Original Article

Role of Calcium Ion and Cyclic Nucleotides in Pheromone Production in *Bombyx mori*

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Injection of cAMP, cGMP and dbcAMP (50, 250 and 500 μg) into decapitated *Bombyx mori* females induced pheromone production in a dose-dependent manner. Among these cyclic nucleotides, cAMP proved to be the most active. Under *in vitro* conditions, however, cyclic nucleotides did not stimulate pheromone production even in a wide range of concentrations (1 μM –10 mM). Likewise, MIX or forskolin, or both (10 μM –1 mM) also failed to stimulate pheromone glands to produce bombykol, whereas, MIX (50 μM) enhanced the action of Bom-PBAN-I and increased bombykol production by 30–60%. In addition, both MIX (100 μM) and forskolin (100 μM) enhanced the action of calcium ionophore in bombykol production by two to three times. The action of PBAN on *in vitro* pheromone production was inhibited in a dose-dependent manner by adding lanthanum ion (100 μM –10 mM) into the medium. Present results provide some evidences for the requirement of calcium ion influx, suggesting the involvement of calcium ion or cAMP, or both in the mode of action of PBAN in the pheromone glands of *B. mori*.

INTRODUCTION

Sex pheromone production in a number of moth species is controlled by pheromone biosynthesis activating neuropeptide (PBAN), which is secreted by the subesophageal ganglion.^{1–5)} The primary structures of PBAN have been reported in two lepidopteran species, *Heliothis zea*^{6–8)} and *Bombyx mori*.^{9,10)}

Recently, a growing number of investigations have been focused on the identification of the target organ of PBAN for a clearer understanding of the mode of action of this hormone. In *Heliothis armigera*, *Bombyx mori* and *Spodoptera litura*, the target organ of PBAN has been demonstrated to be the pheromone gland itself.^{11–14)} Another model has shown that *Heliothis* PBAN stimulates the terminal abdominal ganglion to secrete a neurochemical factor which is responsible for pheromone production in the pheromone gland¹⁵⁾ or direct, neural stimulation induced

by PBAN exists *via* axonal branches innervating the pheromone glands¹⁶⁾ in *H. zea*, *H. virescens* and *H. subflexa*. In addition, reports say that the corpus bursae is the target organ of PBAN in *Argyrotaenia velutinana* and that it produces the “bursa factor” responsible for pheromone production.¹⁷⁾ These findings suggest diverse physiological mechanisms of PBAN action depending on the lepidopteran species.

To date, only a few studies have been conducted on the second messenger system in PBAN action. In *H. armigera*, it has been demonstrated that under *in vitro* conditions, PBAN increased the intracellular level of cAMP in the pheromone gland, resulting in pheromone production.^{11,18)} It has also been revealed for this species that the addition of 8-bromo-cAMP to the incubation media stimulated acetate incorporation into the pheromone.¹¹⁾ This effect was further increased by adding forskolin, a stimulant of

adenylate cyclase¹⁹⁾ and MIX, a potent phosphodiesterase inhibitor,²⁰⁾ into the medium. On the other hand, according to our recent observations on *B. mori*, calcium ionophore mimicked the action of PBAN *in vitro*.¹⁴⁾ Since calcium ionophore is known to stimulate the penetration of divalent cations like the calcium ion,²¹⁾ the stimulating action of calcium ionophore suggests that the influx of calcium ion has a decisive role in the action of PBAN.¹⁴⁾ It has been previously demonstrated that various enzyme activities in diverse systems depend on calcium ion and calmodulin concentrations²²⁾ and that the synthesis or degradation of cyclic nucleotides in insect tissues also depends on calcium/calmodulin.^{23,24)} Therefore, cyclic nucleotides could also be responsible for PBAN induced pheromone synthesis, at rate-limiting step(s), in the pheromone gland of *B. mori*.

The main objective of our present study is to elucidate some further aspects in the second messenger system involved in the mode of action of PBAN in *B. mori* by testing *in vivo* and *in vitro* effects of various cyclic nucleotides as well as some pharmacochemicals such as MIX, forskolin and lanthanum ion, a calcium ion antagonist, which is known to displace specifically the divalent cations from the plasmamembrane.²⁵⁾ Our investigations are based on the previous findings that the target organ of PBAN in *B. mori* is the pheromone gland itself.^{13,14)}

MATERIALS AND METHODS

1. Insects

Bombyx mori (Kinshu & Showa) larvae were reared under a 16L:8D photoperiod at 25°C and fed on artificial diet, as previously described.¹⁴⁾ Newly emerged females were decapitated within 3 hr after moulting and were kept for 24 hr until use.

2. Injection of Cyclic Nucleotides

In *in vivo* cyclic nucleotide dose-dependence experiments, decapitated females of *B. mori* were injected with corresponding doses (50, 250 and 500 μ g) of cAMP, cGMP or dibutyryl cAMP (dbcAMP) (sodium salts; SIGMA) dissolved in 10 μ l of distilled water. The pheromone glands were excised 90–100 min after injection and extracted with 100 μ l *n*-hexane.

A 10 μ l aliquot of the extracts was subjected to pheromone analysis. The amount of bombykol {(10*E*,12*Z*)-10,12-hexadecadien-1-ol} was measured by HPLC as previously reported.¹⁴⁾

3. Incubation of Pheromone Glands with Cyclic Nucleotides

Pheromone glands taken from decapitated females of *B. mori* were damp-dried, cleaned of other tissue remnants and the inside of the glands were carefully washed several times with the respective incubating medium using a microsyringe. The glands were incubated at room temperature, in 150 μ l Grace's medium containing various concentrations of cyclic nucleotides (1 μ M–10 mM) for 90–100 min. After incubation, each gland was taken out from the medium, extracted with 100 μ l *n*-hexane and the amount of bombykol produced in the gland was measured as described above.

4. Incubation of Pheromone Glands with MIX or Forskolin in the Presence of Bom-PBAN-I

Stock solutions of both MIX (3-isobutyl-1-methylxanthine, Sigma) and forskolin (7-*O*-hemisuccinyl-7-deacetyl, Sigma) in DMSO (100 mM) were prepared and diluted in Grace's medium. Dose dependence was studied at different concentrations of MIX or forskolin, or both.

The stimulating effect of either MIX or forskolin, or both was studied in the presence of 0.5 ng or 1 ng synthetic oxidized *Bombyx* PBAN-I²⁶⁾ (*Bom*-PBAN-I)/150 μ l Grace's medium. The glands were incubated and processed as described above.

5. Incubation of Pheromone Glands with MIX or Forskolin in the Presence of Calcium Ionophore

A stock solution (100 mM) of calcium ionophore A23187 (free acid, Sigma) was prepared in DMSO and added to the medium. Calcium ionophore was employed at different concentrations (333, 50 and 10 μ M) in combination with MIX or forskolin. After incubation the glands were processed as above.

6. Incubation of Pheromone Glands with Lanthanum Ion in the Presence of Bom-PBAN-I

The inhibitory action of lanthanum ion, (LaCl_3 , Nacalai) was studied using a series of different doses of PBAN (2, 5 and 10 ng/150 μl Grace's medium) along with a series of various concentrations of lanthanum ion (100 μM –10 mM). Glands were incubated and processed as previously described.

RESULTS

1. Effect of cAMP, cGMP and dbcAMP in Vivo and in Vitro

Injection of cAMP, cGMP or dbcAMP into decapitated female *B. mori* induced bombykol production in a dose-dependent manner (Fig. 1). Among the tested cyclic nucleotides cAMP was found to be the most active. In *in vitro*, however, when the pheromone glands were incubated with these cyclic nucleotides in a wide range of concentrations (1 μM –10 mM), they failed to induce bombykol production (data are not shown).

2. Effect of MIX or Forskolin in the Presence of Bom-PBAN-I in Vitro

As a preliminary experiment, pheromone

Fig. 1 *In vivo* effects of cyclic nucleotides on pheromone production in *B. mori*.

Solid columns represent cAMP, striped ones cGMP, and dotted ones dbcAMP treatments. Different doses of cyclic nucleotides were injected into females, while only distilled water into controls. The columns indicate a mean of minimum 9 measurements of independent gland extracts. Thin bars represent standard deviation.

Fig. 2 *In vitro* effect of MIX or forskolin or both in the presence of Bom-PBAN-I on pheromone production in *B. mori*.

Abbreviations used on figures: F, forskolin; MIX, 3-isobutyl-1-methylxanthine and P, Bom-PBAN-I.

(a) Effect of MIX in the presence of 0.5 or 1 ng Bom-PBAN-I

Control: 100 μM MIX without P; PBAN cont.: 0.5 ng P/vial (black column); 1 ng P/vial (striped column); P+10 μM , P+50 μM , P+100 μM and P+1 mM contain 0.5 or 1 ng PBAN+respective concentrations of MIX indicated. The columns indicate a mean of minimum 9 measurements of independent glands. Thin bars represent standard deviation. Result of P+50 μM MIX significantly differ from that of PBAN cont. at $p < 0.05$ level in Student's *t* test.

(b) Effect of forskolin in the presence of 0.5 ng Bom-PBAN-I

Control: glands in Grace's medium; F control: 100 μM forskolin without P; PBAN cont.: 0.5 ng P/vial; P+50 μM and P+100 μM contain 0.5 ng PBAN+respective concentrations of forskolin. P+MIX+F contains 0.5 ng PBAN+50 μM of both MIX and F. Result of P+MIX+F significantly differs from that of PBAN control at $p < 0.05$ level in Student's *t* test. Otherwise refer to Fig. 2 (a).

glands were incubated either with MIX or forskolin ($10\ \mu\text{M}$ – $1\ \text{mM}$) or both, but pheromone production was not induced (Fig. 2(a) and (b); control and F control).

Incubation of the pheromone glands with $0.5\ \text{ng PBAN}/150\ \mu\text{l}$ medium resulted in $102 \pm 35\ \text{ng}$ of bombykol production (at $10\ \text{ng}$ pheromone production *in vitro* was maximum with $430 \pm 165\ \text{ng/gland}^{14}$). The effect of PBAN on bombykol production was enhanced by addition of MIX to the incubation medium at concentrations between 10 and $100\ \mu\text{M}$. In this range, $50\ \mu\text{M}$ MIX increased bombykol production by 60% (Fig. 2(a)). An addition of $50\ \mu\text{M}$ MIX to the incubation medium containing $1\ \text{ng PBAN}$ increased pheromone production by about 30% (Fig. 2 (a)). In con-

trast, there was no enhancement of bombykol production when forskolin (50 or $100\ \mu\text{M}$) was added to the medium containing $0.5\ \text{ng PBAN}/150\ \mu\text{l}$ (Fig. 2(b)). When both MIX and forskolin ($50\ \mu\text{M}$) were added to the medium, the result was similar to the one when only MIX was employed (Fig. 2(a) and (b)).

3. Effect of MIX or Forskolin in the Presence of Calcium Ionophore *in Vitro*

A twofold elevation was recorded when MIX ($100\ \mu\text{M}$) was added to media containing $50\ \mu\text{M}$ calcium ionophore (Fig. 3(a)).

In a previous study, calcium ionophore stimulated the pheromone gland to produce bombykol *in vitro* and the effect was optimal at a concentration of $333\ \mu\text{M}$.¹⁴ In the present

(a) Effect of MIX in the presence of $50\ \mu\text{M}$ calcium ionophore

Control: glands in Grace's medium; Io cont.: calcium ionophore control at $50\ \mu\text{M}$ concentration; Io+ $10\ \mu\text{M}$, Io+ $100\ \mu\text{M}$, Io+ $1\ \text{mM}$: $50\ \mu\text{M}$ calcium ionophore+respective concentrations of MIX indicated. Otherwise refer to Fig. 2. Result of Io $50+100\ \mu\text{M}$ treatments significantly differs from that of control at $p < 0.01$ level in Student's *t* test.

(b) Effect of forskolin in the presence of calcium ionophore

Control: glands in Grace's medium; Io $333\ \mu\text{M}$, Io $50\ \mu\text{M}$ and Io $10\ \mu\text{M}$: calcium ionophore controls at 333 , 50 or $10\ \mu\text{M}$ concentration; Io $333+10\ \mu\text{M}$, Io $333+100\ \mu\text{M}$, Io $333+1\ \text{mM}$, Io $50+100\ \mu\text{M}$ and Io $10+100\ \mu\text{M}$ contain 333 , 50 or $10\ \mu\text{M}$ calcium ionophore+respective concentrations of forskolin indicated. Otherwise refer to Fig. 2. Results of Io $50+100\ \mu\text{M}$ and Io $10+100\ \mu\text{M}$ treatments significantly differ from those of controls at $p < 0.01$ level in Student's *t* test.

Fig. 3 *In vitro* effect of MIX or forskolin in the presence of calcium ionophore on pheromone production in *B. mori*.

study, forskolin was added in different amounts to the above medium. When 100 μM was added only a slight increase (20%) in bombykol production was obtained (Fig. 3(b)). The stimulative effect, however, was much more profound when calcium ionophore was employed at lower concentrations. With 50 μM ionophore, an addition of 100 μM forskolin gave a twofold increase (Fig. 3(b)). A medium containing 100 μM forskolin and only 10 μM calcium ionophore increased pheromone production more than threefold (Fig. 3(b)).

4. Effect of Lanthanum Ion in the Presence of Bom-PBAN-I in Vitro

The inhibitory action of lanthanum ion was studied with different doses of PBAN along with a series of different lanthanum ion concentrations. The most profound, total inhibitory effect was achieved with a relatively low PBAN dose (2 ng/vial); 100 μM lanthanum ion resulted in 50% inhibition (Fig. 4). With higher doses of PBAN (5 ng/vial or 10 ng/vial) a concentration of 1 or 5 mM lanthanum ion, respectively, was necessary to reach 50% inhibition in pheromone production; 10 mM

lanthanum ion induced about 75% inhibition with both doses (Fig. 4).

DISCUSSION

A series of *in vivo* and *in vitro* experiments was carried out to study the possible involvement of cyclic nucleotides and calcium (or both) in the mode of action of PBAN.

Injection of different cyclic nucleotides into decapitated *B. mori* females revealed dose-dependent action in pheromone production. Cyclic AMP proved to be the strongest stimulant and these observations suggest that cAMP may play a role as a second messenger.

The *in vivo* cyclic nucleotide tests, however, may serve only as guidelines, since direct action in *B. mori* has not yet been proved. The cAMP may act on the whole body indirectly and in a more complex way, or externally, as a hormone does, as was shown by Devroetes.²⁷⁾ Our *in vitro* investigations did not confirm the expected cAMP involvement in PBAN action. The negative results obtained with various nucleotides, MIX and forskolin may be due to poor penetration into the target cells,²⁹⁾ insufficient direct stimulation of *B. mori* pheromone glands under our experimental conditions, inadequate informations from the measurement of pheromone production in which the chemicals are involved as second messenger, *i.e.* the stimulation of cAMP content may have been insufficient to initialize measurable bombykol production or some other unknown factors. It must be noted that in *Manduca sexta*, the pupal prothoracic glands could not be stimulated by forskolin to produce ecdysone, although it has been proved that prothoracicotrophic hormone (PTTH) induces cAMP formation and functions as second messenger for ecdysone production.²⁹⁾ From these results it was concluded that the prothoracic glands apparently have a low level of forskolin-stimulatable adenylate cyclase activity and/or a high level of phosphodiesterase activity (resulting in the quick degradation of cAMP if present). On the other hand, in *Locusta migratoria*, in which cAMP had also been proved to be the second messenger of the antidiuretic hormone, MIX alone did not affect rectal cAMP levels and was ineffective on

Fig. 4 *In vitro* inhibitory action of lanthanum ion in the presence of 2, 5, 10 ng Bom-PBAN-I on pheromone production in *B. mori*.

Solid columns represent 2 ng PBAN, striped ones 5 ng PBAN, and dotted ones 10 ng PBAN/vial. On horizontal axis respective concentrations of lanthanum ion are indicated. Thin bars represent standard deviation.

rectal water reabsorption, whereas forskolin was found to induce noticeable response.³⁰⁾

Remarkable potentiations were observed, however, when MIX was added to medium containing PBAN or when MIX or forskolin was applied in the presence of calcium ionophore. These results suggest that without initial stimulation or triggering of pheromone production, no enhancement could be achieved in *B. mori*. On the other hand, when low pheromone production is initiated (either directly by PBAN or indirectly by calcium ionophore), elevated pheromone production can be measured in the presence of MIX or forskolin.

As far as the stimulation of glands are concerned, there may be differences between species as well. In *H. armigera*, both MIX and forskolin induced a significant increase in cAMP levels in glands and stimulated ¹⁴C-acetate incorporation into the pheromone glands and the major pheromone component.¹¹⁾ When the glands of *S. litura* were incubated *in vitro* with MIX (1 mM) pheromone production increased twofold, while forskolin had no effect (Fónagy, unpublished observations). Regarding the present experiments, the reason still remains unclear as to why forskolin did not exert further effect when incubated with PBAN but affected pheromone production in a positive way when combined with calcium ionophore. A possible interpretation may be that a more precisely coordinated (hormone-second messenger-enzyme activation) system is switched on following PBAN action and a less specific (or a different one?), during only calcium ionophore incubation. The demonstrated stimulating effect of MIX in both cases, however, provides an overall indication for a possible cAMP involvement in the mode of action of PBAN. Actually, similar enhanced effect was observed in pupal *M. sexta* prothoracic glands under *in vitro* conditions where PTTH (0.1 Unit) and MIX (50 μ M) together induced cAMP accumulation, whereas separately these were relatively ineffective.²⁹⁾

Nevertheless, it may be concluded from the present study that different drugs, known to increase intracellular cAMP level, exerted positive/stimulating effect on *in vitro* pheromone production in the presence of PBAN or

calcium ionophore. Further studies are in progress regarding the more precise quantification of the described potentiation effects. Direct measurement (on a time-course basis) of cAMP is required in the same system, since the increased pheromone production observed may be regarded as indirect proof. Moreover, the measurement of cAMP may throw a light on the negative results obtained following only MIX and forskolin treatments.

In the course of study on the mode of action of PBAN and the secondary messenger system involved, we have unequivocal evidence that the hormone requires extracellular calcium and/or calcium influx to initiate *de novo* pheromone production (Fig. 4). A stimulatory role of calcium ion has been already proved for PBAN action in the pheromone glands of *B. mori*, because calcium ionophore mimicked pheromonotropic effect of PBAN.¹⁴⁾ Based on our findings, we suggest that calcium ion has a decisive function instead of a simply permissive role. The positive correlation found between the amount of PBAN and lanthanum ion employed clearly shows relations between PBAN receptor sites and their calcium dependence due to the fact that they can be dose dependently antagonized. Similar dose-dependent antagonizing effect of lanthanum ion has been found in *M. sexta* prothoracic glands incubated with PTTH.³¹⁾

Our experiments have provided some pieces of evidence regarding the sequence of events which take place following PBAN action, like necessary influx of calcium ion, and suggested the involvement of cAMP as second messenger. Further detailed studies are, however, required in this field, since the different physiological mechanisms involved in PBAN action in lepidopterans indicate diversity,¹⁴⁻¹⁷⁾ and the mediation of hormonal effect seems to be more complex than it was originally envisioned.

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要 約

カイコにおける性フェロモンの生産に対する環状ヌクレオチドの影響

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断頭したカイコの雌蛾に cAMP, cGMP, ジブチリル cAMP (50, 250, 500 μ g) を注射すると濃度依存的に性フェロモンであるボンビコールの生産が誘起され, その中でも, cAMP が最も効果があった。しかし, これらのヌクレオチドを含む培地 (1 μ M ~ 10 mM) で PBAN (フェロモン生合成活性化神経ペプチド) の標的器官であるフェロモン腺を培養してもボンビコールの生産は促されなかった。一方, 同じ *in vitro* 条件下で, 環状ヌクレオチドホスホジエステラーゼの阻害剤である MIX (50 μ M) は PBAN によるボンビコールの生産を 30~60% 高め, MIX (100 μ M) あるいは, アデニレートシクラーゼの賦活化剤である forskolin (100 μ M) はカルシウムイオノフォアによるボンビコールの生産を 2~3 倍増加させた。さらに, カルシウムイオンの細胞内への流入を阻止するランタニウムイオン (100 μ M ~ 10 mM) は濃度依存的に PBAN によるボンビコール生産を抑制した。これらの結果から, カイコのボンビコール生産にはフェロモン腺におけるカルシウムイオンの細胞内への流入が重要な役割をなすとともに, cAMP も何らかの役割を担っていることが示唆された。