Many species of female moths produce sex pheromones to attract conspecific males. Most moth species utilize Type I pheromones that consist of straight-chain compounds 10–18 carbons in length with a functional group of a primary alcohol, aldehyde, or acetate ester, and usually with several double bonds. Studies over the past three decades have demonstrated that female moths usually produce sex pheromones as multi-component blends in which the ratio of the individual components is precisely controlled, making it possible to generate species-specific pheromone blends. As for the biosynthesis of Type I pheromones, it is well established that they are de novo synthesized in the pheromone gland (PG) through modifications of fatty acid biosynthetic pathways. However, because many of the molecular components within the PG cells (enzymes, proteins, and small regulatory molecules) had not been functionally characterized, the molecular mechanisms underlying sex pheromone production in PG cells remained poorly understood. To address this, we have characterized some of the key molecules involved in the biosynthesis of the sex pheromone bombykol in the silkmoth, Bombyx mori. Characterization of these molecules has facilitated our understanding of the precise mechanisms underlying lepidopteran sex pheromone production.

Key words: pheromone biosynthesis; pheromone biosynthesis-activating neuropeptide (PBAN) receptor; lipid droplet; store-operated channel; RNA interference

The Insecta, which comprise more than 78% of all animal species, are of great economical and ecological importance. In addition, over 400 million years of evolutionary adaptation, insects have developed diverse biological systems that have been separately selected from those of mammals. Nevertheless, despite large anatomical and physiological differences, there has been remarkable conservation in many functional molecules and mechanisms between mammals and insects. Consequently, basic science associated with insects has greatly contributed to the overall advancement of the biological sciences, as evidenced by the abundant contributions that the fruit fly, Drosophila melanogaster, has made to genetics. By considering and using the advantages exclusive to insects, at our laboratory we have directed our efforts towards elucidating the underlying mechanisms of fundamental biological events exhibited by insects by focusing on the two principal model insects, viz., the silkworm, Bombyx mori, and the fruit fly, D. melanogaster. In this review, I want to highlight our efforts to understand the molecular mechanisms underlying sex pheromone production in moths. In this research endeavor, we have successfully identified and characterized several of the key molecular components in bombykol biosynthesis of B. mori. We feel that our findings are of prime significance because characterization of the key molecules responsible for lepidopteran sex pheromone biosynthesis can significantly contribute to our understanding of the ways a vast number of the species-specific pheromone components are produced. Furthermore, apart from insect physiology, our understanding of the cellular events underlying sex pheromone production in moths also has the potential to provide insight into a number of significant, but unsolved topics relevant to modern cell biology related to lipid uptake, lipogenesis, hormone-regulated lipolysis, membrane trafficking associated with lipid transport/release, intracellular calcium signaling, etc. By characterizing these unsolved mechanisms in pheromone gland cells, we aim to provide a basic, novel understanding of the fundamental aspects of lipid and cellular biology, knowledge that can then be used to elucidate similar mechanisms in organisms of different phyla.

I. Sex Pheromones in Moths

The biological term “pheromone” originates in the insect sciences and was coined by Karlson and Luscher in 1959 when the first pheromone was chemically identified by Adolf Butenandt et al. of the Max-Planck Institute. The first pheromone identified was the sex pheromone bombykol of the silkmoth, B. mori.
To date, sex pheromones from more than 530 moth species have been chemically identified.\(^5\) Most moth species utilize Type I pheromone components that consist of straight-chain compounds 10–18 carbons in length with an oxygenated functional group of a primary alcohol, aldehyde, or acetate ester, and usually with several double bonds.\(^5\) Small numbers of moth species use Type II pheromone components (unsaturated hydrocarbons or hydrocarbon epoxides) as sex pheromones.\(^7\) Studies over the past three decades have demonstrated that female moths usually produce sex pheromones as multi-component blends in which the ratio of the individual components is precisely controlled, making it possible to generate species-specific pheromone blends.\(^5,6\) In addition, a general scheme for the biosynthetic pathway of Type I pheromone components has become apparent, in which the components are synthesized de novo in the pheromone gland (PG) through modifications of fatty acid biosynthetic pathways.\(^5\) The resulting fatty acid intermediates, palmitic and stearic acids, are converted stepwise to the final fatty alcohol, aldehyde, or acetate ester, and usually with several double bonds.\(^5\) Small numbers of moth species use Type II pheromone components (unsaturated hydrocarbons or hydrocarbon epoxides) as sex pheromones.\(^7\) Studies over the past three decades have demonstrated that female moths usually produce sex pheromones as multi-component blends in which the ratio of the individual components is precisely controlled, making it possible to generate species-specific pheromone blends.\(^5,6\) In addition, a general scheme for the biosynthetic pathway of Type I pheromone components has become apparent, in which the components are synthesized de novo in the pheromone gland (PG) through modifications of fatty acid biosynthetic pathways.\(^5\) The resulting fatty acid intermediates, palmitic and stearic acids, are converted stepwise to the final fatty alcohol, aldehyde, or acetate ester, and usually with several double bonds.\(^5\) Small numbers of moth species use Type II pheromone components (unsaturated hydrocarbons or hydrocarbon epoxides) as sex pheromones.\(^7\) Studies over the past three decades have demonstrated that female moths usually produce sex pheromones as multi-component blends in which the ratio of the individual components is precisely controlled, making it possible to generate species-specific pheromone blends.

II. Pheromonogenesis in *B. mori* PG Cells

The sex pheromone bombykol, \((E,Z)-10,12\)-hexadecadien-1-ol, is released by adult female moths immediately after eclosion in the early morning and throughout the remainder of the day. As with many other moth species, sex pheromone production in *B. mori* is regulated by a neurohormone termed pheromone biosynthesis-activating neuropeptide (PBAN).\(^{11–14}\) PBAN originates in the subesophageal ganglion and is released into the hemolymph immediately after eclosion to act directly on the PG, and it stimulates bombykol biosynthesis.\(^{1,15,16}\) The PG of *B. mori* is a functionally differentiated organ that originates from the intersegmental membrane between the eighth and ninth abdominal segments and is distinguishable as a pair of eversible, ventrolateral sacs (sacculi laterales).\(^{17,18}\) We found that bombykol-producing cells, which can be discerned visually by the presence of cytoplasmic lipid droplets (see below), are homogeneous single-layered epidermal cells consisting of about 9,000 cells that are in direct contact with the overlying cuticular surface (Fig. 2A).

We found that numerous cellular events culminating in pheromone production take place in concert before and after eclosion. We named this dynamic process “pheromonogenesis.” Pheromonogenesis can be defined as the dynamic process in which all the machinery required for sex pheromone biosynthesis is generated and organized within the PG cells of the female moth before and after eclosion or PBAN release.\(^3\) In *B. mori*, bombykol-producing cells before eclosion are characterized by an abundance of lipid droplets (LDs) within the cytoplasm; these LDs begin to form 1–2 d prior to eclosion, and they accumulate rapidly on the day of eclosion (Fig. 2B). After eclosion, in contrast, the density of the LDs decreases in response to PBAN stimulation.\(^{17,18}\)

III. Role of Cytoplasmic Lipid Droplets in PG Cells

Staining the cytoplasmic LDs with the fluorescent lipid marker Nile Red made it possible to monitor their
progression throughout pheromonogenesis (Fig. 2B).

After eclosion, the density of the LDs decreases in the course of the day in accordance with female calling behavior, the period when female moths are actively releasing bombykol. The LDs then re-accumulate during the night when the females are inactive. Post-eclosion decrease in the LD density can be prevented by decapitation (inhibition of PBAN release) and re-stimulated with a pheromonotropic stimulus (PBAN injection after decapitation), an indication that the LDs function to store bombykol precursors that are then released upon PBAN stimulation.17) We confirmed this hypothesis by analyzing the chemical composition of the LD contents. HPLC separation of the LD contents and mass-spectrometric structure analyses (FAB-MS and MS-MS) confirmed that they are various triacylglycerols (TAGs) with the bombykol precursor, \( \text{C}_{10,12}\)-hexadecadienoate, predominantly sequestered as a major component at the \( sn-1 \) and/or \( sn-3 \) position of the glycerides.19) These results indicate that the LDs do indeed play a role in storing the bombykol precursor in the form of TAGs and in releasing it for bombykol production in response to PBAN stimulation. It has been found in \( B. \ mori \) that PBAN stimulates fatty acyl reduction, the final step in bombykol biosynthesis.20) Taken together, these results indicate that PBAN effectively stimulates bombykol production by coincidentally activating the two steps essential to bombykol biosynthesis, lipolysis of lipid droplet TAGs (release of stored \( \text{C}_{10,12}\)-hexadecadienoate) and fatty acyl reduction (conversion to \( (E,Z)\)-10,12-hexadecadien-1-ol).

As explained below, knowledge of the roles of cytoplasmic LDs has made a great contribution to understanding of the molecular mechanisms underlying sex pheromone production in \( B. \ mori \).
IV. Identification and Characterization of the Key Molecules Responsible for Pheromone Production

To identify the functional proteins in pheromoneogenesis, we generated a PG expressed-sequence tag (EST) database by constructing a normalized PG cDNA library prepared from newly emerged female moths of an inbred *B. mori* strain p50.10 Expression analyses of EST clones using various tissues and PGs isolated during different developmental stages revealed that a dozen genes were specifically expressed in the PG and up-regulated during pheromoneogenesis. Furthermore, using the public *B. mori* EST databases SilkBase and CYBERGATE, (http://www.ab.a.u-tokyo.ac.jp/silkbase/, http://150.26.71.213/cgi-bin/main_MX), we also screened for PG-specific genes. There were about 11,000 independent clones in SilkBase, and we found 312 clones expressed in the PG. For these clones, we performed expression analyses and then RNA interference (RNAi) screening to identify the genes responsible for bombykol production. We identified a number of intriguing PG-specific and selective genes. In the following sections, I describe several key molecules that we characterized successfully.

1. Bombykol biosynthesis enzymes

Bombykol is synthesized *de novo* in the PG cells through fatty acid biosynthesis and palmitate is converted to bombykol through two desaturation steps and fatty acyl reduction.10 Bombykol biosynthesis (Fig. 1) is a rather simple process that differs from those of many other Type I pheromones in that it does not require chain-shortening or further modification of the terminal hydroxyl group. The first desaturation step appears to be a general step frequently seen in the pheromone biosynthetic pathways of numerous moth species that is presumably catalyzed by a Z11 desaturase.22 In contrast, the second desaturation step is less common in that it generates a conjugated diene system through 1,4-elimination of two hydrogen atoms at the allylic positions of the double bond in the Z11-monoene C16 intermediate. Similar 1,4-desaturation reactions involving monoene acyl precursors have been observed only in a handful of sex pheromone biosynthetic pathways. In experiments designed to characterize the acyl-CoA desaturases responsible for bombykol biosynthesis, we cloned all of the desaturase genes expressed in the *B. mori* PG using a degenerate RT-PCR-based strategy, and functionally expressed them in *S. melonii* cells using a baculovirus expression system.23 The results indicated that a single desaturase, initially referred to as Desat1 but since renamed for clarification as Desat1,24 produced both the Z11 monoene and the Δ10,12 dienes, confirming that this enzyme is the only desaturase necessary for catalyzing the two consecutive desaturation steps in bombykol biosynthesis (Fig. 1). Phylogenetic analysis suggested that this unique bifunctional desaturase diverged from other lepidopteran Δ11 desaturases. Following our publication, desaturases similarly exhibiting dual- or tri-catalytic activity were identified in *Spodoptera littoralis*, *Manduca sexta*, and *Thaumetopoea pityocampa*, suggesting that these multi-functional Δ11 desaturases are likely to represent a new sub-family of lepidopteran desaturases.25-27

In the biosynthesis of Type I pheromones, the key enzyme required for the production of the oxygenated functional groups is a fatty-acyl reductase (FAR) that converts fatty-acyl pheromone precursors to the corresponding alcohols. Depending on the moth species, these alcohols can be acetylated by an acetyl-CoA: fatty alcohol transferase or oxidized to the corresponding aldehydes by an alcohol oxidase. Despite the significant role of FAR in generating the diverse species-specific oxygenated constituents of lepidopteran sex pheromones, this enzyme has yet to be identified and characterized from this large taxonomic group. Furthermore, this type of long-chain fatty-acyl reductase had not been characterized in either vertebrates or invertebrates. Consequently, the quality of our *B. mori* PG EST database proved to be crucial in our efforts to identify and characterize the gene encoding the PG-specific FAR that converts the bombykol precursor fatty acid to its corresponding alcohol. In support of earlier biochemical characterizations,28 this FAR (designated *B. mori* pgFAR) contained the consensus N-terminal NAD(P)/H binding motif observed in other reductases.29 Functional expression in *Saccharomyces cerevisiae* confirmed that *B. mori* pgFAR is an alcohol-generating long-chain FAR, and that *B. mori* pgFAR exhibited a strong substrate specificity for the bombykol precursor fatty acid, (E,Z)-10,12-hexadecadienoic acid. This was an especially surprising finding because reduction of pheromone precursor fatty acids to their corresponding alcohols had been found to be a common step in the biosynthesis of oxygenated sex-pheromone components regardless of the moth species. Although the published pgFAR genes currently in the literature are limited to those of *B. mori* and *Ostrinia scapulalis*,30 our finding implies that pgFARs from different moth species constitute a family of pgFARs of varying substrate specificities. Following our publication, two mammalian FAR isoforms responsible for the synthesis of wax monoesters and ether lipids were identified.31

2. PBAN receptor (PBANR)

Since the discovery by Raina and Klun in 1984 that the sex pheromone biosynthetic machinery of lepidopteran PG cells is triggered by PBAN,31 identification of the cell surface receptor responsible for transmitting the extracellular PBAN stimulus proved elusive. The keys to unraveling the molecular identity of the PBAN receptor (PBANR) were the publication of the *Drosophila* genome sequence in 2000 and the sequence similarities found in the biologically essential C-terminal pentapeptide motifs present in the mammalian peptide neuromedin U (FRPNamide) and PBAN (FSPRlamide). In 2002, based on the assumption that the sequence similarity extended to the receptor as well, Michael Adams’ group at UC Riverside expressed *Drosophila* neuromedin U receptor (NMUR) homologs in *Xenopus* oocytes and indicated that they could be activated by FSPRlamide peptides, including PBAN.32 Consequently, using a homology-based molecular approach, full-length clones encoding the PBANR were independently amplified from the PGs of *Helicoverpa zeae* and *B. mori*.33,34 In this approach, we used degen-
erate primers designed from Drosophila and Anopheles gene sequences exhibiting significant homology with NMURs to clone the B. mori PBANR gene.

The B. mori PBANR gene encodes a 413 amino acid rhodopsin-like G protein-coupled receptor (GPCR) that is predominantly expressed in the PG and is up-regulated on the day preceding adult eclosion. When transiently expressed in insect SF9 cells, this gene mobilized extracellular Ca transiently. When tagged with enhanced green fluorescent protein (GFP), it localized to the cell surface, where it bound a Rhodamine Red-labeled PBAN. This binding was PBAN-specific, because it was inhibited by the addition of an excess amount of unlabeled PBAN but not by unrelated peptides such as B. mori adipokinetic hormone. The B. mori PBANR exhibits 88% sequence similarity with the Heliconypera zea PBANR, but only 53% sequence similarity with known NMURs. A striking difference between the two insect PBANRs is the presence of a 67 amino acid C-terminal extension in the B. mori PBANR that contains a motif essential for receptor internalization, suggesting that the B. mori and H. zea PBANRs are regulated by different mechanisms. PBANR homologs have since been identified in the diamondback moth (Platella xylostella), the Egyptian cotton leafworm (Spodoptera littoralis), the cotton bollworm (Helicoverpa armigera), and the owlet moth (Heliothis virescens), which contains two PBANR isoforms, a short form (342 residues) and a long form (476 residues) that contains the putative internalization motif identified in the B. mori PBANR.

3. Functional relevance of the molecules identified in the PG

We identified and characterized three distinct genes encoding proteins that play key roles in bombykol production (Bmpgdesat1, pgFAR, and PBANR). While the expression of these genes in heterologous systems clearly demonstrated their functionalities, the functional relevance of these genes in the PG itself remained to be determined. This was especially true for PBANR, since heterologous expression could indicate only whether the receptor was activated by PBAN but, given the pleiotropic activities attributed to the essential FXPRLamide sequence, could give no indication as to its true in vivo function.

The gene knockdown effects of RNAi currently offer the best opportunity to resolve this ambiguity and to make possible unequivocal assignment of gene function. While RNAi has been extensively utilized as a means of gene knockdown in diptersans, it has been demonstrated only in a limited number of lepidopteran species. Despite this limitation, we were able to apply RNAi methodologies to determine the in vivo biological relevance of the genes thought to play key roles in the B. mori sex pheromone biosynthetic pathway. Injecting “white” pupae (pupae I d removed from the larval-pupal molt) with dsRNAs for Bmpgdesat1, pgFAR, and PBANR resulted in a dose-dependent knockdown of the corresponding gene transcripts as well as a reduction in bombykol production. Although knockdown efficacy varied among the dsRNAs injected, the reduction of bombykol production to basal levels with Bmpgdesat1 and pgFAR dsRNAs confirmed that

these enzymes were indeed essential to bombykol biosynthesis. As described above, cytoplasmic LDs accumulate just before eclosion and decrease in size and number in response to PBAN. Examination of the cytoplasmic LDs revealed that knockdown of PBANR expression had no effect on the degree of TAG incorporation into the droplets before eclosion but did prevent PBAN-induced lipolysis of the LDs, indicating that the PBANR gene product is functionally relevant in vivo and is the GPCR that mediates the external signal of PBAN. These results further confirmed that the PBAN signal stimulates liberation of the bombykol precursor from LDs.

V. Other Functional Molecules Involved in Pheromone Production

1. Fatty acid transport protein (FATP)

FATPs belong to an evolutionarily conserved family of membrane-bound proteins that facilitate the uptake of extracellular long-chain fatty acids (LCFAs) and/or very long-chain fatty acids across the plasma membrane and catalyze the ATP-dependent esterification of these fatty acids to their corresponding acyl-CoA derivatives. While the presence of FATP homologs in insects has been demonstrated, their functional roles and the molecular mechanisms underlying LCFA transport across the plasma membrane remain to be characterized.

During the course of our search for functional molecules involved in B. mori pheromonogenesis, we found a gene that encodes a FATP homolog of B. mori (BmFATP) predominantly expressed during the adult stage in the PG and undergoes remarkable up-regulation 1 d prior to adult emergence. Furthermore, RNAi treatment for the BmFATP gene in vivo significantly suppressed bombykol production.

As mentioned above, B. mori PG cells accumulate cytoplasmic LDs prior to eclosion. These LDs play a role in storing the pheromone (bombykol) precursor fatty acid in the form of TAG. We have confirmed that fatty acyls sequestered in the TAGs are restricted to five long-chain fatty acyls: two unsaturated C16 fatty acyls (Δ11-hexadecenoate and Δ10,12-hexadecadienoate) and three conventional C18 fatty acyls (oleate, linoleate, and linolenate) with the bombykol precursor, Δ10,12-hexadecadienoate, as a major component. The bombykol biosynthesis precursors, Δ11-hexadecenoate and Δ10,12-hexadecadienoate, are synthesized de novo in PG cells. In contrast, since linoleic and linolenic acids are essential fatty acids also in insects, the conventional C18 fatty acyls are basically derived from dietary fatty acids and must be taken up across the plasma membrane. Because RNAi-mediated gene silencing of BmFATP in vivo significantly suppressed LD accumulation by preventing TAG synthesis, it is obvious that BmFATP plays an essential role in LD accumulation prior to eclosion. Furthermore, in conjunction with the findings that BmFATP stimulates the uptake of extracellular LCFAs and BmFATP knock-down reduces cellular long-chain acyl-CoA synthetase activity, our results indicate that BmFATP plays an essential role in pheromonogenesis by stimulating the TAG synthesis required for LD accumulation via a process similar to the so-called vectorial acylation that
is known to couple the uptake of extracellular fatty acids with activation to CoA thioesters.

2. Acyl-CoA binding proteins (ACBPs)

ACBP is a highly conserved 10-kDa N-acetylated polypeptide that is expressed in a wide variety of species ranging from yeast to mammals, including insects.\textsuperscript{30} We found that two ACBPs, pgACBP and mgACBP, are specifically or selectively expressed in the \textit{B. mori} PG during pheromoneogenesis and undergo up-regulation 1 d prior to adult emergence, although mgACBP is highly expressed also in the midgut during the larval feeding stages.\textsuperscript{51}

Because ACBPs are known to bind specifically straight-chain (C\textsubscript{14}–C\textsubscript{22}) fatty acyl-CoAs esters with high affinity and to protect them from hydrolysis,\textsuperscript{52–55} it was speculated that they function as carriers or cellular deposits of the acyl-CoAs utilized in pheromone biosynthesis. The use of dsRNAs to knock down the expression of pgACBP and mgACBP demonstrated that this was indeed the case, since loss of either the pgACBP or the mgACBP function prevented TAG accumulation within the cytoplasmic LDs, and consequently the availability of the bombykol precursors.\textsuperscript{24} In \textit{Manduca sexta}, ACBP expression is highest during periods of active feeding and lipid transport from the midgut.\textsuperscript{56} The high abundance of mgACBP transcripts in the midgut of larval \textit{B. mori} suggests that the role of mgACBPs in pheromone biosynthesis is secondary to a dietary role, which indicates that mgACBP donates conventional C\textsubscript{18} fatty acyl-CoAs derived from dietary lipids whereas pgACBP specifically donates de novo synthesized pheromone precursor fatty acyl-CoAs. As mentioned above, because BmFATP facilitates both of the uptake of extracellular C\textsubscript{18} fatty acids and conversion to their corresponding C\textsubscript{18} fatty acyl-CoAs via vescicular acylation, our results further imply essential but distinct roles of BmFATP, pgACBP, and mgACBP in the formation of the key organelles of cytoplasmic LDs, which store and release the bombykol precursor fatty acid during pheromoneogenesis.

In conclusion, effective use of an RNAi-mediated loss-of-function approach that we established for \textit{B. mori} not only provides unambiguous evidence regarding the \textit{in vivo} functional relevance of genes previously considered to be essential to the pathway in bombykol biosynthesis, but also indicates the potential of this methodology to dissect the molecular interactions that constitute biosynthetic pathways.\textsuperscript{24}

3. Transcription activating factor for pgACBP

We found that both of the ACBPs are expressed simultaneously in the PG and are up-regulated on the day preceding eclosion.\textsuperscript{51} Based on these observations, we hypothesized that there are physiological cues that trigger transcription and thus regulate ACBP expression in the PG. By monitoring pgACBP transcription, we found that it was regulated by a factor that appears in the pupal hemolymph 1 d prior to eclosion.\textsuperscript{57} Following purification and structure elucidation by high-resolution electrospray-ionization mass spectrometry (HR-ESIMS) and NMR in conjunction with stereochemical analyses using acid hydrolysates, the humoral factor was identified as \textit{\beta}-\textit{d}-glucosyl-\textit{O}-\textit{L}-tyrosine. Examination of the hemolymph titers during development revealed that the amount of \textit{\beta}-\textit{d}-glucosyl-\textit{O}-\textit{L}-tyrosine rose dramatically prior to eclosion and reached a maximum of 5 mg/ml (about 1 mg/pupa) on the day preceding eclosion, which is consistent with the effective dose of \textit{\beta}-\textit{d}-glucosyl-\textit{O}-\textit{L}-tyrosine in stimulating pgACBP transcription \textit{in vivo}. Furthermore, \textit{in vitro} assays using trimmed PGs indicated that \textit{\beta}-\textit{d}-glucosyl-\textit{O}-\textit{L}-tyrosine acts directly on the PG. While these results provided the first evidence that transcription of some ACBPs can be triggered by specific humoral factors, this factor essentially had no effect on the transcription of other PG-specific genes, such as pgFAR and Bmpgdesat1.

VI. Molecular Mechanisms Underlying PBAN Signaling in \textit{B. mori}: from Receptor to Channel

1. Demonstration of extracellular Ca\textsuperscript{2+} influx in response to PBAN stimulation

The molecular mechanisms underlying the way the extracellular signal of PBAN is transmitted into the biological response of sex pheromone production has been the focus of numerous studies. It has been well established that regardless of species, pheromonotropic activity is dependent on extracellular Ca\textsuperscript{2+}, suggesting that PBAN signaling is associated with an influx of extracellular Ca\textsuperscript{2+} into PG cells.\textsuperscript{58,59} This crucial event, however, had not been directly demonstrated in any moth species. To address this, we used fluorescent Ca\textsuperscript{2+} imaging techniques with isolated PGs of \textit{B. mori} and demonstrated that PBAN specifically triggers an influx of extracellular Ca\textsuperscript{2+} (Fig. 3).\textsuperscript{60}

2. Involvement of the store-operated channel and related functional molecules

Because numerous biological processes are mediated by fluctuations in the intracellular levels of Ca\textsuperscript{2+}, entry of Ca\textsuperscript{2+} into the cell is tightly regulated.\textsuperscript{61} The two most pervasive Ca\textsuperscript{2+}-permeable cation channels are voltage-operated channels (VOCs)\textsuperscript{62} and receptor-activated Ca\textsuperscript{2+} channels (RACCs).\textsuperscript{51} In the activation of RACCs, the signal from the extracellular stimulus bifurcates at phospholipase C (PLC) and the enzyme catalyzes the formation of two second messengers, inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG), from phosphatidylinositol (4,5)-bisphosphate. The IP\textsubscript{3} generated by PLC promotes the rapid release of Ca\textsuperscript{2+} stored in the endoplasmic reticulum (ER), which triggers the activation of store-operated channels (SOCs) in the plasma membrane, resulting in a slower, sustained rise in intracellular Ca\textsuperscript{2+}.\textsuperscript{63} Alternatively, a different subset of RACCs can be activated independently of ER Ca\textsuperscript{2+} by DAG or one of its downstream metabolites, such as arachidonic acid.\textsuperscript{64,65}

Early pharmacological studies in heliothine species suggested that PBAN triggers the opening of RACCs, but were unable to differentiate the type of RACC (SOC or DAG-dependent) activated.\textsuperscript{66–68} Recently we expanded on those RACC studies and reported that in \textit{B. mori} the PBAN pathway proceeds \textit{via} SOCs.\textsuperscript{60}

The influx of extracellular Ca\textsuperscript{2+} through SOCs occurs in response to depletion of ER Ca\textsuperscript{2+} stores, which is triggered by an elevation in IP\textsubscript{3} levels following
receptor-linked PLC activation. To examine this aspect of the signal cascade, we analyzed IP$_3$ levels by a conventional method that measures total inositol phosphates by blocking inositol recycling in the presence of LiCl$_2$, an inhibitor of inositol monophosphatase, and found that there was a significant increase in the levels of PG total inositol phosphates following PBAN stimulation (Hull et al., manuscript submitted). Furthermore, the pharmacological profile of PG cells in response to PLC inhibitors suggested the involvement of PLC in PBAN signaling. To examine further which heterotrimeric G protein is involved in PBAN signaling, we sought to clone all of the G protein homologs of PBAN signaling, we identified 2Gs, 1Gq, and 1Go. We have also cloned other related genes, including G protein $\beta$ subunit, $\gamma$ subunit, PLC$\beta$, PLC$\gamma$, IP$_3$R, and targeted all of these genes for disruption by injecting their corresponding dsRNAs directly into the PG on the day of emergence. In conjunction with the findings that sex pheromone production is blocked by classical SOC inhibitors, that activators of diacyl glycerol-dependent channels are ineffective, and that the pheromonotropic effects of PBAN can be mimicked by thapsigargin (Tg), all these data supported the hypothesis that PBAN signaling utilizes the canonical SOC activation pathway.

Recent discoveries have identified two proteins essential to the SOC pathway: stromal interaction molecule 1 (STIM1) and Orai1. STIM1 is a single transmembrane (TM) domain protein that resides in the ER membrane. A change in the conformational state of the STIM1 intramolecular EF-hand motif (the Ca$^{2+}$-sensing region) in response to diminished luminal Ca$^{2+}$ levels promotes the translocation of STIM1 from or within the ER to regions of the plasma membrane where it interacts with Orai dimers to activate store-operated Ca$^{2+}$ entry. Orai (or Ca$^{2+}$ release-activated Ca$^{2+}$ channel modulator 1, CRACM1) is a four-TM domain protein localized in the plasma membrane that is predominantly a dimer under resting conditions. Interaction with the C-terminus of STIM1 induces tetramerization of the Orai dimers to form the Ca$^{2+}$-selective pore. Hence, we delved further into the molecular nature of the PBAN-activated SOC pathway. We have cloned cDNAs from PGs encoding Bombyx homologs of STIM1 (BmSTIM1) and Orai1 (BmOrai1), and functional expression in cultured insect cells as well as knockdown effects on these proteins in the PBAN signaling cascade. 75

VII. Comparative Aspects of Molecular Mechanisms Underlying PBAN Signaling in Moths

While extracellular Ca$^{2+}$ is an absolute requirement for pheromonotropic activity, accumulating evidence suggests that the biochemical processes under PBAN control are species-dependent. This is particularly evident when comparing B. mori and the heliothine species, the two lepidopteran models in which PBAN pheromonotropic activity has been most extensively studied. (i) The B. mori PBANR is extended 67 amino acids more than the heliothine PBANR and undergoes agonist-induced internalization, a regulatory mechanism commonly associated with receptor desensitization. In contrast, the H. zea PBANR, which is truncated upstream of the internalization motif present in the B. mori PBANR, is expected to exhibit significantly different internalization kinetics. (ii) In B. mori, PBAN stimulation results in activation of the lipase activity that releases stored sex pheromone precursors as well as activation of the fatty-acyl reductase that catalyzes the terminal step in sex pheromone biosynthesis. In heliothine moths, however, PBAN regulates a step (most likely acetyl-CoA carboxylase) in fatty acid biosynthesis. (iii) In B. mori, the cyclic nucleotide second messenger, cyclic adenosine-3',5'-monophosphate (cAMP), is not involved in the PBAN signal transduction cascade, whereas in heliothine species the second messenger is a crucial component in PBAN signaling.

VIII. Conclusions

In our endeavour to understand the molecular mechanisms underlying sex pheromone production in the silkmoth B. mori, we have taken advantage of the genomic information available on B. mori and have characterized PG-specific and PG-selective genes (Bmpgdesa1, pgFAR, PBANR, BmFATP, pgACBP, mgACBP, BmSTIM1, and BmOrai1). We have also demonstrated the specific roles of these genes in bombykol biosynthesis in vivo using an RNAi-mediated loss-of-function approach. Despite these efforts, little is known regarding the way the external PBAN signal activates lipolysis of the cytoplasmic TAGs and fatty acyl reduction in bombykol production. To address this, we utilized RNAi screening to clone the PG-specific lipase gene that is most likely responsible for TAG lipolysis. In addition, we found that several proteins in the PG cells are phosphorylated in response to PBAN stimulation (Ohnishi, manuscript in preparation). Based on these data, we propose the following scheme for the molecular mechanisms underlying bombykol production (Fig. 4): Before eclosion, the bombykol precursor is synthesized de novo from acetyl-CoA through the fatty acid biosynthetic pathway. At this time, however, the bombykol precursor is not converted to bombykol, but is rather stored in the cytoplasmic LDs in the form of TAGs. Then, after eclosion, PBAN is released and binds to the cell-surface PBANR. This binding causes SOC activation through the canonical pathway in conjunction with molecular interaction between BmSTIM1 and BmOrai1. Consequently, an influx of extracellular Ca$^{2+}$ accelerates both lipolysis and fatty acyl reduction through a calmodulin/calcineurin-mediated phosphorylation/dephosphorylation cascade culminating in bombykol production, although the precise mechanisms of this cascade have yet to be determined.

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