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Interactions of Ligands with Morphine and Enkephalin Receptors are Differentially Affected by Guanine Nucleotide

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SUMMARY

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The effects of cations and GTP on morphine (μ) and enkephalin (δ) receptors were examined by using binding assays of [^{3}H]naloxone to rat brain membrane preparations and [^{3}H]diprenorphine or [^{3}H]naloxone to neuroblastoma cell membranes. The potencies and Hill coefficients (n) of many opiate agonists and opioid peptides in competing with the binding of the labeled antagonist are reduced by Na^+ (100 mM) and GTP (0.1 mM). These effects are qualitatively similar for both subtypes of opiate receptors. However, quantitatively, the effects of GTP are much more profound for morphine receptors than for enkephalin receptors and the effects of Na^+ are dependent upon the type of labeled antagonist used rather than upon receptor type. Na^+ does not alter the affinity of opiate antagonists. GTP reduces the affinity of naloxone to morphine-binding sites by a factor of 2.5. Mg^{2+} (5 mM) increases the potency of opiate agonists and enkephalins for both receptor sites. The combination of Na^+, GTP, and Mg^{2+} further reduces the affinity of enkephalins and opiate agonists for enkephalin-binding sites and the affinity of Met- and Leu-enkephalin for morphine-binding sites. However, the combination of Na^+, GTP, and Mg^{2+} partially restores the affinity of [D-Ala^2, Leu^5]- and [D-Ala^2, D-Leu^5]enkephalin and morphine for the morphine-binding sites. These differential effects of cations and nucleotide further emphasize the differences that exist between morphine and enkephalin receptors and indicate the complex interactions of cations and nucleotides with opiate-binding sites.

INTRODUCTION

Recently, we have identified two subtypes of opiate receptors in rat brain membrane preparations by using radioactively labeled opiates and enkephalin analogues (1, 2). One of these binds morphine and naloxone with an affinity higher than for enkephalins (referred to as morphine receptors); it can be labeled and characterized by using low concentrations of ^{3}H-labeled dihydromorphine and naloxone (1), or ^{125}I-labeled Sandoz FK 33-824 [Tyr-D-Ala-Gly-N$^\alpha$-Me-Phe-Met(O)ol] (2). The other site, which has preferential affinity for enkephalins, was identified by the specific binding of low concentration of ^{125}I-labeled [D-Ala^2, D-Leu^5]enkephalin (referred to as enkephalin receptors). Lord *et al.* (3) have also identified two subtypes of opiate receptors in guinea-pig brain membrane by using a ^{3}H-labeled opiate antagonist and enkephalin, and they have classified these as μ and δ receptors, respectively.

Cultured neuroblastoma cells provide a useful model for studying the molecular and cellular actions of opiates and enkephalins. Several of these cell lines possess large numbers of opiate receptors. Neuroblastoma-glioma hybrid NG108-15 and neuroblastoma cells N4TG1 have been very useful in studying enkephalin-receptor interactions (4, 5), effects of opiates and enkephalins on adenylate cyclase (6-9), ganglioside synthesis (10), and cluster formation of opiate receptors (11-13). The opiate receptors on these cells resemble enkephalin (δ) receptors observed in brain membranes with respect to their affinity for enkephalins and opiates. Because of the homogeneity of the cultured cells, they are particularly useful in the detailed characterization of enkephalin (δ) receptors.

Opiate antagonists such as naltrexone and naloxone have higher affinity toward morphine than enkephalin receptors (2, 3). Previous studies using low concentrations of [^{3}H]naloxone and [^{3}H]naltrexone have most likely examined only the morphine (μ) receptors. Sodium ions reduce the potency of opiate agonists and opioid peptides in competing with the binding of [^{3}H]naloxone to brain membrane (14-16). GTP increases the dissociation of receptor-bound labeled opioid ligands both in brain membrane (17) and neuroblastoma cell membranes (18). Under certain conditions, GTP preferentially affects

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the labeled agonist binding to brain membrane preparations (19).

[^{3}H]Naloxone binding to rat brain membranes was used as a marker for morphine receptors. Both [^{3}H]-naloxone and [^{3}H]diprenorphine, an opiate antagonist with extremely high affinity to morphine and enkephalin receptors, were employed as markers of enkephalin receptors in neuroblastoma cells. Recently, the binding of many opiates and enkephalins to enkephalin and morphine receptors has been compared by using these two binding assays (20). According to their relative binding potencies and the effects of Na^+ and GTP on the binding to these two receptors, opiate and enkephalin ligands were classified into seven classes (20).

In the present studies, we describe the differential effects of GTP on the potency of opioids in inhibiting the binding of ^{3}H-labeled antagonists to enkephalin receptors in neuroblastoma cells and of [^{3}H]naloxone to brain membrane. These studies reveal that the two major receptor types differ not only in their affinity for certain opiates and opioid peptides, but also in their susceptibility to GTP.

MATERIALS AND METHODS

All enkephalins and opiates were obtained from sources described previously (1, 2, 21). [^{3}H]Naloxone (23 Ci/mmole) and [^{3}H]diprenorphine (9 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.) and Amersham Corporation (Arlington Heights, Ill.), respectively. Rat (Sprague-Dawley) brain membranes were prepared by differential centrifugation in isotonic sucrose solution as described previously (1, 2). Mouse neuroblastoma cells (N4TG1) were grown in Dulbecco's modified Eagle's minimum essential medium with 5% fetal bovine serum. The cells were detached from monolayer after confluence (about 4 days) with 1 mM EDTA for 5 min. Cell membranes were prepared by homogenization using a Polytron PT-10 in 50 mM Tris·HCl (pH 7.7) or isotonic sucrose solution in 10 mM Tris·HCl (pH 7.7) and centrifuged at 40,000 × *g* for 30 min. The pellets were suspended in 50 mM Tris·HCl buffer for binding assays.

Binding assays (24°, 60 min) were performed essentially as described previously by using a filtration method (1, 2). The concentrations of labeled ligand were 0.4 nM of [^{3}H]naloxone to rat brain membranes and 0.5 nM of [^{3}H]diprenorphine or 2 nM of [^{3}H]naloxone to neuroblastoma cell membranes. The total incubation volume was 2 ml. Nonspecific binding was determined in the presence of 1 μM naloxone or [D-Ala^2, D-Leu^5]enkephalin. The binding reaction was stopped by rapidly filtering through GF/C glass filter, followed by washing twice with 10 ml of ice-cold Tris·HCl buffer under vacuum. All assays were performed in duplicate and the variability of the duplicates was usually less than 10% of the mean. Protein concentration was determined by the method of Lowry *et al.* (22), using crystalline bovine serum albumin as a standard.

RESULTS

High-Affinity Binding of Diprenorphine to Enkephalin Receptors

Diprenorphine, a potent opiate antagonist, inhibits the binding of ^{125}I-labeled [D-Ala^2,D-Leu^5]enkephalin to rat brain membrane preparations. The concentration which reduces the binding by 50% (IC_{50} value) is 0.18 nM (Fig. 1), suggesting that diprenorphine is a high-affinity ligand for enkephalin-binding sites. It is also known that diprenorphine binds to morphine receptors with about equally high affinity (23). Its affinity toward enkephalin receptors is about 100 times greater than that of naloxone (1). Hill analysis reveals a Hill coefficient (*n*, slope of Hill plot) value of 1 (Fig. 1).

An equilibrium saturation binding curve (Fig. 2) shows that [^{3}H]diprenorphine binds to neuroblastoma cell membranes with high affinity and in a saturable manner. The Scatchard plot indicates a linear slope and suggests a homogeneous population of binding sites. The apparent K_d value is 0.14 nM and the maximum binding capacity is 420 fmoles/mg of protein. The binding of [^{3}H]diprenorphine to cell membranes is inhibited by low concentrations of enkephalins. The biologically less active stereoisomer, dextrorphan, is about 200 times less active than levorphanol (Table 1). Morphine is about 100 times less active than enkephalin. These data thus suggest that [^{3}H]diprenorphine binds to enkephalin receptors in neuroblastoma cell membranes.

Effect of Na^+, GTP, and Mg^{2+} on the binding of [^{3}H]-diprenorphine to neuroblastoma cell membranes and of [^{3}H]naloxone to brain membranes

Sodium ions (100 mM), GTP (0.1 mM), and Mg^{2+} (5 mM) alone do not alter significantly the binding of [^{3}H]-diphrenorphine to neuroblastoma cell membranes, whereas the combination of Na^+, GTP, and Mg^{2+} significantly decreases the binding (Table 2). In contrast, Na^+ increases and GTP decreases the binding of [^{3}H]naloxone to rat brain membranes. Mg^{2+} and the combination of Na^+, GTP, and Mg^{2+} do not alter the binding of [^{3}H]-naloxone (Table 2) to rat brain membranes.

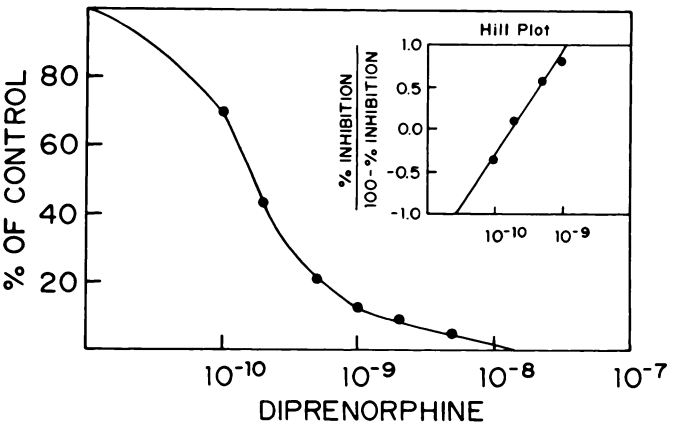

FIG. 1. *Competition curve of diprenorphine in inhibiting the binding of ^{125}I labeled [D-Ala^2,D-Leu^5]enkephalin to rat brain membrane preparations*

^{125}I-Labeled [D-Ala^2, D-Leu^5]enkephalin (0.1 nM) was incubated with rat brain membrane preparation in the absence and presence of various concentrations of diprenorphine. The results are expressed as percentage of the control binding in the absence of diprenorphine. Values represent the mean of duplicates, which are ±5% of the mean. The nonspecific binding is determined in the presence of 1 μM of [D-Ala^2, D-Leu^5]enkephalin. *Inset* is the Hill plot of competition curve.

TABLE 1

Effect of Na^+, Mg^{2+}, and GTP on the potencies of opiates and enkephalins in competing with the binding of [^{3}H]diprenorphine to neuroblastoma cell membranes

IC_{50} values (the concentration of competing ligand causing 50% inhibition of specific binding) were estimated from the competition curves by using a concentration of 0.5 nM [^{3}H]diprenorphine. Hill coefficients (n values) were obtained from the slope of Hill plots, per cent inhibition/100%-per cent inhibition versus ligand concentration. The concentrations of Na^+, GTP, and Mg^{2+} were 100 mM, 0.1 mM, and 5 mM, respectively.

	Tris·HCl		Na^+		GTP		Mg^{2+}		$Na^+ + Mg^+ + GTP$	
	IC_{50}	n	IC_{50}	n	IC_{50}	n	IC_{50}	n	IC_{50}	n
	nM		*nM*		*nM*		*nM*		*nM*	
Met-enkephalin	2.5	1.1	7	0.55	3.8	0.82	1.2	0.92	10	0.72
Leu-enkephalin	3.0	1.0	10	0.60	10	0.80	1.8	0.94		
[D-Ala^2, Leu^5]Enkephalin	2.5	1.0	8	0.55	5.5	0.86	1.6	0.90	30	0.60
[D-Ala^2, D-Leu^5]Enkephalin	2.5	1.0	8	0.52	4.0	1.0	1.6	1.0	30	0.60
Morphine	220	0.92	800	0.68	530	0.85	130	0.90	1,800	0.80
Levorphanol	20	0.90	250	0.70	—		—			
Dextrorphan	4,000	0.95	15,000	0.70	—		—			
Naloxone	100	0.91	90	0.92	120	0.91	110	0.85	60	0.90
Diprenorphine	1.5	1.0	1.3	1.0	1.3	1.0	1.4	1.0	1.5	0.90

Effect of Na^+, Mg^{2+}, and GTP on the potency of opiates and enkephalins

Enkephalin receptors. In neuroblastoma cell membranes, morphine is about 100 times less active than enkephalin in inhibiting the binding of [^{3}H]diprenorphine to neuroblastoma cell membranes (Fig. 3). The potency of other opiates and enkephalins is summarized in Table 1. Levorphanol is 10 times less active than enkephalins. The biologically inactive stereoisomer, dextrorphan, is about 100 times less active than leverphanol. Naloxone is about 40 times less potent than enkephalins. Diprenorphine is 2 times better than enkephalins. This rank order of potency is the same as that obtained with the assay utilizing ^{125}I-labeled [D-Ala^2, D-Leu^5]enkephalin binding to rat brain membrane preparations and neuroblastoma cells (2).

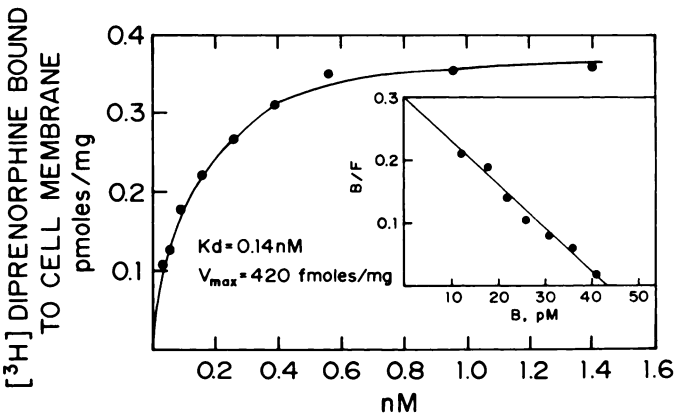

FIG. 2. *Saturation-binding isotherms and Scatchard plots (inset) of the binding of [^{3}H]diprenorphine to neuroblastoma cell membranes*

Two milliliters of neuroblastoma cell (N4TG1) membranes (0.2 mg of protein/ml) are incubated with various concentrations of [^{3}H]-diprenorphine (specific activity, 9 Ci/mmole) at 24° for 60 min. Nonspecific binding is determined in the presence of 1 μM [D-Ala^2, D-Leu^5] enkephalin. Values are expressed as the means of duplicate determinations, which are less than 10% of the mean. The apparent dissociation constant (k_d) was 0.14 nM and the maximal binding (V_{max}) was 420 fmoles/mg of protein.

Addition of Na^+, GTP, or Mg^{2+}, alone or combined, does not significantly alter the potency of opiate antagonists (naloxone and diprenorphine) in competing with the binding of [^{3}H]diprenorphine to cell membranes. The slope of Hill plots of antagonists in inhibiting the binding of [^{3}H]diprenorphine is not affected. Both Na^+ and GTP reduce the potency of enkephalins and opiate agonists by a factor of about 3. They also decrease the Hill coefficient (n) from 1 to 0.5. The effect on the Hill coefficient (n) is more profound with Na^+. GTP (10 μM, 33 μM, 100 μM, and 330 μM) shifts the competition curves of [D-Ala^2,D-Leu^5]enkephalin to the right with "IC_{50} ratios" of 1, 1.8, 2.5, and 2.5, respectively. Mg^{2+} significantly increases the potency of enkephalins and opiate agonists without affecting the Hill coefficient. The combination of Na^+, GTP, and Mg^{2+} further reduces the potency to a factor of about 10 (Table 1), compared with the value obtained with Tris·HCl buffer alone.

These studies confirm the recent data reported by Blume *et al.* (24), who showed that the potency of morphine and enkephalin analogues in competing with the binding of [^{3}H]naltrexone to NG108-15 neuroblastoma cell membranes are slightly reduced by 0.5 mM Gpp(NH)p.[1] The combination of Na^+ and Gpp(NH)p produced much greater reduction in potency. The additional inclusion of Mg^{2+} in our studies may explain the slightly smaller shift than those reported by Blume *et al.* (24) due to the antagonistic effect of Mg^{2+} on monovalent ion (16) and GTP (25).

The effects of Na^+ and GTP on the potency of opioids are further examined by using [^{3}H]naloxone binding to neuroblastoma cell membranes. Because of the low affinity of naloxone to enkephalin receptors, it becomes necessary to use higher concentrations of [^{3}H]naloxone and cell membrane in the binding assay. Only two representative opioids were studied, as shown in Table 3. A much greater reduction in potency by Na^+ is found for both [D-Ala^2, D-Leu^5]enkephalin and morphine. This is consistent

[1] The abbreviation used is: Gpp(NH)p, 5′-guanylylimidodiphosphate.

with the data obtained with [^{3}H]naltrexone in NG108-15 neuroblastoma cell membranes (24). The "Na^+ ratios" do not differ significantly from those for morphine receptors (see Table 4). However, "GTP ratios" and the effect on Hill coefficients for both [D-Ala^2, D-Leu^5]enkephalin and morphine are similar to those values obtained from [^{3}H]-diprenorphine binding assays (Table 4).

Morphine receptors. In the binding assay employing [^{3}H]naloxone and rat brain membranes, the order of potency for enkephalins and opiates is reversed (Fig. 4). Enkephalins are about 10 to 30 times less active than morphine and levorphanol (Fig. 4 and Table 5). Naloxone and diprenorphine are also much more potent than enkephalin (Table 5). Na^+ does not affect the potency of antagonists such as naloxone and diprenorphine. GTP does not affect diprenorphine, but significantly decreases the potency of naloxone by a factor of 2.5. Both Na^+ and GTP profoundly reduce the potency and Hill coefficient (n) of enkephalins and opiate agonists (Table 5.) The effects of Na^+ or GTP on morphine inhibition of [^{3}H]-naloxone binding is greater than that observed on enkephalins. GTP (10 μM, 33 μM, 100 μM, and 330 μM) reduces the potency of morphine by factors of 2, 10, 35, and 33, respectively. The magnitude of GTP effects on morphine receptors is much greater than that seen with neuroblastoma cells (enkephalin receptors) assayed by [^{3}H]naloxone or [^{3}H]diprenorphine. The Na^+ effect is greater than that observed for enkephalin receptors assayed by [^{3}H]diprenorphine but not by [^{3}H]naloxone. Mg^{2+} slightly increases the potency of enkephalins and opiate agonists without altering the Hill coefficient. The combination of Na^+, GTP, and Mg^{2+} further reduces the potency of Met- and Leu-enkephalin while restoring the potency of [D-Ala^2, L-Leu^5]- and [D-Ala^2, D-Leu^5]enkephalin and morphine toward the values observed in the absence of Na^+, GTP, and Mg^{2+} (Fig. 4 and Table 5).

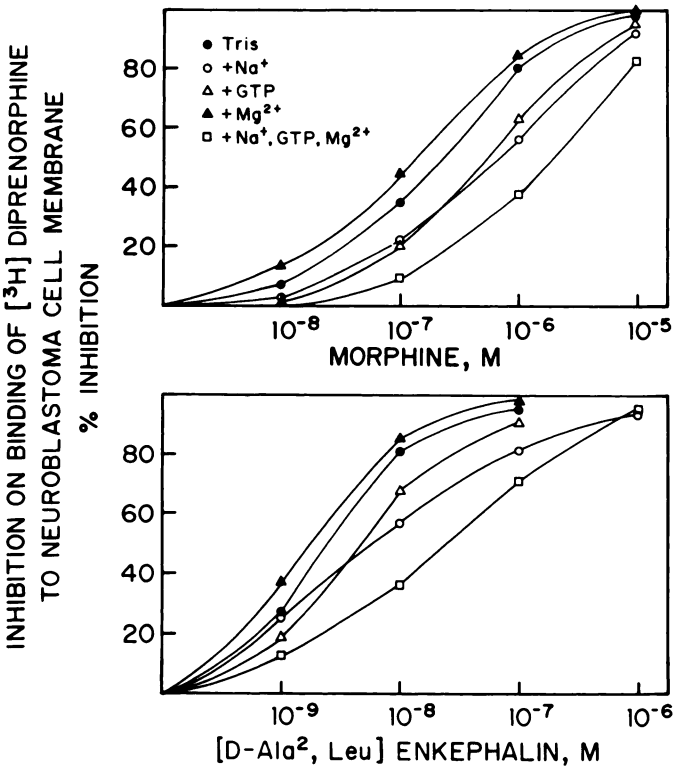

FIG. 3. *Morphine (top) and [* D-*Ala^2, Leu^5]enkephalin (bottom) inhibition of the binding of [^{3}H]diprenorphine to neuroblastoma cell membranes in the absence* (●) *and presence of* Na^+ (○), *GTP* (△), Mg^{2+} (▲), *or* $Na^+ + GTP + Mg^{2+}$ (□)

Two milliliters of neuroblastoma cell membranes were incubated with [^{3}H]diprenorphine (0.5 nM) in the presence of various concentrations of morphine and enkephalin without or with 100 mM Na^+, 0.1 mM GTP, and 5 mM Mg^{2+}. The results are expressed as percentage inhibition. Values represent the means of duplicates, which are ± 5% of the mean.

TABLE 2

Effect of Na^+, GTP, and Mg^{2+} on the binding of [^{3}H]diprenorphine (0.5 nM) to neuroblastoma cell membranes and of [^{3}H]naloxone (0.4 nM) to rat brain membranes

Two milliliters of rat brain membranes (1 mg of protein/ml) and neuroblastoma cell membranes (0.2 mg of protein/ml) were incubated with [^{3}H]naloxone (0.4 nM) and [^{3}H]diprenorphine (0.5 nM), respectively, for 1 hr at 24° in the absence and presence of 100 mM Na^+, 0.1 mM GTP, 5 mM Mg^{2+}, or the combination of Na^+, GTP, and Mg^{2+}. The results are expressed as percentage of the control binding in the absence of ions and nucleotide. Values are expressed as percentage ± standard error of 10–15 separate experiments.

Addition	Binding of [^{3}H]diprenorphine to cell membranes	Binding of [^{3}H]naloxone to brain membranes
	%	%
Tris·HCl	100	100
+Na^+ (100 mM)	95 ± 7	126 ± 4
+GTP (0.1 mM)	97 ± 5	63 ± 3
+Mg^{2+} (5 mM)	95 ± 1	105 ± 4
+Na^+ + GTP + Mg^{2+}	73 ± 5	101 ± 5

DISCUSSION

Morphine (μ) and enkephalin (δ) receptors share many common characteristics. They both bind opiates, enkephalins, and endorphins with high affinity and they show stereospecificity. Na^+, GTP, and Mg^{2+} affect the binding of enkephalins, endorphins, and opiate agonists to both subtypes of opiate receptors (4, 5, 14–19).

The present studies, using [^{3}H]naloxone for rat brain membranes and [^{3}H]diprenorphine for neuroblastoma cell membranes, clearly demonstrate the qualitative similarities and quantitative differences between these two receptor sites. Maximal concentrations of GTP [or Gpp(NH)p] are used in the present studies to ensure the maximal effects and minimize the hydrolysis of guanine nucleotides. No further reduction in the potency of enkephalin and morphine by the increase in the concentration of GTP from 0.1 mM to 0.33 mM suggests that 0.1 mM GTP is above the maximal effective concentration. Furthermore, the effects of GTP are similar to those observed with the metabolically stable analogue Gpp(NH)p. The differential effects are unlikely due to the hydrolysis of GTP. Both sodium ions and GTP reduce the potency and the slope (Hill coefficient) of Hill

TABLE 3

Effect of Na^+ and GTP [or Gpp(NH)p] on the potencies of opiate and enkephalin in competing with the binding of [^{3}H]naloxone to neuroblastoma cell membranes

IC_{50} values were estimated from the competition curves by using a concentration of 2 nM [^{3}H]naloxone. The concentrations of Na^+ and Gpp(NH)p were 100 mM and 0.2 mM, respectively. Similar data were obtained with GTP (0.2 mM).

	Tris·HCl		Na^+		Gpp(NH)p		Na^+ + Gpp(NH)p	
	IC_{50}	n	IC_{50}	n	IC_{50}	n	IC_{50}	n
	nM		*nM*		*nM*		*nM*	
Morphine	150	0.93	2200	0.73	250	0.85	5000	0.65
[D-Ala^2, D-Leu^5]Enkephalin	1.5	1.1	12	0.72	1.8	0.87	50	0.50

plots of the enkephalin and opiate agonist inhibition of labeled ligand binding. These effects are more profound in the [^{3}H]naloxone rat brain membrane-binding assay than in the [^{3}H]diprenorphine-binding assay. To check whether the above quantitative difference in the two binding assays is the reflection of the difference of the intrinsic properties of enkephalin and morphine receptors, [^{3}H]naloxone was used as a label in neuroblastoma cell membranes binding. It is important to emphasize here that the concentrations of neuroblastoma cell membranes and [^{3}H]naloxone are much higher than those in the [^{3}H]diprenorphine binding assays because of the relative low affinity of naloxone to enkephalin receptors. Na^+ ratios of both [D-Ala^2, D-Leu^5]enkephalin and morphine in replacing the [^{3}H]naloxone binding to neuroblastoma cell membranes are much greater than those in replacing the [^{3}H]diprenorphine binding (Table 3). However, both the "GTP ratios" and the GTP effect on Hill coefficients (Table 3) remain small and similar to those in the [^{3}H]diprenorphine-binding assay. These findings suggest that the differential effect of Na^+ is a result of the difference between [^{3}H]diprenorphine-receptor and [^{3}H]-naloxone-receptor complexes, and the differential effect of GTP or Gpp(NH)p is probably due to the difference in intrinsic properties of enkephalin and morphine receptors.

GTP reduces the binding of [^{3}H]naloxone to brain membranes, which is consistent with the reduction of the potency of naloxone to inhibit [^{3}H]naloxone binding (Table 5) and with the data previously reported by Blume (18). However, this reduction is much smaller than that observed for enkephalins and opiate agonists. Mg^{2+} (5 mM) increases the potency of enkephalins and opiate agonists for both receptor sites. The effects of combining Na^+, Mg^{2+}, and GTP are variable. The potency for binding of all ligands to enkephalin receptors is decreased further. The potency of Met- and Leu-enkephalin is also decreased, but there is some restoration of activity for [D-Ala^2, L-Leu^5]- and [D-Ala^2, D-Leu^5]enkephalin, as well as for opiate agonists for morphine receptor sites. This illustrates the complex nature of the interactions between cations and nucleotides on opiate receptors. The additive effect of cations and GTP on reducing the potency of agonists for inhibition of labeled antagonist binding suggests different coupling mechanisms for Na^+ and GTP. Similar conclusions have recently been arrived at by others (24, 25).

It is difficult to compare our data in rat brain membranes with those reported by Childers and Snyder (25), since they employed [^{3}H]diprenorphine. [^{3}H]Diprenorphine labels both morphine and enkephalin receptors equally well since it binds to both receptor types with

TABLE 4

Comparison of Na^+, GTP, and Na^+ + GTP + Mg^{2+} ratios on enkephalin and morphine receptors

The ratios of IC_{50} in the absence and presence of Na^+, GTP, and Na^+ + GTP + Mg^{2+} were calculated from Tables 1 and 5. The values in parentheses were calculated from Table 3 by using [^{3}H]naloxone for neuroblastoma cell membranes.

	Na^+ ratio		GTP ratio		Na^+ + GTP + Mg^{2+} ratio	
	E.R.[a]	M.R.[a]	E.R.[a]	M.R.[a]	E.R.[a]	M.R.[a]
Met-enkephalin	2.8	4.0	1.5	4	4	9
Leu-enkaphalin	3.3	6.7	3.3	7.3		20
[D-Ala^2-Leu^5]Enkephalin	3.2	6.6	2.2	12	12	5
[D-Ala^2,D-Leu^5]Enkephalin	3.3 (8)	5.8	1.6 (1.2)	10	12 (33)[b]	4
Morphine	3.6 (15)	20	2.4 (1.7)	36	8 (33)[b]	4.4
Levorphanol	12.5	18	—	10	—	—
Naloxone	0.9	1.0	1.2	2.5	0.6	3
Diprenorphine	0.87	1.0	0.9	1.0	1.0	0.8

[a] E.R. and M.R., enkephalin and morphine receptors, respectively.

[b] These values are obtained in the presence of Na^+ plus Gpp(NH)p.

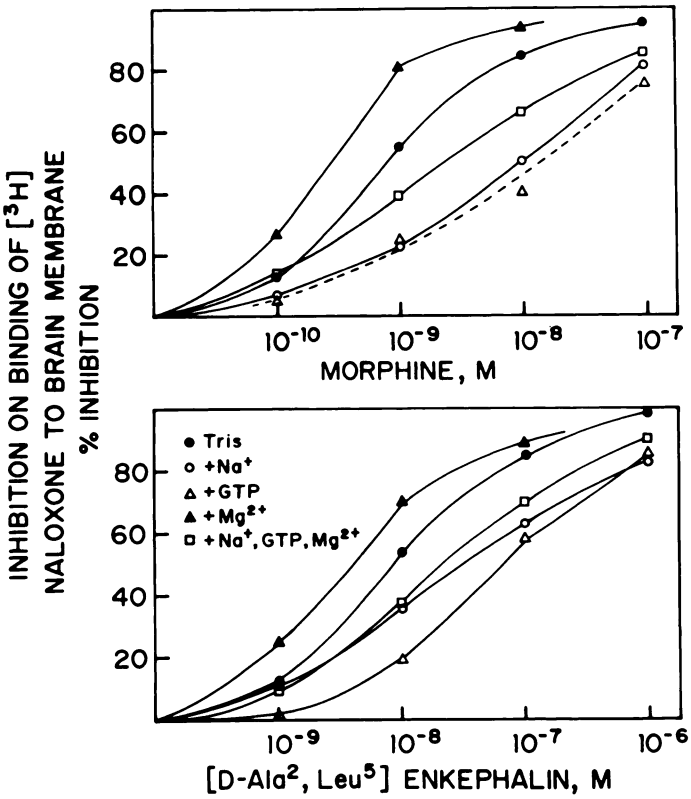

FIG. 4. *Morphine (top) and [D-Ala², Leu⁵]enkephalin (bottom) inhibition of the binding of [³H]naloxone to rat brain membrane preparations in the absence (●) and presence of Na⁺ (○), GTP (△), Mg²⁺ (▲) or Na⁺ + GTP + Mg²⁺ (□)*

Two milliliters of rat brain membrane preparations were incubated with [³H]naloxone (0.4 nM, specific activity, 23 Ci/mmole) without or with 100 mM Na^+, 0.1 mM GTP, or 5 mM Mg^{2+}, or the combination of Na^+, GTP, and Mg^{2+}. The results are expressed as percentage inhibition. Values are the mean of duplicates, which are ±5% of the mean.

about equally high affinity (23). In our studies, we have selected conditions in which [³H]naloxone binds to morphine receptors predominately. However, certain data are consistent with our hypothesis. For instance, GTP ratios for opiates and enkephalins in replacing the [³H]-diprenorphine binding to rat brain membranes (morphine and enkephalin receptors) are intermediate between those obtained by using [³H]naloxone to brain membrane (morphine receptor) and by ³H-labeled antagonists binding to neuroblastoma cell membranes (enkephalin receptors).

Recently, Pert and Taylor (26) described the existence of GTP-sensitive and -resistant subtypes of opiate receptors in rat brain membrane preparations and GTP-resistant receptors in neuroblastoma-glioma hybrid cells. GTP-sensitive and -resistant receptors resemble the morphine and enkephalin receptors, respectively. Our data indicate that the binding affinity of enkephalins and morphine to enkephalin receptors in neuroblastoma cell (N4TG1) is significantly reduced by GTP at 100 μM, although the effect is smaller than that seen with morphine receptors. This difference may be simply quantitative and may result from the higher concentration of GTP used in this experiment. Indeed, it was reported recently (27) that the binding of [³H]dihydromorphine to brain homogenates is much more sensitive to the inhibitory effects of GTP than is the binding of [D-Ala², Met⁵]-enkephalinamide.

GTP is an obligatory regulator for all known hormone receptor-mediated activations of adenylate cyclase. It also regulates the interactions of many hormones and neurotransmitters with their receptors. Recently, Blume and colleagues showed that Na^+ and GTP are required for opiate inhibition of adenylate cyclase in neuroblastoma-glioma hybrid cells (28). Opioid inhibition of adenylate cyclase has also been described in N4TG1 cell membranes (13). The present studies show the differential regulation of enkephalin and morphine receptors by ions and nucleotide and suggest that these two opiate receptors may be regulated quantitatively in a different manner. At present, it is impossible to determine the exact location for the differential effect of GTP on these two binding sites. It could be either directly on the ligand-binding site or on the guanine nucleotide regulatory component, or both.

TABLE 5

Effect of Na^+, Mg^{2+}, and GTP on the potencies of enkephalins and opiates in competing with the binding of [³H]naloxone to rat brain membranes

IC_{50} values were estimated from the competition curves by using [³H]naloxone (0.4 nM). Hill coefficients (*n* values) were obtained from the slopes of Hill plots. The concentrations of Na^+, GTP, and Mg^{2+} were 100 mM, 0.1 mM and 5 mM, respectively.

	Tris·HCl		Na^+		GTP		Mg^{2+}		$Na^+ + Mg^+ + GTP$	
	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*	IC_{50}	*n*
	nM		*nM*		*nM*		*nM*		*nM*	
Met-enkephalin	10	0.95	40	0.60	40	0.72	5	1.1	90	0.56
Leu-enkephalin	15	0.83	100	0.50	110	0.50	5	0.85	300	0.60
[D-Ala², Leu⁵]Enkephalin	5	0.90	33	0.50	60	0.72	3.3	0.90	25	0.65
[D-Ala, D-Leu⁵]Enkephalin	6	0.95	35	0.60	60	0.76	3.0	0.95	25	0.70
Morphine	0.5	0.85	10	0.62	18	0.58	0.4	0.86	2.2	0.55
Levorphanol	0.3	0.80	5	0.64	3	0.97	—		—	
Naloxone	1.0	1.0	1.0	1.0	2.5	0.60	0.9	0.9	3	0.6
Diprenorphine	0.3	1.1	0.3	1.1	0.3	1.0	0.3	1.1	0.25	1.1

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