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EVIDENCE FOR COUPLING OF THE κ OPIOID RECEPTOR TO BRAIN GTPase

Mary J. Clark, Scott D. Levenson and Fedor Medzihradsky

Departments of Biological Chemistry and Pharmacology
The University of Michigan Medical School
Ann Arbor, Michigan 48109-0010

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Summary

In membranes from guinea pig cerebellum, a tissue which predominantly contains κ opioid receptors, exogenous and endogenous κ-selective opioid agonists stimulated low-Km GTPase activity by 11-20% with concentrations for half-maximal stimulation of 3-23 μM. Opioid ligands of the μ and δ type had no effect on GTPase in these membranes. Similar stimulation of GTPase by κ opiates was obtained in rat and monkey brain membranes pretreated with β-funaltrexamine (β-FNA) and cis-(±)-3-methylfentanyl isothiocyanate (superfit) to alkylate the μ and δ receptors, respectively. The stimulation of brain GTPase by κ opiates in both types of membranes was inhibited by naloxone with IC50's of 0.35 μM and 0.40 μM. The results demonstrate the coupling of the κ opioid receptor to high affinity GTPase, the Ni regulatory protein of the adenylate cyclase complex.

Opiates stimulated the activity of a low-Km GTPase in membranes from NG108-15 neuroblastoma-glioma cells (1) and rat brain (2). Conversely, ligand occupancy of the opioid receptor has resulted in the inhibition of adenylate cyclase in NG108-15 hybrid cells (3), striatal membranes (4) and brain slices (5). The two phenomena are causally related by the availability of GTP which is necessary for the coupling of the opioid receptor and Ni, the inhibitory guanyl nucleotide binding protein (6), to adenylate cyclase (2,7). It has recently been shown that purified Ni from bovine brain has the enzymatic properties of a high affinity GTPase (8).

In ongoing work, we have observed that, compared to μ and δ opiates, GTPase stimulation by κ ligands was generally lower (9). Recently, it was shown that, relative to μ and δ opioid ligands, the binding of κ agonists to opioid receptor was less sensitive to GTP regulation (10), and was affected to a lesser degree by Gpp(NH)p, Na^+, Mn^{2+} (11) and by GTP (12). Considering the limited extent of these effects resulting from the interaction of the κ opioid receptor with GTP and Ni, we have in this study evaluated the evidence for coupling of the κ opioid receptor to brain GTPase. We have made use of receptor-selective irreversible opiates to convey specificity toward the κ

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opioid receptor, and have isolated membranes from guinea pig cerebellum, a tissue containing predominantly κ opioid receptors (13). With the κ-specific brain membranes we have described the stimulation of low-Km GTPase in response to ligand occupancy of the κ opioid receptor.

Materials and Methods

Most of the opiates, including β-FNA and superfit, were obtained through the Drug Abuse Basic Research Program, Department of Pharmacology, The University of Michigan. U-50,488 (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide) and the structurally related U-69,593 (14) are prototypes of a recently introduced novel series of opiates with demonstrated κ-agonist activity (15). Mr 2266 is a N-furyl-substituted 6,9-diethyl benzomorphan antagonist of equal potency toward μ and κ agonists (14). ICI-174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; Aib: α-aminoisobutyric acid), is a highly selective antagonist at the δ opioid receptor (16). D-serine-leucine-enkephalin-threonine (DSLET) and dynorphin(1-13) were purchased from Cambridge Research Biochemicals, Atlantic Beach, N.Y., [gamma-^{32}P]GTP was obtained from Amersham Corp., Arlington Heights, IL. The biochemicals for the enzyme assay were purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were of reagent grade.

Membranes from rat brain cerebrum, guinea pig cerebellum and Rhesus monkey midbrain (subcortical cerebrum excluding the striatum and hypothalamus) were prepared as described previously (17). The isolated membranes were suspended in 50 mM Tris.HCl, pH 7.4, to a concentration of 0.6 mg protein/ml, and stored at -70°C until use. Receptor alkylation was carried out by incubation of the membranes for 40 min at room temperature with either 200 μM β-FNA or 100 μM superfit, or both (sequentially). Subsequently, the membranes were diluted with 5 volumes of Tris.HCl, pH 7.4, and centrifuged at 20,000 x g for 15 min at 2°. The washing procedure was repeated twice (shown to be sufficient), and the membranes were suspended in the original volume of Tris buffer. Specific methodological aspects of the direct and protective receptor alkylation, including the Scatchard analysis of ligand binding in these membranes, were reported (18) and will be described elsewhere.

With some procedural modifications, the assay of GTPase activity was carried out as described previously (1,2). The assay is based on the release of inorganic phosphate (Pi) from [gamma-^{32}P]GTP. The reaction mixture included 12.5 mM Tris.HCl, pH 7.5, an ATP-regenerating system, App(NH)p and ouabain to inhibit Na,K-ATPase activity. Each tube contained 1-4 μg membrane protein. After incubation at 37°, the tubes were placed in ice and a suspension of activated charcoal in phosphoric acid was added to absorb excess nucleotide. Released ^{32}Pi was measured by liquid scintillation counting. Release of ^{32}Pi in the absence of membranes was 1-2% of the added [gamma-^{32}P]GTP. In experiments with dynorphin, the assay tubes were treated with bovine serum albumin (BSA) to prevent adsorption of the peptide. Protein was determined according to Lowry et al. (19) using BSA as the standard.

The results shown are averages of data obtained in 2-5 experiments, each done in triplicate. Typical standard deviation or range of variability (with $n<4$) was ± 6% for maximal stimulation of GTPase (Smax) and ± 10% for ligand concentration required for half-maximal GTPase stimulation (Ks). The Ks values for the agonists and the IC50 values for the antagonist were obtained from log-probit plots of the data (Fig. 1).

Results

In membranes from guinea pig cerebellum, the highly selective κ-agonists U-50,488, U-69,593, and dynorphin(1-13), as well as the partially selective agonists bremazocine and ethylketocyclazocine (EKC) (13, 20) stimulated GTPase activity up to 19% (Table I). The obtained Ks values ranged from 3.5 μM for EKC to 23 μM for U-69,593. In contrast, the μ-selective ligands sufentanil and levorphanol, and the δ agonist DSLET had no effect on GTPase activity.

TABLE I

Stimulation of GTPase by opiates in membranes from guinea pig cerebellum.

Opioid ligand	Stimulation of GTPase activity Ks (μM)	Smax (%)
Agonists		
Bremazocine	4.4	19.0
Dynorphin(1-13)	14.0	11.0
EKC	3.5	17.0
U-50,488	12.0	9.8
U-69,593	23.0	19.0
Levorphanol	-	*
Sufentanil	-	*
DSLET	-	*
Antagonists		
Naloxone	-	*
MR-2266	-	*
ICI-174,864	-	*

The experiments and statistical evaluation were carried out as described under Materials and Methods. Ks is the ligand concentration which produced half-maximal GTPase stimulation. Smax is the maximal stimulation of GTPase produced by a ligand.
*: undetectable.

In the experiments with rat cerebral membranes, a tissue containing μ, κ and δ opioid receptors, specific alkylators were used to convey selectivity for the κ opioid receptor. After pretreatment with β-FNA, a μ-selective irreversible ligand (21), and superfit, a δ-specific alkylator (9, 18, 22), levorphanol and DSLET which stimulated GTPase in untreated rat cerebral membranes (9), had no effect on the GTP hydrolizing activity. However, the κ opiates bremazocine and dynorphin(1-13) exhibited Smax of 19% and 10%, respectively, with Ks values in the low micromolar range (Table II). None of the alkylations altered the basal rate of GTPase activity (65 pmol ^{32}Pi/mg protein. min).

In monkey brain membranes, GTPase stimulation by κ opiates was investigated with bremazocine, U-50,488 and U-69,593. In order to block the antagonistic component of bremazocine at the μ receptor (23), these sites were alkylated with β-FNA. On the other hand, considering the lack of

cross-reactivity of the employed μ and κ ligands at the δ opioid receptor, alkylation of these sites with superfit was omitted. Accordingly, in these membranes levorphanol had no effect on GTPase, however DSLET stimulated the enzymatic activity with Smax of 24% and Ks of 15 μM. All three κ selective agonists, bremazocine, U-50,488 and U-69,593 had Smax values between 14% and 20% (Table II).

TABLE II

Stimulation of GTPase by opiates in alkylated brain membranes.

Source and treatment of membranes	Opioid ligand	Stimulation of GTPase activity Kact. (μM)	Smax (%)
Rat cerebrum; treated with 200 uM β-FNA and 100 uM superfit	Bremazocine	12 ± 2.6	19 ± 6.3
	Dynorphin(1-13)	3.6	10.0
	Levorphanol	-	*
	DSLET	-	*
Monkey mid-brain treated with 200 uM β-FNA	Bremazocine	12.0	17.0
	U-50,488	19.0	14.0
	U-69,593	57.0	20.0
	Levorphanol	-	*
	DSLET	15.0	24.0

The experiments and statistical evaluation were carried out as described under Materials and Methods. See also the legend to Table I.
*: undetectable.

In membranes from guinea pig cerebellum, naloxone inhibited GTPase stimulation by the κ agonist U-69,593 with an IC50 of 0.40 μM (Fig. 1), and in the alkylated, κ-specific, rat cerebral membranes with an IC50 of 0.35 μM (not shown). Opioid antagonists with different degrees of receptor selectivity had no effect on GTPase activity (Table I).

Discussion

As shown in this and previous work (2), stimulation of low-Km brain GTPase represents a functional consequence of opioid receptor occupancy by agonists. In addition, the demonstrated reciprocal relationship between GTPase stimulation and inhibition of cyclic cAMP formation (2,5,6), implicates the GTP-hydrolizing activity in the mechanism of opioid receptor coupling to adenylate cyclase, the putative effector. The results of the present study demonstrate the interaction of the κ opioid receptor with brain GTPase. Earlier, the antagonist-reversible interaction of κ opioids with adenylate cyclase in rat brain has been described (5).

The specificity of the observed GTPase stimulation by κ agonists was provided by the selectivity of the employed brain membranes (obtained from three different species) toward the κ opioid receptor, and by the use of selective κ ligands. The findings were substantiated by the good agreement between the data obtained with untreated membranes from a tissue with predominant content of κ opioid receptor, and brain membranes alkylated to block the μ and δ opioid receptors in order to provide selectivity toward the κ receptor. Alkylation also provided a means to eliminate cross-reactivity of one employed κ agonist at the μ opioid receptor. The success of alkylation

was assessed by the ability of highly selective μ, κ and δ ligands to stimulate GTPase before and after the exposure to the irreversible opiates β-FNA and superfit. In addition, the results of direct alkylation described in this work (Table 2) were supported by the finding obtained following protective receptor alkylation with β-CNA (21) in the presence of selective opioid ligands (9, 18). In agreement with previous findings (2), the stimulation of GTPase by opioid agonists was reversible by naloxone. Concerning the discrepancy between receptor binding affinity of opiates and their potency to stimulate GTPase, it should be recalled that the latter is assayed in the presence of GTP and sodium which decrease agonist binding affinity (24). Accordingly, the IC50 of naloxone in inhibiting GTPase stimulation was lower than the Ks values for agonists in enhancing the enzymatic activity. Furthermore, considering the limited extent of GTPase stimulation (Table I) and of adenylate cyclase inhibition (4), an impaired coupling of opioid receptor to effector in isolated brain membranes is apparent.

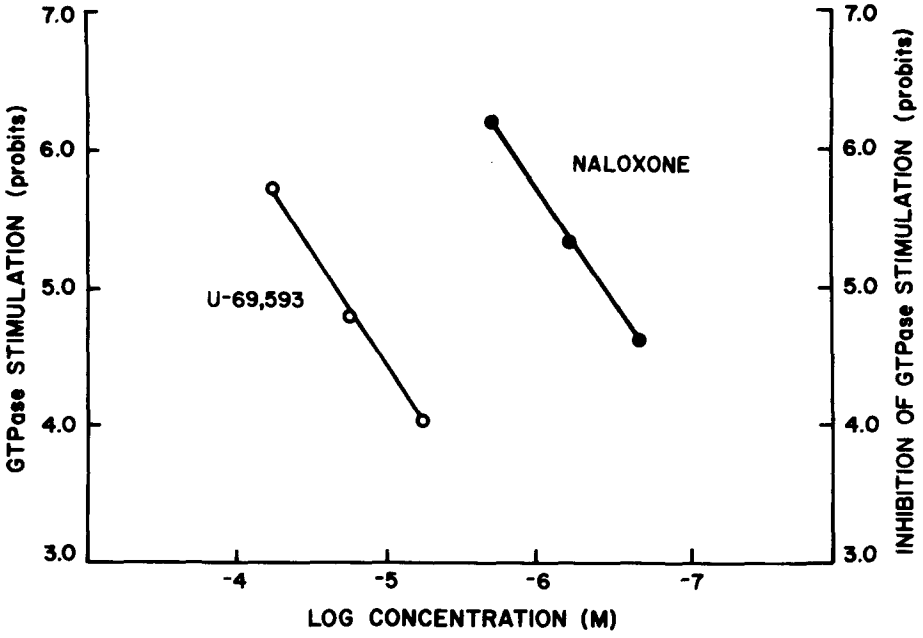

FIG. 1

Log-probit plots of GTPase stimulation by opiates and its inhibition by naloxone. Membranes from guinea pig cerebellum were incubated with different concentrations of U-69,593 as shown. In other experiments, the membranes were incubated with 60 μM U-69,593 and the indicated concentrations of naloxone. Subsequently, in both types of experiments, GTPase activity was determined as described under Materials and Methods. Plotted are GTPase stimulation by the agonist U-69,593 (left ordinate), and the inhibition by naloxone of GTPase stimulation by U-69,593 (right ordinate). Shown are mean values of three experiments.

The generally lower extent of GTPase stimulation by κ opiates relative to the effects of μ and δ ligands, corresponds to the reported lower sensitivity of κ ligand binding toward regulation by GTP (10, 25). It is possible that the κ opioid receptor, in addition to adenylate cyclase, couples to other effectors, e.g., ion channels with (26) or without (27) the participation of the cAMP system.

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