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EFFECT OF δ-OPIOID ANTAGONISTS ON THE FUNCTIONAL COUPLING BETWEEN OPIOID RECEPTORS AND G-PROTEINS IN RAT BRAIN MEMBRANES

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Abstract—It is currently accepted that occupancy of opioid receptors by agonists, but not antagonists, promotes the association of the receptors to guanine nucleotide binding proteins (G-proteins) and stimulates a high affinity GTPase as part of the mechanism that links the receptor–ligand complex to adenylate cyclase inhibition. In this work we report that in rat brain membranes selective δ-opioid antagonists, the peptides *N,N*-Diallyl-Tyr-D-Leu-Gly-Tyr-Leu-OH (Diallyl-G) and *N-N*-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI174 864), inhibit the low K_m GTPase activity in a concentration dependent way. On the other hand the δ-opioid agonists D-Ala^2-D-Leu^5-enkephalin (DADLE) and D-Ser^2-Leu^5-Thr^6-enkephalin stimulate dose-dependently the low K_m GTPase activity in rat brain membranes. This stimulation was blocked in the presence of Diallyl-G, and reciprocally the inhibition induced by Diallyl-G was reversed by DADLE. The inhibitory effect of Diallyl-G as well as the stimulation induced by DADLE were abolished when membranes were exposed to low concentrations of *N*-ethylmaleimide or by ADP ribosylation with pertussis toxin which interferes with the ability of the receptor to couple to G-proteins. These observations indicate that the inhibitory effect of Diallyl-G on GTPase requires a functional G-protein and suggest that certain δ-opioid antagonists exhibit negative intrinsic activity and may have the ability to inhibit the receptor-mediated activation of G-proteins.

The existence of three major types of opioid receptor, designated as μ, δ and κ, is well established [1]. However the molecular events that follow the activation of the opioid receptor subtypes is not yet fully understood. Activation of opioid receptors of μ and δ specificity has been shown to inhibit the adenylate cyclase in NG108-15 hybrid cells and in mammalian brain tissues via coupling to guanine nucleotide binding proteins (G-proteins†) acting as signal transducers [2, 3]. Receptor activation of a G-protein leads to an increase in the rate of exchange of GDP bound to the α subunit of the G-proteins with GTP and subsequent hydrolysis of GTP. Agonist activation of any receptor coupled to G-protein(s) produces a stimulation of GTPase activity [4, 5]. Measurements of the enhanced rate of GTPase activity in response to agonist is therefore a direct assessment of the interaction between a receptor and a G-protein. According to current models of receptor activation, agonists promote the association of receptors to G-proteins whereas antagonists do not produce any effect [6]. Reconstitution experiments with purified receptors and G-proteins as well as studies in various intact membrane systems suggest that agonist-free receptors exhibit more complex effects in catalysing the nucleotide exchange reaction of G-proteins [7, 8]. In addition, in some receptor systems it was observed that certain antagonists may be active by hindering the ability of the receptors to associate spontaneously with G-proteins [9]. Recent studies on the functional coupling between δ-opioid receptors and G-proteins by measuring the low K_m GTPase activity in NG108-15 hybrid cells showed that certain δ-opioid receptor antagonists, such as the peptide *N-N*-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI174864), express a distinct intrinsic activity, determined by their ability to inhibit the high affinity GTPase activity [9, 10]. In this work we report that another δ-opioid antagonist, the peptide *N,N*-Diallyl-Tyr-D-Leu-Gly-Tyr-Leu-OH (Diallyl-G) [11], inhibits the low K_m GTPase activity of a pertussis toxin-sensitive G-protein in rat brain membranes.

MATERIALS AND METHODS

Materials. Radiolabelled compounds [γ-^{32}P]GTP (222 TBq/mmol) and [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADLE) (1.74 TBq/mmol) were from New England Nuclear (Boston, MA). Opioid peptides, DADLE and [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET), and naloxone, ATP, GTP, phosphocreatine, creatine phosphokinase type III, HEPES, EGTA, thymidine, pertussis toxin and dithiothreitol (DTT) were purchased from Sigma. ICI174 864 was purchased from Cambridge Research Biochemicals Ltd (U.K.). Adenosine 5′(β,γ-imido)-triphosphate, NAD and *N*-ethylmaleimide (NEM) were obtained from Pierce. Diallyl-G was generously provided by Dr S. Loukas, Inst. of Biology, NCSR "Demokritos".

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† Abbreviations: G-protein, guanine nucleotide binding protein; Diallyl-G, *N,N*-Diallyl-Tyr-D-Leu-Gly-Tyr-Leu-OH; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesuphfonic acid; NEM, *N*-ethylmaleimide; DADLE, [D-Ala^2,D-Leu^5]enkephalin; ICI174 864, *N,N*-Diallyl-Tyr-Aib-Aib-Phe-Leu-OH; DSLET, [D-Ser^2,Leu^5,Thr^6]enkephalin; DTT, dithiothreitol.

Preparation of membranes. Rat brains without the cerebellum were homogenized with 10 vol. of 10 mM Tris, 0.32 M sucrose pH 7.4 (buffer A). The homogenate was centrifuged at 1500 *g* for 10 min and the supernatant was incubated for 30 min at 25°, and finally centrifuged at 30,000 *g* for 20 min. The pellet was resuspended in buffer A at a protein concentration of 10 mg/mL and stored at −80°. Protein concentration was measured according to Lowry *et al.* [12].

GTPase assay. The experimental procedure used is based on the method of Cassel and Selinger [5]. Brain membranes (3–5 μg) with 5 mM $MgCl_2$, 150 mM NaCl, 1 mM adenosine 5′(β,γ-imido)-triphosphate, 1 mM ATP, 2.5 mM creatine phosphate, 5 U of creatine phosphokinase, 0.5 mM EGTA and 50 mM HEPES–HCl pH 7.4 were incubated in triplicate in a final volume of 50 μL. The reaction was initiated by the addition of 0.15 μM [γ-^{32}P]GTP (50,000 cpm/pmol) and the mixture was incubated at 30° for 10 min. The reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid and the mixture was centrifuged in an Eppendorf centrifuge. The supernatant was removed and diluted 5-fold with 100 mM KH_2PO_4 pH 7.0, and the radioactivity of an aliquot (100 μL) was measured by scintillation spectroscopy in 5 mL Aquasol. To the remaining diluted sample (400 μL) charcoal was added and after centrifugation the radioactivity of an aliquot (100 μL) was measured again. The [γ-^{32}P]GTP hydrolysis was quantitated as a percentage of the total for each sample. The high K_m GTPase activity was measured in the presence of 50 μM unlabelled GTP. The low K_m GTPase activity was calculated by subtracting the amount of ^{32}Pi released from 0.15 μM [γ-^{32}P]GTP from that measured in the presence of 50 μM unlabelled GTP. Under the conditions employed the rate of release of ^{32}P was linear with time and the protein concentration.

N-Ethylmaleimide treatment of membranes. Treatment of the membrane preparation with NEM was carried out as described by Ueda *et al.* [13]. Briefly, membranes (0.5 mg protein) were incubated at 4° for 15 min in the presence of different NEM concentrations ranging from 1 to 100 μM. The reaction was stopped by the addition of 1 mM DTT and after centrifugation the pellet was washed twice with cold 50 mM HEPES pH 7.4. These membranes were used for measuring GTPase activity as described above.

Pertussis toxin treatment of brain membranes. Pertussis toxin was preactivated by incubation with 35 mM dithiothreitol at 35° for 30 min as described by Ribeiro-Neto *et al.* [14]. Membranes (0.5–0.8 mg/mL) in 50 mM Tris pH 7.4, were incubated for 30 min at 32° in the presence of 5 mM NAD, 2.5 mM ATP, 10 mM thymidine and 70 μM DTT (control) or preactivated pertussis toxin (0.01–10 μg/mL). At the end of the ribosylation membranes were diluted with ice-cold 50 mM Tris–HCl pH 7.4, the mixture was centrifuged and the pellet was washed three times with the same buffer. For the GTPase assays membranes were resuspended in 50 mM HEPES and 0.5 mM EGTA pH 7.4.

Statistical analysis. All the results are expressed as means ± SD. Statistical analysis was performed by one way analysis of variance (ANOVA), and Student's *t*-test accepting $P < 0.05$ as significant. For ANOVA if the F values were significant the unpaired two-tailed Student's *t*-test was used to compare the treated and control groups.

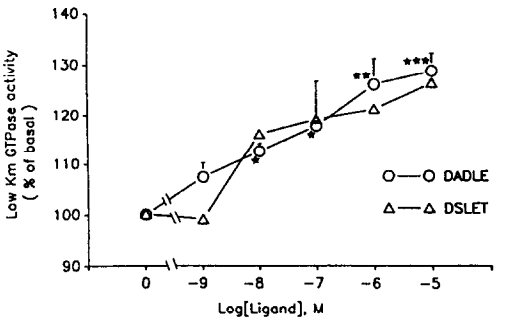

Fig. 1. Effect of δ-opioid agonists on the low K_m GTPase activity in rat brain membranes. Low K_m GTPase was assayed with various concentrations of the opioid agonists DADLE (○) and DSLET (△) as described in Materials and Methods. Data shown for DSLET are representative of two experiments. The results of DADLE are means ± SD from four separate experiments. ANOVA $P < 0.005$. Significant differences from the control values are indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. Data are calculated as % of basal GTPase activity which was 11.7 ± 0.9 pmol/min/mg protein.

RESULTS

The low K_m GTPase activity in rat brain membranes was measured in the presence of 150 mM NaCl. The basal levels of GTPase were 14 ± 4 pmol/min/mg (N = 22). The GTPase activity was stimulated by addition of DADLE in a concentration dependent manner (Fig. 1). Maximal stimulation 28% above control was achieved with 10 μM DADLE. Similarly, the δ-selective agonist DSLET produced 25% stimulation at 10 μM (Fig. 1). In contrast to the stimulation of GTPase by DADLE and DSLET the δ-opioid antagonist Diallyl-G was shown to inhibit the low K_m GTPase activity in brain membranes. This inhibition increased in a dose dependent manner reaching 28% at 1 μM and then increased moderately to 32% at 10 μM Diallyl-G (Fig. 2). Under the same experimental conditions the δ-opioid antagonist ICI174 864 [15], reported to possess negative intrinsic activity by inhibiting the low K_m GTPase activity in NG108-15 hybrid cells [9], inhibited also the basal GTPase activity of rat brain membranes by 12% at 1 μM and 23% at 10 μM ICI174 864 (Fig. 2). On the other hand, naloxone at concentrations of 0.1–10 μM did not produce any significant effect.

Diallyl-G (10 μM) blocked the stimulation of low K_m GTPase activity observed when rat brain membranes were assayed in the presence of increasing concentrations of the opioid agonist DADLE (Fig. 3). Additionally, the inhibition

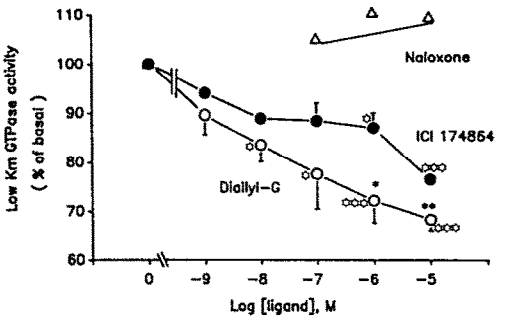

Fig. 2. Effect of δ-opioid antagonists on the low K_m GTPase activity in rat brain membranes. The low K_m GTPase was measured in the presence of various concentrations of Diallyl-G (○) and ICI174 864 (●) as described in Materials and Methods. Results are means ± SD of five independent experiments. ANOVA $P < 0.05$. Significant differences from the control values are indicated as ☆$P < 0.05$, ☆☆☆$P < 0.005$. Significant difference from the corresponding values of the ICI174 864 concentrations are designated as *$P < 0.05$, **$P < 0.01$. Data are calculated as % of basal GTPase activity (14.5 ± 0.7 pmol/min/mg protein).

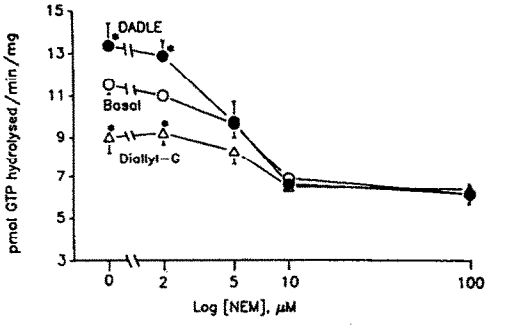

Fig. 4. Effect of NEM treatment on DADLE stimulation and Diallyl-G inhibition of GTPase activity in rat brain membranes. Brain membranes were treated with the indicated concentrations of NEM as described in Materials and Methods. Results represent the low K_m GTPase activity in the absence (basal) (○) or in the presence of 10 μM DADLE (●) or 10 μM Diallyl-G (△). Data are means ± SD values from three separate experiments. *$P < 0.05$, as compared to the corresponding basal values (Student's *t*-test).

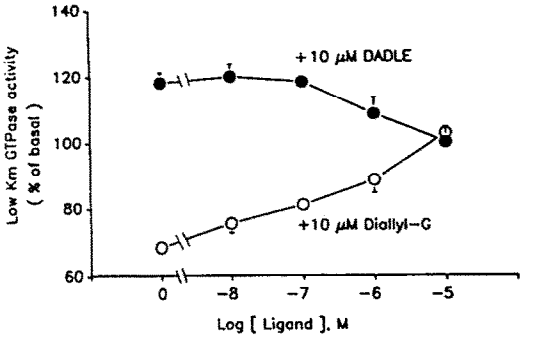

Fig. 3. Reversibility of DADLE-induced stimulation by Diallyl-G and Diallyl-G-induced inhibition in the presence of DADLE. The low K_m GTPase activity was measured with the indicated concentrations of DADLE (0.01–10 μM) in the presence of 10 μM Diallyl-G (○) or with increasing concentrations of Diallyl-G (0.01–10 μM) in the presence of 10 μM DADLE (●). Results represent the means ± SD of four separate experiments and are presented as a percentage of stimulation or inhibition of GTPase activity by the opioid ligands.

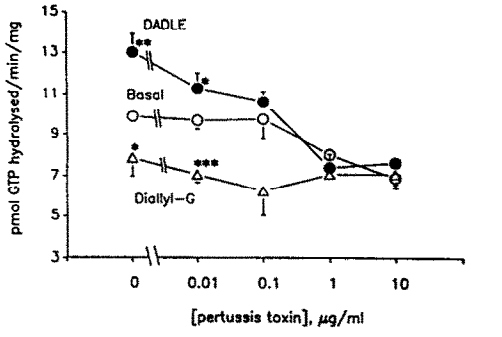

Fig. 5. Effect of pertussis toxin in rat brain plasma membranes on DADLE stimulation and Diallyl-G inhibition of GTPase activity. Brain membranes were exposed to the indicated concentrations of pertussis toxin as described in Materials and Methods, and tested for low K_m GTPase activity in the absence (basal) (○) or presence of 10 μM DADLE (●) or 10 μM Diallyl-G (△). Values are the means ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ as compared to the corresponding basal values (Student's *t*-test).

induced by increasing concentrations of Diallyl-G was reversed in the presence of 10 μM DADLE.

To examine whether the inhibitory effect of Diallyl-G was due to an alteration in the coupling of the δ-opioid receptor to the G-protein, rat brain membranes were treated with low concentrations of NEM, an alkylating reagent known to alkylate critical cysteine residues of G-proteins [16]. As shown in Fig. 4, the stimulation produced by the δ-opioid agonist DADLE was no longer evident above 5 μM of NEM. Similarly, the inhibitory effect of Diallyl-G was also reduced at the same concentrations of NEM, and was completely abolished at higher concentrations. The binding of [^{3}H]DADLE to the same membranes at 2–10 μM NEM concentrations was not affected (data not shown).

In order to assess further the effect of the δ-opioid antagonist Diallyl-G, on the low K_m GTPase activity, rat brain membranes were treated with thiol-activated pertussis toxin and NAD. As indicated in Fig. 5, the basal GTPase activity decreased at

concentrations of pertussis toxin over 0.1 μg/mL, indicating that part of this activity represents an activated form of the G-protein(s) [17]. DADLE stimulated the high affinity GTPase activity in membranes that have been treated with concentrations of pertussis toxin up to 0.01 μg/mL. This stimulation was abolished when membranes were treated with above 1 μg/mL of toxin. On the other hand, Diallyl-G did not exhibit any inhibitory effect on the GTPase activity after pretreatment of the membranes with pertussis toxin (1 μg/mL). The results from Figs 4 and 5 indicate that loss of the ability of G-proteins to interact with opioid receptors abolished the inhibitory effect of Diallyl-G on GTPase activity.

DISCUSSION

In this study we report that in rat brain membranes a δ-selective antagonist, the peptide Diallyl-G, inhibits the low K_m GTPase activity and we present some additional evidence on the interactions between the δ-opioid receptors and G-proteins in rat brain membranes. It has been reported previously that Diallyl-G, an analogue of α-casein exorphin [18], antagonizes the inhibitory effects produced by the δ-opioid agonist DTLET on the electrically stimulated mouse vas deferens, while it is practically without effect on the inhibition induced by the μ-agonist [D-Ala^2-*N*-methyl-Phe^4-Gly-ol]enkephalin in the same bioassay. In addition, the apparent affinity of Diallyl-G for displacing [^{3}H]naloxone in rat brain membranes is enhanced 3-fold in the presence of 100 mM NaCl [11].

Opioids of μ, δ and κ specificity have been demonstrated to stimulate the high affinity GTPase activity in several cultured cells and brain membranes [19–23]. Studies with NG108-15 hybrid cells enriched with δ-opioid receptors showed that agonist-occupied opioid receptors inhibit adenylate cyclase and that this inhibition is reciprocally related to the high affinity GTPase activity of the heterotrimeric G-proteins [3, 19, 20]. In addition, it was demonstrated that the non-selective opioid antagonist naloxone blocks both enzymatic activities [19, 22]. In isolated rat brain membranes the effects of opioid agonists on GTPase and on adenylate cyclase activities exhibit the characteristics of ligand binding to opioid receptor such as stereospecificity and inhibition by antagonists [21, 22].

In this report, we show that in rat brain membranes the δ-opioid agonists DADLE and DSLET stimulate in a concentration dependent manner the low K_m GTPase activity in the presence of 150 mM NaCl. Maximal stimulation of 28% for DADLE and 25% for DSLET is achieved at 10 μM. These values are in agreement with those reported by others for the GTPase activity in rat brain membranes [21]. On the other hand we found that under the same experimental conditions the δ-selective antagonist, Diallyl-G, inhibits in a concentration dependent way the GTPase activity. Maximal inhibition of 28% was reached at 1 μM, while naloxone between 0.1 and 10 μM did not exert any significant effect. Moreover, as shown in Fig. 2 ICI174 864 another δ-selective opioid antagonist [15] inhibited the GTPase activity in rat brain membranes in the presence of NaCl with a similar degree of maximal inhibition as that reported for NG108-15 hybrid cells [9]. These inhibitory effects of Diallyl-G and ICI174 864 have not been reported previously in rat brain membranes.

We further examined the effects of Diallyl-G on the DADLE-induced stimulation of GTPase and found that this stimulation was antagonized by increasing concentrations of Diallyl-G and reciprocally that the inhibition produced by Diallyl-G was reversed by increasing concentrations of DADLE (Fig. 3). These data suggest that δ-opioid receptor stimulation of G-proteins may be inhibited by specific receptor antagonists; thus, one could speculate that Diallyl-G and DADLE possibly compete for the same site on the receptor.

In preliminary experiments we attempted to examine the effects of δ-opioid antagonists on adenylate cyclase in NG108-15 hybrid cells. These experiments have shown that both Diallyl-G and ICI174 864 at concentrations up to 10 μM are without any effect on adenylate cyclase. However, Diallyl-G and ICI174 864 act in a similar manner to naloxone and reverse the inhibition of adenylate cyclase induced by DADLE (100 nM) with half-maximal concentrations of 500 and 60 nM, respectively (unpublished results). From these preliminary data it is difficult to reconcile the effects of the above antagonists on adenylate cyclase and GTPase. Recently, a growing body of evidence supports the notion that βγ like α subunits can interact functionally with different effector systems [24, 25]. Therefore, it is not unreasonable to suggest that these effects in NG108-15 cells are conducted via different subtypes or subunits of G-proteins.

We assessed the ability of Diallyl-G to interfere in the functional coupling between receptors and G-proteins after treatment of the membranes with low concentrations of NEM or by ADP ribosylation with pertussis toxin. Both modifications impaired the ability of G-protein(s) to interact with the receptor and abolished the agonist-induced stimulation and the antagonist-induced inhibition of GTPase. These modifications also reduced substantially the basal GTPase activity indicating that part of this activity is due to activation of G-proteins by unoccupied receptors [17, 26]. Since after pertussis toxin treatment the inhibitory effect of Diallyl-G was eliminated, we can conclude that the G-protein involved is pertussis toxin-sensitive, possibly the Gi or Go [27].

Taken together these findings suggest that antagonists sometimes exert effects *per se* and exhibit a distinct activity possibly interfering in the uncoupling of the receptor with the G-protein(s). A recent report has shown that binding of the antagonist atropine to muscarinic acetylcholine receptor dissociates the receptor–G-protein complexes [28]. Similar regulatory effects of G-proteins by antagonist binding were also observed with the A_1-adenosine [29] and dopamine receptors [30]. Recently, a novel mechanism has been proposed, that antagonists bound to receptors coupled to G-proteins induce conformational changes that favour uncoupling of the receptor from the G-proteins [31].

From the present observations we can conclude

that the GTPase inhibition induced by δ-opioid antagonists Diallyl-G and ICI174864 rat brain membranes is receptor-mediated and not simply a non-specific interaction with the G-proteins. These effects of antagonists with negative intrinsic activity may either be a consequence of hindrance of the spontaneous association of G-proteins with empty receptors, or the fact that antagonist-occupied receptors can block receptor activation and bind more tightly to inactivate forms of G-proteins, such as the GDP-$\alpha\beta\gamma$ conformation, in which case they could inhibit the basal GTPase activity. It is an important question whether antagonist inhibition of GTPase has any physiological significance for signalling systems involving G-proteins. The nature of the mechanism underlying this inhibition of GTPase activity by opioid antagonists is under further investigation.

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