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Identification of cyclic AMP as the response regulator for neurosecretory potentiation: A memory model system

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ABSTRACT **The neural cell line HT4 serves as a model for memory by exhibiting short- and long-term potentiation of neurotransmitter secretion. Previous studies showed that membrane depolarization elicits secretion and that serotonin and *N*-methyl-D-aspartate receptors are involved in potentiation of the response. Adrenergic and adenosine receptors, which are coupled to adenylate cyclase, are also found to induce potentiation. In addition, the direct evaluation of cAMP levels by forskolin, or by addition of dibutyryl cAMP, induces potentiation. In these different types of stimuli, it is the level of cAMP that is the common factor allowing prediction of whether potentiation will be observed or not. The cAMP level therefore qualifies as the response regulator for this phenomenon. Repetitive adrenergic receptor stimulation results in short-term potentiation, while repetitive adenosine stimulation results in long-term potentiation. This difference can be explained by assuming that some precursor that determines the cAMP level exceeds a threshold, to produce long-term potentiation. This threshold is exceeded by adenosine stimulation but not by stimulation of the β-adrenergic receptor.**

The response regulator has been defined as a cellular component (or ratio of cellular components) whose level is identified with the behavioral response of the organism. The response regulator concept was initially proposed to simplify the understanding of the biochemical mechanisms of bacterial memory (1, 2). To qualify as a response regulator, the level of the cellular component should be an accurate predictor of external behavior. External behavior must be defined precisely, and, if a cellular component qualifies, the understanding of both the behavior and the internal biochemistry leading to it are clarified.

Bacteria have evolved a sensory system capable of detecting chemical gradients. Bacteria respond to a change in their environment by altering the direction of flagellar rotation (3, 4). The response regulator for bacterial chemotaxis was postulated to be a molecule whose level correlates directly with swimming behavior. Swimming behavior could be divided into smooth swimming and tumbling, which were in turn correlated with flagellar rotation (1, 2). The chemotactic response regulator has now been identified as the phosphorylated form of the cheY protein (5–8). Identification of the response regulator not only allowed the correlation of a number of apparently diverse phenomena but led to a precise biochemical description of memory storage in the bacterium. The combination of a rapid formation of the response regulator and a slow adaptation process on the receptor gives rise to short-term memory, allowing the bacterium to sense chemical gradients.

To extend this approach to long-term memory, an experimental system other than the bacterium was needed. Neural cell lines provide a homogeneous population of cells that can be manipulated biochemically (9). Recently, neural cell lines showing habituation and potentiation have been used as models of memory processes (10–14).

The particular behavior selected for observation of the output of these cells is neurotransmitter secretion. In PC12 cells (15), the secretion of norepinephrine is found to display a behavior correlated with memory models—i.e., habituation to repetitive stimulation (11, 12). Another neural cell line, HT4, displays a phenomenon associated with learning and memory—namely, potentiation or an increase in neurotransmitter secretion (13). In HT4 cells, the presentation of serotonin increases the cell's responsiveness to membrane depolarization (13). The strength of serotonin stimulation determines the extent of potentiation, with a stronger stimulus resulting in long-term cellular memory lasting many hours.

Identification of a response regulator for neurosecretory potentiation would simplify our understanding of this behavioral system, allowing an extensive analysis of the molecular components that give rise to long-term potentiation. Previously, we found a correlation between the elevation of cAMP levels and the potentiated state of the cell (13), but the cAMP was generated by a single receptor, and therefore the possibility existed that other signal transduction components also correlated with the potentiated state.

MATERIALS AND METHODS

Chemicals and Reagents. Radiolabeled D-[^{3}H]aspartate and ^{125}I-labeled cAMP assay systems were obtained from Amersham. 6-Fluoronorepinephrine, (−)-isoproterenol (IPT), N^6-cyclopentyladenosine, 5′-*N*-ethylcarboxyamidoadenosine (NECA), SKF-38393, bromocriptine mesylate, histamine, and 5-hydroxytryptamine (5HT) were obtained from Research Biochemicals (Natick, MA). HT4 cells were a gift from Ronald McKay (Massachusetts Institute of Technology) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 33°C. For all experiments, cells were differentiated for 3–5 days at 39°C.

Secretion Studies. HT4 cells were differentiated on microcarrier beads (Cytodex 2; Pharmacia). The excitatory amino acid pool was radiolabeled by incubating HT4 cells for 4–6 hr in modified Krebs–Ringer saline containing 4 μCi (1 Ci = 37 GBq) of D-[^{3}H]aspartate per ml. Neurotransmitter secretion was monitored essentially as described by McFadden and Koshland (16).

cAMP Determination. HT4 cells were grown in six-well dishes. The appropriate stimulus was presented in saline. Cells were lysed with 0.4 M $HClO_4$ and the acid extract was neutralized with 1/6th vol of 2.4 M $KHCO_3$. cAMP was determined by competition binding with ^{125}I-labeled cAMP.

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Abbreviations: IPT, (−)-isoproterenol; NECA, 5′-*N*-ethylcarboxyamidoadenosine; 5HT, 5-hydroxytryptamine (serotonin); Bt_2cAMP, dibutyryl cAMP.

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Protein concentrations were determined by Coomassie dye binding with bovine serum albumin as a standard (Pierce).

RESULTS

Receptors Coupled to Adenylate Cyclase. Both long- and short-term secretory potentiation can occur through the activation of 5HT receptors, which in turn elevate cAMP levels (13). To determine whether potentiation of neurosecretion is unique to the 5HT receptor or whether other receptors coupled to adenylate cyclase can induce potentiation, we tested HT4 cells for other receptors that activate adenylate cyclase. Various chemical agonists were used to stimulate specific receptor classes (Table 1). Of the compounds screened, IPT, 5HT, and NECA resulted in elevation of cAMP levels, suggesting that β-adrenergic, 5HT, and A_2-adenosine receptors can stimulate the cAMP second messenger system in HT4 cells.

Epinephrine and Adenosine Receptors Mediate Secretory Potentiation. To test the possibility that adrenergic or adenosine receptors could induce secretory potentiation, neurosecretion was evoked by membrane depolarization before and after the cells were transiently presented with saturating concentrations of either IPT or NECA. A 5-min presentation of 50 μM IPT resulted in the long-lasting potentiation of depolarization-induced secretion (Fig. 1*A*). Neurosecretory potentiation induced by this single 5-min presentation of IPT showed no diminished responsiveness up to 40 min after IPT presentation.

In parallel experiments, cAMP levels were measured after removal of the 5-min presentation of IPT (Fig. 1*B*). Immediately after IPT presentation, cAMP levels increased from basal levels of 9.7 to 75 pmol/mg. cAMP levels decreased with time until they reached a new intermediate steady state of 20 pmol/mg. Cells presented with 5 min of 50 μM IPT maintained this 2-fold higher level of cAMP for the duration of the experiment. This persistent elevation of cAMP is the result of internal changes within the cell, since in parallel experiments IPT is effectively removed from the cells as determined by radioactive tracer washout. As shown previously (13), the temporal correlation between cAMP levels and secretory potentiation suggest involvement of a cAMP-dependent process in potentiation. Thus, the ability of cells to potentiate is not the unique property of serotonin receptors but a general property of cAMP elevation.

The generality of the correlation between cAMP elevation and potentiation is supported by our observation that the stimulation of adenosine receptors by a 5-min presentation of 50 μM NECA also resulted in persistent potentiation (Fig. 2*A*). Potentiation remained 2-fold higher throughout the duration of the experiment. Elevation of cAMP levels also parallels the temporal characteristic of potentiation (Fig. 2*B*). In this case, a 5-min presentation of 50 μM NECA raised the cAMP level to 17.5 pmol/mg and 5 min after the stimulus was removed, it reached a new steady-state level of 9 pmol/mg, approximately twice the basal level. These results show that secretory potentiation can be induced by other receptors coupled to adenylate cyclase. Thus, the elevation of cAMP, either by 5HT (13), epinephrine (Fig. 1), or adenosine (Fig. 2), is capable of inducing long-lasting potentiation.

Table 1. Receptor-mediated elevation of cAMP levels in HT4 cells

Receptor class	Receptor agonist	cAMP levels, pmol/mg
None	Unstimulated, basal	10.2 ± 0.9
α_1-Adrenergic	6-Fluoronorepinephrine	10.1 ± 1.9
β-Adrenergic	IPT	21.4 ± 1.9
A_1-Adenosine	N^6-Cyclopentyladenosine	10.6 ± 0.7
A_2-Adenosine	NECA	43.4 ± 2.5
D_1-Dopamine	SKF-38393	11.1 ± 1.0
D_2-Dopamine	Bromocriptine mesylate	12.7 ± 0.8
Histamine	Histamine	11.0 ± 0.6
5HT	5HT	20.9 ± 1.2

HT4 cells were presented with receptor agonist at a concentration of 100 μM in standard buffered saline for 5 min. The stimulus was removed and the cells were lysed with 0.4 M $HClO_4$. The acid extract was then neutralized with $KHCO_3$ and cAMP levels were assayed by radioimmunoassay. Total cellular protein was determined on a Triton X-100 lysate using Coomassie dye binding. Results are expressed as means ± SE (n = 5).

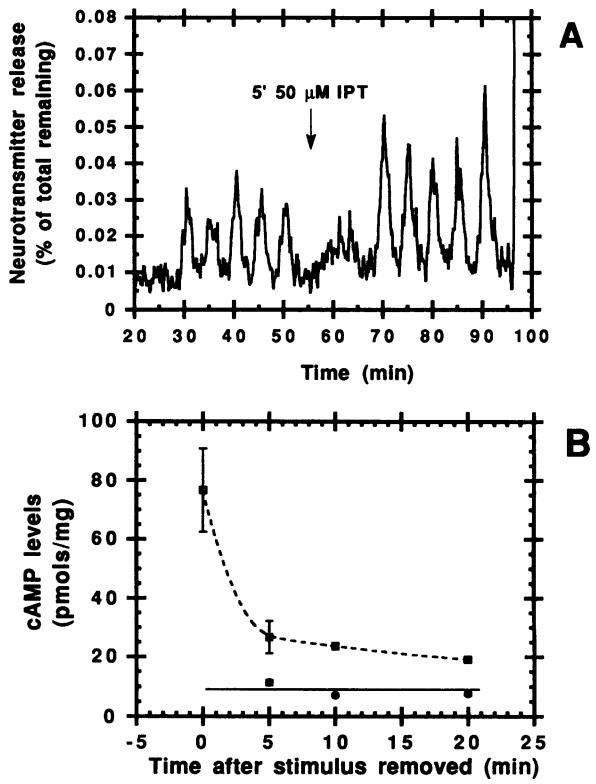

FIG. 1. Effect of IPT on secretory potentiation. (*A*) D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated time, 50 μM IPT was presented for 5 min. Depolarizing stimuli were then presented to the cells for 2 min every 5 min. (*B*) HT4 cells were presented with 50 μM IPT for 5 min. The cells were washed twice and placed in 1 ml of saline for the indicated period of time. The basal, unstimulated level of cAMP was 9.7 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 4).

cAMP as the Response Regulator for Potentiation. To establish further the role of cAMP in secretory potentiation, cAMP levels were generated by means other than receptor activation. Forskolin directly activates adenylate cyclase (17), and in HT4 cells, 50 μM forskolin elevated cAMP levels from 10 to 41.8 pmol/mg. The effect of 50 μM forskolin on depolarization-induced secretion is shown in Fig. 3. Potentiation of secretion was observed throughout the duration of the forskolin presentation, the increase in secretion being ≈2-fold.

Direct elevation of intracellular cAMP concentrations by use of the membrane-permeant and phosphodiesterase-resistant analog of cAMP, dibutyryl cAMP (Bt_2cAMP) also resulted in potentiation (Fig. 4). These results suggest that cAMP is directly responsible for potentiation and that potentiation is not the secondary consequence of some other second messenger pathway. This does not eliminate the

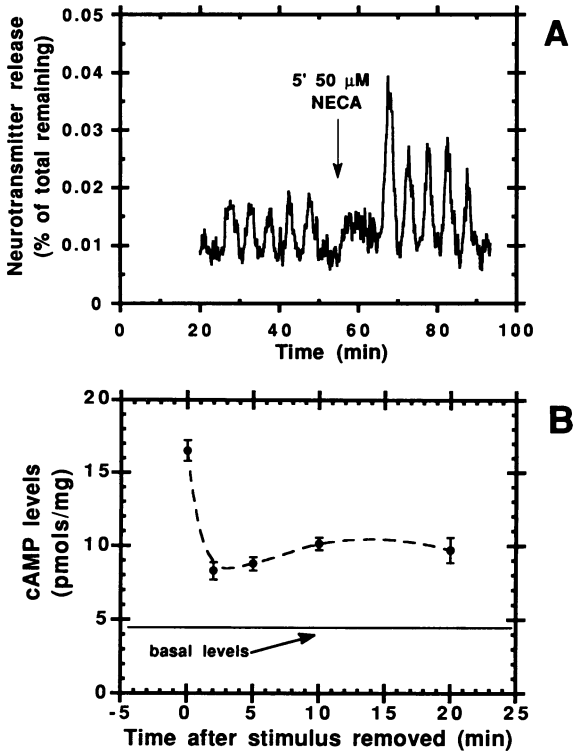

FIG. 2. Effect of NECA on secretory potentiation. (*A*) D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated time, 50 μM NECA was presented for 5 min. Depolarizing stimuli were then presented to the cells for 2 min every 5 min. (*B*) HT4 cells were presented with 50 μM NECA for 5 min. The cells were washed twice and placed in 1 ml of saline for the indicated period of time. The basal, unstimulated level of cAMP was 4.6 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 4).

possibility that some component generated downstream of the cAMP signal is also important for the heightened cellular responsiveness, but the substrate whose level correlates with the increased responsiveness of the cell does appear to be cAMP.

Secretory Potentiation and Repetitive Stimulation. To determine whether secretory potentiation in neural cell lines could be induced by repetitive stimulation of adrenergic receptors, a train of five 1-min presentations of 50 μM IPT at 5-min intervals was presented to HT4 cells, which resulted in

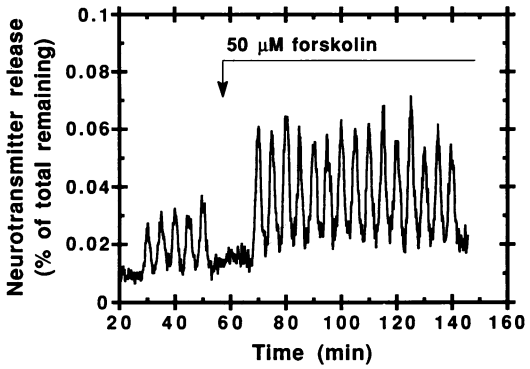

FIG. 3. Effect of forskolin on secretory potentiation. D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated time, 50 μM forskolin was presented for the remainder of the experiment. Depolarizing stimuli were then presented to the cells for 2 min every 5 min for a total of 15 stimulations.

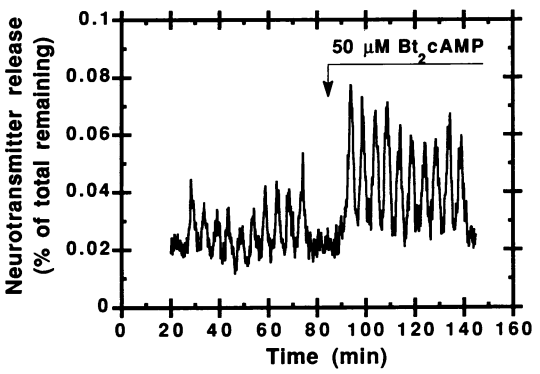

FIG. 4. Effect of Bt_2cAMP on secretory potentiation. D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated time, 50 μM Bt_2cAMP was presented for the remainder of the experiment. Depolarizing stimuli were then presented to the cells for 2 min every 5 min for a total of 10 stimulations.

the transient potentiation of secretion (Fig. 5*A*). After this train of repetitive stimulation, depolarization-induced secretion increased ≈2-fold. However, this increase in cellular responsiveness lasted only 20–25 min, after which secretion relaxed back to basal levels. Thus, only short-term secretory potentiation was expressed by repetitive β-adrenergic receptor stimulation.

The elevation of cAMP levels directly parallels the temporal characteristic of potentiation. Repetitive IPT presentation resulted in the elevation of cAMP. However, as the

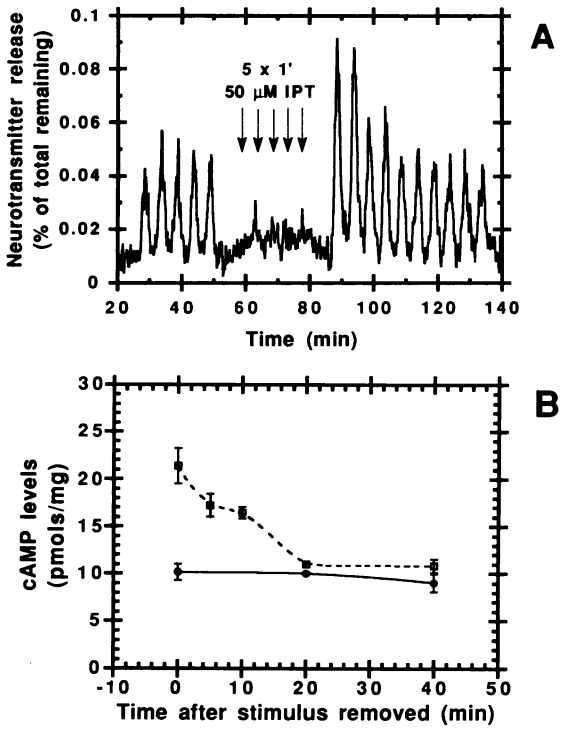

FIG. 5. Effect of repetitive IPT stimulation on secretory potentiation and cAMP levels. (*A*) D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated times, 50 μM IPT was presented for 1 min every 5 min, for a total of five stimulations. Depolarizing stimuli were then presented to the cells for 2 min every 5 min. (*B*) HT4 cells were presented with 50 μM IPT for 1 min every 5 min, for a total of five stimulations. The cells were washed twice and placed in 1 ml of saline for the indicated period of time. The basal, unstimulated level of cAMP was 10.1 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 4).

cAMP levels decreased back to basal levels (Fig. 5B), potentiation of secretion ceased to be expressed.

The same test gave different results with adenosine receptors. Five 1-min presentations of 50 μM NECA every 5 min resulted in persistent or long-term secretory potentiation, with the extent of potentiation lasting at least 1 hr (Fig. 6A). Although there is variation in the actual peak heights of neurotransmitter released, the mean average amount of neurotransmitter secreted is significantly larger after repetitive stimulation by NECA than before.

The elevation of cAMP levels after the final NECA stimulus was removed is presented in Fig. 6B. Once again, cAMP levels increased ≈8-fold and then remained elevated at twice the basal level of cAMP in unstimulated cells. This elevated cAMP corresponds temporally with the potentiation of secretion.

Mechanism for Short- and Long-Term Potentiation by Repetitive Stimulation. To understand why repetitive adrenergic receptor stimulation leads to short-term potentiation, while repetitive adenosine receptor stimulation results in long-term potentiation, we measured cAMP levels in response to repetitive stimulation of these two receptor types. In Fig. 7A, repetitive 1-min presentations of 50 μM IPT were given to HT4 cells and the corresponding elevation of cAMP levels for the last IPT presentation was determined. When HT4 cells were given a single, 1-min IPT stimulus, cAMP levels increased from 10.6 to 81.3 ± 2.2 pmol/mg. A second 1-min stimulus 4 min later only elevated cAMP levels to 59.9 ± 1.8 pmol/mg. By the fifth stimulus, cAMP was elevated only to 15.5 ± 0.4 pmol/mg. Thus, repetitive stimulation of β-adrenergic receptors fails to sustain cAMP levels above a threshold needed for potentiation. This is probably due to the known down-regulation of this receptor (see ref. 18).

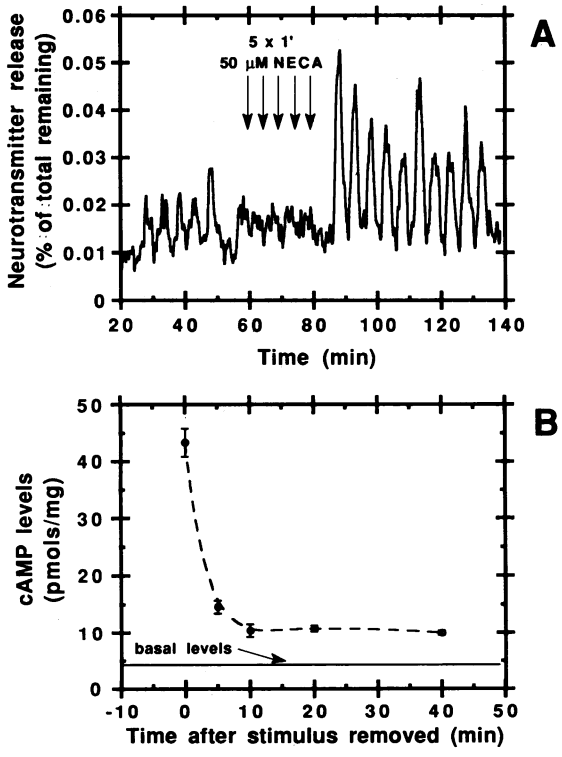

FIG. 6. Effect of repetitive NECA stimulation on secretory potentiation and cAMP levels. (A) D-[^{3}H]Aspartate-loaded HT4 cells were depolarized with a perfusing medium containing high potassium ion (55 mM) for 2 min every 5 min. At the indicated times, 50 μM NECA was presented for 1 min every 5 min, for a total of five stimulations. Depolarizing stimuli were then presented to the cells for 2 min every 5 min. (B) HT4 cells were presented with 50 μM NECA for 1 min every 5 min, for a total of five stimulations. The cells were washed twice and placed in 1 ml of saline for the indicated period of time. The basal, unstimulated level of cAMP was 4.8 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 4).

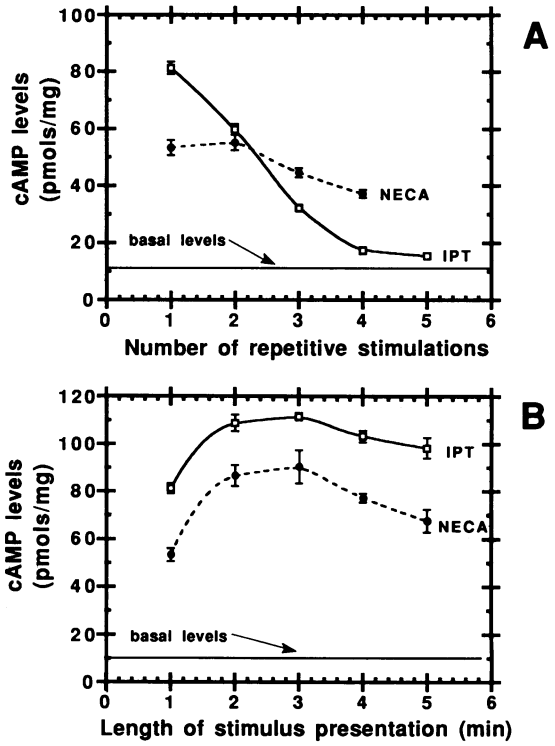

FIG. 7. Characteristics of cAMP elevation. (A) Effect of repetitive stimulation on cAMP elevation. HT4 cells were presented with 50 μM IPT for 1 min every 5 min for up to five stimulations or with 50 μM NECA for 1 min every 5 min for up to four stimulations. At the end of each 1-min stimulation, one set of cells was lysed with 0.4 M $HClO_4$, and cAMP levels were measured on the neutralized extract. The basal, unstimulated level of cAMP was 10.6 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 5). (B) Effect of length of presentation on cAMP elevation. HT4 cells were presented with 50 μM IPT for 1–5 min or with 50 μM NECA for 1–5 min. The basal, unstimulated level of cAMP was 10.6 pmol/mg and is indicated by the solid line. Results are expressed as means ± SE (n = 5).

Repetitive stimulation of adenosine receptors did not result in loss of responsiveness (Fig. 7A). Multiple presentation of NECA resulted in a less-prominent decrease in cAMP levels. Thus, the A_2-adenosine receptor is not subjected to down-regulation as is the β-adrenergic receptor. This indicates that repetitive stimulation of adenosine receptors by NECA can elevate cAMP levels above a particular threshold level to induce persistent secretory potentiation.

Single 5-min presentations of either IPT or NECA resulted in long-term potentiation (Figs. 1A and 2A). In both cases, a single application of agonist does not down-regulate the ability of either adrenergic or adenosine receptors to elevate cAMP levels (Fig. 7B). Thus, cAMP is generated to a level that exceeds the threshold necessary to maintain long-term potentiation. This explains the nonequivalency of single 5-min stimuli and five 1-min stimuli.

DISCUSSION

The response regulator is a useful conceptual term in discussing the biochemistry of cellular memory. The response regulator is defined as a single cellular component (or a parameter based on a simple ratio of cellular components) that is predictive of the behavioral response of the cell.

Conceptually, an observer inside the organism can predict the external behavior of the organism from the level of the response regulator. Applying this concept to potentiation in HT4 cells, the response regulator would be that molecule whose level would reflect whether the cell is in the potentiated or nonpotentiated state. cAMP appears to be the response regulator for potentiation in the HT4 cell, since it can be produced by a variety of different methods and, by its level, predict the responsiveness of the cell to a stimulatory input.

Potentiation induced by β-adrenergic and A_2-adenosine receptors suggests that the 5HT receptor is not the only receptor that can induce potentiation. Since β-adrenergic, A_2-adenosine, and 5HT receptors are coupled to the activation of adenylate cyclase, cAMP level is the common denominator that appears to be responsible for potentiation. Moreover, the direct elevation of cAMP with the diterpene forskolin or with Bt_2cAMP provides further evidence that cAMP is the response regulator for potentiation.

Establishing cAMP as the response regulator for potentiation suggests several possible mechanisms by which cAMP mediates this response. Since the effect of cAMP in mammalian systems is predominantly through the cAMP-dependent protein kinase, a likely mechanism would be the phosphorylation of some component in the secretory apparatus. This could be a secretory vesicle protein, such as synapsin (see ref. 19) or an ion-channel protein, which has been demonstrated by Kandel and co-workers as a concomitant of long-term facilitation in the marine mollusc *Aplysia* (20, 21).

The response regulator concept allows potentiation to be divided into two components: (*i*) the output, which is proportional to the level of the response regulator, and (*ii*) the input pathway, which generates the response regulator. Previously, it was found that a 5-min stimulus of 5HT evoked long-term secretory potentiation; whereas a 2-min stimulus only gave rise to short-term potentiation (13). The length of time for induction (5 min versus 2 min) needed to generate long-term (4 hr) versus short-term (10 min) potentiation suggests the initiation of an ultrasensitive process (22–24)—e.g., one that has the effect of an *n*th power responsiveness. Simple proportionality will not suffice to correlate output with the stimulus time. In other words, a threshold-type phenomenon is necessary for induction of long-term potentiation. Further evidence for such a threshold comes indirectly from the IPT or NECA stimulations. Five 1-min presentations of NECA could induce long-term potentiation, just as observed in a single 5-min presentation. However, repetitive stimulation of β-adrenergic receptors only results in short-term potentiation. The most logical interpretation is that the adaptation of these receptors, known from their desensitization by phosphorylation, prevents the attainment of the threshold necessary to induce long-term potentiation. The next step is to determine the threshold mechanism by which the persistent elevation of cAMP is generated.

Thus, these results indicate that the response regulator for the potentiated state is cAMP; that this cAMP is generated by an ultrasensitive process that exceeds a threshold to generate the long-term potentiation response; and that the threshold is not exceeded when a receptor is subjected to adaptive desensitization, such as the adrenergic receptor. Identification of cAMP as the response regulator for potentiation greatly simplifies the biochemistry that generates this form of cellular memory. For example, it allows the dissection of long-term potentiation into two components—the mechanism by which cAMP becomes persistently elevated and the events after cAMP elevation that lead to enhanced neurotransmitter secretion.

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