MODIFICATION OF GUANINE NUCLEOTIDE-REGULATORY COMPONENTS IN BRAIN MEMBRANES

I. Changes in Guanosine 5'-Triphosphate Regulation of Opiate Receptor-binding Sites¹

SCOTT M. LAMBERT AND STEVEN R. CHILDERS²

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

Received January 4, 1984; Accepted May 4, 1984

Abstract

Guanine nucleotides regulate binding of opiate agonists to membrane receptors by increasing agonist dissociation rates. The current study demonstrates that the ability of guanosine 5'-triphosphate (GTP) and its nonhydrolyzable analogue guanylyl-5'-imidodiphosphate (Gpp(NH)p) to inhibit opiate agonist binding to rat brain membranes can be altered by two methods: by preincubating with EDTA, and by preincubating at pH 4.5. EDTA pretreatment increased the potency of Gpp(NH)p in inhibiting [³H]morphine binding by 4-fold, with little apparent change in the maximum effect of Gpp(NH)p or on levels of binding itself. The effect of EDTA pretreatment was blocked by prior incubation of membranes with excess calcium or manganese but could not be reversed by any divalent cation if the EDTA incubation was longer than 10 min. EDTA pretreatment increased the effects of GTP on dissociation rates of agonists. Pretreatment of membranes at pH 4.5 increased the ability of guanine nucleotides to regulate agonist binding by increasing the maximum effect of Gpp(NH)p from 50% to 80% inhibition of [³H]morphine binding with minor increase in potency of Gpp(NH)p. The actions of EDTA and low pH pretreatments were additive when both were conducted on the same membranes. These results suggest that modification of brain membranes can alter the interaction of receptors with guanine nucleotide-regulatory components which may lead to changes in post-receptor membrane events.

Guanine nucleotides play two important roles in neurotransmitter receptor function, by coupling receptors with adenylate cyclase (Rodbell, 1980) and by decreasing affinity of transmitters and agonists at receptor-binding sites (Rodbell et al., 1971; Mukherjee et al., 1975; U'Prichard and Snyder, 1978; Creese et al., 1979). According to the model of the ternary complex as formulated first for the glucagon and β -adrenergic receptor systems, the agonist binding to a high affinity receptor site catalyzes the displacement of guanosine 5'-diphosphate (GDP) by guanosine 5'-triphosphate (GTP) on the guanine nucleotide-regulatory component (the N-protein), which is then capable of activating or inhibiting adenylate cyclase depending on the nature of the receptor and N-protein system. GTP then increases dissociation rates of the agonist from the receptor binding site (Rodbell, 1980). The action of GTP on receptor binding may be due to interactions of GTP with either the N-proteins or directly with the receptor site itself; reconstitution experiments with β -adrenergic receptors would suggest that the same N-protein mediates both functions of coupling receptors

with adenylate cyclase and regulating binding of agonists (Limbird et al., 1980). Thus, the degree of guanine nucleotide regulation of binding may provide information about the interactions of receptor-binding sites and N-proteins.

Binding of opiate agonists to brain membranes and to neuroblastoma $\times$ glioma cell membranes is regulated by guanine nucleotides in a manner qualitatively similar to those of other neurotransmitter receptors (Blume, 1978a, b; Childers and Snyder, 1978, 1980). GTP and its nonhydrolyzable analogue guanylyl-5'-imidodiphosphate (Gpp(NH)p) increase agonist dissociation rates and thereby decrease equilibrium binding; thus, the degree of regulation by guanine nucleotides can be determined either directly by measuring the decrease in ³H-agonist binding by GTP or by measuring the shift in agonist affinity in experiments displacing ³H-antagonist binding by agonists. In this way, GTP effects have been measured on multiple opiate receptor-binding sites to show that GTP has relatively more effect in inhibiting μ (morphine)-binding sites than δ (enkephalin)-binding sites in brain membranes (Chang et al., 1981). Moreover, the ability of GTP to inhibit opiate agonist binding varies in different brain regions (Zukin and Gintzler, 1980; Zukin et al., 1980). This information may indicate variations in the ability of opiate receptors to interact with guanine nucleotide-regulatory proteins and post-receptor membrane

¹ This work was supported by Grant DA-02094 from the United States Public Health Service. We thank Mr. John Jessup for starting some of the preliminary work of this study.

² To whom correspondence should be addressed.

signaling events, but so far there have been no data to correlate these changes in guanine nucleotide sensitivity with opiate receptor function.

One important facet of the regulation of agonist binding by GTP involves the actions of cations. Sodium, which, like GTP also decreases agonist affinity (Pert and Snyder, 1974; Simon et al., 1975), increases the effect of guanine nucleotides in regulation of binding: the actions of sodium and GTP on binding are additive (Childers and Snyder, 1980). Divalent cations, which antagonize the effects of GTP on other receptor systems such as the α -adrenergic receptor (Rouot et al., 1980), appear to have little effect on guanine nucleotide actions on brain opiate receptors: divalent cations reverse the effects of GTP itself on opiate agonist binding, but this action is caused by an increase in phosphatase activity which hydrolyzes GTP, since the same ions have little effect on the actions of Gpp(NH)p (Childers and Snyder, 1980). The relative insensitivity of guanine nucleotide effects on opiate receptors to divalent cations may not represent a unique feature of opiate receptors but may instead be caused by the fact that brain membranes already contain sufficient divalent cations bound to membrane sites, so that the addition of exogenous cations could have no further effect. To explore the possibility that membrane-bound divalent cations play a role in the interactions of opiate receptors with guanine nucleotide regulatory components, membranes could be stripped of divalent cations with chelating agents and guanine nucleotide effects on agonist binding could be measured. The present study reports on the modification of guanine nucleotide actions on opiate receptors by several treatments of brain membranes, including EDTA incubation and treatment at low pH. The second paper in this series (Childers and LaRiviere, 1984) explores the effects of these membrane treatments on adenylate cyclase in an attempt to correlate changes in GTP regulation in binding with those of GTP coupling of receptors with adenylate cyclase.

Materials and Methods

Opiate receptor-binding assays. Male Sprague-Dawley rats (200 to 250 gm) were decapitated and brains, minus cerebella, were removed and homogenized in 20 vol of 50 mM Tris-HCl, pH 7.7, with a Polytron homogenizer. After centrifugation at $48,000 \times g$ for 10 min, the pellet was resuspended in 20 ml of Tris buffer, incubated at 37°C for 45 min to remove endogenous opiates (Pasternak and Snyder, 1975; Pasternak et al., 1975), and centrifuged again at $48,000 \times g$ for 10 min. The pellet was resuspended in fresh buffer for treatment at low pH or with EDTA as described below, centrifuged at $48,000 \times g$ for 10 min, and resuspended in 100 vol of Tris buffer. Binding assays were conducted as previously described (Childers and Snyder, 1980), using [3 H]morphine, [3 H]-D-Ala²-Met⁵-enkephalinamide (D-Ala enk), or [3 H]-D-Ala²-D-Leu⁵-enkephalin (DADLE) as labeled agonists, [3 H]naloxone as a labeled antagonist, and various concentrations of GTP or Gpp(NH)p to determine levels of guanine nucleotide regulation of binding. Tubes were incubated at 25°C for 40 min, and bound radioactivity was determined by filtration of samples through Whatman GF/B fiber filters followed by liquid scintillation spectrometry in 10 ml of Liquescent scintillation fluid (National Diagnostics, Inc., Somerville, NJ) at 40% counting efficiency. All results are expressed as specific binding, calculated by subtracting nonspecific binding determined in the presence of 1 μ M levallorphan. All assays were performed in triplicate with less than 6% standard deviation between samples.

EDTA pretreatment assays. After preparation of crude membranes from rat brain and preincubation at 37°C as described above, membranes were incubated at 25°C for 20 min in the presence of various concentrations of EDTA (disodium salt) and centrifuged at $48,000 \times g$ for 10 min. The pellets were resuspended in fresh Tris buffer, centrifuged, and resuspended in fresh buffer once more to eliminate EDTA. Membranes were then assayed for opiate receptor binding as described above.

Low pH pretreatment assays. Crude brain membranes prepared through the 37°C preincubation step as described above were resuspended in 20 vol of 50 mM sodium acetate, pH 4.5, and incubated at

25°C for 20 min. The membrane suspensions were diluted with cold 50 mM Tris-HCl, pH 7.7, and centrifuged at $48,000 \times g$ for 10 min. Membranes were resuspended in 100 vol of Tris buffer and assayed for opiate receptor binding as described above.

Materials. [3 H]Morphine (53.6 Ci/mmol), [3 H]DADLE (43.6 Ci/mmol), and [3 H]naloxone (49.3 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). [3 H]-D-Ala enk (35.3 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). GTP was obtained from Calbiochem (La Jolla, CA), and Gpp(NH)p was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were reagent grade materials.

Results

Effect of EDTA pretreatment on regulation of opiate receptor binding by guanine nucleotides. When crude rat brain membranes were incubated with 1 mM EDTA for 30 min at 25°C, washed to remove EDTA, and assayed for [3 H]morphine binding, results showed a minimal effect of EDTA pretreatment on equilibrium binding itself. However, EDTA pretreatment did alter the ability of guanine nucleotides to inhibit [3 H]morphine binding (Fig. 1). In nontreated membranes, Gpp(NH)p inhibited binding to 55% of control levels with an IC₅₀ value of approximately 1 μ M Gpp(NH)p. In EDTA-pretreated membranes, the maximum effect of Gpp(NH)p was not appreciably changed, but the IC₅₀ of Gpp(NH)p was decreased to 0.25 μ M. Similar 4-fold shifts in potency values were observed after EDTA pretreatment for GTP and GDP in inhibiting [3 H]morphine binding, although the absolute IC₅₀ values for the latter guanine nucleotides were approximately 10-fold higher than that of Gpp(NH)p (data not shown). In addition, Figure 1 shows that the Gpp(NH)p inhibition curve was not shifted parallel to the left by EDTA pretreatment. Calculations reveal that EDTA pretreatment decreased the Hill slope of Gpp(NH)p inhibition of binding in various experiments from 0.9 or 1.0 to 0.5 to 0.7. Addition of 200 μ M CaCl₂, MnCl₂, and MgCl₂ in control membranes had no effect on Gpp(NH)p regulation of binding.

The effect of EDTA on guanine nucleotide regulation of opiate agonist binding was observed for several different ligands (Table I). EDTA pretreatment increased the potency of Gpp(NH)p in inhibiting binding of the δ -ligand DADLE and the mixed affinity ligand D-Ala enk as well as the μ -ligand morphine, although the absolute effect of the guanine nucleotides themselves on each ligand varied, with the effect of Gpp(NH)p greatest on [3 H]morphine binding. Such findings are consistent with those of Chang et al. (1981), who showed maximal GTP effects on μ opiate receptor binding. However, in EDTA-pretreated membranes, GTP continued to produce no significant effect on [3 H]naloxone binding in the presence of 100 mM NaCl (Table I). Thus, although guanine nucleotide actions on 3 H-agonist binding were increased, GTP still effectively distinguished agonist and antagonist binding.

The increase in Gpp(NH)p potency by EDTA pretreatment could be produced by EDTA concentrations as low as 10 μ M, when preincubations were conducted at 25°C for 30 min (data not shown). The most consistent effects were observed at EDTA concentrations of 50 to 100 μ M, with no increase in effectiveness seen between 100 μ M and 1 mM EDTA.

The increase in potency of guanine nucleotides in inhibiting agonist binding could be observed only if membranes were preincubated with EDTA, then washed to remove the chelating agent. If EDTA was included during the ligand binding reaction, equilibrium binding results showed that [3 H]morphine binding was reduced by 20 to 30%, as reported previously (Pasternak et al., 1975), with a decrease in the ability of Gpp(NH)p to reduce binding (data not shown). These effects were totally reversed by sufficient wash steps to remove EDTA. Routinely, membranes were washed with a 50-fold dilution of Tris buffer, followed by centrifugation steps sufficient to lower

Figure 1. Effect of EDTA pretreatment of rat brain membranes on the reduction of [^3H]morphine binding by Gpp(NH)p. Membranes were incubated at 25°C for 30 min in the presence and absence of 1 mM EDTA, washed twice, and assayed for binding with 0.78 nM [^3H]morphine in the presence of various concentrations of Gpp(NH)p as described under "Materials and Methods." Data represent percentage of specific binding in nontreated membranes. ●, nontreated membranes; ○, EDTA-pretreated membranes; ■, assayed in the presence of 200 μM CaCl_2 , MnCl_2 , and MgSO_4 in nontreated membranes.

TABLE I

Effect of EDTA pretreatment of rat brain membranes on Gpp(NH)p inhibition of several ^3H -opiate ligands

Rat brain membranes were incubated for 30 min at 25°C in the presence and absence of 1 mM EDTA, washed twice in fresh 50 mM Tris-HCl, pH 7.7, and assayed for binding with 0.78 nM [^3H]morphine, 1.2 nM [^3H]DADLE, 1.0 nM [^3H]-D-Ala enk, or 0.80 nM [^3H]naloxone as described under "Materials and Methods," with 100 mM NaCl added to [^3H]naloxone tubes. Binding was assayed in the presence and absence of 0.5 μM Gpp(NH)p; results are expressed as percentage of specific binding. Data represent mean values $\pm$ SEM of three separate experiments.

^3H -Ligand	Percentages Bound with 0.5 μM Gpp(NH)p	
	-EDTA	+EDTA
[^3H]Morphine	82 $\pm$ 4	70 $\pm$ 6
[^3H]DADLE	90 $\pm$ 5	77 $\pm$ 6
[^3H]-D-Ala enk	91 $\pm$ 3	78 $\pm$ 4
[^3H]Naloxone	99 $\pm$ 4	104 $\pm$ 6

the estimated concentration of EDTA to less than 1 μM before ^3H -ligand incubation was begun.

The specificity of the effects of EDTA pretreatment on guanine nucleotide regulation of binding was examined by studying the actions of EDTA pretreatment on the inhibition of agonist binding by sodium. The sodium effect was of interest since sodium has effects on binding similar to those of GTP and because the actions of sodium and GTP on opiate agonist binding are additive (Childers and Snyder, 1980). Figure 2 shows the effect of various concentrations of sodium on [^3H]morphine binding in the presence and absence of 1 μM Gpp(NH)p. EDTA pretreatment had no effect on the inhibition of binding by sodium alone (Fig. 2, triangles). Addition of Gpp(NH)p in the presence of sodium (Fig. 2, circles) inhibited binding further than sodium alone in both control and EDTA-

pretreated membranes; however, Gpp(NH)p inhibited binding even further in EDTA-pretreated membranes, with appreciable increase in the Gpp(NH)p effect occurring between 0 and 20 mM NaCl. EDTA pretreatment produced no increase in Gpp(NH)p actions at 50 and 100 mM NaCl, but this may represent the fact that binding is too low at these concentrations of sodium to obtain accurate estimations of guanine nucleotide effects.

Effects of divalent cations on guanine nucleotide regulation of binding. The finding that EDTA pretreatment increased the potency of guanine nucleotides in inhibiting opiate agonist binding suggested that divalent cations in brain membranes might play a role in regulating interaction of guanine nucleotide-regulatory sites with opiate receptors. The most direct test of this suggestion was to estimate the effects of divalent cations on the potency of guanine nucleotides in regulating binding in control (not treated with EDTA) membranes. When membranes were incubated with 200 μM manganese, magnesium, or calcium, binding of ^3H -agonists was increased by 10 to 20%, as reported previously (Pasternak et al., 1975). However, as seen in Figure 1, the addition of these ions together to brain membranes had no significant effect on the potency of Gpp(NH)p in inhibiting [^3H]morphine binding. These results agree with the lack of effect found previously with divalent cations on Gpp(NH)p effects on binding (Childers and Snyder, 1980) and suggest either that divalent cations themselves do not affect the guanine nucleotide-regulatory components or that control membranes possess sufficient cations so that addition of exogenous cations produces no further effect.

If EDTA increases potency of guanine nucleotides by simply removing endogenous divalent cations from brain membranes, then its effects might be reversed by adding divalent cations to membranes after EDTA pretreatment. Moreover, such reversal might exhibit a specificity for cations depending on the affinity of EDTA for different divalent cations. For three commonly used cations, EDTA has highest affinity for Mn^{2+} , followed by Ca^{2+} , and weakest affinity for Mg^{2+} (the pK_a value for EDTA-

Figure 2. Effect of EDTA pretreatment on Gpp(NH)p inhibition of [3 H]morphine binding in the presence of sodium. Membranes were pretreated in the presence and absence of 1 mM EDTA, washed twice, and assayed for binding with 0.8 nM [3 H]morphine in the presence and absence of 1 μ M Gpp(NH)p along with 0 to 100 mM NaCl. Results are expressed in terms of specific binding in nontreated membranes. Solid symbols, nontreated membranes; open symbols, EDTA-pretreated membranes. $\blacktriangle$, $\triangle$, assayed without Gpp(NH)p; $\bullet$, $\circ$, assayed in the presence of 1 μ M Gpp(NH)p.

Mn^{2+} is 13.9; for EDTA- Ca^{2+} it is 10.7; and for EDTA- Mg^{2+} it is 8.7) (Kragten, 1978). Table II shows the results of experiments which added various divalent cations both before and after EDTA pretreatment. A low concentration of Gpp(NH)p (0.1 μ M), which did not inhibit [3 H]morphine binding in nontreated membranes, inhibited binding by 15% in membranes pretreated with 50 μ M EDTA. When membranes were incubated with 50 μ M EDTA for 30 min, then 1 mM CaCl_2 , MgCl_2 , or MnCl_2 was added to reverse the reactions and the membranes were washed extensively to remove EDTA and cations, the increased effect of Gpp(NH)p continued, with 0.1 μ M Gpp(NH)p inhibiting binding by 13 to 20% (Table II). Thus, the effect of EDTA pretreatment was not reversed by reaction with a 20-fold excess of divalent cations. Nevertheless, if the same concentrations of divalent cations were added to membranes before addition of 50 μ M EDTA, the effect of EDTA pretreatment was blocked by prior addition of two divalent cations (Ca^{2+} and Mn^{2+}) for which EDTA has high affinity, but not by Mg^{2+} , for which EDTA has a relatively lower affinity.

Since the EDTA effect on increasing potency of Gpp(NH)p inhibition of binding was irreversible after preincubation of membranes with EDTA for 30 min, studies were conducted to determine whether divalent cations could reverse the EDTA effect at shorter incubation periods. The time course of the EDTA pretreatment effect (Fig. 3) was fairly rapid, with 50 μ M EDTA increasing inhibition of [3 H]morphine binding by 0.1 μ M Gpp(NH)p from 8% to 20% within 10 min. Longer incubations up to 30 min produced no further significant increase in Gpp(NH)p regulation of binding. When 1 mM CaCl_2 was added to membranes at various time points after addition of 50 μ M EDTA, the EDTA effect was reversed at 5 and 10 min but

TABLE II
Effect of divalent cations on inhibition of [3 H]morphine binding to rat brain membranes when added before and after EDTA pretreatment

Rat brain membranes were prepared as described under "Materials and Methods." Divalent cations (CaCl_2 , MgCl_2 , and MnCl_2) were added at concentrations of 1 mM either before or after incubation of membranes for 30 min at 25°C with 50 μ M EDTA. Membranes were then washed twice and assayed for binding with 0.8 nM [3 H]morphine in the presence and absence of 0.1 μ M Gpp(NH)p. Results are expressed as percentage of specific binding in the presence of Gpp(NH)p and represent mean values $\pm$ SEM of triplicate determinations in a typical experiment. Each experiment was replicated at least three times.

Treatment	Divalent Cations		Percentage Bound with 0.1 μ M Gpp(NH)p
	Cation type	When added	
None			97 $\pm$ 6
EDTA			85 $\pm$ 5
EDTA	Mg^{2+}	after	72 $\pm$ 5
EDTA	Ca^{2+}	after	80 $\pm$ 4
EDTA	Mn^{2+}	after	77 $\pm$ 1
EDTA	Mg^{2+}	before	80 $\pm$ 6
EDTA	Ca^{2+}	before	94 $\pm$ 2
EDTA	Mn^{2+}	before	98 $\pm$ 5

not after 10 min following EDTA addition (Fig. 3). Similar effects were observed when Ca^{2+} was replaced by Mn^{2+} , but not by Mg^{2+} (not shown). These results suggest that the EDTA effect is at least partially reversible if divalent cations are added within 10 min after addition of EDTA, but that the EDTA effect becomes essentially irreversible after that point.

Figure 3. Time course of EDTA pretreatment of rat brain membranes in the presence and absence of calcium. Membranes were pretreated with 50 μ M EDTA for various periods of time; then the membrane suspension was diluted 25-fold with either buffer alone or with buffer containing 1 mM CaCl_2 . Membranes were washed twice and assayed with 0.85 nM [^3H]morphine in the presence and absence of 0.1 μ M Gpp(NH)p. Results are expressed as percentage of specific binding in nontreated membranes in the absence of Gpp(NH)p. Open bars, treated with EDTA alone; solid bars, calcium added at designated times after EDTA addition.

Other experiments explored the effects of different chelating agents on guanine nucleotide regulation of opiate agonist binding (Table III). Both EDTA and EGTA increased the inhibition of [^3H]morphine binding by 0.1 and 0.5 μ M Gpp(NH)p. For example, both EDTA and EGTA increased inhibition of binding by 0.1 μ M Gpp(NH)p from 8% in control membranes to 25% in chelator-treated membranes. However, 1,10-phenanthroline had no appreciable effect on Gpp(NH)p potency at either concentration of Gpp(NH)p in the binding assay (Table III). Interestingly, both EDTA and EGTA have high affinities for Ca^{2+} and Mn^{2+} (pK_a for EGTA- Ca^{2+} is 11.0; for EGTA- Mn^{2+} it is 12.3), whereas phenanthroline has high affinity for transition metals such as Zn^{2+} and Ni^{2+} ($\text{pK}_a \sim 8$ to 9) but low affinity for Ca^{2+} and Mn^{2+} ($\text{pK}_a \sim 2$ to 3) (Kragten, 1978).

Effects of EDTA pretreatment on kinetics of opiate receptor binding. Because the effects of guanine nucleotides in decreasing agonist binding arise primarily from the ability of the nucleotides to affect kinetics of agonist binding, experiments were conducted to determine the actions of EDTA pretreatment on the effects of Gpp(NH)p on opiate agonist association and dissociation rates. Since the kinetics of [^3H]morphine binding are rapid and difficult to quantitate (Simantov and Snyder, 1976), we used [^3H]-D-Ala enk as the ligand in these studies. Association experiments (not shown) revealed that Gpp(NH)p increased association rates in both control and EDTA-pretreated membranes but that there was no difference in the magnitude of the Gpp(NH)p effect in the two membrane preparations. Therefore, the increased potency of Gpp(NH)p in EDTA-pretreated membranes was not the result of changes in the ability of Gpp(NH)p to affect ligand association rates. However, dissociation experiments revealed a clear effect of EDTA pretreatment on guanine nucleotide actions. EDTA pretreatment had no effect on dissociation of [^3H]-D-Ala enk in the absence of Gpp(NH)p (data not shown) but increased the dissociation rate of [^3H]-D-Ala enk in the presence of 0.5 μ M Gpp(NH)p (Fig. 4). Dissociation of [^3H]-D-Ala enk was biphasic, and EDTA pretreatment increased the $t_{1/2}$ of the fast

TABLE III

Comparison of membrane pretreatment with EDTA, EGTA, and 1,10-phenanthroline of [^3H]morphine binding by Gpp(NH)p

Membranes were incubated at 25°C for 30 min with 1 mM EDTA, EGTA, or 1,10-phenanthroline, washed twice, and assayed for binding with 0.8 nM [^3H]morphine. Inhibition of binding by guanine nucleotides was determined either with 0.1 μ M or 0.5 μ M Gpp(NH)p in the assay. Results are expressed as percentage of specific binding and represent mean values $\pm$ SEM of triplicate determinations in a typical experiment. Each experiment was replicated at least three times.

Membrane Pretreatment	Gpp(NH)p in Assay μ M	Percentage Bound
None	0.1	92 $\pm$ 3
EDTA	0.1	74 $\pm$ 5
EGTA	0.1	75 $\pm$ 5
Phenanthroline	0.1	95 $\pm$ 7
None	0.5	84 $\pm$ 4
EDTA	0.5	69 $\pm$ 3
EGTA	0.5	73 $\pm$ 6
Phenanthroline	0.5	84 $\pm$ 5

component from 4.4 to 1.0 min, while it produced no significant change in the $t_{1/2}$ of the slow component.

Modification of guanine nucleotide regulation of binding by low pH pretreatment. The irreversible actions of EDTA in accentuating the effects of guanine nucleotides on opiate receptor binding suggested that EDTA may be altering some membrane component that interacts between receptors and guanine nucleotide-regulatory proteins by removing important divalent cations. If this hypothesis were true, then it might be possible to produce the same modifications of membrane components by treating brain membranes in different ways. Therefore, a number of experiments were performed to alter membrane structures and integrity to simulate the effect of EDTA pre-

Figure 4. Dissociation kinetics of [3 H]-D-Ala enk binding to nontreated and EDTA-pretreated membranes. Membranes were preincubated in the presence and absence of EDTA as described under "Materials and Methods," washed, and incubated to equilibrium (40 min) with 1.0 nM [3 H]-D-Ala enk. Dissociation was initiated by addition of 1 μ M levallorphan plus 0.5 μ M Gpp(NH)p, and membranes were filtered at designated time intervals. Results are the percentage of specific binding of [3 H]-D-Ala enk at equilibrium in nontreated membranes. ●, dissociated in nontreated membranes; ○, dissociated in EDTA-pretreated membranes.

treatment. One interesting technique involved pretreatment of membranes at low pH. Membranes were preincubated in buffers of various pH values, the reactions were terminated by dilution in regular Tris buffer (pH 7.7), and binding assays were conducted at the normal pH (7.7) at 25°C. Between pH 4.5 and 8.0, preincubation at different pH values had no appreciable effect on binding of [3 H]-D-Ala enk (data not shown). However, after pretreatment of membranes at pH 4.5, a significant increase in the inhibition of [3 H]-D-Ala enk binding by guanine nucleotides was observed (Fig. 5). In control membranes, Gpp(NH)p produced a maximum 35% decrease in [3 H]-D-Ala enk binding with an IC_{50} value of approximately 2 μ M Gpp(NH)p. In low pH-pretreated membranes, the maximum inhibition by Gpp(NH)p was increased to 80% with an IC_{50} value of approximately 0.8 μ M Gpp(NH)p. Thus, the affinity for Gpp(NH)p was slightly increased (less than that observed in the EDTA-pretreated membranes), whereas the maximum effect of Gpp(NH)p was dramatically increased.

Like the actions of EDTA pretreatment on guanine nucleotide regulation of binding, the effects of low pH pretreatment occurred with several opiate agonists (including [3 H]morphine and [3 H]DADLE as well as [3 H]-D-Ala enk) and were observed when both GTP and GDP were used to inhibit agonist binding (data not shown). Moreover, GTP had no effect on [3 H]naloxone binding (assayed in the presence of 100 mM NaCl) in low pH-pretreated membranes. In addition, the actions of the low pH pretreatment were not reversed by multiple wash steps at normal pH or by incubating with divalent cations or with guanine nucleotides after low pH pretreatment (data not shown). Preincubation of membranes at pH values between pH 4.5 and pH 7.7 provided an abrupt effect: the increased GTP inhibition of binding which was evident after treatment at pH

4.5 could not be detected after treatment above pH 5.0 (Table IV). Pretreatments below pH 4.5 caused irreversible loss of 3 H-agonist binding (not shown).

Since both EDTA and low pH pretreatments increased the regulation of agonist binding by guanine nucleotides, these effects were directly compared by treating membranes by both techniques. Membranes were first preincubated with 1 mM EDTA, then diluted with 50 mM sodium acetate, pH 4.5, and incubated at 25°C for 30 min. Results (Fig. 6) showed that the two effects were additive. In control membranes, Gpp(NH)p inhibited [3 H]morphine binding by 40% at maximum effect, with an IC_{50} of 1.0 μ M Gpp(NH)p. In membranes pretreated at pH 4.5, maximum inhibition was increased to 80% with an IC_{50} of 0.8 μ M Gpp(NH)p. In membranes pretreated with 1 mM EDTA and then at low pH, the maximum inhibition effect of Gpp(NH)p remained at 80% inhibition, but the IC_{50} was decreased to 0.3 μ M Gpp(NH)p. Therefore, the EDTA effect in increasing the potency of guanine nucleotide regulation of binding was apparent even in membranes treated at low pH to increase maximum inhibition of guanine nucleotides.

Discussion

These experiments had two principal goals: first, to examine the effects of divalent cations bound to brain membranes on the interaction of opiate receptors with guanine nucleotide-regulatory components (N-proteins), and second, to modify the interaction between receptors and regulatory components so that the relationship between guanine nucleotide regulation of binding and opiate receptor function could be explored. Both of these points were explored by preincubation of rat brain membranes with EDTA to remove endogenous divalent cations. This treatment had no effect on binding when the EDTA was

Figure 5. Effect of low pH pretreatment of rat brain membranes on Gpp(NH)p inhibition of [3 H]-D-Ala enk binding. Membranes were incubated either in 50 mM Tris-HCl, pH 7.7, or in 50 mM sodium acetate, pH 4.5, for 20 min at 25°C. Reactions were terminated by dilution in Tris buffer, and membranes were washed once and assayed for binding with 0.9 nM [3 H]-D-Ala enk in the presence of 0 to 20 μ M Gpp(NH)p. Results represent percentage of specific binding in membranes incubated at pH 7.7. ●, control membranes; ○, low pH-pretreated membranes.

TABLE IV

Effect of preincubation at various pH values on Gpp(NH)p regulation of [3 H]morphine binding

Rat brain membranes were preincubated at 25°C for 20 min in buffers of the indicated pH values (50 mM Tris-HCl for pH 7.7; 50 mM sodium acetate for pH 4.5 to 6.5), washed with 50 mM Tris-HCl, pH 7.7, and assayed for [3 H]morphine binding in the presence of 0.1 to 10 μ M Gpp(NH)p. Results are data from a typical experiment which was repeated twice.

pH Pretreatment	Gpp(NH)p IC ₅₀ μ M	Percentage Bound with 10 μ M Gpp(NH)p
7.7	1.2	54
6.5	1.6	53
5.5	1.0	54
4.5	0.8	20

carefully washed away, but it significantly increased the potency of guanine nucleotides in inhibiting opiate agonist binding. Since studies with other systems revealed that divalent cations antagonized the effect of guanine nucleotides on binding regulation (Rouot et al., 1980), the present results would tend to support such a role because removal of antagonizing cations might be expected to increase the effect of guanine nucleotides. However, it is clear that such a simplistic explanation of the EDTA effect cannot explain the results. If removal of cations were the only effect occurring during EDTA incubation, then the effect should be reversed by adding divalent cations in excess of EDTA concentrations used. In fact, reversal of the EDTA effect by cations could only occur if the reversal took place within 10 min after addition of EDTA; longer incubation periods produced an EDTA effect which was completely irreversible by divalent cations. On the other hand, the

effect of EDTA on GTP potency was somehow related to the chelating properties of EDTA since the EDTA effect could be blocked by prior incubation of membranes with excess manganese or calcium. Moreover, this prior blockade occurred only with those cations for which EDTA has high affinities: for Ca^{2+} and Mn^{2+} , the pK_a values are 10.7 and 13.9, respectively, whereas the affinity of EDTA for Mg^{2+} , which was not effective in blocking the EDTA effect, is two orders of magnitude less than that of calcium ($\text{pK}_a = 8.7$). In addition, EGTA, with affinities for Mn^{2+} and Ca^{2+} similar to those of EDTA, mimicked the EDTA effect, whereas 1,10-phenanthroline, with low affinity for Ca^{2+} and Mn^{2+} , was ineffective. The actions of EDTA also are not the result of inhibition of membrane-bound phosphatase activity, since the effect was observed if either GTP or Gpp(NH)p was utilized in binding assays.

These data are consistent with the suggestion that EDTA increases the potency of guanine nucleotides in inhibiting opiate agonist binding indirectly by removing divalent cations from membrane sites. The act of cation removal may eventually produce irreversible changes in the interaction of N-proteins with receptors, perhaps by causing a conformational change in the N-proteins themselves or by denaturing an unknown membrane component that regulates the interaction of receptors with N-proteins. This suggestion is supported not only by the irreversibility of the EDTA effect but also by the time course of the effect, which occurred within 10 min. If EDTA acted directly on divalent cations without affecting other components, the EDTA effect might be observed within seconds, since the formation of the chelate complex is extremely rapid. An effect that takes several minutes to develop is consistent with a slower process such as protein conformational changes, or physical removal of proteins or lipids from integral membrane sites. The EDTA time course may also be a function of membrane kinetics: the rate-determining step may be the penetration of EDTA into inner membrane sites.

Figure 6. Effect of EDTA and low pH pretreatments together in rat brain membranes on Gpp(NH)p inhibition of [3 H]morphine binding. Membranes were incubated either in Tris buffer alone or with 1 mM EDTA for 30 min at 25°C. Membranes were then diluted in 50 mM Tris-HCl, pH 7.7, or with 50 mM sodium acetate, pH 4.5, for 20 min at 25°C. After washing twice in Tris buffer, membranes were assayed for binding with 0.8 nM [3 H]morphine with 0 to 20 μ M Gpp(NH)p. Results represent percentage of specific binding in control membranes. ●, control membranes; ■, low pH-pretreated membranes; □, membranes treated with EDTA, then low pH buffer.

Whatever the cause of the EDTA effect on guanine nucleotide sensitivity, the result of the membrane treatment is clear. The site of EDTA effect is probably not at the receptor-binding site, since EDTA pretreatment did not produce any appreciable change in agonist binding. Instead, the effect of EDTA pretreatment was confined to the interactions of guanine nucleotides with agonist-binding sites. The actions of EDTA do not affect the maximum inhibition caused by guanine nucleotides but do increase the potency of the nucleotide effects. These results suggest that the change in the N-protein caused by EDTA is associated with an increased affinity for GTP. The decrease in the slope of Gpp(NH)p displacement curves (Fig. 1) may indicate the appearance of a higher affinity class of GTP-binding sites in addition to the normal micromolar affinity class, with a concomitant increase in GTP site heterogeneity. Such findings may perhaps be analogous to the classical effects of GTP on β -adrenergic agonist binding where, by decreasing agonist affinity, GTP changes a heterogeneous agonist displacement curve containing high and low affinity sites to a homogeneous curve with only low affinity sites (Hoffman and Lefkowitz, 1980). By analogy, EDTA pretreatment could cause the appearance of high affinity GTP-binding sites like the β -adrenergic agonist sites seen in the absence of GTP. As discussed above, such changes could result either from direct conformational changes in the N-proteins themselves or from removal (denaturation) of components which normally act to modulate the affinity of N-proteins for GTP. Based on such schemes, we attempted to reverse the EDTA effect by adding back supernatants prepared from membranes during the EDTA incubation, with the idea of reconstituting a GTP-modulating system. These experiments were not successful (S. M. Lambert and S. R. Childers, unpublished results), so that the EDTA effect remains irreversible by a variety of techniques.

The interaction of guanine nucleotide-regulatory components with receptors could also be modified by low pH pretreatment of brain membranes. Superficially, the effect of EDTA and low pH pretreatment was the same: both treatments in-

creased the actions of guanine nucleotides on inhibiting opiate agonist binding. Moreover, the effects of low pH pretreatment were confined to guanine nucleotide interactions with agonist binding, since preincubation at pH 4.5 did not significantly affect 3 H-agonist binding itself. However, the details of the effects are quite different. Instead of increasing potency, low pH pretreatment increased the maximum effectiveness of GTP in inhibiting binding. Again, the effect of low pH pretreatment was not due to inhibition of phosphatases since the effect occurred for both GTP and Gpp(NH)p. Moreover, like the EDTA effect, the effect of low pH pretreatment could not be reversed by washing in normal pH buffers or by cations or nucleotides. Therefore, this action was consistent with the idea that low pH causes denaturation or removal of some membrane component that regulates the interaction of receptors with guanine nucleotide-regulatory components, or alters the conformation of the N-proteins themselves in such a way as to increase the actions of guanine nucleotides. In contrast to the effect of EDTA which increases the affinity of the system for GTP, the actions of low pH pretreatment appear to increase the overall efficacy of the regulatory component with only a small increase in the affinity of the system for GTP. Perhaps low pH pretreatment has removed a modulatory component to allow greater coupling of receptors with regulatory components, or perhaps the N-proteins themselves have changed conformation to allow more efficient coupling. Whatever the mechanism, these experiments demonstrate that membranes can be changed in ways that do not alter receptor-binding sites themselves but can significantly change the interaction between receptors and membrane-regulatory components.

Unfortunately, these studies do not provide any clues as to the effects of these changes on opiate receptor function in membranes; i.e., the ability of opiate agonists to produce a post-receptor response. To explore the effects of these changes on membrane function, the next paper in this series (Childers and La Riviere, 1984) will examine the effects of modification of guanine nucleotide-regulatory components on the activities of

receptor-activated and -inhibited adenylate cyclase in brain membranes.

References

- Blume, A. J. (1978a) Opiate binding to membrane preparations of neuroblastoma-glioma hybrid cells NG108-15: Effects of ions and nucleotides. *Life Sci.* 22: 1843-1852.
- Blume, A. J. (1978b) Interactions of ligands with opiate receptors of brain membranes: Regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 75: 1713-1717.
- Chang, K. J., E. Hazum, A. Killian, and P. Cuatrecasas (1981) Interactions of ligands with morphine and enkephalin receptors are differentially affected by guanine nucleotides. *Mol. Pharmacol.* 20: 1-7.
- Childers, S. R., and G. LaRiviere (1984) Modification of guanine nucleotide-regulatory components in brain membranes. II. Relationship of guanosine 5'-triphosphate on opiate receptor binding and coupling receptors with adenylate cyclase. *J. Neurosci.* 4: 2764-2771.
- Childers, S. R., and S. H. Snyder (1978) Guanine nucleotides differentiate agonist and antagonist interactions with opiate receptors. *Life Sci.* 23: 759-762.
- Childers, S. R., and S. H. Snyder (1980) Differential regulation by guanine nucleotides of opiate agonist and antagonist receptor interactions. *J. Neurochem.* 34: 583-593.
- Creese, I., T. B. Usdin, and S. H. Snyder (1979) Dopamine receptor binding regulated by guanine nucleotides. *Mol. Pharmacol.* 16: 69-76.
- Hoffman, B. B., and R. J. Lefkowitz (1980) Radioligand binding studies of adrenergic receptors: New insights into molecular and physiological regulation. *Annu. Rev. Pharmacol. Toxicol.* 20: 581-608.
- Kragten, J. (1978) *Atlas of Metal-Ligand Equilibria in Aqueous Solution*, Ellis Horwood Ltd., Chichester, England.
- Limbird, L. E., D. M. Gill, and R. J. Lefkowitz (1980) Agonist promoted coupling of the beta-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. U. S. A.* 77: 775-779.
- Mukherjee, C., M. G. Caron, and R. J. Lefkowitz (1975) Catecholamine-induced subsensitivity of adenylate cyclase associated with loss of beta-adrenergic receptor binding sites. *Proc. Natl. Acad. Sci. U. S. A.* 72: 1945-1949.
- Pasternak, G. W., and S. H. Snyder (1975) Opiate receptor binding: Enzymatic treatments that discriminate between agonist and antagonist interactions. *Mol. Pharmacol.* 11: 478-484.
- Pasternak, G. W., A. M. Snowman, and S. H. Snyder (1975) Selective enhancement of [³H]-opiate agonist binding by divalent cations. *Mol. Pharmacol.* 11: 735-744.
- Pert, C. B., and S. H. Snyder (1974) Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.* 10: 868-879.
- Rodbell, M. (1980) The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* 284: 17-21.
- Rodbell, M., H. M. J. Krans, S. L. Phol, and L. Birnbaumer (1971) The glucagon-sensitive adenylate cyclase system in plasma membranes of rat liver. *J. Biol. Chem.* 246: 1872-1976.
- Rouot, B. M., D. C. U'Prichard, and S. H. Snyder (1980) Multiple alpha(2)-noradrenergic receptor sites in rat brain: Selective regulation of high-affinity [³H]clonidine binding by guanine nucleotides and divalent cations. *J. Neurochem.* 34: 374-384.
- Simantov, R., and S. H. Snyder (1976) Morphine-like peptides, leucine enkephalin and methionine enkephalin: Interactions with the opiate receptor. *Mol. Pharmacol.* 12: 987-998.
- Simon, E. J., J. M. Hiller, J. Groth, and I. Edelman (1975) Further properties of stereospecific opiate binding sites in rat brain: On the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* 192: 531-537.
- U'Prichard, D. C., and S. H. Snyder (1978) Guanyl nucleotide influences on ³H-ligand binding to α -noradrenergic receptors in calf brain membranes. *J. Biol. Chem.* 253: 3444-3452.
- Zukin, R. S., and A. R. Gintzler (1980) Guanyl nucleotide interactions with opiate receptors in guinea pig brain and ileum. *Brain Res.* 186: 486-491.
- Zukin, R. S., S. Walczak, and M. H. Makman (1980) GTP modulation of opiate receptors in regions of rat brain and possible mechanism of GTP action. *Brain Res.* 186: 238-244.