

MODULATING A MODULATOR: BIOGENIC AMINES AT SUBTHRESHOLD LEVELS POTENTIATE PEPTIDE-MEDIATED CARDIOEXCITATION OF THE HEART OF THE TOBACCO HAWKMOTH *MANDUCA SEXTA*

K. R. PRIER*, O. H. BECKMAN† AND N. J. TUBLITZ‡

*Department of Biology and Institute of Neuroscience, University of Oregon, Eugene,
OR 97403, USA*

Accepted 6 September 1994

Summary

The central nervous system of the moth *Manduca sexta* contains a group of myoregulatory neuropeptides, the CAPs (Cardioacceleratory Peptides), which cause a physiologically important, dose-dependent increase in heart rate during wing inflation and flight in adult moths. We report here that the response of the adult heart to a subset of the CAPs, the CAP_{2s}, is potentiated nearly twofold in the chronic presence of subthreshold levels of the biogenic amine octopamine or near-threshold levels of the biogenic amine serotonin. Subthreshold levels of the CAP_{2s} fail to alter the response of the heart to octopamine.

We have begun to investigate the molecular mechanisms underlying this potentiation. Previous work on the adult heart has shown that the CAP_{2s} act through an inositol-1,4,5-trisphosphate second-messenger system. Here, we demonstrate that the cardioexcitatory effects of the two amines, in contrast to those of the CAP_{2s}, are both mediated by cyclic AMP. Application to the heart of either 10^{-5} mol l⁻¹ octopamine or 10^{-6} mol l⁻¹ serotonin elicits a threefold increase in intracellular cyclic AMP levels. The CAP_{2s} have no effect on cyclic AMP levels in the heart. These results illustrate a mechanism by which the effectiveness of a neurohormone can be increased with minimal cost to the animal. In *Manduca sexta*, subthreshold levels of octopamine are found in the haemolymph during wing inflation and flight. Thus, it is possible that octopamine up-regulates the effects of CAP₂ via a cyclic-AMP-dependent mechanism during these activities.

Introduction

There is increasing evidence that biogenic amines play a major role as neuromodulators, in addition to their more traditional role as classical neurotransmitters, in many invertebrate

*Present address: Zoologisches Institut der Universität Basel, Rheinsprung 9, 4051 Basel, Switzerland.

†Present address: School of Medicine, Albert Einstein University, Bronx, NY, USA.

‡To whom all correspondence should be addressed.

systems. One biogenic amine frequently implicated in such a role is octopamine. In crustaceans, octopamine enhances phasic flexion of the abdomen, during the crayfish escape response, and also intensifies walking responses (Glanzman and Krasne, 1983). In lobster, octopamine mediates submissive defensive posturing by evoking tonic extension of the extremities and suppressing tonic flexion (Livingston *et al.* 1980).

Similar neuromodulatory effects of octopamine have also been reported in insects. Sombati and Hoyle (1984a) demonstrated a strong dishabituation and sensitization effect of octopamine on fast-type motor neurones in the locust metathoracic ganglion. They further demonstrated that stepping or flight motor patterns could be induced by microinjection of octopamine into separate, narrowly defined areas of the neuropile of the locust metathoracic ganglion (Sombati and Hoyle, 1984b). Octopamine also acts as a peripheral neuromodulator affecting the efficacy of synaptic transmission in the flight muscles of locusts and moths (Whim and Evans, 1988; Klaassen *et al.* 1986).

In all the above cases, biogenic amines act to modify the effects of the primary neurotransmitter at a synapse. Here, we provide evidence that biogenic amines in circulation can also regulate the effectiveness of peptide neurohormones. The heart of the adult moth *Manduca sexta* is regulated by circulating levels of a set of cardioregulatory neuropeptides, the cardioacceleratory peptides (CAPs; Tublitz *et al.* 1991). The five CAPs isolated to date can be separated on the basis of their chromatographic properties into two, biochemically distinct subsets, the CAP₁s (CAP_{1a} and CAP_{1b}) and the CAP₂s (CAP_{2a}–CAP_{2c}; Cheung *et al.* 1992). Both subsets have been demonstrated to be involved in elevating heart rate in adult moths during wing inflation and flight (Tublitz and Evans, 1986; Tublitz, 1989). During these behaviours, the CAPs are released from neurohaemal sites and act as neurohormones. In this paper, we show that subthreshold concentrations of octopamine and near-threshold levels of serotonin act synergistically with the CAPs on the heart. Using bioassay and biochemical techniques, we have characterized this potentiation phenomenon and have started to trace the intracellular interactions of the amines and the peptides at the molecular level.

Materials and methods

Experimental animals

Larvae and developing adult *Manduca sexta* were individually reared on an artificial diet (Bell and Joachim, 1978) under a long day photoperiod (17 h:7 h L:D) superimposed on a 27 °C:25 °C thermal cycle. Pharate adults used for breeding were transferred on the last day of adult development to a breeding cage, with humidity maintained above 50% (Tublitz and Loi, 1993).

Preparation of biogenic amines

Octopamine and serotonin samples were freshly prepared each day from 10^{-3} mol l⁻¹ and 10^{-2} mol l⁻¹ refrigerated stock solutions, respectively. Octopamine stock solution was made up in double-distilled water and serially diluted for use in either a phosphate-buffered saline for bioassay or a Pipes-buffered saline for cyclic AMP assay. Serotonin

stock solution was made up in $10^{-2} \text{ mol l}^{-1}$ acetic acid and was also serially diluted in the appropriate saline.

Composition of experimental salines

The experiments in this study used two different salines, a phosphate-buffered saline and a Pipes-buffered saline. A phosphate-buffered saline of the following composition (in mmol l^{-1}) was used in all bioassays: NaCl, 6.5; KCl, 33.5; MgCl_2 , 16.0; KHCO_3 , 2.5; KH_2PO_4 , 2.5; CaCl_2 , 5.6; and dextrose, 172.9. A Pipes-buffered saline of the following composition (in mmol l^{-1}) was used for dissection and treatment of all tissues for the cyclic AMP assay: NaCl, 6.5; KCl, 23.5; MgCl_2 , 16.0; Pipes (dipotassium salt, Sigma Chemicals), 5.0; CaCl_2 , 5.6; and dextrose, 172.9. Each saline was adjusted to a pH of 6.7 using a concentrated solution of KOH.

Isolation and purification of the CAP₂s

Previous work (Tublitz and Truman, 1985a) determined that CAP bioactivity can be allocated to two distinct peptide groups, CAP₁ and CAP₂, on the basis of their differential retention times in a high-pressure liquid chromatography (HPLC) system. The experiments described in this paper used only CAP₂ purified on the HPLC using the procedure described below. Subsequent work (Cheung *et al.* 1992), performed after the completion of the experiments described in the present paper, demonstrated that CAP₂ is composed of three cardioactive peptides, CAP_{2a}–CAP_{2c}, one of which (CAP_{2a}) is equivalent to crustacean cardioactive peptide (CCAP). Thus, the experiments reported here used a defined mixture of all three CAP₂s and no other known contaminants.

CAP₂s were obtained from the abdominal ventral nerve cords (ANCs) of pharate adult animals. ANCs were removed and frozen at -20°C for up to a few weeks. A few crystals of phenylthiourea were added to the frozen ANCs to prevent melanization by endogenous tyrosinases (Williams, 1959). CAP₂s were purified from frozen ANCs and separated from the CAP₁s as described by Cheung *et al.* (1992). Briefly, frozen ANCs were placed in a small ground-glass tissue homogenizer containing an amount of 0.1 mol l^{-1} acetic acid at least five times greater than the wet mass of the tissue. ANCs were heat-treated for 5 min at 80°C and then homogenized on ice. The homogenate was centrifuged for 3 min at approximately 9000 g (Beckman Microfuge) at 4°C . The supernatant was then drawn off and diluted 1:1 with double-distilled water and lyophilized until dry. Dry samples were resuspended in double-distilled water, applied to a Waters C-18 Sep-pak column, and eluted with increasing step-wise concentrations of acetonitrile. The CAP₂s co-elute with CAP₁s in the 80% acetonitrile fraction, which was lyophilized, resuspended and loaded onto a high-pressure liquid chromatography (HPLC) reverse-phase C-18 column. A two-segment, linear water–acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) as the counter ion was used to separate the CAP₂s from the CAP₁s, as described elsewhere (Cheung *et al.* 1992). The combined CAP₂s were then lyophilized and held at -20°C for future use. CAP₂ solutions were freshly prepared each day from these frozen, lyophilized samples by resuspending them in either Pipes- or phosphate-buffered *Manduca sexta* saline.

Heart dissection

The isolated abdominal heart from pharate adults was used in all experiments. Heart donors were in the last day of adult development, showing advanced breakdown of the pupal cuticle and nearly complete resorption of moulting fluid. The strip of dorsal cuticle containing the entire abdominal heart was removed from the animal, pinned to a dish and immersed in saline. The complete abdominal heart was dissected away from the cuticle, transferred into a dish of saline and either cut into smaller segments for bioassay or preserved intact for biochemical assay. Since the *Manduca sexta* heart is myogenic, all hearts and heart segments continued to beat throughout all experiments until fixed or until the experiment was completed. Individual hearts or segments that stopped beating were discarded.

Heart bioassay

Test substances were assayed on an *in vitro* heart bioassay consisting of the abdominal heart removed from a pharate adult male as described here and elsewhere (Tublitz and Truman, 1985a; Tublitz, 1989). A 0.5 cm segment exhibiting regular contractions was selected and oriented horizontally in a small superfusion chamber. One end of the heart segment was firmly tied with 6-0 suture silk (Ethicon) to an insect pin embedded into the chamber; the other end of the heart was tied to a Bionix F-200 isotonic displacement transducer powered by a Bionix Powerpack ED-1A. The signal from the powerpack was amplified 100-fold and visually displayed on a Tektronix 5113 oscilloscope. The signal was simultaneously fed through a window discriminator and digital-to-analog converter in order to measure instantaneous heart rate. Signals from the transducer and the frequency monitor were recorded onto a Gould 2200 Brush recorder.

An open perfusion system was used for the bioassay, with the open point suspended 5 cm above the superfusion chamber. During each bioassay, phosphate-buffered saline flowed directly from the open point to the superfusion chamber containing the isolated heart. Saline was removed by gravity to a levelling chamber, which allowed precise monitoring of the fluid level in the superfusion chamber (Tublitz and Truman, 1985a). Flow rate through the entire perfusion system was maintained at approximately 60 ml h⁻¹. Samples were applied in pulses at the open point with a 100 μ l Hamilton gas-tight syringe.

For each bioassay, a serial set of concentrations of either the CAP₂s or octopamine was prepared. For the experiments investigating the facilitation of CAP₂ effects by biogenic amines, one or two CAP₂ dose-response curves were established for concentrations of 0.125–1.0 abdominal nerve cord (ANC) unit per 100 μ l, where 1 ANC unit is the amount of CAP₂ extracted from the abdominal portion of one pharate adult ventral nerve cord. The perfusion saline was then changed to saline containing 10⁻⁹ mol l⁻¹ octopamine or serotonin. The heart was allowed to equilibrate for 30–45 min, and two more sets of CAP₂ dose-response curves were obtained. The same procedure was carried out for 100 μ l doses of octopamine ranging from 10⁻⁸ to 10⁻⁴ mol l⁻¹, with 1 ANC unit of CAP₂ per 25 ml of saline used for the second pair of dose-response curves. Threshold for bath application of the CAP₂s was about 1 ANC unit per 20 ml.

Bicinchoninic protein assay

To obtain a reliable measure of intracellular cyclic AMP concentration in individual hearts, measurements needed to be made in units of activity per milligram of total protein in the heart. A bicinchoninic acid (BCA) protein assay developed by Smith *et al.* (1985) was used to determine the average amount of protein in 1 mg of heart tissue.

A BCA solution was made up of 1 % (w/v) disodium BCA, 2 % $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$, 0.16 % disodium tartrate, 0.4 % NaOH and 0.95 % NaHCO_3 . The BCA solution was adjusted to pH 11.25 with either 50 % NaOH or solid NaHCO_3 . A working solution was prepared freshly for each experiment by combining 50 parts of BCA solution with one part of 4 % CuSO_4 solution.

Individual hearts were dissected and cleaned of all fat bodies and as much tracheal tissue as possible. They were then blotted dry, weighed and homogenized in a ground-glass tissue homogenizer with 100 μl of distilled water. The homogenate was removed to an Eppendorf tube and brought up to 1.0 ml with distilled water. 25 μl of the homogenate was then placed into a sample tube with 500 μl of BCA/ CuSO_4 working solution and incubated at room temperature for 2 h. After incubation, the optical density of the sample was measured at 562 nm and compared with a standard curve made from the identical reaction with known amounts of bovine serum albumin (BSA). A new standard curve was run in parallel for each set of hearts tested.

In testing the average protein content of a single *Manduca sexta* heart, 15 separate hearts were tested, with three samples taken from each heart. These measurements revealed an average protein content of 0.1196 ± 0.0045 mg protein mg^{-1} heart tissue (S.E.M.). We therefore measured all cyclic AMP levels in pmol of cyclic AMP per milligram of heart and converted these to pmol of cyclic AMP per milligram protein by dividing by 0.1196.

Cyclic AMP assay

Whole pharate adult hearts were dissected out of the animal and carefully cleaned of all fat bodies and most tracheal tissue, blotted dry and weighed individually. Following dissection, individual hearts were pretreated with 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX) for 10 min to prevent breakdown of cyclic AMP by endogenous phosphodiesterases. After IBMX pretreatment, the hearts were incubated in various experimental solutions for different periods depending on the experiment. Treated hearts were fixed in 100 % ethanol for 2 min and homogenized in a ground-glass tissue homogenizer. The homogenate was then centrifuged for 10 min at about 9000 g (Beckman Microfuge) and the supernatant was retained for assay. The assay was performed using a standard cyclic AMP assay kit (Amersham Corp.). Briefly, the assay consisted of competitive binding of sample cyclic AMP and a fixed amount of ^3H -labelled cyclic AMP to a binding protein, which was then removed *via* an activated charcoal suspension. The residual radioactivity was quantified against a standard curve and the resulting value adjusted by the mass of the heart. Paired male and female hearts were used for each set of experimental values.

Results

The CAP₂s, octopamine and serotonin each cause an increase in heart rate in the isolated Manduca sexta heart

The CAP₂s produce a dose-dependent increase in heart rate when applied either *in vitro* or *in vivo* (Fig. 1A; Tublitz and Truman, 1985*b*; Tublitz and Evans, 1986; Tublitz, 1989; Cheung *et al.* 1992). Threshold for the CAP₂ pulse applied *in vitro* was about 0.1 ANC units per 100 μ l. Application of concentrations greater than 1 ANC unit per 100 μ l generally had detrimental effects on the heart. There was usually little or no corresponding increase in amplitude of contraction or in basal tension of the heart with the application of the CAP₂s, and heart rate returned to normal within a few minutes of wash-out.

Octopamine and serotonin, when pulse-applied, also induced a dose-dependent increase in heart rate similar to that caused by the CAP₂s (Fig. 1B). Pulse threshold of the *in vitro* heart was in the range 10^{-8} to 10^{-9} mol l⁻¹ for octopamine and 10^{-9} to 10^{-10} mol l⁻¹ for serotonin. At higher concentrations, pulse application of either octopamine or serotonin often produced a small transient increase in amplitude of contraction and occasionally an elevation in basal tension (data not shown). These changes in basal tension were difficult to assess quantitatively since they tended to fluctuate gradually even in normal saline. In this study, only changes in heart frequency were taken into account.

The effects of bath applications of both biogenic amines were also analyzed. 10^{-9} mol l⁻¹ octopamine rarely caused any increase in basal heart rate, with the largest increase being 1.4 % ($N=5$). The *in vitro* heart was generally more sensitive to serotonin at all concentrations, with a threshold for bath application of between 10^{-10} and 10^{-9} mol l⁻¹. 10^{-9} mol l⁻¹ serotonin caused an increase in basal heart rate of up to 23 %, with an average increase in rate of about 12 % ($N=5$).

Octopamine and serotonin markedly potentiate CAP₂-induced cardioacceleration

To determine the effects of the biogenic amines on CAP₂-induced cardioexcitation, responses to the CAP₂s were measured in the presence of low levels of either octopamine or serotonin in the bath. These responses were compared with identical measurements obtained by applying the CAP₂s without any other factors in the bath, as described in the Materials and methods section. We found that the cardioacceleratory effect of the CAP₂s was markedly enhanced by the presence of subthreshold concentrations of octopamine (Fig. 2A). On average, the presence of 10^{-9} mol l⁻¹ octopamine in the saline increased the heart's response to the CAP₂s to approximately 170 % of the control value (Fig. 2B). This facilitation was relatively constant for all concentrations of the CAP₂s. Upon return to normal saline, CAP₂ responses declined to normal, pretreatment levels (data not shown). This facilitatory effect cannot be accounted for by the action of octopamine alone, since the presence of 10^{-9} mol l⁻¹ octopamine had little or no effect on the basal heart rate in these experiments, with an average change of -0.64 % and a maximum change of $+2.0$ %. Bath application of 10^{-9} mol l⁻¹ octopamine had no effect on the amplitude of contraction and no detectable effect on the basal tension.

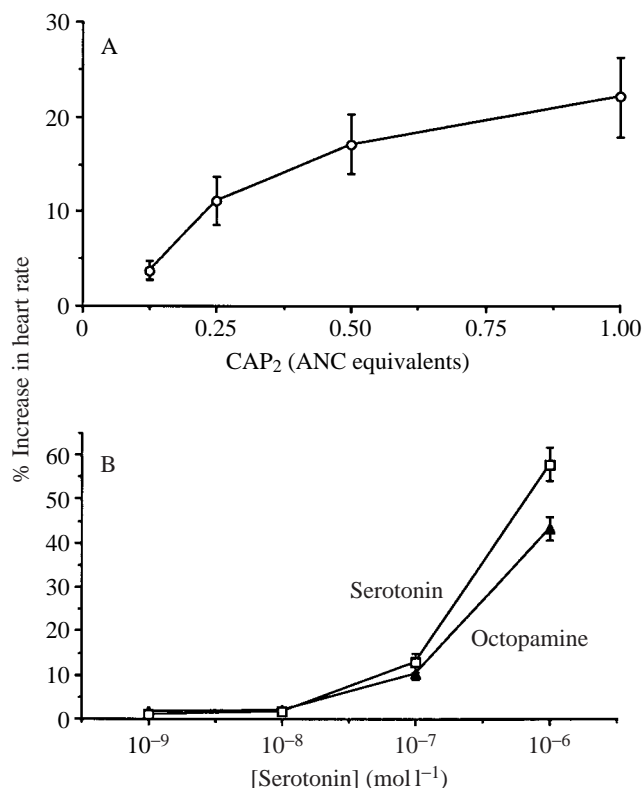


Fig. 1. Dose-response curves of the *in vitro* pharate adult *Manduca sexta* heart to pulse applications of the CAP₂s (A; $N=6-10$), octopamine (B; $N=14$) and serotonin (B; $N=8$). Samples were dissolved in phosphate-buffered saline and applied as described in the Materials and methods section. Each point represents the mean increase in rate. Error bars represent ± 1 standard error of the mean (S.E.M.).

Serotonin was also capable of producing this facilitation. Although not completely subthreshold, $10^{-9} \text{ mol l}^{-1}$ serotonin was used in these experiments for ease of comparison with octopamine. Heart responses to the CAP₂s in the presence of $10^{-9} \text{ mol l}^{-1}$ serotonin were approximately 185% of the control values (Fig. 2B), which is comparable to the effect of octopamine if the effect of serotonin alone on basal frequency is taken into account. As with octopamine, the CAP₂ responses returned to basal, pretreatment levels within 1–2 min after the serotonin had been washed out of the bath. It should be noted that bath application of $10^{-9} \text{ mol l}^{-1}$ serotonin generally caused a small increase in basal tension of the heart, but had no effect on the amplitude of contraction. Thus, both octopamine and serotonin at subthreshold or near-threshold concentrations were capable of enhancing the cardioacceleratory effects of the CAP₂s.

To determine whether this synergism was bidirectional, we measured octopamine responses at a variety of concentrations in the presence of just subthreshold levels of the CAP₂s. The *in vitro* heart was found to be quite sensitive to chronic application of the CAP₂s, with a bath threshold of about 0.1 ANC unit per 2 ml, compared with a threshold

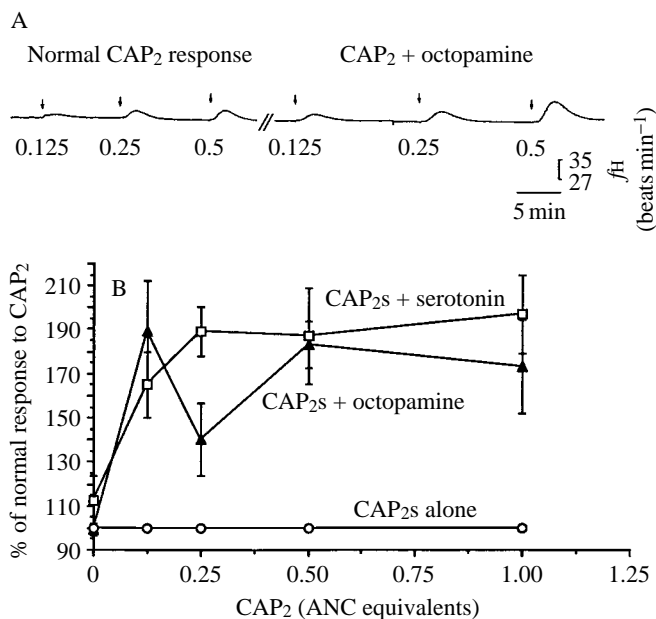


Fig. 2. The effect of $10^{-9} \text{ mol l}^{-1}$ octopamine or serotonin on the response of the *in vitro* pharate adult *Manduca sexta* heart to the CAP₂s. (A) Bioassay traces from a single heart, showing responses to 0.125, 0.25 and 0.5 ANC CAP₂s in the absence (left) and presence (right) of $10^{-9} \text{ mol l}^{-1}$ octopamine. fh, heart rate. (B) Mean effect of $10^{-9} \text{ mol l}^{-1}$ octopamine or serotonin on heart response to the CAP₂s. Each point represents the mean increase in rate from four separate trials. Error bars represent ± 1 S.E.M.

of 0.1 ANC units per 100 μl when pulse-applied. Accordingly, a perfusion concentration of 0.1 ANC unit per 2.5 ml was used to test the effect of subthreshold CAP₂s on the heart's response to octopamine. Dose-response curves were obtained for octopamine both before and during perfusion with subthreshold levels of CAP₂s. There was no significant change found in the heart's reaction to octopamine in the presence of subthreshold levels of CAP₂s (Fig. 3). Serotonin was not tested.

Octopamine and serotonin, but not CAP₂, cause a rise in intracellular cyclic AMP level in the Manduca sexta heart

Having shown that octopamine and serotonin potentiate the effectiveness of the CAP₂s on the heart, we were interested in determining the mechanism underlying this phenomenon. Since octopamine and serotonin act *via* changes in intracellular cyclic AMP concentration in many other insect systems (e.g. Pannabecker and Orchard, 1986, 1987; Fitch and Kammer, 1986), we began by investigating the relationship between cyclic AMP and biogenic amines in this system. Assays of intracellular cyclic AMP using standard techniques described in the Materials and methods section revealed a definite dose- and time-dependent change in cyclic AMP levels in the isolated heart in response to both octopamine and serotonin (Fig. 4A,B). Both octopamine and serotonin, when

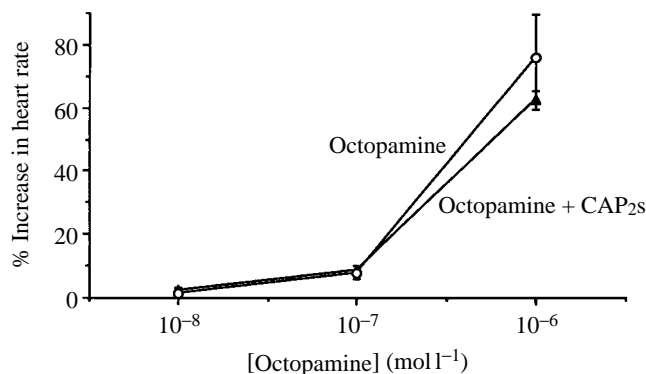


Fig. 3. The effect of subthreshold CAP₂s on the response of the *in vitro* *Manduca sexta* heart to octopamine. Each point represents the mean increase in rate from three separate trials. Error bars represent ± 1 S.E.M.

applied for a 5 min period, induced a dose-dependent elevation in intracellular cyclic AMP levels, with serotonin being much more effective in the range of 10^{-8} to 10^{-5} mol l⁻¹ (Fig. 4A). The dose-response curve for serotonin was also much steeper than that for octopamine, suggesting that the maximal downstream effects of cyclic AMP on the heart are triggered by lower concentrations of serotonin than of octopamine. Temporal measurements of cyclic AMP levels revealed that the heart responded within minutes to biogenic amine stimulation. Intracellular cyclic AMP levels increased to as much as 200% of basal levels after 2 min in 10^{-6} mol l⁻¹ serotonin or 10^{-5} mol l⁻¹ octopamine (Fig. 4B). Cyclic AMP levels remained elevated for the first 5 min of treatment and then slowly declined, even in the continued presence of transmitter. No changes in intracellular cyclic AMP levels were measured in hearts treated with the CAP₂s (Fig. 5), even at high concentrations (1 ANC unit per ml) that provoked a visually obvious, brisk increase in heart rate during treatment.

All cyclic AMP experiments were performed in the presence of 10^{-4} mol l⁻¹ IBMX, a phosphodiesterase inhibitor, which was added to both the pretreatment saline and treatment samples. IBMX doubled both basal and stimulated levels of cyclic AMP (Fig. 6). This IBMX-induced elevation in cyclic AMP concentration made our quantitative measurements more reliable because it put our cyclic AMP values in the linear range of the cyclic AMP assay. Although IBMX significantly boosted cyclic AMP levels, the magnitude of the changes induced by the biogenic amines when compared with basal levels were similar in both IBMX- and non-IBMX-treated samples. The change in basal levels of cyclic AMP due to the addition of IBMX was rapid, but stopped after 2 min and remained fairly constant thereafter (Fig. 6). To prevent our measurements from being adversely affected by the effects of IBMX on cyclic AMP levels within the first 2 min after exposure, hearts were pretreated with IBMX for 10 min before the beginning of any experimental treatment. IBMX had little or no effect on the cyclic AMP time course in response to octopamine or serotonin application (data not shown).

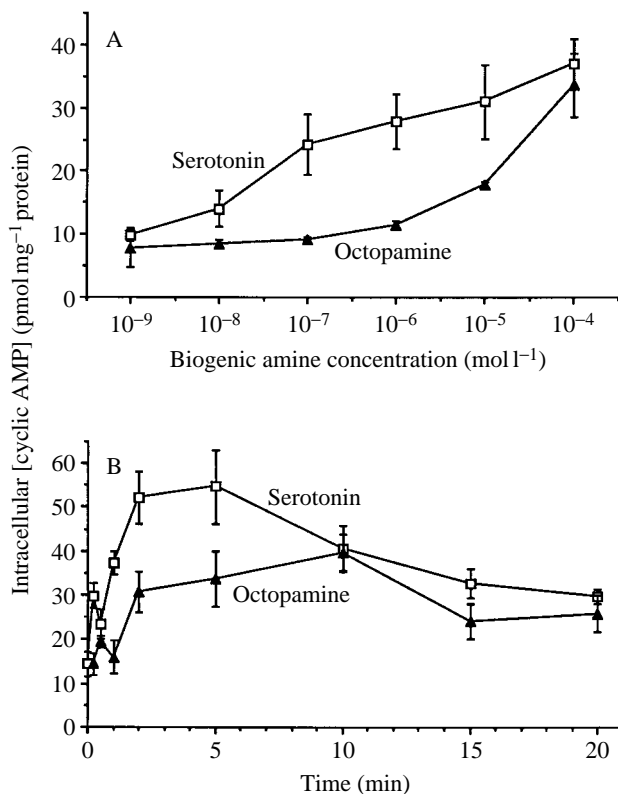


Fig. 4. The effect of octopamine or serotonin on intracellular cyclic AMP levels in the pharate adult heart of *Manduca sexta*. (A) Dose-response curve showing cyclic AMP levels in the adult heart after 5 min of incubation with octopamine ($N=5$) or serotonin ($N=5$). (B) Cyclic AMP time course in the heart in response to either 10^{-5} mol l⁻¹ octopamine ($N=3$) or 10^{-6} mol l⁻¹ serotonin ($N=3$). All measurements were taken in the presence of 10^{-4} mol l⁻¹ IBMX. Each point represents the mean cyclic AMP concentration (pmol cyclic AMP mg⁻¹ heart protein). Error bars represent ± 1 S.E.M.

Discussion

Subthreshold levels of octopamine potentiate CAP-induced cardioexcitation in adult moths

The CAPs are one of the primary modulators of heart rate in the adult moth (Tublitz *et al.* 1991). Released from the transverse nerve, the classical neurohaemal organ in the insect nerve cord, the CAPs act as neurohormones to cause an increase in heart rate during wing inflation of the newly emerged adult (Tublitz and Truman, 1985*b*; Tublitz and Evans, 1986) and also during adult flight (Tublitz, 1989). However, the CAPs are not the only cardioregulatory factors in adult blood. Klaassen and Kammer (1985) measured octopamine levels during adult development and showed that octopamine is in the blood of developing adults at a concentration of 10^{-8} mol l⁻¹, increasing to 10^{-7} mol l⁻¹ in adults. Preliminary attempts to replicate this work suggest that octopamine is indeed present in the haemolymph of newly moulted adults, but at a concentration that does not

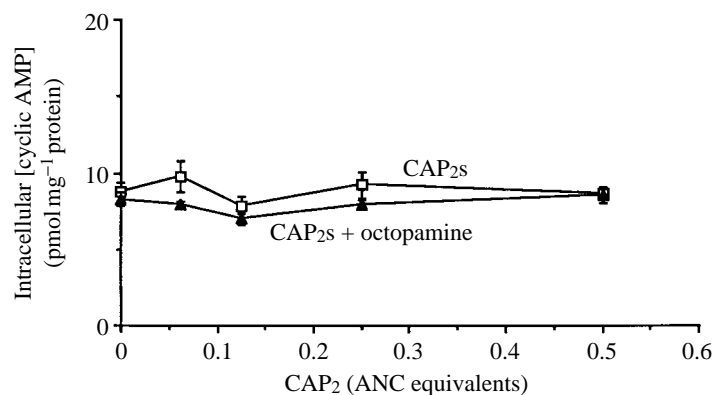


Fig. 5. The effect of the CAP₂s on intracellular cyclic AMP levels in the isolated pharate adult *Manduca sexta* heart in the presence or absence of 10^{-9} mol l⁻¹ octopamine. 10^{-4} mol l⁻¹ IBMX was present in both treatments. Each point represents the mean cyclic AMP concentration (pmol cyclic AMP mg⁻¹ heart protein). $N=6$ for CAP₂ alone; $N=7$ for the CAP₂ + octopamine. Error bars represent ± 1 S.E.M.

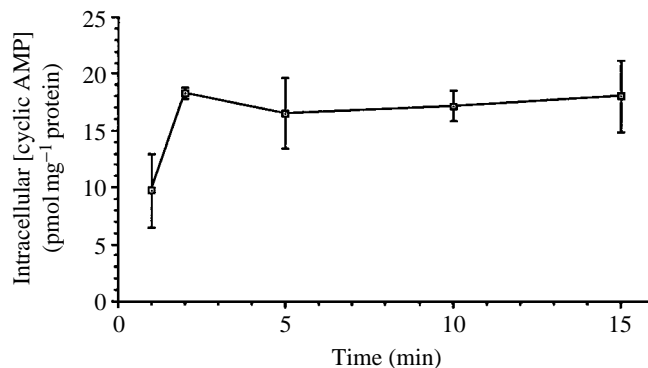


Fig. 6. The effect of 10^{-4} mol l⁻¹ IBMX on cyclic AMP levels in the pharate adult *Manduca sexta* heart. Each point represents the mean cyclic AMP concentration (pmol cyclic AMP mg⁻¹ heart protein) from six separate trials. Although the average cyclic AMP levels in these samples were higher than those in Figs 4 and 5, they fell within the normal range of variability for all IBMX-treated samples. Error bars represent ± 1 S.E.M.

exceed 10^{-8} mol l⁻¹ (N. J. Tublitz, unpublished results). In other invertebrates, there is also biogenic amine release into the haemolymph. For example, serotonin is contained in DUM cell neurohaemal release sites in *Rhodnius prolixus* (Orchard *et al.* 1989) and, in leech, octopamine is released by Leydig cells (Belanger and Orchard, 1988).

The results presented here, combined with the work of Klaassen and Kammer (1985), suggest an unusual cardiomodulatory role for octopamine in the adult. Although present in the blood at subthreshold or near-threshold concentrations, these same levels of octopamine cause a marked potentiation of the CAP-induced cardioacceleration (Fig. 3).

This provides a mechanism by which the animal can increase the gain of a peptide-mediated response with little extra cost to the organism.

Our data indicates that serotonin is also capable of synergizing with the CAP_{2S}. Although there is no evidence in *Manduca sexta* that serotonin is a circulating neurohormone, it does perform a significant hormonal role in many other invertebrates. It acts as a diuretic hormone after a meal in the blood-sucking insect *Rhodnius prolixus* (Maddrell *et al.* 1991), as a stimulant of salivary secretion in flies (Trimmer, 1985) and as an initiator of swimming behaviour in leeches (Kristan, 1983). In addition to their cardioregulatory role in *Manduca sexta* at adult emergence, the CAPs also regulate heart activity during adult flight. We suggest that a synergistic interaction between serotonin and the CAPs, similar to that between octopamine and the CAPs at adult emergence, may exist during flight in adult moths.

This type of interaction, where a biogenic amine modulates an existing neurochemical signalling pathway, has been documented several times in insects, pre- and postsynaptically as well as in hormonal situations. Presynaptically, octopamine works in *Manduca sexta* to increase miniature excitatory junction potentials of adult muscle (Klaassen *et al.* 1986). Octopamine acts postsynaptically in locust visceral (oviduct) muscle to modulate the effect of the normal transmitter (Lange and Orchard, 1986). It also acts on the locust extensor tibiae muscles to increase the relaxation rate of twitch tension after stimulation from the slow or fast motor neurones (Evans, 1985) and on the locust mandibular closer muscle to increase contraction amplitude and rates of contraction and relaxation (Baines *et al.* 1990). Maddrell *et al.* (1993) demonstrated that the action of a peptide hormone in *Rhodnius prolixus* is potentiated in the presence of suprathreshold levels of serotonin. Here, we demonstrate for the first time in an insect that biogenic amines circulating in the blood can act synergistically with a peptide neurohormone in spite of their ineffectiveness on their own.

Molecular mechanisms underlying the interactions between CAPs and octopamine

Although the molecular mechanism underlying this amine-peptide interaction has yet to be completely elucidated, our data indicate that this facilitation is mediated *via* a cyclic-AMP-dependent mechanism (Fig. 4A,B). This is consistent with results from other systems, which have demonstrated a link between octopamine and/or serotonin and cyclic AMP, including Schwann cell activity in the giant squid axon (Reale *et al.* 1986), adipokinetic hormone release in the corpus cardiacum of the locust (Pannabecker and Orchard, 1986, 1987), activity of the oviduct muscle in locust (Lange and Orchard, 1986) and activity in the dorsal longitudinal muscle of adult *Manduca sexta* (Fitch and Kammer, 1986). As all of these other octopamine/cyclic AMP systems also involve calcium, it would not be unreasonable to expect calcium also to be involved in the *Manduca sexta* heart.

Previous work has shown that intracellular levels of inositol-1,4,5-trisphosphate (InsP₃) rise in response to treatment with CAP_{2S}. Furthermore, calcium imaging results, using individual Fura2-filled heart cells, showed a relatively rapid rise in intracellular calcium concentration induced by application of InsP₃ (Tublitz *et al.* 1991). With this in

mind, it appears that the calcium release mechanism is a likely site of interaction of the two pathways.

It is important to note that the presence of octopamine at a subthreshold level has a relatively constant, synergistic effect on the CAP₂ response. That is, the effects seem to be multiplicative rather than additive. It therefore seems probable that the effect is due to a modulation of one or more of the CAP₂ pathway proteins (receptor affinity, calcium channel sensitivity to InsP₃, etc.) rather than to a change in basal levels of a second messenger. This is further supported by the one-way nature of the interaction.

Why have two hormonally mediated signals affecting the same target?

It is always in the best interest of an organism to achieve a maximal response while expending minimal energy. This system illustrates a mechanism by which the adult moth can selectively enhance a physiological response to a neurohormone. Working on their own, the CAPs significantly increase heart rate. However, during some activities, most notably wing inflation, where the animal's survival may depend on a rapid and reliable increase in heart activity, it is vital to have a means of ensuring that the response to the neurohormone is sufficient. One way to do this seems to be through the presence of a second neurohormone that, while present at too low a concentration to have any real effect on its own, can greatly enhance the target tissue's response to the primary hormone. This creates a two-tiered response to the CAP₂s, with a normal response when desired and the ability to maximize the CAP₂ effect when necessary without the need to overproduce any one hormone. Thus, the biogenic amines are used in this system to set the gain of the CAP response.

Our results show a definite, one-way interaction between two neurohormones, each using a separate second-messenger pathway. The synergistic nature of the effect demonstrates a means by which a system can temporarily increase the effectiveness of a neurohormone with minimal cost to the organism. Since the CAP₂s are released into the haemolymph of the adult animal, and not directly onto the heart, it would be very expensive physiologically to produce enough extra CAP₂ to increase the blood concentration significantly. Producing nanomolar amounts of a second, sensitizing transmitter is much more efficient and, since subthreshold levels of octopamine are actually found in the haemolymph of *Manduca sexta* during wing inflation and flight, it is likely that this octopamine is being used to up-regulate the CAP₂ effects during these important activities.

We would like to thank Dr S. H. P. Maddrell for his thoughtful comments on this manuscript and to P. K. Loi and C. C. Cheung for their support and technical assistance. This work was funded by grants from the National Science Foundation, the National Institutes of Health and the Medical Research Foundation of Oregon.

References

- BAINES, R. A., TYRER, N. M. AND DOWNER, R. G. (1990). Serotonergic innervation of the locust mandibular closer muscle modulates contractions through the elevation of cyclic adenosine monophosphate. *J. comp. Neurol.* **294**, 623–632.

- BELANGER, J. H. AND ORCHARD, I. (1988). Release of octopamine by Leydig cells in the central nervous system of the leech *Macrobdella decora* and its possible neurohormonal role. *J. comp. Physiol. A* **162**, 405–412.
- BELL, R. A. AND JOACHIM, F. A. (1978). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. ent. Soc. Am.* **69**, 365–373.
- CHEUNG, C. C., LOI, P. K., SYLWESTER, A. W., LEE, T. D. AND TUBLITZ, N. J. (1992). Primary structure of a cardioactive neuropeptide from the tobacco hawkmoth, *Manduca sexta*. *FEBS Lett.* **313**, 165–168.
- EVANS, P. D. (1985). Regional differences in responsiveness to octopamine within a locust skeletal muscle. *J. Physiol., Lond.* **366**, 331–341.
- FITCH, G. K. AND KAMMER, A. E. (1986). Effects of octopamine and forskolin on excitatory junction potentials of developing and adult moth muscle. *J. Neurobiol.* **17**, 303–316.
- GLANZMAN, D. L. AND KRASNE, F. B. (1983). Serotonin and octopamine have opposite modulatory effects on the crayfish's lateral giant escape reactions. *J. Neurosci.* **3**, 2263–2269.
- KLAASSEN, L. W. AND KAMMER, A. E. (1985). Octopamine enhances neuromuscular transmission in developing and adult moths, *Manduca sexta*. *J. Neurobiol.* **16**, 227–243.
- KLAASSEN, L. W., KAMMER, A. E. AND FITCH, G. K. (1986). Effects of octopamine on miniature excitatory junction potentials from developing and adult moth muscle. *J. Neurobiol.* **17**, 291–302.
- KRISTAN, W. B. (1983). The neurobiology of swimming in the leech. *Trends Neurosci.* **6**, 84–88.
- LANGE, A. B. AND ORCHARD, I. (1986). Identified octopaminergic neurons modulate contractions of locust visceral muscle *via* adenosine 3',5'-monophosphate (cyclic AMP). *Brain Res.* **363**, 340–349.
- LIVINGSTON, M. S., HARRIS-WARRICK, R. M. AND KRAVITZ, E. A. (1980). Serotonin and octopamine produce opposite postures in lobsters. *Science* **208**, 76–79.
- MADDRELL, S. H. P., HERMAN, W. S., FARNDAL, R. W. AND RIEGEL, J. A. (1993). Synergism of hormones controlling epithelial fluid transport in an insect. *J. exp. Biol.* **174**, 65–80.
- MADDRELL, S. H. P., HERMAN, W. S., MOONEY, R. L. AND OVERTON, J. A. (1991). 5-Hydroxytryptamine: a second diuretic hormone in *Rhodnius prolixus*. *J. exp. Biol.* **156**, 557–566.
- ORCHARD, I., LANGE, A. B., COOK, H. AND RAMIREZ, J. M. (1989). A subpopulation of dorsal unpaired median neurons in the blood sucking insect *Rhodnius prolixus* displays serotonin-like immunoreactivity. *J. comp. Neurol.* **289**, 118–128.
- PANNABECKER, T. AND ORCHARD, I. (1986). Octopamine and cyclic AMP mediate release of adipokinetic hormone I and II from isolated locust neuroendocrine tissue. *Molec. cell. Endocr.* **48**, 153–159.
- PANNABECKER, T. AND ORCHARD, I. (1987). Regulation of adipokinetic hormone release from locust neuroendocrine tissue: participation of calcium and cyclic AMP. *Brain Res.* **423**, 13–22.
- REALE, V., EVANS, P. D. AND VILLEGAS, J. (1986). Octopaminergic modulation of the membrane potential of the Schwann cell of the squid giant nerve fibre. *J. exp. Biol.* **121**, 421–443.
- SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. AND KLENK, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* **150**, 76–85.
- SOMBATI, S. AND HOYLE, H. (1984a). Central nervous sensitization and dishabituation of reflex action in an insect by the neuromodulator octopamine. *J. Neurobiol.* **15**, 455–480.
- SOMBATI, S. AND HOYLE, H. (1984b). Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. *J. Neurobiol.* **15**, 481–506.
- TRIMMER, B. A. (1985). Serotonin and the control of salivation in the blowfly, *Calliphora*. *J. exp. Biol.* **114**, 307–328.
- TUBLITZ, N. J. (1989). Insect cardioactive peptides: neurohormonal regulation of cardiac activity by two cardioacceleratory peptides during flight in the tobacco hawkmoth *Manduca sexta*. *J. exp. Biol.* **142**, 31–48.
- TUBLITZ, N. J., BROADIE, K. S., LOI, P. K. AND SYLWESTER, A. W. (1991). From behavior to molecules: An integrated approach to the study of neuropeptides. *Trends Neurosci.* **14**, 254–259.
- TUBLITZ, N. J. AND EVANS, P. D. (1986). Insect cardioactive peptides: Cardioacceleratory peptide (CAP) activity is blocked *in vivo* and *in vitro* with a monoclonal antibody. *J. Neurosci.* **6**, 2451–2456.
- TUBLITZ, N. J. AND LOI, P. K. (1993). Steroid regulation of transmitter phenotype in individual insect peptidergic neurons. II. The prepupal peak of 20-OH ecdysone directly induces bursicon expression. *J. exp. Biol.* **181**, 195–213.
- TUBLITZ, N. J. AND TRUMAN, J. W. (1985a). Insect cardioactive peptides. I. Distribution and molecular characteristics of two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. *J. exp. Biol.* **114**, 365–379.

- TUBLITZ, N. J. AND TRUMAN, J. W. (1985*b*). Insect cardioactive peptides. II. Neurohormonal control of heart activity by two cardioacceleratory peptides in the tobacco hawkmoth, *Manduca sexta*. *J. exp. Biol.* **114**, 381–395.
- WHIM, M. D. AND EVANS, P. D. (1988). Octopaminergic modulation of flight muscle in the locust. *J. exp. Biol.* **134**, 247–266.
- WILLIAMS, C. M. (1959). The juvenile hormone. I. Endocrine activity of the corpora allata of the adult cecropia silkworm. *Biol. Bull. mar. biol. Lab., Woods Hole* **116**, 323–328.