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Intraneuronal guanylyl-imidodiphosphate injection mimics long-term synaptic hyperpolarization in *Aplysia*

(adenosine 3′:5′-cyclic monophosphate/adenylate cyclase activation/phosphodiesterase inhibition)

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ABSTRACT **The phosphodiesterase (3′:5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) inhibitor theophylline enhances both the amplitude and duration of a long-lasting synaptic hyperpolarization in identified neuron R15 in *Aplysia californica*. Intraneuronal injection into R15 of guanylyl-imidodiphosphate, an adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activator, results in a deep and long-lasting hyperpolarization of the cell, similar to that poduced by synaptic stimulation. Biochemical analysis confirms that guanylyl-imidodiphosphate activates adenylate cyclase in *Aplysia californica* nervous tissue, without affecting phosphodiesterase activity.**

These observations suggest that adenosine 3′:5′-cyclic monophosphate plays a role in long-lasting synaptic inhibition and are consistent with a post-synaptic site of action for adenosine 3′:5′-cyclic monophosphate.

The mechanisms underlying long-term modulation of neuronal membrane properties are not well understood. In several preparations, cyclic nucleotide fluctuations in the post-synaptic cell may form a biochemical bridge between synaptic stimulation, and the resulting changes in electrical potential and properties of the neuron (1, 2). Identified neuron R15, in *Aplysia californica*, is an excellent model neuron in which to study long-term changes in electrical properties, because it exhibits a regular pattern of endogenous "bursting" activity consisting of alternating depolarizing and hyperpolarizing membrane oscillations, and is subject to alterations in activity lasting hours after synaptic and hormonal stimulation (3, 4). We have reported that peptide hormones which affect the bursting pattern of R15 alter the levels of cyclic nucleotides in *Aplysia* nervous tissue, and that exposure of R15 to certain phosphodiesterase inhibitors and cyclic nucleotide derivatives causes altered electrical patterns (5), similar to those produced by hormones (3). Furthermore, there is an increased production of adenosine 3′:5′-cyclic monophosphate (cAMP) in *Aplysia* nervous tissue exposed to neurotransmitter substances (6, 7).

In the present report, we examine the possible involvement of cyclic nucleotides in synaptically evoked hyperpolarization of R15. We find that these hyperpolarizations are augmented in the presence of the phosphodiesterase inhibitor, theophylline. To determine whether increased cAMP production in R15 mimics the action of synaptic stimulation, we have injected 5′-guanylyl-imidodiphosphate [Gpp(NH)p], an adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activator (8, 9), directly into the cell.

MATERIALS AND METHODS

Aplysia californica weighing 100–150 g were obtained commercially (Pacific Biomarine, Venice, Calif.), and maintained in aerated artificial sea water (Instant Ocean, Aquarium Systems Inc., Eastlake, Ohio). The abdominal ganglion and attached nerves were removed from the animal through an incision in the foot, and pinned through the connective tissue sheath on a layer of Sylgard (Dow Corning), in a small (3 ml) recording chamber. The preparation was perfused with *Aplysia* medium (10), or *Aplysia* medium containing 1 mM theophylline (Sigma Chemical Co.). Stimulation of the branchial nerve was made through a suction electrode. Intracellular recording was done with 0.6 M K_2SO_4-filled glass micropipettes, and conventional electrophysiological equipment. Traces were recorded on a Hewlett-Packard Oscillographic Recorder. Intracellulr injection was performed as previously described (11, 12), with a double-barreled micropipette; one barrel was used to record membrane potential, and the other to eject the test substance by pressure. Gpp(NH)p (Boehringer, Mannheim) was injected as a 1 mM solution in distilled water.

For adenylate cyclase assays, abdominal ganglia were homogenized in 50 volumes of 5 mM Tris·maleate, 5 mM ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid at pH 7.8. The homogenate was spun at 50,000 × *g* for 15 min, and the pellet was washed twice with 2 mM Tris·maleate at pH 7.8, and finally resuspended in the latter buffer at a concentration of 0.5 mg of protein per ml. Adenylate cyclase activity was assayed as described previously (13), except that GTP was not added. The resuspended membranes could be stored at −70° for at least 6 weeks without loss of adenylate cyclase activity.

To assay cAMP phosphodiesterase (3′:5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) activity, we homogenized ganglia in 1 mM Tris·HCl at pH 7.4 (1 ml per ganglion). cAMP phosphodiesterase activity in the homogenate was estimated by a method similar to that of Eisman and Martin (14), by using 1 μM cAMP as substrate. Protein was measured by the method of Schaffner and Weissmann (15) with bovine serum albumin as the standard.

RESULTS

Stimulation of the branchial nerve results in a biphasic potential in R15 that consists of a fast depolarizing phase, followed by a much slower hyperpolarizing component (16). In all cases (n = 5), the predominant hyperpolarizing phase was augmented in the presence of 1 mM theophylline (Fig. 1a). Theophylline at this concentration had only a minimal effect on the shape of the endogenous bursting rhythm in R15. Both the duration and the amplitude of the response were affected (Fig. 1b), although the magnitude of the effect differed considerably from preparation to preparation; the increase in duration of the hyperpolarizing response ranged from 50 to 400%, while the increase in the amplitude of the hyperpolarization ranged from 10 to 300%. In all cases, there was a lag of at least 20 min before an effect could be seen.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Gpp(NH)p, 5′-guanylyl-imidodiphosphate.

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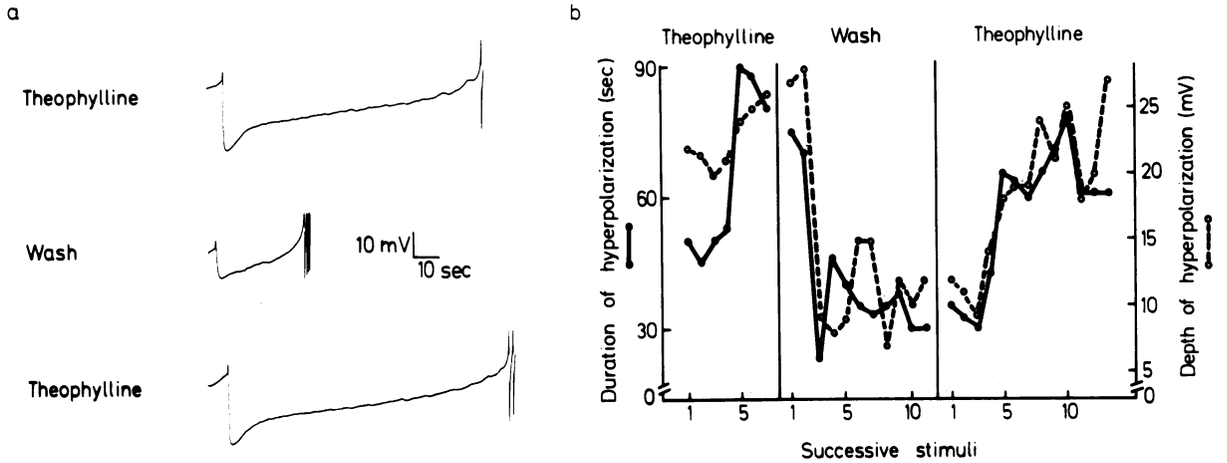

FIG. 1. Effect of 1 mM theophylline on synaptic potential recorded in R15, during branchial nerve stimulation. Successive test stimuli (0.1 msec duration) were applied to the branchial nerve at the same level of membrane potential during the interburst portion of the bursting cycle of R15. (a) Examples of the biphasic potential recorded in R15 after single stimuli to the branchial nerve, 30 min after introduction of 1 mM theophylline; after 90 min of perfusion with theophylline-free medium; 30 min after reintroduction of theophylline. (b) Plot of duration (●—●) and amplitude (O---O) of evoked potentials in R15 during theophylline exposure, wash, and reintroduction of theophylline. Duration is measured as the time from stimulus-induced response until membrane potential has returned to pre-stimulus level. Amplitude is measured as the maximum hyperpolarization of membrane potential in R15 from level at which stimulus occurred. The time between successive stimuli is not identical, but dependent on the bursting rhythm of the cell. Parts (a) and (b) represent data collected from the same cell.

Because methylxanthine phosphodiesterase inhibitors such as theophylline are known to produce a wide variety of side effects (17, 18), we tested the response of R15 to intraneuronally injected Gpp(NH)p, an activator of adenylate cyclase (8, 9). Although Gpp(NH)p may also produce side effects, these are likely to be different from those of the phosphodiesterase inhibitors. In addition to revealing the nature of R15's response to a specific rise in cAMP, this experiment allows an initial determination of the locus of the theophylline-induced synaptic augmentation. Hyperpolarization of the cell would reveal that R15 contains the necessary components of cyclic nucleotide machinery to account for the augmented hyperpolarization seen following branchial nerve stimulation.

In four Gpp(NH)p injections of varying size (the largest injection was approximately 1 nl, which represents 5–10% of R15's cell body volume), R15 showed a distinct hyperpolarization, and diminution of burst activity; in two cases, the cell was hyperpolarized and remained completely silent for hours. Fig. 2 shows a representative experiment in which the onset of hyperpolarization was abrupt (Fig. 2a and b), and resulted in total abolition of burst activity. During the Gpp(NH)p induced hyperpolarization of R15, the spontaneous biphasic potentials which occur in this cell lack a hyperpolarizing phase (Fig. 2c). This result indicates that the membrane potential is close to the equilibrium level of the ionic currents involved in generation of the hyperpolarizing component. Although some recovery was evident after 3.5 hr (Fig. 2d), the cell never fired an action potential during the observation period. Periodic depolarization of the cell by current injection showed the neuron to be undamaged and capable of producing action potentials. The membrane potential in this particular experiment, determined by electrode withdrawal, was −71 mV, and because the cell had recovered by 6 mV from the deepest point of the hyperpolarization, the Gpp(NH)p induced hyperpolarization of R15 to a level of −77 mV. In the other injection which resulted in the complete silencing of R15, the cell hyperpolarized to a membrane potential of −74 mV. These values approach that of potassium equilibrium potential in R15 (4), and are similar to values of membrane potential observed during synaptic hyperpolarization of the cell for very long durations (4). Prior to Gpp(NH)p injection, the membrane potential during the interburst period was between −54 and −59 mV.

We have tested the specificity of Gpp(NH)p action on components of the cyclic nucleotide system in *Aplysia* nervous tissue, and the results of this analysis are shown in the concentration-response curve in Fig. 3. Clearly, adenylate cyclase activity in a membrane preparation from abdominal ganglion is markedly stimulated by Gpp(NH)p. Because other agents which alter electrical activity in R15 (5) are phosphodiesterase inhibitors, it was of interest to examine the effects of Gpp(NH)p on phosphodiesterase activity. cAMP phosphodiesterase activity, measured with 1 μm cAMP as substrate, was unaffected by Gpp(NH)p treatment (Fig. 3). Similar results were obtained when the substrate concentration was 100 μM, or when cyclic GMP phosphodiesterase activity was measured (results not shown).

We also studied the effects of Gpp(NH)p on adenylate cyclase activity in various portions of the *Aplysia* abdominal ganglion. Although the basal cyclase activity appeared to be rather low in membranes prepared from the isolated cell bodies of neurons R2 and R15, Gpp(NH)p stimulated this activity (Table 1). Similarly Gpp(NH)p activated the adenylate cyclase in membranes prepared from the ganglionic neuropil (Table 1), a region devoid of neuronal cell bodies.

DISCUSSION

Although the presence of a cyclic nucleotide link in synaptic transmission has been widely discussed, the complexity of most of the systems under study has precluded analysis at the cellular level. In the current study, we have found that a long-lasting synaptic hyperpolarization in identified neuron R15 is augmented in the presence of a phosphodiesterase inhibitor. This finding suggests that the half-life or increased concentration of a cyclic nucleotide produced during this synaptic stimulation is closely related to the generation of the electrical response. Kandel *et al.* (19) demonstrated a modulation of transmitter release dependent on the presynaptic concentration of cAMP in *Aplysia* abdominal ganglion. It is possible that the augmentation of synaptic hyperpolarization, produced in R15 by

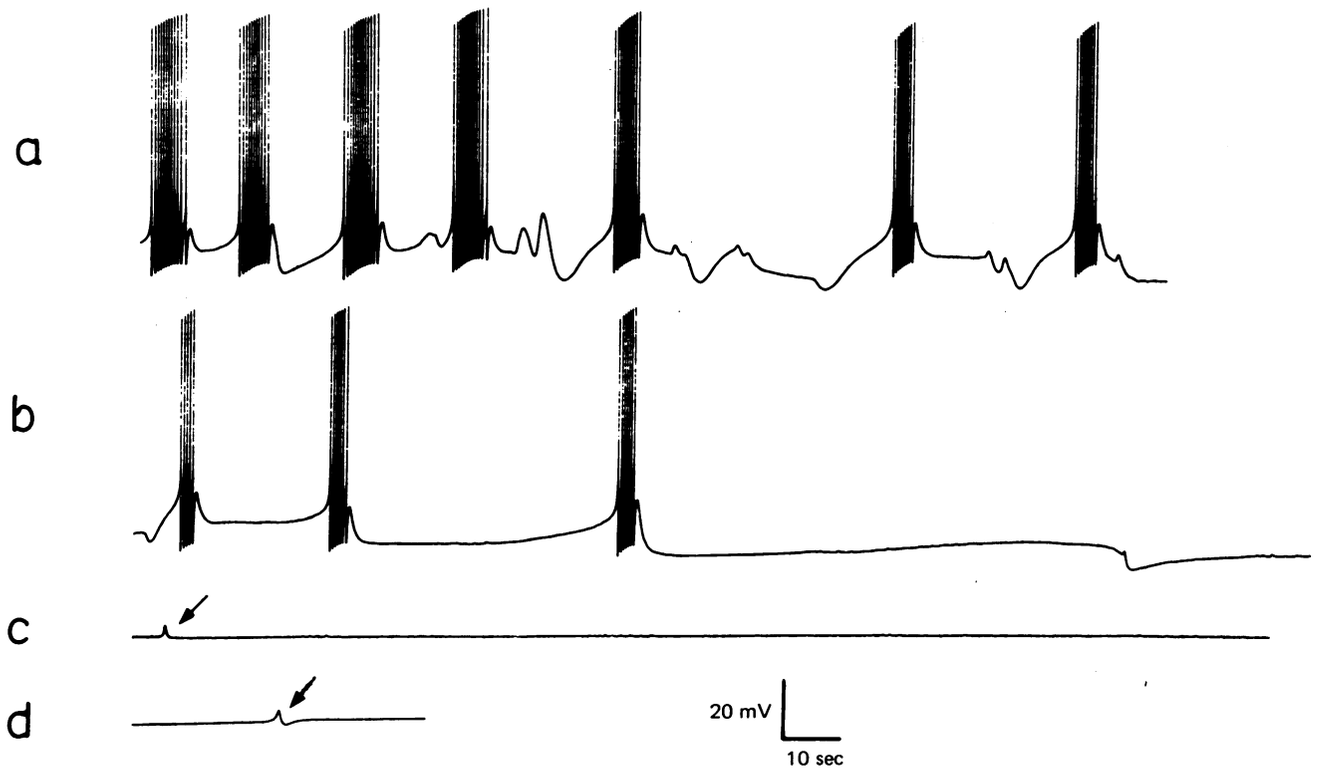

FIG. 2. Response of R15 to intrasomatically-injected Gpp(NH)p. Approximate intracellular concentration of Gpp(NH)p was between 10 μM and 100 μM. (a) Baseline fluctuations caused by spurts of injected Gpp(NH)p are evident immediately preceding the fourth burst of action potentials in trace, and continue for the duration of trace (a). (b) Trace of R15 membrane potential beginning 1.5 min after trace shown in (a), illustrating abrupt hyperpolarization of R15. (c) Trace of membrane potential 2 hr after injection. Arrow marks spontaneous biphasic potential (16) in which the hyperpolarizing phase is absent due to the Gpp(NH)p-induced hyperpolarization of R15. (d) Membrane potential 3.5 hr after injection. Arrow marks spontaneous biphasic potential, in which hyperpolarizing phase is becoming evident as cell recovers from Gpp(NH)p injection.

theophylline, is due to an effect on presynaptic nerve terminals. However, the fact that activation of adenylate cyclase in R15 mimics the hyperpolarization produced by branchial nerve stimulation, is compatible with the hypothesis that a postsynaptic accumulation of cAMP may underlie this long-lasting hyperpolarization. These data, together with our previous report (5) implicating cAMP in the electrical response of R15 to hormonal stimulation, suggest that cyclic nucleotides may play a role in long-term modulation of neuronal electrical activity. The well-defined components of this model system should allow

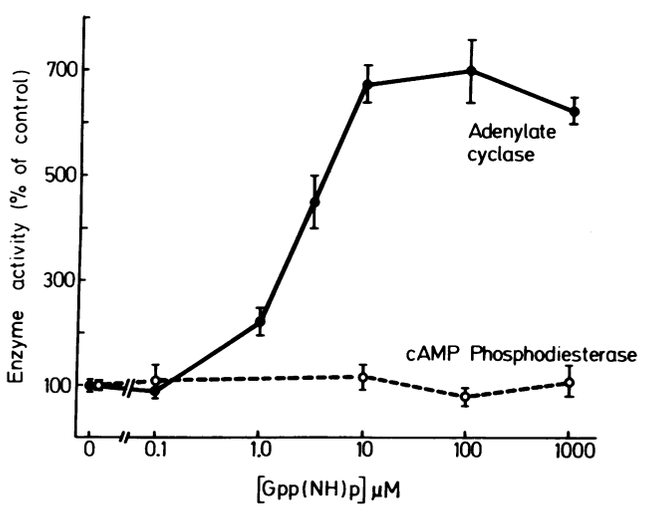

FIG. 3. Effect of different concentrations of Gpp(NH)p on adenylate cyclase and cAMP phosphodiesterase activities in *Aplysia* abdominal ganglion. Adenylate cyclase (●—●) and cAMP phosphodiesterase (O---O) activities were assayed in the presence of different concentrations of Gpp(NH)p. Activity (100%) was 18 ± 2 pmol of cAMP formed/min per mg of protein for adenylate cyclase, and 15 ± 4 pmol of cAMP hydrolyzed/min per mg of protein for cAMP phosphodiesterase.

Table 1. Stimulation of adenylate cyclase activity by Gpp(NH)p in various portions of *Aplysia* abdominal ganglion

	Adenylate cyclase activity, pmol of cAMP formed/min per mg of protein	
Portion of ganglion	Control	Gpp(NH)p (0.1 mM)
Whole ganglion	14 ± 1	158 ± 7
Neuron R2 cell body	5 ± 2	47 ± 7
Neuron R15 cell body	3	22
Neuropil fraction	65 ± 3	473 ± 45

Adenylate cyclase activity was measured, in the presence or absence of 0.1 mM Gpp(NH)p, in membrane preparations from whole ganglion, from pooled cell bodies of neurons R2 and R15, and from a portion of neuropil handled in the same way as the R2 and R15 cell bodies. Values for whole ganglion and neuropil are means ±SEM for eight samples, for R2 means ±SEM for four samples, and for R15 means for duplicate samples. R15 samples contained membranes from eight pooled cell bodies (2.3 μg of protein), R2 samples membranes from three pooled cell bodies (7 μg of protein), and neuropil samples 10 μg of protein. The experiment was performed twice with similar results.

a more definitive elucidation of the locus of cyclic nucleotide action to be made.

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