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ACCELERATED COMMUNICATION

Molecular Cloning and Functional Expression of a μ-Opioid Receptor from Rat Brain

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SUMMARY

Opioid drugs act on specific receptors to modulate a wide range of physiological functions. There are at least three types of opioid receptors, μ, δ, and κ. Using a cDNA probe for a mouse δ-opioid receptor in low stringency hybridization, a clone has been isolated from a rat brain cDNA library. This clone contains an open reading frame of 1194 base pairs, with a deduced polypeptide of 398 amino acid residues. The predicted protein exhibits the structural features of guanine nucleotide-binding protein-coupled receptors and displays a high degree of sequence homology with the mouse δ-opioid receptor. When transfected into COS-7 cells, the cDNA conferred a binding site with subnanomolar affinity for [^{3}H]diprenorphine, a high affinity ligand for all three types of opioid receptors. This site also displayed nanomolar affinity for [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin (DAGO), a μ-selective agonist, whereas its affinities for the δ-selective agonist [D-Pen^{2,5}]-enkephalin and the κ-selective agonist U-50488 were in the micromolar range. Several μ-selective antagonists, including naloxonazine, β-funaltrexamine, and cyprodime, were capable of displacing [^{3}H]diprenorphine binding with nanomolar potency. The pharmacological profile of this binding site thus suggests that it is a μ-type opioid receptor, which we designated MOR-1. In COS-7 cells expressing MOR-1 and stimulated with forskolin, treatment with DAGO decreased the steady state levels of cAMP; this inhibitory effect of DAGO was blocked by naloxonazine. These results suggest that this μ-opioid receptor is functionally coupled to the inhibition of adenylyl cyclase.

Opioid compounds include endogenous peptides and their synthetic analogues, alkaloids derived from opium, and semisynthetic alkaloids. They interact with cell surface receptors and modulate a variety of physiological functions, such as nociception, hormone secretion, neurotransmitter release, feeding, respiratory depression, gastrointestinal motility, and opiate addiction (1). There are at least three types of opioid receptors, μ, κ, and δ, each with a distinct pharmacological profile (2, 3). A major physiological effect attributed to the opioid system is analgesia (4, 5), and opioid drugs are important therapeutic agents in the clinical management of acute pain. The μ-opioid receptor is the major site for the potent effects of opioid analgesics such as morphine and fentanyl. Prolonged use of morphine and related opiates for pain relief leads to the development of tolerance, often necessitating dosage increases to achieve similar levels of analgesia. Recreational use of opioid compounds often results in drug addiction, possibly due to the positive reinforcement associated with the euphoria and to the unpleasant symptoms negatively associated with attempts to withdraw (6). The molecular mechanisms involved in tolerance, dependence, and addiction, however, are not well understood.

The activation of all three opioid receptor types can inhibit adenylyl cyclase and modulate membrane conductances of Ca^{2+} and K^+ (7, 8). The increase in K^+ conductance and the decrease in Ca^{2+} conductance both serve to reduce membrane excitability, as well as secretion of neurotransmitters, and seem to account for the analgesic properties of the opioids (8). The effect of the receptors upon the ion channels requires GTP, involves no diffusible cytosolic molecules, and is considered the more direct route of opiate action. The inhibition of adenylyl cyclase implicates a more complex mode of opioid regulation, which may include controlling the levels of gene expression as well as modulating the activity of cellular kinases and phosphatases (9).

Opioid receptors are thought to belong to a family of membrane receptors that transduce their intracellular signals via G protein-coupled pathways (7, 10, 11). Thus, they are expected to possess the structural features common to G protein-coupled receptors, including multiple (usually seven) hydrophobic re-

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction; bp, base pair(s); DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin; DSLET, [D-Ser^2,Leu^5,Thr^6]-enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; β-FNA, β-funaltrexamine.

gions in the receptor polypeptide chain and amino acid sequence homology within these hydrophobic regions. This was confirmed by the recent cloning of a mouse δ-opioid receptor (12, 13). In this report we describe the cloning of an opioid receptor that contains the structural motifs of G protein-coupled receptors, exhibits the ligand selectivity of μ-type opioid receptors, and mediates an inhibitory coupling to adenylyl cyclase.

Materials and Methods

Isolation of cDNA clones by low stringency hybridization. Two primers, ATCTTCACCCTCACCATGATG and CGGTCCTTCTCCTTGGAACC, were synthesized from the sequence of the mouse δ-opioid receptor (12, 13), corresponding to the third transmembrane domain and the third cytoplasmic loop, respectively. PCR was performed using purified DNA from a rat brain cDNA library (14), in an air Thermo-cycler (Idaho Technology) under modified conditions (94° for 10 sec, 56° for 20 sec, and 72° for 40 sec, for 40 cycles). A fragment of 356 bp was purified and subcloned into the pBLUESCRIPT SK(+) vector. The sequence of three isolates confirmed complete identity with the mouse δ-opioid receptor. The 356-bp fragment was then used to screen the rat brain cDNA library under low stringency conditions (6× SSPE (1.08 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.4), 5× Denhardt solution, 0.5% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA, at 50°). The final wash was carried out in 0.5× standard saline citrate (75 mM NaCl, 7.5 mM sodium citrate), 0.1% sodium dodecyl sulfate, at 50°. Phagemids were rescued from positive λ clones by infection with helper phage. Two independent isolates were used for sequence determination by shotgun cloning into pBLUESCRIPT SK(+). Subsequent sequencing of both strands from each isolate showed these two clones to be identical. Potential post-translational modification sites were identified by using the PCGENE program. Comparison of the MOR-1 sequence with other receptors was performed by using the BLAST program (National Institutes of Health).

DNA transfection of COS-7 cells. A 1.4-kilobase Hind*III* fragment encompassing the open reading frame from the cDNA encoding MOR-1 was cloned downstream of the human cytomegalovirus promoter in the mammalian expression vector pRc/CMV (Invitrogen). COS-7 cells grown in Dulbecco's modified Eagle's medium (Sigma D-5648) supplemented with 10% fetal bovine serum and 2 mM glutamine were either electroporated or transfected with supercoiled DNA by using $CaPO_4$ co-precipitation (15). Electroporation was performed in 0.4-cm cuvettes at 200 V, using 3×10^6 cells in a total volume of 0.5 ml containing growth medium, 40 μg of expression plasmid, and 200 μg of sheared salmon sperm DNA. Cells were harvested 48–72 hr after electroporation or removal of the DNA-$CaPO_4$ co-precipitate.

Preparation of cell membranes. Cells were harvested by scraping into phosphate-buffered saline, pH 7.2, and centrifuged. Cell pellets were resuspended in lysis buffer (20 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and lysed with a Dounce homogenizer fitted with a tight pestle. The suspension was centrifuged for 10 min at 1000 × *g*, and the supernatant was removed to a fresh tube. The pellet was resuspended in lysis buffer and centrifuged as described above. The supernatants were then combined and centrifuged for 20 min at 35,000 × *g*. Membranes were washed in 50 mM Tris·HCl, pH 7.4, and centrifuged for 20 min at 35,000 × *g*. The membrane pellets were then suspended in 50 mM Tris·HCl, pH 7.4. Protein concentrations were determined by the method of Bradford (16).

Binding analysis. Binding of the membrane aliquots (15–50 μg/reaction) from the transfected COS-7 cells was carried out in 50 mM Tris·HCl, pH 7.4, 0.2% bovine serum albumin, at 4° for 90 min. A range of 0.01–2.5 nM [^{3}H]diprenorphine was used in the saturation assay and 0.25 nM was used for the displacement experiment. The reactions were terminated by vacuum filtration through Whatman GF/B filters that had been pretreated with 1% polyethylenimine. Nonspecific binding was determined using 5 μM naloxone.

cAMP assays. COS-7 cells transiently expressing the MOR-1 plasmid cDNA were harvested 48 hr after electroporation and were resuspended in growth medium. Cells were treated with 10 μM forskolin in the presence of 1 mM 3-isobutyl-1-methylxanthine at 37° for 10 min. DAGO (100 nM) and naloxonazine (10 μM) were included during forskolin treatment where indicated. Cells were pelleted and then solubilized in 0.1 N HCl. After extraction with water-saturated ether, the supernatants were lyophilized. cAMP was assayed using the acetylation protocol in the radioimmunoassay kit (DuPont/NEN).

Results and Discussion

We chose to use a strategy of low stringency hybridization for isolating opioid receptors related to the mouse δ-opioid receptor (12, 13) because all three types of opioid receptors may share sequence homology, in view of their overlapping pharmacology, their coupling to G proteins, and their common effect on Ca^{2+} and K^+ channels (1). Oligodeoxynucleotides were synthesized according to the mouse δ-opioid receptor sequence (12, 13) and were used to amplify, by PCR, a sequence fragment from a rat brain cDNA library (14). Sequence analysis of the resulting PCR product revealed complete identity with the corresponding portion of the δ-opioid receptor (12), suggesting a conserved relationship between the δ-opioid receptors from these two species. We then used this PCR product to isolate clones from the same rat brain cDNA library under low stringency conditions. Sequence analysis revealed that one cDNA clone contains an open reading frame of 1194 bp, encoding a protein of 398 amino acids. Hydropathy analysis of the deduced protein indicated seven hydrophobic domains, typical of G protein-coupled receptors (17). This protein, termed MOR-1, shows high levels of homology with the mouse δ-opioid receptor DOR-1 (12) (64%) and rat somatostatin receptors (18, 19) (44%) (Fig. 1). MOR-1 also displays moderate homology (30–32%) with several G protein-coupled receptors, including the angiotensin II receptor, the interleukin-8 receptor, the *N*-formyl peptide receptor, and the C-C chemokine receptor. The sequence homology is lower (≤25%) between MOR-1 and other G protein-coupled receptors, such as the adrenergic and muscarinic receptors (17). At the amino acid sequence level, MOR-1 contains several sites that are conserved among other G protein-coupled receptors (17). Aspartic acid residues proposed to interact with the protonated amine group of various ligands appear in putative transmembrane domains II and III, and two conserved cysteine residues believed to be involved in disulfide bonding occur in the first and second extracellular loop domains (20). Both of these features are conserved between MOR-1 and the δ-opioid receptor (Fig. 1). In addition, MOR-1 displays a cysteine residue in the carboxyl-terminal region that is conserved among many G protein-coupled receptors and that may serve as a target for palmitoylation (17). There are also multiple sites in the second and third intracellular loops as well as the carboxyl-terminal region that may undergo phosphorylation via protein kinase A and protein kinase C. Compared with the mouse δ receptor, MOR-1 contains five instead of two asparagine residues in the amino-terminal region that match the consensus sequence for *N*-linked glycosylation. These glycosylation sites may be important in the modulation of receptor expression and function (21).

To express MOR-1, a cDNA fragment containing the entire protein coding region was subcloned into a mammalian expres-

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                                   ◊
Rat MOR-1                  MDSSTGPGNTSDCSDPLAQASCSPAPGSWL  30
Mouse DOR-1                                MELV-SARAE-  11
Rat Somatostatin Receptor 1          MFPNGTAPSPTS--SSSPGG  20
Rat Somatostatin Receptor 2                      MELTS   5

      ◊      ◊       ◊      ◊
MOR   NLSHVDGNQSDPCGLNRTGLGGNDSLCPQTGSPSMVT AITIMALYSIVC  79
DOR   QS-PLV -L--AFPSAFPSAGA-A-GS-GAR-A--LAL--A-T----A--  60
SOM1  CGEGLCSRGPGSGAADGMEEPGRN-SQNGTL-EGQGS --L-SFI--V--  69
SOM2  EQFNGSQVWIPSPFDLNGS--PSNGSNQTEPYYD-TSN-VL_TFI-FV--  54

                                          *
MOR   VVGLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYL 129
DOR   A---L--V---FG------L------------------------------ 110
SOM1  L---C--SM-I---L--A----------L---I--E-LMLSV--LVTST- 119
SOM2  ____C__T__I___L__A____I_____L___I__E-FMLG___LAMQVA 104
          I                                  II
                =     *
MOR   MGTWPFGTILCKIVISIDYYNMFTSIFTLCT MSVDRYIAVCHPVKALDF 178
DOR   -E-----EL---A-L-------------- -M------------------ 159
SOM1  LRH----AL--RL-L-V-A-------YC- -VL-----V--V--I--ARY 168
SOM2  LVH----KAI-RV-MTV-GI-Q-----C- -V--I---L--V--I-SAKW 153
                              III
        o                              =
MOR   RTPRNAKIVNVCNWILSSAIGLPVMFMATTKYRQGSID CTLTFSHPTWY 227
DOR   ---AK--LI-I-I-V-ASGV-V-I-V--V-QP-D-AVV -M-Q-PS-S-- 208
SOM1  -R-TV--V--LGV-V--LLVI--IVVFSR-AANSDGTVA-NMLMPE-AQR 218
SOM2  -R--T--MI--AV-GV-LLVI--I-IY-GLRSN-WGRSS-TINWPGESGA 203
                         IV
                                        o
MOR   WENLLKICVFIFAFIMPILIITVCYGLMILRLKSVRMLSGSKEKDRNLRR 277
DOR   -DTVT---------VV------------L---R---L---------S--- 258
SOM1  -LVGFVLYT-LMG-LL-VGA-CL--V-I-AKMRM-ALKA-WQQRK-SE-K 268
SOM2  -YTGFI-YA--LG-LV-LT--CL--LFI-IKV--SGIRV--SKRKKSEKK 253
                    V
        ‖
MOR   ITRMVLVVVAVFIVCWTPIHIYVIIKALITIPETTFQTV SWHFCIALGY 326
DOR   ---------GA-V---A----F--VWT-VD-NRRDPLV-AAL-L------ 308
SOM1  --L--MM--M--VI--M-   FYVV Q-VNVFAEQDDATV-QLSV- --- 313
SOM2  V---SI------F--L-FY-FNVSSVSVA-SP-PALKGMFDFVV-_-T- 302
                   VI
                                 ▼          o
MOR   TNSCLNPVLYAFLDENFKRCFREFCIPTSSTIEQQNSTRVRQ NTREHPS 375
DOR   A--S-------------------QL-RTPCGRQ-PGSLR-P--AT---RV 357
SOM1  A---A--I--G--SD----S-QRILCLSWMDNAAEEPVDYY ATALKSRA 362
SOM2  A---A--I--A--SD---KS-QNVLCLVKVSGAEDGERSDSKQDKSRLNE 352
         VII

MOR   TANTVDRTNHQLENLEAETAPLP                           398
DOR   --C-PSDGPGGGAAA                                   372
SOM1  YSVEDFQPENLESGGVFRNGTCASRISTL                      391
SOM2  -TE- Q--LLNGDLQTSI                                369
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Fig. 1. Amino acid sequence alignment of MOR-1 with the mouse δ-opioid receptor (DOR-1) (12) and the rat somatostatin receptors (SOM1 and SOM2) (18, 19). Seven hydrophobic domains are *underlined and numbered I to VII*. –, Amino acids identical to those in MOR-1. *Spaces*, gaps introduced for alignment. ◊, Putative *N*-linked glycosylation sites; ‖, potential site for phosphorylation by cAMP-dependent protein kinase; O, potential sites for phosphorylation by protein kinase C; *, conserved aspartic acid residues proposed to interact with the amine group of ligands; =, conserved cysteine residues that might form a disulfide bond; ▼, potential palmitoylation site. The sequence for the MOR-1 cDNA has been submitted to GenBank (accession number L13069).

sion vector containing the human cytomegalovirus promoter. The plasmid was transiently transfected into COS-7 cells to express MOR-1, and membranes from these cells were prepared. Saturation binding of membranes was performed using [^{3}H]diprenorphine (22), a nonselective opioid antagonist with high affinity for all three types of opioid receptors (Fig. 2). Membranes of COS cells transfected with the MOR-1 plasmid displayed [^{3}H]diprenorphine binding with a dissociation constant (K_d) value of 0.38 ± 0.09 nM (mean ± standard error, five experiments). This is one tenth the K_d value (3.8 nM) reported for the cloned mouse δ-opioid receptor (12).

To characterize the pharmacological features of MOR-1, we used various ligands to displace [^{3}H]diprenorphine binding. The inhibition constant (IC_{50}) values were obtained from three binding experiments for each ligand and are listed in Table 1. Representative data are shown in Fig. 3. The μ-selective agonist DAGO displaced diprenorphine binding with high affinity (IC_{50} = 4.2 nM), whereas the δ-selective agonist DPDPE and

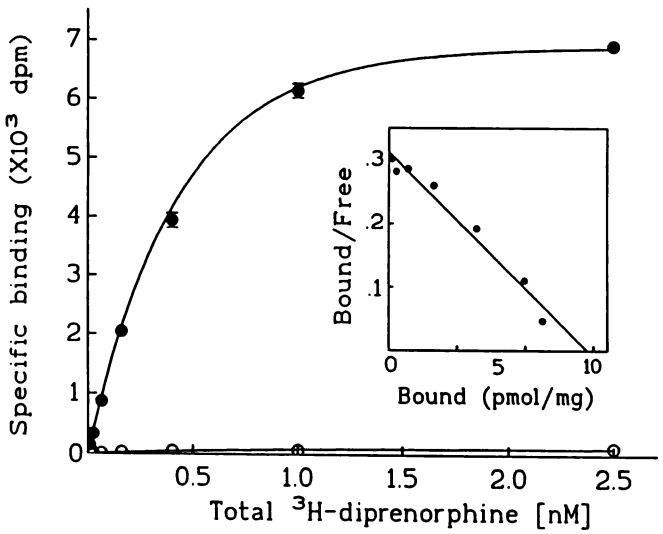

Fig. 2. Saturation binding of [^{3}H]diprenorphine using COS-7 cell membranes. [^{3}H]Diprenorphine binding was determined using membranes prepared from COS-7 cells transfected with either the rat MOR-1 cDNA plasmid (●) or the parental vector (○). Data from a representative experiment are presented and are expressed as mean ± standard error. *Inset*, Scatchard plot analysis of the binding data from MOR-1-transfected cells.

TABLE 1

Ligand binding to MOR-1

IC_{50} values from displacement studies are expressed as mean ± standard error (three experiments for all ligands).

Ligand	IC_{50}
	nM
Agonists	
DAGO	4.2 ± 0.2
DADLE	90 ± 30
DSLET	523 ± 58
U-50488	2,508 ± 607
DPDPE	11,730 ± 1,405
Antagonists and somatostatins	
Naloxone	1.7 ± 1.0
β-FNA	2.1 ± 0.1
Naloxonazine	3.7 ± 1.5
Cyprodime	14.0 ± 4.0
Cyclic somatostatin	10,994 ± 6,777
Somatostatin-1-14	>30,000

the κ-selective agonist U-50488 showed low affinities, with IC_{50} values in the micromolar range (3). DADLE and DSLET, two predominantly δ agonists that have also been shown to interact with μ receptors (23, 24), showed binding to MOR-1 with moderate affinities (IC_{50} = 90 and 523 nM, respectively). The rank order of potency for these opioid agonists is DAGO > DADLE > DSLET > U-50488 > DPDPE, suggesting the pharmacological profile of μ receptors. To examine this possibility further, displacement of diprenorphine binding to MOR-1 was performed with three μ-selective antagonists, i.e., β-FNA, naloxonazine, and cyprodime (25–28). All three ligands exhibited high potency in displacing diprenorphine binding to MOR-1 (Fig. 3), with IC_{50} values in the nanomolar range (Table 1). The rank order of potency for opioid agonists and the nanomolar affinity for μ-selective antagonists suggest that MOR-1 is a μ-opioid receptor.

The sequence homology between the rat μ-opioid receptor encoded by MOR-1 cDNA and the somatostatin receptors is noteworthy. Many somatostatin analogues, especially those of

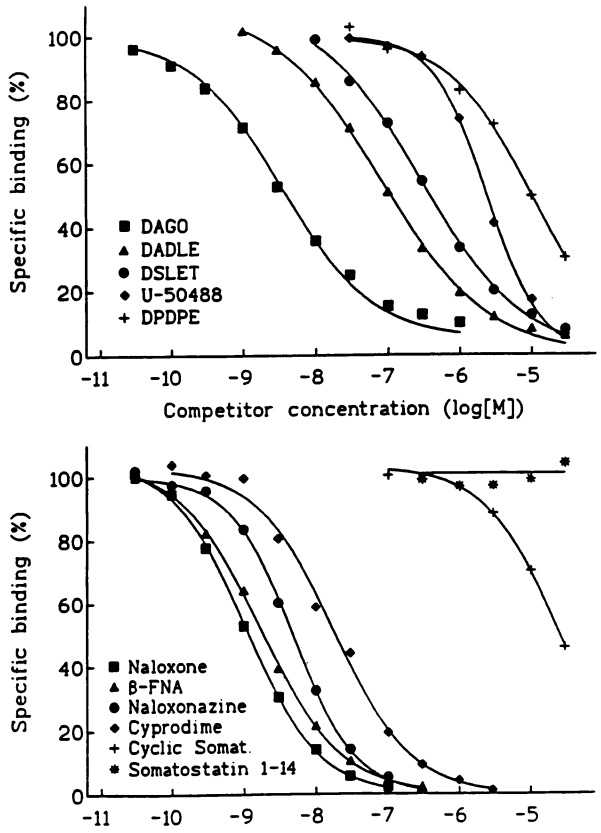

Fig. 3. Displacement of [^{3}H]diprenorphine binding with unlabeled ligands as competitors. Data from a representative experiment are presented for each ligand. *Top*, using opioid agonists as competitors; *bottom*, using opioid antagonists and somatostatins as competitors.

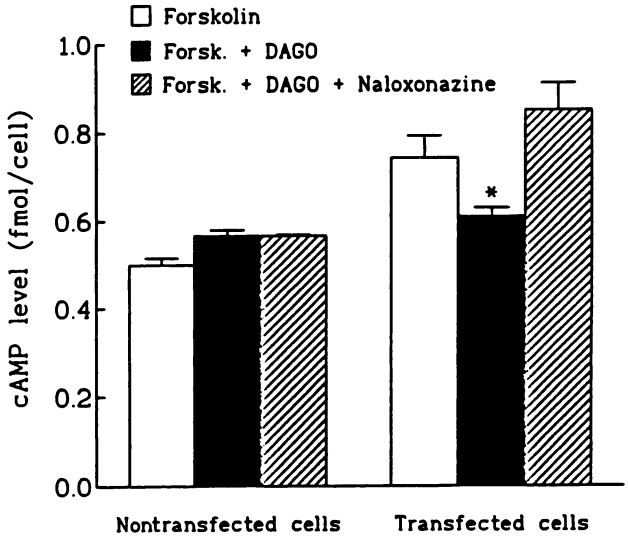

Fig. 4. Functional coupling of MOR-1 to adenylyl cyclase. Parental COS-7 cells (*Nontransfected cells*) or COS-7 cells expressing MOR-1 (*Transfected cells*) were stimulated with forskolin (*Forsk.*) to elevate adenylyl cyclase activity above basal levels. The μ-selective ligands were included during forskolin treatment as indicated. Cellular cAMP levels were determined. Data are expressed as mean ± standard error (four experiments). *, Data are significantly different from the control group (transfected cells treated with forskolin only).

the cyclic form, have been shown to interact with μ-opioid receptors, and some of them have been used as μ-selective antagonists (29). To examine this possibility, displacement binding experiments were performed using two somatostatin ligands, somatostatin-1–14 and cyclic somatostatin. Somatostatin-1–14 did not readily displace [^{3}H]diprenorphine binding to the rat μ receptor encoded by the MOR-1 cDNA, at concentrations as high as 30 μM, whereas cyclic somatostatin competed with diprenorphine binding with an IC_{50} value in the micromolar range (Fig. 3; Table 1).

All three classes of opioid receptors are believed to be coupled to adenylyl cyclase (7, 11, 30). To examine whether the μ receptor is coupled to intracellular signaling pathways, cAMP levels were determined in COS-7 cells after exposure to μ-selective ligands. Results are shown in Fig. 4. In nontransfected COS-7 cells, treatment with these ligands did not cause significant changes in the intracellular cAMP levels. In transfected cells expressing the μ receptor, the μ-specific agonist DAGO reduced cAMP levels significantly (18.1 ± 2.5% reduction from control, $p < 0.05$). This inhibitory effect on adenylyl cyclase activity by DAGO was blocked by the μ-selective antagonist naloxonazine. It has been reported that μ-opioid receptors exert an inhibitory effect on adenylyl cyclase activity (31) and that activation of μ receptors in a human neuroblastoma cell line reduces intracellular cAMP levels by approximately 20% (32). Our data are consistent with these reports and suggest that the μ-opioid receptor encoded by MOR-1 cDNA is functionally coupled to the inhibition of adenylyl cyclase in COS-7 cells.

Considerable progress has been made in characterizing opioid receptors in terms of their pharmacology and physiology. We have described the cloning of a protein that displays both the ligand-binding characteristics and the G protein-coupling capability expected of an opioid receptor of the μ type. The isolation of this receptor will allow additional studies on structure-function relationships of opioid ligand-receptor interactions and will contribute to a better understanding of the molecular basis of opioid physiology.

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