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Dynorphin-Selective Inhibition of Adenylyl Cyclase in Guinea Pig Cerebellum Membranes

CHRISTOPHER S. KONKOY and STEVEN R. CHILDERS

Department of Pharmacology, University of Florida College of Medicine, Gainesville, Florida 32610

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SUMMARY

Guinea pig cerebellum, which contains κ opioid receptors uncontaminated by other opioid receptor types, was chosen to examine whether κ receptors are coupled to adenylyl cyclase. Membranes were prepared from guinea pig cerebellum and pretreated at pH 4.5 to increase inhibitory activity, and adenylyl cyclase was assayed in the presence of dynorphin analogs as prototypical κ agonists. Results showed that several dynorphin analogs inhibited adenylyl cyclase by 30–50%, whereas μ- and δ-preferring agonists had no effect. Dynorphin A and the κ-selective compounds D-Pro^{10}-dynorphin(1–11) and U-50,488H were the most potent agonists, with IC_{50} values of 0.03–0.05 μM, whereas other dynorphin gene products like dynorphin B and α-neo-endorphin were approximately 10-fold less potent. Like other G_i-coupled responses, dynorphin-inhibited adenylyl cyclase required GTP and sodium. Naloxone was a competitive antagonist for dynorphin-inhibited adenylyl cyclase, with 1 μM naloxone shifting the IC_{50} value of dynorphin A by 20-fold. The κ-selective antagonist nor-binaltorphimine was even more potent, with 0.1 μM nor-binaltorphimine shifting the dynorphin IC_{50} value by 50-fold. These results suggest that dynorphin A and its analogs inhibit adenylyl cyclase by binding to a guanine nucleotide-binding protein-coupled opioid receptor whose pharmacological specificity matches those of κ receptors.

The κ opioid receptor was first described by Martin *et al.* (1) as a receptor type that mediated some of the effects of benzomorphans like ethylketocyclazocine. The discovery of dynorphin as a major opioid peptide gene product (2) provided the κ receptor with an endogenous ligand, because dynorphin and its analogs bind with high affinity to κ receptors (3). κ receptors, which may exist in the form of several subtypes (4–7), have been characterized in both brain and smooth muscle of several species (8–10). However, more recent studies have focused on the guinea pig, which contains higher concentrations of κ receptors than rat (11). Of particular interest is the guinea pig cerebellum, a brain area that is almost devoid of μ and δ opioid receptors but that contains a relatively high level of κ receptors (12). Thus, the guinea pig cerebellum can be used as a relatively pure source of κ receptors.

Although binding studies have characterized the pharmacological properties of κ receptors, the coupling of κ receptors to second messenger systems is less clearly understood. The second messenger system most commonly identified with other opioid receptors is inhibition of adenylyl cyclase, characterized for δ receptors in NG108-15 cells (13–15) and identified for μ receptors in SK-N-SH cells (16). Opioid receptor-inhibited adenylyl cyclase is mediated by G_i proteins, because it is blocked by pertussis toxin and requires GTP (17, 18). Moreover, agonist binding to both μ and δ receptors is attenuated by guanine nucleotides (19–21), thus confirming the coupling of receptors to G proteins. However, the question of whether κ receptors are coupled to G proteins has been controversial. Whereas some studies have failed to demonstrate GTP-sensitive ligand binding to κ receptors (22), other studies demonstrated weak effects of guanine nucleotides on κ binding (23, 24). The lack of an effective cell model containing κ receptors (analogous to the δ receptor-containing NG108-15 cells), coupled with the relatively weak response of brain adenylyl cyclase to opioid agonists, meant that the existence of κ receptor-coupled adenylyl cyclase could not be confirmed. A recent study by Attali *et al.* (25) demonstrated that κ agonists inhibited adenylyl cyclase in rat spinal cord. However, it is not known whether this property was shared by dynorphin A and other endogenous κ agonists.

Although opioid-inhibited adenylyl cyclase in brain membranes has been characterized by several groups (26–28), the heterogeneity of brain membranes provides only a small (10–20%) signal of inhibition by opioid agonists. However, studies

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ABBREVIATIONS: G_i, inhibitory guanine nucleotide regulatory protein; nor-BNI, nor-binaltorphimine; D-Ala-enk, D-Ala^2-Met^5-enkephalinamide; DAGO, Tyr-D-Ala^2-*N*-Me-Phe-Gly-ol; DSLET, D-Ser^2-Leu-enkephalin-Thr; DPDPE, D-Pen^{2,5}-enkephalin; U-50,488H, (*trans*-(*dl*)-3,4-dichloro-*N*-methyl-*N*-[2-(l-pyrroldinyl)cyclohexyl]-benzeneacetamide)methane sulfonate; G protein, guanine nucleotide-binding protein; HPLC, high pressure liquid chromatography; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GTP-γS, guanosine-5′-*O*-(3-thio)triphosphate; GDP-βS, guanosine-5′-*O*-(2-thio)-diphosphate.

in our laboratory showed that opioid-inhibited adenylyl cyclase was increased by pretreatment of membranes with a pH 4.5 buffer (29). The low pH pretreatment did not affect receptor binding (30) or basal adenylyl cyclase but eliminated G_s-stimulated activity while increasing maximal opioid inhibition of adenylyl cyclase to 30–40% of basal activity. This low pH pretreatment allowed quantitation of opioid-inhibited adenylyl cyclase in several rat brain regions (31).

If low pH pretreatment is effective in determining properties of opioid-inhibited adenylyl cyclase in rat brain membranes, it might be possible to use this technique to determine whether κ receptors are also coupled to this second messenger system. In this report, we show that dynorphin analogs produce potent and specific inhibition of adenylyl cyclase in membranes from guinea pig cerebellum. Moreover, the pharmacological specificity of this response confirms the conclusion that κ opioid receptors are coupled to inhibition of adenylyl cyclase in this tissue.

Experimental Procedures

Materials. [^{3}H]ATP (33 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Creatine phosphate, creatine phosphokinase, forskolin, and unlabeled ATP were obtained from Sigma (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-BNI was purchased from Research Biochemicals (Natick, MA).

Adenylyl cyclase assay. Female Hartley guinea pigs (Charles River; 450–500 g) were injected intraperitoneally with sodium pentobarbital (130 mg/kg) and decapitated. Cerebella (along with other regions in certain experiments) were quickly dissected on ice and homogenized with a Polytron (setting 3; 15 sec) in Tris-Mg^{2+} buffer (50 mM Tris·HCl, 3 mM $MgCl_2$, pH 7.4). 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 (29). Membranes were resuspended in pH 4.5 buffer (50 mM sodium acetate, 3 mM $MgCl_2$, 1 mM dithiothreitol, pH 4.5) (1 ml of buffer/100 mg original wet weight of brain tissue) and incubated on ice for 10 min. The low pH treatment was terminated by addition of 5 ml of Tris-Mg^{2+} buffer (pH 7.4), and membranes were isolated by centrifugation at 48,000 × *g* for 10 min. Membranes were resuspended in Tris-Mg^{2+} buffer containing 10 mM theophylline and 20 mM NaCl, frozen, and stored in liquid nitrogen.

For assay of adenylyl cyclase, membranes were thawed in a 30° water bath and 20–100 μg of membrane protein were added to tubes containing 10 mM theophylline, 20 mM NaCl, 50 μM GTP, 20 mM creatine phosphate, 10 units of creatine phosphokinase, 30 μM cyclic AMP, 100 μM ATP, and 1 μCi of [^{3}H]ATP, in Tris-Mg^{2+} buffer, in a total volume of 100 μl. Enzyme blanks consisted of identical tubes in which the 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 by immersion in boiling water for 2 min and then tubes were cooled on ice for 5 min. [^{3}H]Adenosine formed from [^{3}H]ATP 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 150 μl of 1 M $Ba(OH)_2$, followed by 150 μl of 1 M $ZnSO_4$, with 5 min between each addition. Tubes were centrifuged at 1000 × *g* for 15 min and supernatants (approximately 320 μl each) were transferred into tubes for automatic sample injection onto the HPLC column.

[^{3}H]Cyclic AMP formed by adenylyl cyclase was assayed by an HPLC method (32) modified from the method of Schultz and Mailman (33). A C-18 reverse phase column (3-μm Microsorb; Rainin Instruments Co., Woburn MA) was connected to a gradient HPLC apparatus together with a C-18 guard column. The mobile phase consisted of 0.8 M sodium acetate, pH 5.0 (prepared by adding 55 ml of glacial acetic acid/liter of acetate and filtering through 0.45-μm nylon filters before use), and 10% methanol at a flow rate of 1 ml/min. Supernatant samples (250-μl aliquots) were injected by an automatic sample injector, 6 min apart. The cyclic AMP peak eluted approximately 4 min after injection and was collected directly into scintillation vials in a fraction collector controlled by an automatic peak detector (Isco, Lincoln, NE), which detected the absorbance of the unlabeled cyclic AMP at 254 nm. Radioactivity was determined by liquid scintillation counting (40% efficiency) after addition of 5 ml of Liquiscint scintillation fluid (National Diagnostics, Somerville, NJ).

All assay samples were conducted in triplicate, with standard deviations between replicates of less than 5%. Activity was expressed as pmol of cyclic AMP formed/mg of protein/min; protein values were determined by the method of Bradford (34). Unless otherwise indicated, results are mean values of triplicate determinations ± standard deviations in representative experiments, which were repeated at least three times. IC_{50} values were defined as the concentration of agonist that produced 50% of maximum inhibition of adenylyl cyclase activity (which ranged from 30 to 40% of basal activity); maximum inhibition levels were determined in concentration-response curves where a 5-fold increase in agonist concentration had no further significant effect on activity.

Results

General characteristics of dynorphin-inhibited adenylyl cyclase. Adenylyl cyclase activity was assayed in membranes that were prepared from guinea pig cerebellum (and pretreated at pH 4.5 before assay at pH 7.4) in the presence of various concentrations of dynorphin A. Results (Fig. 1) revealed that dynorphin A produced significant inhibition of activity, with maximum inhibition of 40% of basal activity. This maximum inhibition varied between different membrane preparations but ranged between 25 and 50% inhibition of basal adenylyl cyclase activity. Among 16 separate experiments, the average maximum inhibition was 32 ± 5%. Compared with the actions of other opioid agonists in inhibiting adenylyl cyclase in rat brain membranes (31), dynorphin A was relatively potent in inhibiting adenylyl cyclase in guinea pig cerebellum membranes, with an IC_{50} value of 0.06 μM. Inhibition of activity was maximal at a concentration of 0.5 μM dynorphin A. Moreover, dynorphin-inhibited adenylyl cyclase was mediated through a genuine opioid receptor, because 1 μM naloxone acted as a competitive antagonist, shifting the dynorphin dose-response

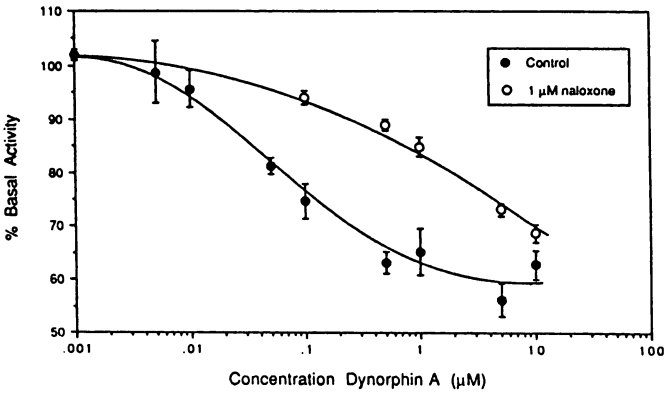

Fig. 1. Inhibition of adenylyl cyclase in guinea pig cerebellum membranes by dynorphin A, in the presence and absence of 1 μM naloxone. Data are expressed as percentage of basal activity (336 pmol of cAMP/min/mg of protein) assayed in the absence of dynorphin.

curve to the right in a parallel fashion and increasing the IC_{50} value for dynorphin A by approximately 20-fold.

Previous studies in rat brain showed that pretreatment of membranes at pH 4.5 increased opioid-inhibited adenylyl cyclase without affecting basal adenylyl cyclase activity or receptor binding (29, 30). To determine whether low pH pretreatment has a similar effect in guinea pig cerebellum, dynorphin-inhibited adenylyl cyclase was compared in pH 7.4- and pH 4.5-pretreated cerebellar membranes (Fig. 2). The results showed that the effects of low pH pretreatment were identical to those seen for opioid-inhibited adenylyl cyclase in rat brain membranes, with an increase in the maximal inhibition by dynorphin A from 15% of basal activity in untreated membranes to 25% in low pH-pretreated membranes. Basal activity was unaffected by the low pH pretreatment. As a result, all further experiments were conducted on low pH-pretreated membranes. However, it is important to note that dynorphin-inhibited adenylyl cyclase can still be detected in untreated membranes. The low pH pretreatment simply allows easier quantitation of this activity.

Receptor-mediated inhibition of adenylyl cyclase occurs through G_i proteins (35) and therefore requires the presence of GTP. Fig. 3 shows the effect of various guanine nucleotides (50 μM) on both basal and dynorphin-inhibited adenylyl cyclase activity. None of the guanine nucleotides produced significant

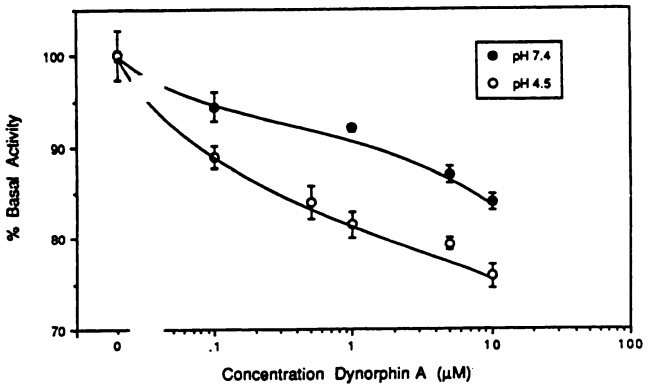

Fig. 2. Effect of pH 4.5 pretreatment on dynorphin-inhibited adenylyl cyclase. Membranes from guinea pig cerebellum were pretreated in either 50 mM Tris·HCl, 3 mM $MgCl_2$ (pH 7.4), or 50 mM sodium acetate, 3 mM $MgCl_2$ (pH 4.5), for 10 min on ice before assay of adenylyl cyclase at pH 7.4 in the presence of indicated concentrations of dynorphin A.

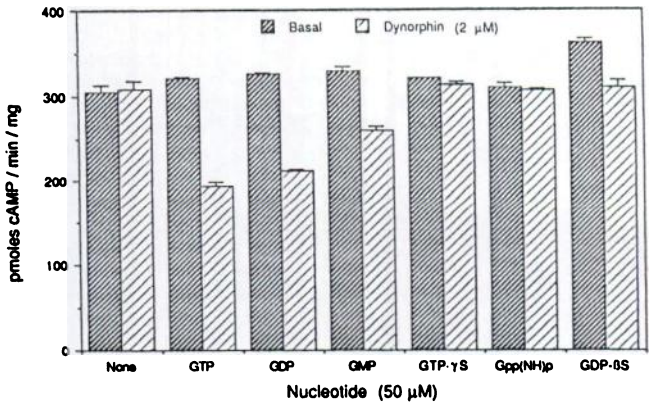

Fig. 3. Guanine nucleotide specificity of dynorphin-inhibited adenylyl cyclase in guinea pig cerebellum membranes. Adenylyl cyclase activity was assayed in the presence and absence of guanine nucleotides (all at 50 μM concentrations), with and without 2 μM dynorphin A.

changes in basal activity (except for GDP-βS, which produced a small 12% increase in activity). This lack of stimulation by guanine nucleotides was caused by the low pH pretreatment of brain membranes, which eliminated G_s-stimulated adenylyl cyclase (29). In the absence of guanine nucleotides, no significant dynorphin-inhibited activity was observed. In the presence of either GTP and GDP, dynorphin A (2 μM) produced 35–40% inhibition of activity. Addition of GMP also supported dynorphin-inhibited activity, but to a lesser extent than GTP and GDP (only 15% inhibition). None of the nonhydrolyzable guanine nucleotide analogs examined, including Gpp(NH)p, GTP-γS, and GDP-βS, supported dynorphin-inhibited activity. These results are identical to the guanine nucleotide specificity observed for opioid-inhibited adenylyl cyclase in rat brain membranes (31).

Receptor-mediated inhibition of adenylyl cyclase can occur either for basal activity (as observed above) or for forskolin-stimulated activity (31, 36). Fig. 4 shows that dynorphin A can inhibit either basal or forskolin-stimulated activity. In the absence of forskolin, dynorphin A (2 μM) inhibited basal adenylyl cyclase by 35%. As increasing concentrations of forskolin stimulated activity, dynorphin A continued to inhibit activity. At low forskolin concentrations (0.01–0.1 μM), dynorphin-inhibited activity was not altered. However, at higher concentrations of forskolin, dynorphin-inhibited activity was attenuated, so that at the 5 μM concentration of forskolin (which stimulated adenylyl cyclase by 175%) maximum dynorphin-inhibited activity was only 10%. Again, these results are identical to those previously observed for opioid-inhibited adenylyl cyclase in rat brain membranes (31).

In addition to guanine nucleotides, receptor-mediated inhibition of adenylyl cyclase also requires sodium (36). The effect of various concentrations of NaCl on adenylyl cyclase activity in guinea pig cerebellum was examined in the absence and presence of 10 μM dynorphin (1–13) (Fig. 5). In the absence of NaCl, dynorphin-inhibited activity was low, with only 15% inhibition observed. NaCl stimulated basal adenylyl cyclase activity, with stimulation peaking at 25 mM NaCl. Because the inhibited levels were not affected by NaCl, dynorphin-inhibited adenylyl cyclase was maximal (at 35% inhibition) with 10–50 mM NaCl. Therefore, all further experiments were conducted in the presence of 20 mM NaCl. Once again, these results are similar to findings obtained with opioid-inhibited adenylyl cyclase in rat brain membranes.[1]

Pharmacological specificity of dynorphin-inhibited adenylyl cyclase. Because dynorphin preferentially binds to κ opioid receptors (3), the selective κ agonist U-50,488H (37) was tested together with several dynorphin peptide analogs of dynorphin A for their ability to inhibit adenylyl cyclase in guinea pig cerebellum (Table 1). All dynorphin analogs (as well as U-50,488H) produced approximately the same degree of maximum inhibition, ranging between 35 and 40% of basal activity. Several agonists demonstrated approximately the same potencies in inhibiting adenylyl cyclase, with IC_{50} values between 0.03 and 0.05 μM. These relatively potent agonists included U-50,488H, dynorphin A, dynorphin(1–13), and the highly κ-selective dynorphin analog D-Pro^{10}-dynorphin(1–11) (38). Other agonists were significantly less potent; these included dynorphin B (with an IC_{50} of 0.13 μM), dynorphin(1–8)

[1] S. R. Childers, unpublished observations.

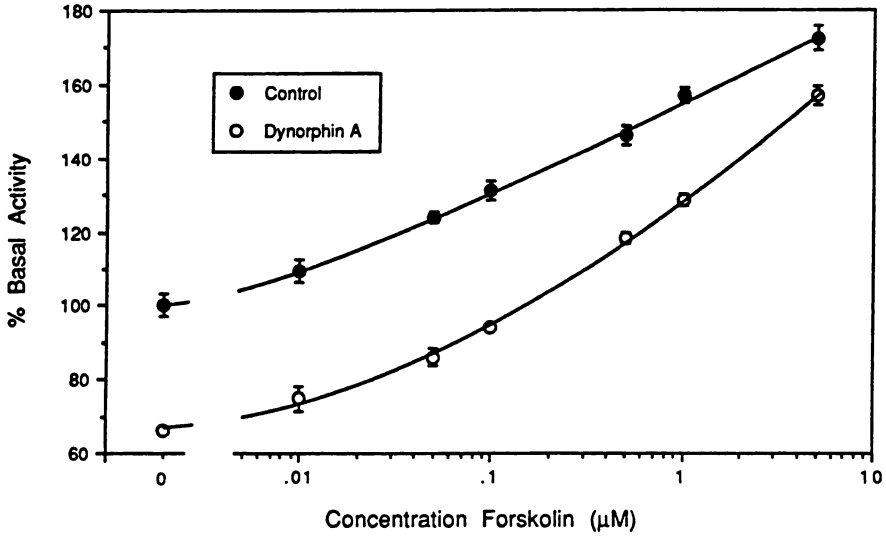

Fig. 4. Dynorphin inhibition of basal and forskolin-stimulated adenylyl cyclase. Guinea pig cerebellum membranes were assayed for activity with 0–5 μM forskolin, in the presence and absence of 2 μM dynorphin A.

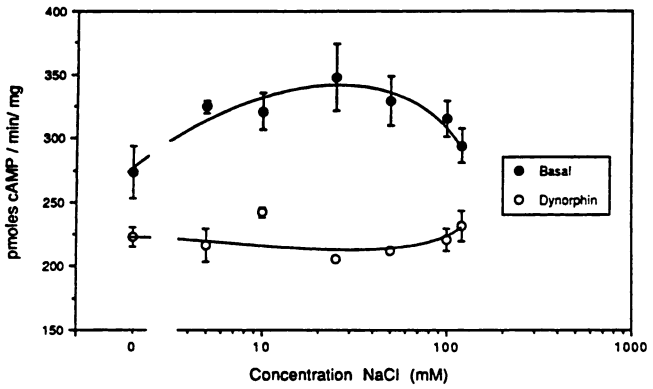

Fig. 5. Effect of sodium on dynorphin-inhibited adenylyl cyclase activity. Guinea pig cerebellum membranes were assayed for adenylyl cyclase with 0–100 mM NaCl, in the presence and absence of 10 μM dynorphin(1–13). All assay components that were normally present as sodium salts (e.g., 20 mM creatine phosphate) were replaced with the corresponding Tris salts and then NaCl was added back to the assay at the indicated concentrations.

TABLE 1

IC_{50} values and efficacies of κ opioid agonists in inhibiting adenylyl cyclase in guinea pig cerebellum

Membranes from guinea pig cerebellum were isolated and pretreated at pH 4.5 before assay of adenylyl cyclase, as described in Experimental Procedures. Agonists were added to the assays at various concentrations; data represent mean values ± standard errors of at least three separate experiments. I_{max} is the maximum percentage of inhibition of adenylyl cyclase for each agonist.

Agonist	IC_{50}	I_{max}
	μM	%
Dynorphin A	0.05 ± 0.009	42 ± 2
Dynorphin(1–13)	0.04 ± 0.006	41 ± 1
D-Pro^{10}-dynorphin	0.03 ± 0.007	41 ± 3
U-50,488H	0.05 ± 0.009	38 ± 7
Dynorphin B	0.13 ± 0.003	37 ± 5
α-Neo-endorphin	0.34 ± 0.083	36 ± 9
Dynorphin(1–8)	0.97 ± 0.24	37 ± 3

(0.97 μM), and α-neo endorphin (0.34 μM). The decreased potency of analogs like dynorphin(1–8), compared with dynorphin A and U-50,488H, is not likely to be caused by proteolysis, because the potency of dynorphin(1–8) in inhibiting cerebellar adenylyl cyclase was not affected by the addition of a cocktail of protease inhibitors that were previously shown to block dynorphin hydrolysis (12) (data not shown).

Other opioid agonists with lower affinity for κ receptors produced little if any effect on adenylyl cyclase in guinea pig cerebellum. Table 2 shows the effects of single concentrations of these agonists. Both β-endorphin and morphine produced weak (20%) inhibition of activity. Other agonists such as D-ala-enk, met-enkephalin, leu-enkephalin, DAGO, DSLET, and DPDPE had no significant effect on adenylyl cyclase activity at 10 μM concentrations, under the same conditions where 1 μM dynorphin A produced 34% inhibition. In contrast, all of these agonists produced significant inhibition of adenylyl cyclase in rat striatal membranes (31). To demonstrate that the lack of effect by these agonists was not due to species differences, their effects on adenylyl cyclase were examined in guinea pig striatal membranes (Table 2). In striatum, dynorphin A continued to be a potent inhibitor of adenylyl cyclase, producing 37% inhibition of basal activity. In contrast to their lack of effect in cerebellum, other opioid peptides did inhibit adenylyl cyclase in striatum. Most effective of these agonists was D-Ala-enk, which produced 25% inhibition of basal activity. More

TABLE 2

Effect of opioid agonists on adenylyl cyclase in guinea pig cerebellum and striatum

Membranes from guinea pig striatum and cerebellum were isolated and pretreated at pH 4.5 before assay of adenylyl cyclase, as described in Experimental Procedures. Agonists were added to the assays at the concentrations listed; data represent mean values ± standard deviations of triplicate determinations. Results are expressed as percentage of basal activity, which was 340 pmol of cAMP/min/mg of protein.

Agonist	Concentration	Inhibition	
		Cerebellum	Striatum
	μM	%	
Dynorphin A	1	36 ± 1	37 ± 2
β-Endorphin	20	20 ± 2	ND*
Morphine	100	16 ± 2	ND
D-Ala-enk	10	4 ± 2	25 ± 2
DAGO	10	4 ± 1	15 ± 1
DSLET	10	0 ± 5	ND
DPDPE	10	0 ± 3	12 ± 2

* ND, not determined.

selective peptides such as DPDPE and DAGO produced less inhibition but still demonstrated higher inhibition in striatal membranes than in cerebellum. Therefore, these results are consistent with the finding of μ, κ, and δ receptors in the striatum, with a selective population of κ receptors in the guinea pig cerebellum (13).

Although Fig. 1 demonstrated that naloxone could act as a competitive antagonist of dynorphin-inhibited adenylyl cyclase in guinea pig cerebellum, naloxone is a μ antagonist with relatively poor potency for κ receptors (8). In order to demonstrate the κ selectivity of the dynorphin-inhibited adenylyl cyclase, the highly selective κ antagonist nor-BNI (39) was compared with naloxone in its ability to block this activity. Results in guinea pig cerebellum (Fig. 6, *top*) compared the effects of nor-BNI and naloxone on inhibition of adenylyl cyclase by dynorphin(1–13). The addition of 0.1 μM nor-BNI shifted the IC_{50} value of dynorphin(1–13) to the right nearly 50-fold, whereas 1 μM naloxone shifted the IC_{50} value by 20-fold. From three separate experiments, the calculated mean K_e values (±SE) for each antagonist in blocking dynorphin-inhibited adenylyl cyclase were 1.9 ± 0.6 nM for nor-BNI and 82 ± 29 nM for naloxone. These results are consistent with the idea that dynorphin-inhibited adenylyl cyclase is acting through κ receptors in the guinea pig cerebellum. On the other hand, nor-BNI was much weaker at antagonizing D-Ala-enk-inhibited adenylyl cyclase in guinea pig striatum (Fig. 6, *bottom*). These results showed that nor-BNI had no effect on the D-Ala-enk inhibition curve at low concentrations (<1 μM) of D-Ala-enk. At higher concentrations (>1 μM) of D-Ala-enk, nor-BNI produced a small (3-fold) shift in the D-Ala-enk inhibition curve. Because the effect of nor-BNI depended on the agonist concentration (a result that is not consistent with competitive antagonism), an accurate K_e value could not be calculated. However, it is clear that the effect of nor-BNI on D-Ala-enk response in the striatum is much less than on dynorphin response in the cerebellum, consistent with the κ selectivity of this antagonist.

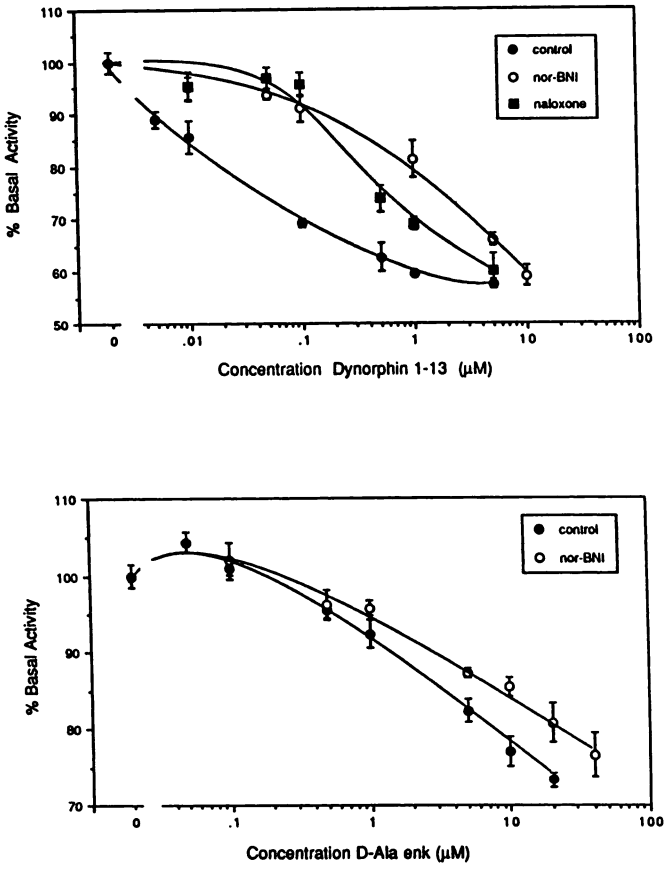

Fig. 6. Effect of antagonists on dynorphin-inhibited adenylyl cyclase in guinea pig cerebellum (*top*) and on D-Ala-enk-inhibited adenylyl cyclase in guinea pig striatum (*bottom*). *Top*, adenylyl cyclase was assayed in cerebellum with various concentrations of dynorphin(1–13), in the presence and absence of 0.1 μM nor-BNI or 1 μM naloxone. *Bottom*, adenylyl cyclase in striatum was assayed with various concentrations of D-Ala-enk in the presence and absence of 0.1 μM nor-BNI. Data are expressed as percentage of basal activity (316 pmol of cAMP/min/mg in the cerebellum and 230 pmol of percentage cAMP/min/mg in the striatum).

Discussion

Dynorphin A and its analogs produced a potent and consistent degree of adenylyl cyclase inhibition in guinea pig cerebellar membranes. This effect is not an artifact of adenylyl cylase inhibition but is instead receptor mediated, because it requires GTP, is maximal in the presence of sodium, occurs in both the presence and absence of forskolin, and is blocked by opioid antagonists like naloxone and nor-BNI. Like other G_i-mediated inhibitory responses in rat brain membranes (29, 31), dynorphin-inhibited adenylyl cyclase is increased by low pH pretreatment. The guanine nucleotide specificity of dynorphin-inhibited adenylyl cyclase is identical to that seen for opioid-inhibited adenylyl cyclase in rat brain membranes. Nevertheless, the finding that both activities are supported not only by GTP but also by GDP (and, to a small extent, GMP) may seem surprising, because classical receptor-mediated adenylyl cyclase specifically requires GTP (35). However, in both guinea pig and rat brain membranes, the effectiveness of GDP and GMP was caused by an artifact of the ATP-regeneration system used in the adenylyl cyclase assays. Control experiments (data not shown) demonstrated that addition of creatine phosphate and creatine phosphokinase phosphorylated both GDP and GMP to GTP, thus providing the assay with sufficient GTP to support receptor-mediated inhibition. In the absence of added regeneration system, opioid-inhibited adenylyl cyclase specifically required GTP, with no effect of either GDP or GMP. The reason why nonhydrolyzable analogs of GTP were not effective in supporting inhibition is not clear; however, the same results have been observed in earlier studies of opioid-inhibited adenylyl cyclase in brain membranes (27, 28, 31).

The dynorphin-inhibited adenylyl cyclase in guinea pig cerebellum is notable in at least two respects, efficacy and potency. The efficacy, or maximal inhibition of adenylyl cyclase by dynorphin, is relatively high, ranging between 30 and 50% inhibition of basal activity. This effect compares with the 25 to 35% inhibition observed in rat (29, 31) and guinea pig striatum (Table 1) for other opioid agonists. It is not clear whether this relatively robust inhibition is caused by a large density of one type of receptor in this tissue or by differences in receptor-adenylyl cyclase coupling efficiencies. However, this efficacy means that quantitation of agonist dose-response curves in the guinea pig cerebellum is straightforward. Furthermore, the potencies of agonists in this preparation are also notable. The most potent agonists had IC_{50} values between 0.03 and 0.05 μM. Although these IC_{50} values are higher than those observed in receptor binding assays conducted in the absence of guanine nucleotides and sodium, they are significantly lower than the

0.5 to 1 μM IC_{50} values observed for μ and δ agonists in rat (31) and guinea pig (Fig. 6, *bottom*) striatum. It is interesting to speculate that the increased potencies of κ agonists in the guinea pig cerebellum are due to the decreased effects of guanine nucleotides and sodium on receptor binding. For both μ and δ receptors, addition of sodium and guanine nucleotides, both of which are required for adenylyl cyclase inhibition, markedly decreases agonist binding affinities (19–21). However, the effect of sodium and guanine nucleotides on κ receptor binding is much smaller, with minimal attenuation of agonist binding compared with μ and δ agonists (22–24). This difference may explain why dynorphin has a higher potency than μ and δ agonists under conditions that allow coupling of receptors to adenylyl cyclase.

Although a complete pharmacological analysis of the adenylyl cyclase response on guinea pig cerebellum has not yet been completed, the results reported in this study strongly suggest that dynorphin-inhibited adenylyl cyclase acts through G protein-coupled κ opioid receptors. This suggestion is supported by previous findings that κ agonist binding can be regulated by guanine nucleotides (23, 24) and that GTPase activity in brain membranes can be stimulated by κ agonists (40). Moreover, the inhibition of adenylate cyclase by κ agonists was recently reported in rat spinal cord (25). The concept that κ receptors can inhibit adenylyl cyclase is supported not only by the fact that guinea pig cerebellum contained a relatively pure population of κ receptors but also by the fact that selective κ agonists such as U-50,488H and D-Pro^{10}-dynorphin(1–11) were among the most potent agonists. Other opioid agonists, including traditional μ- and δ-selective compounds, had no significant effect on activity at concentrations almost 100 times of the IC_{50} of dynorphin analogs. Finally, the κ-selective antagonist nor-BNI was much more potent than naloxone in blocking the agonist response, with an estimated K_e of 2 nM, compared with 80 nM for naloxone. Therefore, these results suggest that assay of adenylyl cyclase in guinea pig cerebellum may be an important screening tool for assay of novel κ agonists and antagonists, because this technique can measure potencies as well as efficacies of novel compounds. From a basic viewpoint, these results are important because they confirm that κ receptors are coupled to inhibition of adenylyl cyclase as a second messenger system. Thus, all three major categories of opioid receptors (μ, δ, and κ) can inhibit adenylyl cyclase in at least some cell systems. This finding does not mean that inhibition of adenylyl cyclase is the only second messenger system associated with these receptors but it does suggest a common link between these different types of opioid receptors.

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Send reprint requests to: Dr. Steven R. Childers, Department of Pharmacology, Box J-267, JHMHC, University of Florida College of Medicine, Gainesville, FL 32610.