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Effects of calcium and glutamate receptor agonists on leaf consumption by lepidopteran neonates

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Abstract

Calcium and glutamate receptor (GluR) agonists affect apple leaf consumption by neonates of the apple pest, the codling moth, *Cydia pomonella* (L.) Initial apple leaf consumption was advanced by the presence of *trans*-1-amino-(1*S*,3*R*)-cyclopentanedicarboxylic acid (*trans*-ACPD), but not by calcium chloride or *N*-methyl-D-aspartate (NMDA). However, during the 3 h following hatch, $CaCl_2$ and NMDA increased the quantity of apple leaf tissue consumed, but *trans*-ACPD had no such effects. Stimulatory effects of $CaCl_2$ and NMDA on leaf consumption were abolished if codling moth larvae were concurrently exposed to calcium chelator EDTA. (*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA) and kainic acid had no effects either on commencement or intensity of leaf consumption. We hypothesize that in codling moth larvae, apple leaf consumption is induced via metabotropic GluR, and sustained feeding is regulated via NMDA GluRs. Practical aspects of this finding are discussed.

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Keywords: Calcium; Codling moth, *Cydia pomonella*; Insects; Feeding; Glutamate receptor; NMDA; AMPA; Kainate

1. Introduction

In unmanaged orchards, the majority of codling moth [*Cydia pomonella* (L.)], eggs is deposited on leaves (Jackson, 1979), rather than the fruit. Soon after hatching, neonate larvae wander in search of fruit and, once found, these larvae burrow into and feed within the fruit until their larval development is complete. However, being polyphagous, the larvae of codling moth can successfully complete their first instar when solely fed apple leaves instead of fruit, and monosodium glutamate (MSG) increases apple leaf consumption (Pszczolkowski et al., 2002). The roles of MSG and L-glutamate in insect taste perception and feeding regulation have not been studied extensively. Apart form our own report (Pszczolkowski et al., 2002), four other reports suggest that L-glutamate functions as a phagostimulant to *Oncopeltus fasciatus* (Dallas) (Fier and Beck, 1963), *Nilaparvata lugens* (Stal) (Sogawa, 1972), *Spodoptera littoralis* (Boisd.) (Ascher et al., 1976), and *Periplaneta americana* (L.) (Sugarman and Jakinovitch, 1986). Interestingly, the addition of MSG increases food intake in rats (Yamaguchi, 1998; Bachmanov et al., 2000; Halpern, 2000) at concentrations corresponding to those shown to increase codling moth apple leaf consumption (Pszczolkowski et al., 2002).

Although molecular mechanisms of MSG contribution to food intake in vertebrates are still unclear, it has been postulated that perception of MSG taste in vertebrates is mediated by glutamate receptors (GluR), localized in taste cells and afferent axons (Bellisle, 1999). These receptors are pharmacologically divided to two major types: ionotropic GluRs and metabotropic GluRs. Ionotropic GluRs are directly coupled to ion channels permeable to extracellular calcium. Metabotropic GluRs are coupled to GTP-binding proteins and, when excited, release Ca^{2+} from internal calcium stores. Elevation of cytosolic calcium level triggers release of neurotransmitters and transduction of signals from taste cell to the brain via afferent axons (Smith and Margolskee, 2001). There are two subtypes of ionotropic GluRs. Receptors of the first subtype, sensitive to *N*-methyl-D-aspartate (NMDA) are NMDA receptors. Receptors of the second subtype, sensitive either to (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepproprionic acid (AMPA) or to

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kainic acid, are AMPA/kainate receptors. Metabotropic GluRs may be excited by (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD) (Nakanishi, 1992). NMDA receptors, AMPA/kainate receptors, and metabotropic GluRs have been found in rat lingual tissue (Chaudhari et al., 1996; Lin and Kinnamon, 1999; Caicedo et al., 2000).

GluRs are present in insects. Moreover, studies on GluR involved in functioning of insect muscles and endocrine glands reveal that insect GluR share their pharmacological characteristics with that of vertebrate GluRs. For instance, GluRs in insect neuromuscular junctions may be excited or blocked by vertebrate GluR agonists or antagonists, respectively (Satelle, 1992; Usherwood, 1994; Zhang et al., 1999). Vertebrate GluR agonists and antagonists also influence calcium influx to cytosol of corpus allatum cells, thereby regulating juvenile hormone synthesis in Hawaiian cockroach, *Diploptera punctata* (Eschscholtz) (Pszczolkowski et al., 1999; Chiang et al., 2002a,b).

A question should be addressed, whether or not codling moth feeding is affected by GluR agonists as it is by MSG. In addition, since calcium chloride exerts the same effects as glutamate on insect cells (Pszczolkowski et al., 1999), it seems justified to test the effects of calcium on codling moth feeding behavior. Using specific receptor ligands in studies on insect feeding behavior is a relatively new technique, previously employed by Mullin et al. (1992) to identify γ-aminobutyric acid (GABA) receptors involved in taste transduction in *Diabrotica virgifera virgifera* (Leconte). It has been discussed elsewhere as a potential tool for studying molecular mechanisms of insect gustation (Mullin et al., 1994). To our knowledge, effects of GluR agonists on insect feeding have not been studied so far. In addition, although mechanisms of calcium taste perception, intake, and influence on appetite have been extensively studied in vertebrates (Tordoff, 2001), very little is known about calcium effects on feeding in insects. Only a few reports showed effects of calcium on insect feeding behavior. Generally, high calcium concentrations were reported to cause cockroaches (Frings, 1946) and lepidopteran larvae (Frings, 1945, 1948) to reject food.

In this study, we assessed the effects of calcium chloride and vertebrate GluR agonists on induction and dynamics of apple leaf consumption by codling moth neonates. We also discuss the practical implications of our findings.

2. Materials and methods

2.1. Insects and feeding substrate

Codling moths [*C. pomonella* (L.)], originating from Yakima, WA, were reared at 25 °C, 70–80% RH, under a L16/D8 light–dark photoregime. The moths, kept in cylindrical cages lined with wax paper as an oviposition surface, were given saturated sucrose solution. The day that larvae hatch was considered as Day 0. In all experiments, 0–0.5-h-old larvae were used.

Larvae were tested on apple leaves of Honeycrisp variety, donated by C&O Nursery, Wenatchee, WA. New foliage (up to 3 days old) was used for all assays.

2.2. Chemicals

Calcium chloride, ethylenediaminetetraacetic acid (EDTA) and Triton X-100 were from Sigma (St Louis, MO). We used general metabotropic GluR agonist, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*trans*-ACPD), and specific ionotropic GluR agonists, NMDA, kainic acid, and (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA). These GluR agonists were from Tocris Cookson (Ballwin, MO). All chemicals were dissolved in double-distilled water, containing 0.02% Triton X-100.

2.3. Feeding bioassay technique

We modified the bioassay previously used by Heron (1965) for monitoring intensity of feeding of spruce budworm larvae. Circular sections of uniform size were removed from leaves by a 12-mm diameter punch. Test solutions (10 μl) were applied to the upper surface of sections, distributed evenly, and the sections were allowed to air dry. Afterward, lower surfaces were treated with an additional 10 μl of test solution and the drying procedure repeated. Treated sections were mounted on a glass microscope slide, between 400 Crepe Liner Double-coated Tape (3M Industrial Tape and Specialties Division, St. Paul, MN) and self-adhesive three-ring binder reinforcement labels (05721 Avery Dennison Office Products, Brea, CA) with a circular opening of 6 mm. Each bioassay station contained one section of leaf infested with one neonate larva, trapped by a glass cover slip and observed to record feeding activity. To prevent dehydration, the microscope slides were kept in Petri dishes with wet filter paper placed on the bottom of each dish. Additional details of this procedure are given elsewhere (Pszczolkowski et al., 2002).

The surface area of leaf disc consumed was determined in ocular units using a stereomicroscope equipped with an ocular square mesh reference scale. Next, surface areas of 33 randomly chosen fragments of leaves were determined by the same method, the leaf fragments were then dried and weighed. Based on this determination, consumed areas of the leaf, expressed in ocular units, were converted to an estimated dry weight of leaf tissue.

2.4. Experimental design

To examine the effect of $CaCl_2$, *trans*-ACPD, NMDA, AMPA, and kainate on feeding induction, experimental larvae were exposed to concentrations varying from

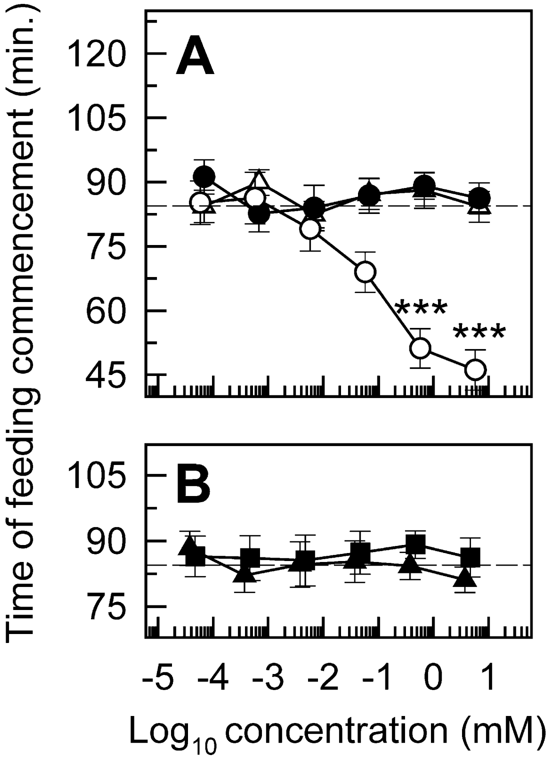

Fig. 1. Effect of calcium chloride (A, open triangles), NMDA (A, solid circles), *trans*-ACPD (A, open circles), kainate (B, solid squares), and AMPA (B, solid triangles) on commencement of post-hatch leaf consumption by codling moth neonates. Controls were exposed to 0.02% Triton X-100. $n=60$ for each data point (mean ± S.E.M.). The average time of leaf consumption commencement by controls is 84.51 ± 4.12 min (dashed lines). $***P<.001$ in comparison with Kruskal–Wallis test of average feeding commencement times between groups exposed to Triton X-100, calcium chloride, or respective GluR agonist.

3.7×10^{-5} to 6.8 mM. Twenty ($n=20$) larvae were exposed individually to each concentration, whereas control larvae ($n=20$) were only exposed to 0.02% Triton X-100. The number of feeding larvae was monitored for 3 h in 15-min intervals. This procedure was repeated three times. The results were expressed as the average time (mean ± S.E.M.) to feeding commencement.

Concentration-dependent effects of $CaCl_2$ and GluR agonists on quantities of leaf consumed were assessed as follows. We treated experimental leaves with test solutions at concentrations varying from 3.7 ± 10^{-5} to 6.8 mM. Control leaves were treated with solvent (0.02% Triton X-100 in double-distilled water) only. For each concentration, 24–32 larvae were used. We monitored feeding during the first 3-h exposure to test solution. The results were expressed as average (mean ± S.E.M.) dry mass of leaf consumed during the assay. Based on results from this experiment, we selected standard concentrations of calcium chloride and GluR agonists to be used in further experiments.

In the third series of experiments, we tested whether or not addition of calcium chelator, EDTA, affects influence of $CaCl_2$ or NMDA on intensity of apple leaf consumption. $CaCl_2$ and NMDA were tested at 6.8-mM concentrations. EDTA was tested at 2.5-, 25-, and 250-μM concentrations. Control larvae were exposed to respective EDTA concentrations or to 0.02% Triton X-100. Each solution was tested in 24 larvae. Results were expressed as average dry mass (mean ± S.E.M.) of leaf consumed (μg/larva) during 3-h assay.

2.5. Statistics

All sets of data on leaf consumption dynamics passed tests for normality with $P<.05$ (GraphPad InStat, GraphPad Software, San Diego, CA). Sample means ± S.E.M. were compared with ANOVA followed by Bonferroni's multiple comparison.

Data on feeding induction did not pass normality tests. Here, mean times of feeding induction ± S.E.M. were compared with Kruskal–Wallis test.

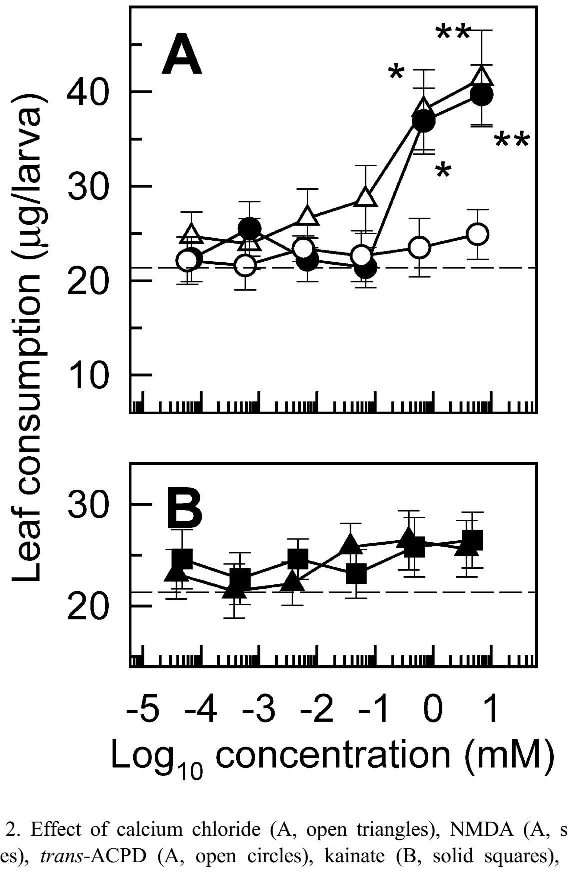

Fig. 2. Effect of calcium chloride (A, open triangles), NMDA (A, solid circles), *trans*-ACPD (A, open circles), kainate (B, solid squares), and AMPA (B, solid triangles) on intensity of post-hatch leaf consumption by codling moth neonates. The average leaf consumption (mean ± S.E.M.) by controls (exposed to 0.02% Triton X-100 only) is 21.34 ± 3.17 μg/larva (dashed lines). $n=24$–32 for each data point. $*P<.05$, $**P<.01$ in comparison with ANOVA followed by Bonferroni's multiple comparison of average leaf consumption among groups exposed to Triton X-100, calcium chloride, or respective GluR agonist.

Table 1
Effect of concurrent exposure to EDTA and calcium chloride or NMDA on leaf consumption by the codling moth

EDTA concentration (μM)	Concurrent treatment		
	None	6.8 mM $CaCl_2$	6.8 mM NMDA
0	22.21 ± 2.07	40.32 ± 5.12 *	38.29 ± 2.23 *
2.5	24.03 ± 2.00	32.46 ± 4.62	28.37 ± 3.67
25	23.12 ± 2.99	28.63 ± 3.56	24.15 ± 3.01
250	25.09 ± 3.06	25.94 ± 2.97	23.84 ± 2.99

$n = 24-32$ for each data point (mean ± S.E.M.).

* $P < .05$ in comparison to average leaf consumption in controls (0.02% Triton X-100, no EDTA, no concurrent treatment) with ANOVA followed by Bonferroni's multiple comparison.

Regardless of the test used, the results were regarded as significantly different at $P < .05$.

3. Results

3.1. The commencement of leaf consumption

Only metabotropic GluR agonist, *trans*-ACPD, altered time of feeding commencement in comparison to controls (Fig. 1). Larvae started to feed significantly earlier in presence of 0.58 and 5.8 mM *trans*-ACPD ($P < .05$, Kruskal–Wallis test, Fig. 1A). Exposure to calcium chloride, NMDA, AMPA, or kainate did not alter the times of leaf consumption commencement in comparison to control groups ($P > .05$, Kruskal–Wallis test, Fig. 1).

3.2. The quantities of leaf consumed

Only calcium chloride and NMDA affected the quantities of leaf consumed. Calcium chloride, at 0.68 and 6.8 mM concentrations, increased leaf consumption by approximately 80% ($P < .05$, ANOVA, Fig. 2A). Similarly, 6.8 mM NMDA increased leaf consumption by approximately 60% ($P < .05$, ANOVA, Fig. 2). Exposure to *trans*-ACPD, AMPA, or kainate had no effect on intensity of leaf consumption ($P > .05$, ANOVA, Fig. 2A,B).

Interestingly, the stimulatory effects of 6.8 mM calcium chloride or 6.8 mM NMDA on leaf consumption were not observed in larvae concurrently exposed to 2.5, 25, or 250 μM EDTA ($P > .05$, ANOVA, Table 1). EDTA alone, at tested concentrations, did not influence leaf consumption ($P > .05$, ANOVA, Table 1).

4. Discussion

This study extends our general knowledge of the physiology regulating insect-feeding behavior by demonstrating that calcium and different GluR agonists affect apple leaf consumption by codling moth larvae. The commencement of leaf consumption was accelerated only by metabotropic GluR agonist, *trans*-ACPD, whereas only calcium and NMDA increased post-hatch leaf consumption. Kainate and AMPA had no effect on either commencement or intensity of leaf consumption.

4.1. Effects on feeding induction

Calcium, at tested concentrations, seems to not play a role in the induction of codling moth feeding. We did not observe either a delay or acceleration of feeding commencement when calcium was present (Fig. 1). This observation agrees with entomological literature on the induction of feeding behavior by Ca^{2+} at concentrations that we used in our study. In fact, Frings (1945, 1946, 1948) reported rejection of calcium chloride by cockroaches, *P. americana* (L.), and caterpillars, *Hyalophora cecropia* (L.) and *Laccophilus maculosus* (Germ.). However, the concentrations tested in Fring's study ranged from 150 mM to 2.4 M, much higher than used in our study. Such high calcium concentrations also caused fluid rejection by rats (Tordoff, 2001). However, even the higher calcium concentrations did not stimulate labellum-located taste receptors in a blowfly, *Phormia regina* (L.) (Dethier and Hanson, 1965). We conclude that our findings are not contradictory to the reports of other authors regarding calcium's effect on the induction of feeding by insects.

The effects of GluR receptor agonists on commencement of the leaf consumption suggest that metabotropic GluRs may mediate feeding induction in codling moth. In our experiments, the general metabotropic glutamate agonist, *trans*-ACPD, induced early feeding. The mean time of leaf intake commencement was approximately 50% shorter in the presence of 1 mg/ml *trans*-ACPD than in the control group (Fig. 1A). Ionotropic GluR agonists (NMDA, kainate, and AMPA) had no effect on feeding commencement (Fig. 1). This finding corresponds well with the reports of Chaudhari et al. (1996, 2000), showing that in rat, immediate response to MSG was mediated by metabotropic, not by ionotropic GluRs. Chaudhari et al. (1996) showed that conditioned taste aversion to MSG was mimicked by metabotropic GluR agonist, L-2-amino-4-phosphobutyrate (L-AP4) but not by NMDA, AMPA, or kainate. In addition, L-AP4 elevated cAMP levels in rat taste bud cells (Chaudhari et al., 2000). Our results, showing that codling moth feeding may be induced with *trans*-ACPD but not with ionotropic GluR antagonists, support our finding that exposure to calcium has no effect on feeding commencement in this species (Fig. 1).

4.2. Effects on leaf consumption

At concentrations of 0.68 and 6.8 mM, calcium chloride had an effect on leaf consumption, the intake of leaf tissue by codling moth neonates is increased (Fig. 2A). We did not find any reports on calcium effects on either food or fluid intake by insects. However, our findings are in accordance with reports on calcium effects in vertebrates. Concentrations ranging from 0.075 to 7.5 mM stimulated fluid intake by rats (Tordoff, 2001) and were preferred to pure water by humans (Zoeteman et al., 1978). Importantly, stimulatory properties of calcium chloride on leaf consumption by codling moth neonates disappear in the presence of increasing concentrations of calcium chelator, EDTA (Table 1), indicating that these are calcium cations, not chloride anions that actually increase leaf consumption.

The effects of GluR receptor agonist, NMDA, on intensity of the leaf consumption suggest that ionotropic NMDA receptors may regulate this process in codling moth neonates. Post-hatch leaf consumption by codling moth neonates was increased by the presence of NMDA, but not *trans*-ACPD, AMPA, or kainate. In light of the recent report by Chiang et al. (2002a) that insect NMDA receptors are coupled to highly permeable calcium channel(s), our result that NMDA increases leaf consumption by the codling moth is supported by our other finding that Ca^{2+} increases leaf tissue intake. Noteworthy, stimulatory properties of NMDA disappeared in the presence of calcium chelator, EDTA (Table 1), indicating that calcium cations were necessary in feeding stimulation caused by NMDA. These results support our hypothesis that NMDA receptors are involved in leaf feeding by codling moth neonates.

It is difficult to interpret stimulatory effects of NMDA on leaf consumption in codling moth and there are no data on how orally administered NMDA affects feeding behavior in any other insect. NMDA receptors, coupled to calcium channels, are present in rat taste cells (Caiceido et al., 2000) and mediate in the perception of signals from glutamate. However, their actual role is still unclear, since rats do not perceive NMDA in conditioned taste aversion assays (Chaudhari et al., 1996). Moreover, no effects of orally administered NMDA on post-ingestive behavior have been reported. It has been shown (Stanley et al., 1993; Duva et al., 2001) that in rats, NMDA stimulates feeding if administered directly to the lateral hypothalamus. It cannot be excluded that ingested NMDA penetrates to codling moth hemolymph, and, further on, exerts some stimulatory effects on feeding control centers. Such an assumption seems plausible in light of the fact that insect blood–brain barrier is highly permeable (Treherne, 1985). However, we have no data on the possible effects of NMDA systemic or central injections in codling moth neonates, and, because newly hatched neonate weighs less than 0.2 mg and measures less than 1 mm, it seems difficult, if not impossible, to produce such data. At the present stage of our study, we limit our report to describing NMDA effects on leaf consumption. Localization of codling moth NMDA receptors might help explain NMDA effects on post-ingestive feeding. Such a study is under way.

Also, our findings have a practical aspect. Recently, we postulated that neonate codling moth larvae might be potential targets for treatment with orally active pesticides enhanced with MSG. Such an enhancement stimulates feeding in the presence of a pesticide, making it possible to reduce the amounts of pesticide's toxic component needed to protect the valued fruit from being damaged (Pszczolkowski and Brown, 2002). However, rain-fastness of hydrophilic MSG is not satisfactory for practical purposes (Pszczolkowski and Brown, 2002), and a hydrophobic alternative for MSG is needed. This study delineates the spectrum of GluR ligands that could serve as a hydrophobic feeding stimulator for codling moth.

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