0026-895X/93/040827-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:827–834

BW373U86: A Nonpeptidic δ-Opioid Agonist with Novel Receptor-G Protein-Mediated Actions in Rat Brain Membranes and Neuroblastoma Cells

STEVEN R. CHILDERS, LYNNE M. FLEMING, DANA E. SELLEY, ROBERT W. McNUTT, and KWEN-JEN CHANG

Division of Cell Biology (K.-J.C.) and Division of Organic Chemistry (R.W.M.), Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709; Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157 (S.R.C., D.E.S.); and Department of Pharmacology, University of Florida College of Medicine, Gainesville, Florida 32610 (L.M.F.)

Received February 22, 1993; Accepted July 31, 1993

SUMMARY

BW373U86 is a potent and highly selective nonpeptidic agonist for δ-opioid receptors. To determine its ability to couple with G protein-linked second messenger systems, this study examined the effects of BW373U86 on the inhibition of adenylyl cyclase and the stimulation of low-K_m GTPase activity. In rat striatal membranes, BW373U86 inhibited basal adenylyl cyclase activity in a GTP-dependent manner, with maximal inhibition levels similar to those of the prototypic δ agonist [D-Ser^2,Thr^6]Leu-enkephalin (DSLET). However, BW373U86 was approximately 100 times more potent than DSLET in inhibiting adenylyl cyclase. Analysis of the inhibitory activity across 10 brain regions revealed that both low and high concentrations of BW373U86 inhibited adenylyl cyclase activity in a manner similar to that of DSLET. Inhibition of adenylyl cyclase by BW373U86 was δ receptor selective, because the δ receptor-selective antagonist naltrindole was significantly more potent than naloxone and the μ receptor-selective antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 was ineffective in blocking BW373U86 inhibition. BW373U86 also inhibited adenylyl cyclase activity in membranes prepared from NG108-15 cells, with an IC_{50} value 5 times lower than that of DSLET. This increased potency was not observed in concentration-effect curves for agonist-stimulated low-K_m GTPase in NG108-15 membranes. BW373U86 is a competitive inhibitor of [^{3}H]diprenorphine at δ receptors of NG108-15 cell membranes. However, unlike DSLET, BW373U86 displacement of [^{3}H]diprenorphine binding to NG108-15 cell membranes was not affected by sodium and guanine nucleotides. This lack of GTP effect on binding apparently produced slow dissociation rates for this agonist, because naltrindole was less potent in blocking BW373U86 inhibition of adenylyl cyclase when membranes were preincubated with this agonist. These results demonstrate the novel finding that the binding of a full agonist to a G protein-coupled receptor is not regulated by GTP, and they also show how the lack of regulation in receptor binding affects agonist potency.

δ-Opioid receptors were first identified as the opioid receptor type that binds enkephalin and related peptides with high affinity (1–3). Agonists that display extremely high selectivity for δ receptors have been prepared; however, in all previous cases, such compounds were structural analogs of traditional opioid peptides (4–6). The recent synthesis of the novel compound BW373U86, (±)-4-[(α-*R**)-α-[(2*S**,5*R**)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide, offers an alternative ligand system for the study of δ receptors (7). This compound is a nonpeptidic derivative and displays selective actions at δ receptors both in mouse vas deferens and in receptor binding assays with brain membranes. This compound demonstrates its agonistic activity to inhibit electrically induced contractions of the mouse vas deferens that are blocked by δ receptor-selective antagonists like NTI and to produce opioid analgesia when injected intrathecally (8). Such a nonpeptidic agonist should be important in delineating properties of δ receptors under circumstances where the limited bioavailibility of peptide analogs prevents their effective use as pharmacological agents.

Like other opioid receptor types, δ receptors are coupled to G proteins. Both sodium and guanine nucleotides inhibit δ receptor binding in a manner typical of G_i-linked receptors (9–11). Moreover, even though δ receptors are coupled through G proteins to other effector systems like potassium channels (12), the best studied second messenger system associated with δ receptors is inhibition of adenylyl cyclase. This reaction was

This research was supported in part by United States Public Health Service Grants DA02904 and DA06634 from the National Institute on Drug Abuse.

ABBREVIATIONS: NTI, naltrindole; DSLET, [D-Ser^2,Thr^6]Leu-enkephalin; Gpp(NH)p, guanylyl-5′-imidodiphosphate; App(NH)p, adenylyl-5′-imidodiphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2.

characterized in NG108-15 neuroblastoma × glioma hybrid cells (13–15), and later studies extended the finding of this system in brain membranes as well (16–20). Although it is still not clear which of the physiological actions of δ agonists are mediated through changes in cAMP levels, it is nevertheless clear that many (if not all) of these actions are mediated through G proteins.

However, in preliminary studies in N4TG1 neuroblastoma cell membranes,[1] BW373U86 demonstrated effects that were not typical of G protein-coupled receptor agonists. Although BW373U86 was potent in binding to δ receptor binding sites, addition of either sodium or guanine nucleotides had no effect on its binding affinity. Thus, in this assay, BW373U86 behaved more like an antagonist than a typical agonist of a G protein-coupled receptor. Because other studies demonstrated that BW373U86 was a full agonist (7), these results suggested either that BW373U86 was not acting through a G protein-coupled system like other δ agonists or that its G protein-coupled properties were different from those of other agonists. The present study was designed to determine whether BW373U86 acted through G proteins, by examining its ability to inhibit adenylyl cyclase and to stimulate low-K_m GTPase. These results show that this novel agonist is indeed coupled to this second messenger system like other δ agonists and that the lack of guanine nucleotide and sodium regulation of binding creates an agonist that is significantly more potent than would be predicted from traditional binding assays.

Experimental Procedures

Materials. [^{3}H]Diprenorphine (~40 Ci/mmol) was purchased from Amersham (Chicago, IL), whereas [α-^{32}P]ATP (25 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]ATP (30 Ci/mmol), and [γ-^{32}P]GTP (30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Sprague-Dawley rats were purchased from Zivic-Miller (Zelienople, PA). NTI was synthesized according to the method of Portoghese *et al.* (21). BW373U86 was synthesized in the Division of Organic Chemistry, Burroughs Wellcome Co. (Research Triangle Park, NC). DSLET and CTOP were purchased from Peninsula Laboratories (Belmont, CA).

Cell culture. NG108-15 cells were cultured at 37° in a humidified atmosphere of 5% $C0_2$/95% air, in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml amphotericin B, 5% fetal bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. To prepare membranes for receptor binding and adenylyl cyclase assays, cells were harvested and centrifuged at 345 × *g* for 10 min. The pellet was resuspended in 50 volumes of 50 mM Tris·HCl, pH 7.4, with 1 mM EGTA, and were homogenized with a Polytron. The homogenate was centrifuged at 500 × *g* for 10 min, and the supernatant was placed on ice. The pellet was resuspended in Tris-EGTA and centrifuged at 500 × *g* for 10 min. The two supernatants were combined and centrifuged at 26,700 × *g* for 20 min at 4°. The resulting pellet was then resuspended in Tris-EGTA, and aliquots were stored at −80°. For low-K_m GTPase assays, membranes were prepared as described by Vachon *et al.* (22), with some modification. Cells were homogenized in 3.2 mM sucrose, 1 mM DTT, in Tris-EGTA (buffer A), with a Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min at 4°, and the supernatant was removed and placed on ice. The pellet was resuspended in buffer A and centrifuged at 1000 × *g* for 10 min. The two supernatants were combined and centrifuged at 27,000 × *g* for 20 min at 4°. The membrane pellet was resuspended in buffer A without sucrose (buffer B) and centrifuged at 27,000 × *g* for 30 min. Membranes were resuspended in buffer B, homogenized with a Dounce homogenizer, and stored at −80°.

[1] K.-J. Chang, M. A. Collins and R. W. McNutt, unpublished observations.

Opioid receptor binding. NG108-15 cell membranes (30 μg of protein) were incubated in buffer C containing 50 mM Tris, pH 7.4, 0.2 mM Tris-EGTA, 0.3 mM DTT, 3 mM $MgCl_2$, and 0.25 nM [^{3}H]diprenorphine, in a final volume of 2 ml. The saturation binding was conducted with nine different concentrations of [^{3}H]diprenorphine, ranging from 0.03 nM to 10 nM, in the absence and presence of 2 nM BW373U86. Nonspecific binding was defined as the difference between total binding and binding in the presence of 10 μM naloxone. Tubes were incubated at 24° for 90 min, and reactions were terminated by rapid filtration through Whatman GF/C glass fiber filters. Bound radioactivity was determined by liquid scintillation counting (50% efficiency) after overnight extraction of the filters in 4 ml of scintillation fluid.

Adenylyl cyclase assays. For the assay of adenylyl cyclase in brain membranes, the method of Childers and LaRiviere (23, 24) was used. Male Sprague-Dawley rats (180–250 g) were decapitated, and striata (along with selected other regions in certain experiments) were quickly dissected on ice and homogenized by using a Polytron (setting 5, 15 sec), in assay buffer (50 mM Tris·HCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.4). Membranes were prepared by centrifugation of the homogenate at 48,000 × *g* for 10 min. Brain membranes were then pretreated at low pH (23) by resuspension in 50 mM sodium acetate, 1 mM DTT, pH 4.5 (1 ml of buffer/100 mg of original wet weight of tissue), and were incubated on ice for 20 min. After addition of 5 ml of assay buffer, membranes were isolated by centrifugation at 48,000 × *g* for 10 min. Membranes (100–200 μg of protein) were resuspended in assay buffer containing 10 mM theophylline, 120 mM NaCl, 50 μM GTP, 20 mM creatine phosphate, 10 units of creatine phosphokinase, 30 μM cAMP, 100 μM ATP, and 1 μCi of [^{3}H]ATP, together with various drug additions, in a total volume of 100 μl. Enzyme blanks consisted of identical tubes in which membranes had been immersed in boiling water for 2 min before addition of substrate. The reaction was initiated by addition of [^{3}H]ATP, and the tubes were incubated at 30° for 10 min. The reaction was terminated in boiling water for 2 min, and then tubes were cooled on ice for 5 min. Before application of samples to the high performance liquid chromatography column (23), [^{3}H]adenosine was removed by incubation with 0.75 units of adenosine deaminase at 30° for 5 min. Tubes were placed back on ice, and remaining ATP was precipitated by the sequential addition of 20 μl of 1 M $Ba(OH)_2$, followed by 20 μl of 1 M $ZnSO_4$ and 250 μl of water, with 5 min between each addition. Tubes were centrifuged at 1000 × *g* for 15 min and supernatants were transferred into tubes for automatic sample injection onto a C-18 reverse phase high performance liquid chromatography column, as described previously (23). Radioactivity was determined by liquid scintillation counting (40% efficiency) after addition of 5 ml of Ecoscint scintillation fluid (ICN, Irvine, CA).

Assay of adenylyl cyclase in NG108-15 cell membranes was performed by the method of Salomon (25). Membranes (70 μg of protein) were incubated for 15 min at 30°, in the presence and absence of various drugs, in assay buffer containing 50 μM ATP, [α-^{32}P]ATP (1.5 μCi), 0.2 mM DTT, 3 mM $MgCl_2$, 0.01% bovine serum albumin, 50 μM cAMP, 50 μM GTP, 120 mM NaCl, 10 mM theophylline, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase, in a final volume of 100 μl. The reaction was terminated by boiling in a water bath for 2 min. [^{32}P]cAMP was isolated by the method of Salomon (25). Radioactivity was determined by liquid scintillation counting (35% efficiency for ^{3}H) after 4 ml of the eluate had been dissolved in 12 ml of Ecolite scintillation fluid (ICN, Irvine, CA).

Low-K_m GTPase assay. NG108-15 cell membranes (3–5 μg of protein) were incubated for 20 min at 30°, with and without various drugs, in assay buffer containing 0.5 μM GTP (including 0.1 μCi of [γ-^{32}P]GTP), 0.2 mM DTT, 3 mM $MgCl_2$, 0.01% bovine serum albumin, 120 mM NaCl, 1 mM ATP, 1 mM App(NH)p, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase, in a final volume of 100 μl. The low-K_m GTPase assay was conducted as described previously (22, 26). Nonspecific ("high" K_m) GTP hydrolysis was determined by the inclusion of 50 μM GTP. In some experiments, 120 mM NaCl was

replaced by 0–120 mM NaCl. The reaction was terminated by boiling in a water bath for 2 min, followed by the addition of 100 μl of 40 mM phosphoric acid. After the addition of 800 μl of a 5% activated charcoal suspension in 20 mM phosphoric acid, the tubes were centrifuged at 2600 × *g* for 30 min. Radioactivity was determined after 700-μl aliquots of the supernatant had been dissolved in 5 ml of scintillation fluid. Nonspecific activity (high-K_m GTPase plus nonenzymatic hydrolysis) accounted for 50–70% of total ^{32}P released.

Miscellaneous assays and data analysis. Protein values were determined by the method of Bradford (27). Unless otherwise indicated, results are mean ± standard error values of separate experiments, each of which was conducted in triplicate. All IC_{50} and Hill slope values were calculated by computer analysis (EBDA).

Results

Effects of BW373U86 on adenylyl cyclase in rat brain membranes. Adenylyl cyclase was assayed in rat striatal membranes that had been pretreated at pH 4.5 before assay at pH 7.4, to increase maximal inhibition of activity by opioid agonists (18, 24). To determine whether BW373U86 possessed the pharmacological properties of a δ agonist in this system, agonist concentration-effect curves were generated for both BW373U86 and the δ agonist DSLET. Results (Fig. 1) showed that, when 50 μM GTP was included in the adenylyl cyclase assay, both DSLET and BW373U86 inhibited adenylyl cyclase with approximately the same efficacies, providing 30–35% inhibition of basal activity. However, in the absence of GTP, neither agonist had any significant effect on basal adenylyl cyclase activity. In addition, BW373U86 was significantly more potent in inhibiting adenylyl cyclase than was DSLET. The IC_{50} value of DSLET was 300 nM (similar to that reported previously in brain membranes (18), whereas the IC_{50} value of BW373U86 was 4 nM (Table 1). Interestingly, the inhibition of adenylyl cyclase by BW373U86 occurred over 4 orders of magnitude of concentration for this agonist. The Hill slope calculated for the concentration-effect curve of DSLET was 0.95, whereas the Hill slope for BW373U86 was 0.41.

To determine whether BW373U86 inhibited adenylyl cyclase in the same brain regions as did DSLET, assays of both low (0.1 μM) and high (10 μM) concentrations of BW373U86, together with a high (10 μM) concentration of DSLET, were performed in 10 different rat brain regions. Results (Table 2) showed that BW373U86 produced regional differences in inhibiting adenylyl cyclase similar to those of DSLET, regardless of the concentration used. For both agonists, relatively high inhibitory levels were observed in striatum, hippocampus, and frontal cortex. Moderate levels were observed in sensomotor cortex, amygdala, hypothalamus, and thalamus. Low levels were observed in colliculus, brainstem, and cerebellum. This distribution is similar to that previously reported for [D-Ala^2,Met^5]enkephalinamide-inhibited adenylyl cyclase in rat brain membranes (18). Not only were the regional differences in inhibition produced by both low and high concentrations of BW373U86 the same as those produced by DSLET, but there was little difference between the levels of inhibition produced by 0.1 μM or 10 μM BW373U86 in any of the regions tested. These results suggested that the increased potency of BW373U86, compared with DSLET, was not restricted to striatum but was observed throughout the brain.

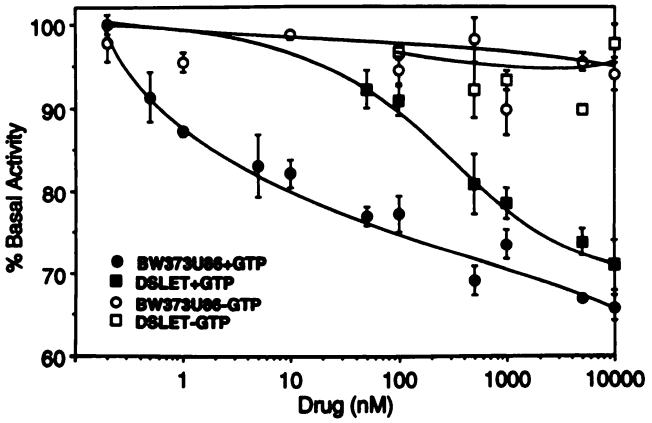

Fig. 1. Inhibition of adenylyl cyclase in rat striatal membranes by DSLET and BW373U86 in the presence and absence of GTP. Striatal membranes were pretreated at pH 4.5, washed, and assayed for adenylyl cyclase with the indicated concentrations of DSLET and BW373U86, with and without 50 μM GTP. Data are expressed as percentage of basal adenylyl cyclase activity (156 pmol/min/mg).

To determine the δ-opioid receptor selectivity of BW373U86, agonist concentration-effect curves were obtained in striatal membranes in the presence of 0.1 μM concentrations of the δ-selective antagonist NTI, 1 μM concentrations of the relatively μ-selective antagonist naloxone, or 1 μM concentrations of the μ-selective antagonist CTOP (28) (Fig. 2). Both NTI and naloxone produced competitive antagonism of the BW373U86 inhibition curves, inasmuch as they increased the IC_{50} value without changing the maximum inhibition level of BW373U86. However, NTI was significantly more potent; at 0.1 μM it increased the IC_{50} value of BW373U86 by almost 3 orders of magnitude, whereas 1 μM naloxone increased the agonist IC_{50} value by approximately 50-fold. Approximate K_e values were 0.1 nM for NTI and 20 nM for naloxone in blocking the inhibitory effect of BW373U86 on adenylyl cyclase. In contrast, CTOP had no significant effect on the potency or maximal inhibition of adenylyl cyclase by BW373U86 (Fig. 2).

Effects of BW373U86 in NG108-15 cells. The surprising finding that BW373U86 was 100 times more potent than DSLET in inhibiting adenylyl cyclase in brain membranes led to the question of its potency in inhibiting adenylyl cyclase in NG108-15 cells. δ agonists are significantly more potent inhibitors of adenylyl cyclase in NG108-15 membranes than in brain membranes (9, 29). Experiments were therefore performed to determine whether BW373U86 displayed an increase in potency, compared with DSLET, in NG108-15 cell membranes equal to that shown in brain membranes. Results of adenylyl cyclase assays (Fig. 3, *top*) revealed that DSLET and BW373U86 inhibited adenylyl cyclase with approximately the same maximal degree of inhibition (approximately 35%). The IC_{50} value of DSLET was 10 nM, and the IC_{50} value of BW373U86 was approximately 5 times lower, at 2 nM (Table 1). Thus, although BW373U86 was more potent than DSLET in inhibiting adenylyl cyclase in NG108-15 membranes, it did not display the dramatic increase in potency observed in brain membranes.

To further explore this relationship, δ receptor-stimulated low-K_m GTPase was assayed in NG108-15 membranes (Fig. 3, *bottom*). In these assays, both BW373U86 and DSLET increased low-K_m GTPase activity to the same extent, by approximately 50%. Interestingly, the two agonists were approximately equipotent in this assay, both exhibiting EC_{50} values of 2–3 nM. The potency of BW373U86 did not change between

TABLE 1

Potencies of DSLET and BW373U86 in brain and NG108-15 membranes

Tissue	Activity	GTP	IC_{50} or EC_{50}	
			BW373U86	DSLET
		μM	nM	
Brain	Adenylyl cyclase	50	4.9 ± 2.1	320 ± 120
NG108-15 cells	Adenylyl cyclase	50	1.9 ± 0.3	9.7 ± 0.6
NG108-15 cells	Low-K_m GTPase	0.5	1.7 ± 0.2	2.7 ± 0.2
NG108-15 cells	Binding, Mg^{2+}	0	1.7 ± 0.2	1.4 ± 0.1
NG108-15 cells	Binding, Mg^{2+} + Na^+ + GTP	50	1.5 ± 0.2	47 ± 4
NG108-15 cells	Binding, Mg^{2+} + Na^+ + Gpp(NH)p	50	1.7 ± 0.3	48 ± 11

TABLE 2

DSLET and BW373U86 effects on adenylyl cyclase in membranes from rat brain regions

Membranes were prepared from various rat brain regions and assayed for adenylyl cyclase in the presence of either 0.1 μM or 10 μM BW373U86 or 10 μM DSLET, as described in Experimental Procedures. Data are presented as percentage of inhibition of basal adenylyl cyclase activity and represent mean ± standard error values from three separate experiments.

Region	Inhibition of basal adenylyl cyclase activity		
	0.1 μM BW373U86	10 μM BW373U86	10 μM DSLET
	%		
Striatum	24.3 ± 2.3	32.7 ± 3.1	27.3 ± 7.6
Hippocampus	24.2 ± 6.5	24.9 ± 6.9	26.2 ± 6.0
Frontal cortex	30.8 ± 5.2	32.3 ± 4.5	19.7 ± 8.3
Sensomotor cortex	21.8 ± 2.7	18.4 ± 0.8	20.6 ± 7.1
Amygdala	15.9 ± 7.1	23.9 ± 4.8	17.7 ± 5.2
Hypothalamus	11.2 ± 2.6	12.1 ± 1.5	15.0 ± 2.1
Thalamus	12.1 ± 4.5	17.2 ± 4.9	17.2 ± 8.9
Superior colliculus	2.0 ± 2.8	8.2 ± 5.5	10.5 ± 3.8
Brainstem	9.1 ± 1.4	9.3 ± 8.0	3.3 ± 5.9
Cerebellum	1.5 ± 5.2	0.1 ± 5.7	0.3 ± 1.3

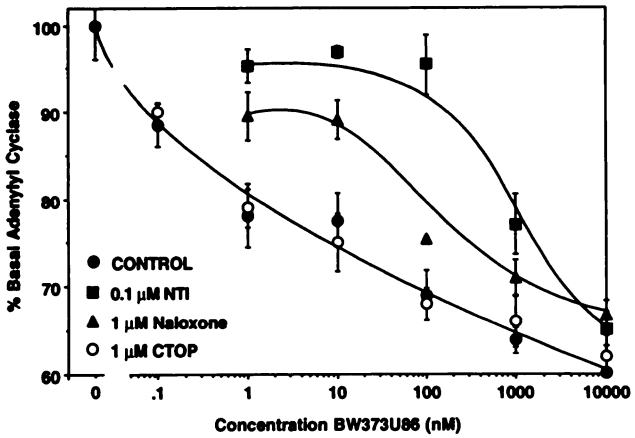

Fig. 2. Effect of opioid antagonists on BW373U86-inhibited adenylyl cyclase in rat striatal membranes. Membranes were assayed for adenylyl cyclase as described in Experimental Procedures, with the indicated concentrations of BW373U86 in the presence of either 1 μM naloxone, 0.1 μM NTI, or 1 μM CTOP. Data are expressed as percentage of basal adenylyl cyclase activity (175 pmol/min/mg).

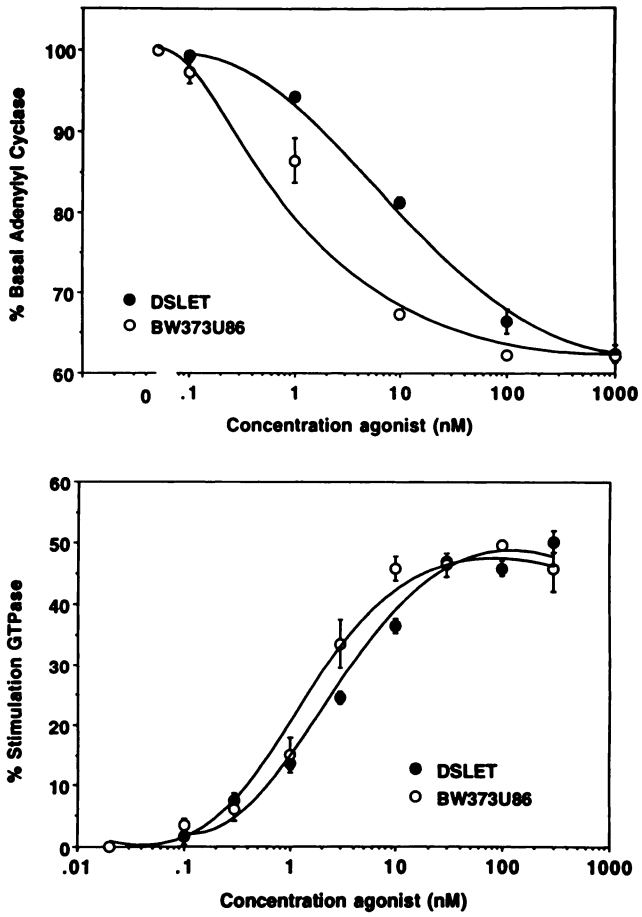

Fig. 3. Comparison of BW373U86 and DSLET inhibition of adenylyl cyclase (*top*) and stimulation of low-K_m GTPase (*bottom*) in membranes from NG108-15 cells. Membranes were prepared from cultured cells as described in Experimental Procedures. Adenylyl cyclase was determined with 50 μM GTP, and low-K_m GTPase was assayed with 0.5 μM GTP, in the presence of various concentrations of DSLET and BW373U86. Data are expressed as percentage of basal adenylyl cyclase activity (11 pmol/min/mg) or as percentage stimulation of basal low-K_m GTPase activity (38 pmol/min/mg).

the two assays, although the potency of DSLET was significantly higher in stimulating low-K_m GTPase than in inhibiting adenylyl cyclase.

One possible explanation for the different potencies of DSLET, compared with BW373U86, in low-K_m GTPase versus adenylyl cyclase assays is the different concentration of GTP utilized in the two assay systems. As indicated in Table 1, the GTPase assay contained 0.5 μM GTP, whereas the adenylyl cyclase assay contained 50 μM GTP. Data in mouse brain and vas deferens membranes (30) indicated that, unlike with other opioid agonists, guanine nucleotides had no effect on the potency of BW373U86 to inhibit [^{3}H]NTI binding. These findings suggested that this unusual property of BW373U86 may explain why varying concentrations of GTP would affect the potencies of DSLET and BW373U86 differently. To confirm these suggestions, opioid receptor binding was assayed in membranes

from NG108-15 cells, using the antagonist [^{3}H]diprenorphine. To mimic the conditions of the adenylyl cyclase assay, [^{3}H] diprenorphine binding was assayed in the presence of either 3 mM Mg^{2+} alone, Mg^{2+} plus 120 mM NaCl and 50 μM GTP (adenylyl cyclase conditions), or Mg^{2+} plus 120 mM NaCl and 50 μM Gpp(NH)p. Fig. 4 (*bottom*) shows the effect of these varying additions on displacement of [^{3}H]diprenorphine binding by DSLET. This analog behaved like a typical agonist binding to a G protein-linked receptor, because the addition of NaCl and GTP, or NaCl and Gpp(NH)p, decreased the IC_{50} value of DSLET by a factor of 40-fold. In contrast (Fig. 4, *top*), NaCl and GTP had no effect on the IC_{50} value of BW373U86. To confirm that the lack of guanine nucleotide and sodium effects was not due to GTP hydrolysis, the same experiment was repeated in the presence of NaCl and Gpp(NH)p, and the results (Fig. 4, *top*) again showed no change in IC_{50} values for BW373U86. Calculated IC_{50} values for both DSLET and BW373U86 are presented in Table 1.

Because the effect of sodium and guanine nucleotides on traditional agonist binding to opioid receptors is to increase agonist dissociation rates (9, 10), the lack of effect of NaCl and GTP on BW373U86 binding (Fig. 4) predicts that the dissociation rate of bound BW373U86 would be relatively slow, compared with that of DSLET, under adenylyl cyclase assay conditions. Direct assay of BW373U86 dissociation rates cannot be determined at this time because this agonist is not yet available as a radioligand. However, an indirect measure of this effect was examined by determining whether the ability of NTI to antagonize agonist inhibition of adenylyl cyclase was affected by pretreatment of NG108-15 cell membranes with agonists. In this experiment (Fig. 5), membranes were preincubated with either agonist or antagonist before initiation of the adenylyl cyclase assay. In the "agonist first" condition, membranes were preincubated for 10 min at 30° with IC_{90} concentrations of DSLET (100 nM) or BW373U86 (20 nM) before addition of various concentrations of NTI. In the "NTI first" condition (Fig. 5), the membranes were preincubated for 10 min at 30° with NTI before the addition of either agonist. The results showed a clear difference in the ability of NTI to block DSLET and BW373U86 inhibition of adenylyl cyclase (Fig. 5). Both DSLET and BW373U86 produced the same (45%) inhibition of activity in the absence of antagonist. NTI decreased DSLET inhibition in a concentration-dependent manner; 1 nM NTI decreased DSLET inhibition approximately 50%, whereas 100 nM NTI completely eliminated DSLET inhibition. Moreover, the effect of NTI on DSLET-inhibited activity was the same whether agonist or antagonist was added first. In contrast, the potency of NTI in blocking BW373U86 inhibition of adenylyl cyclase depended upon the order of agonist and antagonist addition. When the antagonist was added first, the effect of NTI was similar to that observed for DSLET-inhibited activity; 1 nM NTI significantly decreased BW373U86 inhibition, whereas 100 nM NTI completely eliminated BW373U86 inhibition. On the other hand, when membranes were preincubated with BW373U86 before the addition of NTI, the antagonist was significantly weaker; up to 10 nM NTI had no significant effect on BW373U86 inhibition, whereas 100 nM NTI, which completely blocked BW373U86 inhibition when the antagonist was added first, blocked only approximately 20% of BW373U86 inhibition. These results are consistent with the concept that

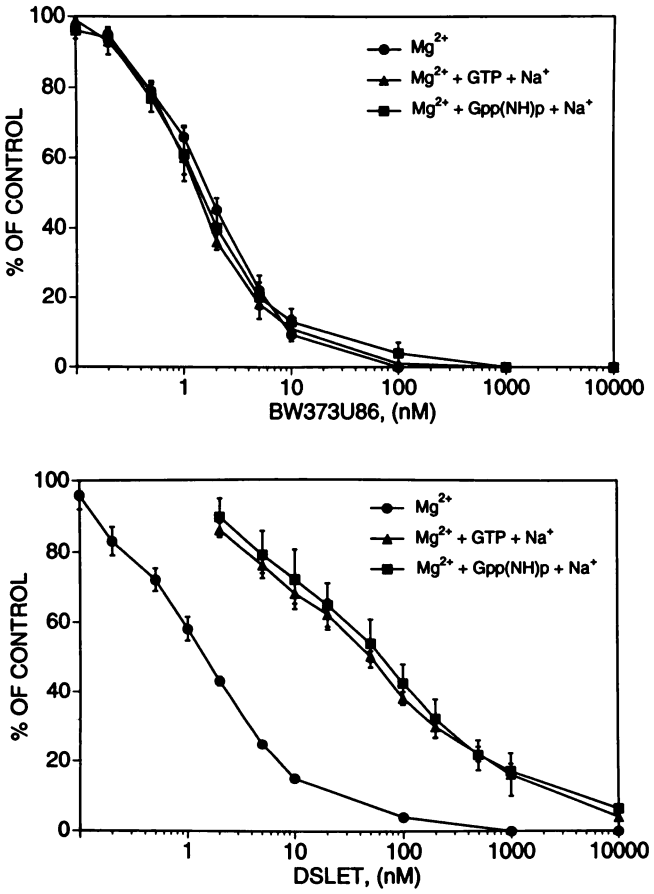

Fig. 4. Effect of NaCl and guanine nucleotides on displacement of [^{3}H] diprenorphine binding in NG108-15 cell membranes by BW373U86 (*top*) and DSLET (*bottom*). Binding was conducted either with 3 mM $MgCl_2$ alone, with Mg^{2+} plus 120 mM NaCl and 50 μM GTP, or with Mg^{2+} plus 120 mM NaCl and 50 μM Gpp(NH)p. Data are expressed as mean ± standard error of percentage of specific [^{3}H]diprenorphine binding, as determined in each condition.

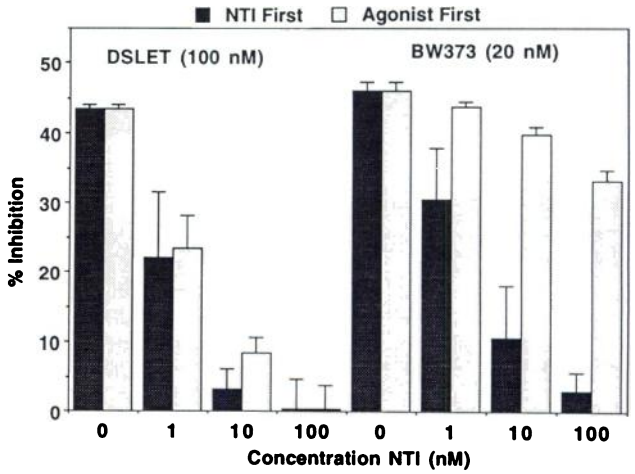

Fig. 5. Effect of agonist pretreatment on NTI blockade of agonist-inhibited adenylyl cyclase in NG108-15 cell membranes. Membranes were preincubated at 30° either with DSLET (100 nM) or BW373U86 (20 nM) for 10 min before addition of NTI (*Agonist First*) or with various concentrations of NTI for 10 min before addition of agonist (*NTI First*). Then [^{32}P] ATP was added and adenylyl cyclase activity was measured as described in Experimental Procedures. Data are expressed as mean ± standard error of percentage inhibition of basal adenylyl cyclase activity (10 pmol/min/mg).

BW373U86 dissociation was relatively slow, compared with that of DSLET.

The finding that guanine nucleotides and sodium had no effect on BW373U86 displacement of [^{3}H]diprenorphine binding (Fig. 4) led to the question of whether this novel agonist actually bound competitively at δ-opioid receptor sites. To answer this question, the Scatchard plot of [^{3}H]diprenorphine saturation binding to NG108-15 cell membranes was obtained in the absence and presence of 2 nM BW373U86 (Fig. 6). The results showed that, indeed, BW373U86 competed for [^{3}H] diprenorphine binding to δ receptors in a competitive manner; the slope of the Scatchard plot was reduced by a factor of 5.6 by the presence of 2 nM BW373U86 and the maximal binding activity (B_{max} value) was not significantly altered. An identical competitive nature of BW373U86 against [^{3}H]diprenorphine binding was observed whether guanine nucleotide (50 μM) and sodium ions (120 mM) were present (Fig. 6) or absent (data not shown).

Discussion

BW373U86 was designed as a nonpeptidic δ-opioid agonist, and a number of studies confirm this activity (7). For example, BW373U86 competed much more potently for radiolabeled δ ligand than for μ, κ, or ε ligands in brain membranes, and it was also potent in inhibiting electrically induced contractions in the mouse vas deferens. The potencies of selective μ and δ antagonists in blocking the agonist effects of BW373U86 in smooth muscle preparations were also consistent with specific δ agonist actions of this novel compound. Finally, BW373U86 produced significant opioid analgesia when injected intrathecally (8), but not centrally, consistent with the spinal mechanisms known to predominate for δ-opioid-mediated analgesia. However, one aspect of BW373U86 effect was not consistent with the actions of known δ agonists; in preliminary assays in N4TG1 neuroblastoma cell and brain membranes, neither sodium nor guanine nucleotides had any significant effect on the binding affinity of BW373U86 at δ-opioid binding sites.[1] The present studies were undertaken to examine whether this compound had effects on G protein-coupled second messenger systems that were typical of other δ agonists.

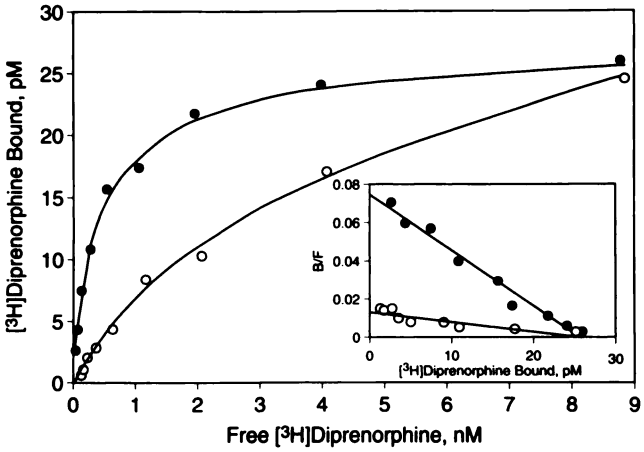

Fig. 6. Effect of BW373U86 on [^{3}H]diprenorphine saturation binding in NG108-15 cell membranes. The saturation curves for [^{3}H]dirpenorphine binding to δ receptors in NG108-15 cell membranes were obtained with nine concentrations of [^{3}H]diprenorphine in the absence (●) and presence (○) of 2 nM BW373U86 and in the presence of sodium ions (120 mM) and Gpp(NH)p (50 μM). *Inset*, Scatchard plots. The apparent K_d values are 0.35 nM and 1.98 nM in the absence and presence of 2 nM BW373U86, respectively. The B_{max} values are 25.7 pM and 25.9 pM in the absence and presence of BW373U86, respectively. Similar competitive inhibition by BW373U86 of [^{3}H]diprenorphine binding was observed in three separate experiments.

In brain membranes, BW373U86 inhibited basal adenylyl cyclase with the same efficacy (30–35% maximum inhibition) as did the prototypic δ agonist DSLET. However, BW373U86 was almost 100 times more potent than DSLET in this assay, thus making it by far the most potent opioid agonist in inhibiting adenylyl cyclase in brain membranes. Previous studies in brain (16, 18, 24, 30) revealed that most opioid agonists inhibited adenylyl cyclase with IC_{50} values between 100 and 500 nM. In guinea pig cerebellum, κ-opioid agonists were more potent in inhibiting adenylyl cyclase, with IC_{50} values of approximately 50 nM (31). Nevertheless, BW373U86 was at least 10 times more potent than these agonists. The explanation for this increased potency was not due to proteolytic degradation of DSLET, compared with the nonpeptide BW373U86, because addition of a protease inhibitor cocktail to the adenylyl cyclase assay had no effect on DSLET potency (data not shown). Furthermore, previous studies demonstrated that DSLET and related opioid peptide analogs were not degraded during the relatively short (10-min) adenylyl cyclase assay (18). The increased potency of BW373U86 was also not produced by a fundamentally higher affinity of this agonist for δ receptors, compared with DSLET. Indeed, when binding assays with [^{3}H] diprenorphine were conducted in NG108-15 cell membranes in the absence of sodium and guanine nucleotides, the IC_{50} values of DSLET and BW373U86 were essentially the same (Table 1).

Another unusual aspect of the actions of BW373U86 on striatal adenylyl cyclase is the appearance of its concentration-effect curve, which was significantly more shallow than that of DSLET or any other opioid agonist in inhibiting adenylyl cyclase (18, 31). One potential explanation is that BW373U86 may inhibit adenylyl cyclase through multiple receptor types. If so, the different subtypes may have different regional distributions in rat brain. To explore this issue, the regional distribution of BW373U86-inhibited adenylyl cyclase in brain, using both low (0.1 μM) and high (10 μM) concentrations of agonist, was compared with inhibition produced by 10 μM DSLET (Table 2). These results did not suggest that low and high concentrations of BW373U86 bound to receptors with different regional distributions, because the inhibition results were identical for the two concentrations. These multiple sites cannot be μ and δ sites, because the μ-selective antagonist CTOP had no effect on inhibition of adenylyl cyclase by BW373U86. Therefore, identification of possible δ subtypes mediating these effects of BW373U86 in brain must await additional experiments.

Assays in NG108-15 cells provided the key data that explained the discrepancies between DSLET and BW373U86 potencies. Binding experiments with the antagonist [^{3}H]diprenorphine showed that, unlike with DSLET and most other opioid agonists, addition of sodium and guanine nucleotides, at the same concentrations used in the adenylyl cyclase assays, had no effect on IC_{50} values of BW373U86. In contrast, the

[1] K.-J. Chang, M. A. Collins and R. W. McNutt, unpublished observations.

same agents increased IC_{50} values of DSLET by 40-fold. If the presence of sodium and guanine nucleotides is a significant factor in DSLET potency, then the potency of DSLET should vary according to the concentration of these agents in each particular assay. This prediction was confirmed by the results summarized in Table 1, where DSLET was most potent in the binding assay in which both sodium and guanine nucleotides were absent. Addition of 50 μM GTP with 120 mM NaCl to the adenylyl cyclase assay decreased potency by 10-fold, whereas addition of a much lower concentration of GTP (0.5 μM) with 120 mM NaCl to the GTPase assay produced only a 2-fold decrease in DSLET potency, compared with its binding potency. At first glance, it may seem surprising that the potency of DSLET in inhibiting adenylyl cyclase in brain membranes is significantly lower than that in the same assay in NG108-15 cell membranes (300 nM versus 10 nM). This discrepancy is probably caused by an increased effect of guanine nucleotides and sodium on DSLET binding in brain, compared with NG108-15 cells. Indeed, the IC_{50} value of DSLET in displacing [^{3}H]diprenorphine binding in NG108-15 cells with NaCl and GTP was 50 nM (Table 1), compared with an IC_{50} value of 400 nM for DSLET in brain membranes under the same conditions (data not shown). The reason for this increased effect of guanine nucleotides and sodium in brain is not clear, but it does explain why opioid agonists are significantly weaker in inhibiting adenylyl cyclase in brain membranes, compared with NG108-15 cell membranes.

This hypothesis also indicates why the binding affinity of BW373U86 is not affected by NaCl and guanine nucleotides and predicts that, unlike that of DSLET, the potency of BW373U86 should be the same regardless of the assay used. Table 1 shows that this prediction is confirmed for BW373U86 in NG108-15 cell membranes; the potency of BW373U86 did not change between the adenylyl cyclase assay (with 50 μM GTP) and the GTPase assay (with 0.5 μM GTP) or between assays measuring displacement of [^{3}H]diprenorphine binding with and without NaCl and guanine nucleotides. Further confirmation was obtained from the adenylyl cyclase assays in brain membranes; the IC_{50} value of 4 nM in brain is approximately the same as the values of 2 nM obtained in various assays in NG108-15 cells. Therefore, the potency of BW373U86 did not change even in a system like brain, where effects of NaCl and guanine nucleotides are much more significant than in cell culture.

A final prediction from these results suggests that BW373U86 would display fundamentally different binding kinetics, compared with traditional agonists, under conditions that allow agonist inhibition of adenylyl cyclase (i.e., in the presence of GTP and sodium). The decrease in agonist binding affinity under these conditions is produced by a large increase in dissociation rates of the receptor-bound agonist (9, 10). Such an increase should not occur for BW373U86; therefore, once this agonist has bound to the receptors, its dissociation should be very slow, compared with traditional agonists, in the presence of sodium and GTP. The finding that NTI was much less effective in blocking BW373U86 inhibition of adenylyl cyclase when membranes were preincubated for 10 min with BW373U86 supports this prediction. These results were not caused by large differences in the K_e values for NTI against BW373U86 and DSLET, because NTI had similar effects on both agonists when antagonist was added first. Furthermore, the effect of BW373U86 was not irreversible, because higher concentrations of NTI began to block BW373U86 inhibition. Finally, the finding (Fig. 6) that BW373U86 is competitive in displacing [^{3}H]diprenorphine confirms that BW373U86 indeed binds to the δ receptor sites and that the novel effects of BW373U86 are not due to binding at allosteric sites on the receptor.

These findings have profound implications at both the practical and mechanistic levels. From a practical point of view, these data suggest that agonist potency is not just determined by the fundamental K_d of the drug in binding studies but also depends on the effect of sodium and guanine nucleotides on binding affinity. Drugs like BW373U86 can be designed so that sodium and guanine nucleotide effects are minimal, to provide the highest level of potency *in vivo*. Equally important, however, are the implications of this finding for the mechanism of receptor-G protein interactions. Since it was originally formulated, the ternary complex model of G protein function (32, 33) has assumed that agonist but not antagonist binding affinities are reduced by guanine nucleotides. Moreover, in the specific case of G_i/G_o-linked receptors, sodium also reduces agonist affinities in an additive fashion with guanine nucleotides (10). The discovery that BW373U86 is a full agonist in a G protein-coupled second messenger system despite the lack of any effect of sodium and guanine nucleotides on binding affinity suggests that this action on binding is not necessary for agonist function. This supports earlier findings from studies that used selective modifications to show that the functions of guanine nucleotides in regulating opioid receptor binding and in supporting opioid-inhibited adenylyl cyclase could be separated from each other (24, 34). This hypothesis also predicts that this discrepancy should not be unique to BW373U86; other opioid agonists could be designed with the same property. One candidate for such an action might be etorphine, which is affected little by sodium and guanine nucleotides despite its known role as a full opioid agonist (10). Recent studies (35) revealed that, unlike other opioid agonists, the potency of etorphine in binding to μ receptors was the same whether it was assayed in isolated membranes without sodium and GTP or in intact cells. The results from the present study predict that BW373U86 would display the same properties.

Acknowledgments

We thank Tammy Sexton and Mark Collins for excellent technical assistance.

References

1. Lord, J. A. H., A. A. Waterfield, J. H. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)* **267**:495–499 (1977).
2. Chang, K.-J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
3. Chang, K.-J., B. R. Cooper, E. Hazum, and P. Cuatrecasas. Multiple opiate receptors: different regional distribution in the brain and differential binding of opiate and opioid peptides. *Mol. Pharmacol.* **16**:91–104 (1979).
4. Gacel, G., M. C. Fournie-Zaluski, and B. P. Roques. Tyr-D-Ser-Gly-Phe-Leu-Thr, a highly preferential ligand for *delta*-opiate receptors. *FEBS Lett.* **118**:245–247 (1980).
5. Mosberg, H. I., R. Hurst, V. J. Hruby, K. Gee, H. I. Yamamura, J. J. Galligan, and T. F. Burks. Bis-penicillamine enkephalins possess highly improved specificity toward *delta* opioid receptors. *Proc. Natl. Acad. Sci. USA* **80**:5871–5874 (1983).
6. Vaughn, L. K., R. J. Knapp, G. Toth, Y. P. Wan, V. J. Hruby, and H. I. Yamamura. A high affinity, highly selective ligand for the *delta* opioid receptor: [^{3}H]-[D-Pen$_2$,*p*-Cl-Phe$_4$,D-Pen$_5$]enkephalin. *Life Sci.* **45**:1001–1008 (1989).
7. Chang, K.-J., G. C. Rigdon, J. L. Howard, and R. W. McNutt. A novel, potent and selective nonpeptidic δ-opioid receptor agonist, BW373U86. *J. Pharmacol. Exp. Ther.*, in press.

8. Wild, K. D., P. J. Horan, Q. Jiang, T. W. Vanderah, E. J. Bilsky, R. W. McNutt, K.-J. Chang, and F. Porreca. Antinociceptive pharmacology of BW373U86: a novel, non-peptidic and selective opioid δ agonist with an atypical profile. *J. Pharmacol. Exp. Ther.*, in press.
9. Blume, A. J. Opiate binding to membrane preparations of neuroblastoma × glioma hybrid cells NG108-15: effects of ions and nucleotides. *Life Sci.* **22**:1843–1852 (1978).
10. Childers, S. R., and S. H. Snyder. Differential regulation by guanine nucleotide of opiate agonist and antagonist receptor interactions. *J. Neurochem.* **34**:583–593 (1980).
11. Chang, K.-J., E. Hazum, A. Killian, and P. Cuatrecasas. Interactions of ligands with morphine and enkephalin receptors are differentially affected by guanine nucleotides. *Mol. Pharmacol.* **20**:1–7 (1981).
12. North, R. A., J. T. Williams, A. Surprenant, and M. J. Christie. μ and δ receptors belong to a family of receptors that are coupled to potassium channels. *Proc. Natl. Acad. Sci. USA* **84**:5487–5491 (1987).
13. Traber, F., R. Gullis, and B. Hamprecht. Influence of opiates on levels of adenosine 3′,5′-monophosphate in neuroblastoma × glioma hybrid cells. *Life Sci.* **16**:1863–1868 (1975).
14. Sharma, S. K., M. Nirenberg, and W. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* **72**:590–594 (1975).
15. Blume, A. J., L. Lichtshtein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for sodium ion and GTP. *Proc. Natl. Acad. Sci. USA* **76**:5626–5630 (1979).
16. Cooper, D. M. F., C. Londos, D. L. Gill, and M. Rodbell. Opiate receptor-mediated inhibition of adenylate cyclase in rat striatal plasma membranes. *J. Neurochem.* **38**:1164–1167 (1982).
17. Law, P. Y., J. Wu, J. E. Koehler, and H. H. Loh. Demonstration and characterization of opiate inhibition of the striatal adenylate cyclase. *J. Neurochem.* **36**:1834–1846 (1981).
18. Childers, S. R. Opiate-inhibited adenylate cyclase in rat brain membranes depleted of G_s-stimulated adenylate cyclase. *J. Neurochem.* **50**:543–553 (1988).
19. Schoffelmeer, A. N., K. C. Rice, M. H. Heijna, F. Hogenboom, and M. H. Mulder. Fentanyl isothiocyanate reveals the existence of physically associated *mu*- and *delta*-opioid receptors mediating inhibition of adenylate cyclase in rat neostriatum. *Eur. J. Pharmacol.* **149**:179–182 (1988).
20. Duman, R. S., J. F. Tallman, and E. J. Nestler. Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J. Pharmacol. Exp. Ther.* **246**:1033–1039 (1988).
21. Portoghese, P. S., M. Sultana, H. Nagase, and A. E. Takemori. Application of the message-address concept in the design of highly potent and selective non-peptide δ-opioid receptor antagonist. *J. Med. Chem.* **31**:281–282 (1988).
22. Vachon, L., T. Costa, and A. Herz. Desensitization of opioid-stimulated GTPase in neuroblastoma × glioma hybrid cells. *Biochem. Biophys. Res. Commun.* **128**:1342–1349 (1985).
23. Childers, S. R. A high-performance liquid chromatography assay of brain adenylate cyclase using [^{3}H]-ATP as substrate. *Neurochem. Res.* **11**:161–171 (1986).
24. Childers, S. R., and G. LaRiviere. Modification of guanine nucleotide-regulatory components in brain membranes. II. Relationship of Guanosine 5′-triphosphate effects on opiate receptor binding and coupling receptors with adenyl cyclase. *J. Neurosci.* **4**:2764–2771 (1984).
25. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–55 (1979).
26. Cassel, D., and Z. Selinger. Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochim. Biophys. Acta* **452**:538–551 (1976).
27. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–252 (1976).
28. Gulya, K., J. T. Pelton, V. J. Hruby, and H. I. Yamamura. Cyclic somatostatin octapeptide analogues with high affinity and selectivity toward *mu* opioid receptors. *Life Sci.* **38**:2221–2229 (1986).
29. Law, P. Y., D. S. Hom, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment: a multi-step process. *Mol. Pharmacol.* **22**:1–4 (1982).
30. Wild, K. D., L. Fang, R. W. McNutt, K.-J. Chang, G. Toth, A. Borsodi, H. I. Yamamura, and F. Porreca. Binding of BW373U86, a non-peptidic δ-opioid agonist, is not regulated by guanine nucleotides and sodium. *Eur. J. Pharmacol.*. **246**:289–292 (1993).
31. Konkoy, C. S., and S. R. Childers. Dynorphin-selective inhibition of adenylyl cyclase in guinea pig cerebellum membranes. *Mol. Pharmacol.* **36**:627–633 (1989).
32. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G-proteins. *Biochim. Biophys. Acta* **1031**:163–224 (1991).
33. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
34. Lambert, S. M., and S. R. Childers. Modification of guanine nucleotide regulatory components in brain membranes. I. Changes in guanosine-5′-triphosphate regulation of opiate receptor binding sites. *J. Neurosci.* **4**:2755–2763 (1984).
35. Toll, L. Comparison of *mu* opioid receptor binding on intact neuroblastoma cells with guinea pig brain and neuroblastoma cell membranes. *J. Pharmacol. Exp. Ther.* **260**:9–15 (1992).

Send reprint requests to: Kwen-Jen Chang, Division of Cell Biology, Burroughs Wellcome Co., 3030 Cornwallis Road, Research Triangle Park, NC 27709.