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Involvement of Calcium, Inositol-1,4,5 Trisphosphate and Diacylglycerol in the Prothoracicotropic Hormone-Stimulated Ecdysteroid Synthesis and Secretion in the Prothoracic Glands of *Bombyx mori*

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ABSTRACT—The objective of this study was to determine which intracellular second messenger systems are activated by prothoracicotropic hormone in the prothoracic glands (PGs) of *Bombyx mori*. Recombinant prothoracicotropic hormone (rPTTH) could stimulate ecdysteroid synthesis and secretion from day 6 PGs of the 5th instar of *Bombyx mori* within 30 min of *in vitro* incubation. However, rPTTH did not stimulate any increases in the glandular content of inositol 1,4,5-trisphosphate and cAMP during this short incubation period. Extracellular Ca^{2+} influenced the basal and rPTTH-stimulated ecdysteroid synthesis and release in a dose-dependent manner. The L-type Ca^{2+} channel antagonist, nitrendipine, inhibited the rPTTH-stimulated ecdysteroid synthesis and secretion (IC_{50} ~28 μM). The phospholipase C inhibitor, 2-nitro-4-carboxyphenyl-N, N-diphenylcarbamate, inhibited the rPTTH-stimulated ecdysteroid synthesis (IC_{50} ~19 μM). The protein kinase C inhibitor, chelerythrine chloride, inhibited the rPTTH-stimulated ecdysteroid synthesis (IC_{50}~14 μM). The protein kinase C activator, phorbol-12-myristate 13-acetate (PMA), could stimulate basal ecdysteroid synthesis and secretion (EC_{50}~1 μM) and its inactive α-isomer (4 α-PMA) was ineffective. The combined results suggest that the PTTH-stimulated ecdysteroid synthesis and release in the PGs of *Bombyx* is dependent on extracellular Ca^{2+} and the bifurcating second messenger signalling cascade of inositol 1,4,5-trisphosphate and diacylglycerol.

Key worsds: prothoracicotropic hormone, ecdysteroid, inositol-1,4,5 trisphosphate, calcium, prothoracic gland, *Bombyx mori*

INTRODUCTION

The synthesis and secretion of ecdysteroid hormone from prothoracic glands (PGs) of insects is regulated by a family of cerebral neuropeptides, the prothoracicotropic hormones (PTTHs) (Gilbert *et al*., 1996). In the tobacco hornworm, *Manduca sexta*, two size variants of PTTH have been identified: big PTTH and small PTTH (Gilbert *et al*., 1996). Both forms of *Manduca* PTTH were shown to mediate their signalling cascade via cAMP as a second messenger (Smith, 1993; Watson *et al*., 1993). The signalling cascade of *Manduca* big PTTH does not involve any increases in glandular inositol phosphate production (Girgenrath and Smith, 1996). Furthermore, phorbol esters (protein kinase C activators) were not found to stimulate basal and big PTTH-stimulated ecdysone synthesis from *Manduca* PGs, although protein kinase C activity was found in the PGs of this insect (Smith, 1993).

In the silkworm, *Bombyx mori*, only the 30,000-dalton PTTH was found to stimulate ecdysteroid synthesis and secretion from the PGs of this insect (Kiriishi *et al*., 1992). In the present study, we used the recombinant form of *Bombyx* PTTH (rPTTH; Ishibashi *et al*., 1994), which was shown to stimulate ecdysteroid synthesis and release from 5th instar PGs (Dedos *et al*., 1999a). Our objective in this study was to determine which intracellular second messenger systems are activated by *Bombyx* PTTH in the PGs and how such activation is regulated. We present evidences to suggest that activation of ecdysone synthesis and secretion by PTTH is dependent on the presence of extracellular Ca^{2+} and on the generation and action of the second messengers, inositol-1,4,5

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trisphosphate (IP_3) and diacylglycerol which are generated from the bifurcating signalling system of receptor activated phospholipase C.

MATERIALS AND METHODS

Animals

All experiments used larvae from the hybrid J106xDAIZO. The larvae were reared on mulberry leaves under a 12:12-L:D photoperiod at 25±1°C and 60% relative humidity. Larvae were staged as we previously described (Dedos *et al.*, 1999a). In this particular hybrid, the 5th instar period lasts about ~208 hr. The onset of wandering behaviour occurs ~144 hr (day 6) after the final larval ecdysis. Female larvae of day 6 were exclusively used in this study.

Reagents

Recombinant *Bombyx* PTTH (rPTTH) (Ishibashi *et al.*, 1994) was dissolved in either Grace's medium (GIBCO-BRL, Grand Island, NY, USA) or Ca^{2+}-free Ringer saline and stored at –20°C until use. Nitrendipine (Calbiochem, La Jolla, CA, USA), chelerythrine chloride (Research Biochemicals International, Natick, MA, USA), 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC) (Sigma, St. Louis, MO, USA), phorbol 12-myristate 13-acetate (PMA) and 4α-phorbol 12-myristate 13-acetate (4α-PMA) (GIBCO-BRL, Grand Island, NY, USA) were prepared as stock solutions in dimethylsulfoxide (DMSO). Ethylene glycol bis(β-aminoethyl ether)- *N,N,N',N'*-tetraacetic acid (EGTA) was purchased from Wako (Osaka, Japan).

In vitro prothoracic gland assay

Larvae were anaesthetized by submersion in water and PGs were dissected rapidly (~2 min /animal) from each larva in sterile saline (0.85% NaCl). The glands were pre-incubated in Grace's medium for 15–30 min. A paired gland design was used in some experiments. One gland of the pair was incubated in 20 μl of medium containing one or more experimental agent(s); the other gland of the pair was incubated in 20 μl medium containing solvent. When DMSO was used as solvent, all PGs were incubated in medium containing 1% DMSO. The presence of 1% DMSO does not affect basal or rPTTH-stimulated ecdysteroid synthesis and release . In experiments where the dose response to an experimental agent was investigated, glands from a pooled group were randomly selected for incubation at the indicated doses. In experiments requiring a Ca^{2+}-free medium, Ringer's saline (Shirai *et al.*, 1994) was prepared using NaCl (4.5 mM) in place of the standard 4.5 mM $CaCl_2$. Hepes buffer (0.01 M, pH 6.8) and either EGTA (0.1 mM) or the indicated amount of Ca^{2+} (in the form of $CaCl_2$) were added prior to use (Hayes *et al.*, 1995). Incubations were carried out at 25±1°C in high humidity in 96-multiwell plates (Wako, Osaka, Japan). After each designated incubation period, the medium was removed, and an aliquot of the medium was subjected to radioimmunoassay for quantification of ecdysteroid content.

Quantification of inositol 1,4,5-trisphosphate and cAMP

The content of inositol 1,4,5-trisphosphate (IP_3) in PGs was quantified by a [^{3}H]radioreceptor assay using the kit and protocol available from New England Nuclear Corp (Boston, MA, USA) as we previously described (Dedos *et al.*,1998). Recovery of IP_3 from the glands was determined to be 90%. Sensitivity of the IP_3 assay system was approximately 0.1 pmol.

The content of cAMP in PGs was quantified by enzyme immunoassay using the kit and protocol available from Cayman Chemical Co. (Ann Arbor, MI, USA), as we previously described (Dedos *et al.*, 1999a).

Radioimmunoassay

The amount of ecdysteroid in the incubation medium was quantified by radioimmunoassay, as we previously described (Dedos *et al.*, 1999a). Radiolabeled ecdysone, [23,24-^{3}H]ecdysone (sp. act. 53 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA).

Statistical analyses

Statistical significance of the results was determined by analysis of variance or Student's t-test. For most experiments, analysis of variance was followed by Tukey multiple comparisons tests. Test results are shown in figure legends. The statistical analyses were done with computer software (GraphPad Prism™ 2.0).

RESULTS

Stimulation of ecdysteroid synthesis and secretion by rPTTH and effects of rPTTH on glandular inositol 1,4,5-trisphosphate (IP_3) and cAMP levels

To determine rPTTH potentiation of ecdysteroid synthesis and release from day 6 PGs, groups of glands were exposed to 1 ng of rPTTH for 30 min. The glands were then transferred to plain Grace's medium and incubated for an additional 90 min (Table 1). The results showed that rPTTH could stimulate ecdysteroid synthesis and secretion from day 6 PGs within the 30 min and even in the subsequent 90 min incubation period (Table 1).

Table 1. Stimulation of ecdysteroid synthesis and release from PGs of *Bombyx mori* by rPTTH

Treatments	Ecdysteroid (ng/gland) 30 min incubation	90 min incubation
Basal	1.83±0.16	2.62±0.28
1 ng rPTTH	3.77±0.24	4.57±0.14

Individual glands from day 6 of the 5th instar were incubated in Grace's medium in the presence of 1 ng rPTTH and their contralaterals in the same medium alone (basal) for 30 min. Then glands from each group were transferred and incubated for an additional 90 min period to Grace's medium in the absence of rPTTH. Each group is the mean±SEM of 6 glands. Tests for the difference between control (basal) and experimental (rPTTH) glands (t-tests) revealed that rPTTH stimulated ecdysteroid synthesis and release in both incubation periods. Results of Tukey multiple comparisons for ecdysteroid synthesis and release (pairs of means enclosed by the range of a bracket are not significantly different, P>0.05): [3.77–4.57].

Next we determined whether rPTTH transduces its signalling cascade through activation of a phospholipase C (PLC), thus generating the second messenger inositol 1,4,5-trisphosphate (IP_3). Groups of day 6 PGs were incubated in the presence of 1 ng rPTTH/gland and their IP_3 contents were determined at various time intervals. As shown in Fig. 1, rPTTH could not stimulate any increase in glandular IP_3 content in time intervals ranging from 30 sec to 2 hr (P=0.95).

In a previous study, we showed that a cerebral prothoracicotropic factor, which is different from *Bombyx* PTTH, stimulates cAMP accumulation in the PGs of this insect (Dedos *et al.*, 1999a). However, both a cerebral prothoracicotropic factor and rPTTH did not stimulate cAMP accumulation in day 6 PGs within a 30 min incubation (Dedos *et al.*, 1999a). Since rPTTH stimulated ecdysteroid synthesis and secretion within

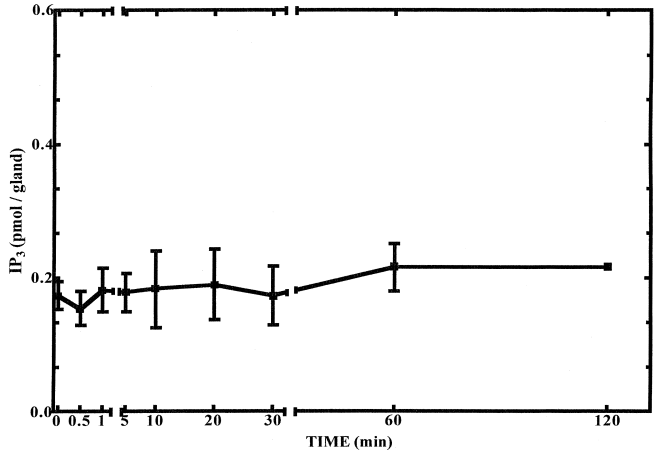

Fig. 1. Effects of rPTTH on prothoracic gland inositol trisphosphate levels. Each point is the mean±S.E. of 4–5 glands. Data were analysed by one-way analysis of variance (P=0.95).

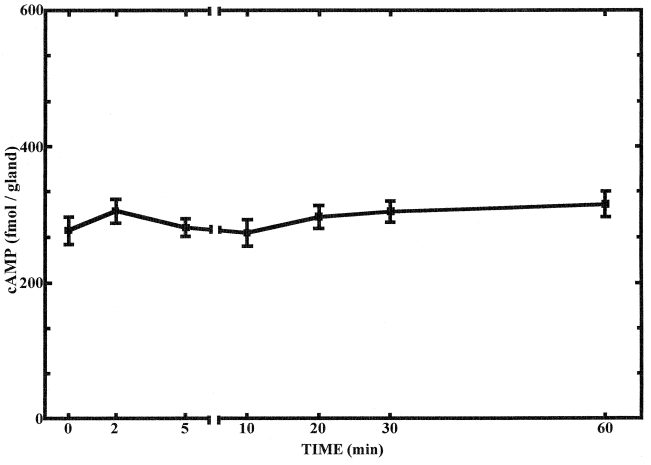

Fig. 2. Effects of rPTTH on prothoracic gland cAMP levels. Each point is the mean±S.E. of 6 glands. Data were analyzed by one-way analysis of variance (P=0.51).

a 30 min incubation (Table 1), we determined whether rPTTH transduces its signalling cascade through activation of an adenylate cyclase at time periods much shorter than 30 min. The results (Fig. 2) showed that 1 ng rPTTH did not change glandular cAMP content during incubation times ranging from 2 min to 1 hr (P=0.51; Fig. 2). The glandular content of another cyclic nucleotide, cGMP, was either not affected by rPTTH in day 6 PGs (data not shown).

Dependence of basal and rPTTH-stimulated ecdysteroid synthesis and secretion on extracellular Ca^{2+}

Prothoracic glands from day 6 were incubated for 2 hr in Ringer's saline, in which the indicated concentration of $CaCl_2$ was added with or without 1 ng rPTTH/gland (Fig. 3). Both, basal and rPTTH-stimulated ecdysteroid secretions were influenced by extracellular Ca^{2+} in a dose-dependent manner. When glands were incubated in Ca^{2+}-free Ringer's saline, the rPTTH-stimulated ecdysteroid synthesis and secretion reached levels similar to the basal ecdysteroid secretion in the absence

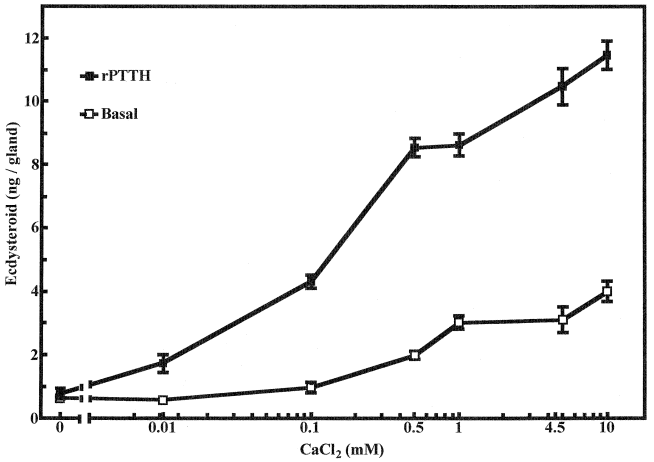

Fig. 3. Effect of external Ca^{2+} on basal and rPTTH-stimulated ecdysteroid synthesis and release by day 6 PGs. Each point is the mean±S.E. of 5 to 6 glands. Results of Tukey multiple comparisons (pairs of means enclosed by the range of a bracket are not significantly different, P>0.05): Basal; [0.56–0.97], [3.01–3.14] [3.14–3.99]; rPTTH; [0.77–1.73], [8.55–8.63], [10.49–11.47].

of extracellular Ca^{2+}. The higher the concentration of $CaCl_2$ in saline, the more rPTTH could stimulate ecdysteroid synthesis and release above basal levels (Fig. 3).

Effects of nitrendipine on basal and rPTTH-stimulated ecdysteroid synthesis and secretion

Prothoracic glands of day 6 were incubated for 2 hr in the presence of various concentrations of the L-type Ca^{2+} channel antagonist, nitrendipine, and 1 ng rPTTH/gland. The results

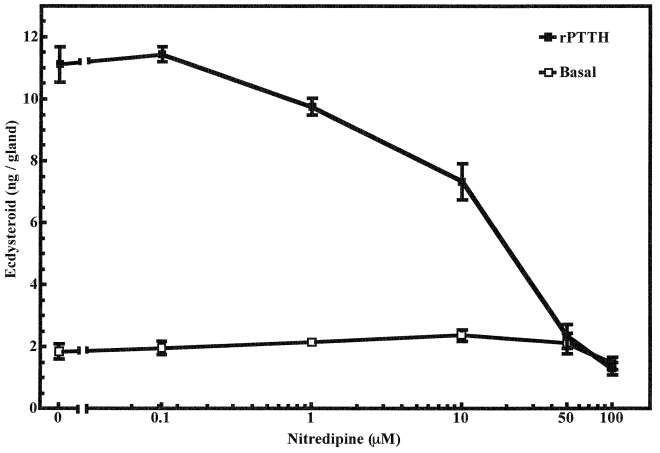

Fig. 4. Effect of nitrendipine on basal and rPTTH-stimulated ecdysteroid synthesis and release by day 6 PGs. Individual glands were incubated for 2 hr with the indicated concentrations of nitrendipine alone (basal) or in the simultaneous presence of 1 ng rPTTH/gland (rPTTH). Each point is the mean±S.E. of 5 to 6 glands. Data for the effects of nitrendipine on basal ecdysteroid synthesis and release were analyzed by one-way analysis of variance (P=0.11). Results of Tukey multiple comparisons for the effects of nitrendipine on rPTTH-stimulated ecdysteroid synthesis and release (pairs of means enclosed by the range of a bracket are not significantly different, P>0.05): [1.28–2.33], [9.74–11.43].

Table 2. Effect of rPTTH on ecdysteroid synthesis and release and IP_3 levels of day 6 larval PGs of *Bombyx mori*

Treatment	Ecdysteroid (ng/gland)		IP_3 (pmol/gland)	
	Basal	Experimental	Basal	Experimental
1 ng rPTTH	2.94±0.23	8.66±0.72	0.31±0.03	0.29±0.02
1 ng rPTTH+50 μM NCDC	2.78±0.22	2.68±0.45	0.27±0.03	0.25±0.01

Glands were incubated for 2 hr in the presence of 1 ng rPTTH/gland or 1 ng rPTTH+50 μM NCDC. After the end of the incubation the IP_3 content of the PGs and the amount of secreted ecdysteroid in the medium were determined. Each value is the mean±SEM of 6 glands. Tests for the difference between experimental and control glands (t-tests) revealed that NCDC inhibited the rPTTH-stimulated ecdysteroid synthesis and release ($P<0.05$). Data of IP_3 determinations were analyzed by one-way analysis of variance ($P=0.48$).

in Figure 4 indicate that nitredipine competetively inhibited the rPTTH-stimulated ecdysteroid synthesis and release *in vitro* (IC_{50}~28 μM). Nitrendipine did not inhibit the basal secretory activity of the glands ($P=0.11$; Fig. 4). The rPTTH-stimulated ecdysteroid synthesis and release was inhibited in a similar way by the phenylalkylamine derivative, verapamil but not by the benzothiazepine, diltiazem (data not shown).

Dependence of rPTTH-stimulated ecdysteroid synthesis and release on the generation of inositol 1,4,5-trisphosphate (IP_3) in the PGs

Two-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC) is known as a potent inhibitor of PLC (Takei *et al.*, 1991). Glands of day 6 were incubated for 2 hr in the presence of 1 ng rPTTH/gland or 1 ng rPTTH+50 μM NCDC (Table 2). Recombinant PTTH stimulated ecdysteroid synthesis and release but NCDC inhibited the rPTTH-stimulated ecdysteroid synthesis and release ($P>0.05$; Table 2). The IP_3 content of the PGs remained unchanged for 2 hr (Table 2; $P=0.48$).

In a different approach, PGs of day 6 were incubated for 2 hr in the presence of various concentrations of NCDC and 1 ng rPTTH/gland. The results in Figure 5 indicate that NCDC competetively inhibited the rPTTH-stimulated ecdysteroid synthesis and release (IC_{50}~19 μM), although NCDC did not inhibit the basal secretory activity ($P=0.11$; Fig. 5).

Effects of chelerythrine chloride on basal and rPTTH-stimulated ecdysteroid synthesis and release

Prothoracic glands of day 6 were incubated for 2 hr in the presence of various concentrations of the protein kinase C (PKC) inhibitor chelerythrine chloride (Herbert *et al.*, 1990) and 1 ng rPTTH/gland. The results in Fig. 6 indicate that chelerythrine chloride competetively inhibited the steroidogenic effect of rPTTH (IC_{50}~14 μM). Chelerythrine chloride did not

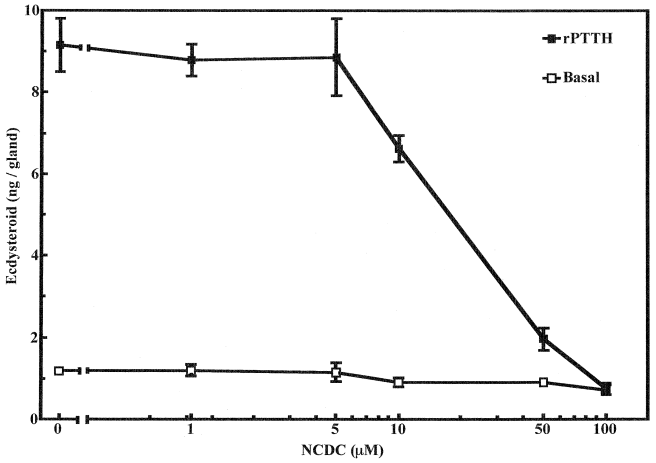

Fig. 5. Effect of NCDC on basal and rPTTH-stimulated ecdysteroid synthesis and release by day 6 PGs. Individual glands were incubated for 2 hr with the indicated concentrations of NCDC alone (basal) or in the simultaneous presence of 1 ng rPTTH/gland (rPTTH). Each point is the mean±S.E. of 5 to 6 glands. Data for the effects of NCDC on basal ecdysteroid level were analyzed by one-way analysis of variance ($P=0.11$). Results of Tukey multiplecomparisons for the effects of NCDC on rPTTH-stimulated ecdysteroid synthesis and release (pairs of means enclosed by the range of a bracket are not significantly different, $P>0.05$): [0.74–1.95], [6.62–8.77], [8.84–9.15].

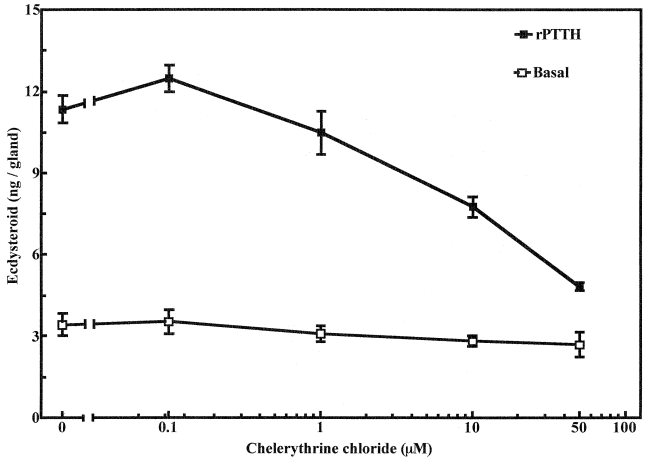

Fig. 6. Effect of chelerythrine chloride on basal and rPTTH-stimulated ecdysteroid synthesis and release by day 6 PGs. Individual glands were incubated for 2 hr with the indicated concentrations of chelerythrine chloride alone (basal) or in the simultaneous presence of 1 ng rPTTH/gland (rPTTH). Each point is the mean±S.E. of 5 to 6 glands. Data for the effects of chelerythrine chloride on basal ecdysteroid level were analyzed by one-way analysis of variance ($P=0.41$). Results of Tukey multiple comparisons for the effects of chelerythrine chloride on rPTTH-stimulated ecdysteroid synthesis and release (pairs of means enclosed by the range of a bracket are not significantly different, $P>0.05$): [4.81–7.76], [10.49–11.33].

inhibit the basal secretory activity of the glands (P=0.41; Fig. 6). Similar results were obtained with another PKC inhibitor, calphostin C (data not shown).

Effect of phorbol 12-myristate 13-acetate (PMA) and 4α-phorbol 12-myristate 13-acetate (4α-PMA) on basal ecdysteroid synthesis and release

In initial experiments, day 6 PGs were incubated in the presence of varying concentrations (0.1 nM ~ 0.1 mM) of PMA and 4α-PMA for 2 hr. During this incubation period, however, no statistically significant change in ecdysteroid synthesis and release was observed with PMA or 4α-PMA (data not shown). Statistical analysis showed that PMA did not alter basal ecdysteroid synthesis and release (P=0.14) and similar results were also observed with 4α-PMA (P=0.11; data not shown). Since the possibility remained that the 2 hr incubation period was too short to observe any PMA-mediated effects on basal ecdysteroid synthesis and release, the incubation period was extended to 5 hr. At 5 hr incubation period, PMA stimulated basal ecdysteroid synthesis and release (EC_{50}~1 μM; Fig. 7). Under similar conditions, 4α-PMA did not alter basal ecdysteroid synthesis and release (P=0.21; Fig. 7). These results were verified using a paired gland design at 5 hr incubation. Glands incubated in 1 μM PMA in Grace's medium secreted 12.3 ng ecdysone/gland while their contralaterals secreted 7.6 ng/gland (n=4), but such an effect was not observed during a 2 hr incubation (data not shown).

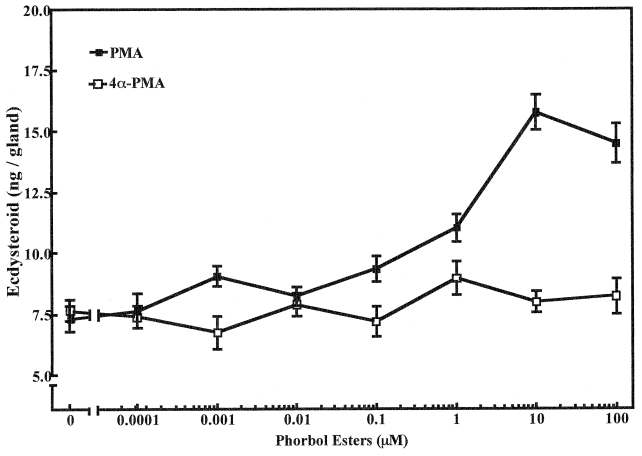

Fig. 7. Effect of phorbol 12-myristate 13-acetate (PMA) and 4α-PMA on basal ecdysteroid synthesis and release by day 6 PGs. Individual glands were incubated for 5 hr with the indicated concentrations of PMA or 4α-PMA. Each point is the mean±S.E. of 5 to 6 glands. Data for the effects of 4α-PMA on basal ecdysteroid level were analyzed by one-way analysis of variance (P=0.21). Results of Tukey multiple comarisons for the effects of PMA on basal ecdysteroid synthesis and release (pairs of means enclosed by the range of a bracket are not significantly different, P>0.05): [7.31–9.35], [9.05–11.03],[14.50–15.70].

DISCUSSION

The results in the present study demonstrate that the steroidogenic effect of *Bombyx* PTTH in PGs is dependent on extracellular Ca^{2+} and the bifurcating second messenger system that generates the two intracellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol.

During a 30 min incubation of day 6 PGs with recombinant PTTH there was a substantial increase of ecdysteroid synthesis and release (P<0.05;Table 1). This stimulation by rPTTH was not mediated through the generation of the second messenger IP_3 since rPTTH did not increase glandular IP_3 content even after 2 hr incubation (Fig. 1, Table 2). Moreover, the increase in ecdysteroid during 30 min incubation (Table 1) was not preceded or accompanied by any rPTTH-mediated increase in the glandular content of the second messenger cAMP, since rPTTH did not increase cAMP content even after 1 hr incubation (Fig. 2). Gu *et al.* (1996), using as source of PTTH medium in which brain complexes of *Bombyx* were incubated, found that PTTH could stimulate cAMP accumulation in the PGs of this insect. However, we observed that this cAMP stimulating activity in *Bombyx* PGs was mediated by another cerebral prothoracicotropic factor different from PTTH (Dedos *et al.*, 1999a). Recombinant PTTH stimulated cAMP accumulation only in day 4 and day 5 *Bombyx* PGs, and did not increase the cAMP content after a 30 min incubation of day 6 PGs (Dedos *et al.*, 1999a). In this study, it was further shown that rPTTH did not stimulate cAMP accumulation in PGs even at incubation periods much shorter than the 30 min used in previous experiments (Fig. 2; Dedos *et al.*, 1999a). Previous research using day 6 *Bombyx* PGs showed that Ca^{2+} and cAMP signaling pathways can cooperatively, as well as independently, stimulate ecdysteroid synthesis and release from the PGs (Dedos and Fugo, 1999b). All these results suggested that there exists a high degree of complexity and stage-specific variability in the signaling cascades that mediate ecdysteroid synthesis and release by the PGs of *Bombyx*. Therefore, in order to explain the signaling cascade of rPTTH-mediated ecdysteroid synthesis and release, it was important to choose, for the present experiments, a developmental stage (day 6, 5th instar) at which the rPTTH-stimulated ecdysteroid synthesis and release does not involve multiple second messenger cascades.

Both basal and rPTTH-stimulated ecdysteroid secretions were found to be affected by the presence of extracellular Ca^{2+} in a dose-dependent manner (Fig. 3). A similar stage-specific dependence of basal ecdysteroid synthesis and release on the presence of extracellular Ca^{2+} was reported for the PGs of *Manduca* (Meller *et al.*, 1990). The rPTTH-stimulated ecdysteroid synthesis was reduced to levels similar to basal release in the absence of Ca^{2+} from the incubation medium as shown in Fig. 3 and also by Gu *et al.* (1998).

These results suggest that a Ca^{2+}-influx pathway into the cytosol of the PG cells may be the primary stimuli in the *Bombyx* PTTH signal transduction cascade. There are several pathways of Ca^{2+} influx activation by receptor agonists through the plasma membrane (Fasolato *et al.*, 1994). Among them, the receptor-operated channels together with the voltage-operated channels provide brief and high intensity bursts

of Ca^{2+} influx into the cytosol, while the store-operated channels provide a much smaller but sustained influx of Ca^{2+} (Berridge, 1997). The existence of receptor-operated Ca^{2+} channels was suggested for the PGs of *Manduca* (Girgenrath and Smith, 1996). These authors suggested that the *Manduca* big PTTH receptor mediates Ca^{2+}-influx through an L-type Ca^{2+} channel based on the ability of the L-type Ca^{2+} channel antagonist, nitrendipine, to inhibit *Manduca* big PTTH-stimulated ecdysteroid synthesis and release (Girgenrath and Smith, 1996). This *Manduca* big PTTH-mediated Ca^{2+} channel was described to be nitrendipine-sensitive and verapamil-insensitive (Girgenrath and Smith, 1996), while *Manduca* small PTTH-stimulated ecdysteroid synthesis was inhibited by verapamil (Hayes *et al*., 1995). In this study, we showed that nitrendipine competitively inhibited only the rPTTH-stimulated ecdysteroid secretion, without affecting basal ecdysteroid synthesis and release in *Bombyx* (Fig. 4). We also suggest that binding of *Bombyx* PTTH to its cellular membrane receptor results in the opening of an L-type Ca^{2+} channel which promotes Ca^{2+} entry into the cytosol of PG cells.

Research on the intracellular Ca^{2+} modulation in the PGs of *Galleria mellonella* and *Manduca* (Birkenbeil, 1996; 1998) revealed that there are differences between insect species in the PTTH-mediated mobilization of intracellular Ca^{2+} in the PGs. For example, it was shown that the PTTH-mediated increase in intracellular Ca^{2+} in the PG cells of *Galleria* was abolished by the removal of extracellular Ca^{2+} or in the presence of the Ca^{2+} channel antagonists nicardipine and verapamil (Birkenbeil, 1996). Similar experiments with the PGs of *Manduca* showed that the PTTH-mediated increase in intracellular Ca^{2+} was not abolished by removal of extracellular Ca^{2+} or in the presence of nicardipine and verapamil (Birkenbeil, 1998). Our results suggest that the PTTH-mediated mobilization of intracellular Ca^{2+} in the PGs of *Bombyx* is different from those of *Galleria* and *Manduca.*

Although rPTTH did not directly stimulate the generation of IP_3 (Fig. 1), its ecdysteroidogenic action was dependent on the generation of this second messenger since the rPTTH-stimulated ecdysteroid secretion was competitively inhibited by NCDC (Table 2 and Fig. 5). These results suggest that the Ca^{2+} that is mobilized by PTTH into the PG cells is sequestered in IP_3-sensitive intracellular Ca^{2+} stores and released in the ecdysteroidogenic process by the action of IP_3. Thus, we believe that the PTTH signalling cascade in the PG cells involves keeping intracellular Ca^{2+} levels elevated during maintained stimulation and speeding the replenishment of intracellular Ca^{2+} stores. By doing so, filled internal Ca^{2+} stores are maintained, and their Ca^{2+} content is ready to be mobilized by IP_3. Because the rPTTH-stimulated ecdysteroid synthesis and release was dependent on external Ca^{2+} (Fig. 3), it appears that an initial PTTH-mediated influx of Ca^{2+} requires the generation of IP_3 (Table 2), and this explains why the NCDC-mediated inhibition of PLC eliminated the rPTTH-stimulated ecdysteroid synthesis and release (Fig. 5). The NCDC-mediated inhibition of PLC may eliminate increases in glandular IP_3 content. This, in turn, would eliminate the IP_3-mediated Ca^{2+} release from intracellular stores which would be the first event in the PTTH-mediated cascade of events that lead to replenishing of intracellular Ca^{2+} stores. Since a similar function in the replenishing of intracellular Ca^{2+} stores has been proposed for store-operated Ca^{2+} channels (Friel, 1996), we believe that if a receptor-operated Ca^{2+} channel is regulated by the *Bombyx* PTTH receptor, then this Ca^{2+} channel is probably in close proximity to IP_3 receptors and it is also regulated by the state of filling of the IP_3-sensitive intracellular Ca^{2+} stores.

Models for conformational coupling mechanisms between Ca^{2+} channels in the plasma membrane and cytosolic Ca^{2+} stores have been suggested to explain receptor-mediated (Rink, 1990; Tsunoda, 1993) and capacitative Ca^{2+} entries (Berridge, 1995; 1997, Parekh, 1997). Capacitative Ca^{2+} entry is the process in which Ca^{2+} enters the cytosol and replenishes intracellular Ca^{2+} stores which are emptied through the action of IP_3 (Friel, 1996). The action of IP_3 is essential for capacitative Ca^{2+} entry through store-operated Ca^{2+} channels (Friel, 1996) in the same way as the action of IP_3 is essential for the PTTH-stimulated ecdysteroid synthesis and release. Moreover, since basal ecdysteroid synthesis and release decreased in the absence of external Ca^{2+} (Fig. 3), it is quite possible that store-operated Ca^{2+} channels exist in the PG cells to facilitate agonist-insensitive Ca^{2+} entry and replenishment of Ca^{2+} stores.

Chelerythrine chloride competitively inhibited the rPTTH-stimulated ecdysteroid synthesis and release (Fig. 6) whereas PMA stimulated basal ecdysteroid synthesis, but only after a 5 hr incubation (Fig. 7). One can assume that the PKC limb of the phosphoinositide signalling pathway is directly involved in the PTTH-evoked Ca^{2+} influx and increase of ecdysteroid synthesis and release. Protein kinase C has disparate and cell type specific effects on store-operated Ca^{2+} influx and voltage-dependent Ca^{2+} channel activity (Shearman *et al*., 1989). Activation of store-operated Ca^{2+} influx by PKC was documented (Tsunoda, 1993; Parekh and Penner, 1997) and this enzyme was found to positively regulate voltage-dependent Ca^{2+} channel activity (Shearman *et al*., 1989). Thus, if a PKC-mediated positive feedback mechanism operates in the PGs, a large rPTTH-stimulated ecdysteroid synthesis and release would not have been expected in the PGs in the presence of PKC inhibitors (Fig. 6). Alternatively, the PKC limb of the phosphoinositide signalling pathway may regulate ecdysteroid synthesis and secretion through an independent pathway. It may also converge with the PTTH-mediated signalling cascade at later stages of the ecdysteroidogenic process for maximal activation of ecdysteroid synthesis and release. Another possibility is that activation of PKC is required for a prolonged and sustained ecdysteroid synthesis and release as suggested for the *Manduca* PGs (Smith, 1993). Such a possibility may explain why a 5 hr incubation was required for PMA to stimulate ecdysteroid synthesis and release by the PGs of *Bombyx* (Fig. 7).

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