to be the predominant opioid receptor expressed by CATH.a cells.

3.3. Adenylyl cyclase activity studies

Based on the finding that δ-opioid receptor couples to CATH.a cell G proteins (Figs. 3 and 4; Tables 2 and 3), experiments were carried out to investigate the functional consequence of δ-opioid receptor activation in intact CATH.a cells. Delta opioid receptor, like other opioid receptor subtypes, inhibits adenylyl cyclase activity via a functional interaction with the inhibitory guanine nucleotide, G_i (Evans et al., 1992; Kieffer et al., 1992; Yasuda et al., 1993). DPDPE maximally inhibited forskolin-stimulated CATH.a cell adenylyl cyclase by approximately 40% with an IC_{50} of 1.86 ± 0.48 nM (Fig. 5A). This IC_{50} value is in general agreement with the K_I of DPDPE determined in competition radioligand binding experiments (Fig. 3; Tables 2 and 3). Inhibition of forskolin-stimulated CATH.a cell adenylyl cyclase activity by DPDPE was fully reversible by the opioid receptor antagonist, naloxone (Fig. 5B), indicating that this inhibi-

Table 3
Regulatory effect of Gpp(NH)p on DPDPE titration of [^{3}H]diprenorphine binding in CATH.a cell membranes

Gpp(NH)p	One-site model	Two-site model				*F*	*P*
	K_I (nM)	Site 1		Site 2			
		$K_{I(H)}$ (nM)	% High	$K_{I(L)}$ (nM)	% Low		
none	–	0.28 ± 0.08	22	2.7 ± 0.4	78	5.1	0.014
300 μM	5.9 ± 0.9	–[a]	–[a]	–[a]	–[a]	2.15	0.15

[a]A two-site model does not provide a statistically significant improvement in fit for these data.
$K_{I(H)}$ and $K_{I(L)}$ correspond to the inhibitor constants for the high and low agonist affinity states, respectively. The *F*-value for improvement in fit was calculated from an analysis of residuals and the *P*-value represents the significance in the improvement of fit when comparing two- and one-site models.

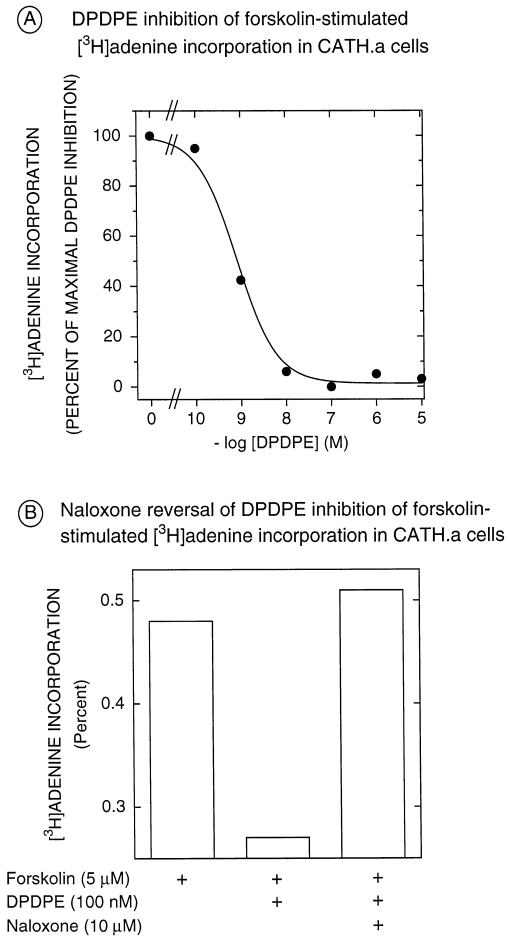

Fig. 5. Inhibition of CATH.a cell forskolin-stimulated adenylyl cyclase activity by DPDPE in CATH.a cells. (A) DPDPE dose-response curve. Forskolin (5 μM) stimulated basal CATH.a cell adenylyl cyclase activity 15-fold (data not shown). Data shown are derived from two pooled experiments in which all points were determined in triplicate. The IC_{50} value for DPDPE inhibition of forskolin-stimulated CATH.a cell adenylyl cyclase activity was 1.86 ± 0.48 nM. (B) 100 nM DPDPE inhibited the forskolin-stimulated adenylyl cyclase activity by approximately 40% and this effect was completely reversed by concomitant treatment of CATH.a cells with 10 μM naloxone.

tion is mediated by an interaction with an opioid receptor. These data corroborate the above studies which identified δ-opioid receptor as the predominant subtype of opioid receptor expressed by CATH.a cells and demonstrate that these receptors are functionally coupled to G_i.

4. Discussion

To our knowledge, this is the first description and pharmacological characterization of endogenous opioid receptors in the catecholaminergic CATH.a cell line. The results of these studies indicate that CATH.a cells express all three of the major subtypes of opioid receptors with the relative level of expression being $\delta > \kappa > \mu$.

CATH.a cell expression of δ-opioid receptor was verified by experiments employing RT-PCR, and by radioligand binding and functional analyses, whereas evidence for κ- and μ-opioid receptor expression in these cells was derived only from RT-PCR analysis. Two parameters were considered in the design of opioid receptor subtype-specific primers for use in RT-PCR. Firstly, all PCR primers were designed to minimize cross-hybridization and priming from cDNA corresponding to other opioid receptor subtypes and this was verified using appropriate plasmid DNA (data not shown). Secondly, all forward and reverse PCR primers used herein were derived from exons 2 (encoding amino acids in the second cytoplasmic loop) and 3 (corresponding to the second extracellular loop), respectively. Thus, all fragments amplified by PCR spanned an exon-intron junction that is present in genes encoding δ-, μ- and κ-opioid receptors. This latter modification prevented, or facilitated detection of, priming from contaminating genomic DNA. As an additional specificity control, receptor subtype-selective probes (derived from the second extracellular loop region of exon 3) were used for Southern analyses under hybridization and washing conditions that minimized cross-hybridization with other opioid receptor subtypes. Together, these modifications facilitated unambiguous identification of and discrimination between amplified fragments derived from the three major subtypes of opioid receptors.

The results of our pharmacological analyses also indicate that the major opioid receptor expressed by CATH.a cells is of the δ subtype. Membranes prepared from CATH.a cells bound the promiscuous opioid antagonist radioligand [^{3}H]diprenorphine with a K_d of 0.98 nM, a value that agrees well with previous studies employing NG108-15 cells (Vachon et al., 1987) and recombinant δ-opioid receptor (Evans et al., 1992), The pharmacological signature of CATH.a cell opioid receptors, as determined by titration of [^{3}H]diprenorphine binding by opioid receptor subtype-selective agonists, was consistent with CATH.a cell expression of predominantly δ-opioid receptor (DPDPE > > > DAMGO > U50488). This agonist titration profile as well as the absolute parameter estimates describing these titration curves were indistinguishable from parallel experiments carried out using membranes derived from CHO cells stably expressing recombinant δ-opioid receptor. Moreover, the existence of CATH.a cell δ-opioid receptor was demonstrated directly using the δ-opioid receptor-selective agonist radioligand [^{3}H]DPDPE. As expected, [^{3}H]DPDPE detected a fraction (22–32%) of the CATH.a cell membrane sites labelled by [^{3}H]diprenorphine corresponding to the subpopulation of CATH.a cell δ-opioid receptors displaying high affinity for unlabelled DPDPE in [^{3}H]diprenorphine titration experiments (Fig. 3; Tables 2 and 3). Finally, DPDPE potently and robustly inhibited forskolin-stimulated CATH.a cell adenylyl cyclase activity in a naloxone-reversible manner demonstrating that δ-opioid receptors are functionally coupled to G_i in CATH.a cell membranes.

In a previous report, Baraban et al. (1995) demonstrated that the κ-opioid receptor-selective compound U50488, but neither μ- nor δ-selective agonists, suppressed a voltage-activated K^+ current in CATH.a cells. Although mRNA encoding κ-opioid receptor is present in CATH.a cells, the abundance of this transcript is approximately 10% that of δ-opioid receptor mRNA (Fig. 1B and data not shown). Radioligand binding experiments employing [^{3}H]diprenorphine to label CATH.a opioid receptors were not suggestive of κ-opioid receptor labelling (see Table 2), however, this may be related to the extremely low expression of this protein in a population of cells grown in a monolayer. In contrast, Baraban et al. (1995), who isolated single CATH.a cells for electrophysiological recordings, may have fortuitously selected a particular subtype of cell from the heterogeneous CATH.a cell population (Qi et al., 1997) that expresses higher levels of κ-opioid receptor. Alternatively, a single cell type in the heterogenous CATH.a cell population may express all three subtypes of opioid receptors with each receptor subtype preferentially associated with specific transduction systems (for example, δ- and κ-opioid receptors may preferentially inhibit CATH.a cell adenylyl cyclase and K^+ channel activity, respectively).

The CATH.a cell line possesses many of the phenotypic properties of noradrenergic neurons of the locus coeruleus including high level expression of tyrosine hydroxylase and production of dopamine and norepinephrine (Suri et al., 1993). The locus coeruleus is composed of at least two cell types that exhibit a catecholaminergic phenotype, intermediate- and medium-sized neurons (Foote et al., 1993). Neither δ-opioid receptor mRNA (Mansour et al., 1995; Mansour et al., 1994) nor protein (Tempel and Zukin, 1987) were detected in locus coeruleus by in situ hybridization and receptor autoradiography, respectively. Nonetheless, it remains possible that δ-opioid receptor is present in locus coeruleus at levels below the limit of detection for these techniques. Indeed, Bagetta et al. (1990) and Elde et al. (1995) have provided pharmacological and immunohistochemical evidence, respectively, for expression of δ-opioid receptor in locus coeruleus. However, we cannot presently exclude the possibility that CATH.a cells may be derived from catecholaminergic neurons outside the locus coeruleus. This cell line was established from a primary tumor that arose in a transgenic mouse expressing SV40 T antigen under the control of the tyrosine hydroxylase promoter. In addition to locus coeruleus, tyrosine hydroxylase is also expressed in discrete nuclei located in olfactory bulb, lateral tegmental area, midbrain and brainstem, any of which may have given rise to the tumor from which CATH.a cells are derived (Suri et al., 1993). Regardless of their locus of origin, CATH.a cells represent a useful cell culture model for study of opioid action and signal transduction mechanisms in the context of a catecholaminergic neuronal cell line.

Acknowledgements

We thank Drs. D. Chikaraishi for CATH.a cells, David Grandy for CHOμ, CHOκ and CHOδ cell lines, and Jane Ishmael for advice and critical reading of this manuscript. The technical assistance of Beth Olenchek is gratefully acknowledged. This work was funded by NIH grant DA10610 to ML.

References

Bagetta, G., De Sarro, G.B., Sakurada, S., Rispoli, V., Nisticò, G., 1990. Different profile of electrocortical power spectrum changes after micro-infusion into the locus coeruleus of selective agonists at various opioid receptor subtypes in rats. Br. J. Pharmacol. 101, 655–661.

Baraban, S.C., Lothman, E.W., Lee, A., Guyenet, P.G., 1995. Kappa opioid receptor-mediated suppression of voltage-activated potassium current in a catecholaminergic neuronal cell line. J. Pharmacol. Exp. Ther. 273, 927–933.

Bundey, R.A., Iredale, P.A., Kendall, D.A., 1997. Corticosteroid modulation of signal transduction in the CATH.a cell line. J. Neurochem. 68, 362–369.

Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M., Low, D.K., Low, G., 1994. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta for kappa opioid receptor type. FEBS Lett. 347, 284–288.

Bunzow, J.R., Zhang, G., Bouvier, C., Saez, C., Ronnekleiv, O.K., Kelly, M.J., Grandy, D.K., 1995. Characterization and distribution of a cloned rat μ-opioid receptor. J. Neurochem. 64, 14–24.

Butour, J.L., Moisand, C., Mazarguil, H., Mollereau, C., Meunier, J.C., 1997. Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogs and opioids. Eur. J. Pharmacol. 321, 97–103.

Chen, Y., Mestek, A., Liu, J., Hurley, J.A., Yu, L., 1993. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol. Pharmacol. 44, 8–12.

Elde, R., Arvidsson, U., Riedl, M., Vulchanova, L., Lee, J.H., Dado, R., Nakano, A., Chakrabarti, S., Zhang, X., Loh, H.H., Law, P.Y., Hökfelt, T., Wessendorf, M., 1995. Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. Ann. NY Acad. Sci. 757, 390–404.

Evans, C.J., Keith Jr., D.E., Morrison, H., Magendzo, K., Edwards, R.H., 1992. Cloning of a delta opioid receptor by functional expression. Science 258, 1952–1955.

Foote, S.L., Bloom, F.E., Aston-Jones, G., 1993. Nucleus locus coeruleus: new evidence of anatomical and physiological specificity. Physiol. Rev., 844–914.

Kieffer, B.L., Befort, K., Ruff-Gaveriaux, C., Hirth, C.G., 1992. The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc. Natl. Acad. Sci. USA 89, 12048–12052.

Law, P.Y., Hom, D.S., Loh, H.H., 1985. Multiple affinity states of opiate receptor in neuroblastoma×glioma NG108-15 hybrid cells. Opiate agonist association rate is a function of receptor occupancy. J. Biol. Chem. 260, 3561–3569.

Lazaroff, M., Dunlap, K., Chikaraishi, D.M., 1996. A CNS catecholaminergic cell line expresses voltage-gated currents. J. Membr. Biol. 15, 279–291.

Lowry, O.H., Rosebrough, J.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mansour, A., Fox, C.A., Burke, S., Meng, F., Thompson, R.C., Akil, H., Watson, S.J., 1994. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in-situ hybridization study. J. Comp. Neurol. 350, 412–438.

Mansour, A., Fox, C.A., Akil, H., Watson, S.J., 1995. Opioid-receptor mRNA expression in rat CNS: anatomical and functional implications. Trends Neurosci. 18, 22–29.

Meng, F., Xie, G.X., Thompson, R.C., Mansour, A., Goldstein, A., Watson, S.J., Akil, H., 1993. Cloning and pharmacological characterization of a rat kappa opioid receptor. Proc. Natl. Acad. Sci. USA 90, 9954–9958.

Meunier, J.-C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerle, P., Butour, J.-L., Guillemot, J.-C., Ferrara, P., Monsarrat, B., Mazargull, H., Vassart, G., Parmentier, M., Costentin, J., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 352, 532–535.

Mollereau, C., Parmentier, M., Mailleu, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G., Meunier, J.C., 1994. ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett. 341, 33–38.

Qi, Y., Wang, J.K.T., McMillian, M., Chikaraishi, D.M., 1997. Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. J. Neurosci. 17, 1217–1225.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J., Civelli, O., 1995. Orphanin FQ: A neuropeptide that activates an opioid-like G protein-coupled receptor. Science 270, 792–794.

Salomon, Y., 1979. Adenylate cyclase assay. Adv. Cyclic Nucleotide Res. 35, 35–55.

Suri, C., Fung, B.P., Tischler, A.S., Chikaraishi, D.M., 1993. Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. J. Neurosci. 13, 1280–1291.

Tempel, A., Zukin, R.S., 1987. Neuroanatomical patterns of μ, δ and K opioid receptors of rat brain as determined by quantitative in vitro autoradiography. Proc. Natl. Acad. Sci. USA 84, 4308–4312.

Tian, J.H., Xu, W., Fang, Y., Mogil, J.S., Grisel, J.E., Grandy, D.K., Han, J.S., 1997. Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. Br. J. Pharmacol. 120, 676–680.

Vachon, L., Costa, T., Herz, A., 1987. GTPase and adenylate cyclase desensitize at different rates in NG108-15 cells. Mol. Pharmacol. 31, 159–168.

Wang, J.B., Imai, Y., Eppler, C.M., Gregor, P., Spivak, C.E., Uhl, G.R., 1993. mu opiate receptor: cDNA cloning and expression. Proc. Natl. Acad. Sci. USA 90, 10230–10234.

Wang, J.B., Johnson, P.S., Imaai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M., Uhl, G.R., 1994. cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett. 348, 75–79.

Yasuda, K., Raynor, K., Kong, H., Breder, C.D., Takeda, J., Reisine, T., Bell, G.I., 1993. Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. Proc. Natl. Acad. Sci. USA 90, 6736–6740.