Signal Transduction in Ecdysis Hormone-induced Secretion of Ecdysis-triggering Hormone

Timothy G. Kinan‡§¶, Richard A. Cardullo¶, and Michael E. Adams‡§

From the Departments of ¶Cell Biology/Neuroscience, §Entomology, and ||Biology, University of California, Riverside, California 92521

Inka cells of insect epitracheal glands (EGs) secrete preecdysis and ecdysis-triggering hormones (PETH and ETH) at the end of each developmental stage. Both peptides act in the central nervous system to evoke the ecdysis behavioral sequence, a stereotype behavior during which old cuticle is shed. Secretion of ETH is stimulated by a brain neuropeptide, eclosion hormone (EH). EH evokes accumulation of cGMP followed by release of ETH from Inka cells, and exogenous cGMP evokes secretion of ETH. The secretory responses to EH and cGMP are inhibited by the broad-spectrum kinase inhibitor staurosporine, and the response to EH is potentiated by the phosphatase inhibitor calcineurin A. Stauroporine did not inhibit EH-evoked accumulation of cGMP. Changes in cytoplasmic Ca$^{2+}$ in Inka cells during EH signaling were monitored via fluorescence ratioing with fura-2-loaded EGs. Cytoplasmic Ca$^{2+}$ increases within 30–120 s after addition of EH to EGs, and it remains elevated for at least 10 min, corresponding with the time course of secretion. Secretion is increased in dose-dependent manner by the Ca$^{2+}$-ATPase inhibitor thapsigargin, a treatment that does not elevate glandular cGMP above basal levels. The secretory response to EH is partially inhibited in glands loaded with EGTA, while cGMP levels are unaffected. These findings suggest that EH activates second messenger cascades leading to cGMP accumulation and Ca$^{2+}$ mobilization and/or influx and that both pathways are required for a full secretory response. cGMP activates a stauroporine-inhibitable protein kinase. We propose that Ca$^{2+}$ acts via a parallel cascade with a time course that is similar to that for cGMP activation of a cGMP-dependent protein kinase.

Growth and differentiation of tissues during development of insects is orchestrated by polyhydroxylated steroids, the ecdysones, and their interplay with the sesquiterpene juvenile hormones, acting through nuclear receptors to direct transcription. An important outcome of this interplay is the production of cuticular exoskeleton. After new cuticle is produced at the end of each stadium, old cuticle must be shed. This shedding of old cuticle, termed ecdysis, occurs in a stereotyped sequence of behaviors (1). These behaviors, most thoroughly studied in the lepidopterous insect Manduca sexta, are initiated and completed in 60–70 minutes by actions of the peptide hormones preecdysis and ecdysis-triggering hormones (PETH and ETH)$^1$ and eclosion hormone (EH) (4). The findings from a number of laboratories suggest that the ecdysis sequence is set in motion following peripheral release of PETH and ETH (2,3), and the behaviors are driven by multiple loci in the CNS (5–7).

ETH is secreted by Inka cells of the segmentally distributed epitracheal glands (3) in response to EH (8). EH is secreted hormonally by peripheral neurohemal endings and centrally from axons, both of the brain-centered “ventromedial” neurosecretory cells (9,10), probably in response to ETH (7,11). Together these findings suggest a model for endocrine events in ecdysis in which ETH and EH are secreted in mutually positive feedback, leading to near depletion in stores of both peptides (8,11). Support for this model comes from several lines of evidence, including the appearance of PETH and ETH in hemolymph at the onset of the behavior (2). It has not yet been possible to adequately determine the timing of EH secretion, because of its very low concentrations in hemolymph. Nevertheless, cGMP, a second messenger in the action of EH (12,13), accumulates in epitracheal glands during the ecdysis sequence (8,11). Since ETH is secreted during EH signaling, elucidating events in the transduction cascade is critical for understanding the endocrinology of ecdysis.

Cyclic GMP increases in epitracheal glands in response to EH, and if added to culture medium, cGMP will evoke a secretory response (8); however, the mechanism by which it causes secretion or if it is even necessary for secretion is not known. In addition, findings to date have not addressed a possible role for Ca$^{2+}$ in ETH secretion; Ca$^{2+}$ is known to participate in secretory events by neurons and neuroendocrine cells (14,15). Here we report the results of pharmacological studies with epitracheal glands showing that cGMP is likely to act in secretion via activation of a protein kinase. In addition, we show that Ca$^{2+}$ levels increase in Inka cells during EH signaling and that this increase is required for a full secretory response.

EXPERIMENTAL PROCEDURES

Materials—Stauroporine and cGMP were obtained from Sigma; calcineurin A and thapsigargin were from Biomol Research Laboratories (Plymouth Meeting, PA). EGTA-AM was obtained from Calbiochem. Inhibitors were dissolved in dry dimethyl sulfoxide (MeSO$_4$) and diluted into Weever’s saline (8) containing 0.3% BSA (Weever’s/BSA) for use; final Me$_2$SO was 0.1% and did not affect basal or evoked secretion. EGTA-AM was diluted in Weever’s/BSA to 0.1 mM, and glands were loaded for 1 h before activation with EH.

Animals and Dissections—Tobacco hornworms (Manduca sexta)

* This work was supported by United States Department of Agriculture Grant CSREES 9802582, National Institutes of Health Grant AI 40555, and National Science Foundation Grant IBN-9514678. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Entomology, University of California, 5419 Boyce Hall, Riverside, CA 92521. Tel.: 909-787-4369; Fax: 909-787-3087; E-mail: tkingan@citrus.ucr.edu.

$^1$ The abbreviations used are: PETH, preecdysis-triggering hormone; ETH, ecdysis-triggering hormone; EH, eclosion hormone; CNS, central nervous system; BSA, bovine serum albumin; AS, anterior shrink stage; PKG, cGMP-dependent protein kinase; PKC, protein kinase C; EG, epitracheal gland.
were reared on artificial diet as described previously (8). Epitracheal glands were removed, attached to a short segment of trachea, from pharate pupae under Weever’s saline, and cultured individually in 40 µl of medium. For most experiments we used glands from insects at “anterior shrink” stage (AS), −3.5−4 h before pupation (16). Enzyme inhibitors were loaded by placing glands in medium with inhibitor for periods of 30–90 min, after which aliquots for ETH assay were removed and EH for activation of glands was added. Glands were cultured for an additional 20–30 min, and the medium was removed for assay. In some experiments glands were first removed from the medium and placed in ethanol:1 N HCl, 100:1, for homogenization and quantification of cGMP; the remaining medium was set aside for ETH determination.

Assays and Data Analysis—ETH and cGMP were quantified by enzyme immunoassay as described previously (8). Values for ETH in the medium after stimulation were corrected for the generally low level of ETH secreted during loading of inhibitor. Statistical comparisons of data sets were done by the Mann-Whitney test of medians.

Ca2+ Imaging—Calcium levels in individual Inka cells were quantified using the calcium indicator dye fura-2 and a video-enhanced microscope system. EGs were dissected from AS pharate pupae. Some glands were selected in which the Inka cell was separated on the trachea from the other gland cells (3, 17). Glands were loaded for 30 min with 2–10 M fura-2-AM (Molecular Probes, Eugene, OR) in Weever’s/BSA medium and then rinsed twice for 5 min in Weever’s/BSA. Glands were loaded on poly-l-lysine-coated “no. 0” coverslips in 90 µl of Weever’s/BSA, the trachea bearing the gland adhered to the coverslip to provide an unobstructed view of the Inka cell.

A Nikon TE-300 inverted microscope equipped with Neufluor optics and a xenon light source was used for fluorescence imaging. The microscope was outfitted with a 10-position filter wheel (Sutter Instruments), which contained both 340- and 380-nm bandpass filters for fura-2 excitation. The filter cube contained a dichroic mirror and a 510-nm longpass filter. Ten microliters of medium containing EH was added and mixed. An intensified charge couple device camera (Princeton Instruments) was used to capture images every 3 or 6 s during excitation at 340 and 380 nm. An image processor (MetaFluor; Universal Imaging) averaged four frames at each wavelength to increase signal-to-noise ratio. The stage of poly-l-lysine-coated coverslips in 90 µl of Weever’s/BSA, the trachea bearing the gland adhered to the coverslip to provide an unobstructed view of the Inka cell.

We previously showed that cGMP accumulates in epitracheal glands during ETH secretion in response to EH exposure and that exogenous cGMP and its 8-bromo analogue activate secretion of ETH by glands in vitro (8). To address the possibility that cGMP acts by regulating the activity of a protein kinase and the phosphorylation state of a protein in the transduction cascade, we tested the action of protein kinase and phosphatase inhibitors in basal and stimulated secretion. We also considered the possibility that protein kinases act upstream of cGMP accumulation. In these experiments, individually cultured glands were removed from medium and extracted for cGMP determination, and the medium was collected for ETH determination.

Staurosporine, an alkaloid from Streptomyces sp., is a potent inhibitor of protein kinase C (18), as well as most other kinases (19). We tested the ability of staurosporine to inhibit EH-evoked secretion of ETH by EGs in vitro. While 0.1 µM staurosporine was without effect, inhibition developed at higher concentrations; the EC50 was 0.2–0.4 M (Fig. 1). Staurosporine did not have a measurable effect on the low basal rate of secretion (data not shown). Because EH evokes an increase in glandular cGMP, and exogenous cGMP evokes a small secretory response (8), we wanted to know if the staurosporine finding reflects inhibition of a cGMP-dependent protein kinase (PKG) required for secretion. As an indirect measure, we tested the action of staurosporine in secretion evoked by exogenous cGMP. While the response was variable (Table I) it appears that staurosporine reduces secretion evoked by cGMP and 8-Br-cGMP, suggesting that elevation of endogenous cGMP during EH stimulation would lead to secretion in a staurosporine-inhibitable manner.

Together, these considerations suggest that EH stimulation leads to activation of a PKG in the transduction cascade, and that this activation plays an important role in secretion.

If phosphorylation of substrates proteins is required in EH signaling, the activity of a protein phosphatase may limit the secretory response. Accordingly, we tested the action of calyculin A, a sponge toxin that inhibits phosphatases (20) with selectivity for types 1 and 2A (21). Calyculin A increases both basal secretion and potentiates, by 5-fold, secretion resulting from threshold doses of EH (Fig. 2). The type II pyrethroid cypermethrin, a protein phosphatase type 2B (calcineurin) inhibitor (22), was without effect in evoked secretion (data not shown). Thus, it is likely that basal and EH-stimulated secretion is quantitatively determined, at least in part, by a balance between the activities of kinases and a protein phosphatase similar to the mammalian type 1 or 2A enzyme.

The suggestion above that staurosporine acts on a PKG does not rule out an action on other kinases. To test the possibility that such a kinase could regulate cGMP production, we measured EH-evoked cGMP accumulation in the presence of staurosporine. As already shown (Fig. 1), staurosporine inhibits evoked secretion (Fig. 3A); however, cGMP accumulation was not reduced (Fig. 3B). This suggests that staurosporine does not inhibit kinases that stimulate synthesis of cGMP.

Exocytotic secretion in neuroendocrine cells and neurons is triggered by elevation of cytoplasmic Ca2+ (14, 23). To begin testing the possibility that Ca2+ also participates in secretion from Inka cells, we investigated the effect of EH on cytoplasmic Ca2+. Fura-2-loaded glands were monitored for fluorescence changes and Ca2+ was measured in single Inka cells. Ca2+ levels were increased by EH ex vivo. The extent of the increase was concentration-dependent and concentra-
with alternate 340 nm and 380 nm excitation. When 340/380 ratios were monitored for 10 min without application of EH, no change was observed (data not shown). In 10 preparations containing a total of 17 Inka cells, 12 cells responded to EH (Fig. 4). Upon application of EH ratios began to increase within 15–195 s, with higher concentrations leading to shorter onsets to measurable increases (Fig. 4, C and F). A plateau in the 340/380 ratio was reached within ~6 min, and it remained elevated for the duration of 10-min experiments. With ×20 magnification the ratio appeared to increase uniformly across the surface of the cell (Fig. 4, A and B). At ×40 magnification, however, it was apparent that the highest ratios were attained at the cell margin, while lower ratios were found in underlying cortical regions (Fig. 4, D and E). In this preparation, a slight reduction of the ratio occurred over the trachea adjacent to the Inka cell (Fig. 4, F and G). Over the Inka cell the ratio increased 60–175% above that found prior to application of EH. Increases of this magnitude, when compared with the calibration set, are found to correspond to ~90–350% increases in Ca⁴⁺. Ratios obtained for Ca²⁺ solutions from a calibration set are shown in Table II. Determinations of absolute values of Ca²⁺ in Inka cells from this set were not made because of an uncertain basis for comparing the environment for fluorescence in the large (200 μm, diameter) Inka cell with that in the saline of the calibration set. Nevertheless, it appeared that Ca²⁺ rose maximally to ~1 μM in the response to EH.

We showed earlier that removal of extracellular Ca²⁺ does not diminish EH-evoked secretion (8). To address the possibility that Ca²⁺ mobilized from intracellular stores drives secretory events in the Inka cell, we tested the effect of thapsigargin, an inhibitor of ATP-dependent Ca²⁺-pumps found in cardiac sarcoplasmic and endoplasmic reticulum (24). While 0.01 μM thapsigargin was without effect, ETH secretion occurred at 0.1 and 1.0 μM inhibitor (Fig. 5A). At 1.0 μM thapsigargin release was already evident at 30 min, and further release apparently did not occur; 2.1 pmol/gland released represents ~15% of the total ETH. In separate experiments we also tested the ability of thapsigargin to affect accumulation of cGMP during evoked secretion. If Ca²⁺ is required as a direct or indirect activator upstream of guanylyl cyclase, an accumulation of cGMP might parallel thapsigargin-evoked secretion of ETH. However, thapsigargin did not significantly alter the amount of cGMP at the end of a 30-min incubation (Fig. 5B). This finding does not rule out a role for a PKC in regulating guanylyl cyclase, however, since production of neutral lipid (diacylglycerol, a second messenger in PKC activation) would not be expected in thapsigargin treatment. These considerations aside, our findings suggest that Ca²⁺ mobilization can evoke ETH release without participation of cGMP.

To test the possibility that mobilization of Ca²⁺ is required in secretion, we preloaded glands with the membrane-permeable ester of EGTA (see “Experimental Procedures”). In the presence of 3 mM Ca²⁺ in the extracellular medium, a small inhibition of release was observed in EGTA-loaded glands (Fig. 6). When glands were incubated in 0 Ca²⁺ medium, EGTA again reduced evoked secretion; the small additional effectiveness of EGTA in the absence of Ca²⁺ may not be significant. Therefore, while Ca²⁺ influx may occur and participate in secretion, it seems not to be required for a full response (Fig. 6; see also Ref. 8). To further address the possibility of cross-talk between Ca²⁺ and cGMP pathways, we tested the effect of EGTA loading on EH-evoked cGMP accumulation. EH (100 pm) evoked

**FIG. 2.** Effect of calyculin A on basal and EH-evoked secretion of ETH. Glands were incubated with or without calyculin A for 30 min and then for an additional 30 min in the presence of 30 pm EH at which time medium from control and stimulated glands was collected. Values shown are average ± S.D.; the number of determinations is shown in parentheses. *p < 0.001; **p < 0.001.

**FIG. 3.** Action of staurosporine in EH-evoked secretion of ETH and accumulation of cGMP in epitracheal glands. Glands from pharate pupae were preincubated for 1 h with 2 μM staurosporine and then stimulated with 0.01 or 0.03 nm EH. At the end of the second period glands were removed for extraction and cGMP determination, and medium was then removed for ETH determination. Values shown are average ± S.D. 10–12 determinations. A, ETH secretion; *, p = 0.0014; **, p = 0.015. B, cGMP accumulation; *, p = 0.03.
accumulation of 87 ± 74 fmol cGMP (S.D., n = 11); when glands were preincubated for 50 min in 0.01 or 0.1 mM EGTA-AM and then rinsed before EH activation, they accumulated 105 ± 70 (n = 11) and 75.1 ± 64 (n = 12) fmol cGMP, respectively; these values are not significantly different from those determined in the absence of EGTA. Therefore, the data suggest that cGMP accumulation is unaffected by EGTA, while ETH secretion is reduced ~36% (Fig. 6).

A summary of our findings and a model for signal transduction in Inka cells is shown in Fig. 7. A cell surface receptor is coupled to a guanylyl cyclase such as that described from *M. sexta* CNS (25). The cyclase produces cGMP in response to EH. A phosphodiesterase limits secretion in unstimulated cells (8), presumably by its action on cGMP that would otherwise accumulate. A PKG, inhibitable with staurosporine, promotes phosphorylation of a substrate protein, while a phosphatase, sensitive to calyculin A, limits basal and evoked secretion, presumably by reversing the action of the PKG. Either in parallel or in sequence with cGMP production, cytoplasmic Ca\(^{2+}\) increases with a time course that is similar to that of cGMP accumulation (8). The Ca\(^{2+}\) increase during secretion plateaus at low micromolar concentrations, suggesting high affinity effectors. This increase may occur either 1) in parallel with activation of guanylyl cyclase, for instance, via separate activation of receptors in intracellular stores leading to mobilization of Ca\(^{2+}\), or 2) in sequence with guanylyl cyclase, by a PKG-mediated activation of a substrate protein, which then participates in Ca\(^{2+}\) mobilization. While we do not yet know if cGMP can affect Ca\(^{2+}\) mobilization, findings from our EGTA and thapsigargin experiments suggest that Ca\(^{2+}\) does not act in a pathway leading to cGMP accumulation. Proteins that function as effectors of second messenger cGMP and Ca\(^{2+}\) have not been identified, but could include effectors of translocation.
of granules to a readily releasable pool as well as mediators of docking and/or fusion immediately prior to exocytosis.

**DISCUSSION**

We have shown that release of ETH from Inka cells of insect epitracheal glands requires participation of a protein kinase that likely is activated by cGMP during EH signaling. The extent of secretion during stimulation is limited by a calyculin A-inhibitable protein phosphatase. In addition, we find that cytoplasmic Ca\(^{2+}\) increases during signaling and that its time course is similar to that of secretion. Thapsigargin, an effective inhibitor of Ca\(^{2+}\)-dependent ATPase in endoplasmic reticulum, increases basal secretion. Intracellular EGTA, a Ca\(^{2+}\)-chelator, blunts stimulated secretion, showing that Ca\(^{2+}\) mobilization participates in secretion.

**A Role for Protein Kinases in EH-evoked Secretion**—Staurosporine is effective in inhibiting EH-evoked secretion \((IC_{50} = 0.2–0.4 \mu M)\), indicating a requirement for a protein kinase in ETH release. Moreover, secretion evoked by exogenous cGMP and 8-Br-cGMP is also inhibited by staurosporine. While the percent inhibition is lower than for EH-evoked secretion (compare Fig. 1, Table I), some of the difference may be accounted for by basal and staurosporine-insensitive secretion in both sets of glands. The current findings with staurosporine, together with our earlier demonstration that cGMP accumulates during EH signaling (8), suggests that a PKG would be activated in EH signaling and participate in the transduction cascade leading to ETH secretion. In an additional test of a role for PKG, we assayed the PKG inhibitors (RR)\(_{3}\)-8-(para-chlorophenylthio)-guanosine-3',5'-cyclic monophosphorothioate and (RR)\(_{3}\)-8-Br-guanosine-3',5'-cyclic monophosphorothioate \((26, 27)\) for activity in inhibiting EH-evoked secretion. However, neither was effective in inhibiting evoked secretion (data not shown). This apparently negative result must be viewed in the context of our observation that 8-Br-cGMP is only weakly active in evoking secretion (Table I), suggesting that insect and mammalian kinases differ in their pharmacological sensitivities or that these exogenous analogues are excluded from access to the enzyme compartment. Additional evidence indicating a role for protein kinases in secretion is the finding that the protein phosphatase inhibitor calyculin A potentiates basal and EH-evoked secretion. Inhibition of phosphatases presumably unmasks or synergizes with a basal level of protein kinase activity, allowing accumulation of a phosphoprotein that is a component in the transduction cascade. We found earlier that the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine potentiates basal secretion (8), presumably by unmasking activity of a guanylyl cyclase. Together these findings indicate that a guanylyl cyclase and one or more protein kinases are active in unstimulated cells, but that secretion is held in check by hydrolytic enzymes. In EH-stimulated cells the kinase is then able to outpace the phosphatase. This conclusion does not rule out, however, the possibility that the phosphodiesterase or phosphatase is negatively regulated to affect secretion in stimulated cells. That phosphatases may be important regulators of secretion was shown in studies with adrenal chromaffin cells, in which calyculin A treatment led to increased phosphorylation of vimentin, redistribution of granules toward the cell periphery, and enhanced basal secretion (28). However, the kinase in these events is likely to be a PKC, rather than a PKG (29).

Characterized PKGs have low \(K_i\) values, 0.05–1.0 \(\mu M\) (30).
cGMP is low in unstimulated Inka cells, 1–3 fmol in 200-μm diameter cells from pharate pupae (8). If contained in the outer 20% of cell volume as suggested by immunocytochemical findings (11), the basal concentration of cGMP would be ~0.5 μM, well within the range for activation of identified PKGs. Activated cells contain 10–50 times greater cGMP.

A role for PKGs in EH signaling in the CNS of M. sexta was suggested earlier by the observation that two 54-kDa proteins are phosphorylated in response to EH and cGMP (31). The identity of these proteins and their role in signaling, or whether they might also be present in epithrochal glands, has not been determined. Identified PKGs in insects include DG2, which has been implicated in regulating foraging behavior in Drosophila melanogaster (32), although substrate proteins for DG2 or the tissue expressing enzyme required for the phenotype have not been identified. In addition, insect PKGs have not been characterized pharmacologically.

Do Kinases Act Up- or Downstream of Guanylyl Cyclase?—In addition to determining the effects of kinase inhibitors on ETH secretion, we also quantified their effects on cGMP accumulation. We found that staurosporine does not affect EH-evoked cGMP accumulation, indicating that its action in ETH release is downstream of guanylyl cyclase. Tamoxifen has also been shown to inhibit EH-evoked cGMP accumulation in M. sexta transverse nerves, a finding that suggested the action of a PKC in regulating ETH-activated guanylyl cyclase (33). However, the specificity of these inhibitors in insect preparations has not been addressed.

A Role for Ca2+ in EH Signaling—EH evoked an increase in Ca2+ in the Inka cell; the time to onset was concentration-dependent. With 5 nM EH the time to onset was ~20 s, while with 0.1 nM onset occurred at ~200 s. The Ca2+ response occurred as a sustained plateau, rising to at most 1–2 μM in concentration. The relatively slow onsets in Inka cells are similar to those observed in a variety of nonexcitable cells in which two receptor classes, either seven-transmembrane domain-containing receptors or receptor tyrosine kinases, are coupled through G-proteins to phospholipase C (34). In Inka cells, the elevation in Ca2+ appeared to sweep uniformly across the surface of the cell during the rising phase. However, use of a x 40 objective clearly revealed higher concentrations of Ca2+ in the cortex of the cell, suggesting either influx or local “hot” areas of mobilization from subplasmalemmal intracellular stores (Fig. 5). We interpret this result with caution, however, because of uncertainty in focal plane thickness and contribution from curvature of the large diameter (200 μm) cell. It will be important to record the response in smaller cells from earlier developmental stages and to use optical sectioning of confocal microscopy to reveal regional details of mobilization. Nevertheless, in other preparations mobilization from intracellular stores activates Ca2+ influx by “capacitative calcium entry” (35), and our observation of higher 340/380 in cortical regions of Inka cells is consistent with the prediction of influx.

We addressed the significance of Ca2+ elevation in two ways: first, we found that thapsigargin treatment leads to secretion of ETH. Thapsigargin treatment leads to elevation of cytoplasmic levels of Ca2+ (36, 37) and activation of effector proteins via its inhibition of ATP-dependent Ca2+ pumps with net release from intracellular stores (38). Second, we showed that EGTA blunts, but does not abolish, EH-evoked secretion. This finding indicates that a portion of secretion is Ca2+-independent (and presumably cGMP-dependent). However, a conclusion on the role of Ca2+ will first require quantifying the effectiveness of EGTA in blunting Ca2+ mobilization during secretion as well as quantifying secretion by and Ca2+ mobilization in individual glands. Nevertheless, our findings indicate that Ca2+ mobilization is both sufficient for partial secretion of available stores and necessary for full secretion in response to EH.

The range of times to onset of Ca2+ mobilization was similar to onset in cGMP increase determined earlier (8), although the temporal resolution in cGMP measurements was relatively crude in comparison with the Ca2+ measurements reported here. These observations lead us to consider the possibility that one second messenger could affect the accumulation of the other. While we have not yet addressed this directly, the lack of effect of thapsigargin and EGTA on cGMP levels suggests that Ca2+ does not directly regulate cGMP accumulation. In this regard, Inka cells differ from other preparations, e.g. rat pancreatic acini, in which thapsigargin does evoke an increase in cGMP (37). In pancreatic acini, however, Ca2+ activates nitric-oxide synthase, which then leads to cGMP production (37), a pathway that does not function in Inka cells (8). These considerations also raise the converse question that has not yet been addressed: does cGMP play a role in Ca2+ mobilization or influx in Inka cells?

The importance of elevated Ca2+ in regulating exocytotic secretion by aminergic and peptidergic endocrine cells has long been known (14, 39). A high degree of cooperativity for the action of Ca2+ in secretion indicates activity at multiple steps, from early events associated with translocation of vesicles to a readily releasable pool (40) to late events following docking and associated with fusion (41). In early events the slower kinetics of rearrangements in cytoskeleton and/or translocation of vesicles to release sites allow for the involvement of both Ca2+ and other second messengers such as cyclic nucleotides (39, 42). Late events are mediated by “sensor” proteins found in vesicle and plasma membranes, the activation of which by relatively high [Ca2+] in neurons or lower [Ca2+] in endocrine or neuroendocrine cells leads to fast fusion of an immediately releasable pool of vesicles.

ETH secretion begins at the onset of preecdysis behavior and increases by positive feedback from EH to maximal activation, driving the behavioral sequence to its conclusion (3, 8, 11). Receptor activation leads to accumulation of cGMP (8, 11) and elevation of Ca2+ (this report). The signaling events leading to Ca2+ elevation have not been identified, but could follow production of inositol 1,4,5-trisphosphate, shown to accumulate in CNS tissue in response to EH (43, 44). Moreover, since cGMP production in CNS is decreased by phospholipase C inhibitors (44), EH receptor activation may lead to cGMP and Ca2+ mobilization through a common PLC, which, once activated, leads to accumulation of each second messenger independent of the other. Earlier work showed that EH is likely to activate a PKG in the CNS (31). Our findings with staurosporine suggest that EH acts similarly in Inka cells. If so, identifying the kinases and their substrates, the Ca2+ effector proteins, and the functions of these proteins in the transduction cascade represents an exciting challenge for the immediate future.
Signal Transduction in EH-induced Secretion of ETH

14. Douglas, W. W. (1968) Br. J. Pharmacol. 34, 451–474
15. Knight, D. E., von Grafenstein, H., and Athayde, C. M. (1989) Trends Neurosci. 12, 451–459
16. Truman, J. W., Taghert, P. H., and Reynolds, S. E. (1980) J. Exp. Biol. 88, 327–337
17. Klein, C., Kallenborn, H. G., and Radlicki, C. (1999) J. Insect Physiol. 45, 65–73
18. Tamaoki, T., Nomoto, H., Takahashi, I., Kate, Y., Morimoto, M., and Tomita, F. (1998) Biochem. Biophys. Res. Commun. 251, 871–877
19. Enan, E., and Matsumura, F. (1992) Biochem. Pharmacol. 43, 1777–1784
20. Morgan, A., and Burgoyne, R. D. (1997) Cell Calcium 21, 141–149
21. Thastrup, O., Cullen, P. J., Drebak, B., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470
22. Shibanaka, Y., Hayashi, H., Takai, M., and Fujita, N. (1993) Eur. J. Biochem. 211, 427–430
23. Morton, D. B., and Simpson, P. J. (1995) J. Comp. Physiol. B 165, 417–427
24. Rutter, G. A., Theler, J.-M., and Wollheim, C. B. (1994) Cell Calcium 16, 71–80
25. Putney, J. W., Jr., and Bird, G. St. J. (1993) Cell 75, 189–201
26. Heemkerk, J. W. M., Feijig, M. A. H., Sage, S. O., and Walter, U. (1994) Eur. J. Biochem. 223, 543–551
27. Xu, X., Star, R. A., Tortorici, G., and Muallem, S. (1994) J. Biol. Chem. 269, 12645–12653
28. Shibanaka, Y., Hayashi, H., Takai, M., and Fujita, N. (1993) Eur. J. Biochem. 211, 427–430
29. Shibanaka, Y., Hayashi, H., Takai, M., and Fujita, N. (1993) Eur. J. Biochem. 211, 427–430
30. Morgan, A., and Burgoyne, R. D. (1997) Cell Calcium 21, 141–149
31. Thastrup, O., Cullen, P. J., Drebak, B., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470
32. Shibanaka, Y., Hayashi, H., Takai, M., and Fujita, N. (1993) Eur. J. Biochem. 211, 427–430
33. Morton, D. B., and Simpson, P. J. (1995) J. Comp. Physiol. B 165, 417–427