Identification and Characterization of Receptors for Ion Transport Peptide (ITP) and ITP-like (ITPL) in the Silkworm Bombyx mori

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Background: Ion transport peptide (ITP) and ITP-like (ITPL) are crustacean hyperglycemic hormone family peptides in insects. Receptors for ITP and ITPL were screened from orphan Bombyx neuropeptide G protein-coupled receptors (BNGRs). BNGR-A2 and -A34 act as ITP receptors, whereas BNGR-A24 functions as an ITPL receptor.

Significance: Receptor identifications provide further understandings of biological events by this neuropeptide superfamily.

Ion transport peptide (ITP) and its alternatively spliced variant, ITP-like (ITPL), are insect peptides that belong to the crustacean hyperglycemic hormone family. These peptides modulate the homeostatic mechanisms for regulating energy metabolism, molting, and reproduction and are specifically conserved in ecdysozoans. Many of the details of the molecular mechanisms by which crustacean hyperglycemic hormone family peptides exert pleiotropy remain to be elucidated, including characterization of their receptors. Here we identified three Bombyx mori orphan neuropeptide G protein-coupled receptors (BNGRs), BNGR-A2, -A24, and -A34, as receptors for ITP and ITPL (collectively referred to as ITPs). BNGR-A2 and -A34 and BNGR-A24 respond to recombinant ITPs, respectively, with EC50 values of 1.1–2.6 × 10−8 M, when expressed in a heterologous expression system. These three candidate BNGRs are expressed at larval B. mori tissues targeted by ITPs, with cGMP elevation observed after exposure to recombinant ITPs. ITPs also increased the cGMP level in B. mori ovary-derived BmN cells via membrane-bound and soluble guanylyl cyclases. The simultaneous knockdown of bngr-A2 and -A34 significantly decreased the response of BmN cells to ITP, whereas knockdown of bngr-A24 led to decreased responses to ITPL. Conversely, transient expression of bngr-A24 potentiated the response of BmN cells to ITPL. An in vitro binding assay showed direct interaction between ITPs and heterologously expressed BNGRs in a ligand-receptor-specific manner. Taken together, these data demonstrate that BNGR-A2 and -A34 are ITP receptors and that BNGR-A24 is an ITPL receptor in B. mori.

Significance: Receptor identifications provide further understandings of biological events by this neuropeptide superfamily.

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5 The abbreviations used are: ITP, ion transport peptide; ITPL, ion transport peptide-like; BNGR, Bombyx mori neuropeptide G protein-coupled receptor; CHH, crustacean hyperglycemic hormone; cGMP, cyclic guanosine 3‘,5‘-monophosphate; mGluR, metabotropic glutamate receptor; TKR, tachykinin receptor.
ITPL is expressed in both the central and peripheral nervous systems in *B. mori*, *D. melanogaster*, and *M. sexta* (5, 6, 13).

Although ITPs share ∼40 N-terminal amino acids, more significant structural differences occur at the C terminus. ITP is C-terminally amidated, whereas the C terminus of ITPL has a free carboxyl group. The C-terminal amide group of ITP is crucial for potentiation of its ileum-stimulating activity in *S. gregaria* (14), consistent with the importance of C-terminal amidation in the biological activities of crustacean CHH family peptides (15, 16).

Studies in both crustacean and insect species have shown that the stimulation of target tissues by CHH family peptides is mediated by cAMP and/or cGMP, with larger alternation of the cGMP level than the cAMP level (17–20). Structure-function studies of CHH family members have identified the essential moieties responsible for the discrimination of different biological activities (21–24). More specific knowledge regarding the in vivo function of CHH family peptides is very limited because pleiotropism and structural similarity among these peptides have largely impeded detailed analyses. Indeed, further understanding of the molecular mechanisms underlying functional activation of CHH family receptors is crucial for elucidating the biological functions mediated by these ligands; however, advances have been largely impeded by the absence of an identified receptor from any species for these peptides.

Most neuropeptide receptors, with few exceptions, belong to the G protein-coupled receptor (GPCR) superfamily (25). Indeed, the estimated molecular weight of receptors for CHH family peptides in crustacean species coincides with those of class A and B GPCRs (26, 27). Recently, much attention has been given to the deorphanization of insect neuropeptide GPCRs predicted by in silico analyses of genomic data (25), including *B. mori* (28). We therefore hypothesized that the biological actions of CHH family peptides in ecdysiozoans are mediated by GPCRs. In the present study, we used Ca\(^{2+}\) imaging to screen 34 orphan *B. mori* neuropeptide GPCRs (BNGRs) (28) for activation by ITPs. Based on this screening, we identified and characterized, using both in vitro and ex vivo experiments, three BNGRs that function as receptors for ITPs. We also discuss the intercellular cGMP-generating pathways utilized by the ITPs and their cognate receptors.

**EXPERIMENTAL PROCEDURES**

**Insects**—Silkworm eggs from a *B. mori* racial hybrid, Kinshu × Showa (Ueda Sanshu), were used for all experiments. Larvae were reared in plastic containers at 26 ± 1 °C with 70 ± 10% relative humidity under long day lighting conditions (16 h light/8 h dark), using the SILKMATE 25 artificial diet (Nihon Nosan Kogyo).

**Preparation of Recombinant Peptides**—Total RNA (1 μg) extracted from the brain of *B. mori* larvae using TRIzol Reagent (Invitrogen) were used for cDNA synthesis using oligo(dT)\(_{12-18}\) primer with SuperScript III (Invitrogen). RT-PCR was carried out with KOD-Plus (Toyobo) using ITP- and ITPL-specific primers designed based on the nucleotide sequences of ITP (GenBank\(^{TM}\) accession number AY950502) and ITPL (GenBank\(^{TM}\) accession number AY950503). The common forward primer (5′-GGCGATATCGAGCTTCTTCCACCTGAGT-

GCAAGGGCG-3′) includes an additional three bases (italic type), EcoRV site (boldface type), start codon (lowercase letters), and subsequent 5′-terminal sequence of ORF of ITPs, with optimization of the codon usage for expression in *E. coli* (underlined). The reverse primer for ITP (5′-GGCGAATTCTtgaGGCAGAAGTCGATATTGGTTG-3′) includes the 3′-terminal sequence of ORF, followed by an additional codon for the amidating donor residue, Gly (underlined), stop codon (lowercase letters), EcoRI site (boldface type), and three additional bases (italic type). The reverse primer for ITPL (5′-GGCGAGAATTCTcaataATCCGCGGGTACGCCCCG-3′) includes the 3′-terminal sequence of ORF, stop codon (lowercase letters), EcoRI site (boldface type), and three additional bases (italic type). The following program was used for amplification: 20 s (140 s for the first cycle) at 94 °C, 30 s at 52 °C, and 30 s at 68 °C for 30 cycles. The PCR product was subcloned into pGEM-T Easy vector (Promega). The resultant plasmid was digested with EcoRV and EcoRI and then ligated with the EcoRV/EcoRI-digested pET32a(+) vector (Novagen).

Competent cells of *E. coli* BL21-CodonPlus strain (Stratagene) were transformed separately with the constructed plasmid containing the cDNA of ITP or ITPL and selected on LB plates with ampicillin (50 μg/ml). The transformed bacterial cells were picked up from a single colony and incubated at 37 °C for 12 h in LB medium with ampicillin (50 μg/ml). The culture was then inoculated into a 50-fold volume of the same medium and incubated at 37 °C until the absorbance at 600 nm reached 0.5–0.6. Then isopropyl-β-D-thiogalactoside was added to the culture at a final concentration of 1 mM. After additional incubation for 4 h, bacterial cells were harvested by centrifugation. The bacterial cells expressing ITPs were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), and 1.8 mM KH\(_2\)PO\(_4\), pH 7.4) with 0.02% lysozyme. After incubation at 37 °C for 1 h and freezing at −80 °C for over 1 h, the suspension was sonicated. The sonicated mixture was centrifuged (4 °C, 5,000 × g, 10 min). Recombinant ITP (rITP)-Gly and recombinant ITPL (rITPL) collected as an inclusion body were dissolved into 7.5 mM guanidine hydrochloride, 10 mM EDTA. After lyophilization, these recombinants were dissolved into 6 mM guanidine hydrochloride, 1 M Tris-HCl (pH 8.0) at a final concentration of ∼0.1 mg/ml. The resultant mixture was diluted with a 3-fold volume of the dilution buffer (0.6 M Tris-HCl, 9.6% glycerol, 2.4 mM reduced form guanidine, pH 8.0). After the addition of oxidized form guanidine to the mixture at a final concentration of 1 mM, the mixture was stirred at 4 °C for 2 days for refolding of the recombinants. The recombinants were then purified by reverse-phase HPLC on a Shodex Asahipak OD-50 column (10-mm inner diameter × 250 mm, Showa Denko) with a 30-min linear gradient of 20–50% acetonitrile, 0.05% TFA at a flow rate of 3 ml/min. The elution was monitored by absorbance at 280 nm. To obtain rITP with C-terminal amidation, the amidating reaction of rITP-Gly was performed as reported previously (14).

**Construction of Expression Plasmids**—The pME18S mammalian expression plasmids containing BNGRs were kind gifts from Dr. N. Yamanaka (26). The pME18S plasmid containing mouse Ga15 (47) was a kind gift from Dr. K. Touhara.
TABLE 1

| Primer name | Sequence (from 5’ to 3’) |
|-------------|--------------------------|
| Bst-A2_Fw   | cccgCACCCGCTCTGGAAGCTGCGAAGAATAAGTACAGC | |
| Bst-A24_Fw  | atacCACCCGCTCTGGAAGCTGCGAAGAATAAGTACAGC |
| Bst-A34_Fw  | cccgCACCCGCTCTGGAAGCTGCGAAGAATAAGTACAGC |
| Xho-*E_Rv   | ccgCTCTGCATGGGTAGCTGCGAAGAATAAGTACAGC |
| Xho-*A2, Rv | ccgCTCTGCATGGGTAGCTGCGAAGAATAAGTACAGC |
| Xho-*A24, Rv| ccgCTCTGCATGGGTAGCTGCGAAGAATAAGTACAGC |
| Xho-*A34, Rv| ccgCTCTGCATGGGTAGCTGCGAAGAATAAGTACAGC |
| A2-E_Fw     | TTTGAACTAAAGATGATGGTGAGCAAGGGCTG |
| A2-E_Rv     | CCGCTCTGCATGGGTAGCTGCGAAGAATAAGTACAGC |
| A24-E_Fw    | ACGCCGAACATACCTGCAACCATGAGGACCTATTTAGT |
| A34-E_Fw    | GAAAAACGAACAGTTTATGGTGAGCAAGGGCTG |
| E/mc-A2_Rv  | GCCCTTGCTCACCATCATCTTTAGTTCCAAAAAGGAGTC |
| E/mc-A24_Rv| GCCCTTGCTCACCATCATCTTTAGTTCCAAAAAGGAGTC |
| E/mc-A34_Rv| GCCCTTGCTCACCATCATCTTTAGTTCCAAAAAGGAGTC |

The gene-specific primers include additional three bases (lowercase letters), a BstXI or XhoI restriction site (italic type), and a stop codon (boldface type). The chimeric primers correspond to the last 15 bases of BNGR ORF and the first 15 bases of EGFP ORF (underlined).

For confocal microscopic examination, we constructed expression plasmids for C-terminally enhanced GFP (EGFP)-fused BNGR-A2, -A24, and -A34 by overlap extension PCR using gene-specific primers indicated in Table 1. The following program was used for amplification: 20 s (140 s for the first cycle) at 94 °C, 30 s at 52 °C and 30–150 s at 68 °C, for 35 cycles. In the case of EGFP-fused BNGR-A2 (pME18S-BNGR-A2-EGFP), the first PCR entailed amplification of the complete coding region of BNGR-A2 minus the stop codon from the plasmid, pME18S-BNGR-A2, by using the gene-specific sense primer, Bst-A2_Fw, and the chimeric antisense primer, A2-E_Rv, with KOD-plus (Toyobo). The second amplification primer, Bst-A2_Fw, and the chimeric antisense primer, A2-E_Rv, with KOD-plus (Toyobo). The second amplification primer, Bst-A2_Fw, and the chimeric antisense primer, A2-E_Rv, with KOD-plus (Toyobo).

Expression Analysis by RT-PCR—Tissues were dissected out in 0.9% NaCl solution from anesthetized *B. mori* larvae at the fifth instar day 2 fed *ad libitum* and after 24-h starvation. Total RNA extraction and subsequent cDNA synthesis were performed as described above. In the case of RNA extraction from BmN cells, partial cDNA fragments of BNGRs, ITP, ITPL, and ribosomal protein L32 (RPL32) (GenBankTM accession number NM_001098282) were amplified with GoTaq DNA polymerase (Promega), using the gene-specific primers indicated in Table 2. The primers used for evaluation of RNA interference efficiency are designated to circumvent the dsRNA-targeting region. The amplification conditions for each gene are presented in Table 2.

RNA Interference—To prepare dsRNAs specific to BNGR-A2, -A24, -A34, EGFP, and BmGyc76C (GenBankTM accession number NM_001043405) (a *B. mori* ortholog to *D. melanogaster* Gyc76c (CG42637; GenBankTM accession number NP_524165)), the cDNAs for the *in vitro* transcription were generated by PCR using KOD-plus with gene-specific sense and antisense primers (Table 3). In the case of three BNGRs and EGFP, the pME18S plasmids containing these ORFs were used as templates. In the case of BmGyc76c, the cDNA subcloned into pGEM-T easy vector was used as a template. The primers include an additional T7 promoter sequence at 5’ termini. Then RNA was synthesized with T7 RNA polymerase (TakaraBio) using 1 μg of the resulting cDNA products, includ-
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**TABLE 2**

| Target gene | Forward/Reverse | Sequence (from 5’ to 3’) | Anneal° | Cycle° |
|-------------|----------------|--------------------------|---------|--------|
| bngr-A2     | Forward        | GCTCTGGATTTGCCTGCTGGAGCAGTGTC | 60       | 30     |
|             | Reverse        | TCTGGCTATCTGCTGGACCTGACCTGCC | 54       | 28     |
|             | Forward*       | TACAAGTTTTTCCAGAGGGGTTGGC | 65       | 30     |
|             | Reverse*       | ACCAGTATCCGAGGAGAGGACCTG | 54       | 28     |
| bngr-A24    | Forward        | ATGGGCTCTCAACCAGTCTCAAAAGCTCG | 60       | 33     |
|             | Reverse        | TTCTGCTTTGAGCCTGCGAGAAGG | 60       | 30     |
|             | Forward*       | CTCAGCCGAGAAATTTAATACG | 54       | 28     |
|             | Reverse*       | CATGAGCCGCGCCAGAAGAAATTTAATAACG | 60       | 30     |
| bngr-A34    | Forward        | TATTTGTATGCTGCTCTTGAGT | 60       | 33     |
|             | Reverse        | TCTGTGCAGTCGCTGCAATG | 60       | 30     |
|             | Forward*       | TTCCACCACTCTAGAGTTGAGAAAG | 54       | 28     |
|             | Reverse*       | CTGAGCCGCGAGAAATTTAATACG | 60       | 30     |
| itp         | Forward        | GCATCTAATTGGTGAGCTCGAAGG | 60       | 30     |
|             | Reverse        | TTAGGCTTTGTCGACAGGAGG | 60       | 30     |
|             | Forward*       | CAGCGCCGCGCCAGAAGAAATTTAATAACG | 60       | 30     |
|             | Reverse*       | CATGAGCCGCGAGAAATTTAATACG | 60       | 30     |
| rpl32       | Forward        | GCTTCACCTGCTGCTCTGTTTTA | 60       | 30     |
|             | Reverse        | TCTTTCCACGATCAGCTTCC | 60       | 30     |

° Primer sets used for expression analyses in BmN cells after gene knockdown.

° Variable parameters of the following amplification conditions are indicated: 20 s (140 s for the first cycle) at 94 °C, 30 s at 60 °C and 45 s at 72 °C, for Y cycles. X and Y are listed in this table.

**TABLE 3**

| Target gene | Forward/Reverse | Sequence (from 5’ to 3’)° |
|-------------|----------------|--------------------------|
| bngr-A2     | Forward        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Forward*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
| bngr-A24    | Forward        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Forward*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
| bngr-A34    | Forward        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Forward*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
| BmGyc76c    | Forward        | gcttcTAATACGACTCACTATAGGCGGACTCTGCTGGTAATGCGAGAG | 60       |
|             | Reverse        | ttggtTAATACGACTCACTATAGGCGGACTCTGCTGGTAATGCGAGAG | 60       |
|             | Forward*       | gcttcTAATACGACTCACTATAGGCGGACTCTGCTGGTAATGCGAGAG | 60       |
|             | Reverse*       | gcttcTAATACGACTCACTATAGGCGGACTCTGCTGGTAATGCGAGAG | 60       |
| egfp        | Forward        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse        | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Forward*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |
|             | Reverse*       | TAAAGACGTCACTAAGGCGGCTGCCGGCTGCTCGT | 60       |

° The gene-specific primers include additional five bases (lowercase letters) and an additional T7 promoter sequence (underlined).
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ligand was then added to the dishes at a final concentration of 100 nM. After incubation at 4 °C for 60 min, the cells were washed twice with an ice-cold fixation buffer (4% formaldehyde in PBS), and then 500 μl of the fixation buffer was added. The fluorescence derived from EGFP and RR was observed using a confocal microscope with the preinstalled dye mode of EGFP and DsRed, respectively. The merged image was obtained using microscope-attached image processing software.

RESULTS

Preparation of Recombinant ITPs—To screen orphan BNGRs for ITP activation, we first prepared B. mori rITP and rITPL (collectively referred to as rITPs) as ligands using a bacterial expression system. Because CHH family peptides require precisely linked intramolecular disulfide bonds for bioactivity, the resulting recombinant peptides, rITP and recombinant ITP-Gly (rITP-Gly), were subjected to refolding. Biologically active rITP was obtained by C-terminal amidation of rITP-Gly. Because little information is available regarding target tissues of ITPs in B. mori, we used BmN cells, a cell line derived from B. mori ovary, to initially assess the biological functionality of the rITPs. Exposure to 100 nM rITP increased the intracellular cGMP levels in BmN cells monotonically over 30 min, although only in the presence of IBMX, a nonspecific inhibitor of phosphodiesterases (data not shown). Measurements of the cGMP levels in BmN cells following 30-min exposure to rITP, rITPL, and rITP-Gly confirmed the biological functionality of rITP and rITPL, although rITP-Gly did not show distinct bioactivity at least at a final concentration of 100 nM (Fig. 1A).

Receptor Screening for ITPs from Orphan BNGRs—Thirty-four class A and class B orphan BNGRs (Fig. 2) were screened via Ca^{2+} imaging for responses to rITPs with simultaneously expressed promiscuous G protein Gαs subunit. BNGR-A2 and -A34 responded to 100 nM rITP, and BNGR-A24 responded to 100 nM rITPL (Fig. 1B, arrow 2); the other BNGRs had no measurable responses (data not shown). Ca^{2+} mobilization by these ligand-receptor combinations was observed in a dose-dependent manner, with EC_{50} values of 1.1–2.6 × 10^{-8} M (Fig. 1C and Table 4). BNGR-A2 and -A34 were not activated by rITPL even at concentrations of 1 μM. In contrast, BNGR-A24 (the rITP-responsive receptor) responded to rITP, albeit with a response curve of approximately 1 order less than the case of rITP (Fig. 1C and Table 4). Responses of BNGR-A2 and -A34 to rITP-Gly were also observed, however, at ~10 times higher concentrations than that of rITP (Fig. 1C and Table 4).

The receptors responding to the recombinant ITPs ligands, BNGR-A2, -A24, and -A34, are class A GPCRs consisting of 352, 392, and 419 amino acids, respectively. The sequence similarities among these receptors are 67% between BNGR-A2 and -A24, 61% between BNGR-A2 and -A34, and 59% between BNGR-A24 and -A34, with all three clustering on discrete branches in the BNGRs phylogenetic tree (Fig. 2, arrows). A BLAST search revealed one BNGR-A34 ortholog in D. melanogaster (an orphan GPCR) and two orthologs from T. castaneum, with 70–79% sequence similarity (Fig. 3C). The only ortholog of BNGR-A2 was found in a solitary lepidopteran species, Danaus plexippus (Fig. 3A). In contrast to BNGR-A2 and -A34, paralogs of BNGR-A24 were found within the same clade of the BNGR phylogenetic tree (BNGR-A32 and -A33; Fig. 2). These paralogs and BNGR-A24 share 81–91% similarity with known and predicted tachykinin-related peptide (TRP) receptors (TKRs) (Fig. 3B).

Functional Analyses in Tissues Expressing ITP Receptors—Data from receptor screening indicated that BNGR-A2 and -A34 were candidate ITP receptors and that BNGR-A24 was a candidate ITPL receptor. To evaluate whether these BNGRs function in vivo, we first analyzed the expression profile of the candidate receptors and ligands in fifth instar day 2 silkworm larvae (Fig. 4A). We found that bngr-A24 was ubiquitously expressed in the larval body. In contrast, whereas bngr-A2 was expressed in a number of tissues, including the brain, prothoracic glands, silk gland, hemocytes, and reproductive tissues, no amplifier was detected in CNS, foregut, or smooth muscle. Expression of the other putative ITP receptor, bngr-A34, was largely restricted to somatic tissues but scarcely in nervous tissues. The expression profile of itp and itpl likewise differed; itp expression was limited to the brain, whereas prominent itpl expression was observed in hemocytes, reproductive tissues, and nervous tissue.

We next measured changes of cGMP levels in tissues isolated from larvae fed ad libitum following exposure to 100 nM rITP or ITPL for 30 min. rITPL led to a significant elevation in cGMP levels in the brain, fat body, and ovary (Fig. 4B), the latter of which was ~30-fold higher than that observed in the vehicle treatment control. rITP elicited a broader tissue response than rITPL, with brain, fat body, Malpighian tubules, abdominal smooth muscle, reproductive organs, and foregut responding; conversely, no rITP-mediated responses were observed in midgut and hindgut from larvae fed ad libitum (Fig. 4B).

Intriguingly, we also noticed that starvation had a significant effect on the basal levels of cGMP in the intestines (Fig. 4C) and that starvation for 24 h potentiated the sensitivity of the foregut, midgut, and hindgut to rITPL (Fig. 4B). In contrast, rITP had no effect on the cGMP levels in these tissues. The increased sensitivity of the alimentary tract tissues to rITPL after the 24-h starvation period (Fig. 4B) coincided with up-regulated expression of both itpl and bngr-A24 (Fig. 4A), suggesting that ITPL and BNGR-A24 mediate the in vivo biological functions associated with these larval tissues.

Effects of BNGR Knockdown and Overexpression on BmN Cell Sensitivity to ITPs—Consistent with rITP-mediated elevation of cGMP levels in ovary-derived BmN cells (Fig. 1A), expression of the three candidate BNGRs was observed in the larval ovary (Fig. 4A) and BmN cells (Fig. 5A). To determine whether the responses to rITPs are mediated by these candidate receptors, we examined the effects of RNAi-mediated knockdown of the endogenous BNGRs (Fig. 5, A and B) on cGMP levels in BmN cells following stimulation by rITPs. We also examined the effects of transient overexpression of these candidate BNGRs on the sensitivity of BmN cells to rITPs. In the absence of stimulation by rITPs, neither knockdown nor overexpression altered the basal cGMP levels compared with mock treatments (data not shown). Although individual knockdown of BNGR-A2, -A24, and -A34 did not significantly alter the response to
rITP, simultaneous knockdown of BNGR-A2 and -A34 significantly suppressed the cGMP level elevated by rITP (Fig. 5C).

However, overexpression of BNGR-A2 and -A34 did not potentiate the response to rITP (Fig. 5D). The knockdown and overexpression of BNGR-A24 effectively decreased and increased the response to rITPL, respectively (Fig. 5C and D). Similar manipulation of BNGR-A2 or -A34 expression levels had no effect on response to rITPL. These data strongly indicate that both BNGR-A2 and -A34 mediate the ITP signal in BmN cells, whereas BNGR-A24 mediates the ITPL signal.

Binding of rITPs to Heterologously Expressed Candidate BNGRs—To demonstrate direct interaction between rITPs and the three candidate BNGRs, we performed an *in vitro* binding assay using RR-labeled rITPs and CHO cells transiently expressing C-terminally fused candidate BNGRs. The EGFP fluorescent signals confirmed that all EGFP-fused candidate BNGRs localized to the CHO cell plasma membrane (Fig. 6A). The merged fluorescence derived from the RR-labeled ligand (*red*) and EGFP-fused receptors (*green*) was observed after incubation with 100 nM RR-labeled rITPs. Co-localization at
the plasma membrane was observed only with the following ligand and receptor combinations: rITP and BNGR-A2; rITP and BNGR-A24; rITPL and BNGR-A24; and rITP and BNGR-A34 (Fig. 6). These combinations are consistent with the data for the distinct receptor responses observed using Ca^{2+}/H_{11001} imaging (Fig. 1B).

Role of Guanylyl Cyclases in the Response Signaling of BmN Cells to rITPs—Consistent with the observations for most CHH family peptides, signaling via ITPs in B. mori proceeds through Guanylyl cyclases.

Class A (30 BNGRs)

| BNGR   | NP_001127717 | NP_001127720 | NP_001127721 |
|--------|---------------|---------------|---------------|
| BNGR-A19 | 217           | 295           | 477           |
| BNGR-A23 | 295           | 892           | 565           |
| BNGR-A7  | 295           | 892           | 565           |
| BNGR-A4  | 477           | 565           | 59            |
| BNGR-A24 | 477           | 565           | 59            |
| BNGR-A32 | 295           | 892           | 565           |
| BNGR-A33 | 295           | 892           | 565           |
| BNGR-A14 | 295           | 892           | 565           |
| BNGR-A15 | 295           | 892           | 565           |
| BNGR-A31 | 295           | 892           | 565           |
| BNGR-A5  | 295           | 892           | 565           |

Class B (4 BNGRs)

| BNGR   | NP_001127726 | NP_001127727 |
|--------|---------------|---------------|
| BNGR-A28 | 1000          | 1000          |
| BNGR-A29 | 1000          | 1000          |
| BNGR-A21 | 1000          | 1000          |
| BNGR-A26 | 1000          | 1000          |
| BNGR-A30 | 1000          | 1000          |
| BNGR-A34 | 1000          | 1000          |
| BNGR-A12 | 1000          | 1000          |
| BNGR-A17 | 1000          | 1000          |

TABLE 4: EC\textsubscript{50} of responses of BNGRs to recombinant ITPs

| Receptor | Ligand | EC\textsubscript{50} (M) |
|----------|--------|--------------------------|
| BNGR-A2  | rITP   | 1.1 \times 10^{-8}       |
|          | rITP-Gly | 8.9 \times 10^{-9}     |
| BNGR-A24 | rITP   | 2.7 \times 10^{-7}       |
|          | rITP-Gly | 2.6 \times 10^{-8}     |
| BNGR-A34 | rITP   | 1.3 \times 10^{-8}       |
|          | rITP-Gly | 4.2 \times 10^{-7}     |

* EC\textsubscript{50} values were calculated from Ca^{2+}/H_{11001} imaging assays shown in Fig. 1C.
a cGMP second messenger cascade (Figs. 1A and 4B). Within this context, membrane-bound and/or soluble guanylyl cyclases (mGCs and/or sGCs, respectively) must be involved in CHH family peptide-evoked cellular signaling to produce the intracellular cGMP. We therefore tested which guanylyl cyclase, mGC or sGC, would contribute to signaling via ITPs in BmN cells. The suppression of BmGyc76c mRNA expression (Fig. 7, A and B), a B. mori mGC ortholog of D. melanogaster mGC Gyc76c, decreased elevated cGMP levels by 100 nM rITP and rITPL to 49.6 and 37.5%, respectively, compared with the mock treatment (Fig. 7C). We next evaluated the involvement of NO-sensitive sGC in signaling via ITPs using ODQ, a selective and potent inhibitor of NO-sensitive sGC. Increases in the cGMP levels by rITPs were significantly inhibited in proportion to the ODQ concentration; notably, 100 μM ODQ completely abolished the cGMP increase following a 30-min exposure to rITPs (Fig. 7D). These results indicate that mGC and/or NO-sensitive sGC may be involved in signaling via ITPs through cGMP production and that NO-sensitive sGC plays a predominant role in ITP signaling in BmN cells.

DISCUSSION

The deorphanization of GPCRs predicted from genomic data is a powerful strategy for identifying the endogenous bioactive ligand for a particular receptor. In the present study, screening of receptors for ITPs from orphan BNGRs revealed that BNGR-A2 and -A34 are ITP receptors, whereas BNGR-A24 is an ITPL receptor in B. mori (Fig. 8). The findings that ITP and ITPL act on a discrete receptor(s) are consistent with the different physiological functions of these neuropeptides (3, 12). The calculated EC_{50} values of these ligand-receptor combinations (Table 4) are 1 order of magnitude lower than that reported for locust ITP on ileal Cl\textsuperscript{-} transport (1–2 × 10\textsuperscript{-9} M) (29), which may be due to differences in the examined species and cell types between our cell line and ileal tissue. rITP showed a much lower affinity for BNGR-A24 than rITPL (Fig. 1C and Table 4), indicating that BNGR-A24 may not function as an ITP receptor in vivo.

Structure-Activity Relationship of Ligands and Receptors—

The presence of discrete receptors for ITP and ITPL indicates that the C-terminal portions of these peptides are important for discriminating between the two ligands by their respective receptors. Similar to the C-terminally amidated crustacean CHH family of peptides, native ITP is C-terminally amidated, which is crucial for its biological activity on locust ileum (14). The response of BNGR-A2 and -A34, identified as B. mori ITP receptors, to rITP-Gly was 1 order of magnitude weaker than that of the C-terminally

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**FIGURE 4.** Tissue distribution expression profile and functional analysis of candidate BNGRs in Bombyx larvae. A, RT-PCR analyses of BNGR, ITP, and ITPL genes. Ribosomal protein L32 (rpL32) was used as an experimental and expression control. Br, brain; CNS, central nervous system; Pg, prothoracic glands; Sg, silk gland; Fg, foregut; Mg, midgut; Hg, hindgut; Mt, Malpighian tubules; Fb, fat body; Ms, smooth muscle from the eighth body segment; Hm, hemocytes; Ts, testis; Ov, ovary. B, intracellular cGMP levels of tissues from fifth instar day-2 larvae either fed ad libitum or starved for 24 h. The isolated tissues were treated with 100 nM rITPs. The data are shown as the ratio of treated to non-treated cGMP levels (mean ± S.D. (error bars); n = 3). Asterisks indicate significant differences compared with the vehicle-treated data (*, p < 0.05; **, p < 0.01; ****, p < 0.001; Dunnett’s test). C, effects of starvation on the basal cGMP level. Foregut (Fg), midgut (Mg), and hindgut (Hg) were dissected out from fifth instar day 2 larvae fed ad libitum or starved for 24 h. The amount of cGMP in tissues was normalized by the amount of tissue proteins. Data are shown as mean ± S.D. (n = 3). Asterisks indicate significant differences from values of vehicle treatment (*, p < 0.05; **, p < 0.01; Dunnett’s test).
amidated rITP (Fig. 1C and Table 4) and was in proportion to the intensity of their biological activities (Fig. 1A). This positive correlation between response of the receptor and bioactivity of the ligand supports these two BNGRs as native receptors for ITP in vivo.

Comparison of BNGR-A2 and -A34 as ITP Receptors—BNGR-A2 and -A34 identified as *B. mori* ITP receptors showed similar ligand specificity; these BNGRs exhibit high affinities to amidated ITP (i.e. rITP) but lower affinities to non-amidated ITP (i.e. rITP-Gly) (Fig. 1C and Table 4). The simultaneous knockdown of BNGR-A2 and -A34 significantly decreased the sensitivity of BmN cells to rITP, whereas the knockdown of one of these two receptors did not (Fig. 5C), indicating that these receptors may functionally overlap. In contrast, the expression profiles of these two BNGRs were different (Fig. 4A), suggesting that the two receptors possess discrete functions. A homology search showed that BNGR-A2 has only one ortholog in a lepidopteran species, whereas orthologs of BNGR-A34 were found in a number of dipteran and coleopteran species (Fig. 3, A and C). Based on these results, we suggest that BNGR-A34 is a conserved ITP receptor across insect species and that BNGR-A2 may have a species-specific role in lepidopteran species.

BNGR-A24 as a Putative TKR—BNGR-A24, which we identified as a *B. mori* ITPL receptor, shares high (86–91%) similarity in amino acid sequence with known and predicted TKRs (Fig. 3B) (for a review, see Ref. 29). It is intriguing that tachykinin and TRPs clearly belong to a different superfamily than the CHH family peptides. In *D. melanogaster*, two isoforms (Drome-TKR-A and -B in Fig. 3B; Takr99D/DTKR) produced from the CG7887 gene and two isoforms (Tark86C/NKD) from the CG6515 gene have been identified as TKRs (31–33). Because BNGR-A24 and -A32 are orthologs of DTKR and NKD, respectively, the five TRPs predicted in *B. mori* (34) may act on these two BNGRs. BNGR-A33, a paralog located in the same phylogenetic clade as BNGR-A24 and -A32 (Fig. 2), is also
a candidate receptor for TRPs. We observed that ITPL acted only on BNGR-A24 and not on BNGR-A32 or -A33, indicating that these three BNGRs may have different modes of recognition for ITPL. The situation is similar to that reported for DTKR and NKD recognition and activation by TRPs in D. melanogaster; DTKR recognizes all six TKPs (35), whereas only one isoform of the six TRPs can activate NKD (36). In this context, BNGR-A24, -A32, and -A33 may also show selectivity for B. mori TRPs. Recently, BNGR-A32 and -A33 have been identified as receptors for novel neuropeptides, natalisins with the C-terminal XXXXRA and YAXXRA (where “a” represents amidation) consensus sequences, respectively (37). In contrast, BNGR-A24 responds to B. mori natalisin 1 with an EC_{50} of over 1 \times 10^{-7} m, indicating that BNGR-A24 may not be a specific natalisin receptor but may share several ligands. Further investigation is needed to determine whether BNGR-A24, -A32, and -A33 can respond to B. mori TRPs and function as TKRs in vivo.

If ITPL and TRP(s) act on the same receptor, these peptides should be functionally related. Indeed, a functional relationship between ITPL and TRPs has been suggested by the previous observation that ITP and TRPs co-localize in some neuroendocrine cells of the brain in D. melanogaster (38). In T. castaneum, the knockdown of ITPL results in a significant reduction in egg number (12), which is also observed when the gene expression of natalisin and its receptor are suppressed (37). These facts demonstrate the participation of both ITPL and TRP signaling systems in the regulation of female fecundity. Our finding that TKR-like BNGR-A24 mediates ITPL signaling in the B. mori larval ovary and BmN cells is consistent with this suggestion. We also found that the sensitivity of the intestinal tissues of 24-h starved larvae to ITPL was strongly potentiated compared with the larvae fed ad libitum (Fig. 4B), indicating that ITPL acts on the intestine according to the nutritional state. Considering that a previous study demonstrated the feeding-stimulatory effects of B. mori TRPs (39), it is possible that the signaling of ITPL and TRP in the modulation of feeding behavior may directly affect one another by interacting with the same receptor, BNGR-A24.

Ion Transport Function of ITPs in B. mori—The fact that the sensitivity to rITP increased in the alimentary tract (i.e. foregut, midgut, and hindgut) by starvation may be due to up-regulated expression of bngr-A24 (Fig. 4, A and B). In contrast to the increased sensitivity, rITP failed to stimulate an elevation in cGMP levels in the midgut and hindgut of both fed and starved larvae (Fig. 4B), although previous studies using locusts have demonstrated the function of ITP on the ileum (1, 17). A possible reason is that endogenous ITP functioned to abolish the effect of exogenous rITP, because basal cGMP levels increased in the intestine of starved larvae (Fig. 4C). Interestingly, 24-h starvation up-regulated the expression of itpl but not that of itp in the intestine (Fig. 4A), consistent with the fact that itp expression is confined to neuroendocrine cells in the brain in several species, including B. mori (5). These results indicate that starvation may potentiate a local ITPL signaling system by up-regulating both the ligand and receptor. Considering that a previous study showed that rITP had no stimulatory effects and reduced the stimulatory effect of synthetic ITP on Cl⁻ transport in the ileum of S. gregaria (40), locally expressed ITPL in intestinal tissues may functionally compete with ITP.

Signaling Pathway of ITPs—Our finding that BmGyc76c participates in signaling via ITPs in BmN cells (Fig. 7C) suggests that this mGC may also function as a receptor for ITPs. In several crustacean species, orthologs of BmGyc76c have been characterized as putative receptors for CHH family peptides (41–43). However, a direct interaction between CHH family peptides and these mGCs has not yet been demonstrated. In addition, a recent study illustrated that D. melanogaster Gyc76c, an ortholog of BmGyc76c, was identified as a receptor for an endogenous peptide ligand, NPLP1-VQQ (44). Moreover, our results showed that NO-sensitive sGC plays a principal role in signaling via ITPs through cGMP production in BmN cells (Fig. 7D). Taken together, it is likely that ITPs do not act directly on BmGyc76c, at least in BmN cells.

This study showed that an unknown NO-sensitive sGC plays a significant role in the elevation of cGMP levels in BmN cells by rITPs (Fig. 7D). This observation differs from a previous report that cGMP of CHH signaling in lobster skeletal muscle is mediated by mGC rather than sGC (45). In contrast, our results are consistent with a report describing the signaling pathway for molt-inhibiting hormone (MIH), a crustacean CHH family peptide, in crab Y-organs (46). In this case (Fig. 8), MIH binds to a GPCR, triggering the production of cAMP via an adenyllyl

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Receptors of Ion Transport Peptides in *B. mori*

**FIGURE 8. Proposed signaling pathway of ITPs in *B. mori***. BNGR-A2 and -A34 function as ITP receptor. BNGR-A24, a putative receptor for TRPs, acts as an ITPL receptor rather than a receptor for ITP because of its low affinity with ITP. ITPs increase intracellular cGMP by activation of both membrane-bound BmGyc76c and NO-sensitive sGC, which is inhibited by ODQ. The signaling pathway via NO-sensitive sGC is shown in reference to the accepted signaling pathway for MIH in decapod crustaceans (46). In brief, activation of receptors for ITPs results in cAMP production by adenyl cyclase (AC) via G protein (G), and subsequent activation of cAMP-dependent protein kinase (PKA). PKA increases intracellular Ca^{2+} via the Ca^{2+} channel, consequently activating calmodulin (CaM). Calmodulin stimulates NO production by NO synthase (NOS), leading to activation of NO-sensitive sGC. Chronically elevated intracellular CAMP and cGMP activate PKA and cGMP-dependent protein kinase (PKG), respectively. These effectors contribute to multiple functions exhibited by ITPs, probably including ion homeostasis.

In conclusion, we identified three class A GPCRs, BNGR-A2, -A24, and -A34, as receptors for two *B. mori* CHH family peptides: ITP and ITPL. Because orthologs of these BNGRs are observed in other ecdysozoan species, including insects, the identification of the receptors will open new avenues to discover novel biological functions and significance of the CHH family peptides in ecdysozoans. Especially, BNGR-A24, identified as an ITPL receptor, has a potential TKR function, suggesting a directly functional connection between the signaling by these neuropeptides. Receptors for the CHH family peptides are likely to have evolved in correspondence with the evolution of the ligands with gene duplication and mutation events (47). In this context, identification of their specific receptors provides a possibility to investigate this neuropeptide family in terms of the model of co-revolution of a ligand and a receptor. In addition, because it enabled us to identify the target cells of CHH family peptides and to manipulate the signaling system of these neuropeptides, our present study is a breakthrough for further understanding the molecular mechanisms underlying the functions of CHH family peptides.

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