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DIFFERENTIAL COUPLING OF Mu-COMPETITIVE AND Mu-NONCOMPETITIVE DELTA OPIATE RECEPTORS TO GUANINE NUCLEOTIDE BINDING PROTEINS IN RAT BRAIN MEMBRANES

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Abstract—The effects of fentanyl isothiocyanate (FIT) and pertussis toxin on the binding of [^{3}H]D-Ala^2, D-Leu^5-enkephalin ([^{3}H]DADLE) to rat brain membranes were compared. The site of action of pertussis toxin was confirmed by the labeling of a 41,000 dalton protein in the presence of [α-^{32}P]NAD. Both reagents produced inhibition of [^{3}H]DADLE binding when binding was assayed in 10 mM Tris–HCl buffer alone. FIT inhibited binding 91% whereas pertussis toxin treatment resulted in 27% inhibition. However, when binding was assayed in 10 mM Tris–HCl containing SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM guanosine triphosphate), inhibition due to both reagents was attenuated markedly: 66% for FIT and 5% for toxin. In addition, both reagents markedly potentiated enhancement of binding by SMG. Thus, the effects of FIT and pertussis toxin on [^{3}H]DADLE binding were qualitatively similar. These results suggest that FIT and pertussis toxin affect binding of [^{3}H]DADLE to the same population of delta receptors. This was further supported by the observation that treatment of membranes with FIT prior to pertussis toxin treatment blocked the effect of toxin on [^{3}H]DADLE binding. FIT selectively eliminates the SMG-insensitive, mu-competitive [^{3}H]DADLE binding site [Rothman *et al.*, *Neuropeptides* **4**, 201 (1984); Rothman *et al.*, *Molec. Pharmac.* **27**, 399 (1985)]. These results indicate that this site is coupled to G protein substrates for pertussis toxin and that it mediates the inhibitory effects of delta ligands on adenylate cyclase. The FIT-insensitive, SMG-sensitive mu-noncompetitive [^{3}H]DADLE site appears not to be coupled to G protein substrates for pertussis toxin and may mediate some other biochemical effects of delta ligands.

We have proposed previously the existence of two distinct [^{3}H]DADLE§ binding sites in rat brain [1–5]. Autoradiographic studies in rat striatum revealed a diffusely distributed site which is insensitive to the ionic environment of the medium [1, 2]. Present in patches and a subcallosal streak is an additional site which is co-localized with mu binding [1]. Binding to this site is enhanced markedly by the presence of SMG [100 mM NaCl, 3 mM manganese acetate, and 2 μM guanosine triphosphate (GTP)]. The density of patch sites is decreased by 6-hydroxy dopamine lesioning of the substantia nigra, while the diffusely distributed sites are unaffected [5].

Rothman and Westfall have examined the mechanism by which mu ligands inhibit binding of delta ligands [6–8] and identified mixed competitive/noncompetitive inhibition. They concluded that a two-site allosteric model best fit the binding data [9]. In light of the autoradiographic results described above, they proposed that [^{3}H]DADLE labels two sites that are distinguished by the mechanism by which mu ligands inhibit binding. Mu ligands are competitive inhibitors at one site but are noncompetitive inhibitors at the other site. The noncompetitive site was postulated to be composed of allosterically coupled mu and delta binding sites (mu/delta opiate receptor complex).

Fentanyl isothiocyanate (FIT) [10] eliminates the high affinity delta binding site by directly acylating a receptor nucleophile. This produces membranes enriched in a low affinity [^{3}H]DADLE binding site [3]. The mu agonist oxymorphone noncompetitively inhibits [^{3}H]DADLE binding to the remaining low affinity site. The low affinity [^{3}H]DADLE binding site is therefore not the "pure" mu receptor and supports the hypothesis that this site is a delta binding site which is allosterically coupled to a mu site. Autoradiographic analysis of striatal sections revealed that FIT eliminates the diffusely distributed [^{3}H]DADLE binding site leaving the patch site intact [4]. Thus, the patch site (originally proposed to be interconvertible with mu sites [1, 11]) is synonymous with the delta binding site of the complex. Co-localization of mu binding to the patch [1] and co-destruction of mu and delta binding by 6-hydroxy dopamine lesioning [5] further support the notion of a receptor complex distinct from a "pure" delta binding site. To reflect the mechanism of interaction

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§ Abbreviations: DADLE, D-Ala^2,D-Leu^5-enkephalin; FIT, fentanyl isothiocyanate; MOPS, 3-[*N*-morpholoino]-propanesulfonic acid; and SMG, 100 mM NaCl, 3 mM manganese acetate, and 2 μM guanosine triphosphate.

of mu and delta ligands at these sites, we have termed the delta binding site at which mu ligands are noncompetitive inhibitors the "mu-noncompetitive delta site" (formerly called type-1-delta). The delta site at which mu ligands are competitive inhibitors is termed the "mu-competitive delta site" (formerly called type-2-delta).

Delta opiate receptors mediate inhibition of cellular adenylate cyclase activity [12, 13] via interaction with the inhibitory guanine nucleotide binding protein, G_i [14]. The existence of two types of delta binding sites in rat brain membranes brings into question which site mediates inhibition of adenylate cyclase. The organism *Bordetella pertussis* produces an endotoxin which has proven to be a useful tool in studies of receptor mechanisms. The effects of pertussis toxin on receptor-regulated adenylate cyclase systems have been well documented. The toxin catalyzes ADP-ribosylation of a 41,000 dalton subunit (α subunit) of G_i [15, 16]. This covalent modification impairs communication of delta opiate receptors with adenylate cyclase, abolishing receptor-mediated inhibition of cyclase activity. In addition, pertussis toxin has effects on ligand binding. Several groups [17–19] have studied the effects of pertussis toxin treatment on opiate binding to NG108-15 neuroblastoma-glioma cell membranes. Toxin treatment decreases binding of opiate agonists but not antagonists. ADP-ribosylation of $G_{i\alpha}$ is believed to prevent coupling of receptor to G_i, producing a lower affinity receptor state [17]. Here we attempt to utilize the selective effects of FIT and pertussis toxin on ligand binding in order to determine which delta binding site of rat brain membranes is coupled to G protein substrates for pertussis toxin.

MATERIALS AND METHODS

Membrane preparation. The crude synaptosomal (P_2) membrane fraction of rat brain was used for these studies. Male Sprague–Dawley rats (Taconic Farms), 180–250 g, were killed by decapitation. Whole brains minus cerebellum were placed in ice-cold 10 mM Tris–HCl, pH 7.4, containing 320 mM sucrose (Tris/sucrose buffer) and homogenized in a Potter–Elvehjem homogenizer by ten strokes of a motor-driven Teflon pestle in a volume of 10 ml/g tissue wet weight. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fractions were saved. The pellets were resuspended by vortexing in 2 ml/g ice-cold Tris/sucrose and centrifuged again at 1000 *g* for 10 min. The combined supernatant fractions were centrifuged at 31,000 *g* for 15 min. The pellets were resuspended by vortexing in 3 ml/g 10 mM Tris–HCl, pH 7.4, and the suspension was allowed to incubate at room temperature for 15 min. Following centrifugation at 31,000 *g*, the pellets were resuspended by gentle Potter–Elvehjem homogenization to a final volume of 1.53 ml/g in 10 mM Tris–HCl, pH 7.4. Aliquots were stored at −80° until use. Protein was determined by the method of Lowry *et al.* [20].

Treatment of membranes with FIT. Membranes were treated with FIT by a method modified from that previously described [3]. Rat brain membranes were suspended to a final concentration of 650 μg protein/ml in a buffer consisting of MOPS, pH 7.4, 3 mM manganese acetate, and 1 or 5 μM FIT hydrochloride. Incubation was carried out for 60 min at 25°. Controls were treated with ethanol, which was solvent for FIT. Membranes were then washed three times by repeated centrifugation and resuspension in 10 mM Tris–HCl, pH 7.4, at 25°.

Treatment of membranes with pertussis toxin. Pertussis toxin was dissolved to a concentration of 2 mg/ml in a vehicle consisting of 0.1 M potassium phosphate buffer, pH 7.4, and 2 M urea. This stock solution was stored at −70° until use. Rat brain membranes (500–600 μg protein/ml) were treated with vehicle or pertussis toxin for 15 min at 37° in 50 mM Tris–HCl, pH 7.4 containing 1 mM ATP, 10 mM thymidine, 10 μM NAD, 0.2 mM GTP, 5 mM dithiothreitol (DTT), and 2 mM $MgCl_2$. Toxin was used at a final concentration of 50 or 100 μg/ml in the treatment medium. After incubation, membranes were washed twice in ice-cold 10 mM Tris–HCl, pH 7.4, and resuspended in 10 mM Tris–HCl, pH 7.4, at 25°. Pertussis toxin was the gift of Dr. J. Adolfo Garcia-Sainz.

[^{3}H]DADLE binding assays. [^{3}H]DADLE binding assays were carried out using 325 μg membrane protein in a final incubation volume of 0.5 ml. The concentration of labeled ligand was 2 nM. Levallorphan (10 μM) was used to determine nonspecific binding. Binding was carried out under two conditions: (a) 10 mM Tris–HCl, pH 7.4, or (b) 10 mM Tris–HCl, pH 7.4, containing SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM GTP). Incubation was for 90 min at 25°. Assays were terminated by dilution of assay mixture with 5.5 ml of ice-cold 10 mM Tris–HCl, pH 7.4, and rapid filtration through Whatman GF/B glass fiber filters. Filters were washed twice with 5.5 ml of ice-cold buffer. Filters were then counted in Ultrafluor (National Diagnostics, Somerville, NJ) scintillation fluid in a Packard scintillation spectrometer following overnight extraction of bound radioactivity from the filters. [^{3}H]D-Ala^2,D-Leu^5-Enkephalin was obtained from New England Nuclear (Boston, MA).

Stastistical analysis. The effect of the reagent (FIT or pertussis toxin) was determined as compared to vehicle-treated controls when [^{3}H]DADLE binding was assayed in the presence or absence of SMG. This value was expressed as percent inhibition. The percent inhibitions were then subjected to a two-sample *t*-test in order to determine whether the value obtained in the absence of SMG differed significantly from that in the presence of SMG.

RESULTS

Table 1 shows the effect of 5 μM FIT treatment on [^{3}H]DADLE binding. Membranes were treated with FIT or vehicle, and [^{3}H]DADLE binding was measured under two assay conditions. The inhibitory effect of FIT was most pronounced when binding was assayed in 10 mM Tris buffer alone. Inhibition was 91% compared to vehicle-treated controls. When SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM GTP) was added to the Tris buffer, [^{3}H]DADLE binding was enhanced in both control and FIT-treated samples. Under this condition, the

Table 1. Effect of FIT on [^{3}H]DADLE binding to rat brain membranes

Treatment	Specific binding (cpm)		% Enhancement by SMG
	Assay condition		
	Tris	Tris + SMG	
Control	359 ± 117	875 ± 224	154 ± 18
FIT	34 ± 11 (−91 ± 3)*	300 ± 38 (−66 ± 5)*	1095 ± 503

Rat brain membranes were treated with 5 μM FIT as described in Materials and Methods. Binding of 2 nM [^{3}H]DADLE was assessed in either 10 mM Tris–HCl, pH 7.4, alone (Tris) or 10 mM Tris–HCl, pH 7.4, containing SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM GTP) as described in Materials and Methods. Values are cpm of specific binding and are averages of three separate experiments ± SE. Each experiment was carried out in triplicate. Numbers in parentheses are percent effect of FIT compared to controls incubated in the absence of FIT (± SE). Though there was some variation in cpm values between experiments, the magnitude of inhibition by FIT across experiments was consistent. Percent enhancement by SMG (average ± SE; N = 3) also varied across experiments but was always greater in FIT-treated membranes.

* Significant difference in the effect of FIT in the presence and absence of SMG (T = 4.45, df = 4, P = 0.011).

Table 2. Effect of pertussis toxin on binding of [^{3}H]DADLE to rat brain membranes

Treatment	Specific binding (cpm)		% Enhancement by SMG
	Assay condition		
	Tris	Tris + SMG	
Vehicle	581 ± 139	1488 ± 359	162 ± 37
Toxin	422 ± 118 (−27 ± 6)*	1417 ± 328 (−5 ± 3)*	259 ± 48

Rat brain membranes were treated with pertussis toxin as described in Materials and Methods. Incubations with 2 nM [^{3}H]DADLE were carried out either in 10 mM Tris–HCl, pH 7.4, alone (Tris) or in 10 mM Tris–HCl, pH 7.4, containing SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM GTP) as described in Materials and Methods. Values are cpm of specific binding and are averages of four separate experiments ± SE. Each experiment was carried out in triplicate. Numbers in parentheses are percent effect of toxin treatment compared to vehicle-treated controls (± SE). Though there was some variation in cpm values between experiments, the magnitude of inhibition by toxin was consistent. Percent enhancement by SMG (average ± SE; N = 4) also varied across experiments but was always greater in toxin-treated membranes.

* Significant difference in the effect of toxin in the presence and absence of SMG (T = 3.88, df = 6, P = 0.008).

Table 3. Effect of pertussis toxin on [^{3}H]DADLE binding to FIT-pretreated membranes

Treatment	Specific binding (cpm)		% Enhancement by SMG
	Assay condition		
	Tris	Tris + SMG	
FIT/Vehicle	213 ± 25	855 ± 60	311 ± 77
FIT/Toxin	255 ± 16 (+22 ± 22)	768 ± 91 (−11 ± 5)	200 ± 17

Membranes were treated with 1 μM FIT, washed, and resuspended in toxin treatment buffer. Either pertussis toxin (100 μg/ml) or vehicle was added, and incubation was carried out as described in Materials and Methods. Binding of 2 nM [^{3}H]DADLE was assessed in Tris buffer alone (Tris) or Tris buffer containing SMG (100 mM NaCl, 3 mM manganese acetate, and 2 μM GTP). Values are cpm specific binding and are averages of two experiments ± SE. Each experiment was carried out in triplicate. Numbers in parentheses are percent effect of pertussis toxin compared to vehicle-treated controls (± SE). Toxin had no significant effect under either assay condition. The difference in effect of toxin between assay conditions was not significant (T = 1.45, df = 2, NS). In each experiment, percent enhancement by SMG (average ± SE; N = 2) was greater in vehicle-treated membranes.

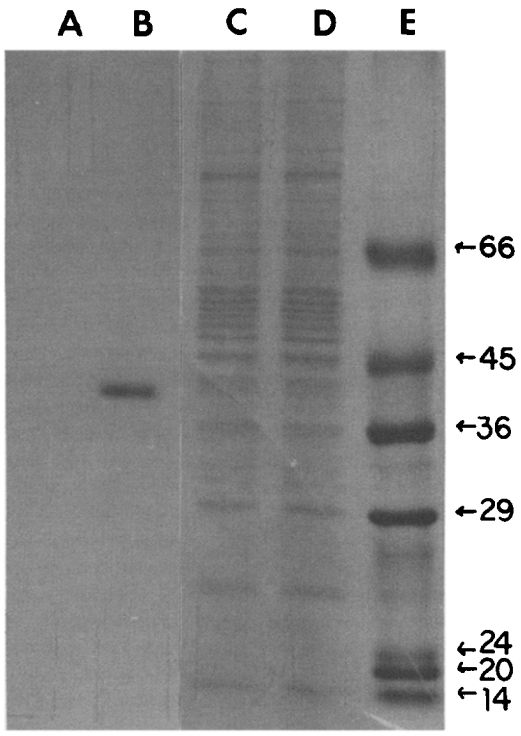

Fig. 1. ADP-ribosylation of G proteins by pertussis toxin. Rat brain membranes (100 μg protein) were treated with vehicle or pertussis toxin (20 μg) for 15 min at 37° in 0.2 ml of 50 mM Tris–HCl, pH 7.4, containing 1 mM ATP, 10 mM thymidine, 10 μM [α-^{32}P]NAD (16.8 Ci/mmol), 0.2 mM GTP, 5 mM DTT, and 2 mM $MgCl_2$. Membranes were then washed by resuspension in 800 μl of 50 mM Tris–HCl, pH 7.4, followed by pelleting in a microcentrifuge. The pellet was solubilized in sample treatment buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [21]. Gel was stained with Coomassie blue, dried onto filter paper, and applied to Kodak X-OMAT X-ray film for autoradiographic analysis. Lanes A + B, autoradiograph; lanes C + D, Coomassie blue staining pattern; and lane E, molecular weight standards (bovine serum albumin, 66,000; egg ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; bovine trypsinogen, 24,000; soybean trypsin inhibitor, 20,100; and bovine α-lactalbumin, 14,200). Treatments were as follows: lanes A + C, vehicle minus toxin; lanes B + D, toxin-treated.

inhibitory effect of FIT was less pronounced. Inhibition was 66% compared to vehicle-treated control. In addition, by virtue of the greater inhibitory effect in the absence of effectors, the enhancement of [^{3}H]DADLE binding by SMG was potentiated markedly by FIT treatment. Enhancement was 154% in control membranes but was increased to 1095% upon treatment with FIT.

The site of action of pertussis toxin in rat brain membranes was verified by radiolabeling experiments in which [α-^{32}P]NAD was used as substrate (Fig. 1). Incubation of rat brain membranes with toxin and [α-^{32}P]NAD, followed by sodium dodecyl sulfate gel electrophoresis of solubilized protein, resulted in labeling of a 41,000 dalton protein. Membranes incubated with [α-^{32}P]NAD in the absence of pertussis toxin showed no such labeling. This corresponds to pertussis toxin substrates found in other cells and confirms that toxin specifically modifies proteins under the conditions used in these studies.

The effect of pertussis toxin treatment on [^{3}H]DADLE binding is shown in Table 2. Membranes were treated with either pertussis toxin or vehicle, and [^{3}H]DADLE binding was then assayed in Tris buffer alone or Tris buffer containing SMG. Compared to vehicle-treated controls, pertussis toxin reduced [^{3}H]DADLE binding 27% when assayed in Tris buffer alone. As observed with FIT treatment, addition of SMG markedly reduced inhibition of [^{3}H]DADLE binding by toxin. Also, enhancement of binding by SMG was potentiated by toxin treatment. Percent enhancement was 162% in vehicle-treated controls but was increased to 259% in toxin-treated membranes.

Thus, the effects of FIT and toxin treatment on [^{3}H]DADLE binding were qualitatively similar. This indicates that the two reagents are affecting the same [^{3}H]DADLE binding site. Table 3 shows the result of the combination of the two reagents. Membranes were first treated with FIT and then incubated with pertussis toxin. In contrast to membranes treated with toxin only, prior treatment of membranes with FIT resulted in no significant effect of toxin under either assay condition. Importantly, the enhancement of [^{3}H]DADLE binding by SMG, though still present, was no longer potentiated by toxin treatment when membranes were first treated by FIT. Thus, the effects of FIT and toxin were not additive, with FIT eliminating the effect of toxin. This supports the notion that FIT and pertussis toxin exert their effects on the same population of delta receptors. Furthermore, this shows that the delta binding sites remaining after FIT treatment are not sensitive to inhibition by toxin treatment.

DISCUSSION

The effects of FIT on [^{3}H]DADLE binding to membranes and slide-mounted sections have been investigated thoroughly [3, 4, 22]. The site eliminated by FIT has the following properties: (1) high affinity site, (2) binding not enhanced by SMG, (3) interacts competitively with mu ligands ("pure" delta site), and (4) diffusely localized in striatal sections. The site which is not affected by FIT has the following properties: (1) lower affinity, (2) binding enhanced by SMG, (3) interacts noncompetitively with mu ligands; allosterically coupled to mu binding site, and (4) discretely localized to striatal patches and subcallosal streak; co-localized with mu sites.

Sensitivity to FIT or ions reveals little about function. Delta opiate receptors are coupled to adenylate cyclase inhibition via G_i [12–14]. ADP-ribosylation of G_i by pertussis toxin inhibits opiate agonist binding to NG108-15 membranes [17–19]. Since the effect of toxin is not at the opiate binding site itself, only that population of receptors coupled to G proteins will be affected. Thus, inhibition of binding by pertussis toxin treatment is a selective criteria for receptors which mediate their effects through G protein sub-

strates for pertussis toxin. Therefore, in combination with the effects of pertussis toxin, the effects of FIT and ions can be used to infer function of these two [^{3}H]DADLE binding sites.

Incubation of rat brain membranes with pertussis toxin in the presence of [α-^{32}P]NAD resulted in labeling of a 41,000 dalton protein. This protein corresponds to the α subunit of G_i and is present in C_6 glioma cells [15, 16] and NG108-15 neuroblastoma-glioma cells [23]. Recently, bovine brain membranes have been shown to contain an additional GTP binding protein, G_0. This protein has a molecular weight of 39,000 daltons and is also ADP-ribosylated by pertussis toxin [24]. G_0 is also present in rat brain membranes. Abood *et al.* [25] showed that pertussis toxin treatment of rat brain membranes in the presence of [α-^{32}P]NAD results in labeling of two proteins, $M_r = 41{,}000$ (G_i) and $M_r = 39{,}000$ (G_0). Since G_0 is the major G protein in brain, both G_i and G_0 may be labeled on our gels but are not resolved under these conditions.

FIT selectively eliminates the mu-competitive, SMG-insensitive [^{3}H]DADLE binding site. Like FIT, pertussis toxin produced differential inhibition of [^{3}H]DADLE binding when assayed in the presence or absence of SMG. In the absence of SMG, binding is largely to the mu-competitive site, whereas in the presence of SMG binding is to both the mu-competitive and mu-noncompetitive sites. The greatest inhibition occurred in the absence of SMG. Also like FIT, pertussis toxin potentiated the enhancing effect of SMG. This is due to a decrease in binding to the population of receptors which is insensitive to SMG, enriching the system with the responsive population. Since FIT selectively eliminated the mu-competitive site, and the effects of pertussis toxin were qualitatively similar to those of FIT, the site affected by pertussis toxin was likely the mu-competitive site. This was confirmed by the observation that pretreatment with FIT eliminated the effect of toxin.

The manner in which pertussis toxin treatment decreased [^{3}H]DADLE binding was not determined. However, it has been shown that pertussis toxin treatment of NG108-15 cells causes a decrease in muscarinic cholinergic and α-adrenergic agonist binding [26] as well as opiate agonist binding [17] by decreasing binding affinity with no effect on receptor number. Presumably, pertussis toxin has the same mechanism in brain membranes. This would be different from the effect of FIT, which drastically decreases the number of mu-competitive delta sites and reflects the difference in the mode of action of the two agents: FIT directly acylating the binding site and pertussis toxin modifying a protein to which the receptor is coupled.

The results of this study suggest differential coupling of the mu-competitive and mu-noncompetitive [^{3}H]DADLE binding sites to G protein substrates for pertussis toxin. Since G_i is a substrate for pertussis toxin and mediates delta inhibition of adenylate cyclase in rat brain, the mu-competitive delta site may be responsible for this activity. This site would therefore be synonymous with the delta receptor of the NG108-15 neuroblastoma-glioma cell line where the effects of opiates on adenylate cyclase activity have been studied extensively. The mu-noncompetitive [^{3}H]DADLE binding site is apparently not coupled to G_i. This binding site may mediate some other biochemical effect of opiates via some other transduction mechanism. This site probably has some specialized function by virtue of its interaction with mu sites.

This contradicts an earlier report [1] in which we suggested that SMG promoted coupling of the striatal patch (mu-noncompetitive) site to adenylate cyclase. This suggestion was based on the observation that conditions which promoted labeling of the patch site with [^{3}H]DADLE were also conditions under which delta receptor-mediated inhibition of striatal adenylate cyclase could be measured. Since both delta receptor types were labeled under these conditions, it was difficult to otherwise associate a receptor type with function. The use of pertussis toxin in the present study allowed a direct effect on binding via a functional component of the transduction system. Therefore, only binding to functionally coupled receptors was affected. It is conceivable that ions and guanine nucleotides could have effects on coupling of the diffusely localized, mu-competitive delta site to adenylate cyclase while simultaneously enhancing binding of [^{3}H]DADLE to the mu-noncompetitive delta site by an independent mechanism [11].

Though delta opiate receptors are found in various regions throughout the rat brain [27, 28], Loh and associates have shown that opiate inhibition of adenylate cyclase activity can be demonstrated only in the striatum and cortex [25, 29]. In addition, G_i and G_0 were found throughout the brain, with the highest levels found in striatum and cortex [25]. Therefore, the effects of pertussis toxin on [^{3}H]DADLE binding may also be the result of ADP-ribosylation of G_0. Using pertussis toxin in conjunction with specific antibodies, Milligan *et al.* [30] have shown that the major G protein in NG108-15 membranes is G_0. Thus, it may be that the mu-competitive site interacts with G_0 in addition to G_i and mediates some as yet unknown function in brain.

Further studies on functional subclassification of delta sites must include direct activation of transduction systems and resolution of their components.

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