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"Effect of Hydrazines upon Cyclic Nucleotide Regulated Neuronal Processes"

The funded project was designed, initially to explore the effects of hydrazines upon cyclic nucleotide regulated neuronal processes. cyclase as it was discovered that hydrazines were potent activators of this enzyme. In order to understand hydrazine actions in the CNS, it was required that more basic knowledge of the adenylate cyclase cascade by accumulated and this study probed some of the distinctions between neural and non-neural adenylate cyclase with that in mind. Specifically, the following has been accomplished during the project period:

1) Interactions between the cytoskeleton and synaptic membrane adenylate cyclase have been probed and we have found a reversible attachment between the GTP-binding proteins regulating adenylate cyclase and that membrane.

2) We have discovered that GTP binding proteins directly interact and may exchange nucleotide with one another, and have hypothesized this mechanism as an intracellular regulator of signal transduction.

3) We have discovered a novel, neural GTP binding protein and are in the process of purification and characterization.

4) We have devised a method for measuring adenylate cyclase in monolayers of permeable cells and have used this method to explore the coupling between receptors and adenylate cyclase GTP-binding proteins.

It is hoped that an increased understanding of the neuronal adenylate cyclase system will lead to an increased understanding of the effects of certain neurotoxins, and to the design of strategies to prevent and/or treat the effects of those compounds.

AFOSR-TR- 87-1310

PROJECT SUMMARY

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Mark M. Rasenick

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I. INTRODUCTION

1.1 Cyclic Nucleotides and Neuronal Function

Despite being the focus of intensive investigation over the past several years there is no clear understanding of the molecular events within the nerve cell during synaptic transmission. Incumbent within the above statement is the fact that there is also no clearly defined role for cyclic nucleotides within this process.

A paradigm which has evolved from molecular endocrinology over the last several years is that cyclic AMP (cAMP) functions as a second messenger for a variety of hormones and neurotransmitters. Although the precise mechanisms are unclear, a variety of hormones and neurotransmitters activate adenylate cyclase which subsequently elevates the level of cAMP. Protein kinases, once activated by cAMP (or cGMP), phosphorylate a variety of soluble and membrane proteins. These proteins, once phosphorylated, might alter transmitter release or ion flux within the nerve cell.

1.2 Adenylate Cyclase

Neuronal adenylate cyclase is responsive to a variety of neurotransmitters which stimulate or inhibit that enzyme. These neurotransmitters exert their effects through at least two membrane-associated GTP-binding proteins, referred to herein as GNs and GNi, denoting, respectively, the stimulatory and inhibitory proteins (see Figure 1). Recently, the alpha, or GTP-binding portion, of GNi has been purified from bovine cerebral cortex and appears to consist of two to three pertussis toxin substrates clustered at about 40 KDa. The alpha subunit of GNs has been purified and appears to be a single polypeptide of about 42-45 KDa, although, in some tissues, a 48-52 KDa form of GNs also appears. [Apparent M_r from SDS-PAGE are cited. Actual M_r of GNs determined from sequence data are 45 and 46 KDa respectively for the small and large forms. GNs and GNi regulate the catalytic activity of the adenylate cyclase enzyme through interaction with a distinct catalytic moiety. The mechanism of this

regulation is unknown, but it has been suggested that the beta and gamma components (36KDa and 8KDa respectively) common to GNs and GNi provide a regulatory function.

1.3 Receptor-Effector Coupling

The coupling of adenylate cyclase refers to the interaction between (or among) neurotransmitter receptor, GNs or GNi and the adenylate cyclase catalytic moiety. Physical interaction among these proteins has been reported and as suggested below, GNs and GNi may interact directly as well. The coupling between the neurotransmitter receptor and GNs or GNi in nerve cells is diminished or lost upon preparation of subcellular fractions. However, as a result of this coupling loss, hydrolysis-resistant guanine nucleotides can profoundly activate or inhibit neuronal adenylate cyclase without benefit of hormone (neurotransmitter). Furthermore, we have demonstrated that coupling between the GN proteins and adenylate cyclase catalytic moiety is augmented by treatments which alter cytoskeletal or membrane composition as well as by chronic antidepressant treatment.

1.4 Possible Mechanism of Tubulin-Mediated Regulation of Adenylate Cyclase

A scheme for the regulation of neuronal adenylate cyclase by tubulin is illustrated in Figure 1. Normally, adenylate cyclase activation proceeds after the binding of a stimulatory agonist to its receptor and the subsequent binding of GTP with GNs. The "charged" GNs then separates from its β γ subunits and activates the catalytic moiety of adenylate cyclase. Receptor-mediated inhibition has been proposed which releases β γ subunits to associate with GNs, therefore inhibiting adenylate cyclase stimulation. Several contradictions to this simple model exist, including the observed hormonal inhibition of the S49 lymphoma variant, cyc$^-$, the apparent interaction of GNi with the catalytic moiety and the possible exchange of GTP between GNi and GNs.

Colchicine or vinblastine, which bind to different sites on the tubulin molecule, both increase adenylate cyclase activity, and that increase appears

localized to the GNs protein. A possible mechanism for this effect involves a steady-state constraint of GNs/catalytic moiety interaction due to the binding of GNs by tubulin. Mitigation of this interaction by colchicine or vinblastine would result in the observed increase in enzyme activity. Increased tubulin association with the membrane might increase constraint upon GNs catalytic moiety interaction, and this has been observed. It is noteworthy that, despite the indication of tubulin as an integral synaptic membrane protein the proposed tubulin/GNs interaction could occur equally well if both tubulin and GNs are considered membrane associated proteins.

There is no indication that tubulin substitutes for GNs or GNi, despite the apparent similarities among these proteins. This molecular similarity might be consistent, however, with an association/dissociation between tubulin and GNs. In fact, an association between microtubules and adenylate cyclase has been reported. Furthermore, recent evidence in our lab has raised the possibility that β tubulin might exchange GTP with the GNi proteins. The time course and extent of this nucleotides exchange are consistent with the observed tubulin-mediated inhibition of adenylate cyclase.

The β and γ subunits are also involved in the regulation of adenylate cyclase activity and may inhibit GNs or GNi interaction with the catalytic moiety of adenylate cyclase. Another possible explanation for tubulin effects might be a tubulin association with $\beta\gamma$ (due to a tubulin/GNs or tubulin GNi homology) and subsequent promotion of adenylate cyclase inhibition. The lack of direct effects of microtubule-disrupting drugs upon adenylate cyclase inhibition makes this unlikely, however.

1.5 Physiological Considerations for Cytoskeletal/GN Association

The possibility that cytoskeletal or membrane components participate in an intracellular regulation of adenylate cyclase is exciting for several reasons. First, the prominence of neurotransmitter receptors linked to GNi in the CNS makes it

likely that such receptors act not only subsequent to GNs-linked neurotransmitters, but on their own as well. Clearly, microtubule-disrupting drugs are not involved in an intracellular process, but enzymes which modify microtuble-associated components, such as Ca^{++}/calmodulin dependent protein kinase (see below), might alter adenylate cyclase activity in a similar manner. Such a mechanism could account for the observed interaction between agents which result in an elevation of intracellular calcium and adenylate cyclase. This is especially relevant in light of the modulation of neural adenylate cyclase by calmodulin to a much greater extent than such modulation occurs in non-neural tissues. Thus, it appears that several components function in a sense of integrated cooperation toward the possible intracellular regulation of neurotransmitter responsiveness. Variations in this system might well result in variations in mental function.

2. SCIENTIFIC PROGRESS

2.1 AAGTP Labelling

In previous studies, we have identified several proteins which appear capable of binding the photoaffinity GTP analog, azidoanilido GTP. These proteins are of the M_r 52,48,42,40,36 and 30 KDa. The 40 KDa proteins, which is also a pertussis toxin substrate, is the dominant AAGTP-binding protein in membranes which have been washed after exposure to AAGTP but prior to UV photolysis. The 40 KDa protein appears to be the inhibitory GTP-binding protein, GNi (which exists in multiple forms, Neer et al., 1984), and the 42 KDa protein appears to be the GNs (see below). Identification of the proteins was made by electrophoretic co-migration with purified G-proteins (provided by Dr. Lutz-Birnbaumer).

Studies on the regulation of GTP binding within the adenylate cyclase system have been attempted using the purified components of that system. In such studies, the specificity of nucleotide binding is unquestioned, but the regulation of that binding cannot be addressed properly. Membrane systems have been employed to study

the binding of the hydrolysis-resistant GTP analog, ^{3}H GppNHp, but low binding specificity and GppNHp binding unrelated to adenylate cyclase make these experiments problematic. The photoaffinity probe, AAGTP, provides an ideal solution, as the binding specificity can be analyzed by PAGE yet the membrane systems remain intact (Rasenick et al., 1984).

2.2 Interaction Between Nucleotide Binding Proteins

In addition to serving as a photoaffinity GTP probe, AAGTP is an hydrolysis-resistant GTP analog which is capable of supporting sustained stimulation or sustained inhibition of adenylate cyclase. Under conditions where exposure of AAGTP to rat cerebral cortex synaptic membranes results in stable inhibition of adenylate cyclase (Figure 2), a subsequent incubation with either GppNHp or NaF can override this inhibition. When ^{32}P labelled AAGTP is employed under these conditions, AAGTP is bound, primarily to the 40 KDa GNi (Figure 2). Under conditions where AAGTP or NaF override the inhibition, ^{32}P AAGTP is increasingly lost from GNi and it appears on GNs. The total AAGTP in GNs and GNi remains constant (Figure 2). (note: the UV irradiation and covalent binding of AAGTP occurs only after the incubation with GppNHp or NaF. Any guanyl nucleotide which will activate synaptic membrane adenylate cyclase will have a similar effect. NaF does not compete with AAGTP for a binding site on GNs or GNi, and other guanyl nucleotides compete slowly.

The observed phenomena appear consistent with a transfer of bound nucleotide between GNi and GNs as adenylate cyclase switches from inhibition to stimulation (Figure 3). The postulated exchange of AAGTP between GNi and GNs is sufficiently rapid that if GTP were the bound nucleotide (as in the case within neurons) exchange could occur before hydrolysis, which could require 2 minutes. It is noteworthy that direct exchange of NAD has been proposed to occur among mitochondrial hydrolases.

2.3 A Novel GTP-Binding Protein

We have also demonstrated the presence of a 32 KDa GTP binding protein. This

protein is not a substrate for ADP ribosylation by pertussis or cholera toxin, and it binds AAGTP loosely yet specifically. A protein of 32 KDa has co-purified with the GNi protein of bovine cerebral cortex but it is not clear whether this protein is identical with the one we have observed. It does not appear that the 32 KDa AAGTP-binding protein is a proteolytic fragment of GNi or GNs, as V8 protease studies yield different fragments for these species (Figure 4). We have observed this protein in brain and platelet, but not liver or kidney membranes, and it is tempting to speculate that it may be involved in some GTP regulated process which is unaffected by cholera or pertussis toxin (Figure 4).

2.4 GNs-Tubulin Interaction

2.4.1 Microtubule Disrupting Drugs Increase Adenylate Cyclase Coupling

Microtubule-disrupting drugs have been shown to increase adenylate cyclase activity in synaptic membranes prepared from rat cerebral cortex (see Fig. 1). Activation of the enzyme by hydrolysis-resistant GTP analogs and NaF is augmented by colchicine or vinblastine (EC_{50} = 5 x 10^{-7} M), while basal and Mn^{++} stimulated (reflecting catalytic-moiety activation) activities are unchanged (Rasenick et al., 1981). These finding suggest that the microtubule-disrupting drugs increase the "coupling" between the GTP-binding protein which stimulates adenylate cyclase (GNs) and the catalytic moiety of that enzyme. When these membranes are treated with colchicine or vinblastine and subsequently washed, activity of the GNs protein is released from the membranes into the supernatants. This release of Ns activity is indicative of facilitated Ns-catalytic moiety coupling and is similar to that observed in the homologous cGMP phosphodiesterase cascade from retinal rod outer segments.

Although microtubule disrupting drugs increase cAMP accumulation in intact cells (Rudolph et al., 1978; Kennedy and Insel, 1978) colchicine or vinblastine enhancement of adenylate cyclase in broken cell preparations, and the release of AAGTP-labelled

Ns, has been observed only in membranes from tissues of neural origin. These data lead to the proposal of a hypothetical Ns-tubulin interaction on the synaptic membrane similar to that suggested for actin and fibronectin. The physiological significance of a putative Ns-tubulin interaction (association/dissociation) might be to intracellularly regulate the amount of cAMP produced in response to a given neurotransmitter.

2.4.2 Tubulin Incorporation Inhibits Adenylate Cyclase

Incubation of tubulin (EC_{50} = 0.5 μg/ml.; 10nM) with synaptic membranes inhibits guanyl nucleotide and NaF activated adenylate cyclase by 50 - 60 percent without altering basal and Mn^{++} activation of the enzyme (Rasenick, 1985).

Tubulin incubation does not inhibit basal or Mn^{++} stimulated adenylate cyclase, thus, only Ns-mediated adenylate cyclase appears to be involved. The "IC_{50}" for this effect is about 10 nM and the effect is maximal at 40 nM tubulin. The ratio of microtuble protein: total synaptic membrane protein required to achieve this effect is about 1:500. Although the nature of tubulin interaction with these membranes is unclear, the effects of added tubulin are maintained after the membranes are washed.

One interpretation of these results might be that addition of tubulin to these membranes results in increased tubulin-Ns association and subsequently, decreased Ns mediated adenylate cyclase coupling. The possibility of a trace impurity in the tubulin preparation having these effects, or of tubulin acting indirectly on Ns cannot be ruled out. Furthermore, observed exchange of some nucleotide between tubulin and GNi is a possible mechanism for this phenomenon.

2.4.3 Release of GNs From The Synaptic Membrane and GNs Binding to Tubulin

As stated above, the photoaffinity GTP analog AAGTP labels both the 42 KDa GNs and the 40 KDa inhibitory GTP-binding proteins GNi on the synaptic membranes (Rasenick et al., 1984). Under conditions where microtubule disrupting drug treatment and subsequent washing releases GNs activity into the supernatant, AAGTP-

labelled 42 KDa protein is also released from those membranes (Rasenick et al., 1984). This labelled 42 KDa protein is retained by an agarose-tubulin affinity column and, when eluted from the column, the protein displays Ns activity (reconstitution of GNs-deficient adenylate cyclase). GTP-dependent (GNi mediated) inhibition of adenylate cyclase is unaltered by colchicine of vinblastine treatment, and the 40 KDa AAGTP labelled protein is not released from the synaptic membrane by these agents.

2.5 PERMEABILIZED INTACT CELLS

We have developed an assay for adenylate cyclase in intact, saponin permeabilized C6 cells. This treatment makes holes of 0.1 to 1 micron in plasma membranes while retaining cell viability (Brooks and Carmichael, 1983). ATP is added to these permeabilized cells and when α ^{32}P ATP is included ^{32}P cAMP produced can be measured (Rasenick and Kaplan, in press). There appears to be a distinct difference in the coupling pattern of the adenylate cyclase system in C6 membranes versus intact C6 cells. This is evidence by a virtually absolute requirement for hormone in order to observe effects of guanine nucleotides in the activation of adenylate cyclase (Figure 5), is marked contrast to membrane. Curiously, inhibition of adenylate cyclase by GTPγS proceeds without a hormone requirement (Figure 6).

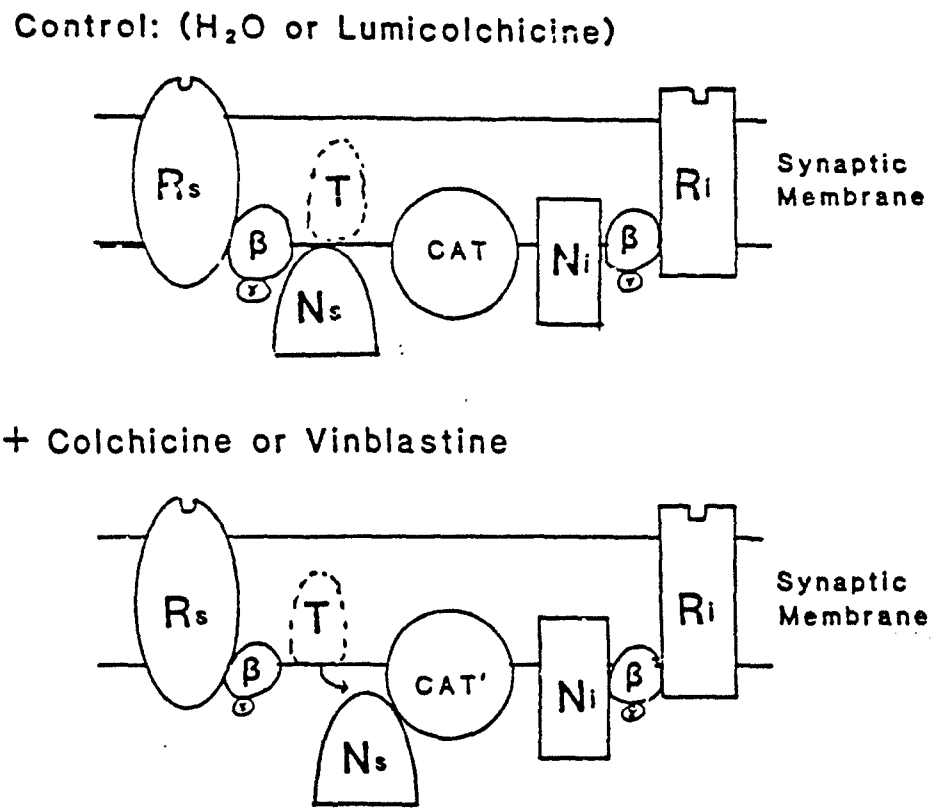

Figure 1: Interaction of Tubulin with Adenylate Cyclase

The above represents a possible format for adenylate cyclase activation or inhibition, and the effects of microtubule disrupting drugs upon that process. Rs and Ri represent receptors for stimulatory (s) and inhibitory (i) hormones or neurotransmitters and GNs or GNi represent the GTP-binding regulatory proteins linked to those receptors. β (35 KDa) and γ (14 KDa) are regulatory proteins which are associated with the GN complex and the catalytic moiety (CAT) produces cAMP from ATP. In this cartoon, tubulin is associated with GNs and colchine or vinblastine dissociate these proteins, resulting in augmented activation of adenylate cyclase by GNs. Additionally, we have indicated recently that infection of guinea pigs with Creutzfeldt-Jakob agent promotes coupling between GNs and the adenylate cyclase catalytic moiety (Rasenick et al., 1986) in basal ganglia membranes prepared from those animals.

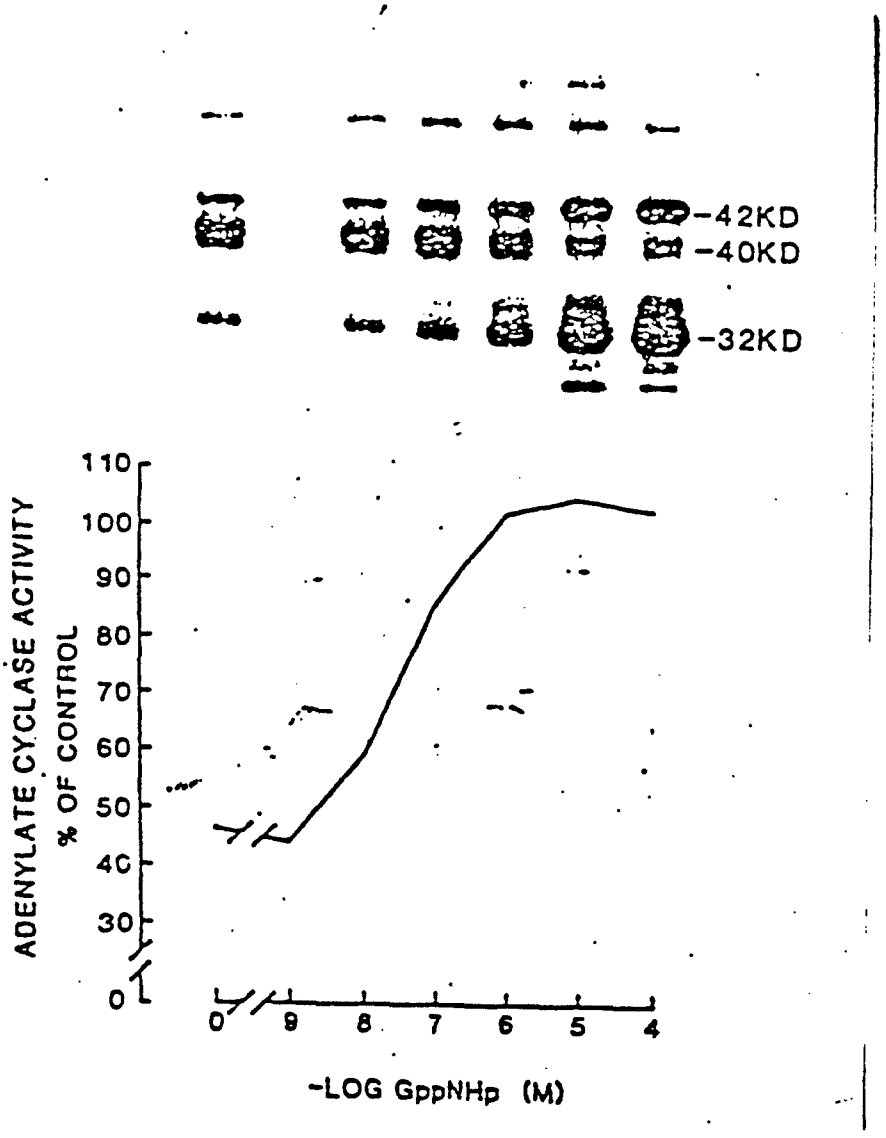

Figure 2. (Upper) SDS/PAGE analysis of AAGTP-labeled synaptic membranes.

Synaptic membranes were incubated with 1.2 x 10^{-7} M [^{32}P]AAGTP as above but after 2nd incubation with GppNHp, reactions were subjected to 20 min of UV photolysis on ice. The membranes were then washed and submitted to SDS/PAGE and autoradiographed.

(LOWER). Adenylate cyclase activity of snyaptic membranes incubated with AAGTP.

Synaptic membranes prepared from rat cerebral cortex were incubated with AAGTP (1.2 x 10^{-4} M) for 3 min at 23°C, subsequently washed and assayed for adenylate cyclase activity with indicated concentration of GppNHp. Adenylate cyclase activity is expressed as a percentage of the control activity of the membranes which were not incubated with AAGTP. Values are means of 3 experiments and basal adenylate cyclase activity in control membranes was 50.4 pmol/mg protein/min. Persistent inhibition of adenylate cyclase was dose-dependent between 10^{-8}-10^{-4} M.

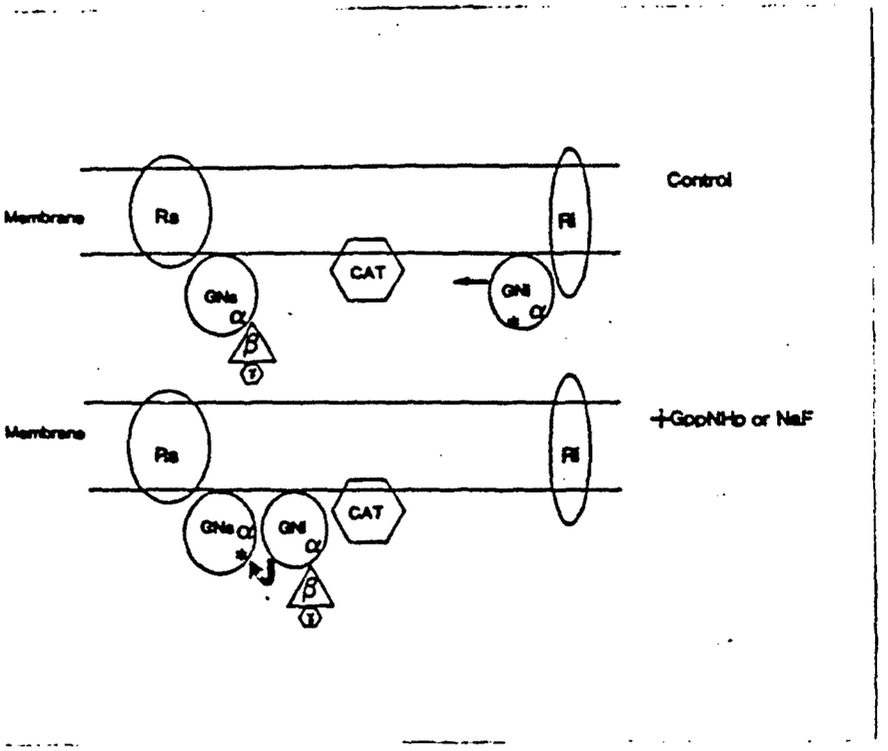

Figure 3. Model for transfer of guanine nucleotides.

Nucleotide is initially bound to GNi when adenylate cyclase is inhibited. As this inhibition is overridden by GppNHp of NaF, nucleotide[*] is transferred to GNs from GNi. Although we depict $\beta\gamma$ transferring from GNi to GNs along this process, we have no data which suggest that this occurs in synaptic membranes.

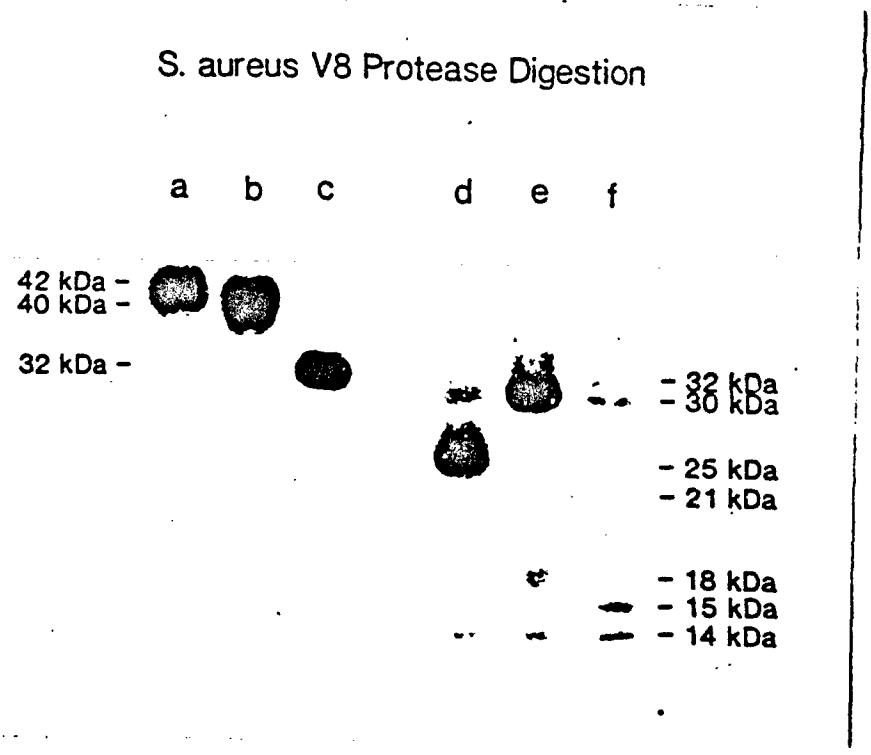

Figure 4.

Autoradiograph of SDS PAGE of Staphylococcus aureus V8 protease digestion products. [^{32}P]AAGTP-labelled bands from SDS PAGE gels were digested without prior elution, by packing gel slices containing these bands in the sample wells of a second SDS gel and then overlaying each slice with S. aureus V8 protease (500 ng/lane). Digestion proceeded directly in the stacking gel during the subsequent electrophoresis. Lane a, b, and c, no protease; lane d, e, and f, S. aureus V8 protease (500 ng/lane). Lane a and d, 42 KDa (GNs) protein; lane c and f, 32 KDa protein.

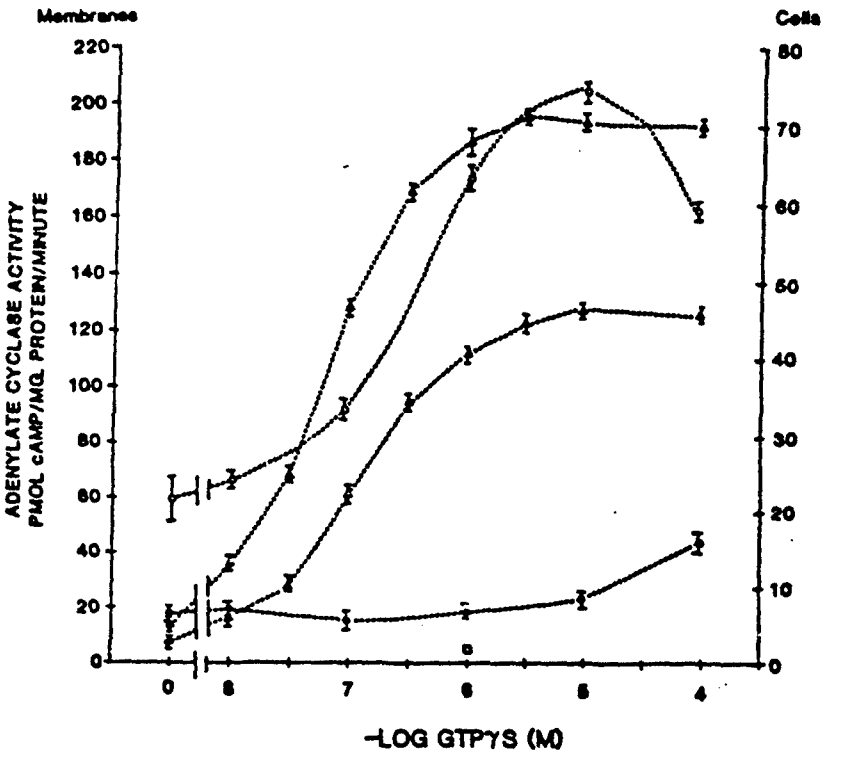

Figure 5. Guanine nucleotide activation of adenylate cyclase in C6 membranes and Permeabilized cells.

C6 membranes were incubated with (Δ) or without (Δ) isoproterenol (1 μM) in the presence of indicated GTP-γS concentrations. Permeabilized C6 cells were assayed for adenylate cyclase activity in the presence (o) or absence (o) of isoproterenol (1 μM) with the indicated concentration of GTP-γS (1 μM).

Means of triplicate determinations (±SEM) from one of three (cells) or four (membranes) similar experiments are depicted above.

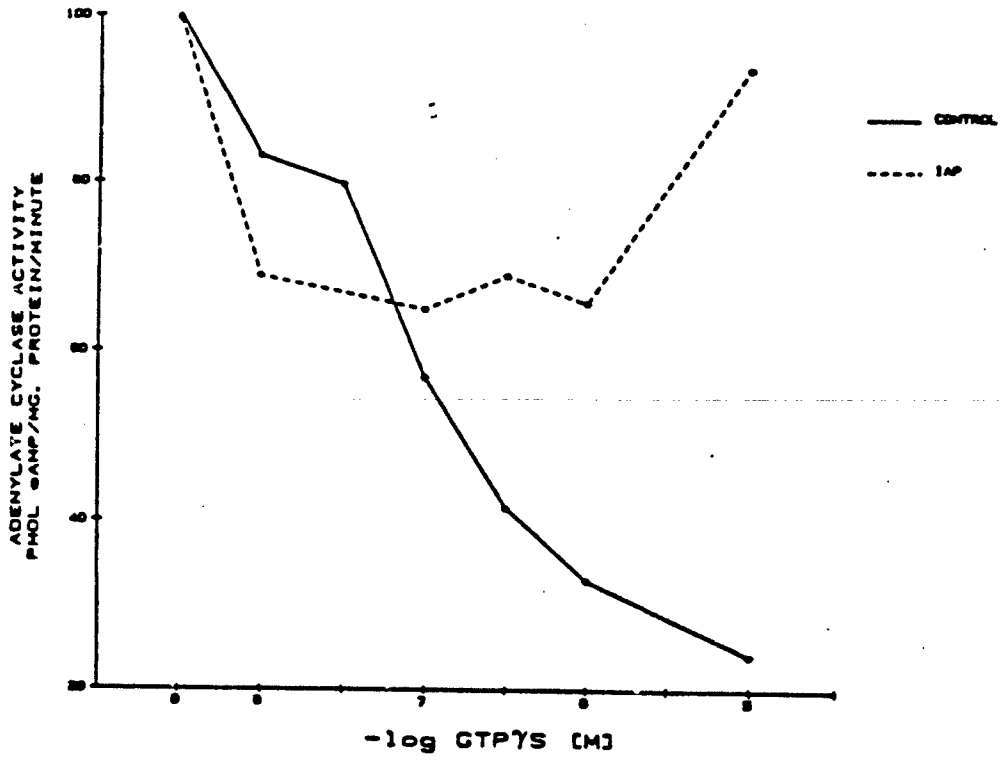

Figure 6. Adenylate cyclase inhibition in permeabilized C6 cells.

C6 cells are incubated with or without pertussis toxin (IAP 10 μg/ml) for 3 hours prior to assay. Cells are then saponin treated and incubated with forskolin (10 μM) plus the indicated GTPγS concentration. Adenylate cyclase activity is determined as listed in the methods.

Publications resulting from AFOSR 83-0249

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Creutzfeldt-Jakob infection increases adenylate cyclase activity in specific regions of guinea pig brain

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Creutzfeldt-Jakob disease is a slow, infectious, progressive neurological disorder which results in human dementia. Synaptic membranes from various brain regions of guinea pigs infected with Creutzfeldt-Jakob disease show increased guanyl nucleotide- or 5-hydroxytryptamine-mediated activation of adenylate cyclase. This increased enzyme activity appears due, primarily, to facilitated 'coupling' between the GTP-binding protein which stimulates adenylate cyclase (GN_s) and the catalytic moiety of that enzyme rather than increased sensitivity to 5-hydroxytryptamine. It is possible that this phenomenon is due to direct effects of the Creutzfeldt-Jakob infectious agent, or a pathological product resulting from that agent, upon synaptic membrane adenylate cyclase.

GTP-binding protein *Receptor-effector coupling* *Oncogene product* *Dementia* *Signal transduction*

1. INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a slow, infectious, progressive neurological disorder which results in human dementia, and may provide a paradigm for more common progressive human dementias such as Alzheimer's disease. CJD is characterized by spongy or membranous changes in affected neuropil, particularly in synaptic regions [1]. Human autopsy material can be used to transmit CJD to rodents [2] and the infectivity is closely associated with a specific, ~~synaptic~~ membrane-associated protein [3,4]. In order to study the possibility that synaptic membrane accumulation of CJD infectious agent, or agent elicited protein, causes clinical and pathological manifestations of CJD, the functional capacity of the neuronal membrane enzyme, adenylate cyclase, was examined.

Note: Various authors have referred to these proteins as N (nucleotide binding protein) and G (GTP binding protein). Here, we refer to these proteins as GN_s or GN_i – GN referring to guanine nucleotide

A variety of hormones or neurotransmitters mediate the activity of adenylate cyclase, however, in neuronal membranes this enzyme can be stimulated or inhibited directly by hydrolysis-resistant guanine nucleotides or F^- and these actions are expressed through separate guanine nucleotide-binding proteins: GN_s (stimulation) and GN_i (inhibition). The coupling of adenylate cyclase refers to the interaction between (or among) neurotransmitter receptor, GN_s or GN_i and the adenylate cyclase catalytic moiety. We have demonstrated previously that neuronal adenylate cyclase coupling might be augmented by treatments which alter cytoskeletal or membrane composition [5,6] and by chronic antidepressant treatment [7]. Thus, we undertook to investigate the possibility that the membrane changes associated with Creutzfeldt-Jakob infection might also effect changes in neuronal adenylate cyclase coupling. Further, recent investigations in rodents infected with scrapie (a spongiform encephalopathy similar to CJD) have detected diminished brain 5-hydroxytryptamine (5HT) levels. In these studies, some of the behavioral aspects of scrapie

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infection were ascribed to 5HT supersensitivity [8,9]. Pursuant to those studies, we chose additionally to examine 5HT-activated adenylate cyclase in brains of normal and CJD-infected guinea pigs.

2. MATERIALS AND METHODS

2.1. *Animals*

We utilized a serially passaged guinea pig model of CJD [10]. Synaptosome-enriched fractions were prepared as described [3] from animals showing typical clinical signs of disease (approx. 20 weeks after i.c. inoculation of young adult guinea pigs). Material from several serial passages was studied (i.e. passages 14–18), and all studies included (i) parietal cerebral cortex, (ii) hippocampus, and (iii) basal ganglia. Regions were rapidly dissected, synaptosomal fractions prepared, and aliquots of prepared membranes stored in liquid nitrogen until assay. Spongy changes are especially apparent in this CJD passaged material in cerebral cortex and basal ganglia [2]. Controls included normal young adult as well as older (2-year-old) guinea pigs of the same strain, and synaptic membrane-enriched fractions were derived identically to those from infected animals.

2.2. *Adenylate cyclase assays*

Membranes were suspended in 20 mM Hepes (pH 7.4) with 5 mM $MgCl_2$, 1 mM DTT and 0.3 mM PMSF and assayed in duplicate for adenylate cyclase as described [5]. Briefly, 20 μg membrane protein was preincubated with 5HT (in 0.02% ascorbate) and/or other agents (as indicated) at 30°C for 20 min. Following this, ATP (500 μM) was added (final incubation volume 100 μl) and the reaction mixtures incubated at 30°C for 10 min. Reactions were stopped by boiling (3 min) and the cAMP produced assayed by protein binding. Some experiments were performed similarly except that the assay was by the method of Salomon [26], and triplicate rather than duplicate determinations were made. These assays were stopped by the addition of 1% SDS rather than by boiling.

3. RESULTS

In all experiments (figs 1,2) there was a clear increase in adenylate cyclase activity in membranes from CJD-infected animals. In membranes from CJD-infected animals, the magnitude of the 5HT response was enhanced (fig.1) without any apparent change in the 5HT sensitivity. Adenylate cyclase activation by 5HT in control membranes was somewhat less sensitive but otherwise comparable to that reported in [11]. Hippocampal membranes from infected animals showed a pattern of increased 5HT-activated adenylate cyclase similar to that observed in the basal ganglia.

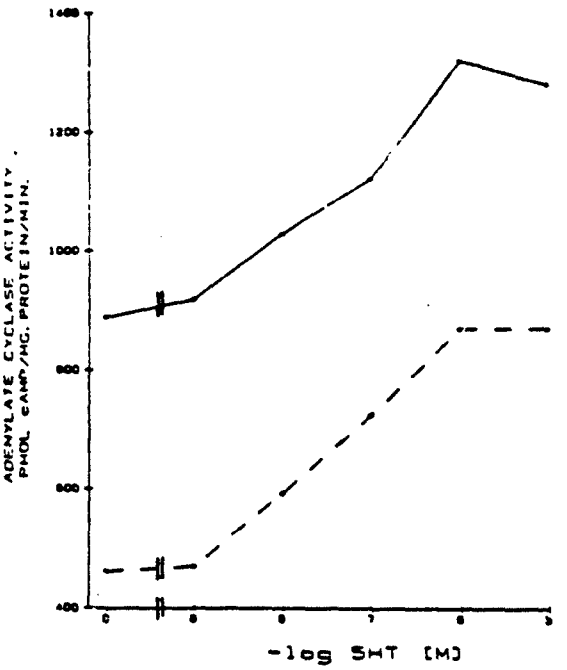

Fig.1. Adenylate cyclase activity in infected and control guinea pig basal ganglia membranes at indicated 5HT concentrations. Guinea pigs were inoculated with a 10% homogenate of CJD passage brain [10] and killed at late clinical stages of the disease. Histological confirmation of the disease was made in parallel cage-mates displaying similar symptomology. Fresh brain (control,– – –; or CJD-infected,——) synaptosome-enriched fractions from each region were prepared and assayed as described, in the presence of the indicated 5HT concentration plus 5 μM Gpp(NH)p. Values expressed are means of duplicate determinations for one of three similar experiments.

Although the results in fig.1 indicate some increased 5HT responsiveness, they cannot be explained on the basis of increased 5HT sensitivity due to the large increase in overall adenylate

cyclase activity in membranes from CJD-infected animals. Therefore, other aspects of adenylate cyclase activation were examined. Basal adenylate cyclase activity was indistinguishable between membranes prepared from infected animals and controls (fig.2, lanes A). Adenylate cyclase activity in the same preparations was also measured in the presence of $MnSO_4$. Mn^{2+} activates the catalytic moiety of adenylate cyclase, and under these assay conditions, is thought to reflect the activity of the enzyme which is independent of GN_s [5,12]. If membranes were pathologically destroyed in CJD, one would expect to see less adenylate cyclase specific activity under these conditions as compared to controls. In both brain regions, the Mn^{2+}-stimulated catalytic moiety activity of adenylate cyclase in CJD groups was comparable (fig.2, lanes B). We then studied the GN_s-dependent activation of adenylate cyclase by using NaF or the hydrolysis-resistant GTP analog, Gpp(NH)p. In the presence of 5×10^{-6} M Gpp(NH)p (fig.2, lanes C) both basal ganglia and hippocampus membranes showed an activation of adenylate cyclase that was significantly greater than the controls. At a submaximal concentration of Gpp(NH)p (10^{-7} M), adenylate cyclase activities in membranes from infected animals were 85% (basal ganglia) and 40% (hippocampus) greater than controls in the illustrated experiment. The mean and standard deviation for 5 experiments indicated an increase in GppNHp-activated adenylate cyclase of 66 ± 17% (mean ± SD) in CJD-infected basal ganglia and 52 ± 22% in CJD-infected hippocampus compared to controls. In each experiment NaF also elicited increased adenylate cyclase activity in basal ganglia and hippocampus in CJD-infected animals (e.g. lanes D, fig.2). Combined data for all experiments showed an increase in NaF-stimulated adenylate cyclase of 100 ± 20% (basal ganglia) and 56 ± 16% (hippocampus) in membranes from CJD-infected animals as compared to controls.

Unlike basal ganglia and hippocampus, cerebral cortex membranes from CJD-infected animals showed a slight decrease in GppNHp-stimulated adenylate cyclase. The cerebral cortex shows marked vacuolization at end stages of CJD, and it is possible that these pathological changes preclude detection of elevated adenylate cyclase in this region.

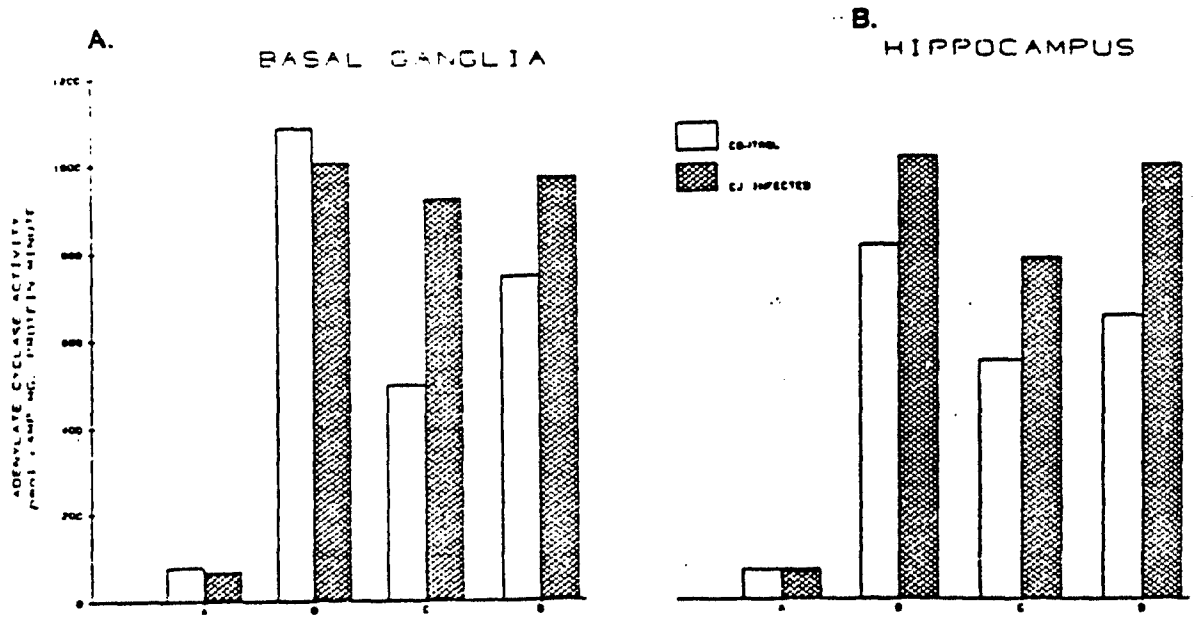

Fig.2. Adenylate cyclase activity in membranes prepared from basal ganglia or hippocampus in CJD-infected and control guinea pigs. Additions to the adenylate cyclase preincubation and assay: (A) H_2O, (B) $MnSO_4$ (10 mM), (C) Gpp(NH)p (5 μM), (D) NaF (20 mM). Points represent the means of duplicate determinations from one of 3–5 similar experiments. Means ± SD of normalized data from all experiments (N = 6–10) are given in the text.

4. DISCUSSION

It is clear from the above studies that the integrity of the adenylate cyclase system of membranes from basal ganglia and hippocampus remains intact in this disease, despite extensive pathological changes at the synaptic level [2,10]. Pathological changes in neurons secondary to CJD infection, and not necessarily entailing a direct or specific effect on neuronal membranes, could lead to the increased adenylate cyclase activation obtained above. In this context, several mechanisms may be considered. For example, disruption of neuronal cytoskeletal integrity, consistent with the focal clearing observed in synaptic processes of CJD-infected animals [12,14,15], may increase adenylate cyclase activity. It has been demonstrated previously [5] that activation of neuronal adenylate cyclase through GN_s is enhanced as a consequence of treatment with microtubule-disrupting drugs or agents which increase membrane fluidity [5,6,15]. Furthermore, selective damage of 5HT-containing neurons can lead to 5HT-stimulated adenylate cyclase supersensitivity in 5HT-responsive neurons. 5HT supersensitivity (due to reduced 5HT levels) has been invoked to explain some of the behavioral changes observed in scrapie-infected rodents [9,10], and an increased response to 5HT may be present with respect to adenylate cyclase in the CJD-infected animals in these studies. Increased 5HT response observed in basal ganglia, which may be consistent with some depletion of 5HT at presynaptic terminals, is dependent upon low (5×10^{-6}) GppNHp concentrations in the adenylate cyclase assay. However, increased response to 5HT does not alone explain increased adenylate cyclase activation in membranes from CJD-infected animals by NaF or GppHNp. Reserpine treatment [11], chronic electroconvulsive shock and chronic treatment with tricyclic antidepressants [7] can also enhance brain adenylate cyclase. The reserpine effect is similar to that observed in CJD where elevated 5HT responsiveness was found at essentially all concentrations tested and is compatible with enhanced coupling of the GN_s protein to adenylate cyclase, rather than (or in addition to) a specific receptor-mediated enhancement. In summary, this work shows an increased interaction (coupling) of GN_s as the predominant consistent finding in CJD membranes from basal ganglia and hippocampus. Additionally, 5HT supersensitivity in basal ganglia may occur secondary to pathological depletion of 5HT terminals in that region.

Both CJD and scrapie are related infectious agents, and similar specific sialoglycoproteins have been identified in both diseases [4,16,18]; these proteins share common antigenic epitopes [4,15,16] and are found in subcellular fractions that are highly infectious. These proteins, as well as infectivity and unique fibrils, cosediment with, and are tightly bound to, synaptosome-enriched or synaptic membrane fractions [3,4]. It is possible that insertion of CJD-specific proteins into neuronal membranes directly alters the interaction of GN_s with the adenylate cyclase catalytic moiety. Another possibility is that a CJD-elicited membrane protein mimics GN_s in the activation of adenylate cyclase. Although at present we have no evidence favoring a direct membrane effect over one secondary to other neuronal pathology (e.g. cytoskeletal changes), it is of interest that recent experiments on *ras* proteins, have indicated that oncogene products (p21-related membrane proteins) can directly bind guanine nucleotides and display GTPase activity [20]. There is some sequence homology between *ras* proteins and adenylate cyclase GN proteins [20,21] and *ras* membrane proteins might substitute for GN_s in the activation of adenylate cyclase [22] associated with transformed growth potentials (this is a matter of some controversy [23]). Although CJD and scrapie are degenerative diseases, there is some evidence that exposure to these agents can also result in altered growth potentials and cell transformation [24,25]. Perhaps a similar protein and similar phenomena account for the observed increase in GN_s activated adenylate cyclase in CJD membranes.

Further investigation of (i) adenylate cyclase response patterns during the long incubation period of CJD (e.g. at early and preclinical stages of infection), (ii) the nature of CJD specific protein(s) interactions with membrane components (i.e. the ability of these proteins to bind guanine nucleotides), and (iii) membrane alterations in CJD-transformed cells should help to clarify the exact target of these 'unconventional' infectious agents and their role in the genesis of encephalopathy.

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Dynamic Aspects of Microtubule Biology
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Regulation of Neuronal Adenylate Cyclase by Microtubule Proteins[a]

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Microtubule-disrupting drugs have been shown to increase adenylate cyclase activity in synaptic membranes prepared from rat cerebral cortex. Activation of the enzyme by hydrolysis-resistant guanosine triphosphate (GTP) analogues and NaF is augmented by colchicine or vinblastine ($EC_{50} = 5 \times 10^{-7}$M), whereas basal and Mn^{++}-stimulated (reflecting catalytic-moiety activation) activities are unchanged.[1] These findings suggest that the microtubule-disrupting drugs increase the "coupling" between the GTP-binding protein that stimulates adenylate cyclase (Ns) and the catalytic moiety of that enzyme. When these membranes are treated with colchicine or vinblastine and subsequently washed, activity of the Ns protein is released from the membranes into the supernatants. This release of Ns activity is indicative of facilitated Ns-catalytic moiety coupling and is similar to that observed in the homologous cGMP phosphodiesterase cascade from retinal rod outer segments.[2]

Incubation of tubulin (EC_{50} = 0.5 μg/ml; 10 n*M*) with synaptic membranes inhibits guanyl nucleotide and NaF activated adenylate cyclase by 50–60% without altering basal and Mn^{++} activation of the enzyme (FIGURE 1). The effects of this tubulin incubation are sustained even after membranes are washed. Furthermore, treatments that diminish the amounts of membrane-associated tubulin increase Ns-mediated adenylate cyclase activation.

The photoaffinity GTP analogue (P^3azidoanilido)-P^1-5′ GTP (AAGTP) labels both the 42 kD Ns and the 40 kD inhibitory GTP-binding protein (Ni) on the synaptic membranes.[3] Under conditions where microtubule disrupting drug treatment and subsequent washing releases Ns activity into the supernatant, AAGTP-labeled 42 kD protein is also released from those membranes (FIGURE 2). This labeled 42 kD protein is retained by an agarose-tubulin affinity column, and when eluted from the column, the protein displays Ns activity (reconstitution of Ns-deficient adenylate cyclase). GTP-dependent (Ni mediated) inhibition of adenylate cyclase is unaltered by colchicine or vinblastine treatment, and the 40 kD AAGTP labeled protein is not released from the synaptic membrane by these agents.

Although microtubule disrupting drugs increase cyclic adenosine monophosphate (cAMP) accumulation in intact cells,[4] colchicine or vinblastine enhancement of adenylate cyclase in broken cell preparations and the release of AAGTP-labeled Ns has been observed only in membranes from tissues of neural origin. These data lead to the proposal of a hypothetical Ns-tubulin interaction similar to that suggested for actin and fibronectin.[5] The physiological significance of a putative Ns-tubulin interaction (association/dissociation) might be to regulate intracellularly the amount of cAMP produced in response to a given neurotransmitter.

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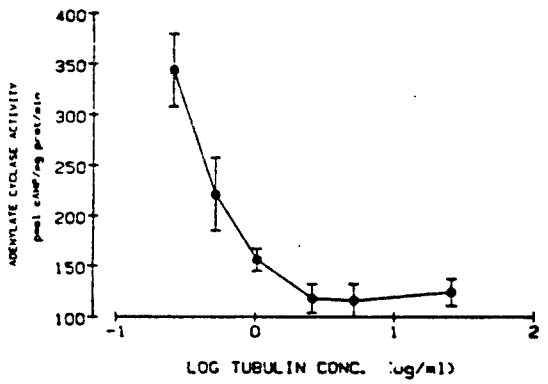

FIGURE 1. Inhibition of Ns-mediated adenylate cyclase by tubulin. Synaptic membranes were washed twice in 20 m*M* Tris HCl (pH7.5) containing 1 m*M* dithiothreitol (DTT), 0.3 m*M* phenylmethyl sulfonyl fluoride (PMSF) and 1 m*M* ethylene diamine tetraacetic acid (EDTA). Following this, membranes were resuspended in the above buffer containing 5 m*M* MgCl [no EDTA], linoleic acid (10 μg/ml), phosphatidylcholine (1 mg/ml), and the indicated tubulin (phosphocellulose purified from rat brain) concentration and incubated for 10 minutes at 37°C followed by 15 minutes on ice. Following this, guanylyl inidodiphosphate (Gpp[NH]p) (10 μ*M*) was added, and the tubes were incubated at 30°C for 10 minutes followed by the addition of adenosine triphosphate (ATP) and a 10-minute assay incubation. Adenylate cyclase activity in the absence of added tubulin plus 10 μ*M* GppNHp was 326 pmol cAMP/mg protein/minute. Basal adenylate cyclase activity (in the absence of added GppNHp) was 36 and 47 pmol cAMP/mg protein/min respectively in the absence or presence of 5 μg/ml added tubulin.

Tubulin incubation did not inhibit basal or Mn^{++}-stimulated adenylate cyclase; thus, only Ns-mediated adenylate cyclase appears to be involved. The "IC_{50}" for this effect is about 10 n*M*, and the effect is maximal at 40 n*M* tubulin. The ratio of microtubule protein:total synaptic membrane protein required to achieve this effect is about 1:500. Although the nature of tubulin interaction with these membranes is unclear, the effects of added tubulin are maintained after the membranes are washed.

One interpretation of these results might be that addition of tubulin to these membranes results in increased tubulin-Ns association and subsequently, decreased Ns-mediated adenylate cyclase coupling. The possibility of a trace impurity in the tubulin preparation having these effects, or of tubulin acting indirectly on Ns, cannot be ruled out.

A. SUPERNATANTS
42K
a. b. c. d.
B. MEMBRANES
42K
40K
35K
a. b. c.

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FIGURE 2. Photoaffinity labeling and colchicine or vinblastine mediated increase in Ns mobility.

A: Synaptic membranes were incubated with 1 μM [^{3}H]AAGTP at 30°C for 20 minutes followed by 20 minutes of UV photolysis on ice. The reaction was quenched with 4 mM DTT, and the membranes were then incubated at 30°C for 10 minutes with 5 μM of either colchicine (lanes b and d) or lumicolchicine (lanes a and c). Following this, the membranes were washed three times with a low ionic strength buffer (2 mM HEPES, pH 7.4, 1 mM MgCl, 2 mM DTT), and the proteins released from the membranes were electrophoresed on 10% polyacrylamide gels and radioautographed. Lanes a and b represent Coomassie blue staining patterns, and lanes c and d are radiofluorographs.

B: Synaptic membranes were treated as above except that [^{32}P]AAGTP was used. Following photolysis, the membranes were treated with H_2O (lane a), 0.1 μM vinblastine (lane b) or 10 μM vinblastine (lane c), and washed as above. These radioautographs represent labeling on the membranes rather than on the supernatants.

Similar experiments using unlabeled AAGTP with subsequent adenylate cyclase assays rather than autoradiography show a vinblastine-mediated loss of Ns activity subsequent to washing without a concomitant loss of Ni activity. Under conditions where colchicine or vinblastine enhance "coupling" of the Ns protein with the adenylate cyclase catalytic moiety, washing membranes with buffer releases about 50% of the Ns activity. This is borne out by comparing [^{32}P]DPM in the 42 kD bands from A, which are 7,445 in lane d and 4,343 in lane c.

Although the coupling of Ns is enhanced by microtubule disrupting drugs, that of Ni is not. Similarly, the 40 kD GTP-binding protein is not released from the membrane under conditions where Ns appears to be released.

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Biochemistry

Exchange of guanine nucleotide between GTP-binding proteins that regulate neuronal adenylate cyclase

(cyclic nucleotides/synaptic membrane/receptor–effector coupling/photoaffinity labeling)

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Communicated by Max Tishler, April 14, 1986

ABSTRACT GTP-binding proteins have been demonstrated to stimulate and inhibit rat brain adenylate cyclase without the prior addition of hormone. Exposure of rat cerebral cortex membranes to hydrolysis-resistant GTP analogs results in inhibition (or stimulation) of adenylate cyclase, which persists subsequent to buffer washing. The hydrolysis-resistant GTP photoaffinity probe P^3-(4-azidoanilido)-P^1-5′ GTP (AAGTP) can promote a similar persistent inhibition of adenylate cyclase, and, after removal of unbound AAGTP and subsequent UV photolysis, AAGTP is covalently linked to the 40-kDa inhibitory GTP binding protein, GN_i (inhibitory guanine nucleotide binding regulatory subunit of adenylate cyclase). Under conditions where the persistent inhibition of adenylate cyclase is overcome by subsequent incubation with 5′-guanylyl imidodiphosphate or NaF, AAGTP bound to the 40-kDa GN_i protein is diminished while that bound to the 42-kDa stimulatory GTP-binding protein (GN_s) is increased. Additionally, we have identified a 32-kDa protein that binds AAGTP with an affinity similar to that of GN_s. This protein does not appear to be a byproduct of proteolysis as demonstrated by *Staphylococcus aureus* V8 protease digestion experiments, and it is not a substrate for ADP-ribosylation by bacterial toxins. The sum of the AAGTP bound by the GN_i and GN_s proteins is constant, and the transfer of nonphotoactivated AAGTP to GN_s from GN_i is stable to buffer washing. Furthermore, this alteration in the AAGTP-labeling pattern corresponds to the shift in adenylate cyclase from inhibition to stimulation. These data raise the possibility that hydrolysis-resistant GTP analogs might be exchanged directly between the GN_i and GN_s and that there exists some interaction between those proteins in the regulation of adenylate cyclase activity.

Neuronal adenylate cyclase is responsive to a variety of neurotransmitters which stimulate or inhibit that enzyme. These neurotransmitters exert their effects through at least two membrane-associated GTP-binding proteins, referred to as GN_s and GN_i, denoting, respectively, the stimulatory and inhibitory guanine nucleotide binding regulatory subunit of adenylate cyclase. The α, or GTP-binding, subunit of GN_i has recently been purified from bovine cerebral cortex and appears to consist of two or three pertussis toxin substrates clustered at about 40 kDa (1, 2). The α subunit of GN_s has been purified and appears to be a single polypeptide of about 42–45 kDa (3), although in some tissues a 48- to 52-kDa form of GN_s also appears (4). GN_s and GN_i regulate the catalytic activity of the adenylate cyclase through interaction with a distinct catalytic moiety of the enzyme. The mechanism of this regulation is unknown, but it has been suggested that the β and γ components (36 kDa and 8 kDa, respectively) common to GN_s and GN_i provide a regulatory function (5, 6).

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Experiments designed to understand the nature of GN_s or GN_i interaction with the adenylate cyclase catalytic moiety have involved the use of purified proteins (3), often inserted into phospholipid vesicles (7, 8). Such systems generally combined a single hormone receptor type, GN_s or GN_i (not both), and a resolved, partially pure adenylate cyclase catalytic moiety. Although such systems have been extraordinarily illuminating with regard to some aspects of GN_s and GN_i function, the membrane systems that provide the natural habitat for these proteins are considerably more complex. Multiple receptor types, the coexistence of GN_s and GN_i, and a myriad of membrane-associated proteins (some of which, such as cytoskeletal proteins, may interact with the adenylate cyclase system; refs. 9 and 10) probably represent the conventional milieu for adenylate cyclase, and the duplication of such an environment is not possible within reconstituted systems.

The regulation of GTP-binding by GN_s and GN_i has proved similarly enigmatic. This has been studied with the purified proteins, where the specificity and the precision of binding is unquestioned, but the regulation of that binding is unclear (3, 6). Membrane systems have been used to study binding of the tritiated hydrolysis-resistant GTP analog 5′-guanylyl imidodiphosphate (p[NH]ppG) (11, 12), but low-binding specificity and p[NH]ppG binding unrelated to adenylate cyclase make interpretation of these experiments problematic. The hydrolysis-resistant photoaffinity GTP probe, P^3-(4-azidoanilido)-P^1-5′ GTP (AAGTP), has been demonstrated to bind to the GN_s of adenylate cyclase from pigeon erythrocytes (13) and rat cerebral cortex synaptic membranes (14). In this manuscript we report a distinction between GN_s and the GN_i complex in relative affinity for AAGTP. We also report that, as the adenylate cyclase complex shifts from the inhibited to the activated state, AAGTP appears to be exchanged between the GTP-binding proteins responsible for regulating those processes.

MATERIALS AND METHODS

Tissue Preparation. Synaptic membrane-enriched fractions were prepared from 21-day-old male Sprague–Dawley rats as described (15, 16) and stored under liquid nitrogen until use.

Adenylate Cyclase Assay. Membranes were thawed and resuspended in a buffer containing 20 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride and were incubated with or without AAGTP at 0.12 or 120 μM at 23°C for 3 min. After incubation the membranes were washed twice and resuspended in the same buffer. Washed membranes (10–20 μg) were incubated

Abbreviations: GN, guanine nucleotide binding regulatory subunit of adenylate cyclase; GN_s, stimulatory GN; GN_i, inhibitory GN; AAGTP, P^3-(4-azidoanilido)-P^1-5′ GTP; p[NH]ppG, 5′-guanylyl imidodiphosphate; GTP[γS], guanosine (5′-O^3)-1-thiotriphosphate; IAP, islet-activating protein.

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for 10 min at 23°C in 100 μl medium containing 15 mM Hepes (pH 7.5); 0.05 mM ATP; [α-^{32}P]ATP (≈5 × 10^5 cpm per tube); 1 mM $MgCl_2$; 1 mM dithiothreitol; 0.05 mM cyclic AMP; 60 mM NaCl; 0.25 mg/ml of bovine serum albumin; 0.5 mM 3-isobutyl-1-methylxanthine; 1 unit of adenosine deaminase per ml; a nucleoside triphosphate-regenerating system consisting of 0.5 mg of creatine phosphate, 0.14 mg of creatine phosphokinase, and 15 units of myokinase per ml; and other reagents as indicated in the text. The reaction was stopped by the addition of 0.1 ml of a "stopping solution" containing 2% $NaDodSO_4$, 1.4 mM cyclic AMP, and 40 mM ATP, and the cyclic [^{32}P]AMP formed was isolated by the method of Salomon (17). Protein was determined by the Coomassie blue binding method (18) with bovine serum albumin as a standard.

Photoaffinity Labeling. [^{32}P]AAGTP was synthesized by the method of Pfeuffer (13). Synaptic membranes were washed and resuspended in 2 mM Hepes, pH 7.4/1 mM $MgCl_2$. Membrane suspensions (3–7 mg of protein per ml) were incubated with 0.12 μM [^{32}P]AAGTP for 3 min at 23°C, 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 incubated with or without guanyl nucleotides or NaF for 10 min at 23°C followed by 20 min of UV photolysis with a Spectroline UV (254 nm, 9 W) lamp on ice at a distance of 3 cm (the photoreaction of AAGTP with membranes was essentially completed after 5 min of UV irradiation, and no incorporation of radioactivity of ^{32}P was observed without UV photolysis). Prior incubation of membranes with hydrolysis-resistant guanine nucleotides also blocks AAGTP incorporation (14). The reaction was quenched with the ice-cold 2 mM Hepes, pH 7.4/1 mM $MgCl_2$/4 mM dithiothreitol, followed by centrifugation at 13,000 × *g* for 10 min. Membrane pellets were dissolved in 3% $NaDodSO_4$ Laemmli sample buffer (19) with 50 mM dithiothreitol. Samples were heated for 4 min at 60°C and electrophoresed in 10% $NaDodSO_4$/polyacrylamide gels by the procedure of Laemmli (19). After electrophoresis, gels were stained with Coomassie blue and autoradiographed with Kodak XAR-5 film. To determine the amount of radioactivity in a particular band, the radiolabeled band of the dried gels was excised and counted in a Beckman LS 5800 scintillation spectrophotometer.

Treatment of Synaptic Membranes with Islet-Activating Protein (IAP), a Pertussis Toxin. ADP-ribosylation of synaptic membranes with IAP was performed at 37°C for 10 min in medium containing 50 μg of IAP per ml, 20 mM Hepes (pH 7.5), 1 mM dithiothreitol, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM ATP, 0.5 mM GTP, 10 mM thymidine, 1 mM EDTA, 1 mM isoniazid, and 10 μM [^{32}P]NAD. Incubation was terminated by dilution with 1 ml of the ice-cold 20 mM Hepes, pH 7.5/2.5 mM $MgCl_2$, followed by centrifugation at 27,000 × *g* for 10 min. The pellet was washed twice by repeating dilution and centrifugation. Washed membranes were electrophoresed in 10% $NaDodSO_4$/PAGE and analyzed by autoradiography.

Protease Digestion. Synaptic membranes were incubated with [^{32}P]AAGTP, washed, and then subjected to electrophoresis and autoradiographed. Gel slices of radioactive bands were excised and placed on top of a discontinuous $NaDodSO_4$ gel system (a 12.5% separating gel and a 3.5% stacking gel). The gel slices were overlaid with 0.125 M Tris (pH 6.8)/30% (vol/vol) glycerol, followed by protease solution containing 5 μg of *Staphylococcus aureus* V8 protease per ml, 0.125 M Tris (pH 6.8), 0.1% $NaDodSO_4$, and 10% glycerol (20). Electrophoresis was performed at a constant voltage (40 V per gel), and the peptides generated were separated in the 12.5% acrylamide separating gel.

Materials. [α-^{32}P]ATP (800 Ci/mmol; 1 Ci = 37 GBq) and [α-^{32}P]NAD (25 Ci/mmol) were purchased from New England Nuclear. [α-^{32}P]GTP was purchased from Amersham. GTP, p[NH]ppG, guanosine (5′-O^3)-1-thiotriphosphate (GTP[γS]), and *Staphylococcus aureus* V8 protease were from Sigma. *p*-Azidoaniline was synthesized by George L. Wheeler. Purified GN_s and GN_i were provided by Lutz Birnbaumer and Juan Codina. IAP was provided by Michio Ui. All other reagents used were of analytical grade.

RESULTS

Stable Inhibition of Adenylate Cyclase Induced by AAGTP in Cerebral Cortex Synaptic Membrane. Hydrolysis-resistant GTP analogs promote inhibition of synaptic membrane adenylate cyclase, which persists after washing of the membranes. In the present study, we have employed AAGTP, a hydrolysis-resistant photoaffinity GTP analog, to examine the stable inhibition of synaptic membrane adenylate cyclase as well as the interaction between GN_s and GN_i during alteration of adenylate cyclase activity. To examine the effect of AAGTP on synaptic membrane adenylate cyclase, the membranes were incubated with AAGTP for 3 min at 23°C with 1 mM Mg^{2+} and subsequently washed, whereupon adenylate cyclase activity was measured in the presence or absence of p[NH]ppG or NaF. AAGTP caused persistent inhibition of adenylate cyclase in the membranes that were not subsequently incubated with p[NH]ppG or NaF (Fig. 1). At 0.12 and 120 μM, AAGTP produced about 15% and 55% inhibition of adenylate cyclase, respectively. When AAGTP-treated membranes were subsequently incubated with increasing concentrations of p[NH]ppG, stable inhibition induced by AAGTP was overcome, and activation of the cyclase was observed at high concentrations [to 100 μM of p[NH]ppG (Fig. 1*A*)]. Intermediate values of AAGTP (1.2 and 12 μM) gave intermediate levels of adenylate cyclase inhibition (35% and 45%, respectively) which were similarly overridden by p[NH]ppG (not shown). Similar results were obtained when membranes were incubated with NaF instead of p[NH]ppG, although inhibition of adenylate cyclase induced by 120 μM AAGTP was not restored fully to control levels (Fig. 1*B*).

A Shift in AAGTP Labeling from GN_i to GN_s During the Release of the Stable Inhibition of Adenylate Cyclase. [^{32}P]AAGTP (0.12 μM) was incubated with membranes under conditions where AAGTP causes the stable inhibition of adenylate cyclase, and AAGTP photoaffinity labeling was analyzed by $NaDodSO_4$/PAGE and autoradiography. Under these conditions, a 40-kDa protein was predominantly labeled with AAGTP, and minor radioactive bands observed corresponded to 42-kDa and 32-kDa proteins (Fig. 2 *Upper*, left lanes; Fig. 3, lanes e and m). We have shown previously that AAGTP binds to the GN_s and GN_i proteins of adenylate cyclase from rat cerebral cortex synaptic membranes (14). AAGTP-labeled 42-kDa and 40-kDa proteins comigrated, respectively, with purified GN_s and GN_i proteins. The 40-kDa protein, which appears to be a doublet, also served as a substrate for IAP-catalyzed ADP-ribosylation (data not shown). As seen in Fig. 2*A*, a 32-kDa protein also was labeled specifically (see Fig. 3) with AAGTP. Proteolytic digestion studies with *Staphylococcus aureus* V8 protease showed that the 32-kDa protein was not a breakdown product from GN_s or GN_i and, in addition, that the 32-kDa protein was not ADP-ribosylated by IAP or cholera toxin (data not shown). It may be possible, therefore, that the 32-kDa protein is a GTP-binding protein that is distinct from GN_s and GN_i, although the character of this protein is not yet clear.

When the AAGTP-treated membranes were exposed subsequently to p[NH]ppG or NaF, AAGTP-labeling of 42-kDa and 32-kDa proteins was enhanced with increasing concen-

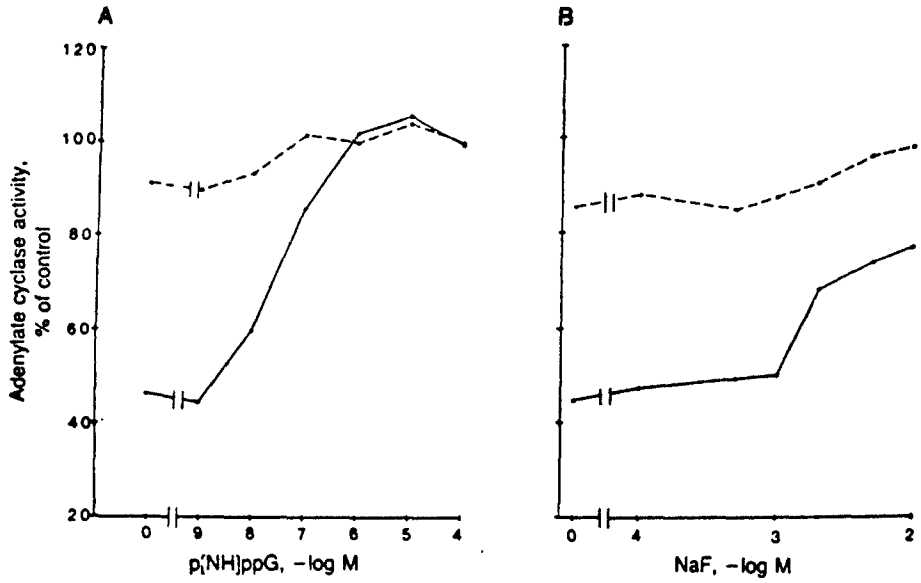

FIG. 1. Adenylate cyclase activity of synaptic membranes incubated with AAGTP. Synaptic membranes prepared from rat cerebral cortex were incubated without or with AAGTP at 0.12 μM (---) or 120 μM (—) for 3 min at 23°C, subsequently washed, and assayed for adenylate cyclase activity with various concentrations of p[NH]ppG (*A*) or NaF (*B*). Adenylate cyclase activity is expressed as a percentage of the control activity of the membranes that were not incubated with AAGTP. Values are means of three experiments, and basal adenylate cyclase activities (without p[NH]ppG or NaF) in control membranes were 50.4 pmol/mg of protein per min in *A* and 45.8 pmol/mg of protein per min in *B*.

tration of p[NH]ppG or NaF, whereas the labeling of the 40-kDa protein was reduced. The changes in AAGTP binding induced by p[NH]ppG or NaF were then quantitated (Fig. 2 *Lower*). The ^{32}P content of the 42-kDa band was increased from 2553 to 5194 cpm (Fig. 2 *Lower A*) and from 239 to 418 cpm (Fig. 2 *Lower B*), while that of the 40-kDa band was decreased from 5327 to 3274 cpm (Fig. 2 *Lower A*) and 828 to 528 cpm (Fig. 2 *Lower B*) in the presence of p[NH]ppG and NaF, respectively. However, total radioactivity of AAGTP bound in (40 kDa and 42 kDa) proteins was essentially constant at various concentrations of p[NH]ppG or NaF used in this experiment. It should be emphasized that after incubation of membranes with [^{32}P]AAGTP, the membranes were washed with buffer to remove unbound [^{32}P]AAGTP prior to exposure to p[NH]ppG or NaF. Only after these treatments was AAGTP binding made covalent by UV

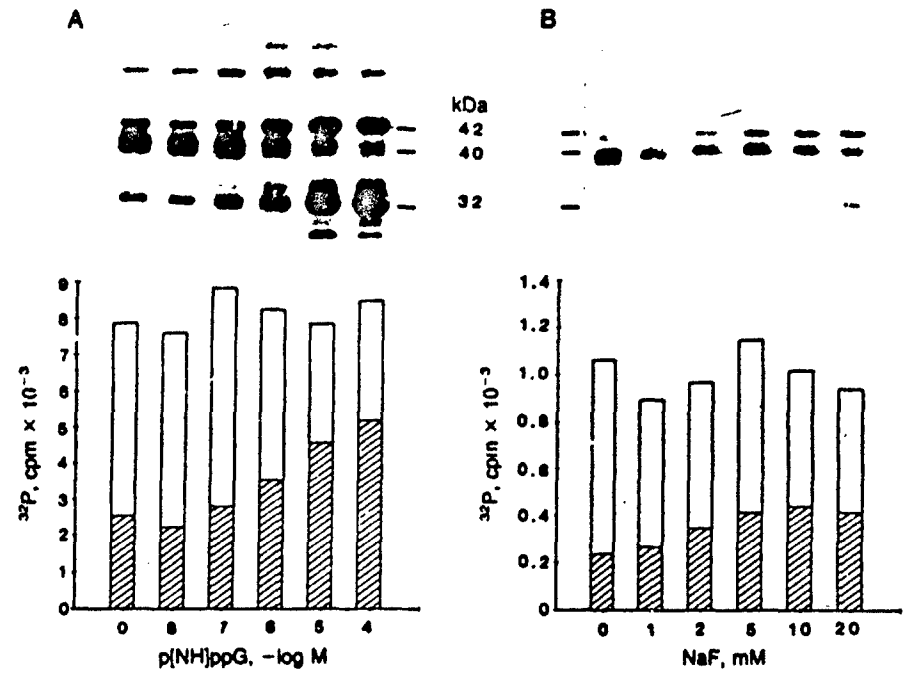

FIG. 2. NaDodSO$_4$/PAGE analysis of AAGTP-labeled synaptic membranes. Synaptic membranes were incubated with 0.12 μM [^{32}P]AAGTP for 3 min at 23°C, and the incubation was terminated by dilution and centrifugation. The washed membranes were then incubated with various concentrations of p[NH]ppG (*A*) or NaF (*B*) for 10 min, followed by 20 min of UV photolysis on ice. The membranes were then washed and submitted to NaDodSO$_4$/PAGE and autoradiographed (*Upper*). Individual radiolabeled bands of the dried gels were cut and counted. (*Lower*) Amount of radioactivity in the 40-kDa (□) and 42-kDa bands (■).

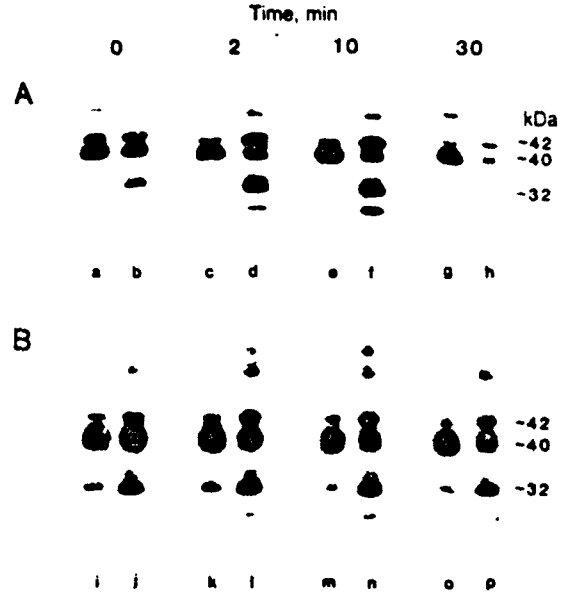

FIG. 3. Time course of the shift in AAGTP labeling from GN_i to GN_s. Synaptic membranes were incubated with [^{32}P]AAGTP as in Fig. 2, washed, and subsequently incubated without or with 5 μM p[NH]ppG (*A*) or 5 mM NaF (*B*). At the indicated times, 50-μl aliquots were withdrawn and photolyzed for 20 min on ice. The irradiated samples then were subjected to $NaDodSO_4$/PAGE and autoradiographed. Lanes: a, c, e, and g, no p[NH]ppG; b, d, f, and h, p[NH]ppG; i, k, m, and o, no NaF; j, l, n, and p, NaF.

exposure; therefore, p[NH]ppG- and NaF-induced changes in AAGTP labeling in 40-kDa and 42-kDa proteins apparently result from a shift of the AAGTP bound from the 40-kDa protein (GN_i) to the 42-kDa protein (GN_s). The magnitude of this shift in AAGTP labeling from GN_i to GN_s increased in proportion to the concentration of p[NH]ppG or NaF. GTP[γS] or unlabeled AAGTP in the second incubation resulted in a similar shift (as p[NH]ppG in Fig. 1*A*) in adenylate cyclase activity from inhibition to activation as well as a similar shift in AAGTP labeling from the 40-kDa protein to the 42-kDa protein. Similar results (although with much less radioactivity) were obtained when 120 μM [^{32}P]AAGTP was used in the initial labeling step.

Time Course of the Shift in AAGTP Labeling from GN_i to GN_s. Synaptic membranes were incubated with [^{32}P]AAGTP, washed, and subsequently incubated in the presence of p[NH]ppG or NaF for periods of 0–30 min. After UV photolysis, AAGTP labeling was estimated by $NaDodSO_4$/PAGE and autoradiography. As shown in Fig. 3, the shift in AAGTP labeling from the 40-kDa protein to the 42-kDa protein by p[NH]ppG or NaF was relatively rapid; it was completed within 2 min after the start of incubation of membranes with the compounds, and it was stable for 10 min. p[NH]ppG-mediated reversal of AAGTP-induced adenylate cyclase inhibition (as in Fig. 1) follows a similar time course. At 30 min of incubation with p[NH]ppG, decline in AAGTP labeling was observed at all bands (Fig. 3*A*, lane h). The extent of reduction in bound AAGTP was greatest at the 32-kDa band, followed by the 42-kDa band and the 40-kDa band. This reduction of AAGTP labeling probably results from a slow competition of p[NH]ppG with [^{32}P]AAGTP for binding sites during the incubation, since decrease in AAGTP labeling was not observed in membranes incubated without p[NH]ppG (Fig. 3*A*, lane g and Fig. 3*B*, lane o) or in the membranes incubated with NaF (Fig. 3*B*, lane p), which does not compete with AAGTP.

Stable Binding of AAGTP to GN_s. Synaptic membranes were exposed to [^{32}P]AAGTP, washed, and exposed to p[NH]ppG or NaF as above. Subsequent to a 10-min incubation, the membranes were washed by dilution and centrifugation prior to resuspension in buffer and UV photolysis. A shift of bound AAGTP from GN_i to GN_s was observed similar to that noted in Fig. 2. This suggests that the AAGTP bound to GN_s was basically stable to washing. However, AAGTP was released from the 32-kDa protein (see Fig. 4) by this treatment.

DISCUSSION

The hydrolysis-resistant GTP analog AAGTP causes an inhibition of synaptic membrane adenylate cyclase that persists after washing of the membranes. Other hydrolysis-resistant guanine nucleotides have been reported to induce a similar phenomenon in membranes from both rat brain (21) and human platelet (6). AAGTP is also a photoaffinity probe; and when adenylate cyclase is inhibited, most of the bound AAGTP is on GN_i. Under conditions where this inhibition is reversed, such as subsequent incubation with p[NH]ppG or NaF, we have observed an apparent displacement of the label from GN_i to GN_s, the sum of AAGTP bound to GN_i and GN_s remaining constant (Fig. 2). Because GN_i binds AAGTP under conditions where GN_s does not (the initial labeling conditions), conditions favoring the activation of adenylate cyclase prompt the removal of AAGTP from GN_i and the concomitant binding of that nucleotide to GN_s. The apparent transfer of AAGTP from GN_i to GN_s persists subsequent to repeated washing of membranes (Fig. 4). The comparable ability of NaF and p[NH]ppG to induce the apparent translocation of AAGTP from GN_i to GN_s renders it likely that the major factor in causing this switch is the ability of a compound to activate adenylate cyclase rather than to compete for a site on the GN protein. NaF does not compete with AAGTP for binding sites (Fig. 3), even though it both activates adenylate cyclase and stabilizes GN_s and GN_i during purification (3). Furthermore, NaF causes the photoreceptor GTP binding protein to assume a conformation similar to that caused by hydrolysis-resistant guanine nucleotides (22). It is possible that a similar "active" conformation of GN_s is induced by NaF.

Although NaF does not compete and p[NH]ppG competes slowly (Fig. 3) with AAGTP for the observed binding sites, it is likely that p[NH]ppG binds to GNs during the observed

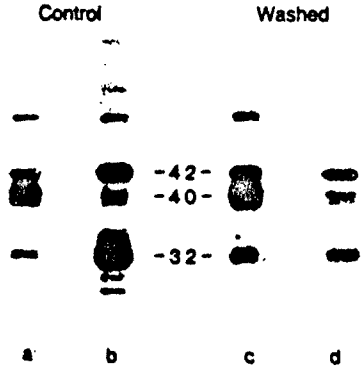

FIG. 4. AAGTP labeling of membranes washed before UV photolysis. Synaptic membranes were incubated with [^{32}P]AAGTP as in Fig. 2, washed, and subsequently incubated with or without 100 μM p[NH]ppG for 10 min. Prior to being subjected to a 20-min UV photolysis on ice, the membranes were washed by dilution and centrifugation with 2 mM Hepes, pH 7.4/1 mM $MgCl_2$ and were resuspended in the same buffer. Control membranes were immediately subjected to UV photolysis without washing with the buffer. AAGTP labeling of membranes was analyzed by $NaDodSO_4$/PAGE and autoradiography. Lanes: a and c, no p[NH]ppG; b and d, p[NH]ppG.

shift (Fig. 1) in adenylate cyclase activity. AAGTP binding to GN_s also increases under these conditions (Fig. 2). One explanation for this apparent discrepancy is the observed existence of two guanine nucleotide binding sites on GN_s (23). It is possible that these sites differ in their affinity for p[NH]ppG and AAGTP.

The shift of AAGTP from GN_i to GN_s is relatively rapid; it appears to be completed within 2 min after the addition of nucleotide or NaF (Fig. 3). p[NH]ppG (10 μM) will compete for bound AAGTP from GN_s, GN_i, and the 32-kDa GTP binding protein, but this process requires ≈30 min. During this period a small portion (≈15%) of the AAGTP is released from the control membranes (Fig. 3), but the binding of AAGTP is relatively stable for at least 60 min under these conditions. Similarly, the tight binding of AAGTP to GN_i and GN_s is revealed by the inability of a second buffer wash (prior to UV irradiation but subsequent to p[NH]ppG or NaF incubation) to release AAGTP from either protein (Fig. 4).

It is possible that certain unique features of the neural adenylate cyclase systems contribute to the phenomena observed here. Neural GN_i has been purified, and it appears to consist of multiple subunits. The larger subunit (40 kDa) is a better substrate for ADP-ribosylation by pertussis toxin, and the smaller (39 kDa) subunit has a more efficient GTPase activity (1). The two GN_i proteins also differ in antigenic properties (24, 25) and, although the 40-kDa protein appears to be the inhibitory GTP-binding protein that functions in the adenylate cyclase system, it is not clear if the 39-kDa GN_i functions in the adenylate cyclase and/or some other system. Both proteins appear to bind AAGTP with equal affinity, and they transfer AAGTP to GN_s to a similar degree. The coupling between the neurotransmitter receptor and the GN proteins in neural membranes is diminished or lost upon preparation of subcellular fractions. Thus, complete stimulation or inhibition of adenylate cyclase in the absence of hormone can be accomplished by simple addition of hydrolysis-resistant GTP analogs to those membranes. This bypass of the hormone receptor allows examination of GN–catalytic moiety interaction or possibly GN_s–GN_i interaction.

During the course of this study we have demonstrated the presence of a 32-kDa GTP binding protein. This protein is not a substrate for ADP-ribosylation by pertussis or cholera toxin, and it binds AAGTP loosely yet specifically. We have demonstrated previously (14) using a different AAGTP labeling protocol that AAGTP may bind to the β subunit of GN_s and GN_i. Under the methods used in this study, AAGTP labels β subunit only weakly, and this 32-kDa protein is distinct from the β subunit as revealed by nitrocellulose blotting experiments (H. Hamm and M.M.R., unpublished observation). A protein of 32 kDa has copurified with the GN_i proteins of bovine cerebral cortex (1), but it is not clear whether this protein is identical with the one we have observed. It does not appear that the 32-kDa AAGTP-binding protein is a proteolytic fragment of GN_i or GN_s, as V8 protease studies yield different fragments for these species (not shown). We have observed this protein in brain and platelet, but not liver or kidney membranes, and it is tempting to speculate that it may be involved in some GTP-regulated process that is unaffected by cholera or pertussis toxin.

Although no other evidence exists for the possibility of direct interaction and exchange of nucleotide between GN_s and GN_i, the data herein appear consistent with such a phenomenon. Physical interaction between some members of the adenylate system has been demonstrated (26, 27), and it is possible that GN_s and GN_i might interact directly. One caveat to this interpretation is the possibility that the AAGTP appearing on GN_s is a result of a "nearest neighbor" association of the nitrene free radical at the terminal phosphate of AAGTP with GN_s, while the AAGTP purine ring remains bound to GN_i. Such interaction might increase when GN_s and GN_i are in greater proximity, as during the crossover from inhibition to stimulation of adenylate cyclase (as in Fig. 1). Such adventitious association of AAGTP seems unlikely because the p[NH]ppG- or NaF-mediated adenylate cyclase activity is altered under these conditions. Another possibility that cannot be discounted is that, during the crossover between inhibition and stimulation of adenylate cyclase, the conformation of GN_s and GN_i is altered so that the efficiency of AAGTP photoinsertion is decreased in GN_i and concomitantly increased in GN_s.

We have demonstrated previously that a soluble GN_s can be released from the synaptic membrane (14, 16), and such appears likely for GN_i as well (1). We have also suggested that, in the synaptic membrane system, cytoskeletal components might regulate adenylate cyclase by reversible association with GN_s (10, 14, 16). Perhaps these elements also contribute to GN_s–GN_i interaction. The possible existence of a GTP exchange process between GN_s and GN_i represents a novel hypothetical switch between the stimulatory and inhibitory regulation of adenylate cyclase, which might function at an intracellular level. Further understanding of this phenomenon awaits further experimentation.

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GTP-BINDING PROTEINS WHICH REGULATE NEURONAL ADENYLATE CYCLASE INTERACT WITH MICROTUBULE PROTEINS

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INTRODUCTION

Adenylate cyclase is a hormone and neurotransmitter-sensitive enzyme associated with the cellular plasma membrane. Receptors for hormones (or neurotransmitters) which stimulate or inhibit adenylate cyclase are linked to the catalytic moiety of the the enzyme through two distinct GTP-binding proteins, which provide for the regulation of adenylate cyclase enzyme activity (Fig.5). There is evidence that physical association of these components is required for hormone or guanine-nucleotide mediated activation of the enzyme (Rodbell, 1980) and agents which increase membrane fluidity and/or membrane protein mobility augment adenylate cyclase activation (Orly and Schramm, 1977).

Agents which disrupt microtubules have also been shown to increase adenylate cyclase activity in intact leukocytes (Rudolph et. al., 1979, Kennedy and Insel, 1979) and neuronal membranes (Rasenick et. al., 1981). Augmentation of adenylate cyclase (or, in the case of intact cells, cAMP accumulation), whether by microtubule disrupting drugs or membrane fluidizers, increased the velocity of adenylate cyclase without altering sensitivity of the drug or neurotransmitter used as an activator for that enzyme. Furthermore, agents which interact with the cytoskeleton have been implicated in the regulation of receptor-mediated desensitization of adenylate cyclase in a variety of cell and tissue types (See Zor, 1983 for review).

The data presented in this paper indicate that microtubule disrupting drugs are capable of enhancing the neuronal adenylate cyclase system by enhancing coupling between the GTP-binding protein which stimulates adenylate cyclase (GNs) and the catalytic moiety of that enzyme. Some interaction between synaptic membrane tubulin and GNs appears likely and recent evidence concerning structural (Gilman, 1984) and functional (Bitensky et. al., 1982) homology among GTP-binding proteins is consistent with this. Cytoskeletal proteins may thus play a role in the regulation of neurotransmitter responsiveness through intracellular modification of adenylate cyclase activity.

EFFECTS OF MICROTUBULE-DISRUPTING DRUGS UPON SYNAPTIC MEMBRANE ADENYLATE CYCLASE.

Considerable evidence from a number of laboratories (See Zor, 1983 for review) suggests that incubation of cells with microbule-disrupting drugs enhances hormone and GNs-mediated activation of adenylate cyclase. Although in most cell types, effects of colchicine or vinblastine upon the cAMP generating system are limited to intact cells, membranes prepared from neuronal cells consistently display augmentation of adenylate cyclase activity after exposure to these agents. The effects of these drugs (See Fig. 1) are apparently mediated through GNs and the augmentation of adenylate cycase produced is not increased further by membrane fluidizing agents (Rasenick, et. al., 1981). This lack of additivity between cis unsaturated fatty acids and microtubule disrupting drugs in the augmentation of adenylate cyclase activity is noteworthy because both classes of compound have been implicated in promoting increased lateral mobility of proteins within limited domains of the plasma membrane (Klausner et. al., 1980). Such a phenomenon is consistent with the observed increase in adenyate cyclase activity which might be attributed to an increase in "coupling" between the adenylate cyclase catalytic moiety and GNs.

Activation of adenylate cyclase by guanyl nucleotides is not reversed after washing the membranes with buffer (See Rodbell, 1980) however, washing colchicine-treated synaptic membranes resulted in a loss of adenylate cyclase activity which has been attributed to a release of GNs from the plasma membrane (vide infra; Rasenick et. al., 1984). Such a release of GTP-binding regulatory protein from the membrane has been previously observed in the analogous rod outer segment phosphodiesterase system and has served as an indication of enhanced coupling of the photoreceptor GTP-binding protein (transducin) with the phosphodiesterase catalytic moiety (Stein et. al, 1982).

PHOTO-AFFINITY LABELLING STUDIES AND COLCHICINE OR VINBLASTINE-MEDIATED RELEASE OF GNS FROM THE SYNAPTIC MEMBRANE

Further investigation of the apparent colchicine or vinblastine-medicated release of GNs from the plasma membrane required direct identification of the GTP-binding proteins. P^3-4 azidoanilido)-P^1 GTP(AAGTP) is an hydrolysis resistant photoaffinity GTP analog (Pfeuffer, 1977) which labels covalently, proteins of 53, 42, 40 and 32 KDa on synaptic membranes (Fig. 2). [Other proteins of 110, 94 and 36 kDa can be labelled as well, but this is dependent upon AAGTP concentration, labelling temperature, and Mg++ ion concentration; Rasenick, Hatta and Marcus, Manuscript in preparation.]

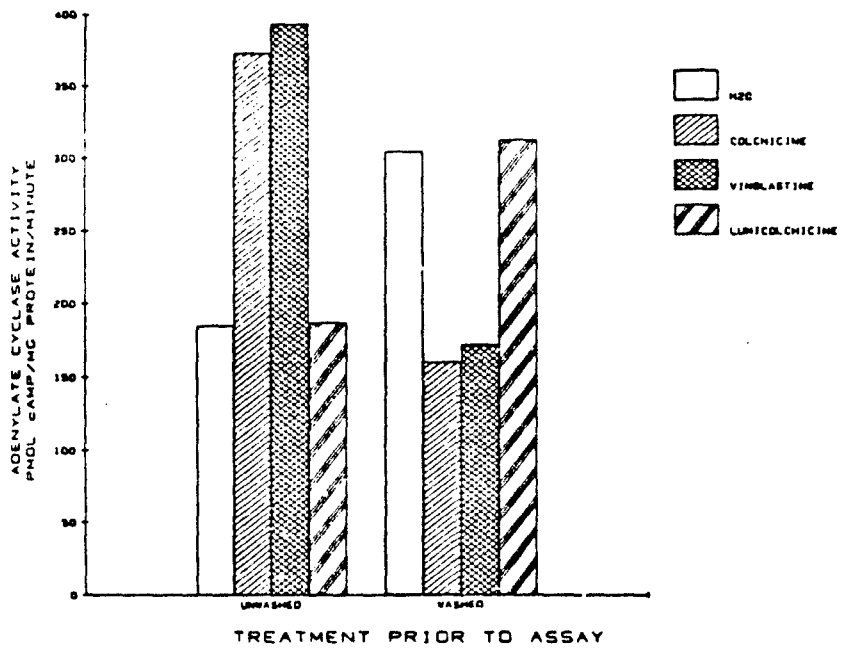


Figure 1: Effects of Microtubule-Disrupting Drugs on Neuronal Adenylate Cyclase

A. UNWASHED. Synaptic membrane enriched fractions from 21-day old male sprauge-dawley rat cerebral cortices were prepared as described previously (Rasenick and Bitensky, 1980) and 50 ug aliquots were incubated with 1 uM colchicine, vinblasine, H^2O or 10 uM lumicolchicine at 30°C for 20 minutes. Following this, membranes were incubated for 20 minutes at 30°C with 2 uM GppNHp (guanylylimidodiphosphate) and assayed (as described; Rasenick et. al. 1981) for adenylate cyclase activity.

B. WASHED. Membranes were treated as above except that following incubation with colchincine, vinblastine, lumicolchicine or H^2O, membranes were washed twice with buffer. Also, GppNHp and ATP were added simultaneously in the 10 minute assay step, eliminating the second 20 minute preincubation (Data and experimental detail from Rasenick et. al., 1981). Neither colchicine or vinblastine altered adenylate cyclase activation by $MnSO^4$, indicating that these agents did not alter the catalytic capacity of the enzyme.

If AAGTP labelled membranes are treated with colchicine and washed the 42 KDa protein (GNs) is preferentially released from the plasma membrane into the supernantant (Rasenick et. al., 1984) Fig. 2). The release of GNs from the membrane coincides with the loss of GNs activity noted above and raises the possibility that GNs (or a subpopulation thereof) is associated with the cytoskeleton. Likewise, fibronectin, which is released from the membrane after treatment with cytochalasin is thought to be linked to the plasma membrane through actin (Ali and Hynes, 1977).

ASSOCIATION BETWEEN TUBULIN AND GNS

The above experiments raise the likelihood that tubulin (or some microtubule associated component) interacts directly with GNs, as both colchicine and vinblastine provoke a similar augmentation of GNs-mediated adenylate cyclase activity. To test this possibility directly, the material released from synaptic membranes by colchicine (with ^{3}H or cold AAGTP covalently bound) was passed over a tubulin affinity column and eluted with NaCl-containing buffer. Most of the material applied to the column did not stick, however all of the GNs activity remained bound and was eluted, along with several other proteins, at .1 M NaCl (Fig. 3).

It is tempting to conclude from this experiment that GNs can complex reversibly with tubulin. However, the presence of microtubule associated proteins (MAPS) both on the column, as well as in the sample applied to the column, cannot be discounted: either with regard to direct binding of GNs or to effects upon tubulin GNs interaction (See below).

Figure 2: AAGTP Photoaffinity Labelling of Synaptic Membranes

Synaptic membranes were incubated with 1 uM [^{3}H] AAGTP at 30°C for 20 minutes followed by 20 minutes of UV photolysis on ice. The reaction was quenched with 4mM DTT and the membranes then incubated at 30°C for 10 minutes with 5 uM of either colchicine (lanes c and e) or lumicolchicine (lanes b and d). Following this the membranes were washed three times with a low ionic strength buffer (2 mM HEPES, pH 7.4, 1mM MgCl, 2mM DTT) and the proteins released from the membranes were electorphoresed on 10% polyacrylamide gels and radioautographed. Lanes d and e represent Coomassie blue straining patterns and lanes b and c are radiofluorographs. Lane a represents a typical ^{32}p AAGTP labelling pattern on synaptic membranes.
^{3}H DPM in the 42 KDa bands is 4343 (b) and 7445 (c).

INHIBITION OF GNS ACTIVATED ADENYLATE CYCLASE BY TUBULIN

Conclusions from the above experiments indicate that colchicine or vinlastine treatment increase adenylate cyclase activity by mitigating a putative tubulin-GNs interation. It follows from this that the addition of tubulin to synaptic membranes under conditions where tubulin might closely associate with (or incorporate into) those membranes, should diminish activation of adenylate cyclase through GNs.

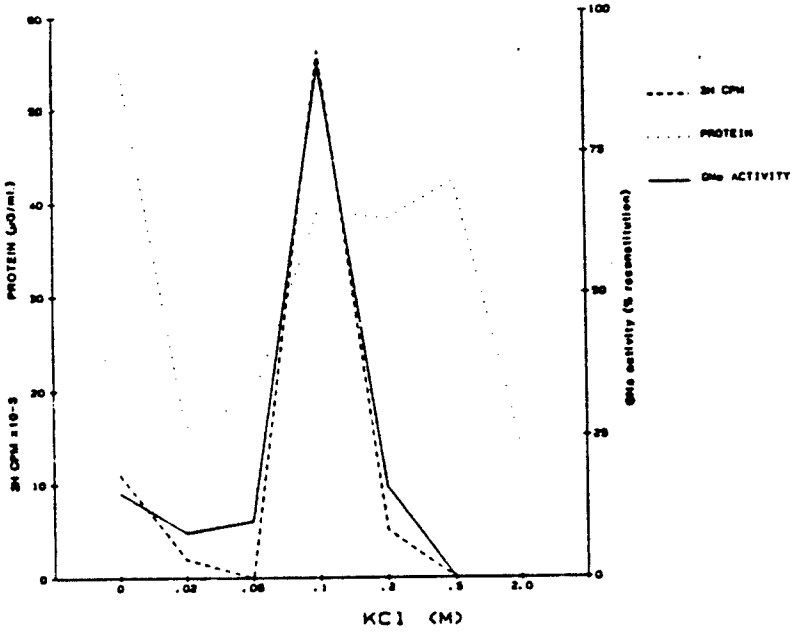


Figure 3: Elution of GNs From Sepharose-Tubulin

Rat brain tubulin (2 cycle Polmerized/depolymerized; Shelansky et. al., 1973) was coupled to CNBR-activated sepharose 4B (Pharmacia) and the column equilibrated in 20 mM Pipes pH 6.9 containing 5 mM MgCl, 2 mM DTT, .3 mM PMSF, .5 mM EGTA and 15% glycerol. Colchicine derived supernatants from synaptic membranes were dialysed free of colchicine and unbound nucleotide (as per Rasenick et. al., 1981) applied to the column and allowed to sit overnight. Fractions were eluted at the indicated KCl concentrations, TCA percipitable radioactivity and protein content were determined and then the fractions were dialysed, concentrated and tested for GNs activity by reconstitution (Rasenick et. al., 1984).

Tubulin added to membranes is capable of inhibiting GNs-mediated activation of adenylate cyclase. Phosphocellulose-purified tubulin (kindly provided by Dr. R. DeLorenzo) saturates at 40 nM tubulin and has an "IC_{50}" of 10 nM (Rasenick, in press). This inhibition is apparent when adenylate cyclase is activated by Gpp (NH)p or NaF (not shown) and occurs whether or not

Table 1: Effects of Tubulin Upon Synaptic Membrane Adenylate Cyclase

	Addition to Membranes: Control	Tubulin	
	*Adenylate Cyclase Activity		%Inhibition
Membrane Pretreatment:			
A. H^2O	100.6	63.6	37
B. Gpp (NH)p	174.2	113.7	35
C. Gpp (NH)p + Tubulin & Washed	485.3	368.1	24

*pmol cAMP/mg. Protein/min.

Synaptic membranes were prepared as described (Rasenick et. al., 1981) and after exposure to 10^{-4M} GppNHp (or H^2O) and subsequent wash were resuspended in 100 mM Pipes pH 6.9, 2 mM MgCl, 0.3 mM PMSF, 1 mM DTT, 1mM EGTA, cis vaccenic acid (10 ug/ml.), phosphatidyl choline (1 mg/ml.), brij 35 (.06%) and tubulin (Final concentration = 250 nM). After incubation at 37°C for 15 minutes, the membranes were washed or placed on ice (as indicated). Gpp (NH)p was added (100 uM) along with ATP and adenylate cyclase was assayed for 10 minutes at 30°C by the method of Salomon et. al., (1974).

adenylate cyclase is preactivated by nucleotide. This would support the likelihood that tubulin is not exerting these effects as a GTP sink (a remote possibility in that guanine nucleotide is present at 1000 fold excess over tubulin). Furthermore, the tubulin added in these experiments is apparently capable of forming a tight association with the plasma membrane, as buffer washing is not effective in reversing the effects of added tubulin.

ROLE OF MICROTUBULE ASSOCIATED PROTEINS:

Despite the apparent ability of added tubulin (including phosphocellulose purified tubulin) to inhibit GNs-mediated adenylate cyclase activity, the contribution of microtubule associated proteins to this process cannot be discounted. MAP's are associated with the synaptic membranes and the addition of tubulin (even if MAP-free) could be attributed to some primary interaction between added tubulin and membrane associated MAP's, followed by an interaction of that complex with GNs.

INTERACTION OF TUBULIN WITH THE INHIBITORY GTP-BINDING PROTEIN

Adenylate cyclase is stimulated by one GTP-binding protein and inhibited

by another (GNi). The above text has attempted to establish an interaction between GNs and tubulin and has not mentioned GNi. In synaptic membranes, Gpp (NH)p can elicit inhibition directly through GNi. Vinblastine does not alter adenylate cyclase inhibition under these conditions (Fig. 4). Colchicine treatment gives similar results (not shown) and, although these compounds augment adenylate cyclase stimulation through GNs, they do not appear to affect adenylate cyclase inhibition through GNi.

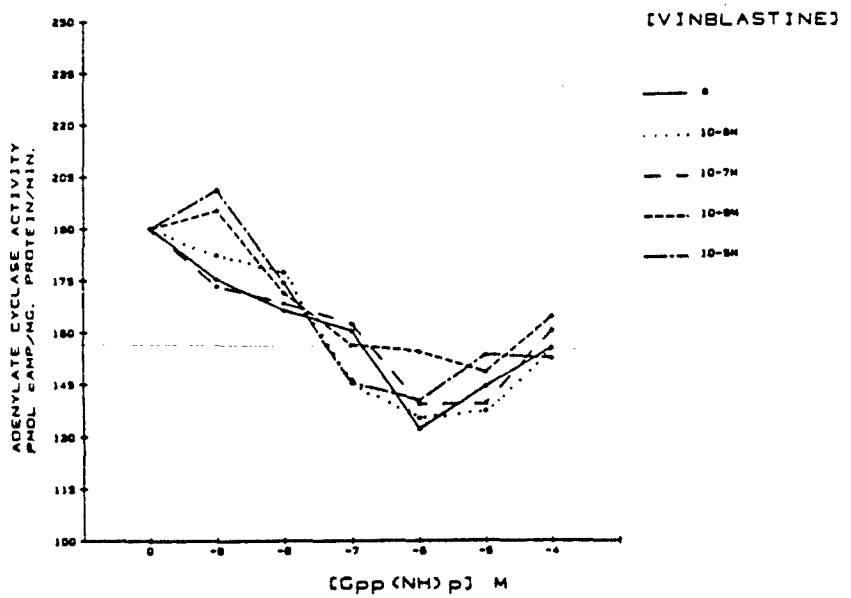


Figure 4: Innibition of Rat Cerebral Cortex Adenylate Cyclase; Effects of Vinblastine

Cerebral cortex membranes prepared as described (Rasenick et. al., 1981) were incubated in 20 mM Hepes pH 7.4 containing 1 mM MgCl, 1 mM DTT, 0.3 mM PMSF and the indicated vinblastine concentration for 10 minutes at 30°C. Following this, Gpp (NH)p was added as indicated along with 25 uM ATP and the membranes were assayed for cAMP for 15 mirutes at 23°C by the method of Salomon (1979). Neurotransmitters which promote elevation of intracellular calcium (e.g. acetylcholine) might modify the respouse of adenylate cyclase-linked neurotransmitters by such a mechanism.

STRUCTURAL AND FUNCTIONAL HOMOLOGY BETWEEN TUBULIN AND GNs

Considerable homology exists among GTP several binding proteins (e.g. ras p21, EFTu, GNs, GNi, transducin, tubulin; see Gilman, 1984; Halliday, 1984). These proteins share a common glycine rich sequence which is the likely GTP binding site, as well as other regions of apparent homology. There also appears to be functional homology among these proteins as transducin and GNs (Bitensky et. al., 1984) or GNi (Cerione et. al., 1985) appear interchang-

able. Although tubulin has not been shown to be interchangable with GNs, it does have GTPase activity, as do GNs or GNi. Cholera toxin catalyzes ADP-ribosylation of GNs and pertussis toxin catalyzes ADP ribosylation of GNi (See Codina et. al., 1984). Recently, tubulin has been reported to be ADP ribosylated by both cholera and pertussis toxins (Lim et. al., 1985).

POSSIBLE MECHANISM OF TUBULIN-MEDIATED REGULATION OF ADENYLATE CYCLASE

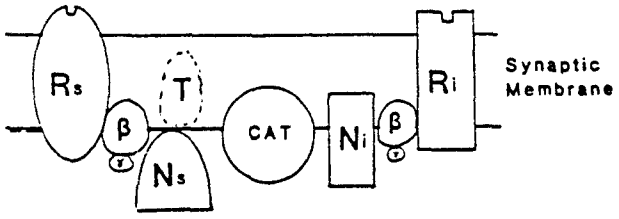
A possible scheme for the regulation of neuronal adenylate cyclase is illustrated in Figure 5. Normally, adenylate cyclase activation proceeds after the binding of a stimulatory agonist to its receptor and the subsequent association of GTP with GNs. The "charged" GNs then activates the catalytic moiety of the enzyme. A similar mechanism may exist for the inhibition of adenylate cyclase, although in that case, an inhibitory agonist would begin the cascade.

Colchicine or vinblastine, which bind to different sites on the tubulin molecule, both increase adenylate cyclase activity, and that increase appears localized to the GNs protein. A possible mechanism for this effect involves a steady-state constraint of GNs/catalytic moiety interaction due to the binding of GNs by tubulin, as might be hypothesized from the results of the experiment in Figure 3. Mitigation of this interaction by colchicine or vinblastine would result in the observed increase in enzyme activity. Increased tubulin association with the membrane might increase constraint upon GNs catalytic moiety interaction, and this has been observed (Table 1). It is noteworthy that, despite the indication of tubulin as an integral synaptic membrane protein (which has been reported, Bhattacharyya and Wolff, 1976; Zisapel et. al., 1980) the proposed tubulin/GNs interaction could occur equally well if both tubulin and GNs are considered membrane associated proteins.

There is no indication that tubulin substitutes for GNs or GNi, despite the apparent similarities among these proteins. This molecular similarity might be consistent, however, with an association/dissociation between tubulin and GNs. In fact, an association between microtubules and adenylate cyclase has been reported (Margolis and Wilson, 1980).

The β and γ subunits are also involved in the regulation of adenylate cyclase activity and may inhibit GNs or GNi interaction with the catalytic moiety of adenylate cycase. Another possible explanation for tubulin effects might be a tubulin association with $\beta\gamma$ (due to a tubulin/GNs or tubulin GNi homology) and subsequent promotion of adenylate cyclase inhibition. The lack of direct effects of microtubule-disrupting drugs upon adenylate cyclase inhibition makes this unlikely, however.

Control: (H_2O or Lumicolchicine)



+ Colchicine or Vinblastine

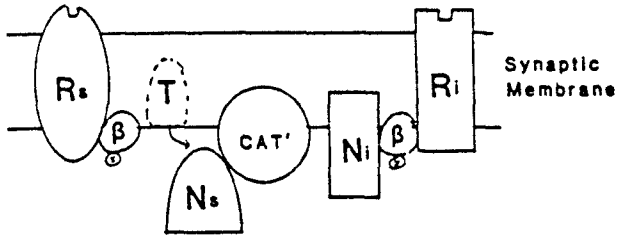


Figure 5: Interaction of Tubulin with Adenylate Cyclase

The above represents a possible format for adenylate cyclase activation or inhibition, and the effects of microtubule disrupting drugs upon that process. Rs and Ri represent receptors for stimulatory (s) and inhibitory (i) hormones or neurotransmitters and GNs or GNi represent the GTP-binding regulatory proteins linked to those receptors. β (35KDa) and γ (14 KDa) are regulatory proteins which are associated with the GN complex and the catalytic moiety (CAT) produces cAMP and ATP. In this cartoon, tubulin is associated with GNs and colchine or vinblastine dissociate these proteins, resulting in augmented activation of adenylate cyclase by GNs.

PHYSIOLOGICAL CONSIDERATIONS:

These data indicate that tubulin may be involved in the regulation of synaptic membrane adenylate cyclase. Although microtubule disrupting drugs augment cAMP accumulation in a variety of leukocytes, these drugs increase membrane adenylate cyclase only in neural tissue. One factor setting neural adenylate cyclase appart from that in other tissues is stimulation by calmodulin (Treisman et. al., 1983). Only 50 percent of synaptic membrane adenylate cyclase is regulated by calmodulin (Brostrom et. al., 1978) and a similar portion of GNs activity is released from the synaptic membrane subsequent to colchicine or vinblastine treatment (Rasenick et. al., 1984). Calcium/-calmodulin protein kinases have been reported to phosphorylate both tubulin and MAPs (See Nestler, and Greengard, 1984). It is possible that such a phosphorylation might alter the interaction between tubulin and GNs, thus affecting adenylate cyclase activity. We are continuing to investigate the apparent regulation of the functional components of neurotransmitter responsiveness by the structural components of the cell membrane.

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Guanine nucleotide activation of adenylate cyclase in saponin permeabilized glioma cells

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We have compared the regulation of adenylate cyclase activity in membrane fractions from C6 glioma cells and in monolayer cultures of C6 cells that had been permeabilized with saponin. Guanine nucleotides (GTP and GTPγS) and isoproterenol increase adenylate cyclase activity in C6 membranes and in permeabilized C6 cells. In C6 membranes, guanine nucleotides activate adenylate cyclase in the presence or absence of isoproterenol; in permeabilized cells, however, guanine nucleotides increase adenylate cyclase activity only in the presence of isoproterenol. We suggest that the properties of the permeabilized cells more closely resemble those of intact cells, and that some component which is present in permeabilized cells but is lost following cell disruption may be important for the normal regulation of adenylate cyclase activity.

GTP-binding protein *β-Adrenergic receptor* *Receptor-effector coupling* *Signal transduction*

1. INTRODUCTION

Determination of the mechanism of adenylate cyclase activation has been a subject of widespread interest. Investigations into that mechanism have encompassed kinetic studies, studies with bacterial toxins, identification and purification of the proteins involved, the reconstitution of those proteins and, now, most recently, the genetics of the adenylate cyclase system. The proteins that have been investigated include receptors for hormones (or neurotransmitters) which stimulate or inhibit adenylate cyclase, the members of the GTP-binding protein cascade which regulate that enzyme and the adenylate cyclase catalytic moiety. Whereas a great deal has been learned about the regulation of the adenylate cyclase system through studies with purified adenylate cyclase components [1] several cellular elements associated with membranes and the cytoskeleton have been observed to regulate adenylate cyclase, especially in neural cells [2,3]. The participation of various other cellular elements in the regulation of adenylate cyclase raised the possibility that this enzyme behaves differently in isolated membranes than in intact cells.

2. MATERIALS AND METHODS

We have developed an assay for adenylate cyclase in saponin-permeabilized C6 cells. This treatment makes holes of 0.1–1 μm in plasma membranes while retaining cell viability [4]. Saponin treatment allows for the free passage of [^{32}P]ATP into cells while attached to wells in monolayers, and, as such, provides for the assay of adenylate cyclase in a nearly intact cell preparation.

C62B cells were maintained in Dulbecco's MEM 4.5 g glucose/l, 10% fetal bovine serum, in a 10% CO_2 atmosphere. Cells were subcultured weekly into 24 well sterile plates. Permeabilization by saponin treatment was achieved by a modification of previously published methods [4]. C6 monolayers (approx. 250000 cells/well) were washed three times with 200 μl complete Locke's solution (154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 10 mM glucose, 2 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) for 5 min at 37°C. 200 μl saponin solution (140 mM potassium glutamate [KG], pH 6.8, 2 mM ATP, saponin [100 μg/ml]) was added for 120 s at room

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temperature. The plates were inverted, saponin solution drained and monolayers washed three times with 200 μl KG buffer. The total time for permeabilization and washing was 5 min. 150 μl [^{32}P]ATP (to give 2 × 10^6 cpm), 0.5 mM ATP, 1 mM $MgCl_2$, 0.5 mM IBMX in Hanks buffer was added to each well and incubated for 3 min at room temperature. Isoproterenol and/or GTPγS was added to each well and incubated 10 min at 37°C. Reactions were stopped with 300 μl ice-cold 15 mM Hepes buffer, pH 7.4, and the entire plate was placed on dry ice for 5 min. Plates were removed from dry ice and allowed to thaw. Cells were then scraped into 1.5 ml microfuge tubes and wells were rinsed with 100 μl Hepes buffer. Tubes were boiled for 8 min in a heat block and then centrifuged at 4°C for 8 min at 15000 × g. Supernatants were then removed and transferred into 12 × 75 borosilicate glass tubes; 100 μl stop solution (2% sodium lauryl sulfate, 45 mM ATP, 1.3 mM 3',5'-cyclic AMP), 50 μl [^{3}H]cAMP (0.02 μCi), and 1 ml dH_2O was added to tubes and supernatants decanted over Dowex columns. [^{32}P]cAMP was isolated and measured by a modification [5] of the method of Salomon [6].

C6 cells were grown to near confluency in 175 cm flasks and harvested by scraping with a rubber policeman. Cells were collected in 15 mM Hepes, 0.25 M sucrose, 0.3 mM PMSF and 1 mM DTT, pH 7.5, and homogenized (8 strokes) in a teflon-glass homogenizer. 600 × g supernatants were collected and centrifuged three times at 40000 × g in the homogenization buffer without sucrose. C6 membrane suspensions (10–20 μg protein/assay tube) were assayed for adenylate cyclase activity by the method of Salomon [6], modified as described [5]. The assay buffer included 15 mM Hepes, pH 7.5, 1 mM DTT, 0.3 mM PMSF, 5 mM $MgCl_2$, 50 μM ATP, and an ATP regenerating system.

3. RESULTS

3.1. *Assay of adenylate cyclase in permeabilized cells*

Addition of [^{32}P]ATP to saponin-treated C6 cells allows measurement of adenylate cyclase activity, rather than cAMP accumulation, in relatively intact cells. Saponin treatment results in 90–95% of the cells taking up trypan blue, and a similar number are likely to have access to the [^{32}P]ATP. Measurement of adenylate cyclase in these cells is dependent upon [^{32}P]ATP permeability induced by saponin, and no measurable

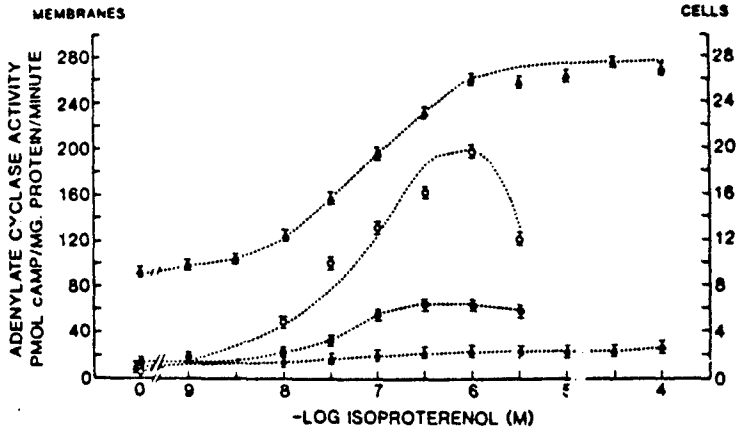

Fig.1. Activation of C6 adenylate cyclase by isoproterenol. C6 cells were permeabilized and assayed for adenylate cyclase as described in section 2. Values are expressed as pmol cAMP formed per mg of total cellular protein (measured by the method of Bradford [7]) at each concentration of isoproterenol in the presence (○---○) or absence (●---●) of GTPγS, and are means of triplicate determinations (± SE) from one of three similar experiments. C6 membranes were assayed for 10 min at 30°C in the presence (△---△) or absence (▲---▲) of 5 μM GTPγS. Each point indicates the mean of a triplicate determination (± SE) in one of 3 similar experiments. Note that specific activity in cells is for total cellular protein as opposed to membrane protein.

[^{32}P]cAMP is generated without saponin treatment. Thus, cellular adenylate cyclase has been assayed in a manner similar to membranes, but results of these assays show distinct differences between cells and membranes in the regulation of that enzyme.

3.2. *Activation of C6 adenylate cyclase by isoproterenol*

Isoproterenol activates adenylate cyclase in permeabilized C6 cells as well as in C6 membranes (fig.1). In the presence of 5 μM GTPγS, the EC_{50} of isoproterenol for activation of C6 membrane adenylate cyclase, derived from the mean of 3 triplicate determinations, is approx. 95 nM which is comparable to that (85 nM) observed in the permeabilized cells. Guanine nucleotides (GTPγS or GTP) do not alter the potency of isoproterenol in either cells or membranes. Activation of adenylate cyclase by isoproterenol is completely inhibited by a 10-fold excess of propranolol (see fig.2). In permeabilized cells, isoproterenol-stimulated adenylate cyclase in the absence of added guanine nucleotide (figs 1,2). In contrast, isoproterenol does not activate C6 membrane adenylate cyclase in the absence of added guanine nucleotide (figs 1,2).

3.3. *Activation of C6 adenylate cyclase by GTPγS*

In the absence of isoproterenol, GTPγS-activated adenylate cyclase in C6 membranes, as has been reported [8]. We demonstrate the potency of GTPγS in fig.2 and observe that the EC_{50} for GTPγS is about 140 nM (average of 4 sets of

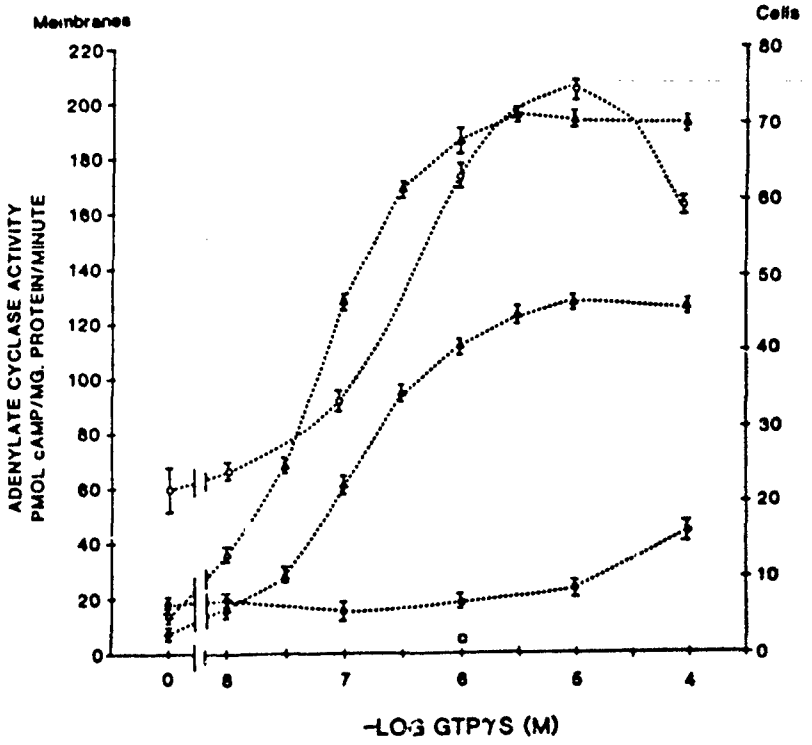

Fig.2. Guanine nucleotide activation of adenylate cyclase in C6 membranes and permeabilized cells. C6 membranes were incubated with (△) or without (▲) isoproterenol (1 μM) in the presence of indicated GTPγS concentrations. Permeabilized C6 cells were assayed for adenylate cyclase activity in the presence (○) or absence (●) of isoproterenol (1 μM) with the indicated concentration of GTPγS. 10 μM l-propranolol was included (□) along with isoproterenol (1 μM) and GTPγS (1 μM). Means of triplicate determinations (± SE) from one of 3 (cells) or 4 (membranes) similar experiments are depicted above.

triplicate determinations). GTP (not shown) is roughly one order of magnitude less potent (1.1 μM). Isoproterenol does not alter the potency of guanine nucleotides, but it does increase the V_{max} of adenylate cyclase activity.

In permeabilized C6 cells, however, GTPγS (fig.2) as well as GTP (not shown) do not increase adenylate cyclase activity (except at 10^{-4} M) in the absence of isoproterenol. In the presence of isoproterenol, GTPγS potentiates adenylate cyclase activity markedly. The potency of GTPγS, in the presence of isoproterenol is comparable ($EC_{50} \approx 150$ nM; mean of 3 sets of triplicate determinations) to that seen in C6 membranes.

3.4. *Direct effects of saponin under adenylate cyclase*

The possibility that the distinctions between permeabilized cell and C6 membrane adenylate cyclase were due to the treatment of cells with saponin required investigation. Saponin treatment of membranes, even at concentrations 10-fold higher than those used in permeabilizing cells, was without effect on adenylate cyclase (table 1). This lack of saponin effect was seen regardless of the presence or identity (GTPγS and/or isoproterenol) of the adenylate cyclase activator employed. Furthermore, when Hank's solution is substituted for the Hepes buffer normally used in assays of C6 membrane adenylate cyclase, the characteristics for the C6 membrane adenylate cyclase remained the same.

Table 1

Effects of saponin on C6 membrane adenylate cyclase

Activator	[Saponin] μg/ml		
	0	10	100
H_2O	9.52	9.39	9.19
Isoproterenol	19.3	19.8	19.8
GTPγS	83.7	83.6	86.6
Isoproterenol + GTPγS	196.9	195.7	196.2

Membranes were incubated with the above concentrations of saponin for 10 min at 30°C and returned to ice. Following this, membrane suspensions were added to tubes containing the adenylate cyclase reaction mixture (see section 2) and the indicated activatory (GTPγS, 5×10^{-7} M; isoproterenol, 10^{-6} M) and assayed for 10 min to determine adenylate cyclase activity (see section 2). The adenylate cyclase activity is expressed as pmol cAMP/mg protein per min. Means of triplicate determinations which vary by less than 7% (see figs 1 and 2) are presented here

4. DISCUSSION

Permeabilized cell techniques have been utilized extensively to investigate the phosphatidylinositol turnover system and the effects of guanine nucleotides on this [9] and other cellular processes [10]. Two previous methods have been reported for the assay of adenylate cyclase in permeabilized cells. One of these methods employed sonication [11] and the other used Lubrol PX or alamethecin [12] to permeabilize cells. Neither method was designed for monolayers of cells, however, and techniques for removing cells, such as trypsin [13] or EGTA [14], affect adenylate cyclase. Saponin treatment has been demonstrated to produce small 'holes' in the membranes of chromaffin cells without affecting the overall appearance or activity of those cells [4]. These results demonstrate that the adenylate cyclase system functions normally (perhaps more normally than in membranes) in saponin-permeabilized C6 cells.

Perhaps the most striking finding is that GTP and its analogs do nothing to activate adenylate cyclase in permeabilized C6 cells even though they activate that enzyme profoundly in C6 membranes. This phenomenon is consistent with the existence of an absolute requirement for β-receptor occupancy for the activation of the stimulatory GTP-binding protein [GN_s] as has been postulated [15]. However, it cannot be determined from these data whether receptor occupancy is required for GTP binding to GN_s, or if GTP binding is normal and the GN_s is inactive for some other reason.

Nucleotide-binding requirements for GN_s interacting with a β-receptor in a reconstituted system have been established [16]. In that system, there is a residual degree of GTP binding by GN_s, the receptor being primarily responsible for the activation of the GTPase rather than the activation of adenylate cyclase. Although we have not addressed the question of the GTPase, preliminary experiments with cholera toxin (Hatta and Rasenick, unpublished) indicate that the lesser potency observed for GTP than GTPγS is due to greater affinity of GTPγS for GN_s, rather than

GTP hydrolysis of the former compound. Thus, intact C6 cells may require β-receptor occupancy by agonist before GTP binds to GN_s and this property may be lost upon preparation of membranes.

The lack of nucleotide effects prior to receptor occupancy appears restricted to GN_s, as preliminary data indicate that guanine nucleotides can inhibit forskolin-stimulated adenylate cyclase in permeabilized C6 cells, independent of agonist (Hughes and Rasenick, unpublished). It is not known whether the inhibitory GTP-binding protein [GN_i] is fundamentally different than GN_s in this regard, or whether the receptor independence of GN_i results from the lack of receptor-mediated inhibition of C6 adenylate cyclase under normal conditions [17].

Isoproterenol is without effect in the stimulation of C6 membrane adenylate cyclase. These results are different from those reported by Katada et al. [8], who observed isoproterenol-activated adenylate cyclase without added GTP. The reasons for this discrepancy may be attributed to our omission of EGTA from the adenylate cyclase reactions [14]. EGTA was omitted to compare the membrane and permeabilized cell system more directly. However, when EGTA is present in the assay, our results resemble those cited above [8].

In both the inositol trisphosphate mediated Ca^{2+} flux in neuroblastoma cells [9] and the release of secretory products from mast cells [10], hydrolysis-resistant guanine nucleotides achieve cellular effects without added hormone. We do not see effects of GTPγS alone, except at 10^{-4} M, on stimulation of the adenylate cyclase system of permeabilized cells. Phosphatidylinositol hydrolysis has been demonstrated in C6 cells [18] and activation of protein kinase C, which follows phosphatidylinositol hydrolysis, appears to increase platelet adenylate cyclase activity by the phosphorylation and subsequent inactivation of GN_i [19]. Such a cascade may account for the 10^{-4} M increase in GTPγS-mediated adenylate cyclase activity. In *Aplysia* neurosecretory cells, pressure injection of GTPγS gives a physiological response similar to dibutyryl cAMP (sustained depolarization) only in conjunction with the extracellular application of 5HT [20]. Intracellular application of GTPγS does, however, potentiate the effects of 5HT, and this is concordant with our findings in C6 cells.

The coupling of receptor and guanine nucleotide-binding proteins in the adenylate cyclase system has been under investigation since Rodbell and his colleagues [21] demonstrated a lag in the GppNHp activation of adenylate cyclase which was partially overcome by stimulatory hormones. This report represents a direct indication that the disruption of cells may significantly alter that coupling process. We have recently proposed that, at least in some systems, the cytoskeleton might provide a constraint to the activation of adenylate cyclase, as cytoskeletal disrupting drugs augment the GN_s-mediated activation of that enzyme [3,22]. Although we have not yet investigated the role of the C6 cytoskeleton in the coupling of the C6 adenylate cyclase system, this provides one possible locus for the discrepancy between the C6 membrane and permeabilized cell data. Certainly, other factors, such as membrane composition [22,23] and calmodulin [14] are involved as well. Thus, activation schemes for the adenylate cyclase system which do not account for these additional factors must be embraced with caution.

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Photoaffinity Identification of Colchicine-Solubilized Regulatory Subunit from Rat Brain Adenylate Cyclase

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Abstract: Five GTP binding proteins in rat cerebral cortex synaptic membranes were identified by photoaffinity labelling with [^{3}H] or [^{32}P](P^3-azido-anilido)-P^1-5' GTP (AAGTP). When AAGTP-treated membranes were incubated with colchicine or vinblastine and subsequently washed, a single AAGTP-labelled protein of 42 kD was released into the supernatant. About 30% of the total labelled 42-kD protein was released into supernatants from membranes pretreated with colchicine or vinblastine compared with 15% released from control membranes. The amount of adenylate cyclase regulatory subunit (G unit) remaining in these membranes was assessed with reconstitution studies after inactivating the adenylate cyclase catalytic moiety with *N*-ethylmaleimide (NEM). Forty to fifty percent of functional G units were lost from membranes treated with colchicine prior to washing. This 40–50% loss of functional G unit after colchicine treatment corresponds to the previously observed 42% loss of NaF and guanylyl-5'-imidodiphosphate [Gpp(NH)p]-activated adenylate cyclase. Release of the AAGTP-labelled 42-kD protein from colchicine-treated synaptic membranes is double that from lumicolchicine-treated membranes. This colchicine-mediated release of 42-kD protein correlates with a doubling of functional G unit released from synaptic membranes after colchicine treatment. These findings suggest multiple populations of the G unit within the synaptic plasma membrane, some of which may interact with cytoskeletal components. **Key Words:** Adenylate cyclase—GTP binding protein—Cytoskeleton—Microtubule protein—Neurotransmitter—Receptors—Photoaffinity labelling. **Rasenick M. M. et al.** Photoaffinity identification of colchicine-solubilized regulatory subunit from rat brain adenylate cyclase. *J. Neurochem.* **43**, 1447–1454 (1984).

Hormone-activated adenylate cyclase is comprised of at least three plasma membrane-associated components: the hormone receptor, the guanyl nucleotide binding regulatory subunit (G unit), and the catalytic moiety. There is evidence that the physical association of these components is required for hormonal activation of adenylate cyclase (Spiegel and Downs, 1981). Recent studies suggest that membrane composition (Orly and Schramm, 1977; Hanski et al., 1979; Rasenick et al. 1981) and/or cytoskeletal elements (Kennedy and Insel, 1979; Rudolph et al., 1979; Hagmann and Fishman, 1980; Rasenick et al., 1981; Sternweis et al., 1981) play an active role in the association of adenylate cyclase components and the subsequent activation of this enzyme. Colchicine and vinblastine, agents that disrupt microtubules, increase adenylate cyclase activity in intact white blood cells (Kennedy and Insel, 1979; Rudolph et al., 1979; Hagmann and Fishman, 1980), but do not alter adenylate cyclase activity in membrane fragments derived from these cells (Kennedy and Insel, 1979; Rudolph et al., 1979).

When synaptic membranes prepared from rat cerebral cortex are incubated with colchicine or vinblastine, adenylate cyclase activation by guanylyl-5'-imidodiphosphate [Gpp(NH)p] or NaF is augmented, whereas activation of the enzyme by $MnSO_4$ [which appears to reflect intrinsic catalytic moiety activity (Spiegel and Downs, 1981)] is unaltered (Rasenick et al., 1981). Furthermore when rat cerebral cortical membranes are incubated with

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Abbreviations used: AAGTP, P^3-(4-azido-anilido)-P^1-5' GTP; DTT, Dithiothreitol; Gpp(NH)p, Guanylyl-5'-imidodiphosphate; GTPγS, Guanosine-5'(5-thio)triphosphate; G unit, Guanyl nucleotide binding regulatory subunit (N_s); HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NEM, *N*-Ethylmaleimide; PMSF, Phenylmethylsulfonyl fluoride.

Gpp(NH)p and colchicine or vinblastine and subsequently centrifuged, the dialyzed supernatants are capable of reconstituting adenylate cyclase activity to heat-inactivated (G unit deficient) synaptic membranes. Adenylate cyclase reconstitution by these synaptic membrane supernatants is similar to that obtained with purified G unit from amphibian photoreceptors (Rasenick et al., 1981). These results are consistent with the hypothesis that these supernatant solutions contain a G unit that may be associated with synaptic membrane tubulin.

In this study, we demonstrate that a 42-kD GTP-binding protein is specifically released from synaptic plasma membranes after treatment with colchicine or vinblastine. Furthermore, evidence is presented that the G-unit activity is lost concurrently with the release of a 42-kD protein from synaptic plasma membranes. The percent removal of G unit into the supernatant after colchicine treatment and subsequent washing correlates with the percent increase of adenylate cyclase activity when membranes are not washed subsequent to colchicine treatment (Rasenick et al., 1981)). As colchicine and vinblastine have a similar effect and lumicolchicine has no effect, these data may implicate tubulin as a modifier of the coupling process within the synaptic membrane adenylate cyclase complex.

MATERIALS AND METHODS

Tissue-preparation and treatment

Enriched synaptic membrane fractions were prepared from 21-day-old male Sprague-Dawley rats as previously described (Rasenick and Bitensky, 1980) and stored under liquid nitrogen until use. Membranes were thawed and resuspended in 10 volumes of 20 m*M* HEPES (pH 7.6), 5 m*M* $MgSO_4$, 1 m*M* dithiotreitol (DTT), 0.3 m*M* phenylmethylsulfonyl fluoride (PMSF) (HMD buffer) and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was resuspended in 0.5 ml of HMD (protein concentration = 4–6 mg/ml) and incubated with 5 μ*M* colchicine, lumicolchicine, or vinblastine and 100 μ*M* Gpp(NH)p, guanosine-5′(5 thio) triphosphate (GTPγS), or GTP, as previously described (Rasenick et al., 1981). After incubation the membranes were washed with 15 volumes of 10 m*M* Tris-HCl (pH 7.6), 5 m*M* MgCl, 1 m*M* DTT, and 0.3 m*M* PMSF (TMD) buffer and centrifuged at 13,000 × *g* for 10 min. The supernatants were reserved and the process (incubation-wash-centrifugation) was repeated. This process released 13–14% of the total membrane protein in the presence or absence of colchicine or vinblastine. The pooled supernatants were filtered through Millipore GV filters and then concentrated and dialyzed in Amicon CF 25 cones. This material, termed colchicine/wash or vinblastine/wash when those agents were present or control/wash when lumicolchicine or water were present, was stored at −70°C until use.

The membranes from above were then split into two groups; half were assayed for remaining adenylate cyclase activity and half were treated with *N*-ethylmaleimide (NEM) (see below).

Photoaffinity labelling and analysis

[^{3}H]P^3-(4-azidoanilido)-P^1-5′ GTP (AAGTP) (36 Ci/mmol) was synthesized by the method of Pfeuffer (1977). Membranes were washed and resuspended in 50 m*M* phosphate buffer (pH 7.5) and 5 m*M* MgCl. Membrane suspensions (0.5–1 ml; 15–20 mg protein/ml) were incubated with or without unlabelled guanyl nucleotide (GMP, GDP, GTP, Gpp(NH)p, GTPγS, or AAGTP 50 μ*M*) for 10 min followed by 2 μ*M* [^{3}H]AAGTP or [^{32}P]AAGTP for 20 min at 23°C in a stirred quartz cuvette under dim red light. These suspensions were then irradiated with 2 Spectroline UV (254 nm) lamps (approximately 5 cm from cuvette) for 20 min at 4°C in the same stirred cuvettes. Following photolysis membranes were resuspended in 15 volumes of ice cold TMD buffer with 4 m*M* DTT (TMDX buffer) and centrifuged at 13,000 × *g* for 10 min to remove unbound AAGTP. Membranes were then resuspended in TMDX buffer at a protein concentration of 3–4 mg/ml, incubated with colchicine, vinblastine, or lumicolchicine (5 μ*M*) for 20 min at 30°C, and subsequently washed with 10 volumes of 10 m*M* HEPES (pH 7.8), 2 m*M* EDTA, and 1 m*M* DTT (HED buffer). Membranes were centrifuged at 13,000 × *g* for 10 min and the supernatants saved. This procedure was repeated twice and the supernatants were pooled and dialyzed vs 6 *M* urea for 6 h followed by H_2O overnight. The supernatants were then lyophilized, resuspended in 3% SDS, and electrophoresed in 10% polyacrylamide gels by the procedure of Laemmli (1970). Membrane pellets were also dissolved in 3% SDS and electrophoresed. Gels were fixed, stained with Coomassie Blue, and fluorographed with Kodak XAR-5 film after (if [^{3}H]AAGTP was used) incubation with EnHance (New England Nuclear) and dried. A Shimadzu model 910 Densitometer-Integrator was employed for densitometric analysis of gels and radiofluorographs. In some experiments each lane of a fixed and stained gel was cut into 1-mm segments, dissolved in 100 μl of 30% H_2O_2, and subjected to liquid scintilation counting.

NEM treatment

Treatment with NEM to inactivate adenylate cyclase catalytic moiety was modified from Orly and Schramm (1977). Membranes were resuspended in HMD buffer (protein concentration = 2.5 mg/ml) plus 50 μ*M* Gpp(NH)p and NEM was added to a final concentration of 5 m*M*. After 30 min on ice, 5 volumes (2.5 ml) of TMDX buffer was added to the membrane suspensions. After an additional 10 min on ice the tubes were centrifuged and resuspended in TMD buffer at a protein concentration of about 1 mg/ml. Membranes were then used for reconstitution with heat-treated synaptic membranes.

Heat inactivation of synaptic membrane G unit

Synaptic membranes were heat-treated as described previously (Rasenick et al., 1981) and immediately used in reconstitution studies. After incubation in the absence of Gpp(NH)p or NaF at 42°C for 45 min, 60–80% of the catalytic moiety activity (as measured by Mn^{2+}) but only 15% of G-unit activity [as measured by Gpp(NH)p or NaF] is preserved.

G-unit assessment by membrane reconstitution

Heat-inactivated synaptic membranes (0.84 mg/ml) were incubated with or without 10 μl of NEM-treated

membranes at 37°C for 10 min in a TMD buffer containing linoleic acid (10 μg/ml), phosphatidyl choline (egg) (1 mg/ml), and polyethylene glycol (2.5 mg/ml). Under these conditions membrane fusion occurs and the G unit and catalytic units from each membrane type are able to interact. Following the initial preincubation a second preincubation at 30°C with H_2O, Gpp(NH)p, NaF, or Mn^{2+} was carried out. Tubes were kept on ice for 30 min and adenylate cyclase was assayed as below.

Adenylate cyclase assays

Adenylate cyclase assays were similar to those described previously (Rasenick et al., 1981). Thirty micrograms of synaptic membranes were incubated with Gpp(NH)p, NaF, and colchicine/wash or control/wash for 20 min at 30°C in a total volume of 50 μl of HMD buffer. Tubes were then placed on ice for 10 min. ATP (500 μ*M*) and an ATP regeneration system were added as described (Rasenick and Bitensky, 1980) and assays were carried out for 20 min at 30°C. Assays were stopped at 90°C for 5 min, and the cAMP produced was determined by a competitive binding assay.

RESULTS

To determine the specificity of AAGTP labelling, synaptic membranes were incubated for 10 min with 10 μ*M* Gpp(NH)p, GTP, GDP, GMP, GTPγS, or unlabelled AAGTP prior to addition of 2 μ*M* [^{32}P]AAGTP. Figure 1 shows the effect of these nucleotides on AAGTP labelling of the unwashed synaptic membranes. Bands at 94 kD, 55 kD, 42 kD, 40 kD, and 36 kD were labelled with AAGTP. Guanyl nucleotides that activate adenylate cyclase, including GTPγS, Gpp(NH)p, and AAGTP, reduce labelling of the 94-kD, 55-kD, 42-kD, and 40-kD bands. Other guanyl nucleotides, which are ineffective in activating synaptic membrane adenylate cyclase (e.g., GTP, GDP),[1] are much less efficient in reducing labelling of these bands. GMP, also ineffective in activating adenylate cyclase, appears not to compete for the 94 + 55-kD bands, but is slightly more effective on chasing label from 42 + 40-kD bands. Furthermore GMP and GTPγS enhance labelling at the 36-kD band, whereas Gpp(NH)p, GTP, and GDP do not significantly alter 36-kD labelling. Unlabelled AAGTP effectively competes (>90%) with [^{32}P]AAGTP at all bands.

[^{3}H]AAGTP-labelled membrane preparations were incubated with colchicine or lumicolchicine and subsequently washed. The supernatants were then analyzed by gel electrophoresis and radiofluorography (Fig. 2). Both the quantity (13–14%) and the variety of proteins (as detected by Coomassie Blue staining) washed from the plasma membrane were similar after colchicine, lumicolchicine, or buffer treatment (Fig. 2, lanes A and B). The only

[1] Although GTP activates adenylate cyclase in most systems, GTP is without effect in synaptic membranes (without a regenerating system).

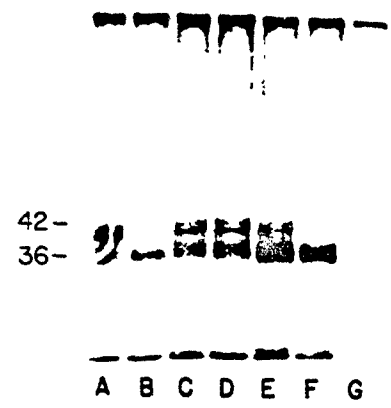

FIG. 1. Competition of AAGTP binding. Membranes were incubated with the indicated compounds (50 μ*M*) for 10 min at 23°C prior to the addition of 2 μ*M* [^{32}P]AAGTP. Gels (8.5%) were run after solubilization of the membrane pellets with 3% SDS (see Materials and Methods) and the radiofluorographs are depicted above. Additions are: lane A, [^{32}P]AAGTP only (control) or [^{32}P]AAGTP plus 50 μ*M*; lane B, Gpp(NH)p; lane C, GTP; lane D, GDP; lane E, GMP; lane F, GTPγS; and lane G, unlabelled AAGTP.

[^{3}H]AAGTP-labelled protein released (detected by radiofluorography) from the synaptic plasma membrane is one of 42 kD. Furthermore a single peak of radioactivity (42 kD) is detected in slices of gels from supernatant fractions. This protein comigrates with the [^{3}H]AAGTP-labelled 42-kD protein from

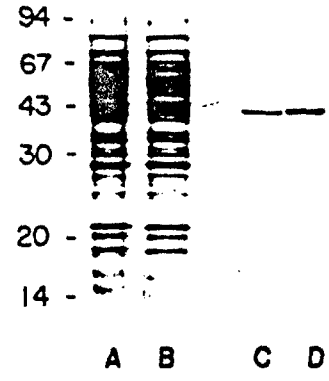

FIG. 2. SDS-PAGE analysis of AAGTP-labelled synaptic membranes. Fifty micrograms of control/wash (lane A) or colchicine/wash (lane B) supernatant were run on a 10% polyacrylamide slab gel. Lanes A and B represent Coomassie Blue staining patterns and lanes C and D represent the radiofluorographic pattern of lanes A and B, respectively, after a 2-week exposure at −70°C. Substitution of 5 μ*M* vinblastine for colchicine yields similar results, whereas lumicolchicine (10 μ*M*) is indistinguishable from the H_2O control (not shown). AAGTP labelling can be competed with prior addition of 10 μ*M* Gpp(NH)p to the membranes. 10 μ*M* GTPγS is equally effective in the competition of AAGTP labelling (see Fig. 1). One of six similar experiments is depicted above.

TABLE 1. *Incorporation of [^{3}H]AAGTP into 42-kD protein of synaptic membranes and supernatants*

Membranes treated with:	Untreated, unwashed membranes[a]	Lumi-colchicine[a]	Colchicine[a]
Pellet	24,816	20,638	16,962
Supernate		4,343	7,445
Total 42 kD dpm	24,816	24,981	24,407

Individual lanes from 10% polyacrylamide gels were cut into 1 mm slices, dissolved in 30% H_2O_2, and counted. Three slices comprise the radioactive peak corresponding to 42 kD (R_f = 0.41–0.43) in both supernatant and pellet gels, and the cumulative radioactivity of those slices is noted above. These data are from one of four similar experiments in which H_2O or lumicolchicine and colchicine or vinblastine gave similar results.
[a] ^{3}H dpm.

the synaptic plasma membrane in one- and two-dimensional SDS gels. The supernatants from colchicine-treated membrane show a distinct increase in the 42-kD AAGTP-labelled protein compared with controls (Fig. 2, lanes C and D). Measurement of the radioactivity in the individual bands indicated that the 42-kD band from the colchicine wash contained 71.4% more radioactivity than the control wash (7445 vs 4343 dpm; Table 1).

Radioactivity in the 42-kD band from membranes that were not incubated and washed subsequent to AAGTP treatment is 24,816 dpm (Table 1). Membranes that were treated (colchicine or control) and subsequently washed showed reduced dpm on the membranes (16,962 and 20,638 dpm, respectively) which appeared in the supernatants (7445 and 4343 dpm, respectively). [The total 42-kD band radioactivity in supernatants and membrane from colchicine or control washed samples equalled that observed in the unwashed membranes (24,407 and 24,971 vs 24,816 dpm, respectively, Table 1).] The differential between the 42-kD band dpm in the supernatants was 1.7× more for colchicine vs control, whereas the 42-kD band dpm on the colchicine-treated membranes was 0.32 times less than the control. The 42-kD band dpm released from synaptic plasma membrane represents 15% of the total labelled 42-kD band protein for the control supernatant and 30% for the colchicine-treated supernatants.

Efficiency of incorporation of AAGTP into the 42-kD band (mol AAGTP in 42-kD band/mol 42-kD protein; assuming a single protein in the band) was 1.5% in the above experiment and varied from 1.5 to 2.3%. The assumption of single 42-kD labelled species was validated by two-dimensional gel electrophoresis (data not shown). This technique revealed a single labelled species of 42-kD with an apparent pI of 5.3–5.4. Within a given experiment, efficiency of incorporation was identical for pellet and supernatant and was independent of treatment of membranes.

Reconstitution studies

In a previous study we demonstrated that about 50% of Gpp(NH)p or NaF activation of adenylate cyclase was lost in membranes that were washed after treatment with colchicine or vinblastine. As it appeared that 70% of the 42-kD protein was retained in these membranes we chose to test the amount of *functional* G unit retained after colchicine treatment and subsequent wash.

Schramm and his colleagues have demonstrated that membranes deficient in receptor or G unit could be fused with membranes made deficient in the catalytic moiety by treatment with NEM (Orly and Schramm, 1977). In experiments of a similar nature (see Fig. 3) we have assessed the ability of colchicine to deplete functional G unit from synaptic membranes. As depicted in Fig. 3 colchicine-treated membranes in which the catalytic moiety has been inactivated NEM can be reconstituted with heat-treated synaptic membranes deficient in function G units. The restoration of Gpp(NH)p-dependent adenylate cyclase activity may be a measure of the quantity of functional G unit remaining in the NEM-treated membranes.

Figure 4 shows that despite the fact that there is little adenylate cyclase activity remaining in NEM-treated membranes (Fig. 4B), they are capable of restoring Gpp(NH)p activation of adenylate cyclase in heat-inactived membranes (Fig. 4A). Addition of NEM-treated membranes restores Gpp(NH)p-dependent adenylate cyclase activity in a linear fashion over the range of 5–50 μg/ml, with maximal reconstitution occurring at 50 μg/ml. Above 50 μg/ml adenylate cyclase activity falls off sharply in NEM-treated membranes (Fig. 5). The ratio of NEM-treated synaptic membrane protein (donor) to heat-treated membrane protein (recipient) is 1:20 at optimal reconstitution. The NEM-treated membrane ratio required for optimal reconstitution remains at about 1:20 when adenylate cyclase assays are not required to measure reconstitution, as Gpp(NH)p was present during initial NEM exposure (Fig. 5).

DISCUSSION

In a previous study we demonstrated that colchicine or vinblastine treatment of synaptic membranes, and subsequent washing, results in a loss of Gpp(NH)p or NaF-activated adenylate cyclase (Rasenick et al., 1981). When supernatants from those washes were dialyzed and concentrated, they were capable of reconstituting adenylate cyclase activity to G-unit-deficient membranes. In this study we demonstrate that colchicine treatment is capable of increasing selectively, the release of a 42-kD AAGTP-labelled protein from the synaptic membrane. Furthermore we demonstrate that the reduc-

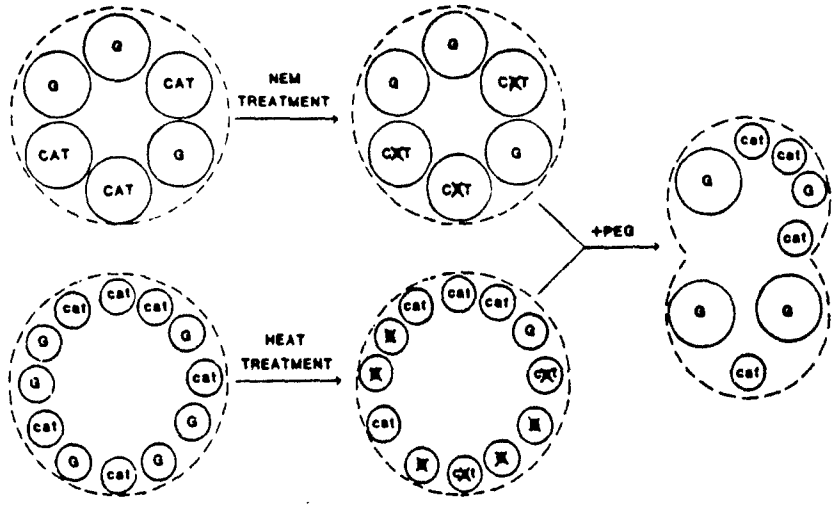

FIG. 3. *N*-Ethylmaleimide (NEM) treatment and reconstitution. The cartoon depicts the effects of NEM in the inactivation of the catalytic moiety and the effects of heating in the inactivation of 86% of the G units. Also depicted are the actions of polyethylene glycol (PEG) in the possible creation of active fused-membrane hybrids. There is no intent to indicate that the G units from membranes treated with NEM are different from those treated with heat.

tion in NaF and Gpp(NH)p-activated adenylate cyclase activity subsequent to colchicine treatment and washing is due to a loss of G unit from the synaptic plasma membrane.

AAGTP specifically labels synaptic membrane proteins of 94 kD, 55 kD, 42 kD, 40 kD, and 36 kD. Compounds that activate adenylate cyclase, i.e., GTPγS, Gpp(NH)p, and unlabelled AAGTP, effectively reduce labelling of the 94-kD, 55-kD, 42-kD, and 40-kD bands. Compounds that are ineffective in activating synaptic membrane adenylate cyclase (GTP and GDP) are much less effective in reducing labelling at these bands. GMP, which has been reported by Downs et al. (1980) to clear the nucleotide occupancy (GTP or GDP) of the G unit while not activating adenylate cyclase, is somewhat more effective than GTP or GDP for competing label from the 42-kD and 40-kD bands. The 55-kD double band appears to comigrate with tubulin, which has been reported both to be tightly associated with the synaptic plasma membrane (Zisapel et al., 1980), and to bind a GTP photoaffinity probe (Geahlen and Haley, 1979). GMP however does not compete with GTP for the nucleotide binding sites of tubulin and thus would be expected to be ineffective at chasing label from the 55-kD band. Our data are consistent with this point.

A 41-kD protein (N_i) can be ADP-ribosylated by the "islet-activating" constituent of pertussis toxin (Katada and Ui, 1982). This protein may represent

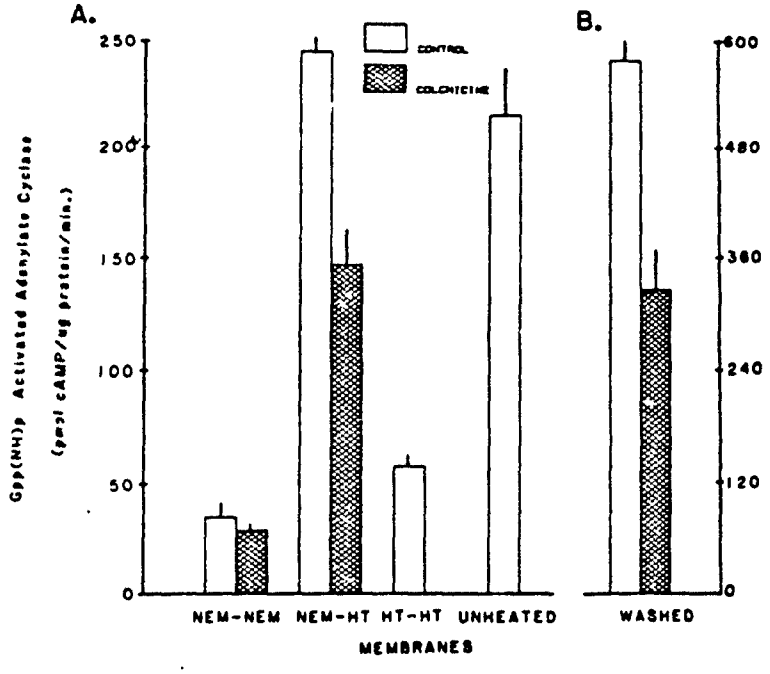

FIG. 4. Effects of colchicine treatment and washing upon Gpp(NH)p-activated adenylate cyclase and upon G unit available for reconstitution. Membranes are incubated with or without colchicine and subsequently washed. Membranes are then split into two groups and either assayed for adenylate cyclase (B) or treated with NEM (A). **A:** Adenylate cyclase activity in the presence of 10 μ*M* Gpp(NH)p for NEM-treated synaptic membranes fused (after polyethylene glycol treatment) with: other NEM-treated membranes (NEM + NEM), heat-treated membranes (NEM + HT). Other controls consist of heat-treated membranes fused with themselves (HT + HT) and unheated membranes fused with themselves (unheated). Final protein concentrations are 40 μg/ml for NEM-treated membranes and 0.8 mg/ml for heat-treated membranes. **B:** Adenylate cyclase activity in the presence of 10 μ*M* Gpp(NH)p for synaptic membranes after treatment with colchicine and washing. Bars represent means of duplicate determinations for one of five similar experiments.

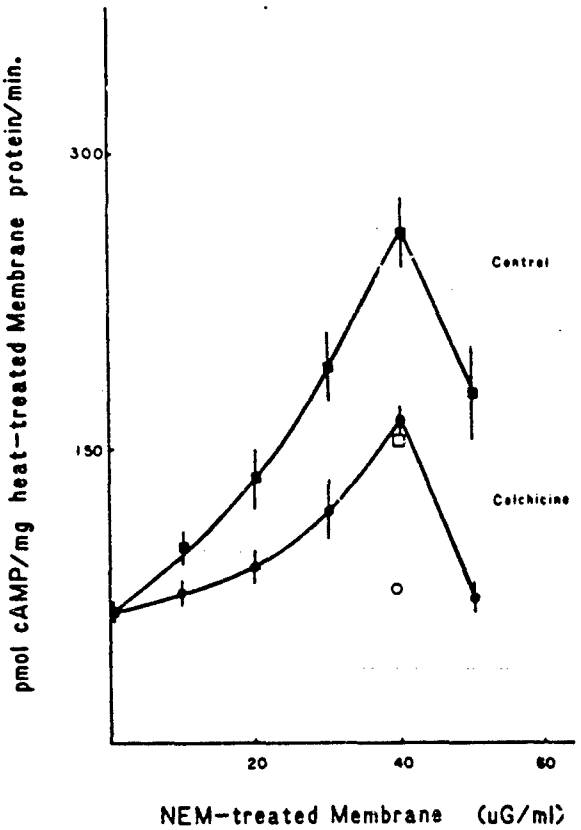

FIG. 5. Reconstitution of Gpp(NH)p-activated adenylate cyclase with NEM-treated synaptic membranes. Membranes are treated as in Fig. 4A. NEM-treated membranes are added to heat-treated membranes to a final protein concentration as indicated. The heat-treated membrane protein concentration is 0.8 mg/ml in each sample. [Adenylate cyclase activity of heat-treated membranes reconstituted with NEM-treated membranes that were previously treated with (○) or without (□) colchicine when Gpp(NH)p is not present in the assay.] Points are means of duplicate determinations from one of three similar experiments.

an inhibitory G unit (N_i) and it would be interesting if the 40-kD AAGTP-labelled protein observed in these studies were similar or identical. [Preliminary indications are that the 40-kD AAGTP-labelled protein is similar or identical to N_i (M. M. Rasenick, unpublished observations).] It is noteworthy, however that under conditions in which colchicine releases the 42-kD protein from the synaptic membrane, the 40-kD protein is not released (Fig. 2). It is currently unclear whether colchicine augments N_i-catalytic moeity coupling (Rasenick and Malina, 1983).

A 36-kD protein has been reported by Gilman and his colleague to be a constituent of the GTP-binding protein(s) in both S49 lymphoma and rabbit liver adenylate cyclase (Sternweis et al., 1981). This protein may also be found in the frog photoreceptor (Fung et al., 1981; Uchida et al., 1981) and in adenylate cyclase from *D. discoideum*, both of which show photoaffinity GTP labelling of a 36-kD protein (Leichtling et al., 1981; Takemoto et al., 1981); Gpp(NH)p, GTP, and GDP do not significantly alter AAGTP labelling of the 36-kD protein. GMP and GTPγS however enhance labelling of the 36-kD protein. Labelling of 36-kD protein may require some additional factor, as when adenylate cyclase G unit or rod outer segment G proteins are purified prior to photoaffinity GTP labelling, labelling is not seen at the 36-kD protein (Uchida et al., 1981; Northup et al., 1982).

We have indicated previously that washing synaptic membranes with buffer diminished Gpp(NH)p and NaF activation of adenylate cyclase, especially when those synaptic membranes had been pretreated with colchicine. In this paper we demonstrate that synaptic plasma membranes in which the catalytic moiety of adenylate cyclase has been destroyed by NEM treatment can sucessfully reconstitute Gpp(NH)p or NaF activation to synaptic membranes in which heating has eliminated most of the G-unit activity. Under these circumstances membranes pretreated with colchicine and subsequently washed were 60% less effective than control members in reconstituting adenylate cyclase activity. Both control and colchicine-treated membranes showed linear reconstitution (subsequent to NEM treatment) in the 5–50 μg/ml range. However colchicine-treated membranes were unable to reach the same level of reconstitution as control-treated membranes, a result that may be due to the presence, in synaptic membranes, of an adenylate cyclase inhibitor protein, as previously reported (Rasenick and Bitensky, 1980). The degree of difference in the ability of colchicine- and control-treated membranes (subsequent to NEM treatment) to reconstitute adenylate cyclase parallels the degree to which colchicine treatment of membranes was previously shown to diminish adenylate cyclase activity subsequent to washing (Figs. 2 and 4). It is noteworthy (see below) that the ratio of control to colchicine reconstitution of NEM-treated membranes is inversely proportional to the appearance of AAGTP-labelled 42-kD protein in the supernatant from those membranes. An important caveat to the above is that our interpretations of the NEM treatment-reconstitution data are based upon G units from the NEM-treated membranes inserting and activating the catalytic moiety from heat-treated membranes (Fig. 3). The possibility that some other factor from the NEM-treated membrane is restoring heat-inactivated G unit cannot be ruled out. This is unlikely, however, for two reasons. First the reconstitution is strictly dependent upon the presence of NEM-treated membranes *and* heat-treated membranes. Neither preparation alone shows significant levels of adenylate cyclase. Second NEM-treated membranes will reconstitute adenylate cyclase in the absence of added

Gpp(NH)p or NaF (Fig. 5). This is likely due to bound Gpp(NH)p (Spiegel and Downs, 1981) and it reduces the possibility that NEM-treated membranes provide some adjuvant for the restoration of G-unit activity to heat-treated membranes.

Analysis of labelling in the 42-kD band from membranes and supernatants revealed that a constant amount of labelling in the 42-kD protein can be distributed between the supernatant and pellet fractions in a manner related to the treatment of the membrane subsequent to AAGTP labelling. Almost twice as much 42-kD protein appears to be released from synaptic membranes after incubation with colchicine or vinblastine compared with H_2O or lumicolchicine. This 2:1 ratio also holds for the degree to which these supernatants can reconstitute adenylate cyclase activity to heat-treated synaptic membranes as indicated in the previous work (Rasenick et al., 1981). Furthermore, the ratio for the reconstitution of adenylate cyclase by NEM-treated membranes was 1:2 for colchicine:control and this is consistent with the above data.

Under control conditions approximately 15% of the 42-kD AAGTP-labelled protein can be washed from the membranes. In the presence of colchicine about 30% of this protein is released from the membranes. When compared with control membranes, however, colchicine-treated membranes show a 50% reduction in Gpp(NH)p or NaF activation of adenylate cyclase (Rasenick et al., 1981). Perhaps 15% of the total G protein in the synaptic membranes is responsible for 50% of the G protein activity. This point will require further investigation.

Although it is commonly assumed that the G unit is an integral membrane protein several investigators have recently demonstrated the possibility that at least some of the G protein might be either soluble or loosely attached to the membrane (Pecker and Hanoune, 1977; Pfeuffer, 1977; Krishna-Bhat et al., 1980; Rasenick et al., 1981; Sayhoun et al., 1981). As extensive washing of colchicine-treated membranes yields a maximum of 30% release of 42-kD AAGTP-labelled protein, it is likely that most of the G protein, even in the synaptic membrane, is either an integral protein or is tightly attached to the membrane. It is possible, however, as we have recently demonstrated (O'Callahan et al., 1983), that at least some of the synaptic membrane G units are associated with tubulin. This finding is consistent with the reported copurification of adenylate cyclase and brain tubulin (Margolis and Wilson, 1979).

Although a mechanistic understanding of the above phenomena awaits further experimentation, a consideration of the photoreceptor phosphodiesterase system may aid in our understanding of a possible role for the cytoskeleton in adenylate cyclase activation. As noted previously, if synaptic membranes are treated with colchicine and not washed, adenylate cyclase activation is enhanced rather than diminished (Rasenick et al., 1981). When light and GTP are added to rod outer segment membranes, PDE is activated. If those membranes are exposed to light and GTP and then washed, the photoreceptor G protein(s) is released (see Stein et al., 1982). This release after washing coincides with increased catalytic enzyme activity that may be physiologically explained by the increased association (coupling) of the G proteins with the catalytic moiety (either adenylate cyclase or rod outer segment phosphodiesterase).

Changes in membrane fluidity and/or in cytoskeletal protein interactions may modulate the expression of adenylate cyclase activity by altering G-unit catalytic moiety coupling (for review, see Zor 1983). Such an apparent increase of G-unit catalytic moiety coupling has recently been noticed in synaptic membranes prepared from rats subjected to chronic antidepressant treatment (Menkes et al., 1983). Addition of colchicine to control membranes has effects similar to those of *in vivo* antidepressant treatment. The mechanism by which either colchicine or antidepressant treatment augment adenylate cyclase is, as yet, unknown, It is possible, however, that cytoskeletal components may act in concert with hormones and guanyl nucleotides to regulate adenylate cyclase activity.

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