Expression Cloning of an Insect Diuretic Hormone Receptor

A MEMBER OF THE CALCITONIN/SECRETIN RECEPTOR FAMILY*

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Insect diuretic hormones and their receptors regulate fluid and ion secretion and thus are attractive targets for the design of novel insect control agents. A complementary DNA clone encoding a corticotropin-releasing factor-related diuretic hormone receptor from the tobacco hornworm Manduca sexta was isolated by expression cloning in COS-7 cells. The receptor consists of 395 amino acids and contains seven putative transmembrane domains. The expressed receptor binds M. sexta diuretic hormone, as well as several related insect diuretic peptides with high affinity. Furthermore, each of these peptides stimulate adenylyl cyclase in COS-7 cells transfected with the receptor. The M. sexta diuretic hormone receptor is homologous to the receptors for calcitonin, secretin, vasoactive intestinal peptide, parathyroid hormone, glucagon-like peptide 1, growth hormone-releasing hormone, pituitary adenylate cyclase-activating polypeptide, and glucagon. The M. sexta diuretic hormone receptor is the first nonmammalian member of this family to be identified.

The control of fluid secretion in insects is crucial for their survival. Diuretic and (presumably) antidiuretic hormones, which primarily exert their effects on the Malpighian tubules (MTs),1 mediate this process. A 41-amino acid diuretic hormone isolated from adult Manduca sexta (Mas-DH) stimulates fluid secretion and cAMP synthesis in the MTs of several insect species (1–4). Furthermore, a recent report suggests that Mas-DH activates a Na⁺/K⁺/2Cl⁻ co-transporter (5). Mas-DH belongs to the corticotropin-releasing factor (CRF)/urotensin/sauvagine peptide family and shares 30% to 39% sequence identity with these peptides. Four CRF-related diuretic hormones/peptides from M. sexta (Mas-DPII), Acheta domesticus (Acd-DH), Locusta migratoria (Lom-DH), and Periplaneta americana (Pee-DP) have been identified and share between 34% and 50% sequence identity with Mas-DH (6, 7).

While considerable effort has been directed toward identifying insect diuretic hormones and their modes of action, a report characterizing an insect diuretic hormone receptor has appeared only recently (8). Mt membranes from M. sexta larvae were found to contain a receptor that bound 3H-labeled Mas-DH with extraordinarily high affinity (Kᵝ = 79 pM). I have used an expression cloning strategy in COS-7 cells to isolate a cDNA that encodes a M. sexta diuretic hormone receptor. The expressed receptor binds Mas-DH with high affinity, stimulates adenylyl cyclase, and is related to the calcitonin/secretin G-protein-coupled receptor family.

EXPERIMENTAL PROCEDURES

Pepitides and Cell Culture—Mas-DH, Mas-DPII, Acd-DH, and Pee-DP were provided by Dr. J. P. Li of Sandoz Agro Inc., Palo Alto, CA. Lom-DH was provided by Dr. David Schooley, University of Nevada, Reno. COS-7 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD) in a 1% CO₂ humidified incubator at 37 °C. Polyadenylated cDNA was prepared by a Stratagene ZAP-cDNA synthesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). The cDNA was size-selected on Sepharose CL-4B, and fractions containing cDNA 1 kb and larger were ligated into the EcoRI and XhoI sites of the mammalian expression vector pRK5 (9). This library was electroplated into Escherichia coli and 5.1 million plaques were spotted onto 612 pools of approximately 2100 transformants each. Plasmid DNA was prepared from each pool by the alkaline lysis method (10).

COS-7 Cell Transfection and Library Screening—COS-7 cells were grown in six-well tissue culture plates and transfected with 1–2 μg of plasmid DNA by the DEAE-dextran method (10). After 3 days, the cells were screened for diuretic hormone binding by incubation with 80–100 pm Bolten-Hunter 3H-labeled Mas-DH (8). After incubation in binding buffer (25 mM potassium phosphate (pH 6.5), 240 mM sucrose, 0.2% bovine serum albumin) for 1 h at 4 °C, the cells were washed three times with ice-cold binding buffer, fixed in phosphate-buffered saline containing 25 mM HEPES (pH 7.4) and 2% glutaraldehyde, dried, and coated with NTB-2 photographic emulsion (Ilford, Leavesden, UK). Plates were exposed for 3 days and developed.

Binding of 3H-labeled Mas-DH to Membranes of COS-7 Cells—COS-7 cells were grown in 150-mm tissue culture flasks and transfected with 15 μg of pRK5/Mas-DH-R by the DEAE-dextran technique and harvested after 3 days with treatment with 1 mM EDTA in phosphate-buffered saline. Cells were homogenized by sonication on ice and centrifuged at 10000 x g. The supernatant was then centrifuged at 107,000 x g, and the pellet was reasuspended in 25 mM potassium phosphate (pH 6.5), 240 mM sucrose, and 1 mM EDTA. The membranes were stored at −70 °C. Binding experiments were carried out as previously described (8) using 10 μg of COS-7 membranes/assay tube in binding buffer.

cAMP Assay—COS-7 cells were grown in 25-mm tissue culture flasks and transfected with 5 μg of PKS/Mas-DH-R by the DEAE-dextran technique. After 3 days, the cells were incubated in cell media (Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin, 0.1% sodium pyruvate, 0.1% glutamine, 1% nonessential amino acids, 0.1% b-mercaptoethanol, and 5% fetal bovine serum) at 37 °C for 15 min. The cDNA encoding the Mas-DH-R was sequenced at both directions by the dideoxy nucleotide chain termination method using Sequenase version 2.0 (U. S. Biochemical Corp.).
A cDNA library was constructed in the mammalian expression vector pKSl (9) from poly(A)+ RNA isolated from the Mt5 of 5th instar M. sexta larvae. The library, containing 1.1 million recombinants, was separated into 512 pools of 2100 clones each. Miniprep plasmid DNA from each pool was transfected into COS-7 cells and screened by 125I-labeled Mas-DH photoemulsion autoradiography. Six pools out of 150 were found to specifically bind 125I-labeled Mas-DH. The positive pool with the greatest number of labeled cells was subdivided until a single clone (pKSl/Mas-DH-R) was obtained. Fig. 1 shows a saturation isotherm of 3H-labeled Mas-DH binding to membranes of COS-7 cells transfected with pKSl/Mas-DH-R. Analysis of the binding data by LIGAND (11) indicated a single specific and saturable binding site displaying an apparent $K_d$ of 56 pm. This value agrees closely with the $K_d$ of Mas-DH receptors prepared from Mt membranes (8).

The clone pKSl/Mas-DH-R contains an insert of 1.6 kb that encodes a protein of 395 amino acids based upon the longest open reading frame (Fig. 2). An in-frame stop codon (nucleotides 143–145) upstream of the proposed initiator AUG codon (nucleotides 248–250) suggests that pKSl/Mas-DH-R contains the entire coding region of the Mas-DH receptor. Hydrophobicity analysis of the amino acid sequence by the method of Kyte and Doolittle (12) suggests the protein spans the membrane seven times. The Mas-DH receptor is the first insect-specific neuropeptide receptor to be cloned and is homologous to a recently discovered family of G-protein-coupled receptors first identified in mammals. It shares sequence identity with the calcitonin (31%), secretin (31%), vasoactive intestinal peptide (51%), parathyroid hormone (29%), glucagon-like peptide 1

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**RESULTS**

**Fig. 1. Specific binding of 3H-labeled Mas-DH to membranes of COS-7 cells transfected with pKSl/Mas-DH-R.** Scatchard analysis is shown in the inset. Results are from four independent experiments performed in duplicate. Analysis of the binding data by LIGAND (11) indicated a single binding site displaying a $K_d$ of 56 pm and $B_{max}$ = 1.1 pmol/mg protein. Binding assays were performed as described (8).

**Fig. 2. Alignment of the Mas-DH receptor (Mas-DH-R) with rat vasoactive intestinal peptide receptor (rVIP-R), rat secretin receptor (rSec-R), porcine calcitonin receptor (pCT-R), opposum parathyroid hormone receptor (opPTH-R), rat glucagon-like peptide 1 receptor (rGLP-1-R), rat growth hormone-releasing hormone receptor (rGRF-R),** and rat glucagon receptor (rGlu-R). Highlighted areas denote amino acids conserved between Mas-DH-R and other receptors. Putative transmembrane domains are indicated by lines above the Mas-DH receptor sequence. Asterisks indicate conserved cysteine residues and potential $N$-glycosylation sites in Mas-DH-R are marked by #. Alignment was performed using the Pileup program in the Genetics Computer Group software package (23).
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DH-R were incubated with 160 pm 3H-labeled Mas-DH and various concentrations of unlabeled peptides. Binding assays were performed as described (8). Results are from at least three independent experiments performed in duplicate (S.E. values were less than 10% of the mean value).

(29%), growth hormone-releasing hormone (28%), pituitary adenylate cyclase-activating polypeptide (27%), and glucagon (27%) receptors (13–20). Areas with the highest degree of sequence identity are located within and adjacent to the putative transmembrane domains, and areas with the least degree of sequence identity are located in portions of the N and C termini. Unlike other members of this family, the Mas-DH receptor has a relatively short N terminus that lacks a signal peptide and cleavage site (21). The Mas-DH receptor has seven potential N-glycosylation sites; six are located in the extracellular N-terminal domain, and the other is located in the first extracellular loop. These sites are not highly conserved with other members of the calcitoninsecretin receptor family. There are seven conserved cysteine residues; 5 are located in the extracellular domains, 1 in the first intracellular loop, and 1 in the seventh putative transmembrane domain. The conserved cysteine residues in the N-terminal domain may be involved in proper folding of the receptor into a structure capable of recognizing the appropriate ligand. Each member of the calcitoninsecretin receptor family that has been identified activates adenylate cyclase; it has been suggested that the amino acids comprising the third intracellular loop are involved in coupling to G-proteins in a manner analogous to the β2-adrenergic receptor (22).

To determine the specificity of ligand binding to the recombinant receptor, several peptides related to Mas-DH were tested in competition with 3H-labeled Mas-DH for binding to the expressed receptor. The known CRF-related diuretic peptides displayed an order of affinity of Mas-DH = Pea-DP = Acd-DH > Lom-DH = Mas-DPII (Fig. 3). The apparent IC50 values for Mas-DH, Pea-DP, and Acd-DH were ~1 nm, while the values for Lom-DH and Mas-DPII were ~8 and ~12 nm, respectively. Neither vasoactive intestinal peptide, growth hormone-releasing hormone, glucagon, nor parathyroid hormone inhibited binding of 3H-labeled Mas-DH to the recombinant Mas-DH receptor at concentrations as high as 1 μM (data not shown).

To determine if the expressed receptor could stimulate adenylate cyclase, COS-7 cells transfected with pKS1/Mas-DH-R were incubated with Mas-DH or related peptides (Fig. 4). All five CRF-related diuretic peptides stimulated cAMP synthesis approximately 22-fold above untreated cells and the order of potency matched the order of affinity. Treatment of COS-7 cells expressing the rat bradykinin receptor with 10 nm Mas-DH did not stimulate cAMP synthesis. The median effective concentration (EC50) for stimulation of cAMP by Mas-DH in COS-7 cells transfected with pKS1/Mas-DH-R was ~0.5 nm. By comparison, the EC50 for stimulation of fluid secretion in adult Mts of M. sexta by Mas-DH is ~0.5 nm.2

Northern blot analysis of poly(A)+ RNA from various insect tissues is shown in Fig. 5. A full-length 32P-labeled cDNA probe of the Mas-DH receptor revealed a single 1.6-kb transcript in Mts of larval M. sexta but not in brain, fat body, or Mts of A. domestica. Thus, although Acd-DH is a potent Mas-DH receptor agonist, the Acd-DH receptor does not appear to have an overall high sequence identity with the Mas-DH receptor.

DISCUSSION

The results presented above indicate that I have cloned a diuretic hormone receptor from the insect M. sexta. The receptor has very high affinity for Mas-DH, is expressed in Mts but not in fat body or brain, and is coupled to a cAMP second messenger system. The receptor possesses seven putative transmembrane domains that are common to other G-protein-coupled receptors. The Mas-DH-R is the first insect (and nonmammalian) receptor identified that is a member of the calcitoninsecretin receptor family. This suggests that, from an evolutionary standpoint, this family is quite old. It is surprising that the other four known CRF-related diuretic peptides are potent agonists of the Mas-DH receptor, especially when one considers that the sequence identity of these peptides to Mas-DH is only in the range of 34–50%. Hence, the receptor binding domains of these peptides should share common structural elements. The secondary structure of Mas-DH has been investigated by circular dichroism studies (8). The peptide appears to exist in a random form in an aqueous environment. However, in a hydrophobic environment, mimicked by trifluoroethanol, Mas-DH undergoes a transition to a helical conformation. It is possible that in vivo the peptide transforms to a helical conformation upon binding to the receptor. In this model, the helical domain of the peptide is positioned in the membrane and surrounded by transmembrane regions of the receptor. Computational analysis of the other known CRF-related diuretic peptides reveal that they also contain long helical regions.3 However, one must await experimental studies of the secondary structure of these peptides to confirm this finding. It is interesting to note that secretin, glucagon, growth hormone-releasing hormone, pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal peptide also assume an ex-

Fig. 3. Displacement of 3H-labeled Mas-DH by Mas-DH and related peptides. Membranes of COS-7 cell transfected with pKS1/Mas-DH-R were incubated with 160 pm 3H-labeled Mas-DH and various concentrations of unlabeled peptides. Binding assays were performed as described (8). Results are from at least three independent experiments performed in duplicate (S.E. values were less than 10% of the mean).

Fig. 4. Stimulation of cAMP synthesis by Mas-DH and related peptides in COS-7 cells transfected with pKS1/Mas-DH-R or pKS1/pBK-R. Transfected cells were treated with various concentrations of peptides as described under "Experimental Procedures." Results are from at least three independent experiments performed in duplicate (S.E. values were less than 15% of the mean value).

2 D. A. Schooley, personal communication

3 J. P. Li and R. L. Carney, unpublished observations.
tended helical conformation in a hydrophobic environment (24). Hence, this common structural element may be important for receptor/ligand interactions for members of the calcitonin/secretin receptor family.

The cloning of the Mas-DH receptor will facilitate our understanding of the mechanism of action of insect diuretic hormones. Furthermore, since the receptor is functionally coupled to a cAMP second messenger system in COS-7 cells, one can utilize this system to study both binding to and activation of the receptor by various analogs of Mas-DH. While receptors for the other known CRF-related diuretic peptides have not been characterized, they are likely to be homologous to the Mas-DH receptor since their ligands are related to Mas-DH and are strong Mas-DH receptor agonists. Hence, use of oligonucleotides derived from conserved regions in the calcitonin/secretin family should facilitate the cloning of other CRF-related insect diuretic hormone receptors.

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Note Added in Proof—While this paper was under review, the cloning of a human CRF receptor was reported that shares 31% sequence identity with the Mas-DH receptor (25).