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μ type opioid receptors in rat periaqueductal gray-enriched P_2 membrane are coupled to G-protein-mediated inhibition of adenylyl cyclase

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The periaqueductal gray (PAG) region of the midbrain has been implicated in both stimulation-produced and opioid-induced analgesia. High-affinity μ-selective opioid-binding sites associated with μ type opioid receptors have been detected in rat PAG-enriched P_2 membranes, and these receptors have been shown to be coupled to guanine nucleotide-binding proteins (G-proteins). In the present study the potential G-protein-mediated coupling of μ type opioid receptors to the inhibition of adenylyl cyclase was examined utilizing in vitro adenylyl cyclase assays. In the presence of Na^+, opioid agonists inhibited adenylyl cyclase in a μ selective, naloxone reversible, dose dependent, and pertussis toxin sensitive manner. Overall the data suggests that μ type opioid receptors in the rat PAG are coupled to G-protein-mediated inhibition of adenylyl cyclase.

Opioid receptor; G-protein; Adenylyl cyclase

1. INTRODUCTION

The periaqueductal gray (PAG) region of the midbrain has been implicated in both stimulation-produced and opioid-induced analgesia. Both focal electrical stimulation within and intracerebral microinjection of opioids into the PAG result in a profound analgesia unaccompanied by general motor or behavioral depression [1,2]. It appears that this opioid-induced analgesia in the PAG is opioid receptor mediated in that it is stereoselective, dose dependent, and reversed by the specific opioid antagonist naloxone [3]. In addition it appears that opioid agonism within the midbrain is important if not essential for the mediation of the analgesia observed after systemic administration of analgesic opioids since the analgesia produced by typical systemic doses of morphine can also be reversibly attenuated by intracerebroventricular microinjection of naloxone into the third ventricle and the vicinity of the PAG in a dose-dependent manner [4].

The heterogeneity of opioid receptors is now widely accepted. μ, κ, δ, and ϵ type opioid receptors have been postulated on the basis of pharmacological data in the chronic spinal dog [5] and in in vitro guinea pig ileum, mouse vas deferens, and rat vas deferens preparations [6,7]. High-affinity μ-, κ- and δ-selective opioid-binding sites have also been subsequently identified in brain tissue [8–10]. However in vitro radioligand-binding studies utilizing homogenized rat PAG tissue have identified only high-affinity μ-selective opioid-binding sites in membrane from this particular rat brain region. High-affinity δ- and κ-selective opioid binding was not detected [11]. Additional binding and biochemical studies utilizing rat PAG membranes have provided strong evidence that these high-affinity μ-selective opioid-binding sites are associated with functional μ type opioid receptors, and that these receptors are coupled to guanine nucleotide-binding proteins (G-

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proteins) in a manner similar to the coupling of other hormone and neurotransmitter cell surface receptors to G-proteins [12].

Such G-proteins are known to mediate the coupling of hormone and neurotransmitter cell surface receptors to a number of different signal transduction, second messenger effector systems [13]. In the NG-108,15 neuroblastoma-glioma cell line, a hybrid cell line which expresses only a single type of opioid receptor, agonism at δ opioid receptors is coupled to the inhibition of adenylyl cyclase activity via a G-protein [14]. It is the purpose of this study to determine if agonist binding at the μ type opioid receptors of the rat PAG are similarly coupled to the adenylyl cyclase signal transduction effector system.

2. METHODS

2.1. *Membrane preparation*

Male Sprague-Dawley rats weighing 180–200 g were used in all experiments. Rats were killed by decapitation, and the brain minus cerebellum were immediately dissected over ice. The PAG region of the midbrain of each rat was initially isolated by three transverse cuts along the main cerebral axis; the first cut caudal to the corpus callosum, the second cut between the superior and inferior colliculi, and the third cut caudal to the inferior colliculi. PAG tissue was further concentrated by removing the white matter surrounding the visible gray matter in the central region of the two inner transverse sections produced by the three previous transverse cuts. The individually isolated PAG regions were pooled, washed in ice-cold 10 mM Hepes/0.32 M sucrose/1.6 mM EGTA buffer (pH 7.4), and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was saved and the pellet resuspended in buffer and again centrifug-

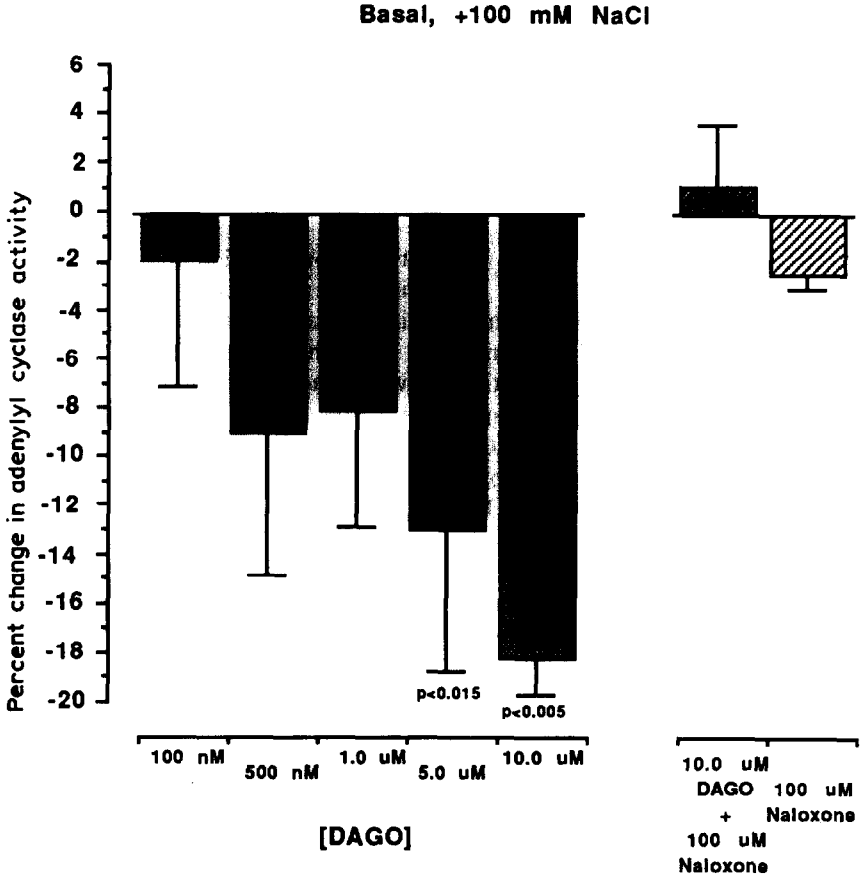

Fig.1. Effects of DAGO on basal adenylyl cyclase activity in the presence of Na^+. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid-treated controls. Each column represents the mean of triplicate determinations. $F = 3.50449532$, $p < 0.055$. Basal activity = 45.04 ± 3.07 pmol [^{32}P]cAMP/min per mg protein.

ed at 1000 × g for 10 min. This pellet was discarded and the pooled supernatant from both 1000 × g spins was centrifuged at 22500 × g for 20 min. The resulting pellet was resuspended in buffer and again centrifuged at 22500 × g for 20 min. The final P_2 pellet was resuspended in 10 mM Hepes/1.6 mM EGTA buffer (pH 7.4), and frozen in aliquots for future use. Typically the PAG-enriched P_2 membrane was resuspended to and stored in an average protein concentration of 4.8 mg/ml, as determined by the Lowry assay [15]. Thawed PAG-enriched P_2 membrane was used once and never refrozen.

2.2. *Pertussis toxin pretreatment of membrane*

PAG-enriched P_2 membrane was pretreated with pertussis toxin utilizing a modified version of a technique described by Kurose et al. [14]. Pertussis toxin was first activated with 100 mM dithiothreitol for 4 h at room temperature. Membrane was incubated with various concentrations of pertussis toxin in a reaction mixture containing 50 mM Tris buffer (pH 8), 20 mM thymidine, 0.5 mM ATP, 20 mM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 20 mM creatine phosphate, 10 U of creatine phosphokinase, 1 mM 1,10-*O*-phenanthroline, and 1 mM NAD at room temperature for 1 h. The ADP-ribosylation reaction was terminated with the addition of 250 μl of ice-cold 25 mM Hepes buffer (pH 7.4), and the samples were centrifuged at 850 × g for 15 min. The resulting pellet was resuspended to an appropriate protein concentration in 10 mM Hepes/1.6 mM EGTA buffer (pH 7.4) for immediate use in adenylate cyclase assays.

2.3. *Adenylyl cyclase assay*

Adenylyl cyclase activity in PAG-enriched P_2 membranes was assayed via the formation of [^{32}P]cAMP utilizing a modified version of the technique described by White and Karr [16]. The reaction mixture of each adenylyl cyclase assay consisted of 10 mM $MgCl_2$, 40 mM creatine phosphate, 2 mM cAMP, 10 U of creatine phosphokinase, 40 mM GTP, 20 mM theophylline, 2 mM 1,10-*O*-phenanthroline, 100 mM NaCl where appropriate, 40 mM Hepes buffer (pH 7.4), and various concentrations of opioid agonists and antagonists to a total volume of 50 μl. The reaction mixture was incubated with 25 μl of PAG-enriched P_2 membrane for 10 min at 0°C. Each sample contained 43.5 or 62.5 μg of protein. The reaction was initiated with the addition of 20 μl of a [α-^{32}P]ATP solution. The final concentration of ATP per assay was 0.1 mM, and each assay contained 1 μCi of [α-^{32}P]ATP. The reaction was terminated with the addition of 150 μl of 1 M $HClO_4$.

10000 cpm of [^{3}H]cAMP was added to each sample as an internal standard for cAMP recovery. Reaction product cAMP was separated from other labeled compounds with double columns of Dowex and alumina as described by White and Karr [16]. Collected cAMP was placed in individual counting vials, scintillation cocktail was added, and the vials were counted. All assays were carried out in triplicate. One way analysis of variance and unpaired Student's *t*-tests were utilized in statistical analysis.

3. RESULTS

A 10 μM concentration of the μ selective opioid agonist [D-Ala^2,*N*-methyl-Phe^4,Glyol^5]enkephalin (DAGO) significantly inhibited both basal and forskolin-stimulated adenylyl cyclase activity in PAG-enriched P_2 membranes. The DAGO inhibition of forskolin-stimulated adenylyl cyclase activity was not consistently dose dependent, was not naloxone reversible, and the opioid antagonist naloxone itself significantly inhibited forskolin-stimulated adenylyl cyclase activity, even when present in low concentration (data not shown).

DAGO inhibition of basal adenylyl cyclase activity was also not consistently dose dependent. However, the DAGO inhibition of basal adenylyl cyclase activity was naloxone reversible, although naloxone itself did not significantly affect adenylyl cyclase activity (data not shown).

In the presence of 100 mM NaCl, DAGO did inhibit basal adenylyl cyclase activity in a more dose-dependent manner with borderline statistical significance. In the presence of 100 mM NaCl DAGO inhibition of adenylyl cyclase activity was

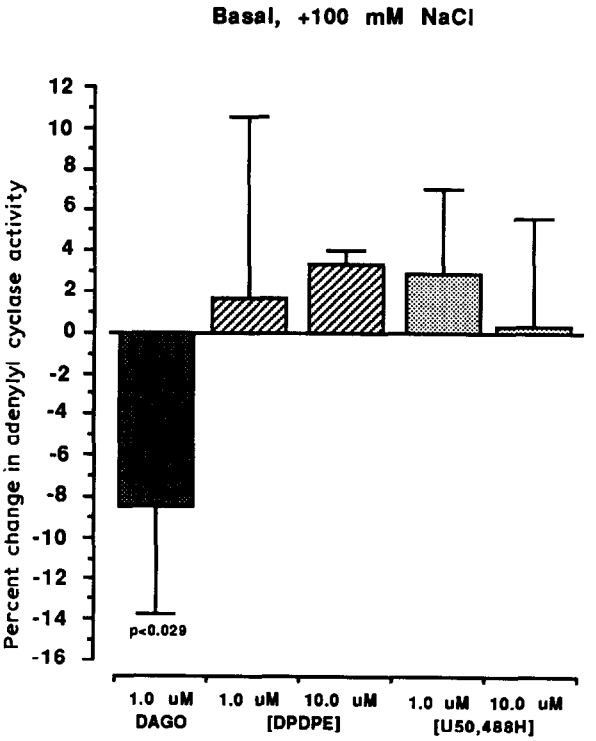

Fig.2. Effects of DAGO, DPDPE, and U50,488H on basal adenylyl cyclase activity in the presence of Na^+. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid-treated controls. Each column represents the mean of triplicate determinations. Basal activity = 58.05 ± 2.47 pmol [^{32}P]cAMP/min per mg protein.

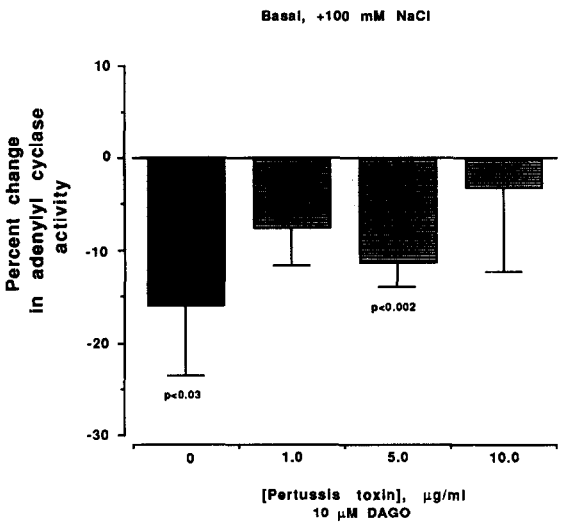

Fig.3. Effects of DAGO on basal adenylyl cyclase activity in the presence of Na^+ following pertussis toxin pretreatment of membrane. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid-treated controls. Each column represents the mean of triplicate determinations. Basal activity after 0, 1.0, 5.0, and 10.0 µg/ml pertussis toxin pretreatment = 57.71 ± 0.89, 65.07 ± 0.65, 60.49 ± 0.74, and 56.99 ± 2.44 pmol [^{32}P]cAMP/min per mg protein respectively.

naloxone reversible. The results are shown in fig.1. This effect is also selective for opioid agonists with a reported μ profile. Neither the δ-selective opioid agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) nor the κ-selective opioid agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, methane sulfonate, hydrate (U50,488H) affected basal adenylyl cyclase activity to a significant extent. The results are shown in fig.2.

Pretreatment of PAG-enriched P_2 membranes with activated pertussis toxin abolished DAGO inhibition of adenylyl cyclase activity. This effect was reproducible but not consistently dose dependent. The results are shown in fig.3.

4. DISCUSSION

The plant-derived diterpine forskolin is believed to directly stimulate the catalytic component of adenylyl cyclase and is commonly used in the study of the adenylyl cyclase enzyme system. Forskolin is a particularly useful tool in the study of the inhibition of adenylyl cyclase in that its presence within an adenylyl cyclase assay produces a higher degree of enzyme activity against which an inhibitory signal may be more easily detectable. However the use of forskolin in the study of opioid inhibition of adenylyl cyclase activity in PAG-enriched P_2 membranes may be undesirable.

The μ-selective opioid agonist DAGO inhibits adenylyl cyclase activity in PAG-enriched P_2 membranes in what initially may seem to be a confusing manner. Both basal and forskolin-stimulated adenylyl cyclase activity are significantly inhibited, but in the presence of forskolin DAGO inhibition of adenylyl cyclase activity is neither dose dependent nor naloxone reversible. Surprisingly, naloxone itself significantly inhibits forskolin-stimulated adenylyl cyclase activity, also in a non-dose-dependent manner. In contrast, DAGO inhibition of basal adenylyl cyclase activity is naloxone reversible, and in the absence of forskolin naloxone itself does not significantly affect adenylyl cyclase activity. It is possible that naloxone somehow interferes with the forskolin stimulation of adenylyl cyclase in a manner unrelated to its antagonism of opioid receptors. Such interference of forskolin stimulation of adenylyl cyclase would appear as inhibition of this enzyme.

Although high doses of DAGO significantly inhibit basal adenylyl cyclase activity in PAG-enriched P_2 membranes in the absence of forskolin, such DAGO inhibition is also not consistently dose dependent. However in the presence of Na^+, a cation which has been shown to be required in the coupling of opioid receptors to G-proteins and adenylyl cyclase in the NG-108,15 neuroblastoma-glioma cell line [18,19], DAGO does inhibit basal adenylyl cyclase activity in both a naloxone reversible and consistent dose-dependent manner. It is possible that in the presence of 100 mM NaCl opioid receptors are more efficiently coupled to the inhibition of adenylyl cyclase in the PAG.

The presence of Na^+ and the absence of forskolin are assay conditions which probably more closely approximate physiological conditions. Under such conditions DAGO inhibits adenylyl cyclase in PAG-enriched P_2 membranes in a manner which is consistent with G-protein-mediated coupling of opioid receptors to this enzyme. In the presence of Na^+ and the absence of forskolin DAGO inhibition of adenylyl cyclase is not only apparently dose dependent and clearly naloxone

reversible, but is also μ opioid agonist selective. δ and κ opioid agonists do not significantly affect adenylyl cyclase activity. In addition after pretreatment of PAG-enriched P_2 membranes with a sufficient concentration of activated pertussis toxin, DAGO does not significantly inhibit adenylyl cyclase activity. While this effect of pertussis toxin pretreatment is not entirely consistent it suggests the involvement of G-proteins in the observed DAGO inhibition of adenylyl cyclase activity in PAG membranes.

The maximal effect of DAGO on adenylyl cyclase activity is small, in the range of 15% to 20%, but it is statistically significant, reproducible, and consistent from tissue preparation to tissue preparation regardless of the amount of basal activity measured.

Opioid agonists have previously been shown to inhibit adenylyl cyclase activity in homogenized rat striatal tissue, but this inhibition is believed to result from agonist activity at δ type opioid receptors as in the NG-108,15 neuroblastoma-glioma cell line [20,21]. Overall the adenylyl cyclase assay data presented in this study provide evidence that μ type opioid receptors of the PAG can also couple to adenylyl cyclase to inhibit the production of the second messenger cAMP. This finding is consistent with a report that μ opioid receptors in rat 7315c pituitary tumor cells are also coupled to the inhibition of adenylyl cyclase via G-proteins [22]. The coupling of μ opioid receptors to additional G-protein-mediated effector systems is possible and warrants further study.

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