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Coupling of the Stimulatory GTP-Binding Protein G_s to Rat Synaptic Membrane Adenylate Cyclase Is Enhanced Subsequent to Chronic Antidepressant Treatment

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SUMMARY

In an attempt to resolve a unified postreceptor mechanism of action for antidepressant therapy, rats were treated with amitriptyline, desipramine or iprindole. Chronic treatment with these antidepressant drugs increased guanylylimidodiphosphate-[Gpp(NH)p-], NaF-, or forskolin-activated adenylate cyclase in synaptic membranes prepared from cerebral cortexes of treated rats. Gpp(NH)p-dependent inhibition of adenylate cyclase was unaffected. Maximal binding of the photoaffinity GTP analog azidoanilido-GTP (AAGTP) to the adenylate cyclase stimulatory (G_{s_α}) and inhibitory (G_{i_α}) G proteins was unaffected by antidepressant treatment. The chemical elimination of G_s (low pH treatment) eliminated all differences between control and antidepressant-treated groups. Further, nonneural tissues from rats receiving chronic antidepressants showed no changes in adenylate cyclase activity or AAGTP binding. The results of these studies suggest that chronic antidepressant administration promoted increased coupling between G_s and catalytic unit of adenylate cyclase. Thus, the molecular locus of antidepressant action may reside at the stimulatory GTP-binding protein, G_s.

There have been many studies concerning TAD effects on neuronal signal transduction, most focusing upon the catecholamine β-receptor adenylate cyclase system (1, 2). A general finding of these studies is that TADs have the ability to inhibit the reuptake or catabolism of monoamines in the presynaptic region (3). A decrease in the number of β-adrenergic receptor sites in various rat brain regions is obtained about 7 days after antidepressant administration (4) and a decrease in β-adrenergic agonist-elicited cAMP production in rat brain slices in observed as well (5). These phenomena parallel the lag time for therapeutic benefit seen in the clinical administration of TADs (4, 5).

Despite the consistency of these findings, it is not clear that reuptake blockade or β-receptor decrease explain the mechanism of antidepressant action; in fact, some TADs (iprindole, mianserin) do not inhibit monoamine reuptake or catabolism (6). Experience with antidepressant therapy has shown that, at therapeutic doses, it usually takes 1 to 3 weeks for a clinical response to occur (7), while reuptake inhibition and monoamine oxidase inhibition can appear immediately (a few hours) after a single dose of the active compounds (3). Furthermore, all drugs that block monoamine uptake or catabolism do not have antidepressant effect (e.g., cocaine and amphetamine) (8). Although it is possible that the decreased number of β-receptors observed may be due to increased monoamine content in the synaptic cleft resulting from blockade of monoamine reuptake or degradation, atypical antidepressants (e.g., iprindole), which do not inhibit the uptake of norepinephrine or 5-hydroxytryptamine, are able to reduce the number of β-receptors after chronic treatment (1). Furthermore, TADs affect several neurotransmitter receptor-effector systems including those for serotonin, dopamine, acetylcholine, γ-aminobutyric acid, and certain neuropeptides (9, 10). It does not seem likely that a single neurotransmitter can account for the common mechanism of TAD action. Thus, we speculate that some mechanisms for TAD action involve postreceptor components or processes that include guanine nucleotide regulatory proteins (G_s and G_i), the adenylate cyclase catalytic unit, and the coupling between G proteins and catalytic unit.

Previous work in this laboratory has already suggested that TADs or electroconvulsive shock may influence coupling of G proteins with the adenylate cyclase catalytic moiety (11). The present study examines the effect of typical or atypical antidepressants on G protein-mediated activation (G_s) or inhibition (G_i) of adenylate cyclase in a membrane preparation from rat cerebral cortex. In this report, we demonstrate a specific in-

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ABBREVIATIONS: TAD, tricyclic antidepressant; G protein, GTP-binding protein; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; AAGTP, P^3-(4-azidoanilido)-5-GTP; Gpp(NH)p, guanylylimidodiphosphate.

crease in the coupling between G_s and the adenylate cyclase catalytic moiety as a result of those treatments.

Materials and Methods

Antidepressant treatment and membrane preparation. Male Sprague-Dawley rats weighing 150–200 g were fed *ad libitum* on a 12-hr light-dark cycle. Desipramine, amitriptyline, or iprindole (10 mg/kg, intraperitoneally, unless noted otherwise) were injected once daily. Animals receiving chronic treatments were injected repeatedly for 21 days and those receiving acute treatments were injected with saline for 20 days followed by a single bolus of drug. Three courses of treatment were performed and each group contained five or six rats. Rats were sacrificed by cervical dislocation 24 hr after the last injection and brains from each group were removed and pooled. Synaptic membrane-enriched fractions were prepared from rat cerebral cortex as described (12) and stored under liquid N_2 until use. Membranes from liver and renal medulla were also prepared from these rats (12) and stored under liquid N_2 until assay.

Adenylate cyclase assay. Synaptic membranes were thawed and resuspended in a buffer containing 20 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT, and 0.3 mM phenylmethylsulfonyl fluoride. Washed membranes (10–20 μg) were resuspended and incubated in the presence of the indicated Gpp(NH)p concentrations for 10 min at 30° in 100 μl of medium containing 15 mM HEPES (pH 7.5), 0.05 mM ATP, [α-^{32}P] ATP (5×10^6 cpm/tube), $MgCl_2$ (1 or 5 mM as noted), 1 mM EGTA (if noted), 1 mM DTT, 0.05 mM cyclic AMP, 60 mM NaCl, 0.25 mg/ml bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 unit of adenosine deaminase/ml, and a nucleotide triphosphate-regenerating system consisting of 0.5 mg of creatine phosphate, 0.14 mg of creatine phosphokinase, and 15 units of myokinase/ml. The reaction, which was linear from 0 to 20 min, in the presence or absence of Gpp(NH)p, was stopped after 10 min by the addition of 0.1 ml of a solution containing 2% sodium dodecyl sulfate, 1.4 mM cyclic AMP, and 40 mM ATP, and the cyclic [^{32}P]AMP formed was isolated by the method of Salomon (13). Protein was determined by the Coomassie blue binding method (14) with bovine serum albumin as a standard. All assays were performed in triplicate.

Low pH treatment. Low pH treatment of synaptic membranes, which reversibly eliminates functional G_s (15), was performed by a modification of the methods of Rasenick and Childers (16). Synaptic membranes were pelleted by centrifugation at 38,000 × *g* and resuspended (at 4–6 mg/ml) in 50 mM sodium acetate (pH 4.5), 5 mM $MgCl_2$, 1 mM DTT (low pH membranes). Control membranes were treated identically but were resuspended in 50 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT. After incubation on ice for 20 min, membranes were diluted with 10 volumes of pH 7.4 Tris buffer, centrifuged at 38,000 × *g*, and resuspended in the HEPES buffer used for adenylate cyclase assays.

Photoaffinity labeling. [^{32}P]AAGTP was synthesized by the method of Pfeuffer (17). Synaptic membranes were washed and resuspended in 2 mM HEPES (pH 7.4) and 1 mM $MgCl_2$ for maximal $G_{i/o\alpha}$ labeling. EDTA (1 mM) was substituted for $MgCl_2$ to achieve maximum $G_{s\alpha}$ labeling. Membrane suspensions (3–7 mg of protein/mg) were incubated with 0.12 μM [^{32}P]AAGTP for 3 min at 23°, and the reaction was terminated by dilution with the above buffer followed by centrifugation at 20,000 × *g* for 10 min to remove unbound [^{32}P]AAGTP. Membranes were washed again and resuspended in the same buffer. Membrane suspensions were then subjected to 5 min of UV photolysis with a Spectroline UV lamp (254 nm, 9 W) on ice at a distance of 4 cm. The reaction was quenched with ice-cold 2 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 4 mM DTT. Samples were heated for 4 min at 60° and electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels by the procedure of Laemmli (18). After electrophoresis, gels were stained with Coomassie blue, dried, and autoradiographed. Radioactive bands corresponding to $G_{s\alpha}$ or $G_{i/o\alpha}$ were excised from the dried gel, immersed in 4 ml of scintillation cocktail, and subjected to counting in a Beckman LS 5800 scintillation counter.

Results

Adenylate cyclase activation. Previous results from this (11) and other (19, 20) laboratories indicated that chronic antidepressant treatment augmented Gpp(NH)p-induced stimulation of adenylate cyclase. Although different assay methods were employed in this study, iprindole and amitriptyline treatment promoted an increase of approximately 2.4- fold in the maximal Gpp(NH)p-induced activation of adenylate cyclase (Fig. 1A). Basal adenylate cyclase activities were nearly identical in membranes prepared from animals in each treatment group.

Adenylate cyclase inhibition. Changes in the adenylate cyclase assay conditions (described in the legend to Fig. 1)

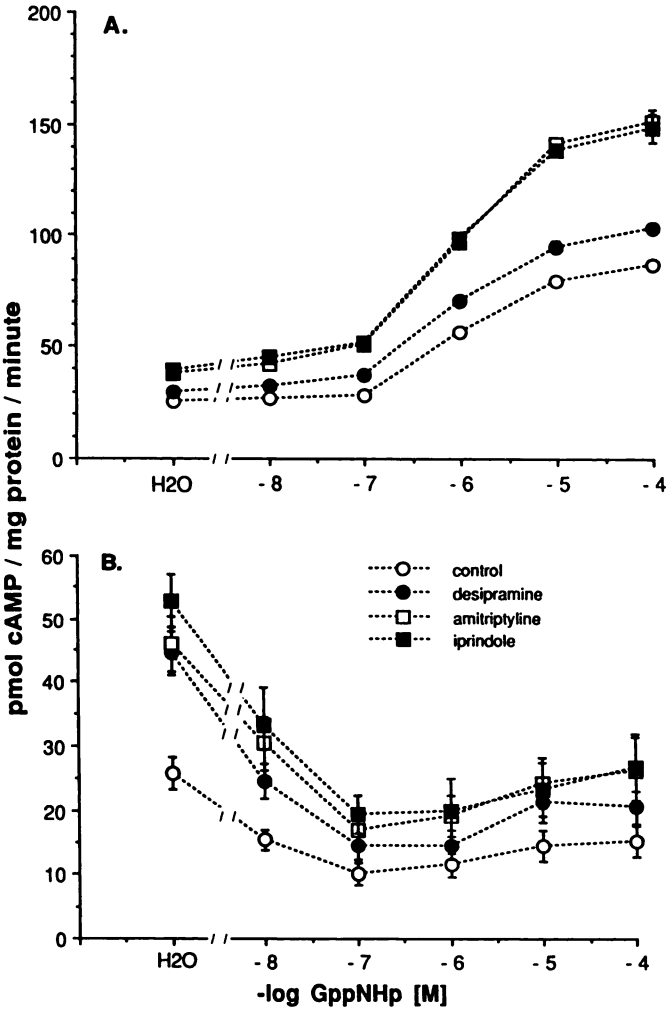

Fig. 1. A, Effect of TAD treatment on the Gpp(NH)p-induced stimulation of adenylate cyclase. Rats were treated with antidepressants as indicated in the text. Membranes were assayed for adenylate cyclase as described in Materials and Methods in buffer containing 5 mM $MgCl_2$ and 1 mM EGTA. Reactions were carried out at 30° for 10 min. The values represent means of three separate experiments (± standard errors), each performed in triplicate. 1B, Effect of TAD treatment on Gpp(NH)p-induced inhibition of adenylate cyclase. Tissue preparation and assays were performed as in A except that the incubation buffer contained 1 mM $MgCl_2$ and not EGTA and was carried out at 23° ("inhibitory conditions"). Results are means of triplicate determinations (± standard errors) from five separate experiments. Membranes prepared from animals receiving a single treatment of any of the drugs displayed Gpp(NH)p-induced adenylate cyclase stimulation of inhibition that was not significantly different from controls (not shown).

cause Gpp(NH)p to inhibit rather than stimulate adenylate cyclase (21). Under these conditions, removal of EGTA from the assay cocktail results in a 30–50% increase (average of all experiments) in basal activity. The basal activity in membranes prepared from animals treated chronically with amitriptyline, desipramic, and iprindole was approximately 2-fold greater than that observed in the membranes prepared from control and single drug treatment groups. The percentage of inhibition induced by Gpp(NH)p, however, was equivalent in all groups (Table 1).

Potency and efficacy of Gpp(NH)p. Data in Fig. 1 were consistent with chronic TAD treatment augmenting coupling between G_s and the catalytic moiety of adenylate cyclase. To investigate whether TADs affected the sensitivity of G_s for GTP analogs, the EC_{50} values for Gpp(NH)p were calculated by linear regression analysis of Michaelis plots made with the data in Fig. 1A. The average EC_{50} for Gpp(NH)p was 1.84 μM and no significant difference was observed in any group. V_{max} values were significantly greater for the amitriptyline and iprindole chronic treatment groups (Table 1). No change was observed in the IC_{50} values for Gpp(NH)p (6.19 nM) or the maximal inhibition elicited by Gpp(NH)p (Table 1).

NaF- and forskolin-stimulated adenylate cyclase. NaF directly stimulates adenylate cyclase by eliciting the active conformation of G_s protein. Forskolin can not only stimulate the catalytic unit of adenylate cyclase but also appears to stabilize the coupling between G_s and adenylate cyclase catalytic unit (22). Under conditions where coupling between G_s and the adenylate cyclase catalytic moiety is enhanced, NaF- and forskolin-stimulated adenylate cyclase should be higher. NaF- or forskolin-stimulated adenylate cyclase was increased in membranes from rats treated chronically with TADs (Fig. 2).

TABLE 1

Gpp(NH)p potency and efficacy for adenylate cyclase stimulation and inhibition: effects of TAD treatment

Separate Michaelis plots were drawn for each of the three experiments depicted in Fig. 1A or five experiments in Fig. 1B. EC_{50}, IC_{50}, V_{max}, and maximal percentage of inhibition were calculated for each experiment and means (± standard errors) of those values appear in the table above.

Adenylate cyclase stimulation (data calculated from Fig. 1A)	EC_{50}[a]	V_{max}[b]
	$\times 10^{-6}$ M	pmol/mg/min
1 × Control	1.13 ± 0.11	56.9 ± 5.5
1 × Desipramine	1.03 ± 0.05	66.1 ± 8.5
1 × Amitriptyline	1.37 ± 0.6	50.1 ± 2.9
1 × Iprindole	1.29 ± 0.19	51.43 ± 3.7
21 × Control	1.71 ± 0.53	79.8 ± 9.3
21 × Desipramine	3.74 ± 2.21	83.1 ± 10.3
21 × Amitriptyline	2.37 ± 1.53	150.8 ± 19.2[c]
21 × Iprindole	2.10 ± 1.31	139.4 ± 15.2[c]
Adenylate cyclase inhibition (data calculated from Fig. 1B)	**IC_{50}[a]**	**Maximal inhibition**
	$\times 10^{-6}$ M	%
1 × Control	6.46 ± 2.67	64.1 ± 0.9
1 × Desipramine	6.99 ± 2.88	66.1 ± 3.8
1 × Amitriptyline	8.82 ± 2.82	68.9 ± 0.1
1 × Iprindole	4.97 ± 1.36	66.5 ± 7.9
21 × Control	4.09 ± 0.36	64.9 ± 2.5
21 × Desipramine	3.68 ± 1.12	71.6 ± 3.3
21 × Amitriptyline	7.68 ± 0.68	73.6 ± 2.4
21 × Iprindole	6.83 ± 0.83	67.9 ± 2.7

[a] Gpp(NH)p.
[b] pmol of cAMP/mg of protein/min after subtraction of basal value.
[c] Significantly different ($p < 0.03$) from controls and acute treatment groups.

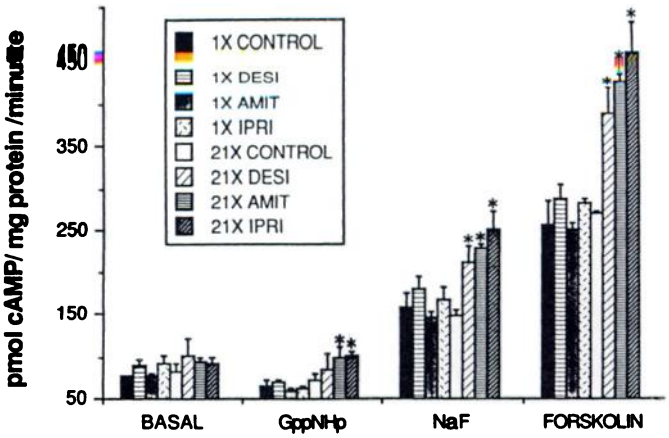

Fig. 2. Effect of TAD treatment on Gpp(NH)p-, NaF-, or forskolin-activated adenylate cyclase. Adenylate cyclase was assayed with 5 mM $MgCl_2$ and 1 mM EGTA at 30° for 10 min in the presence of H_2O (basal), 100 μM Gpp(NH)p, 20 mM NaF, or 100 μM forskolin. EGTA (1 mM) was also present in the assay cocktail. Results are means of triplicate determinations from three separate experiments (± standard errors). Significant differences ($p < 0.05$) from control and acute values are indicated by an *asterisk*. *DESI*, desipramine; *AMIT*, amitriptyline; *IPRI*, iprindole. 1x and 21x refer to days of antidepressant treatment.

Dosage requirements for TAD effects on adenylate cyclase. Although three laboratories (11, 19, 20, 23) have demonstrated changes in adenylate cyclase subsequent to chronic TAD administration, two groups have not observed these effects (24, 25) after chronic treatment of rats with imipramine or desipramine. Data in Fig. 2 indicate that when adenylate cyclase activity was maximized (i.e., in the presence of forskolin) each of the TAD drugs showed similar efficacy. However, data in Fig. 1A and Table 1 indicated that the effects of chronic desipramine administration on Gpp(NH)p-induced adenylate cyclase stimulation, although significant, were not as large as those seen with amitriptyline or iprindole. In order to examine whether the observed difference among the TAD drugs was due to differences in drug potency, rats were treated for 21 days with 5, 10, and 20 mg/kg amitriptyline or desipramine. Whereas maximal enhancement of Gpp(NH)p-stimulated adenylate cyclase by amitriptyline treatment was achieved with 10 mg/kg doses of that drug, 20 mg/kg desipramine was required to achieve a similar result (Fig. 3). These increased doses of either drug did not increase the observed augmentation of forskolin-stimulated adenylate cyclase (data not shown).

GTP binding capabilities of synaptic membrane G_{s_α} and G_{i_α}. Results presented in Figs. 1 and 2 and Table 1 suggested that adenylate cyclase activity after chronic TAD treatment was enhanced due to a change in the nature of G_s or increased coupling between G_s and the catalytic unit of adenylate cyclase. Because the TAD-enhanced stimulation of adenylate cyclase through G_s appeared to be a V_{max} effect, it was important to distinguish whether more functional G_s was induced. The hydrolysis-resistant photoaffinity GTP analog AAGTP has been used as a probe to study the behavior of synaptic membrane G proteins without removing those proteins from the membrane (26, 27). Under conditions where AAGTP binding to G_s is maximized, TAD treatment does not alter the net amount of synaptic membrane $G_{s\alpha}$ available to bind AAGTP. Maximal AAGTP binding of $G_{i\alpha}$ is also unchanged (Table 2).

Functional elimination of G_s from synaptic mem-

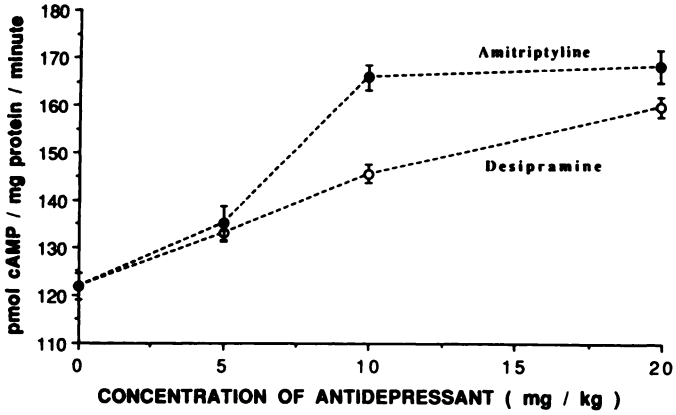

Fig. 3. Potency differences between desipramine and amitriptyline. Rats were treated with the indicated concentrations of desipramine or amitriptyline for 21 days (two treatment series with three animals in each control group and each drug treatment group) before sacrifices and preparation of cerebral cortex membranes. Adenylate cyclase was assayed as described in Fig. 1A, in the presence of Gpp(NH)p (100 μM). Results represent the combined means of triplicate determinations (± standard errors) for two experiments, each for a different treatment series.

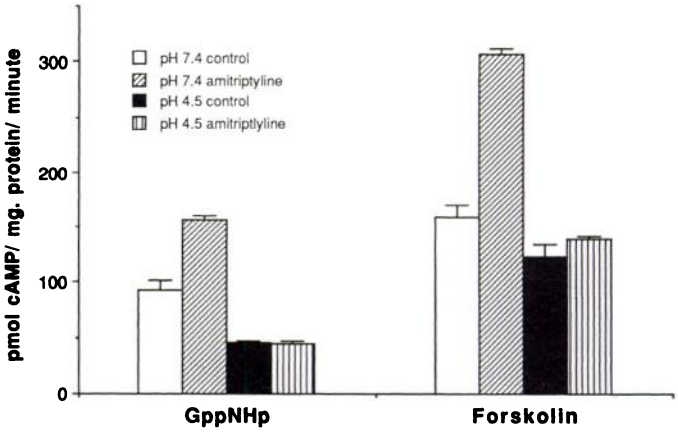

Fig. 4. Antidepressant effects on adenylate cyclase in membranes with "inactive" G_s. Rat cerebral cortex synaptic membranes prepared from animals treated chronically with saline (control) or amitriptyline were thawed and exposed to pH 4.5 acetate buffer or pH 7.4 buffer as described in Materials and Methods. Membranes were then assayed for adenylate cyclase under the conditions described in the legend to Fig. 1A. Gpp(NH)p (100 μM) or forskolin (10 μM) were included in the assay incubation. Values represent means ± standard errors for the combined data from two experiments each performed in triplicate.

TABLE 2

[^{32}P]AAGTP binding covalently to G_s (42 kDa) and $G_{i/o}$ (40 kDa): effects of TSD treatment

Treatment	Total binding[a]	
	G_s[b]	$G_{i/o}$[c]
	fmol/mg of protein	
1 × Control	755 ± 24	818 ± 40
1 × Desipramine	782 ± 56	826 ± 40
1 × Amitriptyline	741 ± 63	799 ± 12
1 × Iprindole	789 ± 31	824 ± 54
21 × Control	833 ± 30	770 ± 91
21 × Desipramine	797 ± 18	753 ± 78
21 × Amitriptyline	801 ± 41	852 ± 77
21 × Iprindole	733 ± 12	730 ± 30

[a] The protein value represents the total applied to each lane of the sodium dodecyl sulfide gel. Values for AAGTP bound represent means ± standard errors for four separate experiments, two from each of two treatment series.

[b] To maximize AAGTP binding to G_s, the incubation contained 1 mM EDTA rather than 1 mM $MgSO_4$. Gel electrophoresis, autoradiography, and quantitation were performed as described in Materials and Methods.

[c] To obtain maximal $G_{i/o}$ binding, membranes were incubated with 10^{-7} M AAGTP as described in Materials and Methods, washed, resuspended, and incubated for 10 min at 23° before UV exposure.

branes. Treatment of rat synaptic membranes from various brain regions with pH 4.5 buffer results in the functional elimination of G_s (15). This effect is reversible and G_{s_α} remains capable of binding guanine nucleotide (16), although the transfer of nucleotide from G_i to G_s is impaired. Furthermore, after low pH treatment of membranes, stable GTP analogs inhibit adenylate cyclase but do not stimulate that enzyme. It was hypothesized that, if chronic TAD treatment enhances coupling between G_s and adenylate cyclase without altering the actions of G_i, then low pH treatment should eliminate any differences in adenylate cyclase between control membranes and those from treated animals. Fig. 4 illustrates that differences in adenylate cyclase activity are not observed in membranes subjected to low pH treatment.

Nonneural tissues. Previous studies from this laboratory indicated that effects of TAD treatment were selective for neural tissues. Membranes were prepared from liver and renal medulla and triplicate adenylate cyclase assays were carried out according to the protocols listed in Fig. 1a [Gpp(NH)p dose response under stimulatory conditions] and Fig. 2 [maximal adenylate cyclase activation by Gpp(NH)p, NaF, or forskolin]. No differences were observed between membranes prepared from control and treated animals (data not shown).

Discussion

Increased interaction between G_s and adenylate cyclase serves as a possible explanation for the previous studies from this and other laboratories (11, 17, 18) that demonstrated that chronic antidepressant treatment enhanced activation of that enzyme. Furthermore, data in this study suggest that the inhibition of adenylate cyclase plays no direct role in the consequences of TAD treatment. This is especially clear in light of the data in Fig. 3, which demonstrate that suppression of G_s activity (low pH treatment of membranes) eliminates differences in adenylate cyclase activity observed in control and TAD-treated membranes.

Receptor desensitization subsequent to chronic TAD treatment has been a consistent experimental finding. Despite this finding, G proteins appear to regulate receptor affinity even after TAD treatment (28). Isoproterenol-elicited increases in cAMP accumulation appear to be lower in slices prepared from brains of animals treated with TADs (1). Furthermore, treatment with various TAD drugs or electroconvulsive shock has led to changes in number or affinity of different receptor types (9, 10). Thus, the generation of a single unifying hypothesis for the molecular mechanisms involved in TAD action is quite difficult, particularly with regard to the adenylate cyclase system.

Because it appeared that changes in receptor parameters do not yield a consistent unifying hypothesis for TAD action, we chose to investigate a "downstream" element involved in neuronal signal transduction. The observation that receptors are down-regulated would, at first, seem to contrast with the observations of increased G_s coupling. However, recent work from this laboratory (29) has indicated that, when the β-adrenergic receptor is coupled to G_s, it actually constrains that protein from activation of adenylate cyclase. Thus, down-regulation of

a G_s-linked receptor might result in increased adenylate cyclase activity within an affected neuron by increasing receptor-independent activation of that enzyme (i.e., increased coupling between G_s and the adenylate cyclase catalytic moiety).

The possibility of intracellular (receptor-independent) activation of neuronal adenylate cyclase has been proposed previously by this laboratory (26, 30, 31). Much of the rationale for such endogenous regulation resides in the fact that, in the mammalian brain, the quantity of neurotransmitter capable of stimulating adenylate cyclase is at least 100-fold lower than the amount of neurotransmitter capable of inhibiting that enzyme. The current paradigms invoked to explain the dual regulation of adenylate cyclase require inhibition of a stimulated enzyme. Thus, neurotransmitters that act by inhibiting adenylate cyclase might be expected to be ineffective without prior exposure of a cell to a stimulatory agonist. Intraneuronal activation of adenylate cyclase could obviate this dilemma.

Although intraneuronal activation of adenylate cyclase may occur independently of receptors coupled to G_s, activation of that enzyme may be a result of the actions of another second messenger system. Neurotransmitters that elevate intracellular Ca^{2+} might cause activation of adenylate cyclase in such a manner. Although the catalytic moiety of adenylate cyclase may interact directly with calmodulin (32), some aspects of calmodulin stimulation of adenylate cyclase are apparently G_s-related (21, 33). It is possible that additional cellular elements are involved in this process (31), and it appears that calmodulin stimulation of adenylate cyclase is restricted to nervous tissues. Because TAD treatment elevates only neuronal adenylate cyclase, there exists a possibility that the calmodulin-stimulated form of the enzyme is the species involved. A more complete understanding of this requires further investigation.

No mechanistic explanation has been offered for the extended time of treatment required to observe TAD effects. In view of the observed increase in G_s "activity," one might expect to see an increased amount of functional G_s. Photoaffinity studies indicate that no increase in G_s binding of AAGTP is seen (Fig. 4; Table 2). Whereas AAGTP cannot be used to give a quantitation of the total G_s present, it does provide an indication of the net, functional, amount of that protein (27).

Enhanced interaction between G_s and the adenylate cyclase catalytic moiety has been seen in synaptic membranes following "fluidization" of the membrane with *cis*-unsaturated fatty acids (34). Whereas chronic TAD treatment facilitated G_s-catalytic moiety coupling in a similar manner, no change in "membrane fluidity" (as measured by fluorescence anisotropy) was observed (11). It is noteworthy, however, that recent data from this and other laboratories suggest the possibility that G_s subunits interact directly (26, 27, 30, 31, 35), raising the possibility that a physical complex composed of different types of G proteins (as well as other elements of the adenylate cyclase and cytoskeletal systems) exists. Observations of AAGTP binding by specific G proteins in the synaptic membrane are consistent with the existence of such complexes (27). Perhaps chronic TAD treatment alters the interactions between G_s and the other members of such complexes on the synaptic membrane. Such a possibility will serve as the basis for further studies of TAD action, as well as for the regulatory mechanism of neuronal signal transduction.

Acknowledgments

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