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Regulation of Synthesis of the Neurosecretory Egg-laying Hormone of *Aplysia*: Antagonistic Roles of Calcium and Cylic Adenosine 3′:5′-Monophosphate[1]

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Abstract

The potential role of cyclic nucleotides and calcium as regulators of neuropeptide biosynthesis was examined in the bag cell neurons of *Aplysia*, which produce and secrete a peptide egg-laying hormone (ELH). Elevated external potassium, which stimulates ELH biosynthesis, increased bag cell cAMP levels when assayed in the presence of a phosphodiesterase inhibitor. Dopamine and serotonin, which increase bag cell cAMP levels, both stimulated ELH synthesis, as did the phosphodiesterase inhibitor isobutylmethylxanthine, the specific adenylate cyclase activator forskolin, and the phosphodiesterase-resistant cAMP analogue 8-benzylthio-cAMP. The stimulatory effect on peptide biosynthesis appears to be specific for cAMP, as bag cell cGMP levels were not altered significantly by high potassium or forskolin, and 8-bromo-cGMP did not stimulate ELH synthesis. In contrast to cAMP, intracellular calcium inhibits ELH production: biosynthesis of the peptide was elevated in a 0 Ca^{2+}/EGTA medium and reduced in the presence of the Ca^{2+} ionophore A23187. Synthesis was also elevated in the presence of the calmodulin inhibitor calmidazolium. Treatment of intact bag cells with 0 Ca^{2+}/EGTA or A23187 did not alter cAMP levels significantly, suggesting that calcium exerts its effect on peptide synthesis independently of cAMP. The antagonistic effects of cAMP and calcium on ELH synthesis parallel their effects on bag cell excitability, suggesting that, in these cells, neuropeptide synthesis and secretion are co-regulated by the same intracellular messengers.

The peptidergic neuron differs from its counterparts which secrete non-peptide transmitters in that its secretory products must be supplied by the protein synthetic and packaging machinery of the cell body, rather than by local synthesis in the secretory terminal. Since activation of such neurons leads to the loss of peptides by secretion, it is of interest to ask whether the production of a neurosecretory peptide is regulated in proportion to its rate of secretion and, if so, by what mechanism(s). Regulation of neuropeptide biosynthesis has been demonstrated in both vertebrate (Russell et al., 1981; Kessler and Black, 1982; Majzoub et al., 1983; Tang et al., 1983) and invertebrate systems (Berry and Arch, 1981; Berry, 1982), but details of the regulatory mechanism(s) are lacking. Regulation of specific peptide biosynthesis is known to occur in several non-neuronal secretory cell types, and in several instances, the regulatory mechanism has been shown to involve receipt of a secretogogue and/or mediation by cAMP (Maurer, 1981; Barinaga et al., 1983; Chen et al., 1983; Eiden and Hotchkiss, 1983). Such a "feed-forward" regulatory mechanism, triggered by receipt of presynaptic transmitter and mediated by an intracellular second messenger, would be particularly advantageous for peptidergic neurons, in which the site of secretion is distant from the site of peptide synthesis. The neurosecretory bag cells of *Aplysia* offer several advantages for the study of neuropeptide regulation, as they form a relatively simple and experimentally accessible system about which there exists a considerable amount of morphological, biochemical, and physiological information. The bag cell organs are paired clusters of apparently homogeneous neurons which produce and secrete a peptide egg-laying hormone (ELH) and other secretory peptides (Arch, 1972; Stuart et al., 1980; Rothman et al., 1983), all of which are generated from a common precursor (Scheller et al., 1983) by a proteolytic processing sequence whose members and kinetics have been studied in detail (Berry, 1981). A major advantage of this preparation is that approximately 50% of total bag cell leucine incorporation is devoted to members of the ELH processing sequence. As a consequence, these proteins may be isolated in essentially radiochemically homogeneous form by one-dimensional electrophoresis (Berry, 1981), facilitating biosynthetic studies.

In addition, ELH synthesis is known to be subject to regulation, both on a short-term (Berry and Arch, 1981) and on a seasonal (Berry, 1982) basis. Exposure of bag cells to elevated external potassium induces a rapid and long-lasting 25 to 30% increase in the relative rate of ELH synthesis (Berry and Arch, 1981). This increase in peptide synthesis is blocked in a low Ca^{2+}/high Mg^{2+} medium and does not occur in isolated somata, suggesting that either receipt of presynaptic transmitter or secretion of the peptide itself triggers the regulatory process. Since both the site of transmitter receipt and the site of peptide release are at some distance from the site of protein synthesis, this raises the possibility that an intracellular messenger might be involved in mediating the regulatory response.

Consideration of the biochemical changes associated with bag cell discharge suggests two candidates as potential regulatory messengers. Synchronous repetitive discharges of the electrotonically coupled clusters can be evoked by brief stimulation of a pleuroabdominal connective nerve. These discharges typically outlast the stimulus by about 30 min, after which the clusters become refractory to further stimulation for a period of hours (Kupfermann and Kandel, 1970). Onset of the discharge is accompanied by, and is probably due to, a transient rise in cAMP levels (Kaczmarek et al., 1978, 1980). Since cAMP has been implicated in the regulation of protein synthesis in a number of systems (Rosenfeld and Barrieux,

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1979), it is conceivable that the potassium-induced elevation of ELH synthesis might be mediated by this nucleotide.

In contrast, calcium is a major inward current carrier in bag cell action potentials (Acosta-Urquidi and Dudek, 1981); and, in addition to its probable role in inducing secretion, calcium influx may be responsible for terminating the discharge and inducing the refractory period (Kaczmarek and Kauer, 1983). Calcium has been implicated in the regulation of specific protein synthesis in some cell types (Martonosi, 1982), and instances of calcium/calmodulin-dependent regulation of cyclic nucleotide levels via activation of phosphodiesterase are known (Klee et al., 1980). Thus, calcium is another potential mediator of the potassium-induced elevation of ELH biosynthesis.

The experiments described here were undertaken to evaluate the role of each of these potential second messengers in the regulation of specific protein synthesis in the bag cells. We asked first whether elevated external K^+ affects bag cell cyclic nucleotide levels. Next, we asked whether the effects of pharmacological treatments on cyclic nucleotide levels were correlated with their effects on ELH synthesis. Finally, we asked whether treatments which should alter intracellular calcium levels influence ELH synthesis and, if so, whether this might be secondary to an alteration of cyclic nucleotide levels. A preliminary report of some of these results has appeared previously (Berry, 1983).

Materials and Methods

Animals. *Aplysia californica*, weighing 150 to 300 gm, were supplied by Pacific Bio-Marine Laboratories (Venice, CA) or by Marine Specimens Unlimited (Pacific Palisades, CA) and kept at 15°C in Instant Ocean (Aquarium Systems, Mentor, OH) for up to 3 weeks before use. The animals were fed Romaine lettuce at 2- to 3-day intervals and were not maintained on a controlled light schedule. Because ELH synthesis is seasonally regulated (Berry, 1982), all ELH synthesis experiments were done only between December and April (1981 to 1984). Cyclic nucleotide levels were assayed throughout the year, and no seasonal variation was found.

Cyclic nucleotide assays. All *in vitro* incubations were carried out at 15°C. The animals were dissected and the abdominal ganglion was removed. After a 30-min preincubation in artificial seawater (ASW; Berry, 1976), the bag cell organs, with pleuroabdominal connective nerves attached, were dissected from the ganglia. One member of the pair of organs was incubated in ASW containing the appropriate experimental agent for 30 min while the other served as a control. Following this incubation, each organ was exposed to 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 2 min, then homogenized in 0.5 ml of ice-cold acid ethanol (Levitan and Norman, 1980). The homogenates were centrifuged and the pellets were stored at −20°C until determination of protein content by a fluorometric assay (Bohlen et al., 1973). The supernatants were lyophilized, resuspended in 100 μl of 0.05 M sodium acetate buffer, pH 6.2, and stored at 4°C. Levels of cAMP or cGMP were measured by radioimmune assay (New England Nuclear, Boston, MA).

Measurement of relative ELH synthesis. ELH production was measured as previously described (Berry and Arch, 1981; Berry, 1982). One member of the pair of bag cell organs was incubated for 3 hr in ASW containing the experimental agent, and the other served as a control. Both organs were then transferred to 0.5 ml of ASW containing 1% glucose and 25 μCi of L-[4,5-^{3}H]leucine (100 Ci/mmol; Amersham, Arlington Heights, IL). After 2 hr, each organ was rinsed briefly in ASW; then the bag cells were dissected from their surrounding sheath, homogenized in sample buffer containing 1% sodium dodecyl sulfate (SDS), and stored at −20°C. Members of the ELH processing sequence were identified by subjecting the homogenates to SDS-polyacrylamide gel electrophoresis (Berry, 1976). These peptides form prominent radiolabeled peaks in the gels, as measured by scintillation counting of 1-mm sequential sections, and these peaks are essentially radiochemically homogeneous (Berry, 1981). Relative ELH synthesis was measured as the ratio of the radioactivity in these peaks to that in the rest of the gel. The validity of this measure of relative hormone synthesis has been discussed at length elsewhere (Berry, 1982), where it was shown that incorporation of label into both ELH-related and nonrelated proteins is linear through at least 8 hr. It should also be noted that the turnover of ELH-related proteins is too small to detect within the time course of these experiments (Berry, 1982), and that turnover is not affected by high K^+ (Berry and Arch, 1981). The term "relative ELH synthesis" will be used for this measure, although at 2 hr, only the precursor and intermediates of the processing sequence have been labeled. For all treatments, the ratio of label in ELH-related proteins to that in an identifiable 70,000-dalton peak was also calculated. Results using this method did not differ from those obtained by the procedure described above.

Chemicals. Forskolin and A23187 were obtained from Calbiochem (La Jolla, CA). Calmidazolium and 8-benzylthio-cAMP (8-BT) were supplied by Boehringer-Mannheim (Indianapolis, IN). Dopamine, serotonin, 8-bromo-cGMP, EGTA, and IBMX were from Sigma Chemical Co. (St. Louis, MO). Forskolin was dissolved in ethanol and stored at 4°C as a 10 mM stock. Bag cells were exposed to a final ethanol concentration of less than 0.1%. A23187 and calmidazolium were dissolved in dimethylsulfoxide (DMSO) and stored as 5 mM stock solutions at −20°C. Final DMSO concentrations never exceeded 1%, and control solutions contained equal amounts of this solvent. In tests where A23187 was used, the bag cells were first exposed to a seawater medium with the ionophore and no calcium. The cells were then transferred to normal ASW with the ionophore, and finally were placed in ASW with no A23187.

Results

Effects of pharmacological agents on cyclic nucleotide levels. We began by confirming that drugs which alter cyclic nucleotide metabolism in other tissues have the expected effects when applied to bag cells. The nonspecific phosphodiesterase inhibitor IBMX elevates bag cell content of both cAMP and cGMP approximately equally (Fig. 1). The effect is dose-dependent, although this compound is approximately 10-fold less potent in our hands than has been reported for other regions of the *Aplysia* nervous system (Levitan and Norman, 1980). Forskolin has been shown to specifically activate adenylate cyclase in several cell types, including *Aplysia* eye (Eskin and Takahashi, 1983), and elevates cAMP content in bag cells without significantly altering cGMP levels (Fig. 1).

The time course of the influence at 1 mM IBMX on bag cell cAMP levels is shown in Figure 2. The 2-min exposure which was routinely applied to both control and experimental organs when cyclic nucleotide levels were to be assayed (to reduce degradation during dissection and homogenization; Kaczmareck et al., 1978) increased cAMP levels approximately 2-fold. The largest increase was seen at 30 min of exposure, with somewhat lower levels occuring after three hours. This may reflect the existence of a compensatory regulatory mechanism in the intact cells, or it could indicate that phosphodiesterase activity was not completely inhibited, even at this concentration of IBMX.

Next, we asked whether elevated external potassium would change bag cell levels of either nucleotide. Following 30-min incubations in 100 mM K^+ medium, bag cell levels of cAMP and cGMP remain essentially unchanged from control values (Fig. 1). However, with IBMX present in both high K^+ and control media, the elevated potassium increases cAMP levels without affecting cGMP content (Fig. 1). Our interpretation of these results is that the high K^+ treatment leads to the activation of adenylate cyclase, but in the absence of a phosphodiesterase inhibitor, the additional cAMP produced is degraded within the time course of our experiments.

Modulation of ELH biosynthesis by cAMP. One explanation of the available data is that elevation of external potassium induces the release of presynaptic transmitter and that receipt of this transmitter induces a cAMP-mediated increase in ELH synthesis. The transmitter responsible for bag cell activation has not yet been identified. However, dopamine elevates bag cell cAMP levels and can prolong bag cell discharges (Kaczmarek et al., 1978). As shown in Figure 3, dopamine causes a dose-dependent increase in ELH biosynthesis. Serotonin, or 5-hydroxytryptamine (5-HT), is known to elevate bag cell cAMP content, but it is electrophysiologically inhibitory (Kaczmarek et al., 1978). This compound also increases ELH synthesis (Fig. 3); moreover, it does not inhibit the effect of high K^+ on synthesis (Fig. 3), suggesting that the biosynthetic effect is mediated by cAMP, rather than by some other factor related to bag cell electrical activity.

Treatments with other agents which either mimic cAMP or elevate bag cell cAMP levels support this interpretation. The cAMP analogue, 8-BT, significantly elevates relative ELH synthesis (Fig. 3). Exposure

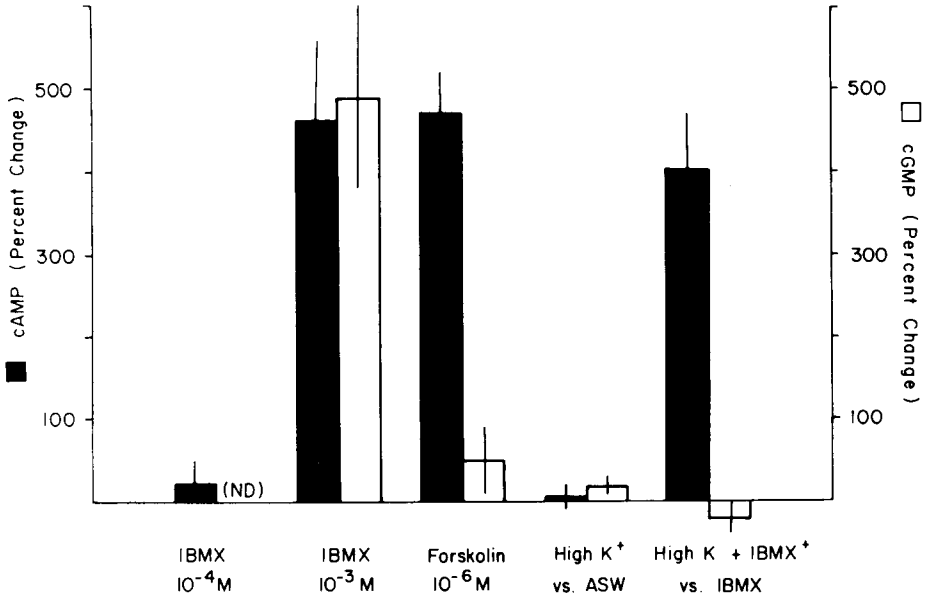

Figure 1. Changes in bag cell cyclic nucleotide levels in response to pharmacological treatments. Histograms represent the mean ± SEM of the difference between levels in paired experimental and control bag cell organs as a percentage of the control value. *N* ranged from 4 to 11. *ND*, not determined.

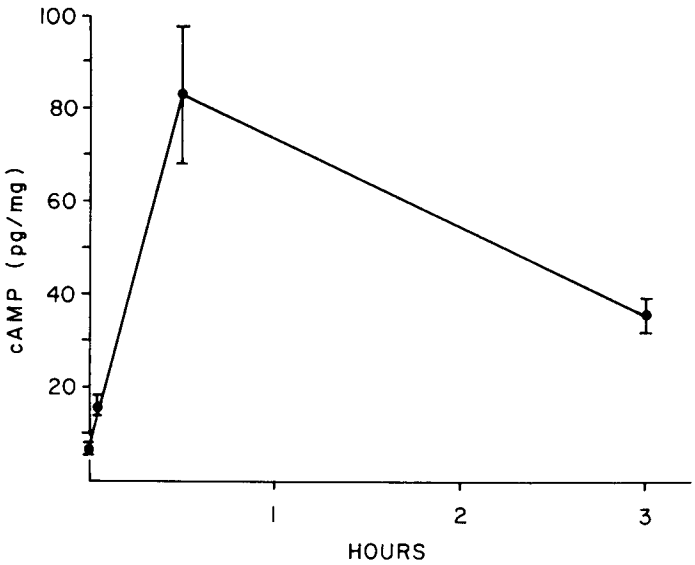

Figure 2. Time course of elevation of bag cell cAMP levels evoked by exposure to 1 mM IBMX. Values are the mean ± SEM of from four to seven experiments.

of the bag cells to IBMX results in a dose-dependent increase in peptide production (Fig. 3) which parallels the effect of this agent on cyclic nucleotide levels (Fig. 1). Forskolin also significantly elevates ELH synthesis (Fig. 3). Since neither forskolin nor high K^+ have any significant effect on cGMP levels, it is unlikely that the regulatory effect on peptide synthesis is mediated by this nucleotide. Confirmation of this conclusion was obtained by exposing bag cells to the phosphodiesterase-resistant cGMP analogue, 8-bromo-cGMP (BrcG). This compound actually causes a decrease in relative ELH synthesis (Fig. 3).

None of these treatments had any significant effect on the incorporation of labeled leucine into non-ELH proteins, indicating both that the effect is specific for ELH and that the observed changes in relative incorporation are due to an increase in incorporation into members of the ELH processing sequence, rather than to a decrease in incorporation into other proteins. Moreover, none of the effective treatments changed the relative proportion of label incorporated into various members of the processing sequence, implying that the rate of precursor processing remains normal and that the extra precursor molecules synthesized as a result of these treatments result in the production of extra secretory products.

Modulation of ELH synthesis by calcium. Our first test of the potential influence of calcium on ELH synthesis was to expose bag cells to ASW in which all of the Ca^{2+} was replaced by Na^+, and which contained 2 mM EGTA. This treatment, which should lower the intracellular Ca^{2+} concentration, resulted in a marked increase in relative ELH synthesis (Fig. 4), suggesting that calcium inhibits synthesis of the peptide. To test this, we treated bag cells with the calcium ionophore A23187 (50 μM), which should raise intracellular calcium levels (Pfeiffer et al., 1978). As predicted, this treatment significantly reduced ELH production (Fig. 4).

In many systems, calcium exerts its second-messenger effect by combining with the regulatory protein calmodulin. Ca^{2+}/calmodulin-dependent protein kinase activity is known to be present in *Aplysia* neural tissue (Novak-Hofer and Levitan, 1983), and calmodulin-dependent effects are subject to inhibition by calmidazolium (Van Belle, 1981). Treatment of bag cells with this compound (50 μM) caused a significant increase in relative ELH synthesis (Fig. 4), suggesting that the inhibitory effect of calcium on ELH synthesis is mediated by calmodulin.

Calcium effects on cAMP levels. Changes in intracellular calcium concentration can have pronounced effects on cyclic nucleotide metabolism, including a change in cAMP levels due to a Ca^{2+}/calmodulin-dependent activation of phosphodiesterase (Klee et al., 1980). Thus, it is conceivable that the observed effects of calcium on ELH biosynthesis are secondary to a calcium-mediated change in cAMP levels. Under this hypothesis, an increase in intracellular Ca^{2+} would lead to phosphodiesterase activation, and the subsequent reduction in cAMP levels would reduce ELH biosynthesis. Accordingly, we assayed bag cell cAMP levels following calcium-altering treatments. As shown in Figure 5, EGTA in calcium-free ASW, A23187, and calmidazolium do change cAMP levels, but these changes are too small to produce a measurable effect on ELH synthesis (cf. data in Figs. 1 and 3). Therefore, we conclude that the effect of calcium on peptide biosynthesis is not likely to be mediated by cAMP.

Discussion

Our results imply a direct and causal relation between bag cell cAMP levels and the rate of biosynthesis of the ELH precursor: agents which elevate cAMP levels stimulate ELH production, and a membrane-permeable and phosphodiesterase-resistant cAMP analogue elevates ELH synthesis. Since a high potassium medium does

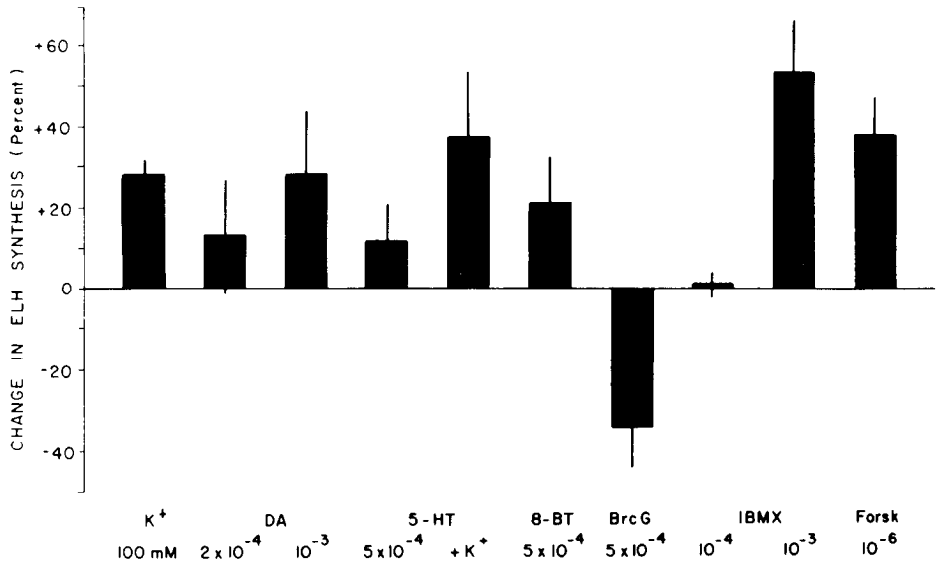

Figure 3. Changes in relative ELH synthesis produced by agents which mimic or alter the metabolism of cyclic nucleotides. *N* ranged from 4 to 8 for various treatments. All differences were significant from at least the $p = 0.05$ level by Wilcoxon's paired sign ranks test except for 2×10^{-4} M dopamine (*DA*) and 10^{-4} M IBMX. The data for high K^+ (Berry and Arch, 1981) and partial data for dopamine and IBMX (Berry, 1983) have appeared previously.

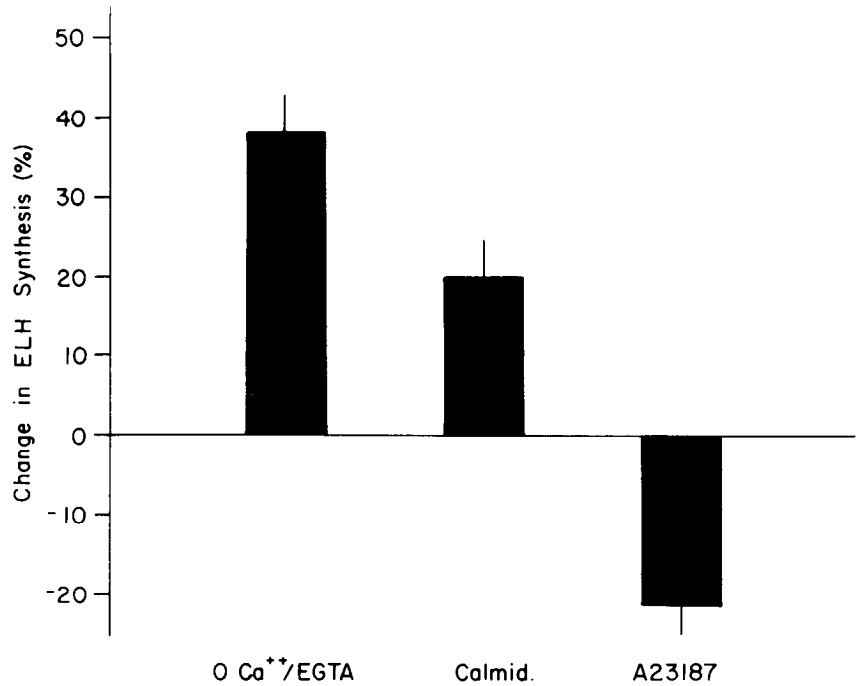

Figure 4. Changes in relative ELH synthesis in response to calcium-altering agents. Mean ± SEM of five or six experiments.

not stimulate ELH synthesis in situations where secretion has been blocked (Berry and Arch, 1981), and since presynaptic stimulation leads to an increase in bag cell cAMP levels (Kaczmarek et al., 1978), we suggest that high K^+ evokes the secretion of presynaptic transmitter, and that receipt of this transmitter is responsible for increasing bag cell cAMP levels. This type of "feed-forward" control, mediated by a second messenger in the target cell, would be particularly advantageous to neurons, as it obviates the necessity of sending a regulatory signal from the site of secretion back to the cell body, which may be some centimeters distant.

The available evidence from other peptide-secreting systems suggests that cAMP-mediated control of peptide synthesis via receipt of a transmitter or releasing factor may be a general regulatory mechanism for governing the rate of production of secretory peptides in concert with their release. There is evidence for cAMP-mediated regulation of proenkephalin production in adrenal chromaffin cells (Eiden and Hotchkiss, 1983); for cAMP-mediated dopaminergic control of prolactin synthesis in the anterior pituitary (Maurer, 1981); and for hormonal or neurotransmitter control of the production of growth hormone in the anterior pituitary (Barinaga et al., 1983), pro-opiomelanocortin in the neurointermediate lobe (Chen et al., 1983), and proenkephalin in the striatum (Tang et al., 1983). In each of these systems, regulatory control has been demonstrated at the transcriptional level. We have no direct evidence bearing on the level of control in the bag cells. However, the earlier suggestion that the rapidity of the onset of the simulatory effect in bag cells made transcriptional control unlikely (Berry and Arch, 1981) may have been premature, since an increase in pro-opiomelanocortin mRNA in the pituitary can be measured within 6 hr of exposure to haloperidol (Chen et al., 1983). It is also worth noting that, although increased intracellular levels of cAMP can induce discharges, cAMP-induced elevation of ELH synthesis cannot be a secondary consequence of bag cell activation, since serotonin does not induce discharges (Kaczmarek et al., 1978). Since protein kinase activity has been demonstrated in this tissue (Jennings et al., 1982), cAMP could be exerting its effect on protein synthesis by this mechanism.

It is noteworthy that 8-BrcG had a significant inhibitory effect on ELH synthesis. There is precedent for antagonistic actions of cAMP and cGMP in regulating various parameters of cellular metabolism (Goldberg et al., 1975) and neuronal electrical activity (Levitan and

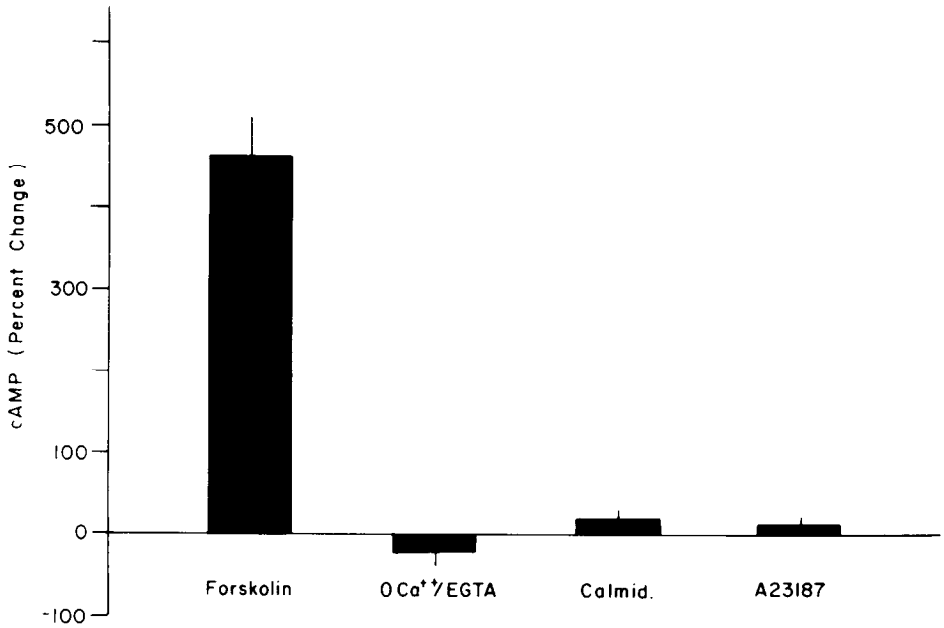

Figure 5. Changes in bag cell cAMP levels produced by calcium-altering agents. *N* ranged from 5 to 7. Forskolin data from Figure 1 are included for comparison.

Norman, 1980). However, there is at present no evidence that cGMP is involved in bag cell activity, and the relevance of the effect of this cGMP analogue on ELH synthesis to events normally occurring in bag cells remains to be established.

Because physiological activation of the bag cells involves a rise in cAMP levels, it is probable that bag cell activation normally leads to an increase in ELH synthesis. It is also probable that this increment is not large enough to be detectable by our methods. The amount of egg-releasing activity secreted during a discharge is a small fraction of the total bag cell content (Dudek et al., 1980). Moreover, although the doubling of cAMP levels that occurs during a discharge is comparable in magnitude to the elevation produced in some of our experiments, cAMP levels return to normal within 4 min of the onset of a discharge. Our 3-hr treatments would presumably have a much greater effect on ELH synthesis. Thus, the effect of an electrically stimulated bag cell discharge on ELH synthesis would probably fall below our detection limit. Consequently, we did not attempt this measurement, relying instead on manipulations which, although unphysiological, produce measurable effects. It is conceivable that these treatments induce biochemical changes influencing ELH biosynthesis that are qualitatively different from those occurring during a physiological discharge. However, it should be noted that the changes in ELH synthesis induced by these treatments are actually somewhat smaller in magnitude than the naturally occurring seasonal changes in this parameter (Berry, 1982).

The results of our experiments with 0 Ca^{2+}, A23187, and calmidazolium are consistent with the hypothesis that Ca^{2+} inhibits ELH biosynthesis specifically. This conclusion is more tentative than for cAMP, because we have tested fewer treatments and because we have yet to confirm that these treatments actually alter intracellular levels of Ca^{2+}. The results of calmidazolium treatment suggest that a Ca^{2+}/calmodulin interaction may be involved. Ca^{2+}/calmodulin-dependent phosphorylation has been demonstrated in the *Aplysia* nervous system (Novak-Hofer and Levitan, 1983), but further evidence will be required before a firm conclusion can be reached with respect to the participation of calmodulin in the Ca^{2+}-dependent regulation of ELH synthesis.

Our data suggest a regulatory interaction between cAMP and calcium in controlling ELH production. Of the several regulatory steps at which such an interaction could occur, one obvious possibility is a Ca^{2+}/calmodulin-dependent activation of phosphodiesterase. However, our results indicate that treatments designed to alter intracellular calcium levels do not alter bag cell cAMP levels to the extent necessary to account for their influence on ELH synthesis. The existence of proteins which are phosphorylated both by cAMP-dependent and Ca^{2+}-dependent protein kinases has been demonstrated in the *Aplysia* nervous system (Novak-Hofer and Levitan, 1983), leaving open the possibility that this is the point of interaction in the regulation of ELH biosynthesis.

If the results of this investigation are considered in conjunction with the physiological roles of cAMP and calcium in the bag cell discharge, a coherent picture emerges which suggests a strict and coordinated mechanism for co-regulation of peptide synthesis and secretion. Kaczmarek et al. (1978, 1980) have provided evidence that the early transient rise in cAMP levels, presumably due to the receipt of presynaptic transmitter, is responsible for initiating the discharge which results in ELH secretion. Our results imply that this rise in cAMP also leads to an enhanced rate of ELH production. Bag cell action potentials have a major calcium component and increase in duration during a discharge, presumably augmenting ELH release (Acosta-Urquidi and Dudek, 1981). Increased intracellular Ca^{2+} is also thought to be responsible for the termination of the discharge and the initiation of the subsequent refractory period (Kaczmarek and Kauer, 1983). Our results suggest that the increased intracellular Ca^{2+} would also reduce ELH synthesis, preventing an overproduction of the hormone. Under this hypothesis, coordinate control of peptide synthesis and secretion would be achieved by a combination of feed-forward enhancement mediated by cAMP and feedback reduction mediated by calcium.

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