

## Pharmacological Effects of Calcium Channel Antagonists on Juvenile Hormone Acid Release and Intracellular Calcium Level in the Corpora Allata of Adult Male *Mythimna loreyi* (Lepidoptera: Noctuidae)

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**Yi-Chung Hsieh, Ying-Ru Chen, Err-Lieh Hsu and Rong Kou (2003)** Pharmacological effects of calcium channel antagonists on juvenile hormone acid release and intracellular calcium level in the corpora allata of adult male *Mythimna loreyi* (Lepidoptera: Noctuidae). *Zoological Studies* 42(2): 336-345. The pharmacological effects of calcium channel antagonists on the in vitro release of juvenile hormone acids (JHAs) and the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in corpora allata (CA) of *Mythimna loreyi* adult males were investigated in this study. Newly dissected CA were first incubated in medium 199 for 1 h for equilibration, CA were then transferred to fresh medium containing  $Ca^{2+}$  channel antagonists for another 3-h incubation. Most antagonists of the voltage-dependent calcium channel (VDCC) subtypes (L-, N-, P-, Q-, R-, and T-types), at physiological doses, were unable to inhibit JHA release, except for diazoxide. Among the non-specific calcium channel antagonists, cadmium ( $Cd^{2+}$ ), cobalt ( $Co^{2+}$ ), and lanthanum ( $La^{3+}$ ) were effective inhibitors of JHA release. The effects of VDCC antagonists (i.e., diazoxide and verapamil) and non-specific calcium channel antagonists on  $[Ca^{2+}]_i$  were also measured in individual CA cells with Fura-2. Excised glands were first loaded with 20  $\mu M$  fura-2/AM in lepidopteran saline for 45 min at 28°C in the dark. After hydrolysis, the  $[Ca^{2+}]_i$  was observed for 30 min. Diazoxide ( $10^{-4}$  M) initially caused a significant depression of  $[Ca^{2+}]_i$ , which then gradually increased to a normal level. Among the non-specific calcium channel antagonists, both  $Cd^{2+}$  and  $La^{3+}$  caused a significant depression of  $[Ca^{2+}]_i$ . Our results suggest the possible existence of some kind of calcium channel in the plasma membrane of CA cells. <http://www.sinica.edu.tw/zool/zoolstud/42.2/336.pdf>

**Key words:** *Mythimna loreyi*, Calcium channel antagonist, Corpora allata, Juvenile hormone acid, Intracellular calcium concentration.

$Ca^{2+}$  is well recognized as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger or for the maximum activation of other enzymes in the signal cascade (Putney 1993, Berridge 1997). In insects, the role of  $Ca^{2+}$  in the modulation of juvenile hormone (JH) or JH acid (JHA) biosynthesis and release by the corpora allata (CA) was investigated in the adult female cockroach *Diploptera punctata* (Kikukawa et al. 1987, Rachinsky and Tobe 1996), the adult female locust *Locusta migratoria* (Dale and Tobe 1988), the larvae of the tobacco hornworm *Manduca sexta* (Allen et al. 1992), the adult male cricket *Gryllus bimaculatus* (Klein et al.

1993), and the adult male *loreyi* leafworm *Mythimna loreyi* (Kou and Chen 2000a b). In *D. punctata*, JH production showed a dose dependence on extracellular  $Ca^{2+}$  in the incubation medium. Voltage-dependent  $Ca^{2+}$  channel (VDCC) antagonists such as verapamil and nifedipine were able to modulate JH production (Kikukawa et al. 1987), and the existence of VDCC in the plasma membrane of CA cells in *D. punctata* was later demonstrated by McQuiston et al. (1990). Also in *D. punctata*, non-specific  $Ca^{2+}$  channel antagonists such as lanthanum effectively inhibited JH release, and the thapsigargin (the calcium-mobilizing drug which mimics the effect of

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IP<sub>3</sub> to release calcium from intracellular stores (Smith and Gallacher 1994))-induced Ca<sup>2+</sup> response in the CA was suppressed by the allatostatin, Dip-Ast 7 (Rachinsky and Tobe 1996). In *L. migratoria*, high rates of JH release were precluded from prolonged incubation in Ca<sup>2+</sup>-free medium, but incubation with the ionophore, A23187, resulted in a substantial stimulation of JH release (Dale and Tobe 1988). In *M. sexta*, the larval CA required extracellular Ca<sup>2+</sup> concentrations of > 10<sup>-4</sup> M for maximal JH biosynthesis, whereas the production of JHA by glands after pupal commitment was independent of extracellular Ca<sup>2+</sup> (Allen et al. 1992). Also in *M. sexta*, intracellular Ca<sup>2+</sup> levels were found to be highest when the CAs were biosynthetically active (Allen et al. 1992). In *G. bimaculatus*, elevated Ca<sup>2+</sup> concentrations in the incubation medium stimulated JH III synthesis in vitro in a dose-dependent manner (Klein et al. 1993). In *M. loreyi*, allatotrophic activities of both the subesophageal ganglion (SOG) and corpora cardiaca (CC) extract were dependent on the Ca<sup>2+</sup> concentration in the medium (Kou and Chen 2000a b).

In our previous study of the *loreyi* leafworm, *M. loreyi*, we found that adult males possess giant CA of an uncapsulated, isolated cell type (each gland is composed of a cluster of approximately 40 semi-transparent, spherical, isolated single cells held together by fine tracheae and nerve fibers (Kou et al. 1995)), and that JHA III and iso-JHA II are its major biosynthetic products (Ho et al. 1995, Kou et al. 1995). Both nervous and allatotrophic controls of the CA in adult males of this insect species were also demonstrated recently (Kou and Chen 2000a b). Since allatotrophic activities of both the subesophageal ganglion (SOG) and corpus cardiacum (CC) extracts showed a dose dependence on extracellular Ca<sup>2+</sup> in the incubation medium, the possible existence of Ca<sup>2+</sup> channels in the plasma membrane should be further explored.

In most endocrine cells, VDCCs are the primary route of Ca<sup>2+</sup> entry across plasma membranes (Hille 1992, Walker and De Waard 1998), and VDCCs have been classified according to their electrophysiological and pharmacological properties into 5 essential groups, termed L, N, P/Q, R, and T (Fox et al. 1987, Tsien et al. 1987, Miller 1992, Usowicz et al. 1992, Ellinor et al. 1993, Walker and De Waard 1998, Triggler 1999). The L-type channels (Nowycky et al. 1985), which are activated by high voltage (Carbone and Lux 1984) and conduct a long-lasting current of large conductance (~ 25 pS), are widely distributed in heart and

smooth muscles, and are highly sensitive to benzothiazepines (BTZs), dihydropyridines (DHPs), and phenylalkylamines (PAAs) (Spedding and Paoletti 1992). The N-type channels (Nowycky et al. 1985, Fox et al. 1987), which are activated at relatively high voltages and conduct a relatively transient current of intermediate size (~15 pS), are largely restricted to neurons and are irreversibly blocked by  $\omega$ -Conotoxin ( $\omega$ -CgTx) GVIA (Mori et al. 1996). The P-type channels, first identified in Purkinje cells (Linass et al. 1989), are activated by high voltage and are selectively blocked by  $\omega$ -Agatoxin ( $\omega$ -Aga) and funnel-web spider toxin (FTX) (Mintz et al. 1992). The T-type channels (Nowycky et al. 1985, Fox et al. 1987) are activated at low voltages and are characterized by transient currents with small conductance (~ 9 pS).

In the present study, the pharmacological effects of specific L-, N-, P/Q-, R-, and T-type VDCC antagonists on JHA release and intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) were investigated. Also the effects of non-specific Ca<sup>2+</sup> channel antagonists such as Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, and La<sup>3+</sup> were also tested.

## MATERIALS AND METHODS

### Insects

Larvae of *Mythimna loreyi* were reared on an artificial diet (Shorey and Hale 1965) at 26-28°C under a 16-h light/8-h dark photoregime. Sexes were separated at the pupal stage. Pupae were collected daily, and adults were allowed to emerge in screened cages. Moths emerging within 8 h were placed in the same age group. The life span of adults was 10-12 d.

### Chemicals

Components of medium 199, cadmium chloride (CdCl<sub>2</sub>), cobalt chloride (CoCl<sub>2</sub>), lanthanum chloride (LaCl<sub>3</sub>), and nickel chloride (NiCl<sub>2</sub>) were purchased from Sigma (St. Louis, MO, USA). Amiloride, diazoxide, dopamine, ethosuximide, flunarizine, histamine, nifedipine, nitrendipine, verapamil,  $\omega$ -Agatoxin VIA ( $\omega$ -Aga VIA),  $\omega$ -Conotoxin GVIA ( $\omega$ -CgTx GVIA), and  $\omega$ -CgTx MVIIC were purchased from RBI/Sigma (Natick, MA, USA). Fura-2 acetoxymethylester (AM) and the detergent Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). [2-<sup>14</sup>C] acetate sodium salt (specific activity 2.0 mCi/mmol) was from

New England Nuclear (Boston, MA, USA).

### In vitro assay of CA activity

The JHA release rate was determined by an in vitro radiochemical assay (Wennauer and Hoffmann 1988, Cusson et al. 1993). In our previous study, CA from 3-d old adult males showed higher rates of JHA release than did those from the other age groups under in vitro conditions (Kou and Tu 1998). CA from this age group were dissected in lepidopteran saline (3 mM CaCl<sub>2</sub>, 170 mM glucose, 21 mM KCl, 9 mM KOH, 18 mM MgCl<sub>2</sub>, 12 mM NaCl, 5 mM PIPES; pH 6.6) (Jones and Yin 1989). The rate of JHA release was approximately the same for right and left CA from the same pair during 4 h of incubation (Kou and Chen 2000a b). A single CA, either the right or the left of a CA pair was transferred into 40 µl of medium 199 (pH 6.9; with Hank's salt, L-glutamine, buffered with 25 mM HEPES, but without glucose or sodium acetate) supplemented with 5 mM CaCl<sub>2</sub> and 2.4 mM [2-<sup>14</sup>C] acetate, sodium salt (modified from Cusson et al. 1993). Medium 199 was used as the incubation medium, since it had been previously used for the incubation of lepidopteran CA of *Lacanobia oleracea* (Audsley et al. 1998) and the tobacco hornworm *M. sexta* (Kataoka et al. 1989).

For detecting JHA release, glands were removed from the media after incubation, and the incubation media were extracted with chloroform. JHA was separated by thin-layer chromatography using hexane: ethyl acetate: acetic acid, 66: 33: 1 (v/v) as the solvent system (Share and Roe 1988, Kou and Chen 2000a b). Radioactivity was determined by liquid scintillation counting using the Ready Safe™ liquid scintillation cocktail. Because the stoichiometry of incorporation of acetate into the carbon skeleton of JHA has not been determined, JHA released into the incubation medium was simply expressed as dpm.

### Measurements of [Ca<sup>2+</sup>]<sub>i</sub>

Before measuring [Ca<sup>2+</sup>]<sub>i</sub>, excised glands were first loaded with 20 µM fura-2/AM (containing 0.4% Pluronic F-127) (Tsien 1989) in lepidopteran saline for 45 min at 28°C in the dark. Loading efficacy was initially estimated by recording the increase in fluorescence during incubation over 120 min. After loading, the hydrolysis of fura-2/AM by intracellular esterases was allowed to continue in a 15-min rinse in order to liberate the Ca<sup>2+</sup>-sensitive free fura-2 within the CA gland cells, in nor-

mal lepidopteran saline. The normal loading and rinse times were 45 and 15 min, respectively. The standard [Ca<sup>2+</sup>]<sub>i</sub> observation time was 30 min.

For monitoring real-time [Ca<sup>2+</sup>]<sub>i</sub> levels, the method of high-performance ratio fluorescence with the MERLIN (Life Science Resources Inc., UK) ratio imaging system, which possesses an LSR SpectroMASTER™ monocromatic illuminator, was used (Mason et al. 1998). Dual wavelength measurements were made with an AstroCam camera system connected to a Zeiss Axiovert S100TV fluorescence microscope. Emitted fluorescence was collected through an aperture adjusted to the size of the CA cells. Background fluorescence was determined from an area where there were no cells. Autofluorescence was estimated with unloaded cells. The measurements of fluorescence intensity at 2 excitation wavelengths (340 and 380 nm) were used to calculate [Ca<sup>2+</sup>]<sub>i</sub> according to Grynkiewicz et al. (1985), by the following equation:  $[Ca^{2+}] = \beta \times Kd \times (R - R_{min}) / (R_{max} - R)$ , where R is the ratio of the experimentally measured fluorescence intensities; R<sub>min</sub> is the measured fluorescence ratio in the absence of Ca<sup>2+</sup>. R<sub>max</sub> is the measured fluorescence ratio of Ca<sup>2+</sup>-saturated dye. β is the ratio of the fluorescence intensities at the wavelength chosen for the denominator of R (e.g., 380 nm excitation for fura-2) in 0 and saturated [Ca<sup>2+</sup>]<sub>i</sub>; and Kd is the dissociation constant of the indicator for Ca<sup>2+</sup>.

As described in the 2nd paragraph of the "Introduction section", the gland is originally uncapsulated, each CA is composed of 40 or more isolated single cells, and the diameter of each cell is about 200 µ (Kou et al. 1995). The [Ca<sup>2+</sup>]<sub>i</sub> was usually measured in 9 to 12 cells of each gland.

### Effect of Ca<sup>2+</sup> channel antagonists on JHA release

To determine if a disruption in calcium flux will modulate JHA release, newly dissected glands were first incubated in medium 199 for an initial hour of equilibration, CA were then transferred to fresh medium with or without (control) the Ca<sup>2+</sup> channel antagonists for another 3 hours incubation. JHA release was examined from the later 3-h incubation. Ca<sup>2+</sup> channel antagonists were used in this study.

1. The VDCC antagonists included the dihydropyridine (DHP)-sensitive L-type antagonists nifedipine and nitrendipine, the phenylalkylamine (PAA)-sensitive L-type antagonist verapamil; the N-type antagonist ω-CgTx GVIA; the N- and

P/Q-type antagonist  $\omega$ -CgTx MVIIC; the P-type antagonist  $\omega$ -Aga VIA; the T-type antagonists amiloride and ethosuximide, the L- and N-type antagonist dopamine, and the L-, N-, and T-type antagonist flunarizine. Other L- type antagonists such as diazoxide and histamine are also tested.

2. Non-specific Ca<sup>2+</sup> channel antagonists which includes Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> and La<sup>3+</sup>.

### Time course of the effect of non-specific Ca<sup>2+</sup> channel antagonists on JHA release and CA recovery

Since Cd<sup>2+</sup>, Co<sup>2+</sup>, and La<sup>3+</sup> showed significant ( $p < 0.01$ ) antagonistic effect on JHA release, the time course of the antagonistic effect of these non-specific Ca<sup>2+</sup> channel antagonists and that of CA recovery were further investigated. Newly dissected glands were incubated in medium 199 containing 1 mM Cd<sup>2+</sup>, Co<sup>2+</sup>, or La<sup>3+</sup> for 2 h; CA were then transferred to fresh medium 199 without the anta-

gonist for another 4-h incubation to see if there was a reversibility, when the fresh medium was changed hourly.

### Effect of Ca<sup>2+</sup> channel antagonists on [Ca<sup>2+</sup>]<sub>i</sub>

To measure the effect of Ca<sup>2+</sup> channel antagonists on the level of [Ca<sup>2+</sup>]<sub>i</sub>, newly dissected glands were loaded with 20  $\mu$ M fura-2/AM in lepidopteran saline for 45 min, and the Ca<sup>2+</sup> channel antagonist was then added during the 15-min rinse period. Glands were then observed for changes in [Ca<sup>2+</sup>]<sub>i</sub> during a 30-min incubation in the lepidopteran saline. The VDCC Ca<sup>2+</sup> channel antagonists used were verapamil (10<sup>-4</sup> M) and diazoxide (10<sup>-4</sup> M). The non-specific Ca<sup>2+</sup> channel antagonists used were Cd<sup>2+</sup> (1 mM), Co<sup>2+</sup> (1 mM), and La<sup>3+</sup> (1 mM).

### Calculations

All values are means  $\pm$  SE of individuals indi-

**Table 1.** Effect of VDCC antagonists on JHA release in the corpora allata of 3-d-old adult male *Mythimna loryei*

Antagonist	Concentration tested (M)	Inhibitory effect <sup>a</sup>	Subtype of VDCC (or pharmacological property)
Amiloride	10 <sup>-4</sup>	-	T-type Ca <sup>2+</sup> channel blocker
Diazoxide	10 <sup>-4</sup>	+	K <sup>+</sup> channel opener, closing the L-type Ca <sup>2+</sup> channel
	10 <sup>-5</sup>	-	
	10 <sup>-6</sup>	-	
Dopamine	10 <sup>-5</sup>	-	L-, T-type Ca <sup>2+</sup> channel blocker
	10 <sup>-6</sup>	-	
Ethosuximide	10 <sup>-2</sup>	-	T-type Ca <sup>2+</sup> channel blocker
	10 <sup>-3</sup>	-	
	10 <sup>-4</sup>	-	
	10 <sup>-5</sup>	-	
Flunarizine	10 <sup>-5</sup>	-	L-type Ca <sup>2+</sup> channel blocker; Ca <sup>2+</sup> /Na <sup>+</sup> channel blocker; anti-Bay K 8644
	10 <sup>-6</sup>	-	
	10 <sup>-7</sup>	-	
Histamine	10 <sup>-5</sup>	-	L-type Ca <sup>2+</sup> channel blocker
	10 <sup>-6</sup>	-	
Nifedipine	10 <sup>-6</sup>	-	L-type Ca <sup>2+</sup> channel blocker; vasodilator
	10 <sup>-7</sup>	-	
	10 <sup>-8</sup>	-	
Nitrendipine	10 <sup>-6</sup>	-	Ca <sup>2+</sup> channel blocker; antihypertensive
	10 <sup>-7</sup>	-	
	10 <sup>-8</sup>	-	
Verapamil	10 <sup>-4</sup>	-	L-type Ca <sup>2+</sup> channel blocker
	10 <sup>-5</sup>	-	
	10 <sup>-6</sup>	-	
$\omega$ -AGA VIA	10 <sup>-6</sup>	-	P-, and R-type Ca <sup>2+</sup> channel blocker
	10 <sup>-7</sup>	-	
$\omega$ -CTX GVIA	5 x 10 <sup>-6</sup>	-	N-type Ca <sup>2+</sup> channel blocker
	10 <sup>-6</sup>	-	
$\omega$ -CTX MVIIC	10 <sup>-6</sup>	-	P-, Q-, N-, and R-type Ca <sup>2+</sup> channel blocker
	10 <sup>-7</sup>	-	

<sup>a</sup>+, statistical significance at  $p < 0.05$  level.

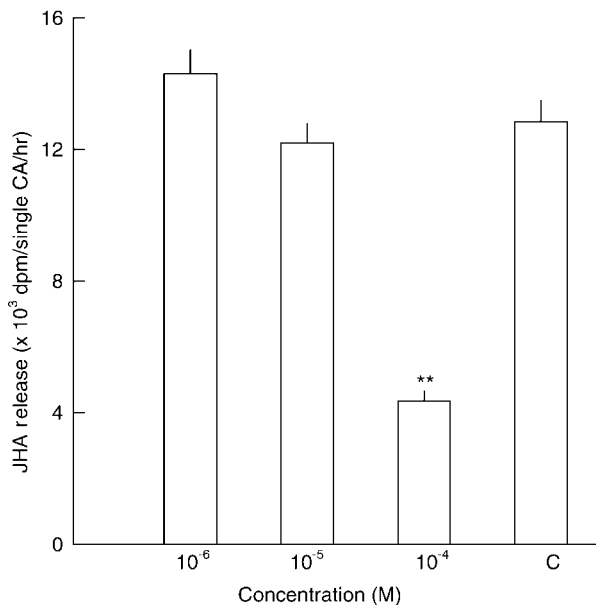
cated in the legend of each figure. One-way ANOVA (SAS Institute 1990) was used in statistical treatment of the data ( $p < 0.05$ ).

## RESULTS

### Effect of $\text{Ca}^{2+}$ channel antagonists on JHA release

A variety of more or less specific VDCC antagonists, listed in table 1, were tested for their pharmacological effects on JHA release. Among all tested VDCC antagonists, only diazoxide showed a significant ( $p < 0.01$ ) inhibitory effect on JHA release at a concentration of  $10^{-4}$  M (Table 1; Fig. 1).

On the other hand, almost all tested non-specific  $\text{Ca}^{2+}$  channel antagonists showed a certain degree of inhibitory effects on JHA release.  $\text{Cd}^{2+}$  significantly inhibited JHA release at the concentrations of  $10^{-2}$  M ( $p < 0.01$ ),  $10^{-3}$  M ( $p < 0.01$ ),  $10^{-4}$  M ( $p < 0.01$ ), and  $10^{-5}$  M ( $p < 0.05$ ) (Fig. 2A).  $\text{Co}^{2+}$  significantly inhibited JHA release at the concentrations of  $10^{-2}$  M ( $p < 0.01$ ),  $10^{-3}$  M ( $p < 0.01$ ), and  $10^{-4}$  M ( $p < 0.05$ ) (Fig. 2B).  $\text{La}^{3+}$  significantly inhibited

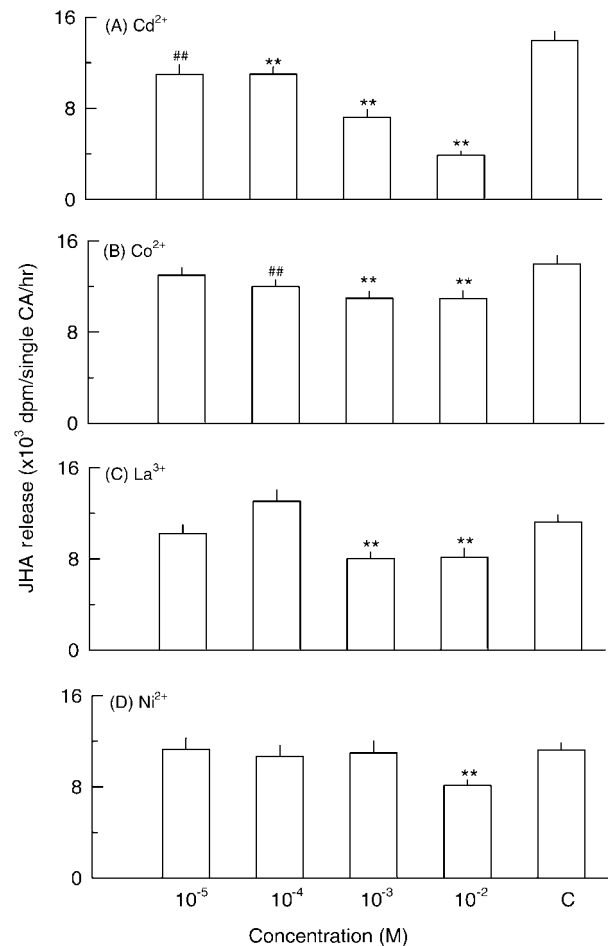


**Fig. 1.** Effect of diazoxide ( $10^{-4}$  M) on JHA release. CA from 3-d-old virgin males were used for the assay. After an initial 1-h incubation for equilibration, CA were transferred to the medium with or without (control) diazoxide for another 3 consecutive h of incubation. JHA release was determined from this later 3-h incubation. Values are the mean of 8-10 determinations  $\pm$  SE. \*\*  $p < 0.01$  vs. the control.

ted JHA release at the concentrations of  $10^{-2}$  M ( $p < 0.01$ ) and  $10^{-3}$  M ( $p < 0.01$ ) (Fig. 2C).  $\text{Ni}^{2+}$  significantly inhibited JHA release only at the concentration of  $10^{-2}$  M ( $p < 0.01$ ) (Fig. 2D).

### Time course of the effect of non-specific $\text{Ca}^{2+}$ channel antagonists on JHA release and the CA recovery

Since the non-specific  $\text{Ca}^{2+}$  channel antagonists  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{La}^{3+}$ , at a concentration of  $10^{-3}$  M, showed significant inhibitory effect on JHA release, the time courses of these inhibitory effects and of CA recovery were further investigated. The



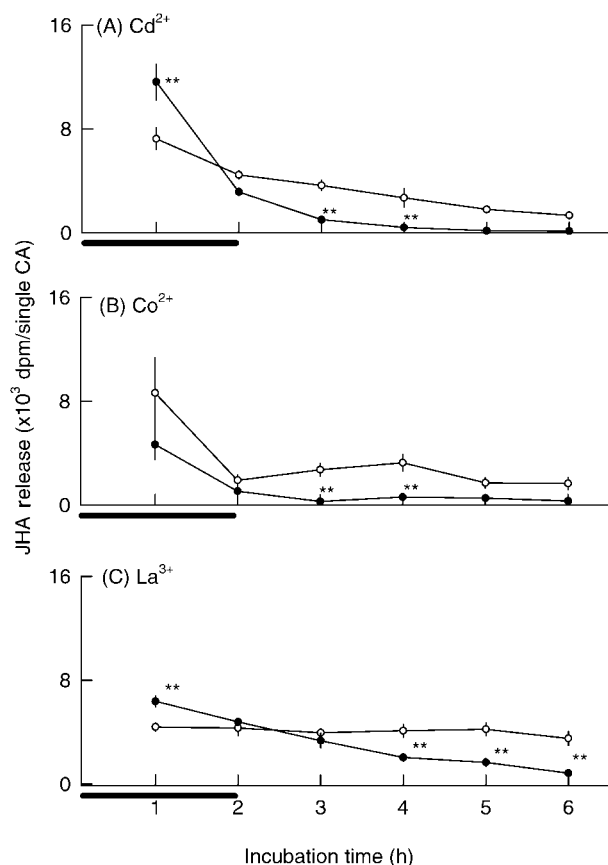
**Fig. 2.** Effect of different concentrations of (A)  $\text{Cd}^{2+}$ , (B)  $\text{Co}^{2+}$ , (C)  $\text{La}^{3+}$ , and (D)  $\text{Ni}^{2+}$  on JHA release. CA from 3-d-old virgin males were used for the assay. After an initial 1-h incubation for equilibration, CA were transferred to the medium with or without (control) certain concentrations of  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{La}^{3+}$ , and  $\text{Ni}^{2+}$ , respectively, for another 3 consecutive h of incubation. JHA release was determined from this later 3-h incubation. Values are the mean of 8-10 determinations  $\pm$  SE. C: control. ##  $p < 0.05$ , \*\*  $p < 0.01$  vs. the control.

results showed that, with Cd<sup>2+</sup> treatment, JHA release was significantly ( $p < 0.05$ ) increased in the first incubation hour, but sharply decreased at the 2nd incubation hour, and was significantly ( $p < 0.01$ ) inhibited in the 3rd incubation hour (i.e. 1 hour after being transferred to the fresh medium without Cd<sup>2+</sup>) (Fig. 3A). With Co<sup>2+</sup> treatment, JHA release also showed an inhibited pattern: the significant ( $p < 0.01$ ) inhibitory effect was exerted from the 3rd incubation hour (i.e. 1 h after being transferred to fresh medium without Co<sup>2+</sup>) (Fig. 3B). With La<sup>3+</sup> treatment, JHA release was significantly increased ( $p < 0.01$ ) in the 1st incubation hour, but was significantly ( $p < 0.01$ ) decreased to a very low level in the 4th incubation hour (i.e. 2 h after being transferred to fresh medium without

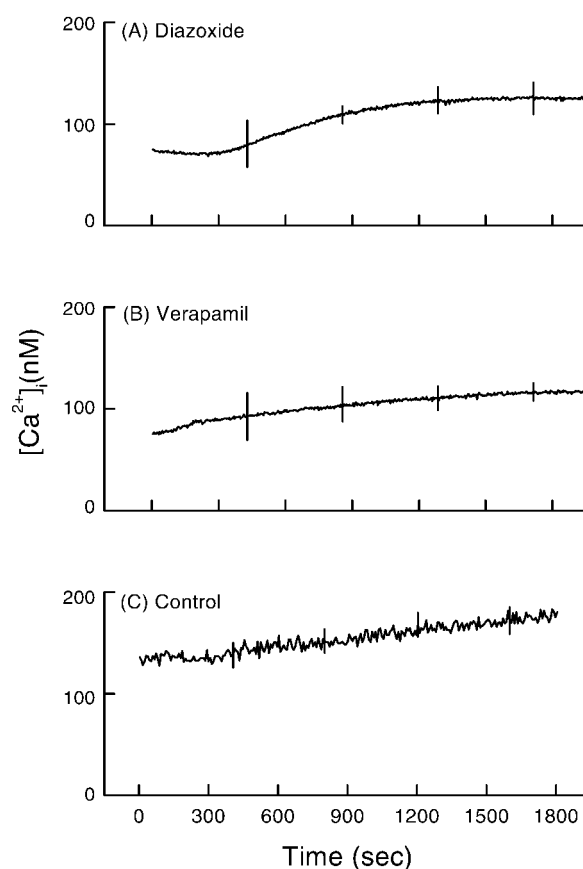
La<sup>3+</sup>), and this significant inhibitory effect lasted until the end of the 6 h of incubation (Fig. 3C). No recovery of JHA release was observed for any of the tested antagonists (Fig. 3A-C).

### Effect of Ca<sup>2+</sup> channel antagonists on [Ca<sup>2+</sup>]<sub>i</sub>

Since the VDCC antagonist diazoxide showed a significant ( $p < 0.01$ ) inhibitory effect on JHA release at a concentration of 10<sup>-4</sup> M (Table 1; Fig. 1), its effect on [Ca<sup>2+</sup>]<sub>i</sub> was further investigated. The results showed that, compared with the control (the [Ca<sup>2+</sup>]<sub>i</sub> of the control was around 140–170 nM (Fig. 4C)), diazoxide (10<sup>-4</sup> M) treatment first caused a significant ( $p < 0.01$ ) depression of [Ca<sup>2+</sup>]<sub>i</sub> to around 75 nM for the first 300 s, then the [Ca<sup>2+</sup>]<sub>i</sub> gradually recovered to 120 nM (Fig. 4A). For comparison, the effect of another VDCC



**Fig. 3.** Time course and reversibility of the inhibition of JHA release by (A) Cd<sup>2+</sup>, (B) Co<sup>2+</sup> and (C) La<sup>3+</sup>. Single CA from 3-d-old males was incubated for 2 h in 1 mM Cd<sup>2+</sup>, in 1 mM Co<sup>2+</sup>, in 1 mM La<sup>3+</sup>, or in control medium. CA were then transferred to normal medium containing 5 mM Ca<sup>2+</sup>. Fresh medium was changed hourly during the heavy metal treatment and the normal medium incubation. □: time period of heavy metal treatment. ●: heavy metal treatment. ○: control. Values are the mean of 9–12 determinations ± SE. \*\*  $p < 0.01$  vs. the control at the indicated time point.



**Fig. 4.** Effect of (A) diazoxide and (B) verapamil on the [Ca<sup>2+</sup>]<sub>i</sub> level in CA gland cells. CA from 3-d-old virgin males were first loaded with 20 μM Fura-2/AM for 45 min in lepidopteran saline, in diazoxide (10<sup>-4</sup> M), or in verapamil (10<sup>-4</sup> M); or (C) lepidopteran saline (control) was added during the 15-min rinse period. CA were then transferred to normal lepidopteran saline for [Ca<sup>2+</sup>]<sub>i</sub> observation.

antagonist, verapamil ( $10^{-4}$  M), which showed no inhibitory effect on JHA release (Table 1), on  $[Ca^{2+}]_i$  was also investigated. The result also showed that, with verapamil ( $10^{-4}$  M) treatment, although the  $[Ca^{2+}]_i$  was initially significantly ( $p < 0.01$ ) lower than the control, it gradually increased to 120 nM by the end of the observation time (Fig. 4C).

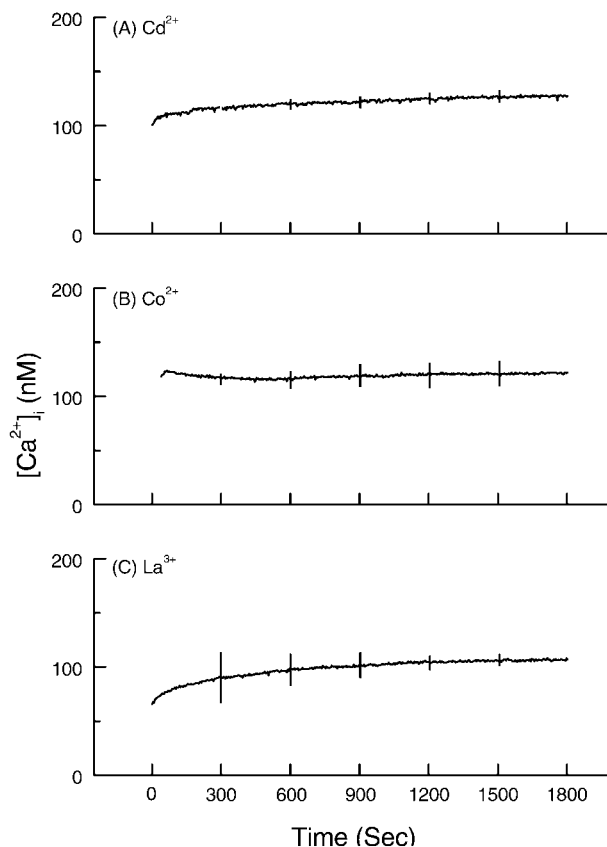
The results of non-specific  $Ca^{2+}$  channel antagonist treatment showed that,  $Cd^{2+}$  ( $10^{-3}$  M) treatment resulted in a significantly ( $p < 0.01$ ) depressed  $[Ca^{2+}]_i$  level of around 100~120 nM through the entire observation period (Fig. 5A).  $Co^{2+}$  ( $10^{-3}$  M) treatment also resulted in a  $[Ca^{2+}]_i$  level of around 120 nM (Fig. 5B). With  $La^{3+}$  ( $10^{-3}$  M) treatment, the  $[Ca^{2+}]_i$  level was initially significantly ( $p < 0.01$ ) depressed to around 70~90 nM for the first 300 s, then gradually increased to 100

nM by the end of the observation time (Fig. 5C).

## DISCUSSION

It is well recognized that  $Ca^{2+}$  is an essential element for many cellular processes, and the major entry pathway for  $Ca^{2+}$  is via plasma membrane  $Ca^{2+}$  channels (Walker and De Waard 1998). In excitable cells, such as endocrine cells, VDCCs are the primary route of  $Ca^{2+}$  entry across the plasma membrane, which serve as the only link for transducing depolarization into activities controlled by excitation in diverse cellular processes; the basis of this pivotal role of  $Ca^{2+}$  channels is the transient increase in intracellular  $Ca^{2+}$  concentration evoked by the gating of the channels (Mori et al. 1996, Walker and DeWaard 1998). In this study, the type(s) of the  $Ca^{2+}$  channel involved in JHA release in the CA cells was investigated using the pharmacological effects of both VDCC antagonists and non-specific  $Ca^{2+}$  channel antagonists.

Among all the tested VDCC antagonists (Table 1), most channel antagonists showed little or no effect on JHA release. Diazoxide, at a concentration of  $10^{-4}$  M, was the only antagonist which showed a significant ( $p < 0.01$ ) inhibitory effect on JHA release. In adult female *Diptera punctata*, although VDCC antagonists such as verapamil and nifedipine were able to modulate JH production, VDCCs were not suggested as the means of access extracellular  $Ca^{2+}$  to the cell (Kikukawa et al. 1987). Diazoxide, which is a  $K^+$  channel opener, hyperpolarizes the plasma membrane, and closes L-type calcium channels (Quast 1992). When the effect of diazoxide on  $[Ca^{2+}]_i$  was further investigated, a significant depression of  $[Ca^{2+}]_i$  in the initial 300 s was observed, so the effect of diazoxide on CA cells might be exerted by blocking L-type calcium channels. In fact, in our previous studies (Hsieh et al. 2001 2002), we have hypothesized that L-type, N-type, and T-type VDCCs may be involved to different degrees in the depolarization-activated JHA release and transient  $[Ca^{2+}]_i$  increase in individual CA cells of the adult male *Mythimna loreyi*. The precise inhibitory mechanisms of diazoxide on both JHA release and  $[Ca^{2+}]_i$  still require further study. In prothoracic gland cells of *M. sexta*, all tested VDCC antagonists were ineffective in blocking the PTHH-induced  $[Ca^{2+}]_i$  increase except for the T-type  $Ca^{2+}$  channel antagonist amiloride, so T-type  $Ca^{2+}$  channels were suggested as the only pathway for increased  $[Ca^{2+}]_i$  in response to PTHH (Birkenbeil 1998). The effect of



**Fig. 5.** Effect of (A)  $Cd^{2+}$ , (B)  $Co^{2+}$ , and (C)  $La^{3+}$  on the  $[Ca^{2+}]_i$  level in CA gland cells. CA from 3-d-old virgin males were first loaded with 20  $\mu$ M fura-2/AM for 45 min in lepidopteran saline, then 1 mM  $Cd^{2+}$ , 1 mM  $Co^{2+}$ , or 1 mM  $La^{3+}$  was added during the 15-min rinse period. CA were then transferred to normal lepidopteran saline for  $[Ca^{2+}]_i$  observation. For the control, see figure 4C.

diazoxide treatment seemed to imply a correlation between JHA release and [Ca<sup>2+</sup>]<sub>i</sub> in CA cells. So the results of another VDCC antagonist, verapamil, which did not inhibit JHA but decreased [Ca<sup>2+</sup>]<sub>i</sub>, seems to conflict the results for diazoxide. In fact, the degree and duration of [Ca<sup>2+</sup>]<sub>i</sub> depression caused by verapamil were less than those caused by diazoxide (Fig. 4), and this may explain the different effects of these 2 compounds on JHA release.

The ineffectiveness of VDCC antagonists on JHA release might be attributed to the following reasons: generally, one or more subtypes of VDCCs may exist in a particular cell type, and different VDCC subtypes may serve different functions (Hosey and Lazdunski 1988); so the disruption of only one type of the VDCC channel might not exert obvious effects on cellular events such as JHA release. Another factor which might affect the VDCC antagonist effect is the method of antagonist treatment. In another experiment of ours, newly dissected CA were first incubated for 1 h in Ca<sup>2+</sup>-free medium in the presence of the antagonist; CA were then transferred to normal medium without the antagonist. The results showed that nifedipine and ω-CgTx GVIA were able to significantly inhibit JHA release (Hsieh et al. 2002). The exact mechanisms underlying “the different methods of antagonist treatments resulting in quite different results” is worthy of further investigation.

In this study, the effects of non-specific Ca<sup>2+</sup> channel antagonists (Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, and La<sup>3+</sup>) on JHA release and [Ca<sup>2+</sup>]<sub>i</sub> were also investigated. Among all tested non-specific calcium channel antagonists (Table 1), Cd<sup>2+</sup> (10<sup>-3</sup> M), Co<sup>2+</sup> (10<sup>-3</sup> M), and La<sup>3+</sup> (10<sup>-3</sup> M) showed significant (*p* < 0.01) inhibitory effects on JHA release, while Ni<sup>2+</sup> was less effective. In *M. sexta*, both JH and JHA syntheses during the larval and pupal stages, respectively, were significantly decreased by Cd<sup>2+</sup> at concentrations above 10<sup>-4</sup> M (Allen et al. 1992). Our present results show that both JHA release and [Ca<sup>2+</sup>]<sub>i</sub> level were reduced by Cd<sup>2+</sup> treatment. In vertebrates, the most sensitive cellular targets of Cd<sup>2+</sup> seem to be ion transport and cellular signal transduction (Souza et al. 1996). Cellular effects of Cd<sup>2+</sup> include intracellular mobilization of 2nd messengers such as IP<sub>3</sub> and Ca<sup>2+</sup> (Smith et al. 1989), the inhibition of plasma membrane Ca<sup>2+</sup> channels (Hughes et al. 1989), and inhibition of Ca<sup>2+</sup>-ATPases of both sarcoplasmic reticula and cell nuclei (Hechtenberg et al. 1993). Cd<sup>2+</sup> can also substitute for Ca<sup>2+</sup> and modulate the activity of

calmodulin (Cheung 1984) and enhance nuclear translocation of protein kinase C (Block et al. 1992). Whether the reduced [Ca<sup>2+</sup>]<sub>i</sub> in *M. loreyi* CA cells was due to inhibition of plasma membrane Ca<sup>2+</sup> channels or due to Ca<sup>2+</sup> substitution or other related cellular responses is worthy of further investigation.

Co<sup>2+</sup>, also a divalent Ca<sup>2+</sup> channel antagonist, significantly reduced JHA release in this study. When the effect of Co<sup>2+</sup> (1 mM) on [Ca<sup>2+</sup>]<sub>i</sub> was further investigated, no significant effect was observed, although the [Ca<sup>2+</sup>]<sub>i</sub> was reduced. In *D. punctata*, although Co<sup>2+</sup> treatment resulted in a slight stimulation of JH release, it blocked the effect of the ionophore A23187 (Kikukawa et al. 1987). In another cockroach, *Nauphoeta cinerea*, Co<sup>2+</sup> was also reported to inhibit vitellogenin uptake into follicles through the mechanism of Ca<sup>2+</sup> channel inhibition (Kindle et al. 1990). Ionic antagonists, such as heavy metals used in this study, usually affect not only Ca<sup>2+</sup> channels but most ion channels. Determining whether the effect of Co<sup>2+</sup> on JHA release was exerted through some channels other than Ca<sup>2+</sup> channels still requires further study.

La<sup>3+</sup>, which usually blocks calcium influx and calcium-related metabolic functions such as transmembrane Ca<sup>2+</sup> transport within excitable tissues, is responsible for the inhibition of stimulus-coupled secretion in endocrine cells (Evans 1983, Fitzpatrick 1990). In *D. punctata*, JH release was effectively inhibited by La<sup>3+</sup> (Kikukawa et al. 1987). In *M. sexta*, both JH and JHA syntheses during the larval and pupal stage, respectively, were significantly decreased by La<sup>3+</sup> (Allen et al. 1992). But in *L. migratoria*, La<sup>3+</sup> did not reduce JHA release (Dale and Tobe 1988).

The results of our present study show that except for reduced JHA release, La<sup>3+</sup> also significantly (*p* < 0.05) reduced the [Ca<sup>2+</sup>]<sub>i</sub>. In prothoracic gland cells of *M. sexta*, pharmacological studies of the PTTH effect with Ca<sup>2+</sup> channel blockers revealed that the increase in [Ca<sup>2+</sup>]<sub>i</sub> was totally blocked by Cd<sup>2+</sup>, and partially inhibited by Ni<sup>2+</sup> and La<sup>3+</sup> (Birkenbeil 1998). In thymocytes, La<sup>3+</sup> produced a concentration-dependent increase in cytoplasmic free Ca<sup>2+</sup> concentrations by enhancing Ca<sup>2+</sup> influx (Segal 1986). But in LLC-PK1 cells, La<sup>3+</sup> may affect cellular Ca<sup>2+</sup> homeostasis by actions, such as releasing Ca<sup>2+</sup> from Golgi complexes, other than as a simple Ca<sup>2+</sup> channel blocker (Zha and Morrison 1995). In this study, JHA release with both Cd<sup>2+</sup> and La<sup>3+</sup> treatments was significantly increased during the 1st

incubation hour, although those were reduced later in the incubation. Finding a way to couple the activation effects on JHA release and the reduced  $[Ca^{2+}]_i$  during the initial period is a good topic for further study of *M. loreyi* CA cells.

From all the above information, although our data suggest the possible existence of certain  $Ca^{2+}$  channels in the plasma membrane of CA gland cells in *M. loreyi*, the possible involvement of some other channels in JHA release is not excluded, since non-specific ionic  $Ca^{2+}$  channels, such as the heavy metals used in this study, usually affect most channels not only the  $Ca^{2+}$  channels. Determining whether the effects of these antagonists on JHA release are exerted through some other channels is still worthy of further investigation.

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