

# Intracellular Injection of Guanyl Nucleotides Alters the Serotonin-induced Increase in Potassium Conductance in *Aplysia* Neuron R15

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**ABSTRACT** The effects of the adenylate cyclase inhibitor GDP $\beta$ S on the response of *Aplysia* neuron R15 to serotonin (5HT) were investigated. Previous studies have demonstrated that 5HT causes an increase in K<sup>+</sup> conductance in R15 and that the response is mediated by cAMP. At concentrations in the micromolar range, GDP $\beta$ S inhibits the stimulation of adenylate cyclase by 5HT in particulate fractions from *Aplysia* ganglia. When micromolar concentrations of GDP $\beta$ S are injected into neuron R15, there is no effect on the resting membrane conductance, but the increase in K<sup>+</sup> conductance normally elicited by 5HT is completely inhibited. Furthermore, the decrease in inward current normally elicited by dopamine (DA), which does not appear to involve cAMP, is not affected by micromolar concentrations of GDP $\beta$ S. In addition, application of 8-benzylthio cAMP to R15 can evoke an increase in K<sup>+</sup> conductance even after the injection of GDP $\beta$ S, which indicates that events subsequent to the activation of adenylate cyclase are not inhibited by the GDP analogue. In contrast, when millimolar concentrations of GDP $\beta$ S are injected into R15, direct effects on membrane conductance are observed and the response of R15 to 5HT is enhanced. Although these effects of high concentrations of GDP $\beta$ S are only poorly understood, the results with micromolar concentrations are consistent with the hypothesis that stimulation of adenylate cyclase is necessary for the 5HT-induced increase in K<sup>+</sup> conductance in neuron R15.

## INTRODUCTION

The mechanism by which neurotransmitters alter the membrane properties of target cells is a topic of wide current interest. Many actions of neurotransmitters, such as that of acetylcholine at the vertebrate neuromuscular junction, are rapid in onset and rapidly reversible. Such effects may be due to rapidly reversible conformational changes in ion channels, which are closely associated with the neurotransmitter binding site (Neher and Sakmann, 1976). Other actions of neurotransmitters are slower in onset and longer in duration, and it has been

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proposed (Greengard, 1978) that in such cases intracellular second messengers such as cyclic nucleotides or calcium ions may mediate relatively long-lasting changes in the properties of ion channels. Although definitive evidence for a role for second messengers in the physiological actions of neurotransmitters has been difficult to obtain, it has become clear in recent years that cAMP can regulate the electrical properties of certain excitable cells (Tsien et al., 1972; Tsien, 1973; Treisman and Levitan 1976*a, b*; Brunelli et al., 1976; Kaczmarek et al., 1978; Pellmar, 1981). Furthermore, in several different molluscan neurons, cAMP appears to mediate changes in K<sup>+</sup> conductance elicited by the neurotransmitter serotonin (5HT) (Klein and Kandel, 1978; Klein et al., 1982; Drummond et al., 1980*a*; Deterre et al., 1981; Siegelbaum et al., 1982). In the identified *Aplysia* neuron R15, 5HT causes an increase in an inwardly rectifying K<sup>+</sup> current (Drummond et al., 1980*a*; Benson and Levitan, 1983). A series of biochemical, pharmacological, and electrophysiological experiments have satisfied all the criteria (Robinson et al., 1971; Greengard, 1978) necessary to implicate cAMP as an intracellular second messenger for this response (Drummond et al., 1980*a*).

Although correlative experiments such as those described above have provided strong evidence for a role for cAMP, the lack of effective adenylate cyclase inhibitors has made it difficult to test directly whether cyclase activation is a necessary step in the sequence of events leading to the change in membrane conductance. Recently, Eckstein et al. (1979) have synthesized a GDP analogue, GDP $\beta$ S, which can be phosphorylated or hydrolyzed only very slowly. This analogue can bind to the GDP binding site on the regulatory (nucleotide binding) subunit of adenylate cyclase and can prevent the stimulation of the cyclase by hormones or neurotransmitters (Cassel et al., 1979). We report here that micromolar concentrations of GDP $\beta$ S inhibit *Aplysia* adenylate cyclase activity and also selectively inhibit the K<sup>+</sup> conductance increase elicited by 5HT.

#### MATERIALS AND METHODS

##### *Materials*

$\alpha$ [<sup>32</sup>P]ATP (5–20 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England). 5-Hydroxytryptamine (5HT) creatinine sulphate and dopamine (DA) hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). 8-Benzylthio-cAMP (8BTcAMP) was purchased from ICN K&K Laboratories (Irvine, CA). Guanosine diphosphate (GDP), guanosine triphosphate (GTP), and guanylylimidodiphosphate (GppNHp) were from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany), as were creatine phosphate and creatine kinase. Guanosine 5'-O-(2-thiodiphosphate) (GDP $\beta$ S) was a generous gift of F. Eckstein (Gottingen, Federal Republic of Germany).

##### *Animals*

Marine snails, *Aplysia californica*, were purchased from Marine Specimens Unlimited (Pacific Palisades, CA) and maintained in artificial seawater at 18–20°C.

##### *Enzyme Preparation*

Adenylate cyclase activity was assayed in crude particulate fractions prepared from *Aplysia* abdominal ganglia (Levitan et al., 1978). Ganglia were homogenized in 2 mM Tris/2 mM

EGTA, pH 8.0 (20 ml/g wet weight of tissue), using a ground-glass homogenizer. Nondisrupted pieces of connective tissue were removed with forceps, and the homogenate was centrifuged at 50,000 *g* for 15 min. The pellet was washed once with 2 mM Tris-maleate, pH 8 (without EGTA), and was resuspended by homogenization in the latter buffer at a concentration of 3–4 mg protein/ml. The resuspended membranes were used immediately.

#### *Adenylate Cyclase Assay*

Adenylate cyclase activity was measured by a modification of the method of Salomon et al. (1974). The assay mixture contained 25 mM Tris-maleate, 0.6 mM EGTA, 1 mM isobutylmethylxanthine, 5 mM MgCl<sub>2</sub>, 1 mM cAMP, 20 mM creatine phosphate, 80 U/ml creatine phosphokinase, and 0.5 mM α[<sup>32</sup>P]ATP (specific activity 150–250 mCi/mmol, to give 5–10 × 10<sup>6</sup> cpm in the 50-μl assay volume). The pH was 7.8. The reaction was initiated by the addition of 15–25 μg of membrane protein; incubation was at 30°C for 3 min, and the reaction was terminated by the addition of stopping solution containing 2% sodium dodecyl sulfate (SDS), 40 mM ATP, and 1 mM cAMP. The [<sup>32</sup>P]cAMP was isolated by the sequential Dowex and Alumina column method of Salomon et al. (1974) and was counted in a LS-355 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Protein was estimated by the method of Schaffner and Weissmann (1973), using bovine serum albumin as the standard.

#### *Receptor Binding Studies*

The number and affinity of serotonin and dopamine receptors in membrane fractions from *Aplysia* ganglia were measured as described by Drummond et al. (1980*b, c*), using [<sup>3</sup>H]LSD as the receptor ligand. [<sup>3</sup>H]LSD binds to a mixture of serotonin and dopamine receptors in this system, but under appropriate conditions the binding to each receptor can be measured independent of the other (Drummond et al., 1980*b, c*).

#### *Recording*

The abdominal ganglion was pinned dorsal side up in a Sylgard dish and neuron R15 was penetrated with one or two microelectrodes (3–10 MΩ) for recording and voltage clamping. In most of the experiments described here, a single-electrode voltage clamp was used (Wilson and Goldner, 1975; design modified by W. B. Adams, unpublished). Another microelectrode, filled with GDPβS for intraneuronal injection, was then inserted into R15 and removed immediately after pressure injection. The ganglion was continuously perfused with *Aplysia* medium (Levitan and Barondes, 1974) at 20–22°C, with additions or modifications as described for individual experiments. The cell was voltage clamped by conventional techniques, the membrane potential was swept between –120 and –40 mV at a rate of 4 mV/s, and the resulting current was recorded. This sweep time is long relative to the membrane time constant and short relative to the duration of the effects of 5HT, DA, and cAMP analogues reported below.

#### *Intracellular Injections*

Cells were injected as described by Lemos et al. (1982). Using a Hamilton (Reno, NV) syringe, 50 nl of GDPβS or GppNHp, dissolved in distilled H<sub>2</sub>O at ~50× the final desired intracellular concentration, was placed in the tip of a microelectrode pulled from Microstar capillary tubing (Radnoti Glass Technology, Inc., Monrovia, CA). The solution (0.5–2 nl, estimated by movement of the meniscus in the electrode tip) was injected into voltage-clamped neuron R15 (estimated cell body volume 20–60 nl) under pressure using N<sub>2</sub> gas. Such injections did not alter the current-voltage relationship of R15.

## RESULTS

*GDP $\beta$ S Effects on Aplysia Adenylate Cyclase Activity*

Table I shows the effects of guanyl nucleotides on basal and 5HT-dependent adenylate cyclase activity in membranes prepared from *Aplysia* abdominal ganglia. GTP alone can stimulate the enzyme activity in a dose-dependent manner. Although 5HT ( $10^{-5}$  M) alone has no effect, when coupled with  $10^{-4}$  M GTP it can stimulate the cyclase activity as much as 400%. GDP $\beta$ S inhibits both the basal and the 5HT/GTP-stimulated enzyme activity. In contrast, GDP either

TABLE I  
*Effects of Guanyl Nucleotides on Aplysia Nervous System Adenylate Cyclase Activity*

Additions to assay	Percent basal cyclase activity
$10^{-4}$ M GTP	161 $\pm$ 15
$10^{-5}$ M GTP	125 $\pm$ 16
$10^{-6}$ M GTP	115 $\pm$ 8.5
$10^{-4}$ M GDP $\beta$ S	48 $\pm$ 5
$10^{-5}$ M GDP $\beta$ S	40 $\pm$ 4
$10^{-6}$ M GDP $\beta$ S	50 $\pm$ 3.5
$10^{-5}$ M 5HT	101 $\pm$ 7
$10^{-5}$ M 5HT + $10^{-5}$ M GTP	357 $\pm$ 14
$10^{-5}$ M 5HT + $10^{-4}$ M GDP $\beta$ S	36 $\pm$ 3
$10^{-5}$ M 5HT + $10^{-4}$ M GDP $\beta$ S + $10^{-5}$ M GTP	167 $\pm$ 10
$10^{-4}$ M GDP	170 $\pm$ 4
$10^{-5}$ M 5HT + $10^{-4}$ M GDP	393 $\pm$ 9
$10^{-6}$ M GppNHp	214 $\pm$ 6.5
$10^{-5}$ M 5HT + $10^{-6}$ M GppNHp	367 $\pm$ 10

Adenylate cyclase activity was assayed by the conversion of  $\alpha$ [ $^{32}$ P]ATP to [ $^{32}$ P]cAMP using membranes prepared from *Aplysia* abdominal ganglia as described previously (Drummond et al., 1980b, c). Basal adenylate cyclase activity of  $141 \pm 10$  cpm was set equal to 100%. All effects are expressed as percentages (%)  $\pm$  SEM of this basal cyclase activity.

alone or with 5HT stimulates the cyclase activity, because of the rapid conversion of GDP to GTP by the ATP-regeneration system which is included in the assay. This will also occur in a living cell, which emphasizes the necessity of using a nonphosphorylatable analogue such as GDP $\beta$ S in vivo. GppNHp is a more potent stimulator than GTP and at micromolar concentrations can act synergistically with 5HT to stimulate the enzyme activity three- to fourfold (Table I). Fig. 1 shows the dose-dependent inhibition by GDP $\beta$ S of the basal adenylate cyclase activity (A) and of the 5HT/GTP stimulation of the enzyme (B). GDP $\beta$ S never totally inhibits (maximally only 60%) the basal activity and its effects plateau at  $\sim 10^{-6}$  M. The same is true for its effects on 5HT/GTP activation: maximal inhibition is 57% at  $10^{-6}$  M.

*Intraneuronal Injection of Low Concentrations of GDPβS*

When the final intracellular concentration of GDPβS is in the micromolar range, no direct effect on the current-voltage relationship of R15 is observed (see Fig. 2A). This lack of effect on steady state conductances continues for as long as 12 h after the initial injection of GDPβS at the highest (2 μM) concentration tested

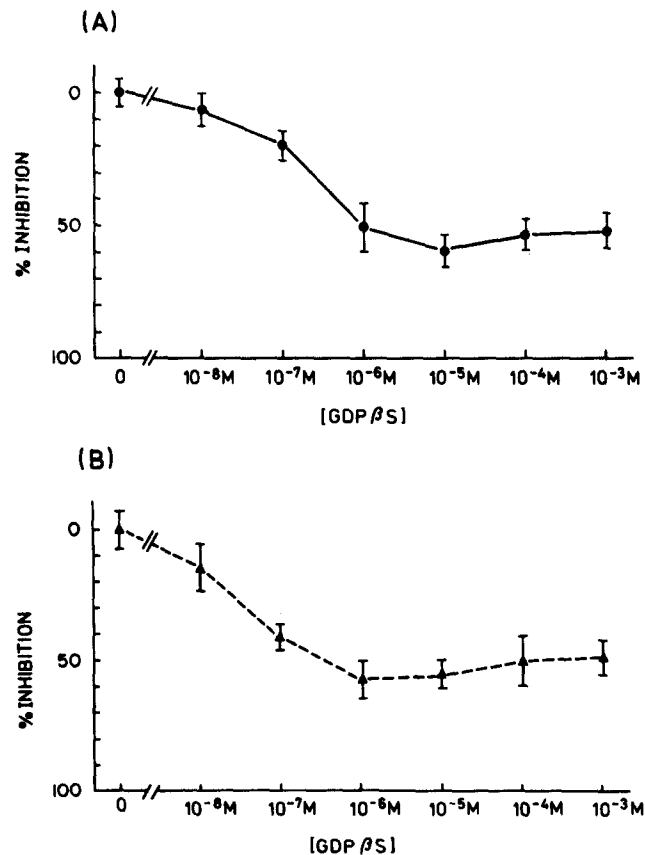


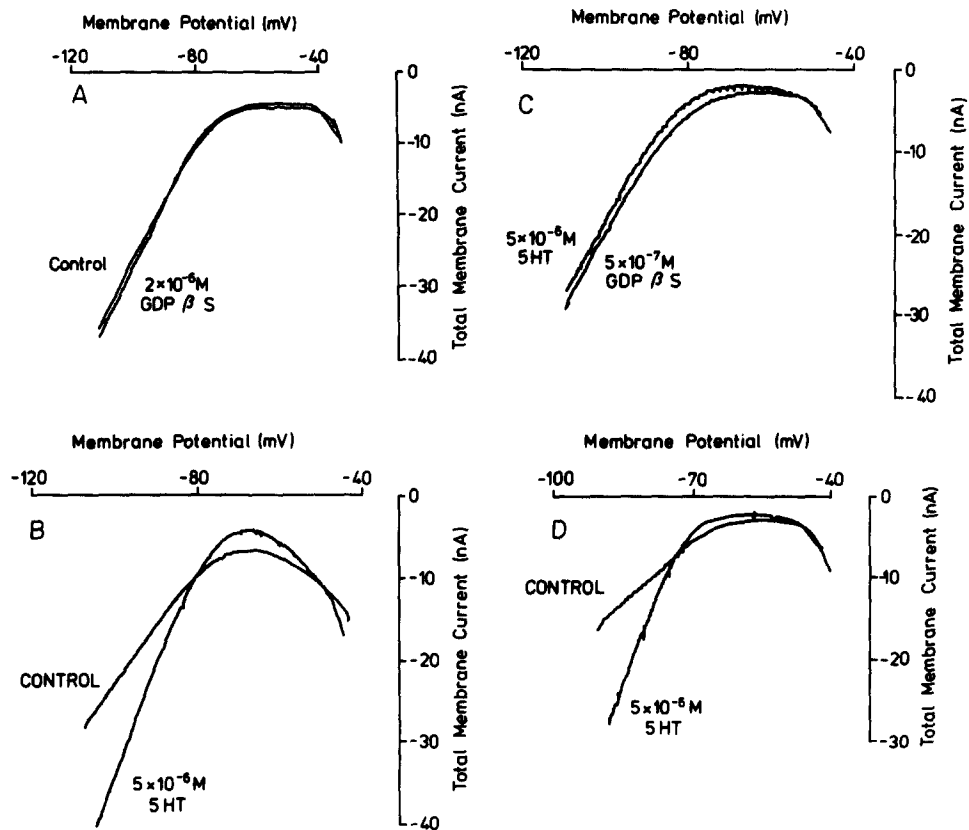
FIGURE 1. Dose-response curves for GDPβS inhibition of adenylate cyclase activity. (A) Adenylate cyclase activity was measured as in Table I using a range of GDPβS concentrations. Basal activity (no additions) was set to 0% inhibition. All values are means  $\pm$  SEM from triplicate assays. (B) Adenylate cyclase activity in the presence of 10<sup>-5</sup> M 5HT and 10<sup>-5</sup> M GTP was measured as in Table I and set to 0% inhibition. The inhibition of this stimulated activity was measured using a range of GDPβS concentrations. Values are means  $\pm$  SEM for triplicates.

in this series of experiments (see below for effects of millimolar concentrations of GDPβS).

*5HT Response*

Perfusion of the abdominal ganglion with  $5 \times 10^{-6}$  M 5HT normally elicits a maximal increase in K<sup>+</sup> conductance (Fig. 2B); in contrast, 5HT had no effect

on R15's previously injected with GDP $\beta$ S at intracellular concentrations as low as 0.5  $\mu$ M (Fig. 2C). This blocking of the 5HT-evoked K<sup>+</sup> conductance increase was observable within 5 min after GDP $\beta$ S injection and persisted for at least 12 h. GDP $\beta$ S, as discussed previously, is hydrolyzed only at very slow rates. By 18–24 h after intracellular injection, however, this GDP analogue seems to be



**FIGURE 2.** Effects of low intracellular concentrations of GDP $\beta$ S. (A) Direct effects. Steady state current-voltage (*I-V*) curves before (control) and 2 h after intraneuronal injection of  $2 \times 10^{-6}$  M GDP $\beta$ S. The membrane potential was swept between  $-120$  and  $-35$  mV ( $dV/dt = 4$  mV/s), and the total membrane current was measured in nanoamperes (nA). (B–D) Effect of micromolar concentrations of GDP $\beta$ S on the 5HT response in R15. (B) Normal response of R15 to 5HT. Steady state *I-V* curves from an uninjected neuron before (control) and 20 min after perfusion of  $5 \times 10^{-6}$  M 5HT. Notice the reversal of sign of the elicited current at about  $-80$  mV, the K<sup>+</sup> equilibrium potential for R15. (C) Block of the 5HT response. Steady state *I-V* curves from the same R15 as in B, after injection of  $5 \times 10^{-7}$  M GDP $\beta$ S. After 20 min of perfusion with  $5 \times 10^{-6}$  M 5HT, no change in the total membrane current was observed. (D) Recovery of the 5HT response. Steady state *I-V* curves from the same R15 as in B and C, 24 h after the injection of GDP $\beta$ S. Perfusion with  $5 \times 10^{-6}$  M 5HT again elicits a current which reverses sign near the K<sup>+</sup> equilibrium potential.

appreciably broken down since 5HT can then elicit a normal increase in K<sup>+</sup> conductance (Fig. 2D). It was not possible to check for such breakdown directly because there was so little GDPβS in a single injected R15.

#### *Dopamine Response*

Both dopamine (DA) (Ascher, 1972) and 5HT (Drummond et al., 1980a) hyperpolarize R15 and inhibit its normal bursting activity, but they do so by

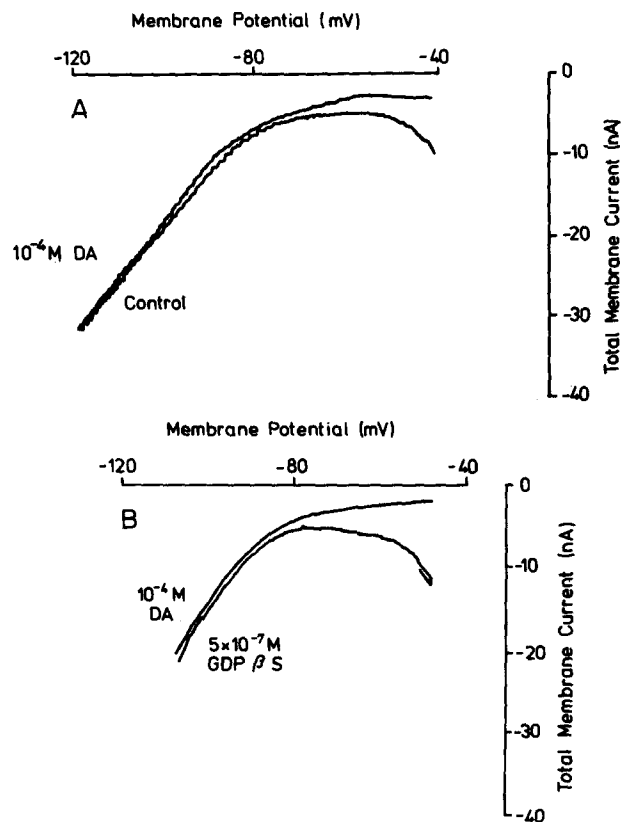


FIGURE 3. Effect of micromolar concentrations of GDPβS on the DA response in R15. (A) Normal response of R15 to DA. Steady state *I-V* curves are from an uninjected R15, before (control) and 20 min after perfusion of the ganglion with 10<sup>-4</sup> M DA. Notice the decrease in inward current, and the lack of effect on the hyperpolarized region of the *I-V* curve, in contrast to 5HT. (B) Response to DA after GDPβS. Steady state *I-V* curves are from R15 injected with a final intracellular concentration of 5 × 10<sup>-7</sup> M GDPβS. Perfusion with 10<sup>-4</sup> M DA still elicits a decrease in inward current after 20 min.

affecting different conductances. 5HT increases the conductance of the anomalously rectifying K<sup>+</sup> current (Benson and Levitan, 1983), while DA appears to decrease a Na<sup>+</sup> and/or Ca<sup>++</sup> current (Wilson and Wachtel, 1978) (Fig. 3A). This change in Na<sup>+</sup>/Ca<sup>++</sup> current is not mimicked by cAMP (Drummond et al.,

1980a), and thus the molecular mechanisms underlying the DA and 5HT responses appear to be different. In addition, a DA-sensitive adenylate cyclase could not be detected in *Aplysia* (Drummond et al., 1980b), although a recent report suggests that high concentrations of DA can increase cAMP levels in R15 (Bernier et al., 1982).

Perfusion of the abdominal ganglion with a maximal concentration ( $10^{-4}$  M) of DA elicits a normal response in cells previously injected with up to 2  $\mu$ M GDP $\beta$ S (Fig. 3B). Under these conditions, the 5HT response is totally blocked (Fig. 2C). Furthermore, this effect of DA washes off within 30 min, as it does in uninjected cells.

#### *cAMP Response*

The  $K^+$  conductance increase elicited by 5HT in R15 can be mimicked by injection or perfusion of nonhydrolyzable cAMP analogues such as 8BTcAMP (Drummond et al., 1980a). (Fig. 4A). If GDP $\beta$ S blocks the 5HT effect by selectively inhibiting the activation of adenylate cyclase, it should be possible to bypass the block by application of 8BTcAMP. Fig. 4 shows that this is indeed the case; when 8BTcAMP is perfused over an R15 which had previously been injected with GDP $\beta$ S, there is an increase in  $K^+$  conductance (Fig. 4B) identical to that elicited by 8BTcAMP in an uninjected cell (Fig. 4A). Thus, GDP $\beta$ S does not appear to alter any of the events in the cAMP cascade subsequent to adenylate cyclase activation.

To further test the possibility that GDP $\beta$ S might be having multiple effects, we examined its action in several in vitro assays. We found that GDP $\beta$ S, at concentrations up to 1 mM, did not change the activity of cAMP or cGMP phosphodiesterase or of cAMP-dependent protein kinase, measured in *Aplysia* ganglion homogenates. We also measured 5HT and DA receptors in membranes from *Aplysia* ganglia using [ $^3$ H]LSD binding (Drummond et al., 1980b, c). Although guanine nucleotides, particularly GTP, can under some conditions influence receptor affinity (Rodbell, 1980), GDP $\beta$ S did not alter the affinity of 5HT or DA for their receptors, as measured by displacement of [ $^3$ H]LSD from the membranes by 5HT and DA (Table II).

#### *Intraneuronal Injection of High Concentrations of GDP $\beta$ S*

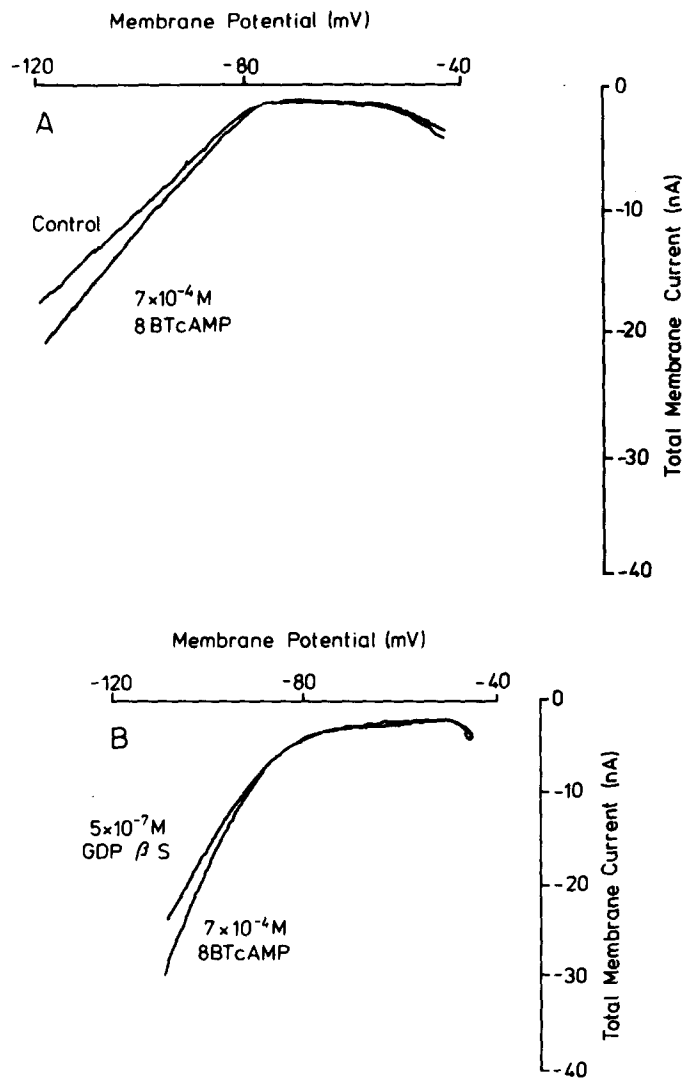
When the final concentration of GDP $\beta$ S is in the millimolar range, there is a direct effect on the current-voltage relationship of R15 (Fig. 5). At these concentrations, GDP $\beta$ S elicits a decrease in inward current similar to that observed with dopamine. This effect becomes apparent 1–2 h after injection and persists for at least 20 h.

#### *5HT Response*

5HT perfused at a concentration of  $10^{-5}$  M, which normally elicits a maximal increase in  $K^+$  conductance, has even larger effects on those R15's that have previously been injected with millimolar concentrations of GDP $\beta$ S (Fig. 5). In contrast to the blocking of the 5HT response seen with micromolar GDP $\beta$ S, high GDP $\beta$ S actually potentiates the conductance increase by severalfold. This



potentiation is seen within 5 min after injection of high GDP $\beta$ S, and the conductance increase cannot be reversed even after 20 h of washing (Fig. 5). Potentiation can also be observed with concentrations of 5HT as low as  $10^{-8}$  M, which normally do not affect R15 (Fig. 6A); when the cell has previously been



**FIGURE 4.** Effect of micromolar concentrations of GDP $\beta$ S on the cAMP response in R15. (A) Normal response of R15 to 8BTcAMP. Steady state *I-V* curves from voltage-clamped uninjected R15, before (control) and 20 min after perfusion with  $7 \times 10^{-4}$  M 8BTcAMP. (B) Response to 8BTcAMP after GDP $\beta$ S. Steady state *I-V* curves from voltage-clamped R15 injected with  $5 \times 10^{-7}$  M GDP $\beta$ S. 8BTcAMP elicits an increase in K<sup>+</sup> conductance comparable to that in A. The experiments in A and B were on two separate cells. Note the difference in the current scales.

TABLE II  
Effects of GDP $\beta$ S on *d*-[<sup>3</sup>H]LSD Binding to Serotonin and Dopamine Receptors in the *Aplysia* Nervous System

Parameter tested	Control	10 <sup>-4</sup> M GDP $\beta$ S
<i>fmol/mg protein</i>		
Total specific [ <sup>3</sup> H]LSD binding		
(a) to serotonin site	104±23	95±16
(b) to dopamine site	71±5	79±11
<i>IC<sub>50</sub> (μM)</i>		
Half-maximal displacement of [ <sup>3</sup> H]LSD		
(a) from serotonin site by serotonin	1.6	2.3
(b) from dopamine site by dopamine	6.4	5.1

The binding of *d*-[<sup>3</sup>H]LSD to membranes derived from *Aplysia* ganglia was measured as described by Drummond et al. (1980*b, c*), in the presence or absence of 10<sup>-4</sup> M GDP $\beta$ S. The IC<sub>50</sub> value is the concentration of serotonin or dopamine required to inhibit [<sup>3</sup>H]LSD binding to the serotonin or dopamine site, respectively, by 50%. GDP $\beta$ S had no significant effect on any of the binding parameters measured.

injected with millimolar GDP $\beta$ S, 10<sup>-8</sup> M 5HT can elicit a large conductance increase that does not reverse upon washing (Fig. 6*B*).

#### DA Response

To examine the specificity of these effects, ganglia were perfused with DA after the injection of high concentrations of GDP $\beta$ S into R15. Up to several hours after the injection, DA was still capable of evoking a decrease in inward current

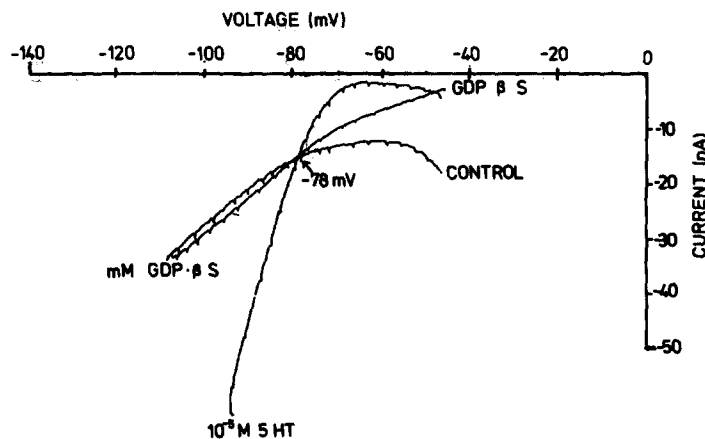


FIGURE 5. Direct effects of high intracellular concentrations of GDP $\beta$ S, and potentiation of the 5HT response. *I-V* curves from voltage-clamped R15 before (control) and 2 h after intraneuronal injection of GDP $\beta$ S at millimolar concentration. GDP $\beta$ S causes a decrease in inward current at depolarized potentials. Subsequent perfusion of 10<sup>-5</sup> M 5HT elicits a large increase in K<sup>+</sup> current within 10 min. This response does not reverse upon washing with normal medium for 20 h (as long as we could hold this cell).

(data not shown). However, if DA was applied after GDP $\beta$ S had already decreased the inward current (see Fig. 5), it did not elicit any further decrease, which suggests that DA and millimolar GDP $\beta$ S may be affecting the same inward current.

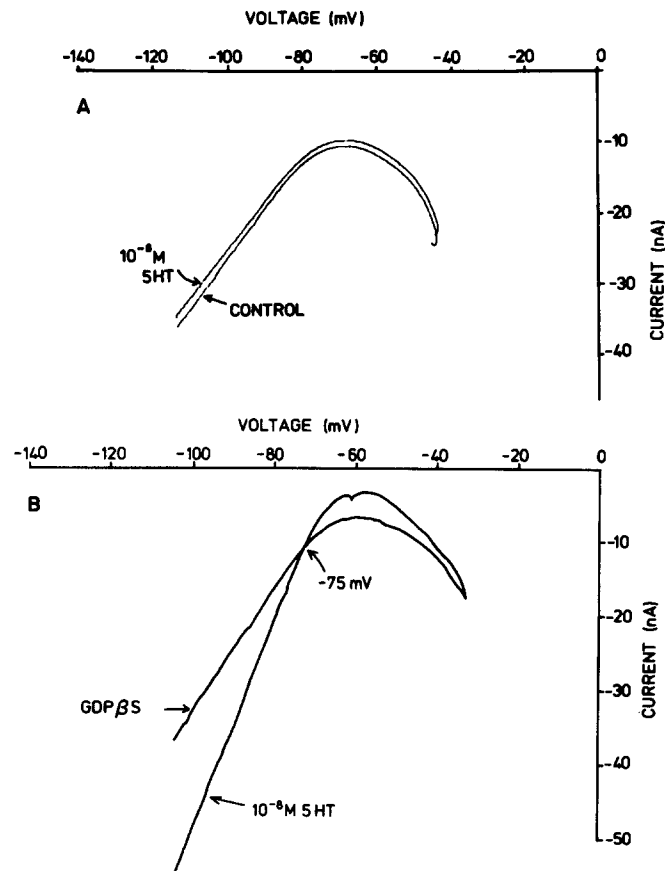


FIGURE 6. Effects of millimolar GDP $\beta$ S on the response to subthreshold concentrations of 5HT. (A) Steady state  $I$ - $V$  curve from un.injected R15 before (control) and after perfusion with  $10^{-8}$  M 5HT. This concentration is normally subthreshold and does not elicit any increase in current. (B) Response after high GDP $\beta$ S. Steady state  $I$ - $V$  curves from voltage-clamped R15 15 min after injection of millimolar GDP $\beta$ S (control) and 20 min after subsequent perfusion with  $10^{-8}$  M 5HT. 5HT elicits a large increase in K<sup>+</sup> current which does not reverse upon washing for 6 h.

#### *cAMP Response*

We also found that 8BTcAMP could elicit a normal increase in K<sup>+</sup> conductance in R15's that had been injected with high concentrations of GDP $\beta$ S (Fig. 7). Thus, the potentiation of the 5HT response described above is not due to an effect of GDP $\beta$ S directly on the 5HT/cAMP-sensitive K<sup>+</sup> channel; rather, high

GDP $\beta$ S must be affecting some step in the 5HT response prior to the rise in intracellular cAMP.

#### *Intraneuronal Injection of GppNHp*

Treistman and Levitan (1976*b*) have shown that injection of a GTP analogue resistant to hydrolysis, GppNHp, into cell R15 can cause it to hyperpolarize, and that GppNHp is a potent activator of *Aplysia* nervous system adenylate cyclase. Treistman (1981) subsequently found that GppNHp injection produces an increase in conductance, presumably to K<sup>+</sup>. These experiments were conducted

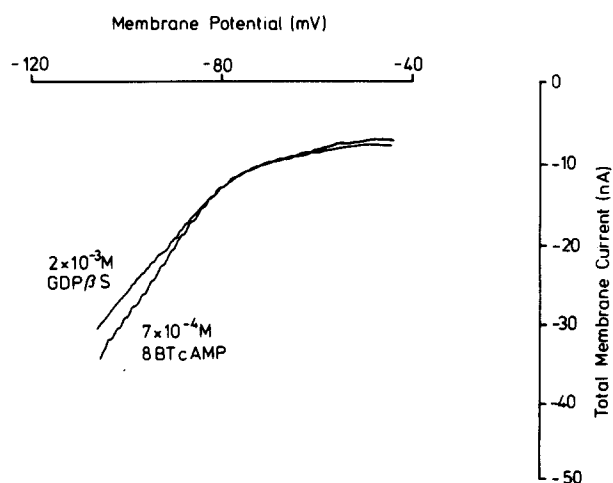


FIGURE 7. cAMP response after high GDP $\beta$ S. Steady state  $I$ - $V$  curves from R15 2 h after injection with GDP $\beta$ S (final intracellular concentration 2 mM). Perfusion with  $7 \times 10^{-4}$  M 8BTcAMP elicits a normal increase in K<sup>+</sup> current. By this time GDP $\beta$ S has decreased the inward current (compare with Fig. 5). Perfusion with  $10^{-4}$  M DA did not decrease this current any further.

using intracellular concentrations of GppNHp in the millimolar range. In order to test the possibility that the high GDP $\beta$ S effects might be due to contamination by a GTP analogue, we tested low (micromolar) concentrations of GppNHp on the 5HT response of R15. Intracellular concentrations of 0.2–1  $\mu$ M GppNHp mimic the 5HT response (Fig. 8) within 2–5 min after injection, confirming the former results (Treistman, 1981). Furthermore, the cell is unable to respond to 5HT for at least 6 h after GppNHp injection, presumably because the K<sup>+</sup> conductance is maximally activated by GppNHp. These results are markedly different from those produced by high GDP $\beta$ S, and thus it is unlikely that the effects of high GDP $\beta$ S are due to contamination by an agonist such as GppNHp.

We also studied the effects of GppNHp and low concentrations of GDP $\beta$ S injected together into R15. The increase in K<sup>+</sup> conductance normally elicited by GppNHp (Fig. 8) is absent when GDP $\beta$ S is injected at the same time (Fig. 9). No conductance changes were observed in cells monitored as long as 4 h after such injections. Furthermore, no 5HT response can be elicited in cells injected with

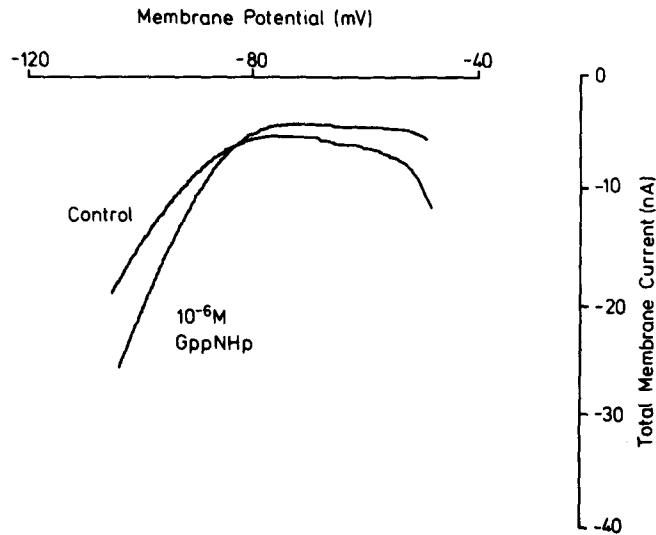


FIGURE 8. Effects of micromolar GppNHp. Steady state  $I$ - $V$  curves from voltage-clamped R15 before (control) and after intraneuronal injection of  $10^{-6}$  M GppNHp. Note the reversal of the evoked current near  $E_K$ .

the mixture of GppNHp and GDP $\beta$ S. These results confirm that GDP $\beta$ S can antagonize the effects of a GTP analogue *in vivo* as well as *in vitro* (Table I), and indicate that GDP $\beta$ S inhibition of the 5HT response is indeed due to competition with intracellular GTP.

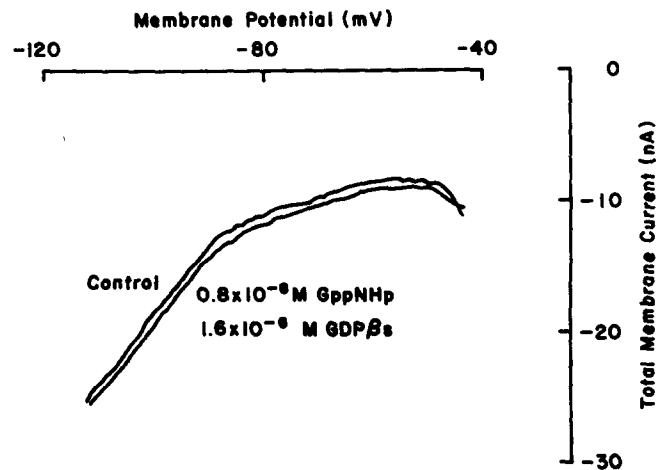


FIGURE 9. Effects of GppNHp and GDP $\beta$ S injected together. Steady state  $I$ - $V$  curves from voltage-clamped R15 before (control) and 20 min after injection of  $0.8 \times 10^{-6}$  M GppNHp together with  $1.6 \times 10^{-6}$  M GDP $\beta$ S. The increase in K<sup>+</sup> conductance normally elicited by GppNHp (see Fig. 8) does not occur when GDP $\beta$ S is also injected. The  $I$ - $V$  curve did not change appreciably during the period (>3 h) after injection that the cell was monitored.

## DISCUSSION

It is becoming evident that many actions of neurotransmitters on membrane ion conductances involve metabolic modifications in the target cell. Many of these metabolically mediated effects exhibit slow kinetics, and the ion conductances affected are often voltage dependent (Nicoll, 1982), in contrast to the better-understood fast actions of transmitters on voltage-independent conductances. One widely studied metabolic modification is the neurotransmitter-induced activation of adenylate cyclase. Although a number of workers have used activators of adenylate cyclase to mimic neurotransmitter responses, only recently has a specific inhibitor of the enzyme, which could be utilized to determine whether cAMP production is necessary for a neurotransmitter response, become available. Eckstein et al. (1979) synthesized GDP $\beta$ S, an analogue of GDP, and showed it to be only slowly hydrolyzed and partially (5%) phosphorylated under in vivo conditions. Furthermore, they showed it to be a specific inhibitor of adenylate cyclase in turkey erythrocytes and a partial agonist (at high concentrations) in the rat parotid. GDP $\beta$ S inhibits the cyclase by interacting with the guanyl nucleotide binding protein (N protein), which is necessary for the neurohormone-dependent activation of the enzyme (Rodbell, 1980). The model of Cassel et al. (1979) hypothesizes that receptor occupancy by an agonist is necessary for the release of GDP from the N protein and the subsequent binding of GTP to the same site. The GTP-N protein complex can then activate adenylate cyclase and increase cAMP levels within the cell. The adenylate cyclase is inactivated by the hydrolysis of GTP to GDP by a GTPase closely allied to the N protein, and GDP $\beta$ S apparently inhibits the cyclase by competing with both GTP and GDP for the guanyl nucleotide binding site on this protein. Once bound, GDP $\beta$ S is not easily dissociated and prevents the activation of the enzyme by GTP (Cassel et al., 1979).

In order to determine whether the stimulation of adenylate cyclase by 5HT is a necessary step in the activation of the anomalously rectifying K<sup>+</sup> channel in neuron R15, it was first necessary to test whether GDP $\beta$ S can inhibit adenylate cyclase in the *Aplysia* nervous system. Our results show that GDP $\beta$ S partially inhibits both the basal and 5HT-stimulated adenylate cyclase activities in membranes prepared from *Aplysia* abdominal ganglia. It is important to note that the inhibitory effects of GDP $\beta$ S are already maximal at micromolar concentrations.

If adenylate cyclase stimulation is necessary for the 5HT response, then GDP $\beta$ S at the appropriate concentration should be able to block the increase in K<sup>+</sup> conductance usually elicited in R15 by 5HT. It is possible to inject known quantities of specific probes (Lemos et al., 1982; Adams and Levitan, 1982) into R15 without disturbing the steady state *I-V* relationship of the cell. GDP $\beta$ S, when injected into this neuron at micromolar concentrations, had no direct effect on steady state conductances for at least 20 h. (See Table III for a summary of GDP $\beta$ S effects on R15.) However, at concentrations as low as 0.5  $\mu$ M, GDP $\beta$ S totally blocked the 5HT activation of K<sup>+</sup> conductance as early as 5 min after its injection into R15. This block persisted for up to 12 h, but the cell was eventually able to show a normal response to 5HT after 18–24 h. The fact that the same cell could respond normally both before and many hours after GDP $\beta$ S injection

shows that the cell is capable of responding to 5HT whenever GDP $\beta$ S is not present at appropriate concentrations. It is somewhat surprising that GDP $\beta$ S can completely block the 5HT-induced increase in K<sup>+</sup> conductance but only partially inhibits adenylate cyclase activity. We do not have a good explanation for this finding, although it may reflect the general problem of comparing *in vitro* with *in vivo* experiments. For example, it seems possible that GDP $\beta$ S can act as a partial agonist at micromolar concentrations *in vitro*, as it does in rat parotid (Cassel et al., 1979), but can only exhibit partial agonist properties at higher concentrations *in vivo*.

The effects of millimolar concentrations of GDP $\beta$ S are more difficult to explain. One possibility is that GDP $\beta$ S produces side effects because of actions on guanyl nucleotide-dependent enzyme systems not related to adenylate cyclase. In this regard it is worthy of note that its "DA-like" action (a decrease in inward

TABLE III  
*Summary of the Effects of High and Low Concentrations of GDP $\beta$ S on Neuron R15*

Parameter measured	Micromolar GDP $\beta$ S	Millimolar GDP $\beta$ S
Resting <i>I-V</i> relationship	No effect	Decrease in inward current (DA-like effect)
5HT-evoked increase in K <sup>+</sup> conductance	Blocked	Potentiated and does not reverse
cAMP-evoked increase in K <sup>+</sup> conductance	No effect	No effect
DA-evoked decrease in inward current	No effect	No effect

current) on the steady state *I-V* relationship of R15 is also elicited by millimolar concentrations of GppNHp (Treisman, 1981). Another possibility is that, at high concentrations, GDP $\beta$ S can act as a partial agonist *in vivo* as discussed above; this might account for the enhanced response to 5HT observed in cells injected with millimolar concentrations of GDP $\beta$ S. These effects of high GDP $\beta$ S will have to be explored further in order to determine whether they are simply nonspecific side effects, or are indeed relevant to our understanding of the physiological regulation of ion conductance in R15.

The GDP $\beta$ S block is specific for the cAMP pathway since DA, which appears not to act via this intracellular messenger in R15, is able to produce normal conductance changes in the presence of the same concentrations of GDP $\beta$ S that block the 5HT response. Furthermore, the block occurs before the elevation of cAMP levels since it can be circumvented by perfusion of 8BTcAMP, a nonhydrolyzable analogue of cAMP. Thus, the anomalously rectifying K<sup>+</sup> channels are still capable of being activated by cAMP in GDP $\beta$ S-injected R15's. The action of the analogue, therefore, must be in the chain of events leading up to the elevation of cAMP, i.e., the binding of 5HT to its receptor and the subsequent stimulation of adenylate cyclase. Since our results demonstrate that GDP $\beta$ S does not affect the number or affinity of 5HT receptors in *Aplysia*, we conclude that

the block is at the adenylate cyclase step, as is found in vitro. These findings using micromolar concentrations of GDP $\beta$ S are entirely consistent with the suggestion that adenylate cyclase activation is a necessary step in the sequence of events leading to activation of a specific K<sup>+</sup> channel in neuron R15.

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