Eur. J. Entomol. **96**: 295–298, 1999
ISSN 1210-5759

Intracellular calcium in PTTH-stimulated prothoracic glands of *Manduca sexta* (Lepidoptera: Sphingidae)*

HEINER BIRKENBEIL

Sächsische Akademie der Wissenschaften zu Leipzig, AG Neurohormonale Wirkungsmechanismen, Erbertstr.1, Postfach 100322, D-07743 Jena, Germany; e-mail: b7bihe@pan.zoo.uni-jena.de

Key words. Calcium, ecdysteroid synthesis, *Manduca sexta*, prothoracic gland, PTTH

Abstract. Larval *Manduca* prothoracic gland cells in vitro responded to prothoracicotropic hormone (PTTH) from neurosecretory cells of the brain with an increase of intracellular free calcium. This effect is reversible and dose-dependent. Preincubation of the glands with TMB-8 and dantrolene, which inhibit the release of calcium from intracellular stores, did not decrease the PTTH-stimulated increase in calcium, indicating that intracellular calcium stores are not involved in the control of ecdysteroidogenesis. Pharmacological studies of the PTTH effect with calcium channel blockers revealed that the increase in calcium was totally blocked by cadmium, partially inhibited by nickel and lanthanum and by amiloride, an antagonist of T-type calcium channels. All other inhibitors tested were ineffective, suggesting that the increase in cytosolic calcium is induced by opening of calcium channels, presumably of the T-type, in response to PTTH. The action of PTTH on these channels may be mediated by a G-protein as shown by the effect of mastoparan, a G-protein activator, which increased the concentration of cytosolic calcium comparable to that evoked by PTTH.

INTRODUCTION

Moulting during post embryonic development of insects is triggered by the moulting hormone 20-hydroxyecdysone. The prohormone of this steroid, ecdysone or 3-dehydroecdysone, is predominantly secreted by the prothoracic glands, which are under stimulatory control of the prothoracicotropic hormone (PTTH) produced in neurosecretory cells of the brain. The mechanism of this stimulation has been studied mainly in the tobacco hornworm, *Manduca sexta*, and includes stimulation of a calcium-dependent increase in cAMP synthesis, the activation of a cAMP-dependent protein kinase, phosphorylation of a ribosomal protein and an increase in protein synthesis (Smith & Gilbert, 1989; Rybczinski & Gilbert, 1994; Kulezsa et al., 1994; Smith, 1995; Song & Gilbert, 1995). Calcium-free medium completely inhibited the PTTH-stimulated increase in ecdysone secretion whereas the stimulating effect of PTTH was mimicked by the calcium ionophore A 23187 which suggests that extracellular calcium is necessary in this process (Smith et al., 1985). The elevation of intracellular free calcium by PTTH was directly recorded by microfluorometry with Fura 2 in prothoracic glands of *Galleria mellonella* and *Manduca sexta* (Birkenbeil, 1996, 1998) and further characterised using last larval *Manduca* prothoracic glands in vitro.

MATERIALS AND METHODS

Larvae of the tobacco hornworm, *Manduca sexta*, were reared on an artificial diet at 25°C, > 60% r.h. under a photoperiod of 16L : 8D and staged by the last larval moult and by the beginning of wandering behaviour on day 5 of the instar.

PTTH was extracted from brains of larvae at the wandering stage (day 5) according to Watson et al. (1989) ("crude PTTH"). Aliquots in saline (21 mM KCl, 16 mM NaCl, 3 mM $CaCl_2$, 4 mM glucose, 100 mM trehalose, 5 mM Hepes; pH 6.6; 350 mosm) were stored frozen at –22°C. Fura 2/AM, amiloride and dantrolene were purchased from Calbiochem (Bad Soden, Germany), all other reagents from Sigma (Deisenhofen, Germany).

Loading of prothoracic gland cells with Fura 2/AM in vitro, microfluorometry, and calibration were essentially as described by Birkenbeil (1996). The concentration of free intracellular calcium, $[Ca^{2+}]_i$, was measured in 10 identified cells of a prothoracic gland and was recorded as the mean ± SEM. Addition of reagents was performed by replacement of the whole bathing medium or by addition in 10 µl samples with a pipette. Crude PTTH was always added in 10 µl saline.

RESULTS

PTTH added to *Manduca* prothoracic glands in vitro induces an immediate increase in the concentration of free intracellular calcium, $[Ca^{2+}]_i$, which is reversible by washing the glands with saline (Birkenbeil, 1998). Due to this reversibility it was possible to determine the dose-response relationship of the calcium-increasing effect of PTTH using the same 10 identified cells of a single prothoracic gland in vitro by subsequent addition of increasing amounts of PTTH after washing with saline (Fig. 1). Significant ($p < 0.01$) elevation of 29.6 ± 6.8 nM calcium is shown to be induced by PTTH equivalents to 0.1 brain, whereas 2 brain equivalents evoke an increase in $[Ca^{2+}]_i$ of 299.6 ± 33.4 nM.

The calcium increasing action of PTTH on prothoracic gland cells was studied throughout the last larval instar of *Manduca*. Fig. 2 shows that the gland cells responded to

* This paper is based on a lecture presented at the 6th European Congress of Entomology held in České Budějovice, Czech Republic, August 1998.

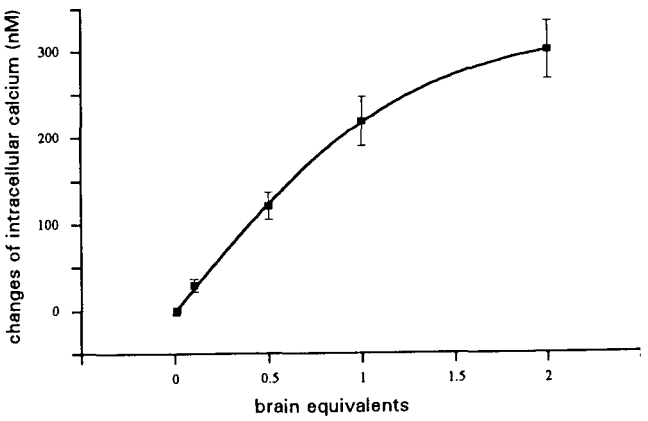

Fig. 1. Dose-response relationship of the effect of PTTH in elevating $[Ca^{2+}]_i$ of a prothoracic gland in vitro. Increasing concentrations of PTTH were subsequently added to a single prothoracic gland after 5 washes with saline. $[Ca^{2+}]_i$ was recorded in 10 identified cells of the gland. Mean ± SEM.

the addition of one brain equivalent of PTTH with an increase of $[Ca^{2+}]_i$ every day during the instar, demonstrating that there are no refractory stages. This means that at least the receptors for PTTH are present in the plasma membrane at all times during the instar. Even in glands on day 7, which have been PTTH-stimulated in vivo and are secreting ecdysteroid at the maximal rate, $[Ca^{2+}]_i$ was elevated by the addition of PTTH from 118.2 ± 4.3 nM to 242.7 ± 6.1 nM.

Prothoracic gland cells of *Manduca* are connected by numerous gap junctions which are assumed to transfer a PTTH-induced intracellular signal to all cells of the gland and to synchronize the secretory activity (Dai et al., 1994). In order to determine whether the intracellular calcium signal is propagated, the bathing medium of the anterior part of a prothoracic gland in vitro was separated from that of the posterior part by a barrier of vaseline in a tight chamber. PTTH was added to only one compartment and the changes in $[Ca^{2+}]_i$ were recorded in 10 identified

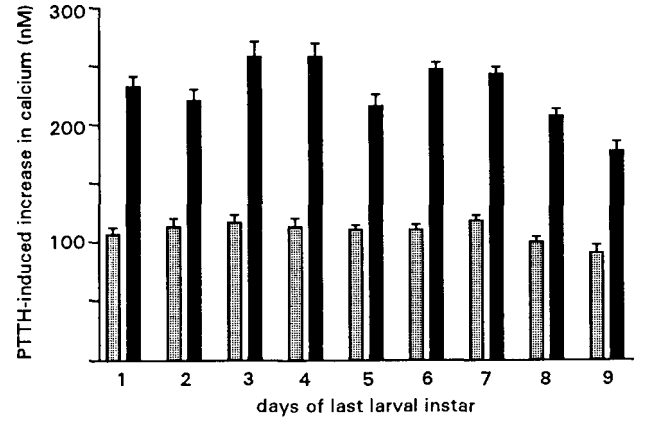

Fig. 2. The calcium-increasing effect of PTTH on prothoracic glands from larvae during the last larval instar. The left column of each pair shows the calcium concentration before (control) and the right (black) column after addition of PTTH (1 brain equivalent). It is demonstrated that the glands respond to PTTH throughout the whole instar. Mean ± SEM; n = 40–60 cells from 4–6 glands.

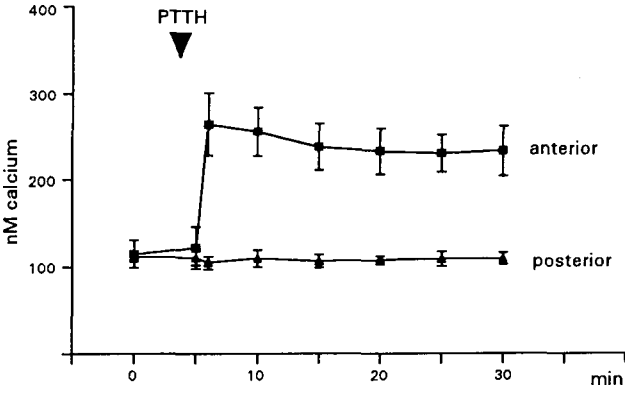

Fig. 3. The intracellular calcium signal induced by PTTH is not propagated throughout the entire prothoracic gland. Two compartments of the bathing medium of a gland were separated by a barrier of vaseline and PTTH (1 brain equivalent) was added only to the anterior compartment. $[Ca^{2+}]_i$ was recorded in 10 identified cells of both parts before and after addition of PTTH. Mean ± SEM.

cells of each compartment. Fig. 3 demonstrates that $[Ca^{2+}]_i$ increased in the gland cells only in the compartment to which PTTH was added, indicating that there is no propagation of the calcium signal throughout the gland.

The involvement of intracellular calcium stores, which are part of the endoplasmic reticulum, in the PTTH-evoked increase of $[Ca^{2+}]_i$ was studied by using inhibitors of calcium release from these stores in conjunction with the addition of PTTH. The inhibitor of IP_3-dependent calcium release, TMB-8 (Birkenbeil, 1998), as well as dantrolene, an inhibitor of ryanodine-sensitive receptors at the membranes of the calcium stores, did not block the rise of calcium levels by PTTH (Fig. 4). On the other hand, preincubation of the glands with cadmium to block calcium channels in the plasma membrane totally prevented the PTTH-induced calcium increase, suggesting extracellular calcium to be responsible for the increase in $[Ca^{2+}]_i$ (Birkenbeil, 1998).

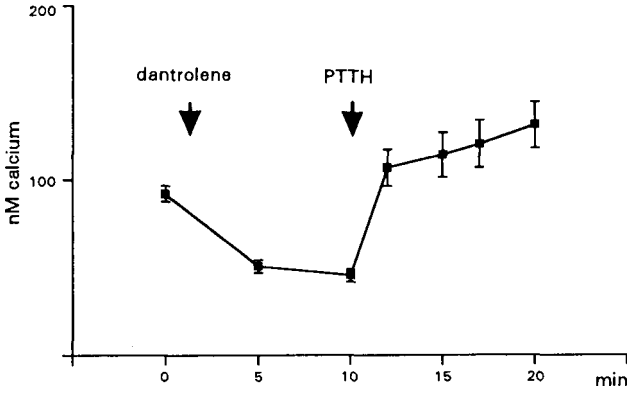

Fig. 4. The effect of dantrolene, an inhibitor of ryanodine-sensitive intracellular calcium stores on the calcium-increasing effect of PTTH. Preincubation of a prothoracic gland with 10 µM dantrolene decreased $[Ca^{2+}]_i$ but did not inhibit the PTTH-stimulated (1 brain equivalent) elevation of calcium. Mean ± SEM; n = 10.

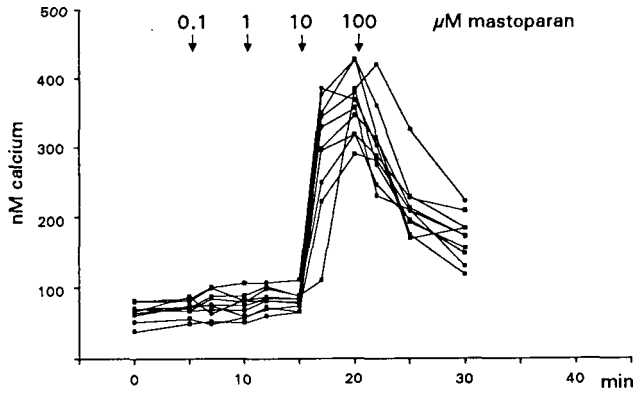

Fig. 5. The effect of mastoparan on $[Ca^{2+}]_i$ of prothoracic glands. The addition of increasing concentrations of mastoparan, a peptide stimulating G-proteins, shows that 10 µM mastoparan evoked a conspicuous increase of intracellular calcium in 10 identified cells of a prothoracic gland in vitro. This effect is decreased by subsequent addition of 100 µM mastoparan.

A variety of selective calcium channel antagonists were used to characterise pharmacologically the plasma membrane calcium channels involved in the PTTH signal transduction process (Birkenbeil, 1998). It has been shown that the less specific inorganic channel blockers nickel and lanthanum, partially, and cadmium totally, inhibit the effect of PTTH on intracellular calcium. Antagonists of L-, P-, and N-type channels did not influence the calcium increase by PTTH. The single more specific blocker exhibiting an inhibitory effect on PTTH action was amiloride. Amiloride is known to inhibit calcium channels of the T-type. Therefore, it is concluded that PTTH exerts its stimulatory action on ecdysteroidogenesis by opening plasma membrane calcium channels, presumably of the T-type.

Control of these calcium channels was studied by the addition of mastoparan to prothoracic glands in vitro. Mastoparan, a peptide that activates G-proteins, induced an elevation of $[Ca^{2+}]_i$ comparable to that evoked by PTTH (Fig. 5), suggesting that a G-protein is involved in the control of the calcium channels.

DISCUSSION

The requirement for extracellular calcium in the stimulation by PTTH of ecdysteroidogenesis in prothoracic glands of *Manduca sexta* has been shown by Smith et al. (1985). More recent studies of prothoracic glands in vitro from larvae of *Galleria mellonella* (Birkenbeil, 1996) and of *Manduca* (Birkenbeil, 1998) have shown that an early step in the chain of events in the stimulatory mechanism triggered by PTTH is a conspicuous increase in $[Ca^{2+}]_i$. In *Manduca* glands this increase of calcium is reversible by washing with saline.This allows the determination of the dose-response relationship with 10 identified cells of a single gland (Fig. 1). The curve shows a steep increase in the response to PTTH from 0.1 to 1 brain equivalents and saturation at a concentration of 2 brain equivalents.

The effect of PTTH on intracellular calcium was studied in prothoracic glands in vitro from larvae throughout the last larval instar to determine whether there was a refractory phase. However, all gland cells responded to the addition of PTTH with an elevation of $[Ca^{2+}]_i$ in nearly the same degree (Fig. 2). This shows that the receptors for PTTH and the calcium-activating mechanisms are present at the prothoracic gland plasma membranes during the entire instar and agrees with the finding that PTTH can stimulate ecdysteroidogenesis throughout the last larval instar (Smith, 1995). In contrast, other insect species are refractory to stimulation of ecdysteroid production with PTTH early in the instar, e.g. *Bombyx mori* (Okuda et al., 1985; Gu et al., 1996) and *Schistocerca gregaria* (Li et al., 1997).

Prothoracic gland cells from *Manduca* are connected by numerous gap junctions which are thought to transfer cellular signals from cell to cell in order to synchronize the secretory activity of all cells of a gland (Dai et al., 1994). In addition, calcium-dependent action potentials have been elicited by electrical stimulation with microelectrodes in prothoracic gland cells of *Manduca* (Eusebio & Moody, 1986) and are propagated across many, if not all, cells of a gland indicating an electrical coupling of the cells. However, the PTTH-evoked calcium signal is not propagated across the entire gland, as demonstrated in Fig. 3. suggesting that the action potentials are propagated electrotonically by opening of voltage-dependent calcium channels without involvement of an intracellular calcium signal. On the other hand, a later signal in the stimulatory chain of events, e.g. the increase in cAMP, may be responsible for intercellular communication of prothoracic gland cells via gap junctions, as proposed by Dai et al. (1994).

Previous studies demonstrated that IP_3-sensitive intracellular calcium stores are not involved in PTTH-stimulated elevation of both $[Ca^{2+}]_i$ (Birkenbeil, 1998) and of ecdysteroidogenesis of *Manduca* glands (Smith, 1993; Girgenrath & Smith, 1996). Treatment of prothoracic glands in vitro with dantrolene, an inhibitor of ryanodine-sensitive receptors, did not block the action of PTTH on intracellular calcium (Fig. 4), demonstrating that ryanodine-sensitive intracellular calcium stores do not contribute to the PTTH-induced calcium signal. This was further corroborated by the complete inhibition of the PTTH-induced increase of calcium by cadmium, which is known to block plasma membrane calcium channels, thus suggesting that extracellular calcium is the source of the additional calcium. It has been shown (Birkenbeil, 1998) that, in addition to the inorganic antagonists cadmium, nickel and lanthanum, only amiloride could decrease the effect of PTTH. The antagonists of L-, N-, and P-channels as well as SKF 96365, an antagonist of receptor-operated channels, and charybdotoxin, an inhibitor of calcium-activated potassium channels, failed to inhibit this effect. The effect of amiloride was dose-dependent with a maximal response at 100 µM (Birkenbeil, 1998). Amiloride is an inhibitor of vertebrate T-type calcium channels (Miller & Fox, 1990; Tsien & Tsien, 1990) and has been shown to inhibit insect calcium channels (Gielow et al., 1995) and to inhibit sodium

channels and Ca^{2+}-Na^{+}-exchange. Regarding the total block of PTTH action by the calcium channel antagonist cadmium and the antagonistic effect of amiloride the stimulation of ecdysteroid production by PTTH may be initiated by opening of T-type calcium channels in the plasma membrane of the prothoracic gland cells, although other inhibitors of T-channels, bepridil and flunarizine, did not have an effect.

A further question regards the control of these channels – are they directly operated by the receptors or is a G-protein as an intermediate link involved? Choleratoxin, catalysing the ADP-ribosylation of G-protein, was shown to stimulate ecdysteroidogenesis in pupal prothoracic glands of *Manduca*, whereas pertussis toxin had no effect (Girgenrath & Smith, 1996), although neither had an effect on cytosolic calcium in larval glands (Birkenbeil, 1998). However, mastoparan, a peptide that activates G-proteins, induced a conspicuous elevation of $[Ca^{2+}]i$ in larval prothoracic glands comparable to that evoked by PTTH (Fig. 5) suggesting that a G-protein activates the calcium channels.

ACKNOWLEDGEMENTS. The author thanks S. Kaltofen for excellent assistance and D. Wicher for valuable discussions.

REFERENCES

Birkenbeil H. 1996: Involvement of calcium in prothoracicotropic stimulation of ecdysone synthesis in Galleria mellonella. *Arch. Insect Biochem. Physiol.* **33**: 39–52.

Birkenbeil H. 1998: Intracellular calcium in prothoracic glands of Manduca sexta. *J. Insect Physiol.* **44**: 279–286.

Dai J.-D., Costello M.J. & Gilbert L.I. 1994: The prothoracic glands of Manduca sexta: a microscopic analysis of gap junctions and intercellular bridges. *Invertebr. Reprod. Dev.* **25**: 93–110.

Eusebio E.J. & Moody W.J. 1986: Calcium-dependent action potentials in the prothoracic gland of Manduca sexta. *J. Exp. Biol.* **126**: 531–536.

Gielow M.L., Gu G.G. & Singh S. 1995: Resolution and pharmacological analysis of the voltage-dependent calcium channels of Drosophila larval muscles. *J. Neurosci.* **15**: 6085–6093.

Girgenrath S. & Smith W.A. 1996: Investigation of presumptive mobilization pathways for calcium in the steroidogenic action of big prothoracicotropic hormone. *Insect Biochem. Mol. Biol.* **26**: 455–463.

Gu S.-H., Chow Y.-S., Lin F.-J., Wu J.-L. & Ho R.-J. 1996: A deficiency in prothoracico-tropic hormone transduction pathway during the early last larval instar of Bombyx mori. *Mol. Cell. Endocrinol.* **120**: 99–105.

Kulesza P., Lee C.-Y. & Watson R.D. 1994: Protein synthesis and ecdysteroidogenesis in prothoracic glands of the tobacco hornworm (Manduca sexta): stimulation by big prothoracicotropic hormone. *Gen. Comp. Endocrinol.* **93**: 448–458.

Li W.,Vedrová A. & Sehnal F. 1997: Humoral stimulation of the larval and adult prothoracic glands in Schistocerca gregaria. *Arch. Insect Biochem. Physiol.* **36**: 85–93.

Miller R.J. & Fox A.P. 1990: Voltage-sensitive calcium channels. In Bronner F. (ed): *Intracellular Calcium Regulation.* Alan R. Liss Inc., New York, pp. 97–183.

Okuda M., Sakurai S. & Ohtaki T. 1985: Activity of the prothoracic gland and its sensitivity to prothoracicotropic hormone in the penultimate and last-larval instar of Bombyx mori. *J. Insect Physiol.* **31**: 455–461.

Rybczynski R. & Gilbert L.I. 1994: Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of Manduca sexta. *Insect Biochem. Mol. Biol.* **24**: 175–189.

Smith W.A. 1993: Second messengers and the action of prothoracicotropic hormone in Manduca sexta. *Am. Zool.* **33**: 330–339.

Smith W.A. 1995: Regulation and consequences of cellular changes in the prothoracic glands of Manduca sexta during the last larval instar: a review. *Arch. Insect Biochem. Physiol.* **30**: 271–293.

Smith W.A. & Gilbert L.I. 1989: Early events in peptide-stimulated ecdysteroid secretion by the prothoracic glands of Manduca sexta. *J. Exp. Zool.* **252**: 264–270.

Smith W.A., Gilbert L.I. & Bollenbacher W.E. 1985: Calcium-cyclic AMP interactions in prothoracicotropic hormone stimulation of ecdysone synthesis. *Mol. Cell. Endocrinol.* **39**: 71–78.

Song Q. & Gilbert L.I. 1995: Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of Manduca sexta. *Insect Biochem. Mol. Biol.* **25**: 591–602.

Tsien R.W. & Tsien R.Y. 1990: Calcium channels, stores, and oscillations. *Annu. Rev. Cell Biol.* **6**: 715–760.

Watson R.D., Thomas M.K. & Bollenbacher W.E. 1989: Regulation of ecdysteroidogenesis in prothoracic glands of the tobacco hornworm Manduca sexta. *J. Exp. Zool.* **252**: 255–263.

Received January 4, 1999; accepted March 11, 1999