Pharmacological study of signal transduction during stimulation of prothoracic glands from *Manduca sexta*

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Abstract

Cytosolic free calcium, [Ca$^2+$], measured in individual prothoracic gland cells of *Manduca sexta* with Fura-2 was increased by prothoracicotropic hormone, PTTH, and by mastoparan, a wasp venom peptide, activating G proteins. The effect on [Ca$^2+$] of mastoparan and of PTTH was inhibited by cadmium and the antagonist of T-type calcium channels, amiloride, and not influenced by the L-type calcium channel blocker nitrendipine, suggesting that the same or similar plasma membrane channels are involved in the action of mastoparan and of PTTH. Pertussis toxin prevented the mastoparan-induced increase of [Ca$^2+$], whereas the effect of PTTH is not influenced by pertussis toxin. Intracellular addition of GDP-β-S failed to inhibit the PTTH-stimulated increase in [Ca$^2+$] suggesting that G proteins are not involved in the stimulatory mechanism of PTTH. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Moulting in the postembryonic development of insects is controlled by the steroid hormone 20-hydroxyecdysone, the prohormone of which is ecdysone (or 3-dehydroecdysone) released by the prothoracic or molting glands into the hemolymph. Biosynthesis and release of ecdysone by the prothoracic glands is stimulated by neuropeptides from the brain, the prothoracicotropic hormones, PTTH (Gilbert et al., 1997). In *Manduca sexta* this stimulation was found to be dependent on extracellular calcium as shown by complete inhibition of PTTH-stimulated ecdysteroid secretion by prothoracic glands in calcium-free medium and by stimulation of ecdysteroid production with the calcium ionophore A23187 (Smith et al., 1985). Microfluorometric studies with the calcium-sensitive dye Fura-2 demonstrated that PTTH increases the concentration of free intracellular calcium, [Ca$^2+$], in the prothoracic glands of *Galleria mellonella* (Birkenbeil, 1996) and *Manduca sexta* (Birkenbeil 1998, 1999). Inhibitors of calcium release from intracellular stores, TMB-8 and dantrolene, did not interfere with the action of PTTH on [Ca$^2+$], of prothoracic glands of *Manduca*. Blockers of calcium channels in the plasma membrane, e.g. cadmium and amiloride, inhibited the effect of PTTH on calcium indicating that PTTH exerts its stimulatory action by opening of, presumably T-type, plasma membrane calcium channels (Birkenbeil 1998, 1999). In order to explore the possibility that the plasma membrane PTTH receptors are coupled by a G protein to the calcium channels, the effect of mastoparan, a wasp venom peptide activating G proteins (Chahdi et al., 1998), on [Ca$^2+$], was studied with *Manduca* prothoracic glands in vitro (Birkenbeil, 1999). Mastoparan increased [Ca$^2+$], indicating that G-protein-coupled calcium channels are at least present in *Manduca* prothoracic gland plasma membranes, but their involvement in the stimulatory mechanism of PTTH should be further investigated.

2. 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 16 h light:8 h dark and staged by the last larval moult and by the beginning of wandering behaviour on day 5 of the last larval instar. Experimental animals were used in the last instar, 1 day after wandering.

PTTH was extracted from brains of last instar larvae at the wandering stage (day 5) (“crude PTTH”) according to Watson et al. (1989). Aliquots in saline (Birkenbeil, 1998) were stored frozen at -80°C. Fura-2, amiloride, guanosine-5’-O-(2-thiodiphosphate) (GDP-β-S), mastoparan, nitrendipine, pertussis toxin, pyridoxalphosphate-6-azophenyl-2’-4’-disulfonic acid (PPADS) and suramin were purchased from Calbiochem (Bad Soden, Germany), all other reagents from Sigma (Deisenhofen, Germany).

Loading of prothoracic gland cells in vitro with Fura-2, microfluorometry, calibration, in vitro culture of glands and radioimmunoassay (RIA) were essentially the same as described previously (Birkenbeil, 1998). The concentration of free intracellular calcium, [Ca^{2+}]_i, was measured in 10 identified cells of a prothoracic gland and was given as mean±SEM. Addition of reagents was made by replacing the whole bathing medium (50 μl) or by addition in 10 μl samples with a pipette. Crude PTTH was always added in 10 μl saline.

3. Results

The dose-dependent increase by mastoparan of [Ca^{2+}]_i, in Manduca prothoracic glands (Birkenbeil, 1999) was the reason to study the effect of mastoparan on the ecdysteroid secretion of prothoracic glands in vitro. Fig. 1 shows that mastoparan did not increase ecdysone production, but instead there was a dose-dependent decrease in ecdysteroid secretion: 50 μM mastoparan induced a decrease of ecdysone production to 50.3±13.7% (contralateral control glands of the same larvae=100%). Therefore, calcium channel antagonists were used in order to compare their effect on the [Ca^{2+}]_i, increasing action of mastoparan and of PTTH. Preincubation of prothoracic glands with cadmium, an inorganic calcium channel blocker, totally prevented an increase of [Ca^{2+}]_i, by subsequent addition of mastoparan (n=20 cells) (Fig. 2). Also, preincubation of the glands with amiloride, an inhibitor of T-type calcium channels, blocked the calcium-increasing effect of mastoparan (n=20 cells) (not shown). On the other hand, nitrendipine, an inhibitor of L-type calcium channels, did not influence the effect of mastoparan in increasing [Ca^{2+}]_i, of Manduca prothoracic gland cells (n=50 cells) (Fig. 3). It is seen that these calcium channel blockers behave just the same way in the effect of mastoparan and PTTH on [Ca^{2+}]_i, cadmium and amiloride inhibiting and nitrendipine not influencing the increase of [Ca^{2+}]_i, suggesting that the same or similar calcium channels are involved as well in the action of PTTH and mastoparan.

Nitrendipine, which did not inhibit the effect of PTTH on [Ca^{2+}]_i, was shown to inhibit steroidogenesis induced by PTTH (Girgenrath and Smith, 1996). In the stimulatory chain of events leading to increased ecdysteroid production, the second-messenger calcium was assumed to act before cAMP, as demonstrated by stimulation of ecdysteroid secretion in Manduca prothoracic glands by cAMP in calcium-free medium (Smith et al., 1985). Fig. 4 shows that, instead of the elevation of ecdysteroid secretion by prothoracic glands in vitro treated with db-cAMP, there is a decrease in ecdysteroid production in the contralateral glands from the same larvae challenged with db-cAMP together with 100 μM nitrendipine, sug-

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**Fig. 1.** Dose-response relationship of the effect of mastoparan on the ecdysteroid secretion of prothoracic glands in vitro. Pairs of prothoracic glands were incubated for 80 min in 50 μl Grace’s medium. One gland served as control whereas the contralateral gland was challenged with mastoparan. The amount of ecdysteroid secreted by the control gland=100%. n=5–7 gland pairs. Mean±SEM.

**Fig. 2.** Preincubation of prothoracic glands with cadmium prevents the mastoparan-induced increase in [Ca^{2+}]_i. Addition of mastoparan (16.7 μM) to a prothoracic gland resulted in elevation of intracellular calcium (■). The [Ca^{2+}]_i of the contralateral gland from the same larva, preincubated with cadmium (1 mM), did not show any effect of mastoparan (▲), indicating that plasma membrane calcium channels are responsible for the increase in [Ca^{2+}]_i, evoked by mastoparan.
Fig. 3. Nitrendipine did not inhibit the increase in [Ca\(^{2+}\)]\(_i\) stimulated by mastoparan. A prothoracic gland in vitro was incubated with the L-type calcium channel antagonist nitrendipine (100 μM). Subsequent addition of mastoparan (16.7 μM) resulted in increased [Ca\(^{2+}\)]\(_i\), suggesting that L-type calcium channels do not take part in the action of mastoparan.

Fig. 4. Effect of nitrendipine on db-cAMP-stimulated ecdysteroid secretion of prothoracic glands in vitro. Pairs of prothoracic glands were incubated for 80 min in 50 μl Grace’s medium (control). In a second incubation period of 80 min, one gland of a pair was treated with db-cAMP (10 mM) and the contralateral gland with db-cAMP (10 mM) together with nitrendipine (100 μM). Incubation with db-cAMP alone stimulated the ecdysteroid secretion from 4.76±1.38 ng (control) to 11.09±2.28 ng, whereas additional treatment with nitrendipine resulted in a decrease from 4.68±1.72 ng (control) to 2.61±0.71 ng. Mean±SEM; n=10 gland pairs.

Fig. 5. Pertussis toxin does not inhibit the action of PTTH on [Ca\(^{2+}\)]\(_i\). One prothoracic gland of a pair was treated with pertussis toxin (4.17 μg/ml) (■). Subsequent addition of PTTH (1 brain equivalent) resulted in a similar increase in [Ca\(^{2+}\)]\(_i\), as seen in the contralateral gland treated with PTTH only (▲). Mean±SEM; n=10 cells.

Fig. 6. The effect of mastoparan on prothoracic gland [Ca\(^{2+}\)]\(_i\) is pertussis toxin-sensitive. One prothoracic gland of a pair was preincubated with pertussis toxin (4.17 μg/ml) (■). Subsequent treatment with mastoparan (16.7 μM) failed to increase [Ca\(^{2+}\)]\(_i\) (■). The [Ca\(^{2+}\)]\(_i\) of the contralateral control gland from the same larva was increased by addition of mastoparan alone (▲). Mean±SEM; n=10 cells.

Some G proteins are inhibited by pertussis toxin, uncoupling them from their receptors by ADP-ribosylation. Therefore, the effects of PTTH and mastoparan on [Ca\(^{2+}\)]\(_i\), were compared with or without preincubation of the prothoracic glands with pertussis toxin. Fig. 5 shows that pertussis toxin did not influence the increase in [Ca\(^{2+}\)]\(_i\), evoked by PTTH (n=40 cells), whereas the action of mastoparan on prothoracic gland [Ca\(^{2+}\)]\(_i\), is completely abolished by preincubation with pertussis toxin (n=30 cells) (Fig. 6), indicating that different G proteins or different mechanisms are involved.

The non-hydrolysable GDP analog, GDP-β-S (0.5 and 1 mM), injected into prothoracic gland cells in vitro, did not suppress the [Ca\(^{2+}\)]\(_i\)-elevating effect of PTTH, supporting the assumption that G proteins do not take part in the stimulatory action of PTTH (Fig. 7).

Suramin, besides other effects, was shown to inhibit purinergic receptors coupled to calcium channels (Mateo et al., 1998; Taschenberger et al., 1999) and to interfere with the binding of various growth factors to their cell-surface receptors (Benini et al., 1999; Zangani et al., 1999). Addition of suramin to Manduca prothoracic glands in vitro prevented the PTTH-induced increase in [Ca\(^{2+}\)]\(_i\), (Fig. 8). The calcium increase evoked by masto-
4. Discussion

PTTH stimulates ecdysteroid biosynthesis in prothoracic glands by opening plasma membrane calcium channels resulting in an increase in intracellular calcium $[\text{Ca}^{2+}]$, which acts as an intracellular mediator activating the stimulatory chain of events including calmodulin, cAMP, protein kinase A and phosphorylation of a ribosomal protein (Gilbert et al., 1997; Birkenbeil 1998, 1999). An increase in $[\text{Ca}^{2+}]$, in prothoracic gland cells of Manduca, induced by the calcium ionophore A 23187, was shown to stimulate ecdysone biosynthesis (Smith et al., 1985). Mastoparan, a wasp venom peptide activating G proteins, also increased $[\text{Ca}^{2+}]$, of Manduca prothoracic glands (Birkenbeil, 1999), but this increase did not result in an increase in ecdysteroid secretion. Moreover, there was a dose-dependent decrease in ecdysteroid production by prothoracic glands in vitro (Fig. 1). These two effects of mastoparan, an increase of $[\text{Ca}^{2+}]$, and a decrease in ecdysteroid secretion, can be compared with the activation by octopamine or tyramine of the cloned Drosophila octopamine–tyramine receptor, leading to an inhibition of adenylate cyclase and an elevation of $[\text{Ca}^{2+}]$, by separate G-protein-coupled pathways, the former effect being pertussis toxin-sensitive, the latter insensitive (Robb et al., 1994). Consequently, the decrease of ecdysteroid secretion by mastoparan could be explained by an inhibition of adenylate cyclase via a G protein activated by mastoparan, whereas another G protein, also activated by mastoparan, controls the opening of calcium channels. Increased activity of adenylate cyclase and increased cAMP-titer have been shown to accompany the stimulation by PTTH of ecdysteroid production in Manduca prothoracic glands (Smith et al., 1985).

Calcium channel antagonists were used to characterize the elevation by mastoparan of $[\text{Ca}^{2+}]$, in Manduca prothoracic glands in vitro. The nonspecific inorganic calcium channel blocker cadmium totally prevented any increase of $[\text{Ca}^{2+}]$, evoked in the contralateral control gland by mastoparan alone, indicating that plasma membrane calcium channels are responsible for this increase (Fig. 2). Also amiloride, a T-type channel antagonist inhibiting the calcium increasing action of PTTH (Birkenbeil, 1998), prevented the calcium elevation by mastoparan, whereas nitrendipine, an inhibitor of L-type channels, did not interfere with the effect on calcium of mastoparan (Fig. 3) or PTTH (Birkenbeil, 1998). In conclusion, the increase in $[\text{Ca}^{2+}]$, by mastoparan seems to be regulated by the same or a similar type of, presumably T-type, calcium channels as those involved in the action of PTTH.

The L-type calcium channel antagonist nitrendipine was demonstrated to be effective in inhibiting stimulation by PTTH of ecdysteroid secretion by Manduca prothoracic glands (Girgenrath and Smith, 1996),

paran was not inhibited by suramin, indicating that the stimulation of G protein by mastoparan is not abolished by suramin. The inhibition by suramin of the action of PTTH is perhaps due to an interfering of suramin with the binding of PTTH to its receptor. An involvement of purinergic receptors in the action of PTTH can be excluded, because PPADS (100 μM; 1 mM), a further antagonist of these receptors, did not prevent the effect of PTTH on $[\text{Ca}^{2+}]$, (n=20 cells) and because the agonist ATP (1 mM) did not show any effect on prothoracic gland calcium (not shown).
whereas it did not prevent the increase in \([\text{Ca}^{2+}]\), by PTTH (Birkenbeil, 1998). Therefore, prothoracic glands in vitro were stimulated with db-cAMP with and without nitrendipine. cAMP acts as second messenger in the stimulatory chain behind calcium, as demonstrated by its stimulation of ecdysteroid biosynthesis in calcium-free medium (Smith et al., 1985). It is shown (Fig. 4) that addition of nitrendipine together with db-cAMP resulted in a decrease of ecdysteroid secretion, whereas incubation of the glands with db-cAMP alone led to an increase in production of ecdysteroid. This means that L-type calcium channels are indeed involved in the stimulation of ecdysteroid production, but obviously not in the calcium increase primarily evoked by PTTH, but perhaps in a calcium-dependent excytotic release of the ecdysteroids (Birkenbeil, 1983; Hanton et al., 1993).

Some, but not all, G proteins are sensitive to pertussis toxin (PTX) which ADP-ribosylates the \(\alpha\)-subunits uncoupling the G protein from the receptor. Preincubation of prothoracic glands with PTX did not prevent the increase in \([\text{Ca}^{2+}]\), by PTTH (Fig. 5). This is in accordance with Girgenrath and Smith (1996) demonstrating that pretreatment with PTX of Manduca prothoracic glands did not affect ecdysone secretion stimulated by PTTH. However, preincubation of the glands with PTX is shown to abolish the rise of \([\text{Ca}^{2+}]\), effected by mastoparan (Fig. 6) indicating that pertussis toxin-sensitive G proteins are indeed present in Manduca prothoracic gland plasma membranes as shown previously (Girgenrath and Smith, 1996), but they are obviously not involved in the action of PTTH.

The non-hydrolysable GDP analog guanosine 5′-O-(2-thiodiphosphate) (GDP-\(\beta\)-S), which inhibits G-protein activation, was injected into prothoracic gland cells. The effect of PTTH in increasing \([\text{Ca}^{2+}]\), was the same in injected and control cells (Fig. 7), indicating that GDP-\(\beta\)-S does not inhibit the action of PTTH. Therefore, it is suggested that G proteins do not take part in the stimulation of ecdysteroidogenesis by PTTH in Manduca glands.

A further experiment demonstrating the different mechanisms of increasing \([\text{Ca}^{2+}]\), by mastoparan and PTTH was made by preincubation of prothoracic glands in vitro with suramin. The mastoparan-induced rise of \([\text{Ca}^{2+}]\), was not abolished by suramin, whereas the effect of PTTH on \([\text{Ca}^{2+}]\), was completely prevented (Fig. 8). Suramin is known as an antagonist of purinergic receptors coupled to calcium channels (Mateo et al., 1998; Taschenberger et al., 1999). However, the involvement of such receptors is not probable because a further inhibitor of purinergic receptors, PPADS, did not show any effect on the action of PTTH. Moreover, the agonist of these receptors, ATP, did not influence \([\text{Ca}^{2+}]\), of prothoracic gland cells. Another effect of suramin described concerns its inhibition of the binding of growth factors (Benini et al., 1999; Zangani et al., 1999) and of FSH (Stevis et al., 1999) to the extracellular domains of their receptors. It is suggested that suramin prevents the binding of PTTH to its receptor, thus abolishing the increase in \([\text{Ca}^{2+}]\), whereas the elevation of \([\text{Ca}^{2+}]\), by mastoparan via an activation of G protein is not affected by suramin.

In conclusion, the stimulatory chain of events leading to increased ecdysteroid secretion by prothoracic glands, which is triggered by PTTH, starts, after binding of PTTH to its receptor, by opening of plasma membrane calcium channels resulting in elevated \([\text{Ca}^{2+}]\). G proteins do not seem to be involved in the control of the calcium channels, which are assumed to be directly operated by the PTTH receptor.

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**References**


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