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 GDPβS on the response of Aplysia neuron R15 to serotonin (5HT) were investigated. Previous studies have demonstrated that 5HT causes an increase in K+ conductance in R15 and that the response is mediated by cAMP. At concentrations in the micromolar range, GDPβS inhibits the stimulation of adenylate cyclase by 5HT in particulate fractions from Aplysia ganglia. When micromolar concentrations of GDPβS are injected into neuron R15, there is no effect on the resting membrane conductance, but the increase in K+ 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 GDPβS. In addition, application of 8-benzylthio cAMP to R15 can evoke an increase in K+ conductance even after the injection of GDPβS, which indicates that events subsequent to the activation of adenylate cyclase are not inhibited by the GDP analogue. In contrast, when millimolar concentrations of GDPβS 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 GDPβS 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+ 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
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; Treistman and Levitan 1976a, b; Brunelli et al., 1976; Kaczmarek et al., 1978; Pellmar, 1981). Furthermore, in several different molluscan neurons, cAMP appears to mediate changes in K⁺ conductance elicited by the neurotransmitter serotonin (5HT) (Klein and Kandel, 1978; Klein et al., 1982; Drummond et al., 1980a; Deterre et al., 1981; Siegelbaum et al., 1982). In the identified *Aplysia* neuron R15, 5HT causes an increase in an inwardly rectifying K⁺ current (Drummond et al., 1980a; 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., 1980a).

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, GDPβS, 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 GDPβS inhibit *Aplysia* adenylate cyclase activity and also selectively inhibit the K⁺ conductance increase elicited by 5HT.

**MATERIALS AND METHODS**

**Materials**

α[³²P]ATP (5–20 Ci/mm mol) 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) (GDPβS) 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 MgCl₂, 1 mM cAMP, 20 mM creatine phosphate, 80 U/ml creatine phosphokinase, and 0.5 mM [³²P]ATP (specific activity 150–250 mCi/mmol, to give 5–10 × 10⁶ 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 [³²P]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. (1980b, c), using [³H]LSD as the receptor ligand. [³H]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., 1980b, 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 GDP/βS 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 GDP/βS or GppNHp, dissolved in distilled H₂O 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 N₂ gas. Such injections did not alter the current-voltage relationship of R15.
RESULTS

GDPβS 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%. GDPβS inhibits both the basal and the 5HT/GTP-stimulated enzyme activity. In contrast, GDP either

| Additions to assay | Percent basal cyclase activity |
|-------------------|-------------------------------|
| 10⁻⁴ M GTP        | 161±15                        |
| 10⁻⁵ M GTP        | 125±16                        |
| 10⁻⁶ M GTP        | 115±8.5                       |
| 10⁻⁴ M GDPβS      | 48±5                          |
| 10⁻³ M GDPβS      | 40±4                          |
| 10⁻⁵ M GDPβS      | 50±3                          |
| 10⁻⁴ M 5HT        | 101±7                         |
| 10⁻³ M 5HT + 10⁻⁵ M GTP | 357±14                     |
| 10⁻³ M 5HT + 10⁻⁴ M GDPβS | 36±3                  |
| 10⁻³ M 5HT + 10⁻⁴ M GDPβS + 10⁻⁵ M GTP | 167±10             |
| 10⁻⁴ M GDP        | 170±4                         |
| 10⁻⁵ M 5HT + 10⁻⁴ M GDP | 395±9                    |
| 10⁻⁴ M GppNHp     | 214±5.5                       |
| 10⁻⁵ M 5HT + 10⁻⁴ M GppNHp | 367±10                  |

Adenylate cyclase activity was assayed by the conversion of [³²P]ATP to [³²P]cAMP using membranes prepared from Aplysia abdominal ganglia as described previously (Drummond et al., 1980b, c). Basal adenylate cyclase activity of 141 ± 10 cpm was set equal to 100%. All effects are expressed as percentages (%) ± 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 GDPβS 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 GDPβS of the basal adenylate cyclase activity (A) and of the 5HT/GTP stimulation of the enzyme (B). GDPβS never totally inhibits (maximally only 60%) the basal activity and its effects plateau at ~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 ± SEM from triplicate assays. (B) Adenylate cyclase activity in the presence of $10^{-5}$ M 5HT and $10^{-5}$ 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 ± SEM for triplicates.

5HT Response

Perfusion of the abdominal ganglion with $5 \times 10^{-6}$ M 5HT normally elicits a maximal increase in K⁺ conductance (Fig. 2B); in contrast, 5HT had no effect
on R15's previously injected with GDPβS at intracellular concentrations as low as 0.5 μM (Fig. 2C). This blocking of the 5HT-evoked K⁺ conductance increase was observable within 5 min after GDPβS injection and persisted for at least 12 h. GDPβS, as discussed previously, is hydrolyzed only at very slow rates. By 18–24 h after intracellular injection, however, this GDP analogue seems to be

![Diagram](image-url)
appreciably broken down since 5HT can then elicit a normal increase in K+ 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 affecting different conductances. 5HT increases the conductance of the anomalously rectifying K+ current (Benson and Levitan, 1983), while DA appears to decrease a Na+ and/or Ca++ current (Wilson and Wachtel, 1978) (Fig. 3A). This change in Na+/Ca++ current is not mimicked by cAMP (Drummond et al.,

**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^{-4} 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 \times 10^{-7} M GDPβS. Perfusion with 10^{-4} M DA still elicits a decrease in inward current after 20 min.
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 uM GDPβS (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 GDPβS 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 GDPβS, there is an increase in K⁺ conductance (Fig. 4B) identical to that elicited by 8BtCAMP in an uninjected cell (Fig. 4A). Thus, GDPβS does not appear to alter any of the events in the cAMP cascade subsequent to adenylate cyclase activation.

To further test the possibility that GDPβS might be having multiple effects, we examined its action in several in vitro assays. We found that GDPβS, 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 [³H]LSD binding (Drummond et al., 1980b, c). Although guanine nucleotides, particularly GTP, can under some conditions influence receptor affinity (Rodbell, 1980), GDPβS did not alter the affinity of 5HT or DA for their receptors, as measured by displacement of [³H]LSD from the membranes by 5HT and DA (Table II).

**Intraneuronal Injection of High Concentrations of GDPβS**

When the final concentration of GDPβS is in the millimolar range, there is a direct effect on the current-voltage relationship of R15 (Fig. 5). At these concentrations, GDPβS 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 GDPβS (Fig. 5). In contrast to the blocking of the 5HT response seen with micromolar GDPβS, high GDPβS actually potentiates the conductance increase by severalfold. This
potentiation is seen within 5 min after injection of high GDPβS, 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

![Graph A](image1.png)

**Figure 4.** Effect of micromolar concentrations of GDPβS 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 x 10^{-4} M 8BTcAMP. (B) Response to 8BTcAMP after GDPβS. Steady state I-V curves from voltage-clamped R15 injected with 5 x 10^{-7} M GDPβS. 8BTcAMP elicits an increase in K^+ 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 GDPβS on d-[³H]LSD Binding to Serotonin and Dopamine Receptors in the Aplysia Nervous System

| Parameter tested                             | Control | 10⁻⁴ M GDPβS |
|----------------------------------------------|---------|-------------|
| Total specific [³H]LSD binding (fmol/mg protein) |         |             |
| (a) to serotonin site                         | 104±23  | 95±16       |
| (b) to dopamine site                          | 71±5    | 79±11       |
| Half-maximal displacement of [³H]LSD (µM)     |         |             |
| (a) from serotonin site by serotonin          | 1.6     | 2.3         |
| (b) from dopamine site by dopamine            | 6.4     | 5.1         |

The binding of d-[³H]LSD to membranes derived from Aplysia ganglia was measured as described by Drummond et al. (1980a, c), in the presence or absence of 10⁻⁴ M GDPβS. The IC₅₀ value is the concentration of serotonin or dopamine required to inhibit [³H]LSD binding to the serotonin or dopamine site, respectively, by 50%. GDPβS had no significant effect on any of the binding parameters measured.

Injected with millimolar GDPβS, 10⁻⁸ M 5HT can elicit a large conductance increase that does not reverse upon washing (Fig. 6B).

**DA Response**

To examine the specificity of these effects, ganglia were perfused with DA after the injection of high concentrations of GDPβS 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 GDPβS, and potentiation of the 5HT response. I-V curves from voltage-clamped R15 before (control) and 2 h after intraneuronal injection of GDPβS at millimolar concentration. GDPβS causes a decrease in inward current at depolarized potentials. Subsequent perfusion of 10⁻⁸ M 5HT elicits a large increase in K⁺ 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).](image)
(data not shown). However, if DA was applied after GDPβS had already decreased the inward current (see Fig. 5), it did not elicit any further decrease, which suggests that DA and millimolar GDPβS may be affecting the same inward current.

**Figure 6.** Effects of millimolar GDPβS on the response to subthreshold concentrations of 5HT. (A) Steady state $I-V$ curve from uninjected 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 GDPβS. Steady state $I-V$ curves from voltage-clamped R15 15 min after injection of millimolar GDPβS (control) and 20 min after subsequent perfusion with $10^{-8}$ M 5HT. 5HT elicits a large increase in K$^+$ current which does not reverse upon washing for 6 h.

cAMP Response

We also found that 8BTeAMP could elicit a normal increase in K$^+$ conductance in R15's that had been injected with high concentrations of GDPβS (Fig. 7). Thus, the potentiation of the 5HT response described above is not due to an effect of GDPβS directly on the 5HT/cAMP-sensitive K$^+$ channel, rather, high
GDPβS must be affecting some step in the 5HT response prior to the rise in intracellular cAMP.

Intraneuronal Injection of GppNHp

Treistman and Levitan (1976b) 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⁺. These experiments were conducted using intracellular concentrations of GppNHp in the millimolar range. In order to test the possibility that the high GDPβS 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 μM 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⁺ conductance is maximally activated by GppNHp. These results are markedly different from those produced by high GDPβS, and thus it is unlikely that the effects of high GDPβS are due to contamination by an agonist such as GppNHp.

We also studied the effects of GppNHp and low concentrations of GDPβS injected together into R15. The increase in K⁺ conductance normally elicited by GppNHp (Fig. 8) is absent when GDPβS 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 GDPβS. These results confirm that GDPβS can antagonize the effects of a GTP analogue in vivo as well as in vitro (Table I), and indicate that GDPβS inhibition of the 5HT response is indeed due to competition with intracellular GTP.

FIGURE 9. Effects of GppNHp and GDPβS 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 GDPβS. The increase in $K^+$ conductance normally elicited by GppNHp (see Fig. 8) does not occur when GDPβS 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 GDPβS, 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. GDPβS 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 GDPβS apparently inhibits the cyclase by competing with both GTP and GDP for the guanyl nucleotide binding site on this protein. Once bound, GDPβS 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+ channel in neuron R15, it was first necessary to test whether GDPβS can inhibit adenylate cyclase in the Aplysia nervous system. Our results show that GDPβS 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 GDPβS are already maximal at micromolar concentrations.

If adenylate cyclase stimulation is necessary for the 5HT response, then GDPβS at the appropriate concentration should be able to block the increase in K+ 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. GDPβS, 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 GDPβS effects on R15.) However, at concentrations as low as 0.5 μM, GDPβS totally blocked the 5HT activation of K+ 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 GDPβS injection
shows that the cell is capable of responding to 5HT whenever GDPβS is not present at appropriate concentrations. It is somewhat surprising that GDPβS can completely block the 5HT-induced increase in K⁺ 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 GDPβS 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 GDPβS are more difficult to explain. One possibility is that GDPβS 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 current) on the steady state $I-V$ relationship of R15 is also elicited by millimolar concentrations of GppNHp (Treistman, 1981). Another possibility is that, at high concentrations, GDPβS 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 GDPβS. These effects of high GDPβS 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 GDPβS 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 GDPβS 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⁺ channels are still capable of being activated by cAMP in GDPβS-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 GDPβS does not affect the number or affinity of 5HT receptors in Aplysia, we conclude that

| Parameter measured | Micromolar GDPβS | Millimolar GDPβS |
|-------------------|-----------------|-----------------|
| Resting $I-V$ relationship | No effect | Decrease in inward current (DA-like effect) |
| 5HT-evoked increase in K⁺ conductance | Blocked | Potentiated and does not reverse |
| cAMP-evoked increase in K⁺ conductance | No effect | No effect |
| DA-evoked decrease in inward current | No effect | No effect |
the block is at the adenylate cyclase step, as is found in vitro. These findings using micromolar concentrations of GDP\$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\(^+\) channel in neuron R15.

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REFERENCES

Adams, W. B., and I. B. Levitan. 1982. Intracellular injection of protein kinase inhibitor blocks the serotonin-induced increase in K\(^+\) conductance in Aplysia neuron R15. Proc. Natl. Acad. Sci. USA. 79:3877–3880.

Ascher, P. 1972. Inhibitory and excitatory effects of dopamine on Aplysia neurones. J. Physiol. (Lond.). 225:173–209.

Benson, J. A., and I. B. Levitan. 1983. Serotonin increases an anomalously rectifying K\(^+\) current in the Aplysia neuron R15. Proc. Natl. Acad. Sci. USA. 80:3522–3525.

Bernier, L., V. F. Castellucci, E. R. Kandel, and J. H. Schwartz. 1982. Facilitatory transmitter causes a selective and prolonged increase in cAMP in sensory neurons mediating the gill and siphon withdrawal reflex in Aplysia. J. Neurosci. 2:1682–1691.

Brunelli, M., V. Castellucci, and E. R. Kandel. 1976. Synaptic facilitation and behavioral sensitization in Aplysia: possible role of serotonin and cyclic AMP. Science (Wash. DC). 194:1178–1181.

Cassel, D., F. Eckstein, M. Lowe, and Z. Selinger. 1979. Determination of the turn-off reaction for the hormone-activated adenylate cyclase. J. Biol. Chem. 254:9835–9838.

Deterre, P., D. Paupardin-Tritsch, J. Bockaert, and H. M. Gerschenfeld. 1981. Role of cyclic AMP in a serotonin-evoked slow inward current in snail neurones. Nature (Lond.). 290:783–785.

Drummond, A. H., J. A. Benson, and I. B. Levitan. 1980a. Serotonin-induced hyperpolarization of an identified Aplysia neuron is mediated by cyclic AMP. Proc. Natl. Acad. Sci. USA. 77:5013–5017.

Drummond, A. H., F. Bucher, and I. B. Levitan. 1980b. Distribution of serotonin and dopamine receptors in Aplysia tissues: analysis by \(^{3}H\)LSD binding and adenylate cyclase stimulation. Brain Res. 184:163–177.

Drummond, A. H., F. Bucher, and I. B. Levitan. 1980c. \(^{3}H\)LSD binding to serotonin receptors in the molluscan nervous system. J. Biol. Chem. 255:6679–6686.

Eckstein, F., D. Cassel, H. Levkowitz, M. Lowe, and Z. Selinger. 1979. Guanosine 5\'O(2-thiodiphosphate): an inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. J. Biol. Chem. 254:9829–9834.

Greengard, P. 1978. Phosphorylated proteins as physiological effectors. Science (Wash. DC). 199:146–152.

Kaczmarek, L., K. Jennings, and F. Strumwasser. 1978. Neurotransmitter modulation, phosphodiesterase inhibitor effects, and cyclic AMP correlates of afterdischarge in peptidergic neurites. Proc. Natl. Acad. Sci. USA. 75:5200–5204.

Klein, M., J. Camardo, and E. R. Kandel. 1982. Serotonin modulates a specific K\(^+\) current in
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the sensory neurons that show presynaptic facilitation in Aplysia. Proc. Natl. Acad. Sci. USA. 79:5713–5717.

Klein, M., and E. R. Kandel. 1978. Presynaptic modulation of voltage-dependent Ca²⁺ current: mechanism for behavioral sensitization in Aplysia californica. Proc. Natl. Acad. Sci. USA. 75:3512–3516.

Lemos, J. R., I. Novak-Hofer, and I. B. Levitan. 1982. Serotonin alters the phosphorylation of specific proteins inside a single living nerve cell. Nature (Lond.). 298:64–65.

Levitan, I. B., and S. H. Barondes. 1974. Octopamine- and serotonin-stimulated phosphorylation of specific protein in the abdominal ganglion of Aplysia. Proc. Natl. Acad. Sci. USA. 71:1145–1148.

Levitan, I. B., E. Bergstroem, and M. Simonet. 1978. Adenylate cyclase in Helix and Aplysia ganglia. J. Neurochem. 31:1553–1559.

Neher, E., and B. Sakmann. 1976. Single channel currents recorded from membrane of denervated frog muscle fibers. Nature (Lond.). 260:799–802.

Nicoll, R. A. 1982. Neurotransmitters can say more than just yes or no. Trends Neurosci. 5:369–374.

Pellmar, T. 1981. Ionic mechanism of a voltage-dependent current elicited by cyclic AMP. Cell. Mol. Neurobiol. 1:87–97.

Robison, G. A., R. Butcher, and E. Sutherland. 1971. Cyclic AMP. Academic Press, Inc., New York.

Rodbell, M. 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature (Lond.). 284:17–22.

Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58:541–548.

Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. 56:502–514.

Siegelbaum, S. A., J. S. Camardo, and E. R. Kandel. 1982. Serotonin and cAMP close single K⁺ channels in Aplysia sensory neurons. Nature (Lond.). 299:413–417.

Treistman, S. 1981. Effect of adenosine 3',5'-monophosphate on neuronal pacemaker activity: a voltage clamp analysis. Science (Wash. DC). 211:59–61.

Treistman, S. N., and I. B. Levitan. 1976a. Alteration of electrical activity in molluscan neurones by cyclic nucleotides and peptide factors. Nature (Lond.). 261:62–64.

Treistman, S. N., and I. B. Levitan. 1976b. Intraneuronal guanylylimidodiphosphate injection mimics long-term synaptic hyperpolarization in Aplysia. Proc. Natl. Acad. Sci. USA. 73:4689–4692.

Tsien, R. W. 1973. Adrenaline-like effects of intracellular iontophoresis of cAMP in cardiac purkinje fibres. Nat. New Biol. 245:120–122.

Tsien, R. W., W. Giles, and P. Greengard. 1972. Cyclic AMP mediates the effects of adrenaline on cardiac Purkinje fibers. Nat. New Biol. 240:181–183.

Wilson, W., and M. Goldner. 1975. Voltage clamping with a single microelectrode. J. Neurobiol. 6:411–422.

Wilson, W., and H. Wachtel. 1978. Prolonged inhibition in burst firing neurons: synaptic inactivation of the slow regenerative inward current. Science (Wash. DC). 202:772–775.