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RELATIONSHIP BETWEEN KAPPA$_1$ OPIOID RECEPTOR BINDING AND INHIBITION OF ADENYLYL CYCLASE IN GUINEA PIG BRAIN MEMBRANES

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Abstract—Previously, we showed that κ-selective ligands inhibit adenylyl cyclase in guinea pig cerebellar membranes. The present studies explore the relationship between κ_1 binding sites (as determined with [^{3}H]U-69,593 binding) and κ_1-inhibition of adenylyl cyclase (using U-50,488H) in guinea pig brain membranes. Various κ opioids displaced [^{3}H]U-69,593 binding at a single site with subnanomolar affinities. These agonists were several hundred-fold weaker in inhibiting adenylyl cyclase, but for most agonists the rank order of adenylyl cyclase inhibition paralleled the displacement of κ_1 binding. The correlation of IC_{50} values for both adenylyl cyclase and binding was significant except for α-neo endorphin, which was relatively weak at inhibiting adenylyl cyclase despite a K_i value of 0.08 nM versus κ_1 binding. Comparison between κ_1 binding and κ_1-inhibited adenylyl cyclase across eleven guinea pig brain regions revealed that κ_1-inhibited adenylyl cyclase was highest in the cerebellum, absent in thalamus and superior colliculus, and moderate in other regions. In most regions, κ_1 binding correlated with the efficacy of κ_1-inhibited adenylyl cyclase. However, the hippocampus had high levels of κ_1-inhibited adenylyl cyclase despite low levels of κ_1 binding, while cortex exhibited a high density of κ_1 sites but a relatively low level of κ_1-inhibited adenylyl cyclase. Reaction of cerebellar κ receptors with β-chlornaltrexamine (β-CNA) blocked both κ_1 binding and κ_1-inhibited adenylyl cyclase. The effect of β-CNA on κ_1-inhibited adenylyl cyclase was to inhibit efficacy with little decrease in agonist potency, thus suggesting no significant level of κ receptor reserve for this effector system.

Kappa (κ) receptors were first identified as a separate type of opioid receptor by physiological effects, radioligand binding assays, and peripheral bioassays [1–3]. More recent reports have provided evidence for κ receptor multiplicity [4–8] and have demonstrated marked differences in receptor binding densities between rat and guinea pig brain [7, 9]. The κ_1 sites, which are found predominantly in guinea pig brain, display high affinity for the dynorphins [7], which serve as endogenous ligands for the κ receptor [10–11], and for the arylacetamide derivatives U-50,488H§ and U-69,593 [12–13]. In contrast, κ_2 sites exhibit low affinity for the benzeneacetamide compounds but retain high affinity for the dynorphins. The κ_3 sites exhibit high affinity for ketocyclazocine but relatively low affinity for both dynorphin A and U-50,488H, and, according to Pasternak and colleagues [14] are the predominant opioid subtype in rat brain.

The guinea pig cerebellum serves as a relatively pure source of κ receptors, with only 15% of total opioid binding sites being μ and δ types [15]. This receptor homogeneity has enabled studies which address the coupling of κ receptors to effector systems. Kappa receptors are coupled to stimulation of low K_m GTPase in membranes prepared from guinea pig cerebellum [16]. We have shown previously that analogs of dynorphin A inhibit adenylyl cyclase in guinea pig cerebellar membranes, and that this inhibition is dependent upon the presence of sodium and GTP [17]. Moreover, dynorphin-inhibited adenylyl cyclase is blocked by nor-binaltorphimine (nor-BNI), a selective κ antagonist. Together, these data suggest that κ receptors are negatively coupled to adenylyl cyclase via a G protein-linked mechanism.

The relationship between high affinity opioid receptor binding and opioid-inhibited adenylyl cyclase has been studied with specific receptor alkylating agents. One such agent is β-chlornaltrexamine (β-CNA), a site-directed alkylating agent synthesized by Portoghese and colleagues [18]. β-CNA is a mustard analog of the opioid antagonist naltrexone. Numerous studies have addressed the physiological and biochemical responses which follow irreversible blockade via alkylation of receptors outside the CNS [19–24]. Moreover, Chavkin and Goldstein [25] used β-CNA to demonstrate κ receptor reserve in the guinea pig myenteric plexus. Irreversible antagonists have been used to demonstrate the existence of spare receptor populations for effector responses, such as the inhibition of adenylyl cyclase and the stimulation of

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§ Abbreviations: nor-BNI, nor-binaltorphimine; DAMGO, Tyr-D-ala^2-*N*-Me-Phe-Gly-ol; DPDPE, D-Pen^{2,5}-enkephalin; U-50,488H, (*trans*-(*dl*)-3,4-dichloro-*N*-methyl-*N*-[2-(*l*-pyrrolidinyl)cyclohexyl]-benzeneacetamide) methane sulfonate; β-CNA, β-chlornaltrexamine; and EKC, ethylketocylazocine.

phosphoinositide turnover [24]. To our knowledge, however, no reports have examined the relationship between receptor occupancy and effector responses within the CNS.

The aim of the present study was to correlate κ_1 receptor binding with the effector response best characterized for opioid systems, namely the inhibition of adenylyl cyclase. First, the potencies of dynorphin analogs in inhibiting adenylyl cyclase were correlated with their affinities at the κ_1 (U-69,593-sensitive) site. Second, the density of κ_1 sites was correlated with U-50,488H-inhibited adenylyl cyclase across several guinea pig brain regions. A final set of experiments provide evidence that there is no significant receptor reserve for κ_1-inhibited adenylyl cyclase in guinea pig cerebellum.

MATERIALS AND METHODS

Chemicals. [^{3}H]ATP (33 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Creatine phosphate, creatine phosphokinase, (*trans*-(*dl*)-3, 4-dichloro - *N* - methyl - *N* - [(2 - [*l* - pyrrolidinyl) cyclohexyl]-benzeneacetamide methane sulfonate, naltrexone (U-50,488H), unlabeled ATP, and protease inhibitors were obtained from the Sigma Chemical Co. (St. Louis, MO). Opioid peptides were obtained from Sigma and from Peninsula Laboratories (Belmont, CA). Guanine nucleotides and cyclic AMP were purchased from Boehringer Mannheim (Indianapolis, IN). Nor-binaltorphimine and β-chlornaltrexamine were purchased from Research Biochemicals Inc. (Natick, MA).

Adenylyl cyclase assay. Female Hartley guinea pigs (400–500 g, Sasco) were anesthetized with halothane and decapitated. Brain regions were quickly dissected on ice and stored at −80° for up to 1 month. Brain regions were homogenized in cold Tris buffer (50 mM Tris·HCl, pH 7.4) with a Polytron (setting 6; 15 sec). Membranes were isolated by centrifugation at 48,000 *g* for 10 min and pretreated at pH 4.5 by a modification of the low pH treatment previously described [26]. Membranes were resuspended in pH 4.5 buffer (50 mM sodium acetate, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 4.5) and incubated on ice for 10 min. The low pH treatment was terminated by the addition of a 10-fold excess volume of cold Tris buffer (pH 7.4), and membranes were centrifuged at 48,000 *g* and rewashed with cold Tris buffer. Aliquots were then frozen for subsequent receptor binding assays. For adenylyl cyclase assays, membranes were isolated by centrifugation at 48,000 *g* for 10 min, and then resuspended in cyclase assay buffer (50 mM Tris·HCl, 3 mM $MgCl_2$,10 mM theophylline, pH 7.4).

Adenylyl cyclase was assayed as previously described [17]. Briefly, 20–100 μg membrane protein was added to tubes containing 50 mM NaCl, 50 μM cyclic AMP, 100 μM GTP, 20 mM creatine phosphate, 10 U of creatine phosphokinase, 100 μM ATP, and 1 μCi of [^{3}H]ATP, in cyclase assay buffer, in a total volume of 100 μL. The reaction was initiated by the addition of [^{3}H]ATP, and the tubes were incubated for 10 min at 30°. The reaction was terminated by immersion in boiling water; enzyme blanks were boiled prior to the 30° incubation. [^{3}H]-Adenosine formed from [^{3}H]ATP was removed by incubation with 0.75 U of adenosine deaminase at 30° for 5 min. Remaining ATP was precipitated by the sequential addition of 50 μL of 1 M $Ba(OH)_2$, followed by 50 μL of $ZnSO_4$, with 5 min between each addition. The tubes were centrifuged at 3000 *g* for 15 min, and 250 μL of supernatant were automatically injected onto a Microsorb C-18 HPLC column. [^{3}H]Cyclic AMP was assayed by a method modified from the procedure of Schultz and Mailman [27] as previously described [28]. [^{3}H]Cyclic AMP eluates were quantified by liquid scintillation spectrophotometry in 5 mL Ecolite scintillation fluid (ICN, Irvine, CA) with 35% efficiency. Results were expressed as percentages of basal adenylyl cyclase activity (100–200 pmol/mg/min). K_e values for antagonists were calculated by the following relationship:

$$K_e = \frac{[\text{Ant}]}{\text{IR} - 1}$$

where [Ant] is the antagonist concentration, and IR is the inhibition ratio, defined as the agonist IC_{50} value in the presence of antagonist divided by the IC_{50} value in the absence of antagonist.

β-CNA treatments. Frozen cerebella were homogenized in cold Tris buffer with a Polytron (setting 6; 15 sec). Aliquots were placed into tubes containing various concentrations of β-CNA, and the tubes were incubated at 25° for 15 min. The reaction was terminated by the addition of a 15-fold excess of cold Tris buffer. Membranes were isolated by centrifugation at 48,000 *g* and treated in pH 4.5 buffer for 10 min (see above). Membranes were washed by centrifugation with cold Tris buffer one additional time. Some aliquots were frozen and stored at −80° for receptor binding assays, while other aliquots were resuspended in theophylline buffer (see above) for assay of adenylyl cyclase. To control for the reversible binding component of β-CNA, equal concentrations of naltrexone were added to other tubes. Control experiments (see Results) revealed that these washing procedures sufficed to remove up to 10 μM naltrexone.

Receptor binding assays. All binding experiments except for the β-CNA studies were conducted in untreated (pH 7.4) membranes. The β-CNA experiments were conducted in low pH pretreated membranes so that binding and adenylyl cyclase results could be compared in the same membranes. Experiments comparing the displacement of [^{3}H]U-69,593 binding by various agonists revealed no effect of low pH pretreatment of membranes on binding or agonist IC_{50} values (see Results). Frozen brain membranes were thawed in a 30° water bath, and then were centrifuged at 48,000 *g* for 10 min. For experiments which compared the receptor binding affinities of κ agonists, membranes were resuspended in Tris buffer and preincubated at 37° for 15 min. Membranes were centrifuged at 48,000 *g* for 10 min, and then resuspended in a cocktail containing bestatin, leupeptin, pepstatin A, and aprotinin (all 1 μg/mL) in Tris buffer. This final membrane suspension was added to tubes containing Tris buffer, opioid drugs (0.01 to 100 nM), and unlabeled

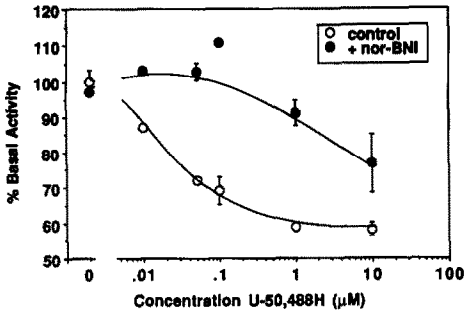

Fig. 1. Effect of the κ-selective antagonist nor-BNI on U-50,488H-inhibited adenylyl cyclase in guinea pig cerebellar membranes. Membranes were pretreated at pH 4.5, washed, and assayed for adenylyl cyclase with various concentrations of U-50,488H in the presence and absence of 0.1 μM nor-BNI as described in Materials and Methods. Data are expressed as a percentage of basal adenylyl cyclase activity, which was 158 pmol/min/mg protein; results are mean values ± SD of triplicate determinations of a typical experiment which was repeated twice.

DAMGO (Tyr-D-Ala^2-*N*-Me-Phe-Gly-ol, 10 nM) and unlabeled DPDPE (D-Pen^{2,5}-enkephalin, 10 nM) to eliminate binding to μ and δ sites, respectively, in a total volume of 1 mL. For all receptor binding assays, [^{3}H]U-69,593 (1–3 nM) was added to initiate the reaction. Membrane suspensions were incubated at 25° for 60 min, and then rapidly filtered through Whatman glass fiber filters which had been soaked in polyethyleneimine (0.1% in Tris buffer) for at least 2 hr. Non-specific binding was defined with 1 μM U-50,488H. The filters were immersed in 5 mL of Ecolite scintillation fluid overnight and then radioactivity was determined by liquid scintillation spectrophotometry (50% efficiency). Protein was determined by the method of Lowry *et al.* [29]. For the comparison of κ agonists, K_i values were calculated from a computer analysis using LIGAND. For experiments that quantified receptor binding following β-CNA pretreatments, data were analyzed as dpm [^{3}H]U-69,593 bound/mg protein for untreated controls, naltrexone-treated membranes and β-CNA-treated membranes. Data were expressed as the percentage loss of κ_1 receptors relative to naltrexone-treated membranes.

Data analysis. For experiments that examined the effect of β-CNA pretreatments on U-50,488H-inhibited adenylyl cyclase, Student's unpaired *t*-test was used to determine the level of significance.

RESULTS

Pharmacological characteristics of κ-inhibited adenylyl cyclase in guinea pig cerebellum membranes. We previously showed that dynorphin analogs inhibit adenylyl cyclase activity in guinea pig cerebellum membranes. Moreover, this inhibition by dynorphin is blocked by naloxone as well as the selective κ antagonist nor-BNI [17]. The present study confirms the κ-selective nature of this second messenger response by showing 30–40% inhibition of adenylyl cyclase by the selective agonist U-50,488H (Fig. 1). Addition of nor-BNI (0.1 μM) effectively blocked U-50,488H-inhibited adenylyl cyclase, resulting in a nearly 100-fold shift in the agonist concentration–response curve. The calculated K_e value for nor-BNI was 1.5 nM, a value in close agreement with previous calculations for the antagonism of dynorphin-inhibited adenylyl cyclase in these membranes.

To explore the relationship between κ-inhibited adenylyl cyclase and κ receptor binding, several κ agonists were compared with regard to their binding affinities at the U-69,593-sensitive (κ_1) site and their potencies in inhibiting adenylyl cyclase activity (Table 1). Each agonist was potent in displacing [^{3}H]U-69,593 binding with K_i values of less than 1 nM. Hill slope values were not significantly less than unity, and computer analysis revealed that all agonists displaced [^{3}H]U-69,593 binding at a single site. Assay of adenylyl cyclase revealed that all agonists inhibited adenylyl cyclase to the same degree (approximately 30–40% of basal activity). In general, the rank order of agonist potencies were parallel in displacing [^{3}H]U-69,593 binding and inhibiting adenylyl cyclase. For example, D-pro$_{10}$ dynorphin$_{1-11}$ was the most potent agonist in both systems, while dynorphin$_{1-13}$ and U-50,488H were relatively potent and dynorphin B was relatively less potent in both assays. However, two agonists did not follow this general trend. Dynorphin$_{1-8}$ was six times less potent than dynorphin B in inhibiting adenylyl cyclase, although both peptides were equipotent in displacing [^{3}H]U-69,593 binding. In addition, α-neo endorphin was eight times weaker than dynorphin$_{1-13}$ in inhibiting adenylyl cyclase, although both peptides had approximately equal K_i values in displacing [^{3}H]U-69,593 binding. To confirm that the low pH pretreatment of membranes used in adenylyl cyclase assays did not change agonist displacement of binding, we directly compared the ability of α-neo endorphin to displace [^{3}H]U-69,593 binding in both control and pH 4.5 pretreated membranes. Results (data not shown) indicated that prior treatment of membranes at pH 4.5 had no effect on the displacement by α-neo endorphin of [^{3}H]U-69,593 binding. Therefore, the anomalous behavior of α-neo endorphin could not be explained as an artifact of this treatment.

All of these agonists were several hundred-fold weaker in inhibiting adenylyl cyclase than in displacing [^{3}H]U-69,593 binding, with IC_{50} values ranging from 30 nM to 1 μM (Table 1). However, the adenylyl cyclase assay was conducted in the presence of Na^+ and GTP, where agonist binding was shifted to low affinity and no significant [^{3}H]U-69,593 binding was observed. Because no selective labeled κ_1 antagonist is available at this time, we used the non-selective opioid antagonist [^{3}H]-diprenorphine (with DAMGO and DPDPE to block μ and δ sites) to assay receptor binding under the same conditions as adenylyl cyclase assays. However, results (data not shown) revealed that displacement of [^{3}H]diprenorphine binding by U-50488H and α-neo endorphin was complex, with Hill slopes of 0.66 and 0.44, respectively, in the absence of GTP and Na^+. These multiple sites were also observed in the presence of GTP and Na^+. The likelihood that [^{3}H]diprenorphine was binding to

Table 1. κ_1 Receptor binding versus κ_1-inhibited adenylyl cyclase: Agonist comparisons

Agonist	[^{3}H]U-69,593 binding		Adenylyl cyclase inhibition	
	Hill slope	K_i (nM)	IC_{50} (nM)	I_{max} (%)
Dynorphin$_{1-13}$	1.05 ± 0.22	0.11 ± 0.04	40 ± 6	41 ± 1
Dynorphin$_{1-8}$	1.01 ± 0.10	0.46 ± 0.09	970 ± 240	37 ± 3
D-pro$_{10}$ dynorphin$_{1-11}$	0.98 ± 0.02	0.043 ± 0.01	30 ± 7	41 ± 3
Dynorphin B	0.95 ± 0.22	0.45 ± 0.34	130 ± 3	37 ± 5
α-Neo endorphin	1.08 ± 0.09	0.082 ± 0.09	340 ± 83	36 ± 9
U-50,488H	0.83 ± 0.05	0.22 ± 0.06	50 ± 9	38 ± 7

[^{3}H]U-69,593 binding (with 1.7 nM [^{3}H]U-69,593) and U-50,488H-inhibited adenylyl cyclase (using 1 μM U-50,488H as the maximally inhibitory concentration) were determined in guinea pig cerebellar membranes as described in Materials and Methods. Each value is the mean value ± SEM of at least three experiments, each repeated in triplicate.

Table 2. κ_1 Receptor binding versus κ_1-inhibited adenylyl cyclase: Comparison across guinea pig brain regions

Region	[^{3}H]U-69,593 binding		U-50,488-inhibited adenylyl cyclase	
	fmol/mg protein	% of max. region	I_{max} (%)	% of max. region
Striatum	32.1 ± 2.6	84 ± 7	29.1 ± 3.4	91 ± 11
Cerebellum	31.0 ± 2.8	81 ± 7	32.0 ± 0.8	100 ± 2
Thalamus	8.5 ± 1.1	22 ± 3	5.0 ± 1.7	16 ± 5
Hypothalamus	4.8 ± 1.6	13 ± 4	11.1 ± 0.5	35 ± 2
Frontal cortex	35.0 ± 3.6	91 ± 9	14.0 ± 0.7	44 ± 2
Sensomotor cortex	38.4 ± 6.0	100 ± 16	11.2 ± 3.7	35 ± 12
Superior colliculus	12.0 ± 0.6	31 ± 2	6.0 ± 2.0	19 ± 6
Brainstem	14.6 ± 2.5	38 ± 6	10.1 ± 5.1	32 ± 16
Hippocampus	5.5 ± 0.5	14 ± 1	21.7 ± 2.8	68 ± 9
Amygdala	29.8 ± 5.9	77 ± 15	24.2 ± 1.9	76 ± 6
Inferior colliculus	33.7 ± 2.8	88 ± 7	21.3 ± 3.9	67 ± 12

[^{3}H]U-69,593 binding (with 1.2 nM [^{3}H]U-69,593) and U-50,488H-inhibited adenylyl cyclase (using 1 μM U-50,488H as the maximally inhibitory concentration) were determined in membranes from the indicated guinea pig brain regions as described in Materials and Methods. Binding data are expressed as femtomoles [^{3}H]U-69,593 bound per milligram protein, while adenylyl cyclase data are expressed as I_{max} (the maximal inhibition of basal activity by U-50,488H). In each case data are also expressed as the percentage of the region of highest binding (sensomotor cortex) or inhibited adenylyl cyclase (cerebellum). Each value is the mean ± SEM of at least three experiments, each performed in triplicate.

sites other than κ_1 sites precluded further comparison with [^{3}H]U-69,593 binding data.

Kappa receptor binding and κ-inhibited adenylyl cyclase were compared further by their regional distributions in guinea pig brain. The density of κ_1 sites (by [^{3}H]U-69,593 binding) was compared to the maximal inhibition of adenylyl cyclase by U-50,488H (10 μM) across eleven regions of the guinea pig brain (Table 2). Binding of κ_1 receptors was highest in the cortex, and low in both thalamus and hypothalamus (less than 15% of the density of κ_1 sites in sensomotor cortex), a result consistent with previous reports [7, 9]. Inhibition of adenylyl cyclase by U-50,488H was highest in the cerebellum, absent in the thalamus and superior colliculus, and moderate in the other regions.

To correlate the density of κ_1 sites to that of U-50,488H-inhibited adenylyl cyclase for each region, both values were normalized with respect to the region which exhibited the highest value (Table 2). These results revealed that receptor density paralleled inhibitory activity across eight of the eleven brain regions. In other words, regions that had a high density of [^{3}H]U-69,593-sensitive sites (e.g. striatum) also supported a high degree of U-50,488H-inhibited adenylyl cyclase, whereas regions which had a low density of binding sites supported little inhibitory activity. Exceptions to this trend were frontal and sensomotor cortices (which displayed high receptor densities but supported relatively sparse inhibition of adenylyl cyclase), and hippocampus (which had a relatively small receptor density but supported significant inhibitory activity). Owing to these exceptional regions, the binding of [^{3}H]U-69,593 across eleven brain regions correlated poorly ($r = 0.48$) with that of U-50,488H-inhibited adenylyl cyclase (Fig. 2, top). However, if κ_1 binding and U50,488H-inhibited adenylyl cyclase were compared with the exclusion of hippocampus and cortical regions, the correlation became significant ($r = 0.89$, Fig. 2, bottom).

Irreversible blockade of κ receptors with β-CNA.

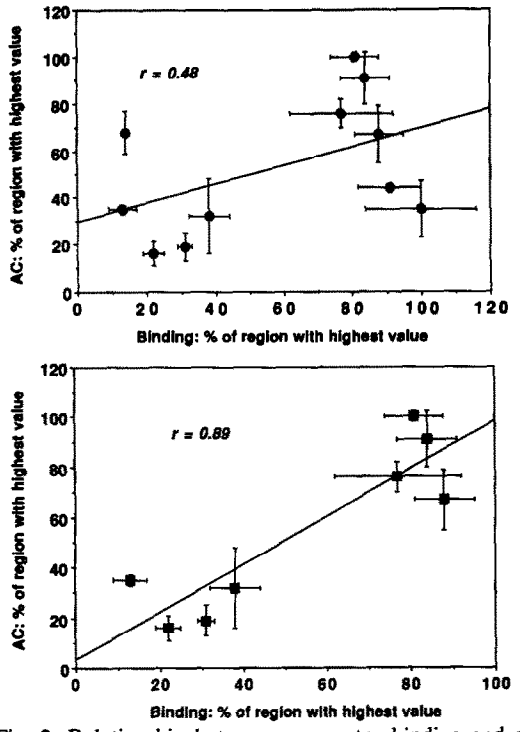

Fig. 2. Relationship between κ receptor binding and κ-inhibited adenylyl cyclase (AC) across guinea pig brain regions. Data are expressed as a percentage of the region having the highest value. Top: correlation with eleven brain regions. Bottom: correlation without cortical regions and hippocampus. Results are mean values ± SEM of three separate experiments, each of which was conducted in triplicate.

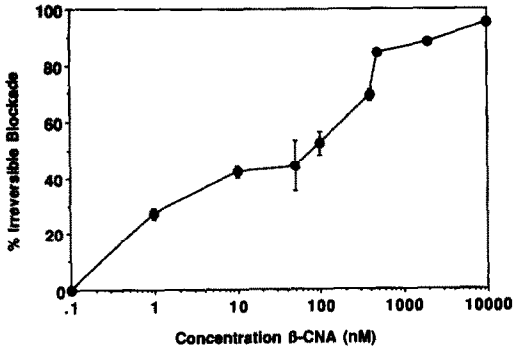

Fig. 3. Irreversible blockade of [^{3}H]U-69,593 sites following preincubation of cerebellar membranes with β-CNA. Membranes were preincubated with the indicated concentrations of β-CNA for 15 min at 25°, then washed, and assayed for [^{3}H]U-69,593 binding as described in Materials and Methods. Data are expressed as the percentage of [^{3}H]U-69,593 binding lost following preincubation of membranes with various concentrations of β-CNA. The percentage of irreversible blockade by each concentration of β-CNA was calculated relative to the amount of [^{3}H]U-69,593 binding in membranes preincubated with equal concentrations of naltrexone. Data are mean values ± SEM of separate experiments, each of which was conducted in triplicate.

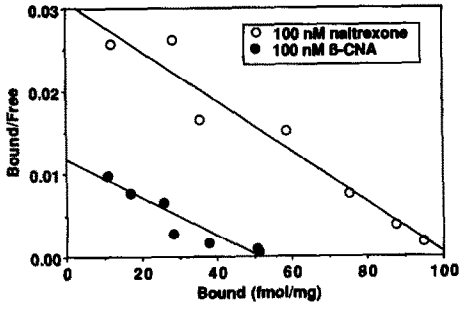

Fig. 4. Scatchard plot of the effect of β-CNA on [^{3}H]U-69,593 binding in guinea pig cerebellar membranes. Membranes were preincubated with naltrexone (100 nM) or β-CNA (100 nM), washed, and incubated with various concentrations of [^{3}H]U-69,593, as described in Materials and Methods. Data are mean values ± SD of triplicate determinations in a typical experiment which was repeated twice.

The use of the general opioid alkylating agent β-CNA enabled a further comparison of κ receptor binding and κ-inhibited adenylyl cyclase. In particular, β-CNA offered an opportunity to use the method of Furchgott [20] to examine the role of spare receptors (or receptor reserve) in mediating κ responses in guinea pig cerebellum. First, it was important to show that our washing procedures eliminated the reversible binding component of β-CNA. To control for this possibility, naltrexone was chosen as a reversible control drug since β-CNA is an analog of naltrexone. Binding assays (results not shown) demonstrated that pretreatment of cerebellar membranes with up to 2 μM naltrexone had no significant effect on the density of κ_1 sites in comparison to untreated control membranes. In general, a slight increase (10–20%) in [^{3}H]U-69,593 binding was seen following pretreatment of membranes with naltrexone, similar to the effect of naloxone on μ and δ binding in rat brain membranes [30]. Preincubation of cerebellar membranes with β-CNA resulted in a concentration-dependent loss of [^{3}H]U-69,593 binding sites (Fig. 3). This loss in binding ranged from 20% with 1 nM β-CNA to 95% with 10 μM β-CNA. Further, Scatchard analysis (Fig. 4) showed that pretreatment of cerebellar membranes with 100 nM β-CNA decreased the B_{max} of [^{3}H]U-69,593 (51 pmol/mg protein for β-CNA-pretreated membranes vs 101 pmol/mg protein in naltrexone-pretreated membranes) without appreciably affecting the calculated K_D value (0.9 nM for β-CNA-pretreated membranes vs 1.2 nM for naltrexone-pretreated membranes).

The next experiments examined the effect of β-CNA on κ_1-inhibited adenylyl cyclase. Pretreatment of membranes with up to 10 μM naltrexone, followed by low pH treatment and subsequent washings, had no effect on the potency or efficacy of U-50488H in inhibiting adenylyl cyclase when compared to untreated tissue (results not shown). Pretreatment

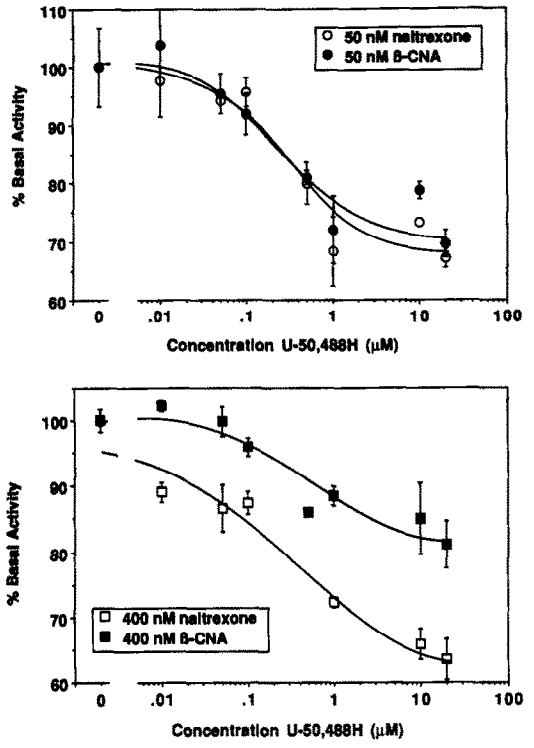

Fig. 5. Effect of β-CNA on U-50,488H-inhibited adenylyl cyclase in guinea pig cerebellar membranes. Membranes were preincubated with either 50 nM (top panel) or 400 nM (bottom panel) β-CNA or naltrexone prior to assay of adenylyl cyclase with U-50,488H. Data are mean values ± SD of triplicate determinations of a typical experiment which was repeated three times.

of membranes with 50 nM β-CNA, a concentration which inactivated nearly 50% of the [^{3}H]U-69,593 binding sites, had no effect on the potency or efficacy of U-50488H in inhibiting adenylyl cyclase when compared to membranes pretreated with 50 nM naltrexone (Fig. 5, top). When β-CNA was increased to 400 nM (Fig. 5, bottom), U-50,488H-inhibited adenylyl cyclase was decreased by approximately 50% (36% inhibition in naltrexone-pretreated membranes vs 19% inhibition in β-CNA-pretreated membranes). However, the IC_{50} value for U-50,488H was affected to only a small extent (0.14 μM in naltrexone-pretreated membranes vs 0.43 μM in β-CNA pretreated membranes). A binding experiment from this membrane preparation revealed that 74% of [^{3}H]U-69,593-sensitive sites were inactivated following the β-CNA pretreatment.

Table 3 compares the effects of four concentrations of β-CNA on κ_1 receptor binding and κ_1-inhibited adenylyl cyclase. Pretreatments with both 50 nM and 100 nM β-CNA had no effect on κ_1-inhibited adenylyl cyclase despite a loss of approximately 50% of κ_1 binding sites. Increasing the β-CNA concentration to 400 nM produced a 40% loss in agonist efficacy with a slight (2-fold) increase in the IC_{50} value. Interestingly, increasing β-CNA to 10 μM produced no further loss in κ-inhibited adenylyl cyclase despite total (95%) loss in κ_1 binding sites.

DISCUSSION

The κ_1 receptors have been defined on the basis of their high selectivity toward the arylacetamides. U-50,488H and U-69,593 potently displace [^{3}H]U-69,593 from its binding site, referred to as the κ_1 site [7], but are relatively weak in displacing either [^{3}H]bremazocine or [^{3}H]EKC (ethylketocylazocine) from the U-69,593-insensitive site, now referred to as the κ_2 site [6,7,9,31]. At least three observations suggest that the inhibition of adenylyl cyclase by the dynorphin peptides in guinea pig cerebellar membranes was mediated through κ_1 receptors. First, κ-inhibited adenylyl cyclase was relatively high in guinea pig cerebellum, an area in which receptor autoradiography indicates that κ_1 sites predominate over κ_2 sites [9]. Second, U-50,488H and U-69,593 both were potent in inhibiting adenylyl cyclase and were equal in efficacy to the inhibitory activity elicited by the dynorphin analogs. Finally, the selective κ antagonist nor-BNI displayed over 1000-fold greater affinity for the [^{3}H]U-69,593-sensitive site in comparison to its affinity for the [^{3}H]EKC site [6]. The calculated K_i value (0.7 nM) for the displacement of [^{3}H]U-69,593 by nor-BNI is in close agreement with the K_e value (1.5 nM) calculated for the blockade by nor-BNI of U-50,488H-inhibited adenylyl cyclase activity in the present study. Guinea pig cerebellum is an ideal tissue to study κ-inhibited adenylyl cyclase: although μ and δ receptors comprise 15% of total opioid receptors in this tissue, selective μ and δ agonists in concentrations of up to 10 μM have no effect on adenylyl cyclase [17]. Thus, although unlabeled μ and δ ligands were included in binding assays so that such sites would not interfere with [^{3}H]U-69,593 binding, the inclusion of blocking agents was unnecessary in adenylyl cyclase assays.

In general, the rank order of potencies of agonists in inhibiting adenylyl cyclase in guinea pig cerebellar membranes paralleled that of agonist binding affinities at the κ_1 site. Dynorphin$_{1-8}$ and α-neo endorphin were exceptions to this trend; the latter displayed high affinity for the κ_1 binding site, yet was relatively weak in inhibiting adenylyl cyclase. Dynorphin$_{1-8}$ and dynorphin B were equipotent in displacing [^{3}H]U-69,593 binding, yet dynorphin$_{1-8}$ was six times weaker in inhibiting adenylyl cyclase. Several possibilities could explain the anomalous behavior of these compounds. First, it is possible that these peptides were degraded by proteases more readily than the other agonists. However, this is unlikely since protease inhibitors having a broad spectrum of activity were included in the incubation. It is also possible that α-neo endorphin and dynorphin$_{1-8}$ discriminate between subtypes of κ_1 receptors. Pasternak and co-workers [14] have identified two subtypes of the κ_1 site, referred to as κ_{1a} and κ_{1b}, based upon the biphasic displacement by α-neo endorphin and dynorphin B of [^{3}H]U-69,593 binding in guinea pig cerebellar membranes. Our results, however, indicate that the binding of these compounds are best fit to a one site model. It is still conceivable that two highly conserved binding

Table 3. Effects of β-CNA on κ_1 receptor binding and κ_1-inhibited adenylyl cyclase

β-CNA (nM)	% [³H]U-69,593 Bound	U-50,488H-inhibited adenylyl cyclase			
		IC_{50} ratio	I_{max} (%) Naltrexone	I_{max} (%) β-CNA	% Loss of I_{max} by β-CNA
50	56 ± 9	0.70 ± 0.21	28 ± 7	28 ± 2	0
100	48 ± 4	1.88 ± 0.40	31 ± 2	28 ± 1	10
400	31 ± 2	2.35 ± 0.57	32 ± 2	19 ± 2*	41
10,000	5 ± 1	ND†	37 ± 3	19 ± 2*	49

[³H]U-69,593 binding and U-50,488H-inhibited adenylyl cyclase were determined in guinea pig cerebellar membranes that had been pretreated for 15 min at 25° with the indicated concentrations of β-CNA or equal concentrations of naltrexone as described in Materials and Methods. Binding data are expressed as percent of [³H]U-69,593 bound in β-CNA-pretreated membranes compared to membranes pretreated with the same concentrations of naltrexone. For adenylyl cyclase data, the IC_{50} ratio is the IC_{50} value of U-50,488H in inhibiting basal adenylyl cyclase activity in β-CNA-pretreated membranes divided by its IC_{50} value in naltrexone-pretreated membranes. Adenylyl cyclase data are also expressed as I_{max} (the maximum inhibitory effect of 10 μM U-50,488H on basal activity) in both naltrexone- and β-CNA-pretreated membranes. Each value is the mean ± SEM of at least three separate experiments, each performed in triplicate.

* $p < 0.05$ vs naltrexone.

† Not determined.

sites are indeed present, only one of which is coupled to the inhibition of adenylyl cyclase. If α-neo endorphin and dynorphin B differed in their affinities for the two sites only to a small degree, then significantly weaker inhibitory activity might be observed for these compounds despite their apparent homogeneity in binding to κ_1 sites.

A third possibility is that α-neo endorphin and dynorphin B differed from the remaining compounds in terms of their sensitivities toward sodium and GTP. Kosterlitz *et al.* [32] showed that a group of κ agonists, including both dynorphin peptides and benzomorphans, exhibited large differences in their sensitivities to the inhibition by Na^+ of κ receptor binding in the guinea pig cerebellum. Recent research with novel δ agonists* has revealed that agonists whose binding isotherms were not greatly affected by Na^+ and GTP were significantly more potent in inhibiting adenylyl cyclase than other agonists. Unfortunately, since in the present study [³H]U-69,593 binding had to be measured in the absence of Na^+ and GTP, the influence of ions and nucleotides on the receptor binding of agonists used in this study cannot be assessed with [³H]agonist binding. The attempt to use [³H]diprenorphine to address this issue was not successful because of the non-selective nature of this ligand. Although neither Na^+ nor GTP had any effect on the binding of [³H]diprenorphine, thus confirming the antagonist nature of this ligand [33], the displacement of [³H]diprenorphine binding by U-50,488H and α-neo endorphin was complex, even in the presence of DAMGO and DPDPE to block μ and δ sites. Therefore, unlike [³H]U-69,593 binding where these agonists bind to a single site, these agonists probably displaced [³H]diprenorphine at multiple κ sites. We concluded that with the lack of selective κ_2 and κ_3 ligands to block non-κ_1 sites, the nonselective opioid antagonist diprenorphine could not be used to clarify whether GTP and Na^+ altered the affinity of α-neo endorphin for the κ_1 site.

The present study confirms earlier reports [7, 9] that high densities of κ_1 sites are present in cortex, striatum, and cerebellum, whereas low densities are present in thalamus and hippocampus. Brain regions which supported a high degree of U-50,488H (κ_1)-inhibited adenylyl cyclase included cerebellum, striatum, hippocampus, amygdala, and inferior colliculus. Thalamus and superior colliculus supported little or no inhibition, and the remaining regions supported moderate inhibition. The correlation between κ_1 receptor binding and κ_1-inhibited adenylyl cyclase was significant for eight of the eleven regions. Certain regions, however, exhibited either a preponderance of κ_1 binding relative to κ_1-inhibited adenylyl cyclase (cortex), or only a small amount of κ_1 binding relative to κ_1-inhibited adenylyl cyclase (hippocampus). One explanation for this apparent lack of correlation may be interregional differences in receptor reserve. Thus, in cortex, it is possible that only a small proportion of κ_1 binding sites must be occupied in order to elicit maximal inhibition of adenylyl cyclase. Conversely, one could reason that in the hippocampus the occupation of κ_1 binding sites is more tightly coupled to this effector system. An alternative possibility is that an excess of κ_1 binding sites relative to negatively coupled adenylyl cyclase might imply the presence of other second messenger systems. One report demonstrated that high concentrations of κ agonists stimulated phosphoinositide turnover in rat brain slices [34], while another report demonstrated that U-50,488H inhibits GTP-stimulated phospholipase C activity in synaptic membranes from guinea pig cerebellum [35]. Moreover, Gross *et al.* [36] have shown dynorphin-mediated inhibition of Ca^{2+} conductances

* Childers S and Chang K, manuscript in preparation.

in the presence of exogenously supplied cAMP, implying that inhibition of adenylyl cyclase might not be required for κ receptor coupling to ion channels.

To explore whether receptor reserve plays a significant role in the coupling of κ receptors to adenylyl cyclase, we utilized the general opioid receptor alkylating agent β-CNA. It is thought that β-CNA initially binds to opioid receptors in a reversible manner, and then forms a covalent bond via an aziridinium ion, thus irreversibly inactivating the receptor [18]. The action of β-CNA is relatively specific for opioid receptors [22], but does not discriminate among opioid receptor subtypes [37]. In rat brain membranes, β-CNA is more potent in blocking μ sites as compared to δ and κ sites,* but micromolar concentrations of β-CNA are sufficient to block >90% of κ binding sites. The ability of β-CNA to inactivate receptors was confirmed in Fig. 3, which showed that membrane pretreatment with β-CNA (100 nM) reduced maximal κ_1 receptor density without having an effect on the K_D for [^{3}H]U-69,593 binding. The reduction by β-CNA of opioid receptor density concomitant with the absence of an effect on agonist affinities is consistent with an earlier report [22]. Figure 4 reveals that the inhibition of [^{3}H]U-69,593 binding by β-CNA was shallow, thereby suggesting that the loss of κ_1 sites may be biphasic in nature. However, since the inactivation of κ_1 sites by β-CNA is non-competitive, further analysis of this curve assuming equilibrium conditions, i.e. determination of IC_{50} values and Hill coefficients, was not practical. Moreover, the finding in Table 1 that κ agonists displaced [^{3}H]U-69,593 binding at a single site suggests that this shallow inhibition curve is probably not the result of multiple binding sites.

Numerous studies have demonstrated a high degree of receptor reserve for receptors coupled to physiological responses in the peripheral nervous system [19–21, 23, 38]. For example, Chavkin and Goldstein [25] showed that only 10% of κ receptors in the guinea pig ileum preparation had to be occupied in order to elicit a maximal inhibition of electrically induced contractions. Further, it was shown that a larger receptor reserve could account for the 10-fold greater potency of dynorphin in the guinea pig ileum bioassay relative to the potency of dynorphin in the mouse vas deferens bioassay [25]. Clark and Medzhiradsky [39] demonstrated significant receptor reserve for the stimulation of brain GTPase coupled to δ opioid receptors. To our knowledge, however, the present study is the first to examine the relationship between receptor occupancy and an effector response within the brain.

Stephenson [40] was the first to describe the phenomenon of receptor reserve or "spare receptors": that is, in certain receptor-mediated events only a fraction of available receptors must be occupied by an agonist in order to elicit a maximal response. We prefer the term receptor reserve since the entire receptor population under study is coupled to the effector response; "spare receptors" might imply the presence of uncoupled receptors. Furchgott analysis [20] enables the quantitation of agonist affinities for receptor-mediated responses, as long as rightward shifts in the concentration–response curve can be measured following inactivation of a fraction of the receptor population. In the present study, no shift in the IC_{50} value for κ_1-inhibited adenylyl cyclase could be detected without an accompanying loss in the maximal response. An estimate, however, of κ receptor reserve negatively coupled to adenylyl cyclase in the guinea pig cerebellum can be obtained by simple substitution into the mass-action equation. For example, a 3-fold rightward shift in the IC_{50} value for U-50,488H-inhibited adenylyl cyclase would be expected if only one-half occupancy of κ_1 receptors was required for maximal inhibition. Pretreatment of membranes with 400 nM β-CNA resulted in only a 2-fold shift in IC_{50} values, with a significant loss of the maximal inhibitory response. Thus, receptor reserve in this system is less than 50% (the calculated IC_{50} ratio of 2.35 gives a value of 40% receptor reserve). It must be emphasized that this calculation represents an upper limit to the degree of receptor reserve since we could not identify a concentration of β-CNA which produced a significant shift in the IC_{50} ratio without reducing the maximal level of inhibition. Indeed, since no shift in agonist IC_{50} values was observed without loss in I_{max}, it is likely that no significant receptor reserve exists for κ_1-inhibited adenylyl cyclase in guinea pig cerebellum. This result is surprising compared to earlier reports [24] which showed that less than 5% of receptors negatively coupled to adenylyl cyclase had to be occupied for the maximal inhibitory response. It is important to note that the relationship between receptor binding and response is often complex. For example, Blume and colleagues [22] showed that blockade of 95% of delta opioid binding sites in NG108-15 cells has no effect on either IC_{50} or I_{max} values of delta receptor-inhibited adenylyl cyclase, and data from our laboratory [41] have demonstrated that no correlation exists between high-affinity opioid receptor binding sites and opioid-inhibited adenylyl cyclase in rat brain membranes. Together with the observation that endogenous κ opioids possess remarkably high affinities for the κ_1 site, the relatively small degree of κ_1 receptor reserve may have interesting implications for κ receptor function in the guinea pig cerebellum.

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