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MODIFICATION OF GUANINE NUCLEOTIDE-REGULATORY COMPONENTS IN BRAIN MEMBRANES

II. Relationship of Guanosine 5′-Triphosphate Effects on Opiate Receptor Binding and Coupling Receptors with Adenylate Cyclase[1]

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Abstract

Guanine nucleotides couple receptors to stimulate or inhibit adenylate cyclase as well as regulate binding of neurotransmitters. To explore the relationship between these different functions of guanosine 5′-triphosphate (GTP), rat brain membranes were preincubated in 50 mM sodium acetate, pH 4.5, which increased GTP regulation of ^{3}H-opiate agonist binding. Assay of adenylate cyclase in the low pH-pretreated membranes revealed no loss of basal activity but a dramatic loss in fluoride- and guanylyl-5′-imidodiphosphate-stimulated activity, thus suggesting a loss in stimulatory guanine nucleotide coupling function. Manganese stimulation, which presumably occurs on the catalytic subunit of adenylate cyclase directly, was not affected by low pH treatment. In striatum, dopamine-stimulated adenylate cyclase was eliminated, but inhibition of adenylate cyclase by D-Ala^2-Met^5-enkephalinamide (D-Ala enk) was increased by low pH treatment. The effect of low pH on sodium fluoride-stimulated and D-Ala enk-inhibited adenylate cyclase could be reversed by addition of either *cis*-vaccenic acid or phosphatidylcholine to treated membranes, but the effect on GTP regulation of binding was not reversed by lipid incorporation. These results suggest that fundamental differences exist between membrane components which couple receptors to adenylate cyclase and those that regulate neurotransmitter binding.

Guanine nucleotides perform dual roles in neurotransmitter function by coupling receptors to adenylate cyclase and by regulating binding of neurotransmitters to receptors (Rodbell, 1980). Moreover, in recent years it has become clear that neurotransmitters can affect adenylate cyclase in two ways: either to stimulate or to inhibit formation of cAMP. These two latter classes of GTP function may be mediated by separate guanine nucleotide-binding proteins: N_s for stimulatory receptors and N_i for inhibitory receptors (Rodbell, 1980). Although N_s has been well characterized in several systems as a GTP-binding protein and a GTPase (Cassel and Selinger, 1978), less information is known about N_i. Several recent studies (Jakobs and Schultz, 1983; Jakobs et al., 1983) have provided evidence that N_s and N_i represent different proteins which can at least be differentiated by labeling with different toxins: cholera toxin for N_s (Cassel and Pfeuffer, 1978) and pertussis toxin for N_i (Katada and Ui, 1982). However, the precise relationship between these two proposed proteins is not clear, nor is the mechanism by which these subunits interact with various receptors to regulate binding understood in any detail.

Several kinds of membrane treatments can alter the coupling of adenylate cyclase with neurotransmitter receptors through N_s. For example, N_s activity in brain membranes can be inactivated by heat or can be removed from membrane sites by incubation with colchicine (Rasenick et al., 1981). In erythrocytes, coupling efficiency of N_s can be increased by addition of fatty acids to decrease membrane microviscosity (Hanski et al., 1979), while in brain, N_s-stimulated adenylate cyclase can be increased by phospholipase A_2 (Reese and Hoss, 1983).

The finding that treatment of brain membranes at pH 4.5 increased guanine nucleotide regulation of ^{3}H-opiate agonist binding (Lambert and Childers, 1984) has provided an interesting opportunity to explore the relationships between different functions of GTP. The function of N_s in these membranes can be studied by assay of adenylate cyclase in the presence of sodium fluoride or guanylyl-5′-imidodiphosphate (Gpp(NH)p, the nonhydrolyzable analogue of GTP), agents which stimulate the enzyme by interacting directly at N_s (Ross et al., 1978; Rasenick and Bitensky, 1980). The function of N_i can be studied by assaying adenylate cyclase in the presence of opiate agonists which have been shown to inhibit the enzyme with GTP-dependent reactions in brain membranes (Collier and Roy, 1974; Law et al., 1981; Cooper et al., 1982) and in neuroblastoma × glioma hybrid cells (Klee and Niremberg, 1976; Goldstein et al., 1977; Blume et al., 1979; Law et al., 1982;

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McGee and Kenimer, 1982). The present study explores the action of these agents on adenylate cyclase activity in both normal and low pH-pretreated rat brain membranes.

Materials and Methods

Adenylate cyclase assay. Whole brains from male Sprague-Dawley rats (150 to 180 gm) were homogenized in 20 vol of cyclase buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and 2 mM EDTA) in a Teflon-glass Potter-Elvejhem homogenizer, centrifuged at 48,000 × *g* for 10 min, and resuspended in 10 vol of cyclase buffer or pH 4.5 buffer (50 mM sodium acetate, pH 4.5, containing 5 mM $MgCl_2$, 2 mM EDTA, and 1 mM dithiothreitol). Membranes were incubated for 10 to 20 min on ice, 10 ml of cyclase buffer (pH 7.4) were added, and tubes were centrifuged at 48,000 × *g* for 10 min. Membranes were resuspended in cyclase buffer and added to assay tubes which contained 20 mM creatine phosphate, 10 units of creatine phosphokinase, 10 mM theophylline, 700 μg of bovine serum albumin, 1 mM ATP along with 100 to 200 μg of membrane protein and various drug additions in a total volume of 100 μl. Reactions were initiated by addition of ATP, and tubes were incubated at 30°C for 10 min and immersed in boiling water for 2 min. After adding 400 μl of cAMP assay buffer (50 mM Tris-HCl, 8 mM theophylline, 1 mM dithiothreitol, pH 7.4), assay tubes were centrifuged at 1,000 × *g* for 15 min, and aliquots of the supernatants were removed for cAMP assay by a modification (Brostrom and Kon, 1974) of the method of Brown et al. (1971), utilizing bovine adrenal cortex as a source of cAMP-binding protein and [^{3}H]cAMP as ligand. Adenylate cyclase tubes prepared in triplicate were assayed for cAMP content in duplicate, and the results were mean values of six cAMP determinations. Results were calculated from standard curves using six concentrations of unlabeled cAMP, with the limit of sensitivity approximately 0.1 pmol of cAMP. Aliquots from adenylate cyclase assays were chosen so that cAMP content would occur approximately in the middle of the standard curve. All additions to adenylate cyclase assays, including nucleotides and drugs, were checked for possible interference in the cAMP-binding protein assay, and all were found to have no significant effect at the concentrations used.

For determination of opiate-inhibited adenylate cyclase, striata were dissected from fresh rat brains, homogenized, and treated at low pH as described above for whole brain membranes. Adenylate cyclase assay tubes contained 50 μM GTP and 100 mM NaCl in addition to the components listed above; other assay steps were identical to those in whole brain.

For lipid reversal experiments, membranes from whole brain or striatum were homogenized and treated at low pH as described above. Membranes were added to assay tubes containing cyclase buffer, regeneration system, and lipid (either *cis*-vaccenic acid or phosphatidylcholine, prepared by dissolving 100 mg of lipid in 1 ml of ethanol, diluting in cyclase buffer, and sonicating the suspension for 30 sec). (The total quantity of ethanol added to membranes during these experiments was less than 2% and had no effect on adenylate cyclase activity.) The tubes were incubated at 25°C for 20 min and then placed on ice. The remaining assay components were added to the tubes, ATP was added to initiate the reaction, and tubes were assayed for cAMP content as described above.

Assay of membrane fluidity. Microviscosity of brain membranes was estimated by the fluorescence polarization method of Hanski et al. (1979). Membranes treated at normal and low pH, then incubated in the presence and absence of lipids as described above, were diluted 10-fold in cyclase buffer. The fluorescent hydrocarbon, 1,6-diphenyl-1,3,5-hexatriene (DPH) was dissolved in tetrahydrofuran, added to a stirring solution of cyclase buffer (Shinitzky et al., 1971) at a concentration of 6 mM, and stirred for 45 min. DPH (100 μl) was added to 900 μl of membranes and the suspension was incubated at 30°C for 30 min. After centrifugation at 48,000 × *g* for 10 min, membranes were suspended in fresh cyclase buffer, fluorescence measurements were made, and microviscosity calculations were determined according to the method of Hanski et al. (1979). All measurements were performed in triplicate and all experiments were repeated at least three times.

Other assays. Determination of opiate receptor binding was performed as previously described (Lambert and Childers, 1984), using [^{3}H]-D-Ala^2-Met^5-enkephalinamide (D-Ala enk) as ligand and GTP at concentrations ranging from 0.5 to 50 μM for determination of guanine nucleotide regulation of opiate agonist binding. Protein concentration was determined by the method of Lowry et al. (1951).

Materials. [^{3}H]cAMP (12.6 Ci/mmol) was obtained from ICN (Irvine, CA); [^{3}H]-D-Ala enk (29.1 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). GTP was obtained from Calbiochem (La Jolla, CA), and other nucleotides, creatine phosphate, creatine phosphokinase, DPH, *cis*-vaccenic acid, and phosphatidylcholine were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Results

Effect of low pH treatment on stimulation of adenylate cyclase by N_s. As demonstrated in the previous paper in this series (Lambert and Childers, 1984), treatment of brain membranes at pH 4.5 increased regulation of opiate receptor binding by guanine nucleotides. To explore the effect of this low pH treatment on other guanine nucleotide functions associated with neurotransmitter receptors, adenylate cyclase was assayed in the presence and absence of sodium fluoride and Gpp(NH)p, which stimulate adenylate cyclase directly through the N_s subunit. In preliminary studies, pretreatment of brain membranes at pH 4.5 caused appreciable loss (greater than 40%) in basal adenylate cyclase activity. Later experiments prevented the loss of basal activity by incubation at pH 4.5 on ice instead of at room temperature, and by including magnesium and dithiothreitol in the pH 4.5 buffer. This buffer continued to produce the same increase in guanine nucleotide regulation of opiate agonist binding as the previous low pH treatment (data not shown).

Figure 1 shows the results of pH 4.5 preincubation on adenylate cyclase activity in whole brain membranes. In control (treated at pH 7.4) preparations, sodium fluoride- and Gpp(NH)p-stimulated activity increased to 195% and 250% of basal levels, respectively, while manganese, which bypasses the

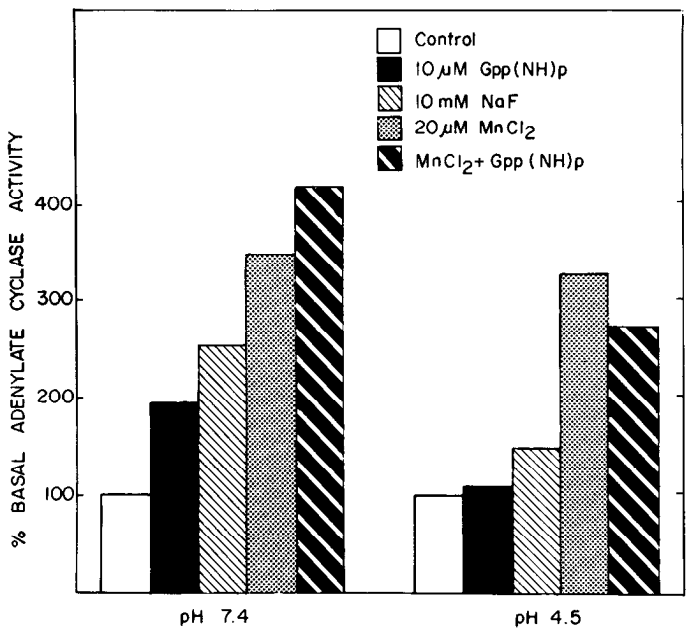

Figure 1. Assay of adenylate cyclase activity in whole rat brain membranes before and after pretreatment of pH 4.5. Crude membranes were incubated either in cyclase buffer, pH 7.4, or in low pH buffer (50 mM sodium acetate, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 4.5) on ice for 10 min as described under "Materials and Methods." After washing in cyclase buffer, membranes were assayed for adenylate cyclase activity at pH 7.4 in the presence of 10 μM Gpp(NH)p, 10 mM sodium fluoride, 20 μM $MnCl_2$, or Gpp(NH)p plus $MnCl_2$. Results are the means of six different determinations of cAMP and are expressed as percentage of basal activity in control membranes. Control adenylate cyclase activity was 68 pmol of cAMP/min/mg.

N_s subunit and stimulates the catalytic unit of adenylate cyclase directly (Limbird et al., 1978), increased activity to 350% of basal levels. In pH 4.5-treated membranes, basal activity was not significantly changed compared to control membranes. However, much of sodium fluoride- and Gpp(NH)p-stimulated adenylate cyclase was lost, with increases to only 105% and 140%, respectively. On the other hand, manganese stimulation was largely unchanged compared to control preparations, with an increase to 340% of basal activity. When adenylate cyclase was assayed in the presence of both Gpp(NH)p and manganese, the increase was to 290%, a level which was even less than manganese-stimulated activity alone. These results suggest that the low pH treatment caused a selective loss in N_s-stimulated adenylate cyclase, with no significant loss in catalytic unit activity.

The time course for the low pH effect on Gpp(NH)p-stimulated adenylate cyclase was rapid (Fig. 2). Although no significant loss of stimulated activity could be seen after 2 min of preincubation at pH 4.5, full loss of activity was seen after 5 min, with no further loss in activity up to 30 min of preincubation. Beginning at 30 min, significant loss of basal activity (greater than 20%) began to be observed (data not shown); therefore, most experiments utilized a 10-min incubation time for determination of low pH effects.

Effect of low pH treatment on adenylate cyclase in rat striatum. Although whole brain membranes were useful in determining effects on agents that stimulate N_s directly (such as sodium fluoride and Gpp(NH)p), specific brain regions must be utilized to study effects of low pH treatment on specific neurotransmitter receptor-coupled adenylate cyclase. Therefore, membranes were prepared from rat striatum to determine activity of dopamine-stimulated and opiate-inhibited adenylate cyclase. Results (Fig. 3) demonstrated that, as in whole brain membranes, the low pH pretreatment did not reduce basal adenylate cyclase activity. In control striatal membranes, sodium fluoride stimulated activity to 220% of basal levels, while dopamine increased activity to 140%. In our hands, opiate agonists had very little effect on basal adenylate cyclase activity in untreated crude membranes from striatum; an inhibition to 90% of basal levels was a typical finding. In low pH-pretreated membranes, as in whole brain, sodium fluoride-stimulated activity was decreased to 105%; Gpp(NH)p-stimulated activity was similarly decreased (not shown). In addition, dopamine-stimulated adenylate cyclase was completely eliminated in low pH-pretreated striatum. However, opiate inhibition of adenylate cyclase became very evident in treated membranes: 10 μM D-Ala enk inhibited activity to 60% of basal levels. Thus, as the function of N_s was attenuated by treatment at low pH, the function of N_i appeared to be increased.

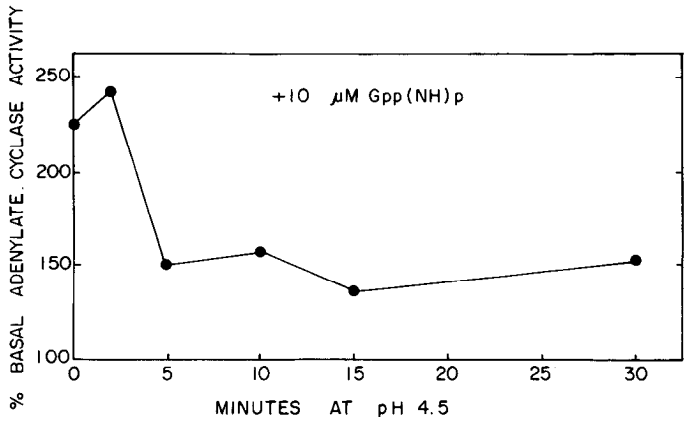

Figure 2. Time course of low pH-induced decrease in Gpp(NH)p-stimulated adenylate cyclase in rat brain membranes. Membranes were incubated in pH 4.5 buffer on ice for various periods of time, washed with cyclase buffer, and assayed for adenylate cyclase activity at pH 7.4 in the presence and absence of 10 μM Gpp(NH)p. Results are expressed in terms of percentage of basal activity in control membranes (72 pmol/min/mg).

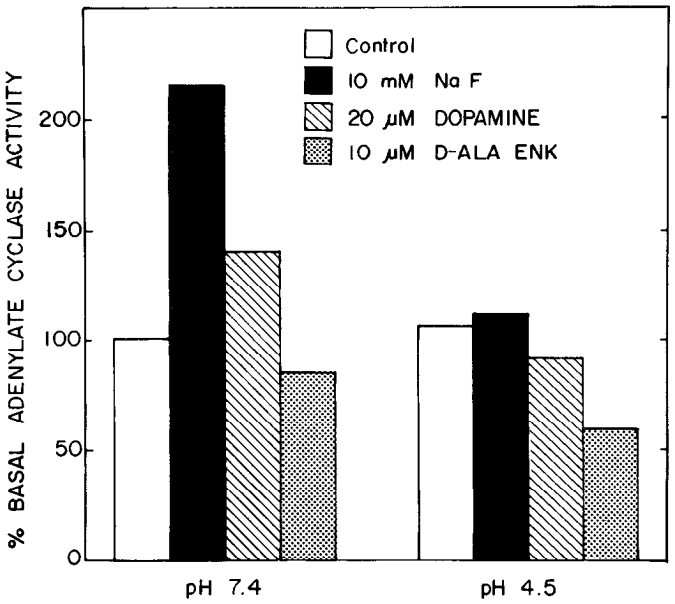

Figure 3. Effect of low pH pretreatment on adenylate cyclase activity in rat striatal membranes. Crude striatal membranes were pretreated at pH 7.4 or 4.5, washed, and assayed at pH 7.4 in the presence of 10 mM sodium fluoride, 20 μM dopamine, or 10 μM D-Ala enk. Results are expressed as percentage of basal activity in pH 7.4-treated membranes (86 pmol of cAMP/mg/min).

A dose response curve for D-Ala enk inhibition of adenylate cyclase in low pH-pretreated striatal membranes is seen in Figure 4. These results demonstrate that D-Ala enk produced a maximum inhibition to 60% of basal activity, with an ED_{50} of approximately 0.2 μM. This affinity is similar to those previously observed for opiate inhibition of adenylate cyclase in brain membranes (Law et al., 1981) and neuroblastoma × glioma cells (Blume et al., 1979). The dose response curve for D-Ala enk was shifted to the right in the presence of 0.2 μM naloxone, which completely antagonized enzyme inhibition up to 1 μM D-Ala enk. Therefore, the inhibition caused by D-Ala enk appears to be genuinely mediated through opiate receptors.

Reversal of low pH effects on adenylate cyclase with lipid incorporation. Several possible mechanisms could be responsible for the apparent loss of N_s function in brain membranes after pretreatment at pH 4.5. One explanation could be that low pH removes some fraction of N_s proteins from membrane sites. However, preliminary attempts to reconstitute membranes with supernatants from low pH incubations failed to restore any significant stimulation of adenylate cyclase by sodium fluoride or Gpp(NH)p (data not shown). Another possibility is that low pH could be denaturing membrane components and altering membrane fluidity to decrease coupling of N_s with adenylate cyclase. Experiments with β-adrenergic receptors in erythrocytes have shown that coupling of receptors with adenylate cyclase depends on membrane fluidity and that membrane-fluidizing agents such as *cis*-vaccenic acid can increase coupling (Hanski et al., 1979). To explore this possibility, whole brain membranes pretreated at pH 4.5 were incubated with different concentrations of *cis*-vaccenic acid and then assayed for sodium fluoride-stimulated adenylate cyclase activity. Results (Fig. 5) showed that addition of 1 μmol of *cis*-vaccenic acid/mg of protein to low pH-pretreated membranes

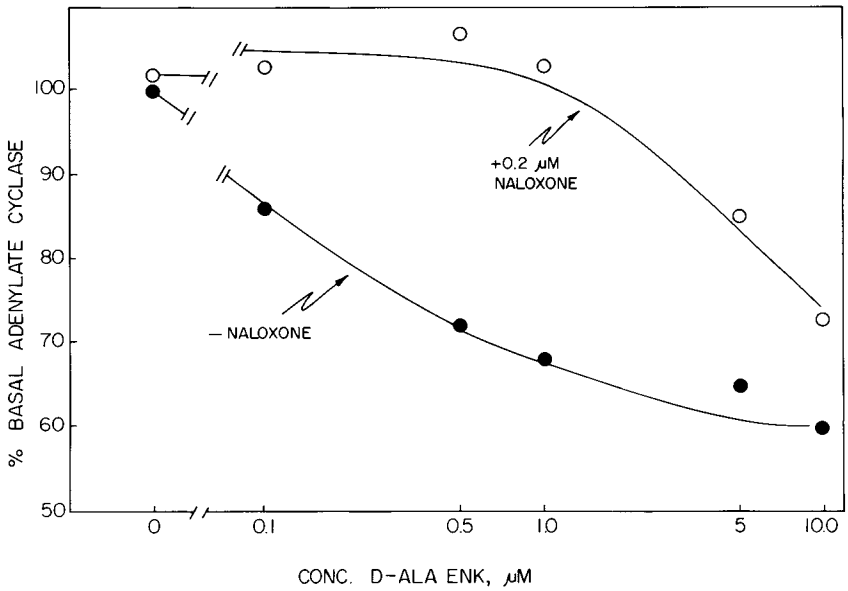

Figure 4. Opiate-inhibited adenylate cyclase in rat striatum after pretreatment at pH 4.5. Striatal membranes were incubated at pH 4.5 as described in the legend to Figure 1, washed, and assayed at pH 7.4 in the presence of various concentrations of D-Ala enk with (O) and without (●) 0.2 μM naloxone. Results are expressed as percentage of basal activity in the absence of D-Ala enk (81 pmol of cAMP/mg/min).

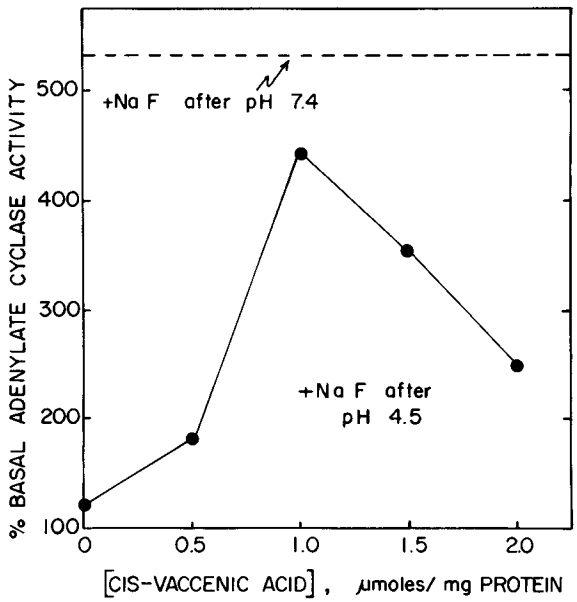

Figure 5. Effect of *cis*-vaccenic acid on low pH-induced decrease in sodium fluoride-stimulated adenylate cyclase in whole brain membranes. Membranes were pretreated at pH 4.5 as described in the legend to Figure 1, washed, and placed in adenylate cyclase assay tubes with the indicated concentrations of *cis*-vaccenic acid. Tubes were incubated for 20 min at 25°C, cooled on ice, and then assayed for adenylate cyclase activity as described under "Materials and Methods." Results are expressed as percentage of activity in control membranes (63 pmol of cAMP/mg/min).

restored most of the sodium fluoride-stimulated activity observed in control membranes. Higher concentrations of *cis*-vaccenic acid were less effective in reversing the low pH effect, and 2.0 μmol/mg of *cis*-vaccenic acid actually inhibited basal activity by 30% as well (not shown). Incubation of control brain membranes themselves with *cis*-vaccenic acid had no reproducible effect on either basal adenylate cyclase activity or on activity stimulated by sodium fluoride (data not shown).

An important question was whether *cis*-vaccenic acid could also reverse the low pH effect on guanine nucleotide regulation of opiate receptor binding in brain membranes. Experiments were performed on low pH-treated membranes, assaying both adenylate cyclase and [^{3}H]-D-Ala enk binding in the same membrane preparations (Fig. 6). As seen earlier (Fig. 5), *cis*-vaccenic acid had no effect on sodium fluoride-stimulated activity in control membranes, but in low pH-pretreated membranes, *cis*-vaccenic acid restored sodium fluoride-stimulated adenylate cyclase nearly to control levels (Fig. 6*A*). In contrast, addition of *cis*-vaccenic acid had no significant effect on opiate receptor binding (Fig. 6*B*). The effect of 10 μM Gpp(NH)p was increased from 36% to 60% inhibition of [^{3}H]-D-Ala enk binding by low pH pretreatment; the Gpp(NH)p effect remained at 60% inhibition after incorporation of *cis*-vaccenic acid into low pH-treated membranes. *cis*-Vaccenic acid had no effect on binding itself or on Gpp(NH)p inhibition of binding in control membranes (Fig. 6*B*). It is important to note that cyclase and binding assays were accomplished in the same membranes; thus, low pH-pretreated membranes which had their N_s function restored by lipid incorporation still exhibited an increase in GTP regulation of agonist binding.

The next question was whether lipid incorporation could reverse the increased opiate inhibition of adenylate cyclase caused by low pH pretreatment. Studies on whole brain revealed that, in addition to *cis*-vaccenic acid, phosphatidylcholine also restored sodium fluoride-stimulated adenylate cyclase activity in pH 4.5-pretreated membranes (not shown). Therefore, adenylate cyclase was assayed in low pH-pretreated striatal membranes in the presence and absence of phosphatidylcholine. Results (Fig. 7) showed that, as before, inhibition of adenylate cyclase by D-Ala enk was minimal in control membranes and was significant (52% of basal levels) in pH 4.5-pretreated membranes. Sodium fluoride-stimulated activity again was lost after low pH pretreatment. In low pH-pretreated membranes incubated with 1 μmol of phosphatidylcholine/mg of protein, sodium fluoride-stimulated activity was restored to

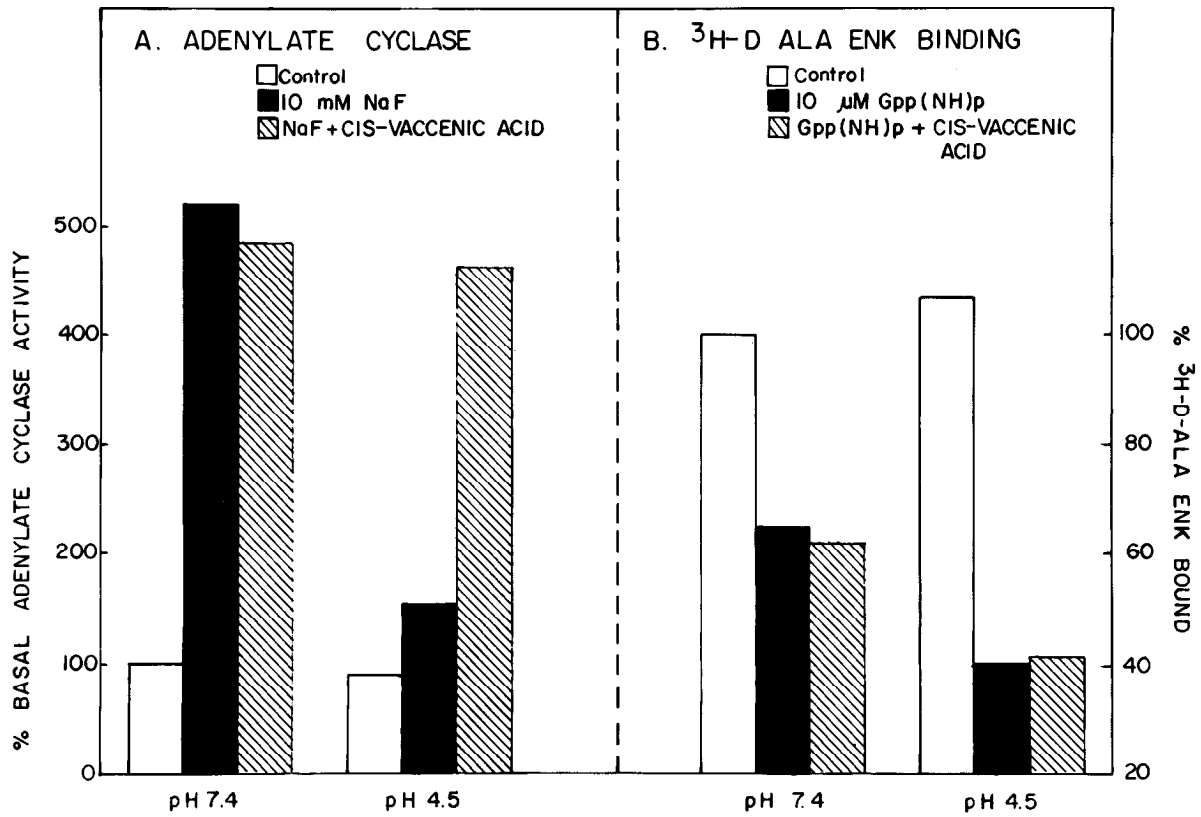

Figure 6. Effect of *cis*-vaccenic acid incorporation on adenylate cyclase activity (*A*) and [^{3}H]-D-Ala enk binding (*B*) in whole brain membranes. Membranes were incubated at pH 7.4 or 4.5, washed, incubated with 1 μM *cis*-vaccenic acid as described in the legend to Figure 5, and then divided into two aliquots for adenylate cyclase and receptor-binding assays. For the adenylate cyclase assay, membranes were resuspended in cyclase buffer and assayed in the presence of 10 mM sodium fluoride; results are expressed as percentage of basal activity in control membranes (82 pmol of cAMP/mg/min). For the receptor-binding assay, membranes were resuspended in 50 mM Tris-HCl, pH 7.7, and assayed with 0.8 nM [^{3}H]-D-Ala enk in the presence of 10 μM Gpp(NH)p; results are expressed as percentage of specific binding in control membranes (6.5 fmol of [^{3}H]-D-Ala enk bound/mg of tissue).

80% of control levels, whereas D-Ala enk-inhibited activity was decreased to 40% of the inhibition seen in pH 4.5-pretreated membranes without phosphatidylcholine. When several concentrations of phosphatidylcholine were used to confirm these effects, results showed that as sodium fluoride-stimulated activity was progressively restored by lipid treatment, opiate-inhibited activity was progressively lost. These results are demonstrated in Figure 8, which is a correlation diagram between adenylate cyclase activity in the presence of 10 mM sodium fluoride or 5 μM D-Ala enk, assayed in low pH-pretreated striatal membranes with several concentrations of phosphatidylcholine. The data clearly show an inverse correlation between sodium fluoride-stimulated activity and D-Ala enk-inhibited activity ($R = 0.95$). Therefore, as the function of N_s decreases, the function of N_i increases proportionately.

Determination of membrane microviscosity. To determine whether the effect of low pH is actually due to changes in membrane viscosity, fluorescence polarization measurements were made on whole brain membranes with DPH as a probe (Table I). Addition of phosphatidylcholine decreased membrane microviscosity in both normal and low pH-pretreated membranes, with 10 μmol of phosphatidylcholine/mg decreasing viscosity by approximately 50% in both membrane preparations. However, there was no significant difference in microviscosity in normal and low pH-pretreated membranes either in the presence or absence of lipid. Therefore, if low pH effects are caused by changes in membrane fluidity, such changes could not be confirmed using DPH as a probe. Similar results were obtained using two other fluidity probes, perylene and 2-methylanthracene (Hanski et al., 1979), in fluorescence polarization studies (data not shown). The finding that low pH effects were not directly associated with membrane fluidity was supported by the result (not shown) that *trans*-vaccenic acid, which does not increase membrane fluidity (Hanski et al., 1979), was as potent as *cis*-vaccenic acid in restoring sodium fluoride-stimulated adenylate cyclase in low pH-treated membranes.

Discussion

Treatment of brain membranes at pH 4.5 is clearly not a specific procedure and undoubtably leads to denaturation of a number of different membrane components. However, some specificity was seen in the effect of such treatment on the neurotransmitter systems studied. As reported earlier (Lambert and Childers, 1984), low pH pretreatment did not decrease specific binding of opiate ligands and, as shown in Figure 1, did not significantly affect adenylate cyclase catalytic units themselves, suggested by the maintenance of basal activity and manganese-stimulated activity after treatment. In contrast, the functions of guanine nucleotides were dramatically affected by this treatment, which increased GTP regulation of opiate agonist binding and decreased stimulation of adenylate cyclase through the N_s subunits. It is clear that stimulation of adenylate cyclase by sodium fluoride or Gpp(NH)p are only indirect measures of N_s function. Direct assay of N_s sites might be a more specific method. However, such methods are not yet practical in tissues like crude brain membranes where cholera toxin reacts with several components other than N_s. Moreover, the loss of N_s-stimulated activity is not caused by physical loss

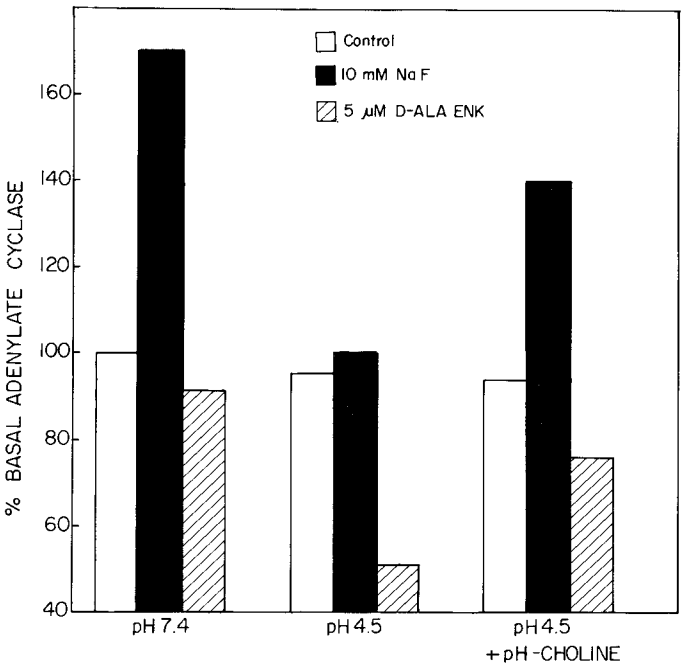

Figure 7. Effect of phosphatidylcholine incorporation on sodium fluoride-stimulated and D-Ala enk-inhibited adenylate cyclase in rat striatum. Membranes were treated at pH 7.4 or pH 4.5, washed, incubated with 1 μmol of phosphatidylcholine/mg of protein as described in the legend to Figure 5, and assayed for adenylate cyclase activity in the presence of 10 mM sodium fluoride or 5 μM D-Ala enk. Results are expressed as percentage of basal activity in pH 7.4-treated membranes (79 pmol of cAMP/mg/min).

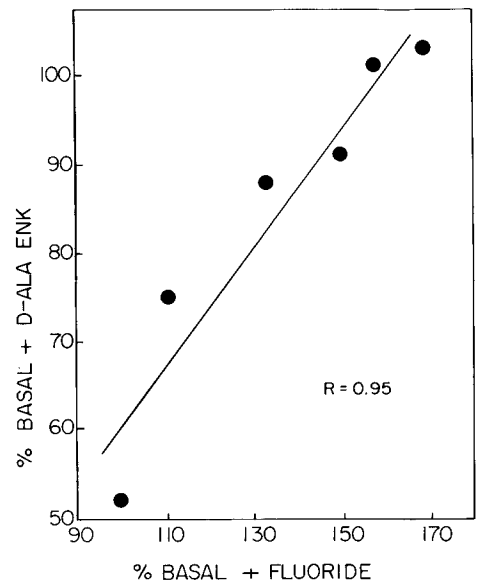

Figure 8. Correlation between sodium fluoride-stimulated and D-Ala enk-inhibited adenylate cyclase activity in low pH-pretreated striatal membranes. Membranes were treated at pH 4.5, incubated with various concentrations of phosphatidylcholine, and assayed in the presence of 10 mM sodium fluoride or 5 μM D-Ala enk. The *line* is a linear regression fit of the data points.

of the proteins themselves, since the loss of sodium fluoride-stimulated activity could be effectively reversed by addition of lipids to membranes.

The various functions of guanine nucleotides in neurotransmitter receptor actions are summarized in Table II. The four possible functions are listed separately, which is not to imply that they are mediated by different components but simply to identify the functions that can be measured by receptor binding and adenylate cyclase assays. According to this scheme, $N_s(C)$ refers to coupling-stimulatory receptors with adenylate cyclase; $N_s(R)$ refers to GTP regulation of stimulatory receptor binding; $N_i(C)$ refers to the coupling of inhibitory receptors with adenylate cyclase; $N_i(R)$ refers to GTP regulation of inhibitory receptor binding. The two papers in this series have examined three of the four functions listed; $N_s(R)$ must await further characterization. Nevertheless, the present experiments can reveal some interesting information about the relationship between these three functions of GTP. Treatment of membranes at pH 4.5 increases $N_i(C)$ function but decreases $N_s(C)$ function; in fact, according to Figure 8, there is a significant inverse correlation between these two functions. This result might suggest the existence of two separate N proteins for stimulatory and inhibitory receptors or, alternatively, might be explained by a single N site for which different receptors compete for coupling. According to this latter scheme, low pH-pretreated membranes would have stimulatory receptors that can no longer compete for N sites which are then taken up by inhibitory receptors. The available biochemical evidence so far argues against this hypothesis, since a number of experiments have provided clear evidence of separate $N_s(C)$ and $N_i(C)$ sites, including the use of specific toxins to differentiate the sites (Jakobs et al., 1983) and the finding that S 49 lymphoma cyc(−) variants possess at least some form of N_i without any significant N_s (Jakobs and Schultz, 1983). If two separate proteins exist, the inverse correlation between $N_s(C)$ and $N_i(C)$ may reflect

TABLE I

Effect of phosphatidylcholine on miroviscosity in normal and pH 4.5-treated brain membranes

Membranes were preincubated at pH 7.4 or 4.5, washed, and incubated with phosphatidylcholine for 20 min at 25°C. DPH was added to membranes (final concentration, 0.5 μM), the suspension was incubated at 30°C for 30 min and centrifuged, and fluorescence polarization measurements were assayed as described under "Materials and Methods." Results were expressed as the mean values of three separate determinations ± SEM.

Phosphatidylcholine	Microviscosity (poise)	
	pH 7.4 Membranes	pH 4.5 Membranes
μmol/mg		
0.0	4.95 ± 0.28	4.65 ± 0.47
0.2	4.37 ± 0.68	4.47 ± 0.17
1.0	3.15 ± 0.33[a]	2.76 ± 0.29[b]
10.0	2.44 ± 0.21[a]	2.69 ± 0.63[b]

[a] $p < 0.05$ versus pH 7.4 control.
[b] $p < 0.05$ versus pH 4.5 control.

TABLE II

Summary of results: Effect of low pH treatment on various functions of guanine nucleotides

Receptor Type	Function	Term	Effect of pH 4.5	Reversed by Lipid
Stimulatory	Cyclase coupling	$N_s(C)$[a]	Decrease	Yes
Stimulatory	Binding regulation	$N_s(R)$	?	?
Inhibitory	Cyclase coupling	$N_i(C)$	Increase	Yes
Inhibitory	Binding regulation	$N_i(R)$	Increase	No

[a] $N_s(C)$, coupling-stimulatory receptors with adenylate cyclase; $N_s(R)$, GTP regulation of stimulatory receptor binding; $N_i(C)$, coupling of inhibitory receptors with adenylate cyclase; $N_i(R)$, GTP regulation of inhibitory receptor binding.

efficiency of coupling: in normal membranes, the stimulatory function may be more efficient and therefore predominates. It is only when the stimulatory actions are attenuated that the N_i functions begin to predominate. It is important to note that these speculations are based on data of ony one N_i system, opiate receptors, which may not behave like other N_i systems. Experiments are in progress to test other N_i receptors to determine whether this correlation holds for receptors other than opiate receptors. Conclusions about effects on $N_s(C)$ are more confident, since three separate $N_s(C)$ functions were examined: sodium fluoride, Gpp(NH)p, and dopamine.

Another interesting relationship was observed between the binding-regulatory function $N_i(R)$ and the adenylate cyclase-coupling functions $N_s(C)$ and $N_i(C)$. Since low pH pretreatment increases $N_i(R)$ and decreases $N_s(C)$, these sites appear to be different. The same kind of reasoning mentioned above could be applied here to hypothesize the same N site competing for different receptors; however, the lipid incorporation experiments which reversed the low pH effect on $N_s(C)$ but had no effect on $N_i(R)$ in the same membranes effectively rule out that possibility. It is difficult to picture how lipid incorporation could restore one function and not the other if both functions were mediated by the same component.

The same reasoning applies to the relationship between $N_i(R)$ and $N_i(C)$, the two actions of guanine nucleotides on opiate receptor systems. Although both of these functions were increased by low pH pretreatment, only one, the coupling function $N_i(C)$, was affected by lipid incorporation. This finding sets up the interesting possibility that the coupling and binding-regulatory functions of GTP on opiate receptors are mediated by different sites. Although this conclusion is counter to most of the findings on guanine nucleotide function with the β-adrenergic receptor, where reconstitution of solubilized receptors with N_s subunits can restore both coupling and binding-regulatory functions to the reconstituted system (Limbird et al., 1980), there is abundant evidence to suggest differences between coupling and binding-regulatory effects of guanine nucleotides. For example, the nucleotide specificity of the two effects are different, with GDP being completely ineffective in coupling receptors to adenylate cyclase while it is equipotent to GTP in decreasing agonist affinity (Rodbell et al., 1971). In addition, Gpp(NH)p acts irreversibly to stimulate adenylate cyclase through $N_s(C)$, but its effect on agonist binding is easily washed from membranes (Lefkowitz, 1974; Pfeuffer and Helmreich, 1975).

As discussed before (Lambert and Childers, 1984), the mechanisms of the modifications of guanine nucleotide functions in these studies are not clear. The ability of *cis*-vaccenic acid and phosphatidylcholine to reverse the effects on adenylate cyclase would suggest that the principal action of low pH treatment is to decrease membrane fluidity. However, that assumption is not supported by the fluorescence polarization experiments which showed no correlation between membrane microviscosity and ability of sodium fluoride to stimulate adenylate cyclase. It seems more likely that low pH could be denaturing some unknown lipid-containing component; incubation with lipids may be able to restore the environment of this component to near normal conditions. More experiments are planned with other kinds of lipids to study the specificity of the reversal reaction. Clearly, however, lipid reversal did not affect GTP regulation of [^{3}H]-D-Ala enk binding; therefore, the low pH effect on that function either occurs through a different mechanism or, because of different membrane geometry around the regulatory site, the lipids used in these studies were not effective in restoring the chemical environment. Whatever the mechanism of the low pH effect, the finding that these membranes contain increased inhibitory receptor function suggests that these membranes may be useful in determining characteristics of receptor-inhibited adenylate cyclase in tissues like mammalian brain which so far have yielded little information on this class of receptors.

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