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Insect Cardioactive Peptides: Cardioacceleratory Peptide (CAP) Activity Is Blocked *In Vivo* and *In Vitro* with a Monoclonal Antibody

Nathan J. Tublitz and Peter D. Evans

AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, United Kingdom

We demonstrate here that a specific monoclonal antibody can be utilized as a physiological tool to analyze neuropeptide function. Two cardioacceleratory peptides (CAPs) have been recently isolated from the CNS of the tobacco hawkmoth, *Manduca sexta* (Tublitz and Truman, 1985a), and it has been suggested that they act as cardioregulatory neurohormones during adult emergence and wing inflation (Tublitz and Truman, 1985b). Evidence is presented here indicating that a monoclonal antibody, 6C5, selectively and specifically precipitated the biological activities of both CAPs. *In vivo* injections of 6C5 markedly reduced CAP hemolymph titers in newly emerged adults. The 6C5 treatment also blocked the primary physiological effect of the CAPs, the increase in cardiac activity seen during adult wing expansion. In addition, removal of the postemergence CAP pulse with 6C5 prolonged the duration of wing-inflation behavior. Thus, by neutralizing CAP hemolymph activity with a CAP-specific antibody, we have shown that the CAPs are involved in cardioregulation in newly emerged moths.

Neuropeptides are utilized by the nervous system in a variety of physiological processes, in which they act as neurotransmitters (Adams and O'Shea, 1983; Jan and Jan, 1982; Konishi et al., 1981; Lundberg et al., 1979; Takahashi et al., 1974), neuromodulators (Abrams et al., 1984; Brownell and Mayeri, 1979; Kreiger et al., 1983; Lloyd et al., 1984; Mayeri and Rothman, 1982), and neurohormones (Guillemin, 1978; Kupfermann, 1970; Lloyd, 1978; Pfaff, 1973; Truman, 1980). Yet unequivocal identification of a functional role for many peptides remains elusive because of the stringent physiological, pharmacological, and anatomical criteria that must be met (Barker, 1977), many of which are similar to those used in the identification of conventional neurotransmitters (Werman, 1966). One condition that is seldom met is that the absence of the neuropeptide under investigation prevents the onset of the proposed physiological response. Attempts at satisfying this criterion for neurotransmitters other than neuropeptides have usually utilized pharmacologically similar compounds that act as antagonists by binding to a membrane-bound receptor (Evans, 1981; Langer, 1980). In the case of neuropeptides, such experiments have generally not been possible due to the lack of a compound that specifically blocks the peptide receptor (Hanley, 1982). One possible solution to this dilemma often used by neuroendocrinologists is to extirpate the tissue responsible for the synthesis and/or release of the neuropeptide. Unfortunately, this procedure is not always feasible in many neurosecretory systems because of anatomical considerations or because of the diffuse nature of the neurohemal release site. We have taken a different approach to this problem, and in this report we show that neuropeptide effects can be blocked *in vivo* and *in vitro* through the use of a specific monoclonal antibody.

We have been investigating this problem in relation to the action of the cardioacceleratory peptides (CAPs) in the tobacco hawkmoth, *Manduca sexta* (Tublitz, 1983). Two cardioacceleratory neuropeptides, known as cardioacceleratory peptide$_1$ (CAP_1) and cardioacceleratory peptide$_2$ (CAP_2), have been physiologically localized to individually indentifiable neurons in the ventral nerve cord (VNC) of the pharate adult moth (Tublitz and Truman, 1985a, c, d). The CAP-containing neurons project to and terminate in blind neurohemal endings along the segmentally repeated perivisceral organs (PVOs). The PVOs are the primary neurohemal release site of the insect VNC and in *Manduca* are located on each of the abdominal and thoracic transverse nerves. Previous reports (Tublitz and Truman, 1985b) suggested the possibility that the CAPs might act as cardioregulatory neurohormones during adult wing-spreading (WS) behavior, facilitating the inflation of the adult wings by markedly increasing the heartbeat. This conclusion was based on several lines of evidence, much of which was indirect and of a correlative nature. The experiments presented here represented an attempt to verify the above conclusion using a more direct, empirical approach.

Materials and Methods

In vitro Manduca *heart bioassay*

CAP bioactivity was measured using an isolated *Manduca* heart bioassay as previously described (Tublitz and Truman, 1985a). In short, a portion of the abdominal heart was removed from a pharate adult male, pinned into a small superfusion chamber, and attached to a force transducer (Bionix F-200 Isotonic Displacement Transducer powered by a Bionix Powerpack Ed-1A). The signal from the transducer was amplified and fed through a window discriminator and digital-to-analog converter to measure instantaneous heart rate. Normal *Manduca* saline was used for all experiments (Tublitz and Truman, 1985a, b).

Immunoprecipitation

Equal aliquots of CAP_1 or CAP_2 purified by high-pressure liquid chromatography (HPLC) were incubated for 30 min at 0°C in *Manduca* saline (Tublitz and Truman, 1985a, b) either alone or in the presence of antibodies 6C5 or 2C7 at a dilution of 1 part antibody: 10 parts saline. All but 1 aliquot then received an excess of *S. aurenus* (Cowen strain) whole cells (10 mg/ml) containing Protein A (Sigma). After a second 30 min incubation at 0°C, each aliquot was spun in a microfuge (MSE MicroCentaur) at 12,000 × *g* for 5 min and the supernatant bioassayed on the isolated pharate adult *Manduca* heart as previously described (Tublitz and Truman, 1985a, b).

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Correspondence should be addressed to Dr. Nathan J. Tublitz, Institute of Neuroscience, University of Oregon, Eugene, OR 97403.

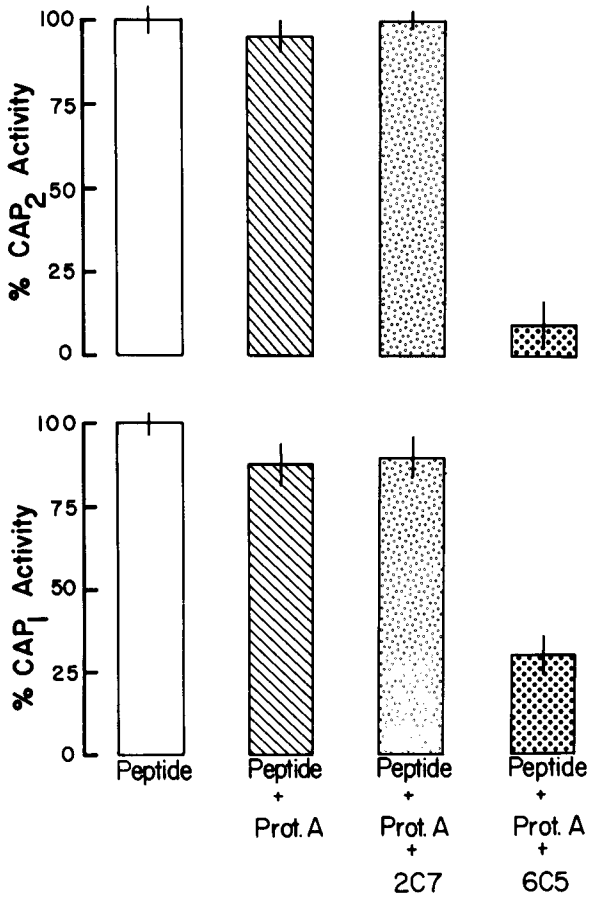

Figure 1. Immunoprecipitation of CAP_1 and CAP_2 activity by antibody 6C5. Antibody 2C7 nonspecifically stains all neurons in the *Manduca* CNS (Taghert et al., 1983; P. Taghert, personal communication) and was used as a control in these experiments. Activity is expressed as a percentage of peptide activity remaining compared to the untreated (*Peptide*) controls. Error bars on the untreated controls refer to the variability between individual samples. No measurable loss of peptide activity was measured in the untreated controls. Each bar represents the mean ± SEM of at least 5 separate determinations.

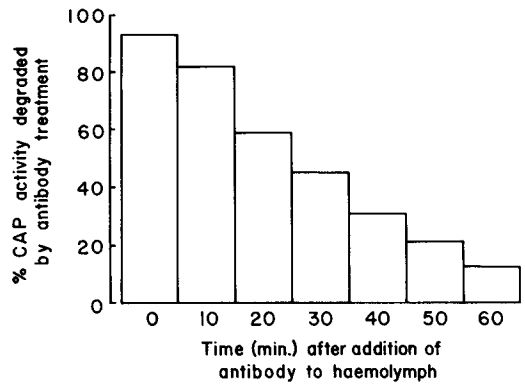

Figure 2. Kinetics of antibody degradation in *Manduca* hemolymph. The antibody 6C5 was incubated in pharate adult hemolymph for various times and then tested for its ability to precipitate CAP_2 activity. Data are presented as a percentage of CAP activity inactivated by antibody treatment compared to controls containing no antibody. Each histogram represents the mean of 2 determinations.

CAP purification

The abdominal portions of the ventral nerve cord (ANC) were removed from pharate adults and stored at −20°C. A few phenylthiourea crystals were added to the frozen tissues to prevent melanization by endogenous tyrosinases (Williams, 1959). Tissues were homogenized in a small ground-glass homogenizer containing 100% methanol at least 5 times greater than the wet weight of the tissue. The homogenate was centrifuged for 5 min at approximately 12,000 × *g* in a microfuge. The resultant supernatant was diluted 1:1 with double-distilled water and lyophilized. The dried sample was resuspended in double-distilled water and applied to a Waters C-18 Sep-pak. CAP_1 and CAP_2 activities were eluted with 80% acetonitrile, freeze-dried, resuspended, and applied to a reverse-phase HPLC C-18 column. A linear water–acetonitrile gradient separated CAP_1 from CAP_2, and final purification was achieved by eluting under isocratic conditions.

In vivo *heart activity*

Continuous *in vivo* heart recordings from intact pharate adult and adult *Manduca* were obtained using an impedance converter (Biocom Corp., Model 2991) as previously described (Tublitz and Truman, 1985b). The heart of the device is a load-sensitive 50 kHz oscillator that converts an impedance change at the recording electrodes into an output voltage (Miller, 1973). The leads from the impedance converter were inserted into 2 holes that had been made in a descaled portion of the metathoracic medial scutellar plate and fixed into place on either side of the heart using beeswax. The signal from the impedance converter was amplified and recorded onto a Rickadenki chart recorder for subsequent analysis. After electrode implantation, animals were placed inside a small wire mesh container that permitted *in vivo* heart recordings to be taken during eclosion and WS behaviors (Tublitz and Truman, 1985b).

In vivo *antibody injections*

Pharate adult males were monitored throughout the final day of adult development and were chosen for these experiments only after they had entirely resorbed their molting fluid. At this developmental stage, the pupal cuticle was extremely dry and brittle, indicating that adult emergence was imminent. Each animal then received a 100 μl injection from a Hamilton syringe of either antibody 6C5 or mouse serum dissolved in *Manduca* saline, resulting in an *in vivo* hemolymph antibody dilution of approximately 1:10. Controls received injections of the saline carrier. Occasionally the physical manipulation of the injection procedure was sufficient to induce eclosion, but usually it was necessary to remove gently the pupal cuticle around the head to ensure the intiation of adult ecdysis after antibody application. Because the results from experiments described in Figure 2 indicated that the antibody was degraded *in vivo* by the hemolymph within 30 min, animals received antibody booster injections (1:10 final antibody dilution) every half-hour. There was no apparent effect of repeated saline injections on the duration or quality of eclosion and WS behaviors (Fig. 5).

For the *in vivo* heart recording experiments, initial and booster injections of either antibody 6C5 or mouse serum were applied according to the protocol described above, except that great care was taken not to induce premature eclosion by the injection procedure. As before, boosters were administered at 30 min intervals.

CAP hemolymph titers

CAP titers were determined by collecting hemolymph at various times after eclosion and bioassaying for CAP activity on the *in vitro* pharate adult *Manduca* heart as previously described (Tublitz and Truman, 1985a, b). In short, hemolymph was obtained by inverting a decapitated animal into an ice-cold glass vial. After collection, hemolymph was heat-treated at 80°C for 5 min and centrifuged in a microfuge at 12,000 × *g* for 3 min. The supernatant was then removed and either bioassayed immediately or stored at −20°C for later testing.

Results

Immunoprecipitation of CAP activity

As previously detailed, monoclonal antibodies directed against the CAPs were raised in mice using 3000 pharate adult PVOs as the primary antigenic source (Taghert et al., 1983). After isolation and immortalization of individual clones, each antibody was histologically screened on the *Manduca* CNS (Taghert et al., 1983). Those antibodies that proved positive in the his-

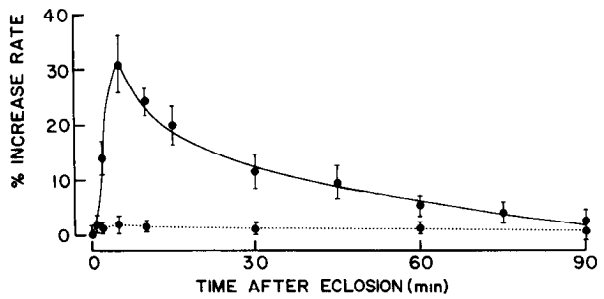

Figure 3. Effect of antibody 6C5 on the CAP hemolymph titer during eclosion and WS in the tobacco hawkmoth, *M. sexta.* Data are expressed as a percentage increase in basal heart rate. Each point indicates the mean ± SEM of at least 6 separate determinations. Time 0 represents samples taken within a few seconds after the successful completion of ecdysis. *Dotted curve,* 6C5 treatment; *solid line,* mouse serum.

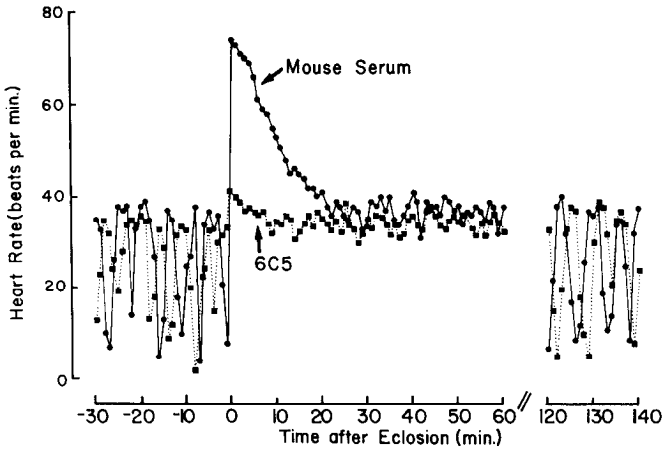

Figure 4. Effect of antibody 6C5 on *in vivo* heart rate during eclosion and WS in the tobacco hawkmoth, *M. sexta.* As described earlier (Tublitz and Truman, 1985b), the behavior of the heart changes dramatically at eclosion (time 0). A significant increase in heart rate is accompanied by a cessation of heartbeat reversals, a cardiac behavior characterized by periodic changes in beat frequency and direction of flow that occurs routinely during the pharate adult and post-WS stages (Tublitz and Truman, 1985b). Note that 6C5 treatment dramatically reduces the posteclosion rise in heart rate. *Squares,* 6C5; *circles,* mouse serum.

tological assay were further tested for specificity using both enzyme-linked immunosorbent assay (ELISA) and biological assay systems (Taghert et al., 1984; Taghert and Tublitz, unpublished observations). With the recent development of a purification procedure for both CAPs, one of the putative anti-CAP antibodies, 6C5, was tested for its ability to precipitate HPLC-purified CAP_1 and CAP_2 bioactivity. As shown in Figure 1, the bioactivity levels of both CAPs were markedly reduced in the presence of the 6C5 antibody. The antibody showed a relatively greater preference for CAP_2 than for CAP_1. CAP levels were relatively unaffected in those aliquots containing either Protein A alone or another monoclonal antibody (2C7) derived from the same fusion. The 6C5 antibody did not immunoprecipitate the biological activities of biogenic amines, 5-HT, or octopamine, nor 2 known insect peptide hormones, eclosion hormone and bursicon.

Kinetics of antibody degradation in the hemolymph of Manduca

To determine the functional lifetime of 6C5 activity in the hemolymph, antibody was incubated at 0°C in whole blood extracted from pharate adults. This developmental stage was chosen because it was previously demonstrated that pharate adult hemolymph contained no detectable amount of either CAP_1 or CAP_2 (Tublitz and Truman, 1985b). At various times after the addition of antibody, aliquots of hemolymph were removed and incubated with a small amount of HPLC-purified CAP_2 for 10 min at 0°C. Each sample was immunoprecipitated using the procedure described in Materials and Methods and the resultant supernatant bioassayed for CAP activity.

The ability of the antibody 6C5 to bind to CAP_2 was inversely proportional to the amount of time the antibody was incubated in pharate adult hemolymph (Fig. 2). 6C5 immunobinding activity slowly declined in a linear fashion over the 60 min testing period. The functional half-life of 6C5 in the hemolymph was approximately 30 min. These results were taken into account in all subsequent experiments that required prolonged antibody exposures.

Effect of antibody treatment of CAP hemolymph titers

The 6C5 antibody was injected *in vivo* into pharate adults just prior to adult emergence to investigate its effects on the rise in the CAP hemolymph titer normally seen immediately after adult emergence (Tublitz and Truman, 1985b). Animals treated with 6C5 did not exhibit the characteristic increase in CAP hemolymph levels (Fig. 3). Their levels remained close to basal throughout the 90 min following adult ecdysis, during which time the adult wings are inflated. In contrast, CAP titers were unaffected and followed their normal time course in those animals injected with mouse serum (Fig. 3). In addition, no effect on the posteclosion rise in CAP levels was noticed in those animals treated with the antibody 2C7, another monoclonal from the same fusion (data not shown).

Effect of antibody treatment on in vivo *heart rate*

Having demonstrated that an *in vivo* injection of the 6C5 antibody was capable of blocking the rise in CAP hemolymph titers immediately following eclosion, we were interested in determining whether 6C5 treatment also blocked one of the physiological effects of the CAPs, e.g., the posteclosion increase in heartbeat frequency. In these experiments, *in vivo* heart rate was monitored with an impedance converter. The 6C5 injected animals exhibited only a slight rise in heart rate *in vivo* following adult emergence (Fig. 4, Table 1), whereas controls treated with mouse serum showed a dramatic posteclosion increase in heartbeat (Fig. 4, Table 1). No measurable difference in maximal heartbeat rate after eclosion was noted between those controls that were injected either with saline or mouse serum (Table 1), and these values did not differ from those obtained with untreated animals (Tublitz and Truman, 1985b). Antibody treatment had no apparent effect on the behavior of the *in vivo* heart in the pharate adult before adult emergence nor after WS was successfully completed (Fig. 4). These data show that the post-

Table 1. Effect of the monoclonal antibody 6C5 on *in vivo* heart rate in newly emerged *M. sexta* adults

Treatment	Increase in heart rate at eclosion (%)
Saline	87.6 ± 2.9
Mouse serum	89.2 ± 4.6
6C5	15.0 ± 3.2

Experimental protocol for these experiments is as described in Materials and Methods. Heart rate was continuously measured prior to and throughout eclosion and WS. Maximal heart rate after eclosion was divided by the beat frequency of the heart immediately prior to adult emergence and expressed as a percentage. Each value represents the mean ± SEM of 6 separate determinations.

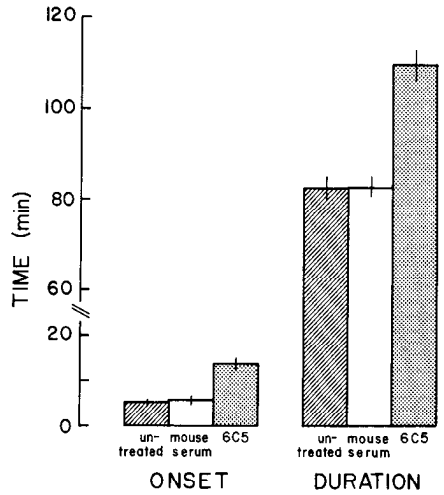

Figure 5. Effect of 6C5 injections on the onset and duration of WS behavior in *M. sexta*. Injections were carried out according to the procedure described in Materials and Methods. Antibody and mouse serum blood titers were maintained at a dilution of approximately 1:10 relative to hemolymph volume. *Onset* and *Duration* refer to the time intervals (min) between adult emergence and the initiation of WS behavior, and from the commencement to the completion of WS, respectively. *Untreated controls* received no injections. Each value represents the mean ± SEM of 10 separate determinations.

eclosion rise in heart rate is greatly reduced by the presence of the 6C5 antibody in the hemolymph.

Effect of antibody treatment on the initiation and duration of WS behavior

We were also interested in ascertaining whether 6C5 treatment had any significant behavioral effects on adult emergence and WS. Animals injected with 6C5 eclosed normally and successfully inflated their wings. There were, however, 2 significant behavioral differences, as determined by measuring the time from eclosion to the onset of wing inflation and the total duration of WS behavior, between control and 6C5-treated animals. As shown in Figure 5, the primary effect of the antibody was to extend markedly the time needed to complete WS behavior. Animals receiving 6C5 injections required 33% more time to inflate their wings fully in comparison to either uninjected controls or those treated with mouse serum (Fig. 5). In addition, antibody treatment produced a substantial effect on the onset of WS compared to controls treated with mouse serum. Based on observation, other common adult behaviors such as walking, preflight warm-up, and flight appeared to be qualitatively unaffected by the antibody treatment.

Discussion

Molecular specificity of antibody 6C5

All of the experimental manipulations of CAP activity described in this paper utilized a monoclonal antibody to specifically block CAP activity *in vivo*. To interpret the *in vivo* antibody injection experiments properly, the antibody used in these experiments, 6C5, was subjected to several tests to determine its molecular specificity.

Earlier work (Taghert et al., 1984; Taghert and Tublitz, unpublished observations) using an ELISA plate assay system indicated that one antibody, 6C5, was capable of binding to both CAP_1 and CAP_2 that had been partially purified using gel-filtration chromatography. Further experiments using partially purified material suggested that 6C5 was also able to immunoprecipitate the biological activities of both CAPs (Taghert et al., 1984). The experiments presented in this paper using HPLC-purified material corroborated our initial findings. 6C5 immunoprecipitated both CAPs, although it appeared to have a greater affinity for CAP_2 activity. 6C5 had no effect on several other known insect cardioregulatory substances, e.g., octopamine and 5-HT, and did not interfere *in vitro* with the activities of eclosion hormone and bursicon, 2 insect neuropeptides known to be present in the hemolymph of *Manduca* at the time of CAP release. The fact that the biological activities of both CAPs decreased when incubated with 6C5 suggested that the 2 CAPs share a similar antigenic recognition site. Based on these results, we concluded that the 6C5 antibody is specifically directed against both CAPs.

CAP_1 and CAP_2 are cardioregulatory neurohormones

Inflation and expansion of the wings after adult emergence in *Manduca*, as in all Lepidoptera, is brought about by a complex series of behavioral and physiological responses (Wigglesworth, 1972). In short, hemolymph is pumped into the wings by the relatively simple insect circulatory system assisted by a large increase in hemocoelic pressure due to a tonic contracture of the abdominal tergosternal muscles. Several investigations have reported that there is a dramatic change in the patterned behavior of the heart during adult emergence and wing inflation (Moreau and Lavenseau, 1975; Tublitz and Truman, 1985b; Wasserthal, 1976).

Since the discovery of the CAPs (Tublitz and Truman, 1980), our working hypothesis on the function of these cardioregulatory neuropeptides has been that one of their actions is to facilitate inflation of the wings by increasing the pumping rate of the circulatory system during this crucial developmental period (Tublitz and Truman, 1985b). The empirical basis underpinning this hypothesis came from several different lines of evidence, described in detail in an earlier publication (Tublitz and Truman, 1985b). To summarize, *in vivo* heart recordings from pharate and newly emerged adults revealed a marked increase in heart rate associated with WS behavior. Bioassay of whole blood taken from WS animals indicated the presence of 2 blood-borne, cardioacceleratory factors and several biochemical analyses showed that they coeluted with the 2 CAPs. In addition, measurements of blood CAP levels indicated that the peak CAP titers were coincident with the initiation of WS behavior. These data taken together indirectly suggest, but do not prove, a causal relationship between the CAPs and the postemergence increase in heart rate.

The data presented here provide evidence of a more direct nature in support of our working hypothesis. *In vivo* injections of 6C5 (Fig. 3) completely abolished the rise in CAP hemolymph titers normally seen immediately after adult emergence (Tublitz and Truman, 1985b), indicating that antibody treatment was pharmacologically able to depress CAP levels. That this immunoneutralization effect is not simply due to a blockage of the cardiac peptide receptor is based on the results from preliminary experiments in which CAP_2 activity was not diminished when applied on the *in vitro* heart bioassay that had been exposed to 6C5 (Tublitz, unpublished observations). 6C5 treatment also blocked most of the increase in heart rate seen during adult emergence and WS behaviors (Fig. 4, Table 1), clearly showing that the postemergence excitation of the *Manduca* heart *in vivo* is a physiological result of the elevated CAP levels. Moreover, antibody-treated animals without high levels of CAP in their hemolymph took much longer to inflate their wings successfully (Fig. 5). These results confirm our hypothesis that the primary function of the CAPs is to facilitate the successful completion of WS behavior by elevating beat frequency of the circulatory system.

Are the CAPs responsible for all the changes in heart rate during eclosion and WS?

The behavior of the heart in pharate adult and adult *Manduca* is exceedingly complex (Tublitz and Truman, 1985b). Prior to adult emergence, the heart is characterized by 2 distinct alternating modes of activity, commonly known as heartbeat reversals. At emergence, heartbeat reversals cease, and there is an almost immediate rise in heartbeat frequency. Heart rate slowly declines during WS behavior, and heartbeat reversals resume only after the wings are fully inflated.

The results from the experiments described in this paper clearly indicate that the CAPs are not responsible for all of the postemergence changes in the behavior of the heart. Antibody application failed to prevent the termination of heartbeat reversals at eclosion and also did not completely inhibit the rise in heart rate (Fig. 4, Table 1). It is possible that the local release of the CAPs around the cardiac musculature was not accessible to the antibody procedure. Alternatively, the CNS might be responsible for these effects via direct neural innervation. The latter interpretation is corroborated by data from both this and previous studies (Tublitz and Truman, 1985b), which have shown that the peak CAP titers occur after heart rate has reached its maximal level. Studies in other insects (Heinrich, 1970, 1971) have clearly shown that the CNS plays an important role in the regulation of the heart. As the innervation of the lepidopteran heart is not well known at present, this hypothesis of direct neural control must await testing.

Monoclonal antibodies as pharmacological tools

It is clear that monoclonal antibodies have other uses besides their more established ones as cellular markers (Valentino et al., 1985). They have been utilized as physiological tools in recent studies on developing (Steinhart and Alderton, 1982), neurosecretory (Kenigsberg and Trifaro, 1985), and neuronal tissues (Lundberg, 1981). Here, we have shown that a specific monoclonal antibody to the CAPs is capable of blocking neuropeptide activity both *in vivo* and *in vitro* in the tobacco hawkmoth, *M. sexta,* under well-defined conditions. As demonstrated in this report, the advantages of monoclonal antibodies for pharmacological and physiological investigations should not be underestimated (Valentino et al., 1985), especially in cases where pharmacological blockers are difficult, if not impossible, to obtain (e.g., Hanley, 1982). The use of antibodies in these situations will surely enhance our ability to elucidate the physiological role played by neuropeptides and other neuroeffectors.

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