Activation of Membrane Guanylate Cyclase by an Invertebrate Peptide Hormone*

Michael F. Goy

From the Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27599-7545

Peptide hormones can stimulate cyclic GMP synthesis through either of two general mechanisms: some peptides activate the cytoplasmic form of guanylate cyclase via a coupling factor called EDRF (endothelium-derived relaxation factor), while others activate the membrane form by interacting directly with an extracellular binding domain of the cyclase molecule itself. We have investigated the mechanism(s) by which crustacean hyperglycemic hormone (CHH), a neuropeptide that regulates energy metabolism in crustaceans, elevates cyclic GMP levels in lobster muscle. Phosphodiesterase inhibitors potentiate the response in intact tissue. This indicates that the primary effect of the peptide is to activate a cyclase rather than inhibit a phosphodiesterase. Methylene blue, a specific inhibitor of the EDRF pathway, does not block the actions of CHH. In addition, nitroprusside, an agent that directly activates the EDRF pathway, in vertebrate animals, does not activate guanylate cyclase either in intact or homogenized lobster muscle. This indicates that the EDRF pathway, although prominent in vertebrate muscle, is not found in crustaceans and further suggests that the membrane cyclase is the most likely target of CHH. Membrane and soluble cyclases can be isolated from homogenates of lobster muscle (in a 3.5:1 ratio), and both are stimulated by Mn2+ and inhibited by Ca2+. CHH has no effect on the soluble enzyme. Coupling of CHH receptors to the particulate cyclase, however, remains intact in isolated membranes, thus providing a new model system for the study of receptor/cyclase interactions.

Cyclic GMP is an important second messenger in vertebrate and invertebrate muscle. Mammalian vascular smooth muscle, mammalian cardiac muscle, and the skeletal muscles of invertebrates, like the lobster, are enriched for cyclic GMP-dependent kinase and its substrates (Casnellie and Greengard, 1974; Rapoport et al., 1982; Lincoln and Johnson, 1984; Kuo and Greengard, 1970; Goy et al., 1984). Furthermore, specific stimuli elevate cyclic GMP levels in these tissues. In vertebrate animals, a variety of vaso-dilating hormones raise cyclic GMP levels and subsequently induce vascular and cardiac relaxation (reviewed in Waldman and Murad, 1987; Tremblay et al., 1988; and Fischmeister and Hartzell, 1987). In the lobster and other crustaceans, a peptide hormone called crustacean hyperglycemic hormone (CHH) 1 raises muscle cyclic GMP levels (Sedlmeier and Keller, 1981; Goy et al., 1987a; Pavloff and Goy, 1990), stimulates muscle glycogen metabolism (Keller and Andrew, 1973), and may also relax hormone- or depolarization-induced muscle contraction (Goy et al., 1987b).

In vertebrates, at least three different enzymes control tissue levels of cyclic GMP. 1) A membrane-specific form of guanylate cyclase is regulated in many tissues, including vascular smooth muscle, by the members of the atrial natriuretic peptide (ANP) family (Waldman et al., 1985; Leitman and Murad, 1987). The family includes a set of homologous peptides produced by the atrium of the heart and related forms produced in the brain (Seidah et al., 1984; Tanaka et al., 1984, Saper et al., 1985; Sudoh et al., 1988). These peptides selectively turn on the membrane cyclase, probably by interacting with an extracellular binding domain of this membrane-spanning enzyme (Schulz et al., 1989). 2) A Cytoplasmic form of guanylate cyclase is activated in many tissues, again including vascular smooth muscle, by an endogenous regulatory molecule called endothelium-derived relaxation factor (EDRF). In the case of smooth muscle, EDRF is produced in the vascular endothelium in response to a variety of hormones, including the peptides bradykinin, cholecystokinin, substance P, thrombin, and calcitonin gene-related peptide (Griffith et al., 1988). Once produced by the endothelium, EDRF diffuses into the cytoplasm of nearby smooth muscle cells (limited primarily by the relatively short half-life of the EDRF molecule, where it directly stimulates the soluble cyclase. 3) A cyclic GMP-specific phosphodiesterase is regulated by light in vertebrate photoreceptors (reviewed in Stryer, 1986). The capture of a photon of light activates the phosphodiesterase via a GTP binding protein (transducin), which in turn produces a decrease in photoreceptor cyclic GMP levels.

The present study is directed toward identifying the mechanisms by which CHH regulates cyclic GMP metabolism in lobster exoskeletal muscle. Because of similarities between this tissue and vertebrate vascular smooth muscle, we were particularly interested in seeing whether CHH would act through the membrane cyclase, as do the peptides in the ANP family, or via EDRF and the soluble cyclase, as do other vertebrate peptides such as bradykinin. We have also investigated the possibility that CHH raises cyclic GMP levels in lobster muscle by inhibiting the cyclic GMP-specific phosphodiesterase (the converse of the effects of light in retinal photoreceptors).

* This work was supported by National Institutes of Health Grants NS21290 and NS25915. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: CHH, crustacean hyperglycemic hormone; EDRF, endothelium-derived relaxation factor; BSA, bovine serum albumin; IBMX, isobutylmethylxanthine; HPLC, high pressure liquid chromatography; ANP, atrial natriuretic peptide(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
MATERIALS AND METHODS

Preparation of Crude and Purified CHH—CHH is produced by the sinu gland, a well known crustacean neurohemal organ (Keller et al., 1985). In the lobster, as in other crustaceans, two forms of CHH have been identified. In the lobster, the total activity, appears to be closely related to the major form, studies) has been purified (Pavloff and Goy, 1990) and its sequence sinuses gland, a well known crustacean neurohemal organ (Keller et al., 1986). These two isoforms of CHH are the only molecules present in the supernatant with significant stimulatory effects on cyclic GMP metabolism.

For the current studies, sinuses glands were identified and dissected as described previously (Goy et al., 1987a). Crude CHH-containing extracts were prepared by homogenizing the glands in a bioassay medium consisting of lobster saline (462 mM NaCl, 16 mM KCl, 26 mM CaCl2, 8 mM MgCl2, 11 mM glucose, 10 mM Tris maleate, pH 7.4) with 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM o-phenanthroline as protease inhibitors, and 1 mg/ml bovine serum albumin (BSA). Before use, the homogenate was centrifuged for 20 min in a Beckman Microfuge (8000 x g) to remove particulate material.

Purified CHH was prepared from crude sinu gland extracts by sequential ion exchange and reverse phase high pressure liquid chromatography (HPLC), as described by Pavloff and Goy (1990). Purified material from the final HPLC step was diluted into distilled water containing BSA, lyophilized to dryness to remove HPLC solvents, and resuspended in bioassay medium or Buffer A, as described below.

The approximate concentration of CHH was determined from the estimated molecular weight of the peptide and the results of amino acid analysis on an aliquot of the purified material.

Bioassay in Intact Muscle—The effects of CHH on cyclic GMP metabolism in intact muscle tissue were evaluated using a previously described assay (Goy et al., 1987a). To compare the response in the presence and absence of phosphodiesterase inhibitor, individually dissected pieces of muscle were preincubated for 2 h either in lobster saline or in saline containing 0.5 mM isobutylmethylxanthine (IBMX). This preincubation was performed to allow cyclic GMP levels to reach steady state after IBMX treatment (about 3- to 5-fold above control), so that changes in cyclic GMP levels due to the phosphodiesterase inhibitor would not be superimposed on those due to the peptide.

Each piece of tissue was then transferred to bioassay medium (approximately 0.2 ml/muscle) containing a specified concentration of CHH, either with or without IBMX and other pharmacological reagents, as appropriate. After an additional period of incubation (as indicated in the figures), the pieces of tissue were briefly rinsed in saline (to wash away BSA from the incubation medium), frozen, and homogenized in trichloroacetic acid. Each homogenate was then centrifuged, to yield a soluble fraction (containing cyclic GMP) and an insoluble fraction (containing protein).

The cyclic GMP levels were determined by radioimmunoassay (reagents and procedures obtained from Bioscience Technologies Incorporated, Stoughton, MA) and corrected for the size of the piece of tissue (determined by measuring protein in the trichloroacetic acid pellet, according to the method of Lowry et al. (1951)).

The effects of the guanylate cyclase inhibitor (methylene blue) were evaluated in intact tissue by preincubating pieces of muscle for 10 min in saline containing 0.5 mM IBMX, followed by an additional 15 min in saline with IBMX and various concentrations of methylene blue (ranging from none to 2 mM). Muscle pieces were then transferred to bioassay medium containing CHH and the appropriate concentration of methylene blue. After an additional 90 min, the muscle pieces were frozen and processed as above.

The effects of the guanylate cyclase activator (sodium nitroprusside) were determined by treating muscle (preincubated with IBMX) for times ranging from 1 min to 90 min with concentrations of nitroprusside ranging from none to 50 mM.

Bioassay in Intact Muscle—The effects of CHH on cyclic GMP metabolism in muscle homogenates, the assay of Leitman et al. (1987) was adapted. For this assay, either tail muscle or the opener muscle of the dactyl of the walking leg was used. After dissection under cold saline, muscles were rinsed once with cold buffer B (60 mM HEPES, pH 7.4, with 1 mM EDTA, 0.01% bacitracin, 0.1 mM phenylmethylsulfonyl fluoride) and then homogenized in ice-cold buffer B (1 ml of buffer/0.5 g of tissue wet weight) using a motor-driven Potter-Evvelheim homogenizer with a nylon pestle. After removing a portion of this material (crude cyclase), the remainder was centrifuged for 80 min at 100,000 g (4 °C). The upper two-thirds of the supernatant were carefully removed (to yield the soluble cyclase preparation). Earlier studies have shown that the cytoplasmic fraction generated by this procedure contains less than 1% of the membrane lipid initially present in the crude homogenate (Goy et al., 1984). The remainder of the supernatant was then discarded and Buffer C (Buffer B + 100 mM KCl) was used to resuspend the pellet (5 ml of buffer/0.5 g of initial tissue wet weight). The resuspended pellet was then again centrifuged (for 80 min at 100,000 g), and the new pellet was resuspended by homogenization in Buffer B (3 ml/0.5 g of initial tissue wet weight) and subjected to a third centrifugation for 60 min at 100,000 × g (4 °C). This final pellet (membrane cyclase) was resuspended in Buffer B to the same volume as the soluble cyclase fraction. All resuspensions were by homogenization.

Protein content was determined on an aliquot of each fraction by the method of Lowry et al. (1951). The amounts of protein obtainable from 0.5 g wet weight of tissue (the approximate wet weight of a single dactyl opener muscle) are 11.18 ± 2.3 mg of protein as a crude homogenate (n = 5, ±SE), or 24.8 ± 3.4 mg in the soluble preparation plus 51.2 ± 4.0 mg in the membrane preparation (n = 8, ±SE).

In some cases, large membrane preparations were divided into aliquots and rapidly frozen on dry ice. Frozen aliquots could be stored up to 12 months and thawed without noticeable loss of activity. No consistent differences were observed between tail and dactyl opener muscle or between fresh and frozen tissue. We did note some deterioration if membranes were held on ice for periods of hours, and this deterioration appeared to accelerate if the temperature was raised.

To initiate cyclase activity, aliquots of each of the muscle fractions were rapidly mixed with equal volumes of freshly made, room temperature Buffer A (60 mM HEPES, pH 7.4, with 8 mM MgCl2, 2 mM IBMX, 5 mM GTP, 0.5 mg/ml bovine serum albumin, 46 units/mg proteinase, 185 units/mg kinase, and 1 mg/ml BSA). At various times thereafter, 40-μl samples were removed, pipetted into 100 μl of 6% trichloroacetic acid (w/v), vortexed immediately, and placed on ice. Trichloroacetic acid was used as a stopping agent, because the boiling procedure of Leitman et al. (1987) was found to generate artifacts. A “zero” time point was prepared for each condition by preincubating 20 μl of Buffer A with 100 μl of trichloroacetic acid, and then adding 20 μl of the appropriate muscle fraction. When the effects of ions, methylene blue, nitroprusside, or purified CHH were tested on homogenate fractions, these agents were added to the Buffer A diluent where appropriate, at twice the desired final concentration. Final free concentrations of all reagents were calculated using the molar program (Biosoft, Cambridge UK), with dissociation constants from Martell and Smith (1974, 1975).

The cyclic GMP content of each time point was determined by radioimmunoassay, as in the procedure described above for intact tissues. Each sample was extracted four times with 3 volumes of 2.5% trichloroacetic acid, to remove phosphodiesterase inhibitor, and centrifuged for 60 min at 100,000 g (4 °C). The supernatant was discarded, and the final pellets were resuspended and resuspended in Buffer A to the same volume as the membrane preparation. These homogenized preparations were quite reproducible. The average variability of duplicate points in 80 randomly chosen determinations was 7.5%, approximately half the variability observed with intact tissues. The variation from experiment to experiment is also significantly

3 M. F. Goy, manuscript in preparation

4 Prolonged incubation in IBMX has no deleterious effects on nerve or muscle as judged by measurement of membrane potential, input resistance, or the pattern of spike potentials. An isolated nerve-muscle preparation of the crayfish has been prited, with recording of synaptic potential amplitude, and exposure to IBMX alone does not produce any unusual side effect due to lengthy exposure to IBMX, we have also measured the time course of the cyclic GMP response after simultaneous exposure to IBMX and peptide. When the effects due to IBMX alone are subtracted, the response is identical with that of Fig. 1b (data not shown).
decreased. This improvement in reproducibility is probably due to the elimination of muscle to muscle or animal to animal differences.

Vertebrate Tissues—A 2-cm piece of aorta (0.11 g wet weight) was removed from an adult male Sprague-Dawley rat under urethane and α-chloralose anesthesia (495 and 100 mg/kg intraperitoneally). The tissue was homogenized in 1 ml of cold Buffer B, and the crude homogenate was used at once in the guanylate cyclase assay described above.

Dorsal root ganglia were removed from embryonic rats (day E16-17), dissociated, and grown for 7 days in tissue culture, as described by Gannoun et al. (1989). Cultures were placed in room temperature and treated for 1 min with either control saline (145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4) or saline containing 0.3 mM nitroprusside. The saline was then replaced with 1.5 ml of 6% trichloroacetic acid, cells were scraped from the dish, and the trichloroacetic acid soluble and insoluble fractions were analyzed for cyclic GMP and protein, respectively, as described above.

Chemicals and Supplies—EDTA, HEPES, bacitracin, phenylmethylsulfonyl fluoride, o-phenanthroline, IBMX, GTP, creatine kinase, phosphocreatine, methylene blue, nitroprusside, and bovine serum albumin were obtained from Sigma; HPLC solvents and trichloroacetic acid from J. T. Baker and Fisher; and radioimmunoassay kits for cyclic GMP from Biomedical Technologies Incorporated. M&B2948 was the generous gift of Dr. H. Gerebenboki (Recole Normale Superieure, Paris, France). All other chemicals and solvents were reagent grade, obtained from commercial sources. Lobsters were purchased from commercial suppliers and held at 12 ± 1 °C in artificial seawater, usually for at least a week prior to use.

RESULTS
Phosphodiesterase Inhibitors Amplify the Effects of CHH in Intact Tissues

A first step toward understanding regulation of cyclic GMP metabolism in lobster tissues is to define the mechanism(s) by which CHH increases cyclic GMP levels. In general, CHH might act either by stimulating the rate of synthesis of cyclic GMP (by activating one of the forms of guanylate cyclase) or by reducing its rate of breakdown (by inhibiting phosphodiesterase). One way to distinguish these two classes of mechanisms is to compare the response of the target tissue in the presence and absence of a phosphodiesterase inhibitor. If the peptide stimulates cyclase, then the magnitude of the response should be increased by the inhibitor, whereas if it inhibits phosphodiesterase the response should be decreased.

As seen in Fig. 1a, the response to CHH by intact muscle is greatly potentiated by the phosphodiesterase inhibitor IBMX, both at saturating and subsaturating levels of peptide. A similar result (not shown) was obtained with a second phosphodiesterase inhibitor, M&B2948, thought to be more selective for the form of phosphodiesterase that preferentially breaks down cyclic GMP (Hergstrand et al., 1977). Thus, the primary effect of the peptide is probably to stimulate cyclase rather than to inhibit phosphodiesterase.

The initial rate of the response to CHH in intact tissues is roughly linear, both in the presence and absence of IBMX (Fig. 1b). Over a period of 20–50 min, however, a plateau is attained. Presumably, this represents the new equilibrium level attained as a result of enhanced synthesis in the presence of the peptide. The plateau level is maintained with only a slight decline for as long as the peptide is present (up to 180 min). This suggests that if the CHH receptor desensitizes, it does so only at a slow rate.

When the peptide is washed away, the elevated levels of cyclic GMP begin to fall (Fig. 1c). The time course of recovery, however, is not simple. Initially, the rate of cyclic GMP breakdown after peptide washout is relatively rapid. This is followed by an extended period (commencing about 50 min after washout) in which the rate of decline is much slower. The biochemical basis for the biphasic nature of the recovery process is not understood. We have noted, however, that during the late phase the tissue is capable of responding normally to a new application of peptide (added at the arrow in Fig. 1c).

Because CHH is a very hydrophobic molecule (Pavloff and Goy, 1990), we considered the possibility that the washing procedure might fail to completely remove the peptide from the binding site within the tissue. If so, then subsequent slow release of nonspecifically bound peptide could have produced a low level of free peptide, thereby keeping the level of cyclic GMP elevated above baseline. However, when we subjected tissues to a second washing step 25 min after the initial wash, the subsequent decline of cyclic GMP levels could not be distinguished from that of tissues that had only received the initial wash (compare filled and open circles at 260 min in Fig. 1c). Thus, the late phase of the recovery process probably does not result from incomplete removal of the peptide.

In all of the experiments in Fig. 1 we have used both purified CHH (square symbols) and an unpurified CHH-containing extract made from the sinus gland (circular symbols). The results are essentially identical in either case, which suggests that there are no additional hormonal agents produced by the sinus gland whose function is to modify the CHH response.

CHH Activates a Guanylate Cyclase in Muscle Homogenates

Establishing an Assay for Lobster Guanylate Cyclase—Because the results in the preceding section suggest that CHH is likely to act by increasing the rate of cyclic GMP synthesis, we attempted to directly measure guanylate cyclase activity in fractionated lobster tissues. The assay that we used is based on methods that have been successfully applied to homogenates of vertebrate tissues (Leitman et al., 1987). The ability to quantitatively measure cyclase activity is demonstrated by several features of the assay. 1) Cyclic GMP synthesis is linear for at least 30 min (Figs. 2a and 4). We believe that this approximates the true rate of synthesis, since high levels of IBMX significantly block the enzymatic contribution of phosphodiesterases. Although it is possible that the lobster preparation contains an IBMX-insensitive phosphodiesterase, we consider this unlikely because no such enzyme has been reported in the literature (Beavo, 1988). 2) The response reflects the rate at which the cyclase enzymes are working rather than limitations imposed by the availability of substrate. As shown in Fig. 2a, cyclic GMP synthesis is not stimulated by doubling the levels of GTP in the reaction mixture. 3) Synthesis can be stimulated, however, by increasing the amount of tissue in the incubation mix. Fig. 2b shows the rate of synthesis obtained as the concentration of purified membranes is varied. The response is proportional to the relative dilution, as expected if the assay accurately reflects the level of enzyme activity. All other experiments reported in this study were performed at rates of synthesis below 0.2 pmol of cyclic GMP/μl/min/40-μl sample, well within the range of demonstrated linearity. 4) As in vertebrate animals, the activity of the lobster enzymes can be enhanced by Mn²⁺ (Figs. 2c and 3). This further establishes that cyclic GMP synthesis under basal conditions is not substrate-limited.

Characterization of Membrane and Cytoplasmic Forms of Cyclase—When high speed centrifugation is used to prepare soluble and particulate fractions of muscle homogenates, the fractions each contain substantial guanylate cyclase activity. The specific activity of the membrane cyclase is 0.275 ± 0.065 pmol of cyclic GMP synthesized/min/mg of protein (mean ± S.E., n = 29), while that of the cytoplasmic cyclase is 0.158 ± 0.030 pmol of cyclic GMP synthesized/min/mg of protein (mean ± S.E., n = 19). Since there is approximately twice as much...
Peptide Stimulation of Particulate Guanylate Cyclase

![Diagram](image)

**Fig. 1.** Effects of IBMX on the magnitude and time course of the CHH response in intact muscle. a, IBMX potentiates the response to sinus gland extracts (O—O) or purified CHH (O—O) at both saturating and subsaturating levels. Muscles were preincubated either with (filled symbols) or without (open symbols) IBMX and then treated with peptide at the indicated dilutions. Levels of cyclic GMP were determined after 90 min of additional incubation, as described under “Materials and Methods.” Each determination was made in duplicate (crude extract) or triplicate (purified peptide), and the error bars report the range and the S.E. The lines were fitted by eye. b, time course of the response in the presence and absence of IBMX. Muscles were preincubated as in (a), then exposed to a subsaturating dose of sinus gland extract (O—O), at 0.08 gland/ml or purified peptide (O—O), at 7 pmol/ml, either with (filled symbols) or without (open symbols) IBMX. At the times indicated, individual muscles were removed and their cyclic GMP content was determined. Each point is the mean ± S.E. of 3 to 10 determinations, pooled from 3 separate experiments. The individual experiments, each of which was performed with muscles from a single animal, closely resemble the composite data. The lines were fitted by eye. c, reversal of the response following removal of the peptide. A detailed time course was determined only in the absence of IBMX, since, as expected from its role as a phosphodiesterase inhibitor, IBMX dramatically retards the recovery process (data not shown). Muscles were stimulated for 40 min with a subsaturating dose of sinus gland extract (O—O) or purified peptide (C—C). At the end of the stimulation period (time = 0), muscles were washed for 3 min with saline (3 rinses at 10 ml/rinse), in order to remove the peptide, and samples were taken at the indicated intervals. The solid line was fitted by eye. For purified and crude peptide, respectively, each point is the mean ± S.E. of 9 (or 12) determinations, pooled from 3 (or 4) separate experiments. As in b, each individual experiment closely resembles the composite. In all 7 experiments, the last data point remains significantly above baseline (indicated by the horizontal dashed line). To see if this prolonged effect on cyclic GMP levels is due to residual peptide not removed by the washing procedure, we subjected 3 muscles to an additional 3 washes 25 min after the initial wash and measured their cyclic GMP levels 245 min later (O—O); see text. In one experiment, we also subjected three washed muscles to a second exposure to purified peptide at 160 min (upward arrow) and measured their cyclic GMP levels 40 min later (O—O).
Peptide Stimulation of Particulate Guanylate Cyclase

**Fig. 2. Basic properties of the guanylate cyclase assay in muscle homogenates.** Data are from experiments with isolated membranes. Similar data (not shown) may be obtained with crude homogenates or with isolated cytoplasm. a, linearity of the response at standard (2 mM, O—O) and elevated (4 mM, □—□) GTP levels. Each data point for the time course at 2 mM GTP is the mean (±S.E.) of 3 to 6 determinations from 3 separate experiments. The data points at 4 mM GTP are the mean (±S.E.) for 3 determinations made during one of the 2 mM experiments. The line was fitted by linear regression to the pooled 2 and 4 mM data (for incubation times of 60 min or less), with a correlation coefficient (R² value) of 0.978. When the two sets of data were fitted separately, there was a small decrease (12%) in the rate at 4 mM GTP compared to that at 2 mM.

We do not know if this slight apparent inhibition is significant. b, the rate of cyclic GMP synthesis is dependent on the amount of membrane added to the assay. Each point gives the rate of synthesis at a single concentration of protein. Rates were obtained by measuring cyclic GMP levels in duplicate at 0, 10, and 20 min of reaction time, then determining the slope of a line fitted to these data points by linear regression. In all cases, the correlation coefficients for the fitted lines were greater than 0.95. Data were obtained on 2 days, using aliquots frozen from a single batch of membranes with particularly high cyclase activity (approximately 0.4 pmol/min/mg of protein). The line drawn in the figure was determined by linear regression analysis of the rates as a function of protein concentration (correlation coefficient = 0.93). c, the rate of cyclic GMP synthesis is stimulated by Mn²⁺ (■—■) and inhibited by Ca²⁺ (Δ—Δ). Each point is the mean (±range) of duplicate determinations made in a single experiment. Lines were fitted by linear regression.

**Fig. 3. Comparison of the Ca²⁺ (■—■, □—□) and Mn²⁺ (□—□, ○—○) sensitivity of the membrane cyclase (filled symbols) and soluble cyclase (open symbols).** As in Fig. 2b, rates were obtained from linear regression analysis of several time points in the 0–20 min range. The correlation coefficients of the fitted lines were usually above 0.90 and always greater than 0.05. The control rate (no added ions) is defined as 100%. The figure shows the averaged results of 3 experiments for each ion (mean ± S.E.).

These results, therefore, provide strong evidence that the peptide can activate a membrane-bound form of guanylate cyclase. The properties of the response of the purified membranes are consistent with those in the intact cell, except that the efficiency of coupling of receptors to cyclase appears to be reduced in the homogenate. In intact cells, when tested at the EC₅₀, the peptide can cause 50- to 100-fold increases in cyclic GMP levels (Fig. 1, and Goy et al., 1987a). In contrast, at the EC₅₀, the peptide causes only 2- to 3-fold stimulation of cyclic GMP synthesis in homogenates (see above and Fig. 4). A similar disparity in the magnitude of the response of intact compared to homogenized tissues has been noted for the ANP-sensitive cyclase obtained from vertebrate sources (Hamet et al., 1986).

**Investigation of a Possible Contribution of the Soluble Cyclase**

The results in the preceding section demonstrate that CHH can activate membrane-bound cyclase, but do not rule out the possibility that, in the intact cell, it also activates cytoplasmic cyclase. The failure to observe an effect on the soluble enzyme in the homogenate studies may simply reflect the fact that the receptors for the peptide are associated with the membrane, and hence cannot interact with soluble cyclase after fractionation. This idea is somewhat attractive, since it could explain why the response in the intact cell is larger than the response in purified membranes.

In vertebrates, the only definitively characterized route for activation of the soluble cyclase is the EDRF pathway. The identity of EDRF is somewhat controversial. Among the leading contenders are nitric oxide (Palmer et al., 1987; Ignarro et al., 1987) and nitrocysteine (Myers et al., 1990). Once released, EDRF appears to stimulate the activity of the soluble cyclase through the transfer of electrons, perhaps to a flavin group associated with the enzyme (Craven and DeRubertis, 1978). Several pharmacological tools have proven useful in the investigation of EDRF-dependent processes (Furchgott and Vanhoutte, 1989). Methylene blue, a potent electron acceptor, can be used to intercept receptor-induced EDRF molecules before their electrons reach and activate the cyclase.
Peptide Stimulation of Particulate Guanylate Cyclase

molecule. Nitroprusside, which breaks down spontaneously to produce nitric oxide, can be used to activate the pathway in a receptor-independent manner. Therefore, if CHH acts in part through the generation of EDRF, then methylene blue should inhibit that part of the response while nitroprusside should mimic it.

Fig. 5a shows the effect of methylene blue on the response to CHH. Not only is there no block of the peptide-induced cyclic GMP increase, but, somewhat surprisingly, methylene blue actually potentiates the response in intact tissue. The potentiation is not due to an unexpected stimulatory effect of methylene blue on the cyclase, because methylene blue alone appears to decrease the basal level of cyclic GMP (Fig. 5a, +methylene blue). We do not know why methylene blue enhances the response to CHH. However, despite this complication, it is quite clear that no inhibition occurs. This result is consistent with earlier experiments demonstrating that CHH-stimulated cyclic GMP synthesis in intact muscle is Ca²⁺-independent (Goy et al., 1987a), while EDRF-stimulated cyclase activity in intact vertebrate tissues requires the presence of extracellular Ca²⁺ (Waldman and Murad, 1987). Taken together, the Ca²⁺ and methylene blue results strongly suggest that the EDRF pathway does not mediate the effects of CHH.

In further support of this idea, Fig. 5b shows that intact lobster muscle fails to elevate cyclic GMP levels when treated with nitroprusside at a concentration more than 50-fold above that needed to activate the soluble cyclase in vertebrate tissues. We have also tested nitroprusside on intact lobster muscle at a variety of lower concentrations and for times ranging from 1 to 90 min, in all cases without effect (data not shown). As a control for the possibility that nitric oxide was produced by the nitroprusside but was unable to enter the cytoplasm to interact with the soluble cyclase, we applied nitroprusside to a broken cell preparation. Fig. 5c shows that the activator has no effect in a crude homogenate of lobster muscle (which contains both membrane and soluble cyclases). As positive controls, we have demonstrated that nitroprusside has the expected effect on cyclic GMP synthesis both in intact rat sensory neurons (Fig. 5b) and in homogenized rat smooth

---

**FIG. 4.** Comparison of the CHH sensitivity of the membrane and soluble cyclases. Time course for the membrane (a) and soluble (b) cyclases with (■—■, ■—■) and without (○—○, □—□) a subsaturating dose of purified CHH (squares) or sinus gland extract (circles). Each point is the mean ± range for 2 determinations. These results have been confirmed by many additional experiments. See text for details.

---

**FIG. 5.** Effects of methylene blue and nitroprusside on cyclic GMP metabolism in various cell types. a, failure to inhibit the response to CHH in intact lobster muscle with methylene blue (see "Materials and Methods" for details). Each bar is the mean ± S.E. of 3 to 9 determinations from 3 separate experiments. Crude and purified peptides were used at a subsaturating level, to ensure that inhibition, if it occurred, would be apparent. The concentration of methylene blue (1 mM) is 10- to 100-fold above that needed to cause complete inhibition of EDRF-mediated responses in vertebrate tissues (Gruetter et al., 1979; Garthwaite et al., 1988). b, cultured rat sensory (DRG) neurons or intact lobster muscles were exposed to nitroprusside at 0.3 mM (+NP) or a control solution (−NP) for 5 min. Cells were then treated with trichloroacetic acid, and cyclic GMP levels were measured as described under "Materials and Methods." Each bar is the mean ± S.E. for 3 determinations. We have tested nitroprusside on lobster muscle at many additional times and concentrations, without effect in all cases. See text for details. c, homogenates of rat aorta or lobster muscle were tested in the standard assay for nitroprusside sensitivity. The bars show net synthesis of cyclic GMP after 10 min in the presence (+NP) or absence (−NP) of 0.3 mM nitroprusside (mean ± range for duplicate determinations).
muscle (Fig. 5c). These tissues have been shown in other studies to contain a functioning EDRF pathway (Wood et al., 1988; Furchgott and Vanhoutte, 1989). The nitroprusside data suggest strongly that the EDRF pathway not only does not mediate the effects of CHH, but, in fact, is probably not even expressed in lobster muscle.

**DISCUSSION**

From previous studies, many lines of evidence suggest that cyclic GMP metabolism plays a significant regulatory role in crustacean muscle. 1) The cytoplasmic levels of cyclic GMP in lobster muscle are much higher than the levels in comparable vertebrate tissues (Goy et al., 1987a). 2) The tissue content of cyclic GMP dependent protein kinase is among the highest measured in any species (Kuo and Greengard, 1970; Kuo and Shoji, 1982). 3) Endogenous substrates for cyclic GMP-dependent protein kinase are readily demonstrated in lobster muscle (Goy et al., 1984), although they have been difficult to find in vertebrate animals (Nairn and Greengard, 1983). 4) The sinus gland, a prominent lobster neurosecretory organ, contains a peptide (CHH) that dramatically increases the cyclic GMP levels of lobster muscle, and other lobster tissues as well (Sedlmeier and Keller, 1981; Goy et al., 1987a). 5) CHH regulates glycogen metabolism in crustacean muscle (Keller and Andrew, 1973). Preliminary data also suggest that CHH may alter the contractility of lobster muscle, perhaps by modifying excitation-contraction coupling (Goy et al., 1987b).

The experiments in this paper have addressed the mechanism by which CHH mobilizes cyclic GMP metabolism in the neuromuscular target. The observations in Fig. 1 are consistent with the idea that in lobster muscle, as in other tissues, the level of cyclic GMP at any time reflects the relative rates of the cyclase and phosphodiesterase enzymes. The equilibrium level can be raised by inhibiting the phosphodiesterase with IBMX. It can also be raised by treating the tissue with CHH, either in the presence or absence of the phosphodiesterase inhibitor. The enhancement of the peptide's actions by IBMX suggests that the peptide acts primarily by stimulating one or both of the cyclases rather than by inhibiting a phosphodiesterase enzyme. The data do not, however, rigorously exclude the phosphodiesterases from all aspects of the response. Parallel regulation of the phosphodiesterase could, for example, serve to amplify the cyclase response (or act in a negative feedback loop to limit it).

One of the primary contributions of this work is the demonstration that CHH is capable of stimulating guanylate cyclase activity in isolated membranes (Fig. 4). This provides a plausible mechanism by which the peptide might carry out its biochemical actions in intact cells. It also places the peptide in the company of a relatively small number of biological activators of the cyclic GMP cascade. To date, only the mutually homologous members of the ANP family (Leitman and Murad, 1987; Song et al., 1988), ACTH (Nambi and Sharma, 1981), and a pair of sea urchin peptides called speract and resact (Hansbrough and Garbers, 1981; Suzuki et al., 1984), have been shown to activate the membrane cyclase. A variety of cross-linking, purification, and cloning studies suggest that the receptors for these peptides in fact comprise an extracellular domain of the cyclase enzyme itself (reviewed in Schulz et al., 1989). This ligand binding domain appears to communicate with an intracellular catalytic domain by means of a single membrane-spanning helix. It will be important to determine whether the same is true of the CHH receptor in the lobster. Since there is no sequence homology between CHH and the peptides of the ANP, ACTH, or speract/resact families, it is apparent that the peptide recognition component associated with the lobster cyclase cannot be identical with those in vertebrates or sea urchins. However, the fact that the sequence of the catalytic component of the cyclase is maintained across all species tested suggests that the conserved regions of the molecule could be used to clone the lobster cyclase gene(s). This would establish a novel member of the guanylate cyclase family for comparative structure/function analysis and would provide an additional vantage point for evaluating evolutionary constraints on the structure of the membrane cyclase.

We have tried to address the question of whether CHH might, in addition, make use of the cytoplasmic form of guanylate cyclase, which is reasonably abundant in lobster muscle. Although we cannot entirely exclude a contribution of the soluble cyclase to the receptor-mediated response, Fig. 5 strongly suggests that if coupling occurs between peptide receptors and the soluble cyclase then it does not occur by means of the EDRF pathway. In fact, the EDRF pathway appears not to function at all in lobster muscle, since a dose of nitroprusside that causes more than an 80-fold increase in the cyclic GMP level of cultured rat sensory neurons has no effect on isolated lobster muscle. This is somewhat unexpected, since the ability to respond to nitroprusside is one of the hallmarks of the vertebrate cytoplasmic cyclase. It remains to be seen whether the lobster-soluble enzyme displays other significant differences from the vertebrate enzyme.

If the EDRF pathway is not active in the lobster, then the existence of significant levels of cytoplasmic cyclase is somewhat puzzling. The cytoplasmic enzyme is clearly distinct from the membrane cyclase, since it does not sediment at 100,000 X g, is not coupled to CHH receptors, and does not show the time-dependent inactivation that is characteristic of the membrane form. In this regard, we do not know whether the lobster soluble cyclase is the product of a unique gene, or a degradation product resulting, for example, from proteolytic cleavage of the membrane cyclase. However, in either case, it seems likely that the soluble form is a natural component of intact tissue, rather than an artifact of homogenization, since the ratio of membrane to soluble cyclase remains constant even if we greatly prolong the interval between homogenization and centrifugation. This raises the question of the functional significance of the soluble cyclase in intact tissue. Several possibilities should be considered. 1) Perhaps CHH (or some other lobster hormone) stimulates the soluble cyclase by means of a coupling factor distinct from EDRF. 2) Perhaps the soluble cyclase is tonically active in a resting cell, but is inhibited following exposure to agents that raise intracellular Ca" (as in Figs. 2e and 3), thus leading to a decrease in cyclic GMP levels. 3) Perhaps the cytoplasmic form is an intermediate in the degradation of the membrane cyclase and serves no other biological role.

Whatever explanation proves to be correct, it is clear that describing the properties of the cyclic GMP system is essential to our basic understanding of metabolic regulation in the lobster. It is also apparent that structural and functional comparison of the guanylate cyclases of the lobster (both soluble and membrane) to those of vertebrate animals remains a fruitful area for further inquiry.

**Acknowledgments**—I am greatly indebted to Rémy Coyeaux, David L. Ingham, and Nelson Sinback for expert technical assistance. I thank Sara Raulerson, Cara Geary, and Dan McGehee for performing some of the experiments reported here, and also I thank Kathleen Dunlop, Alan Willard, and Dan McGehee for reviewing an early draft of the manuscript.
REFERENCES

Beavo, J. A. (1988) Ado. Second Messenger Phosphoprotein Res. 23, 1-38
Bergstrand, H., Kristoffersson, J., Lundquist, B., and Schurmann, A. (1977) Mol. Pharmacol. 13, 38-43
Casnellie, J. E., and Greengard, P. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1891-1895
Craven, P. A., and DeRubertis, F. R. (1978) J. Biol. Chem. 253, 8430-8443
Fischmeister, R., and Hartzell, H. C. (1987) J. Physiol. 387, 453-472
Goy, M. F., and Kravitz, E. A. (1989) J. Neurosci. 9, 369-379
Goy, M. F., Schwarz, T. L., and Kravitz, E. A. (1984) J. Neurosci. 4, 611-626
Goy, M. F., and Morel, P. (1989) J. Neurochem. 53, 95-101
Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988) Nature 336, 385-388
Goy, M. F., Mandelbrot, D. A., and York, C. M. (1987a) J. Neurochem. 48, 954-966
Goy, M. F., Kuo, M. H.-F., Pavloff, M., and Kravitz E. A. (1987b) Neurosci. Abstr. 13, 1237
Griffith, T. M., Lewis, M. J., Newby, A. C., and Henderson, A. H. (1981) J. Am. Coll. Cardiol. 12, 797-806
Kuo, J. F., and Greengard, P. (1970) J. Biol. Chem. 245, 2493-2498
Kuo, J. F., and Shoji, M. (1982) Handb. Exp. Pharmacol. 58, 393-424
Leitman, D. C., and Murad, F. (1987) End. Metab. Clin. North Am. 16, 79-105
Leitman, D. C., Agnost, V. L., Tuan, J. J., Andresen, J. W., and Murad, F. (1987) Biochem. J. 244, 69-74
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
Martell, A. E., and Smith, R. M. (1974) Critical Stability Constants, Vol. 1, pp. 204-206, Plenum Publishing Co., New York
Martell, A. E., and Smith, R. M. (1976) Critical Stability Constants, Vol. 2, p. 290, Plenum Publishing Co., New York
Myers, P. R., Minor, R. L., Jr., Guerra, R., Jr., Bates, J. N., and Harrison, D. G. (1990) Nature 345, 161-163
Nairn, A. C., and Greengard, P. (1983) Fed. Proc. 42, 3107-3113
Nambi, P., and Sharma, R. K. (1981) Endocrinology 108, 2025-2027
Newcomb, R. W. (1983) J. Comp. Physiol. 153, 207-221
Palmer, R. M. J., Ferrige, A. G., and Moncada, S. (1987) Nature 327, 524-529
Pavlopoulos, M. S., and Goy, M. F. (1990) J. Neurochem. 55, 788-797
Rapoport, R. M., Dranin, M. B., and Murad, F. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6470-6474
Saper, C. B., Standaert, D. G., Currie, D. G., Schwartz, D., Geller, D. M., and Needleman, P. (1985) Science 227, 1047-1049
Sudoh, T., Kamakawa, K., Minamino, N., and Matsuo, H. (1988) Nature 332, 78-81
Sudoh, T., Namihira, H., Radany, E. W., Ramarao, C. S., Ward, G. E., Bentley, J. K., and Garbers D. L. (1984) J. Biol. Chem. 259, 14874-14879
Tanaka, I., Misono, K. S., and Ingami, T. (1984) Biochem. Biophys. Res. Commun. 124, 663-668
Tremblay, J., Gerster, K., and Hamet, P. (1988) Adv. Second Messenger Phosphoprotein Res. 22, 319-383
Waldman, S. A., and Murad, F. (1987) Pharmacol. Rev. 39, 163-196
Waldman, S. A., Rapoport, R. M., Piscus, R. R., and Murad, F. (1985) Biochem. Biophys. Acta 845, 295-303
Wood, J. N., Coote, P. R., Minhas, A., Mullaney, L., McNeill, M., and Burgess, G. M. (1989) J. Neurochem. 53, 1203-1211