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Spontaneous and evoked release of prothoracicotropin from multiple neurohemal organs of the tobacco hornworm

(*in vitro* neurosecretion/brain/corpora cardiaca/corpora allata/prothoracic glands)

GRANT M. CARROW, RONALD L. CALABRESE, AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT Release of neurohormone from putative cephalic neurohemal organs was directly demonstrated in an insect. The prothoracicotropic hormone (PTTH) of the tobacco hornworm, *Manduca sexta*, was measured indirectly by its ability to stimulate the secretion of α-ecdysone by inactive prothoracic glands; the ecdysone was measured by radioimmunoassay. The PTTH released spontaneously from intact brain–retrocerebral complexes was localized to the retrocerebral complex by placing a waxy barrier across the nerves connecting the corpora cardiaca to the brain. Isolated corpora allata spontaneously released much more PTTH than did either isolated corpora cardiaca or isolated brains. Media containing 100 mM potassium stimulated PTTH release from both isolated corpora allata and isolated corpora cardiaca. In calcium-free media, spontaneous PTTH release was diminished and release could not be stimulated by high potassium. These results indicate that depolarization of the neurosecretory cells is correlated with calcium-dependent neurohormone release and that there are multiple neurohemal organs for PTTH. The biological activities of stored and circulating PTTH are compared.

Scharrer (1) traced the path of brain neurosecretions in insects by observing the distribution of neurosecretory material after transection of the nerves from the brain to the paired corpora cardiaca (CC) and corpora allata (CA). Because neurosecretory material accumulated proximal to the site of nerve section and the CC were subsequently depleted of material, the CC appeared to serve as neurohemal organs for the release of cephalic neurohormones. The CC have been implicated, for example, as the site of release of the brain's major neuroendocrine product, the prothoracicotropic hormone (PTTH), which is known to play a central role in initiating molting and metamorphosis (2–4). Yet, in the Lepidoptera, PTTH activity has been recovered from the CA (5–8). In the absence of any direct demonstration of neurohormone release from either of these organs, little can be said about the actual sites for release of identified brain neurohormones.

Depolarization of neurosecretory cells has been correlated with the release of several vertebrate neurohormones (9, 10). The diuretic hormone of the bug *Rhodnius* was released from abdominal nerves by depolarization with potassium-enriched media (11). In this case, induction of release was successfully used as a probe for localizing neurohemal areas as well as for studying release mechanisms.

The PTTH secretory activity of isolated brains (lacking putative neurohemal organs) has been previously studied *in vitro* by coincubation with its target, the prothoracic glands (PG) (12, 13). Ecdysone secreted by the PG in response to PTTH was measured by *in vitro* bioassay. These studies established that PTTH activity from brains could be detected *in vitro*. However, because of the lack of information on the activity of intact brain–retrocerebral complexes (brain and attached pairs of CC and CA) or of putative neurohemal organs, no conclusions could be made concerning the normal mechanisms of PTTH release. Recently, an *in vitro* bioassay for the ready quantification of PTTH extracts was developed based on the tropic action of the neurohormone on cultured PG and measurement of the secreted ecdysone by a radioimmunoassay (RIA) (14).

In the present investigation we sought to identify the sites and mechanisms of brain neurohormone release in the tobacco hornworm, *Manduca sexta*, by studying both the spontaneous and induced release of PTTH *in vitro*. These determinations became feasible after our development of a rapid and highly sensitive alternate form of the *in vitro* bioassay for PTTH.

MATERIALS AND METHODS

Donors. Larvae of the tobacco hornworm were reared at 25°C on an artificial diet (15, 16). All tissue donors were maintained in individual plastic containers under a 12-hr light/12-hr dark photoperiod; lights off is denoted by 2400 (0000) arbitrary *Zeitgeber* time (AZT). The first day of feeding after ecdysis was designated day 1.

PG donors were day-3 fifth-instar larvae with a live weight in the range 5.25–6.25 g. All PG were extirpated between 0900 and 1400 AZT, just prior to their normal activation as determined by monitoring their activity *in vitro* (unpublished data).

Brain–retrocerebral complexes were excised from day-5 fifth instars between 1200 and 1600 AZT; this is just prior to a presumed peak time of PTTH release (17).

Dissection and Incubation of Tissues. All tissues were dissected from CO_2-anesthetized animals that were immersed in a lepidopteran saline (21 mM KCl/12 mM NaCl/3 mM $CaCl_2$/18 mM $MgCl_2$/170 mM glucose/5 mM piperazinediethanesulfonic acid/9 mM KOH, pH 6.6; 300 mosmol/liter) modified from that described by Weevers (18); the solution was autoclaved and kept sterile until used.

PG were washed in 50 μl of Grace's medium (GIBCO) for 30–45 min; they were then incubated individually in uncovered acrylic culture wells (Linbro) in 25 μl of Grace's medium at 26°C in an Aquatherm water bath with shaking at 50 rpm. The bath's atmosphere was saturated with water vapor to eliminate evaporation.

Brains and neurohemal organs were treated similarly except that they were washed for 15–30 min and incubated in either commercial Grace's medium (GIBCO) or freshly prepared medium. The latter consisted of the same mixture of amino acids and vitamins (K C Biological, Lenexa, KS) as in Grace's medium with added inorganic salts, organic acids, and sugars (pH 6.6). All chemicals were reagent grade. Osmolarity, as determined by freezing-point depression on a Fiske osmometer (model G-62), was adjusted to 350 mosmol/liter by varying the sucrose concentration.

Abbreviations: PTTH, prothoracicotropic hormone; CC, corpus cardiacum or corpora cardiaca; CA, corpus allatum or corpora allata; PG, prothoracic gland(s); AZT, arbitrary *Zeitgeber* time; RIA, radioimmunoassay.

Collection of Released PTTH. Brains, CC, and CA were incubated in 5 or 10 μl of medium separately, with their nervous connections intact (intact complexes), or as intact complexes lacking CA. At the end of the 4-hr incubation period, the tissues were discarded. The incubation medium, presumably containing PTTH, was stored at −23°C in the acrylic culture wells. Subsequently, the medium was thawed and its volume was adjusted to 25 μl with fresh Grace's medium; a single PG was then added.

Extracts of PTTH. Brains, CC, or CA were homogenized in Grace's medium and transferred to a micro test tube. The tube was suspended in boiling water for 5 min and centrifuged (12,800 × *g*) for 10 min (Brinkman, model 5412). The supernatant was collected and stored at −23°C; subsequently, the solution was thawed and serially diluted, and aliquots were added to medium bathing single PG as described.

Ecdysone Radioimmunoassay. The rabbit anti-α-ecdysone antiserum utilized was a generous gift from Timothy Kingan (Columbia University). After incubation, the ecdysone-containing medium bathing the PG was collected and diluted with borate buffer (8.27 g of boric acid, 12.24 g of borax, and 4.22 g of NaCl in 1 liter of water, pH 8.4) and stored at −23°C. The RIA was then performed by using a modification of described procedures (19, 20). The secreted ecdysone in 50 μl of borate buffer and α-[23,24-^{3}H] ecdysone (15 nCi; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) in 50 μl of borate buffer were added to a 1.5-ml polypropylene tube. The anti-α-ecdysone antiserum, diluted in a 1:10 solution of control rabbit serum and borate buffer, was added to the tube. The mixture was vortexed and stored at 4°C for 12–16 hr. Bound label was precipitated upon addition of 200 μl of saturated ammonium sulfate with vortexing. After 1 hr at 4°C, the suspension was centrifuged (12,800 × *g*) for 5 min, the supernatant was discarded, and the pellet was rinsed with 50% ammonium sulfate. The pellet was redissolved in 100 μl of water and 1 ml of scintillation solution (Aquasol-2; New England Nuclear) and vortexed. Radioactivity was determined by using a Beckman scintillation counter (model LS-230 or LS-233).

Under these conditions the counting efficiency was 33%. The binding curve for solutions of crystalline α-ecdysone (Simes, Milano) was log-linear between 25 and 500 pg of ecdysone ($r = 0.999$). Although the product of PG is α-ecdysone (21, 22), the antibody was crossreactive with other ecdysteroids. Consequently, all data are expressed as α-ecdysone equivalents.

Data Analysis. RIA data were corrected for nonspecific binding; they were then transformed and analyzed by using a DEC PDP 11/70 computer. All data are expressed as mean ± SEM. Tests of differences between means were done by *t* test on original data before transformation to brain equivalents. Tests for significance of regression were performed according to the procedure for model I regression (23).

RESULTS

Stimulation of PG with PTTH. Individual PG were washed and incubated in fresh medium, and aliquots were taken after 3 and 6 hr. Fig. 1 is a cumulative record of the amount of α-ecdysone in the medium as a function of time. During the first 3 hr of incubation, an unstimulated PG typically secreted about 2.5 ng of ecdysone; during the next 3 hr, only an additional 0.5 ng was secreted. When an extract of a day-7 fifth-instar brain was added after the first 3 hr of incubation, a dramatic increase in ecdysone secretion was observed during the ensuing 3 hr. The increase was about 30-fold compared to controls. This rapid activation of substantially inactive PG provided the basis for a swift and sensitive assay for PTTH.

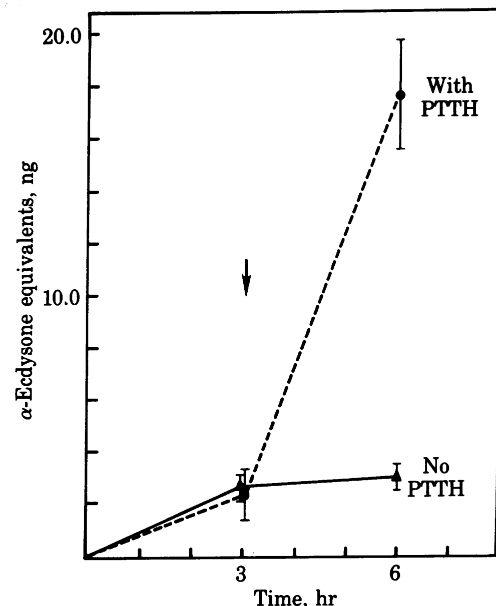

FIG. 1. Time course of accumulation of α-ecdysone in the medium bathing individual PG from *M. sexta*. Aliquots were taken at 3 hr and replaced either with Grace's medium containing the extract of one brain (●) or with extract-free Grace's medium (▲). Further aliquots were taken at 6 hr. Arrow denotes the time of PTTH addition. Each point is the mean ± SEM ($n = 10$ for ▲; 4 for ●).

Dose–Response of PG to Brain Extracts. Individual PG were washed and then preincubated for 3.5 hr in 50 μl of Grace's medium. They were then transferred to 25 μl of fresh Grace's medium with or without added PTTH extract and incubated for 3 hr. The dose–response of PG to extracts of day-7 fifth-instar brains is illustrated in Fig. 2. As little as 2% of the PTTH extractable from a single brain was sufficient to cause a significant increase in the secretion of ecdysone when compared to controls. The response was log-linear over a 100-fold range of PTTH concentration ($r = 0.86$).

This protocol allowed rapid quantification of PTTH activity in experimental samples containing unknown amounts of released PTTH. The PTTH in the sample was estimated from the standard curve and expressed in terms of extracted PTTH activity (brain equivalents).

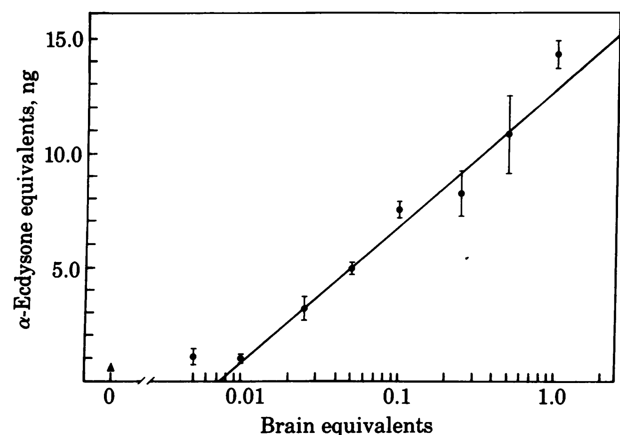

FIG. 2. Ecdysone production by PG in response to a 3-hr exposure to dilutions of an extract of brains. Each point (●) is the mean ± SEM ($n = 5$–9). The single point (▲) represents the mean ± SEM for PG ($n = 34$) bathed in the absence of extracts. The line is the regression for all points ($n = 42$) between 0.01 and 1.0 brain equivalents ($r = 0.86$).

Spontaneous Release of PTTH. Intact complexes or reduced preparations were incubated in Grace's medium for 4 hr; then, the medium was collected and assayed for PTTH activity. Intact complexes released significant amounts of PTTH; by contrast, brains alone, complexes lacking CA, or isolated CC released only small amounts of PTTH (Fig. 3). It is noteworthy that isolated pairs of CA released as much PTTH as did intact complexes ($P \gg 0.05$).

Localization of Sites of PTTH Release. Intact complexes were studied in further detail in order to determine the sites of PTTH release. To this end, intact complexes were incubated in glass Petri dishes with a barrier (CENCO Tackiwax and mineral oil, 1:1) interposed between the brain and the CC. In this manner, the medium bathing the CC and CA was isolated from that bathing the rest of the brain. The media in the two pools were collected and assayed separately for PTTH activity. After PTTH collection, all preparations were checked with the dye amaranth for the absence of leaks between pools.

PTTH was recovered only from the pool containing the CC and CA (0.08 ± 0.023 brain equivalent, $n = 6$). The activity recovered from the pool bathing the brain (0.01 ± 0.001 brain equivalent, $n = 6$) was not significantly greater than background.

K^+-Evoked Release of PTTH from CA. Depolarization of the cells was accomplished by incubation of isolated pairs of CA in Grace's medium containing 100 mM K^+ (350 mosmol/liter). About 5 times as much PTTH was released from these CA compared to CA incubated in normal Grace's medium (Fig. 4). The same high-K^+ medium did not stimulate ecdysone production by isolated PG (0.25 ± 0.035 ng α-ecdysone equivalents, $n = 7$).

Ca^{2+}-Dependence of PTTH Release. Pairs of CA were incubated in Ca^{2+}-free Grace's medium containing 100 mM K^+. In no case did high K^+ stimulate PTTH release in Ca^{2+}-free media (Fig. 4). Indeed, less PTTH was released in Ca^{2+}-free media than in normal medium.

The viability of the CA after exposure to Ca^{2+}-free conditions was ascertained by preincubating pairs of CA in Ca^{2+}-free medium for 4 hr followed by transfer to high-K^+, Ca^{2+}-containing medium for 2 hr. PTTH release could still be evoked after this preincubation period (Fig. 4).

K^+-Evoked Release of PTTH from CC. The experiments were repeated using pairs of CC (Fig. 5). As with the CA, the CC released much more PTTH in high-K^+ medium than in normal medium. However, the amount of PTTH evoked from CC was only about a fifth of that evoked from CA. Here again, the K^+-stimulated release of PTTH could not be achieved in Ca^{2+}-free medium. As with the CA, exposure to high-K^+, Ca^{2+}-containing medium after preincubation for 4 hr in Ca^{2+}-free medium resulted in evoked release of PTTH.

Dose–Response of PG to Released PTTH. Groups of 10 CA (five pairs) were incubated for 4 hr in 10 μl of high-K^+ Grace's medium. The media were collected, pooled, and serially diluted, and the PTTH was bioassayed in the usual way. The dose–response of PG to the released PTTH is illustrated in Fig. 6. As with brain extracts, the response is log-linear ($r = 0.92$). Furthermore, the slope of this curve (5.0) is similar to that in Fig. 2 (6.0) in that the 95% confidence intervals of the slopes overlap.

DISCUSSION

In the current study we have demonstrated the spontaneous and evoked, calcium-dependent release of PTTH from the CC and the CA of the tobacco hornworm. Although the CC and the CA have been individually implicated as neurohemal organs in several species of insects (1, 5–8, 24), the actual release of a neurohormone from either of these organs has not been previously demonstrated.

Although spontaneous release of neurohormones *in vitro* has been reported in vertebrates (9, 10), such release has not been detected in invertebrates. Detection and quantification of the

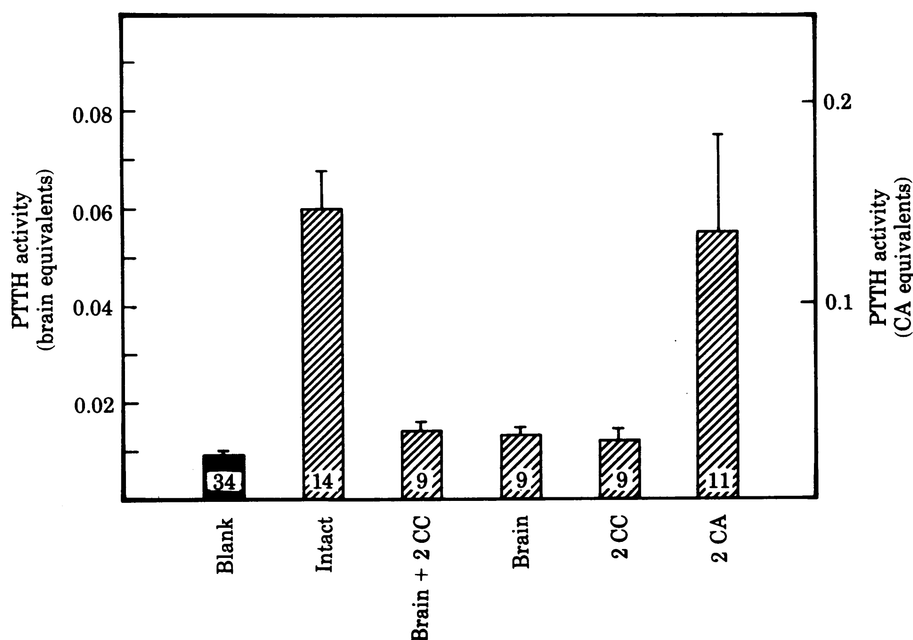

FIG. 3. Spontaneous release of PTTH by day-5 fifth-instar organs during 4-hr incubation period. The means ± SEM are plotted; the number in each bar denotes the number of trials for the organ type. Intact, intact brain–retrocerebral complexes; brain + 2 CC, intact but lacking the pair of CA; brain, isolated brain; 2 CC, a pair of CC; 2 CA, a pair of CA. Blank, background due to low-level ecdysone production by PG. The left ordinate denotes PTTH activity in brain equivalents as determined from Fig. 2. The right ordinate denotes PTTH activity in CA equivalents (day 5) as determined by the assay of three dilutions ($n = 10$) of extracts of CA.

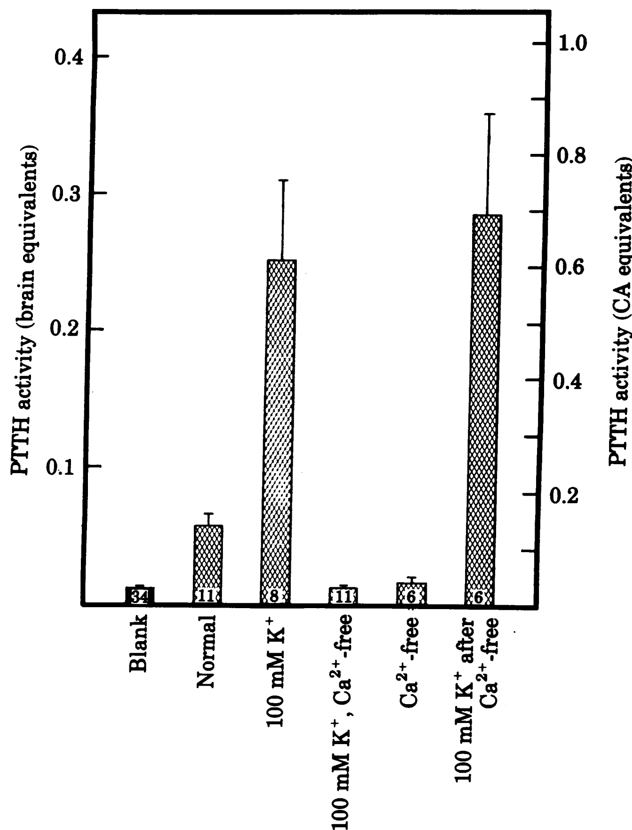

FIG. 4. PTTH release from isolated pairs of CA evoked by high K^+ and Ca^{2+} dependence of release. The Grace's media represented on the abscissa were isosmotic (350 mosmol/liter). The two bars at the right represent PTTH released from pairs of CA preincubated for 4 hr in Ca^{2+}-free, normal-K^+ medium followed by incubation for 2 hr in high-K^+, normal-Ca^{2+} medium. All other variables were as in Fig. 3.

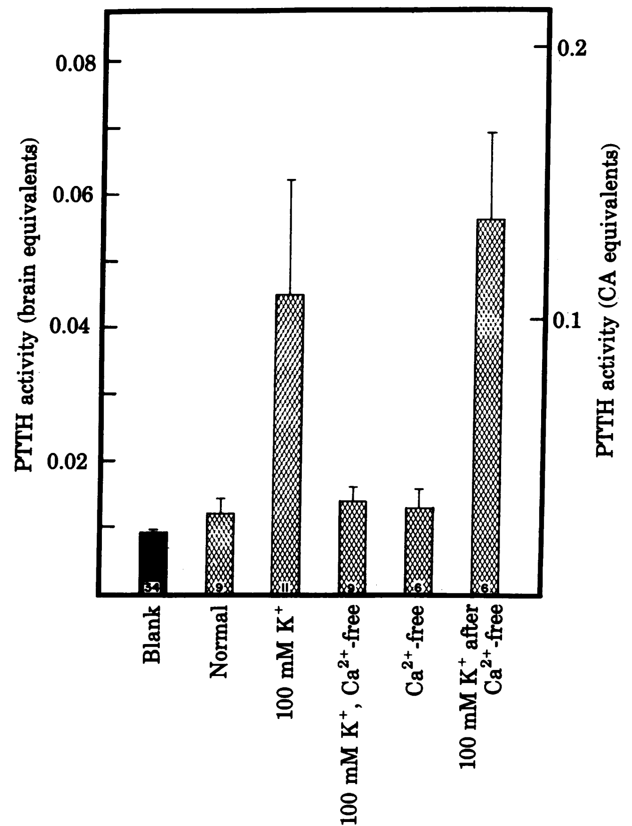

FIG. 5. PTTH release from isolated pairs of CC evoked by high-K^+ media and Ca^{2+} dependence of release. Details are as in Fig. 4.

small amounts of PTTH released spontaneously *in vitro* was facilitated by our development of an *in vitro* bioassay for PTTH with improved sensitivity and efficiency over that previously reported (14). The use of single, inactive PG described here obviated the need to make separate determinations on active, paired homologues and permitted the detection of as little as 2% of the PTTH extractable from a single brain. Moreover, the response showed relatively low variance and was linear over a 100-fold range of PTTH concentration so that the PTTH activity in individual samples could be determined directly from the dose-response curve. Thus, the need to generate a separate dose-response curve for each sample was eliminated.

PTTH release was localized to the retrocerebral complex (CC–CA) in cultures of intact brain–retrocerebral complexes with a barrier used to separate the medium bathing the brain from the medium bathing the CC–CA. All the PTTH was recovered from the pool containing the CC–CA. Furthermore, the data presented here show that isolated pairs of CA spontaneously released as much PTTH as did intact complexes and released much more than did isolated brains, isolated CC, or complexes lacking CA. These data suggest that the CA is the primary site for PTTH release in the tobacco hornworm. This conclusion is in line with previous findings that more PTTH could be extracted from the CA than from the CC of this species (7, 8).

High K^+ concentrations are known to depolarize neurons (25) and to stimulate the release of vertebrate neurohormones *in vitro* (9, 10); in invertebrates, high K^+ was shown to cause the release of diuretic hormone *in vitro* in the bug *Rhodnius* (11). In the present study, PTTH release from isolated CA incubated in high-K^+ solutions was increased more than 5-fold over the basal level. Similarly, PTTH release from isolated CC was stimulated with K^+-enriched media, although the quantity released was far less than in the case of the CA. This is circumstantial evidence that depolarization of the axons or terminals of the PTTH neurosecretory cells can stimulate the release of the neurohormone.

Furthermore, the release of stored PTTH upon depolarization of either the CC or the CA indicates that both the CC and the CA are PTTH neurohemal organs in the tobacco hornworm.

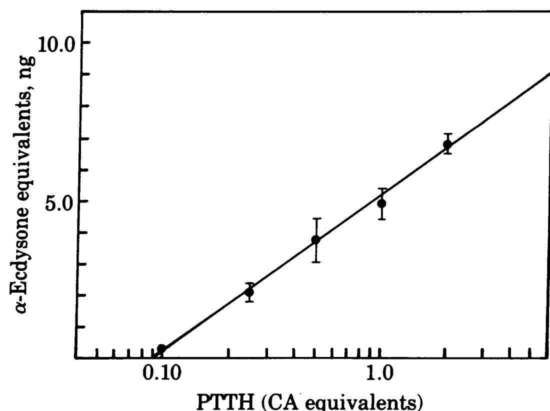

FIG. 6. Dose–response curve for dilutions of PTTH released from isolated CA in response to high K^+. Each point is the mean ± SEM (n = 3–5). The line is the regression (r = 0.92) for all points (n = 22).

By using cobalt back-filling and precipitation, Nijhout (26) showed that many axons from the brain pass through the CC to the CA in the tobacco hornworm. Thus, the CC may contain specializations of the same cell which passes through it to terminate in the CA; by repeated branching of axons, each neurosecretory cell can have many axon endings (24). Alternatively, these two neurohemal organs may contain the terminals of different PTTH-producing cells. This concept of multiple neurohemal areas is reminiscent of the multiple release sites for some vertebrate neurohormones such as somatostatin (27).

When the CA or the CC were incubated in calcium-free media, release of PTTH could not be stimulated with high K^+, and basal activity was diminished or absent. This finding is in accordance with the general belief that exocytosis of neurohormones requires extracellular calcium (28). In the present study, PTTH release was not always completely abolished in Ca^{2+}-free media. For comparison, in vertebrates, spontaneous release proceeds in Ca^{2+}-free media (9, 10). The basal activity in Ca^{2+}-free media in these cases might be attributed to a small amount of release by means of the mobilization of intracellular calcium. Nevertheless, the calcium-dependence of depolarization-induced release of vertebrate (9, 10) and invertebrate (11, 29) neurohormones has been documented. The calcium dependence of PTTH release reported here suggests that release of neurohormones upon the influx of extracellular calcium may be a generalized phenomenon in insects as well as in vertebrates.

The dose-response curves for PTTH extracts of brain and of CA and for PTTH released from CA were all log-linear and had similar slopes. The specific activities and degree of homogeneity of the stored and circulating forms cannot be determined in the absence of a means of purifying PTTH. However, the similarity of the biological activities of the materials examined here suggests a common origin.

The development of the model system described here makes possible further study of the control of neurohormone release, particularly by neurotransmitters and hormones. In addition, study of the synthesis and transport of an identified brain neurohormone is now feasible. Finally, high-K^+ depolarization provides a clean source of the circulating form of PTTH.

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