THE CHARACTERIZATION, FUNCTIONAL EXPRESSION, AND LOCALIZATION OF THE FIRST ARTHROPOD MYOKININ RECEPTOR FROM THE SOUTHERN CATTLE TICK, *Boophilus microplus* (ACARI: IXODIDAE)

A Dissertation

by

STEVEN P. HOLMES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Entomology

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Approved as to style and content by:

Patricia V. Pietrantonio (Chair of Committee)	Robert C. Burghardt (Member)
Andrew C. Chen (Member)	Nancy H. Ing (Member)
Pete D. Teel (Member)	Kevin M. Heinz (Head of Department)

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ABSTRACT

The Characterization, Functional Expression, and Localization of the First Arthropod Myokinin Receptor from the Southern Cattle Tick, *Boophilus microplus* (Acari: Ixodidae). (December 2003)

Steven P. Holmes, B.S., Brigham Young University;

M.S., Texas A&M University

Chair of Advisory Committee: Dr. Patricia V. Pietrantonio

Myokinins are invertebrate neuropeptides with myotropic and diuretic activity. The lymnokinin receptor from the snail *Lymnaea stagnalis* was the only previously identified myokinin receptor. A cDNA encoding a neuropeptide receptor was cloned from the southern cattle tick, *Boophilus microplus*. The deduced amino acid sequence was 40 % identical to the lymnokinin receptor. The receptor transcript is present in all tick life stages as determined by semiquantitative RT-PCR. When expressed in mammalian CHO-K1 cells, myokinins at nanomolar concentrations induced increases in intracellular calcium as measured by fluorescent cytometry. The rank order of potency for peptides tested was FFFSWS-NH2≥FFFSWG-NH2≥FFSWG-NH2>FYSWG-NH2>muscakinin>lymnokinin>>APTGFFGVR-NH2. The receptor coupled to a pertussis toxin insensitive G protein. Absence of extracellular calcium did not inhibit the calcium response, indicating the release of Ca^{2+} from intracellular stores. Receptor transcript was detected by RT-PCR in the dissected synganglia, ovaries, salivary glands,

guts and Malpighian tubules of partially engorged adult female ticks. It is concluded that the *B. microplus* receptor is the first myokinin receptor cloned from an arthropod, and the first neuropeptide receptor known from the Acari. The presence of this receptor transcript in multiple tissues and all life stages suggests a multifunctional role in ticks.

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CHAPTER I

INTRODUCTION

Background

Overview and definition of G protein-coupled receptor (GPCR) superfamily

The fundamental ability of cells to interact and adjust to their environment is accomplished primarily through cell surface receptors and channels that respond specifically to a variety of compounds (Uings & Farrow, 2000). G protein-coupled receptors (GPCRs) are the largest single class of eukaryotic receptors (Iismaa *et al.*, 1995), and are the largest superfamily of proteins in the human body and constitute the majority of receptors on the plasma membrane (Gether, 2000; Watson & Arkinstall, 1995). Over one thousand GPCRs were known in 1998 (Vaughan, 1998) and additional ones are characterized each year. GPCRs consist of a single polypeptide chain and share a seven transmembrane (TM) domain topology (Fig. 1), and are named based on their ability to recruit and activate heterotrimeric G proteins, although they are not the only receptors that can activate G proteins (Iismaa *et al.*, 1995).

GPCRs vary significantly in overall length, although the length of primary sequence comprising the transmembrane domains is fairly constant. The smallest known GPCR, the human adrenocorticotropic receptor, is only 297 amino acid residues in length while the metabotropic glutamate receptor mGluR1a is 1180 residues (Iismaa *et al.*, 1995). There is no overall sequence homology that can be used to define the

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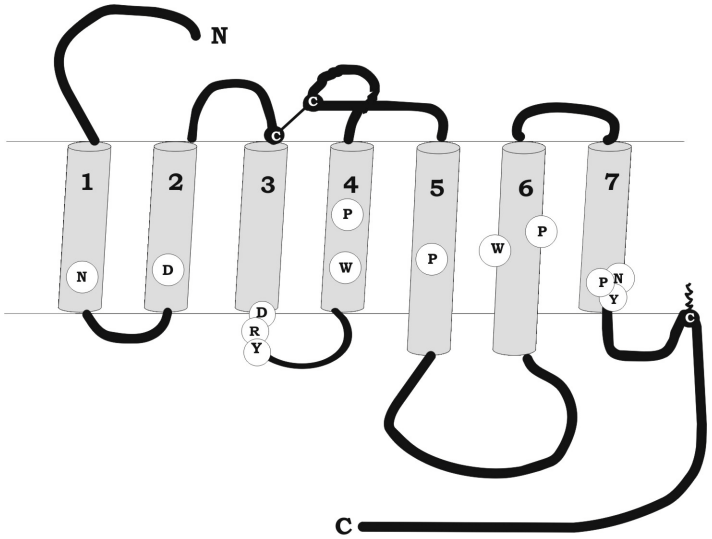
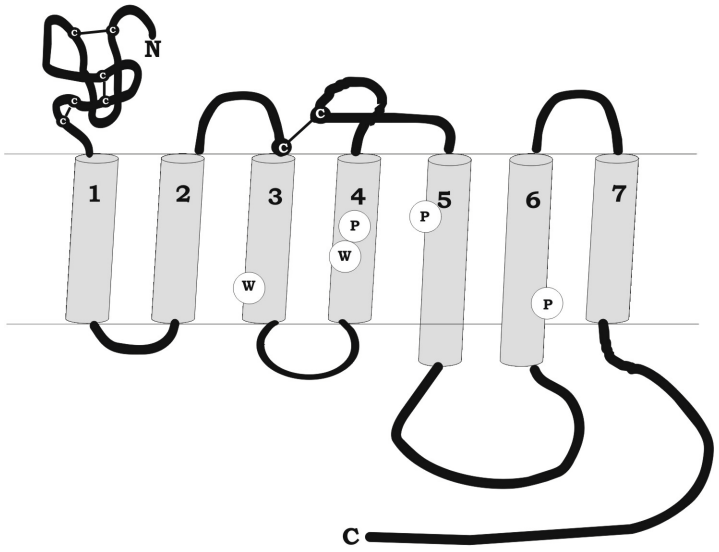
This dissertation follows the style and format of *Insect Molecular Biology*.

superfamily of GPCRs, although there is significant similarity within some individual subfamilies of GPCRs (Gether, 2000). However, some subfamilies share surprisingly little homology. For example, among the three mammalian neuropeptide Y receptors, subtypes Y1 and Y2 have only 31% overall sequence identity (Larhammar, 1996). This low homology and the relative low abundance of many GPCRs in tissues has made them difficult to clone or to characterize by biochemical methods. However the recent completion of genome projects for several species, including the fruit fly *Drosophila melanogaster*, and the malaria mosquito *Anopheles gambiae* have revealed genes for many previously unknown GPCRs (Adams *et al.* 2000; Holt *et al.*, 2002). This new wealth of genomic information will surely accelerate investigation and identification of GPCR gene candidates, as already seen by the identification of over one hundred genes encoding fora novel family of putative odorant receptors (Vosshall *et al.* 1999) and for the drosokinin receptor (Radford *et al.*, 2002).

The first GPCRs to be characterized were the rhodopsin receptor and the β-adrenergic receptor (Hargrave & McDowell, 1992; Shorr *et al.*, 1981). Much of what is known of GPCR structure is based on these two receptors. The seven TM structure common to GPCRs was predicted based on studies of rhodopsin, because this receptor is available in high quantities and easily purified from rod cells (Hargrave & McDowell, 1992). Low resolution electron crystallography structures of frog and bovine rhodopsins, resolved down to 6 and 9.5 angstroms, respectively, revealed an asymmetric clockwise arrangement of the TMs as viewed from the interior of the cell. Helices 1-3 and 5 are tilted while 4, 6, 7 are nearly perpendicular (Schertler *et al.*, 1993), (Schertler

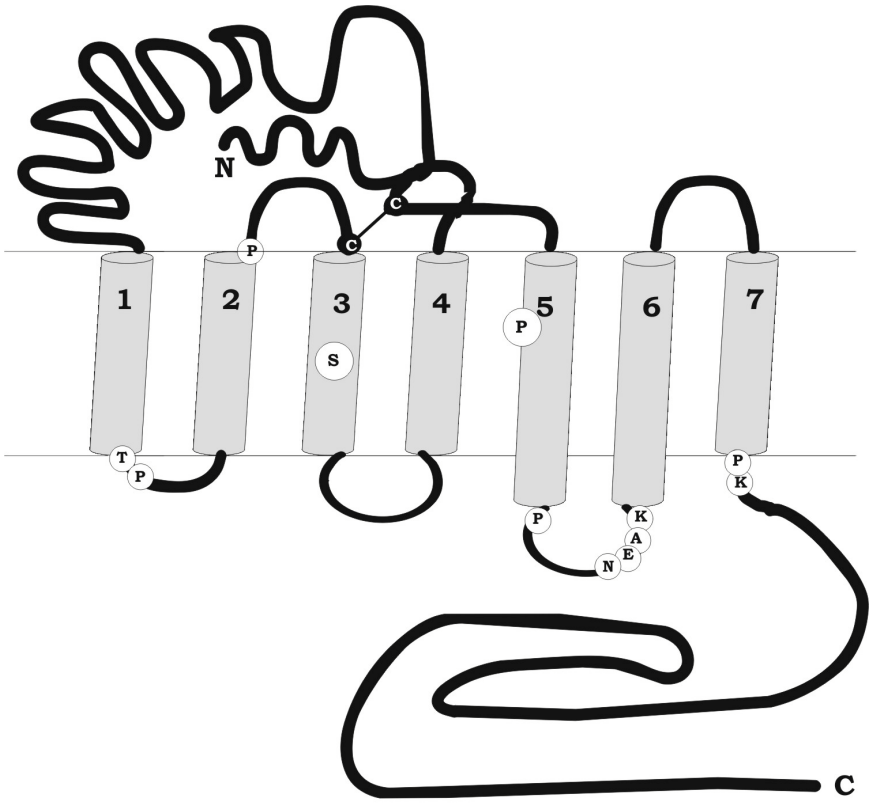


Family A. Rhodopsin/B2 adrenergic receptor-like



Family B. Glucagon/VIP/Calcitonin receptor like

Figure 1. The three major classes of GPCRs. A diagram of a typical member of each class is shown. In Class A receptors, conserved features include a disulfide bridge between Cys residues on extracellular loops 1 and 3, a DRY sequence on the 2nd intracellular loop, and a palmitoylated Cys in the C-terminal tail. Redrawn from Gether (2000) by Balu Jegganathan.



Family C. Metabotropic neurotransmitter/ Calcium receptors

Figure 1. Continued.

& Hargrave, 1995), (Unger & Schertler, 1995). Recently, high resolution x-ray diffraction of the 3D crystal structure of bovine rhodopsin in inactive conformation at 2.8 angstrom has confirmed the predicted seven transmembrane structure (Palczewski *et al.*, 2000).

GPCR diversity of function and ligands

GPCRs are the most common signal transduction system in animals (Bouvier, 2001). Their endogenous ligands perform or mediate a wide range of functions, including neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, and may act as neuropeptides, polypeptide hormones, and inflammatory mediators. These receptors respond to a variety of bioactive molecules, including biogenic amines, peptides, glycoproteins, lipids, nucleotides, amino acids, and ions (Gether, 2000; Iismaa *et al.*, 1995; Bouvier, 2001). An emerging family of GPCRs, the protease-activated receptors, has also been discovered. Following a specific proteolytic cleavage of the N-terminus, the new N-terminal region interacts with other domains to activate the receptor (Derian *et al.* 2002). The majority of bioactive molecules, hormones, and neurotransmitters transduce their signal through specific interactions with GPCRs which activate cellular signal transduction mechanisms (Iismaa *et al.*, 1995; Gether & Kobilka, 1998). GPCRs are the principal signal transducers for the sensation of external stimuli such as light, odors, and taste (Gether & Kobilka, 1998), (Gether, 2000).

Overview of signaling

GPCR signal transduction pathways classically consist of three elements: the receptor, a heterotrimeric G protein (consisting of α, β, and γ subunits), and downstream effector components. Upon binding an external ligand, GPCRs transmit a signal to the interior of the cell. Ligand binding promotes allosteric interactions with a cytosolic G protein. The GPCR activates the heterotrimeric G protein, resulting in its dissociation into separate monomeric α and heterodimeric βγ subunits, which modulate the effector units in a signal transduction pathway that involves the release or production of other second messengers (Bouvier, 2001). Each receptor may activate a large number of G proteins before the signal is terminated, allowing amplification of the external signal (Uings & Farrow, 2000). Upon ligand binding, GPCRs undergo a conformational change that allows them to bind and activate a specific class of heterotrimeric G-proteins. Although the mechanisms effecting the conformational changes in the receptor upon ligand binding are not well understood, certain key elements have been elucidated. Current research suggests that activation changes the relative orientation or position of several TM segments, which exposes previously inaccessible key residues on the intracellular surface of the receptor critical for interacting with specific G proteins (Wess, 1997). Studies suggest that activation of rhodopsin involves a small outward movement of TM III and a clockwise rotation of TM VI along with an outward movement of the cytoplasmic end (Wess, 1997).

Upon activation, the G protein exchanges a bound molecule of GDP for GTP, and dissociates into the activated α and βγ subunits. The α subunit is known to activate

downstream elements in a signal transduction pathway, including adenylate cyclase, guanylate cyclase, phospholipase C, phospholipase A2, phosphodiesterases, and Ca^{2+} and K^{+} channels (Strader *et al.*, 1989). The role of the βγ subunit in cell signaling is more complex and not as well understood as that of the α subunit. In addition to direct signaling it has also been shown to modulate signal transduction by receptor crosstalk. Stimulation of the Gαi-coupled adenosine A1 and α2c-adrenergic receptors in *Cercopithecus* Origin-deficient SV40 transformed (COS) cells leads to the enhancement of inositol phosphate signaling from Gαq coupled receptors that is mediated by Gβγ exchange (Quitterer & Lohse, 1999).

Classes of GPCRs

GPCRs have been classified into 3 major (A, B, C) and 3 minor (D, E, F) classes or families. Family A is the largest and consists of the receptors related to the rhodopsin and β-adrenergic receptors, including most kinin receptors and the tick myokinin receptor. Family A receptors are commonly modeled based on the structure of rhodopsin, which is intermediate in size among Family A and is considered typical of this family. Family A receptors have a disulfide bridge between cysteine residues in the 1st and 2nd extracellular loop, and the highly conserved DRY sequence (or similar, see page 12) on the amino-terminal end of the 2nd intracellular loop (Fig. 1). Family B is made up of approximately 20 receptors related to the glucagon receptor, and includes receptors for several peptide hormones (Gether, 2000). Family B is characterized by a long exracellular N-terminus of approximately 100 residues that contains several

cysteines, which are assumed to form a network of disulfide bonds (Ulrich *et al.*, 1998). Family C consists of the metabotropic neurotransmitter receptors and related receptors, such as the γ-aminobutyric acid (GABA) receptors, calcium receptors, and some mammalian pheromone receptors. This family is characterized by a long amino-terminus that is 500-600 residues in length (Gether, 2000).

Structure and function of Class A receptors

GPCRs are all comprised of a single polypeptide chain and have seven stretches of mostly hydrophobic residues of 20-30 amino acids, connected by alternating cytoplasmic and extracellular hydrophilic loops of varying length. The hydrophobic stretches are expected to form transmembrane domains which occur as α-helical "cylinders" traversing the membrane and are arranged such that a central pore is formed on the extracellular surface (Iismaa *et al.*, 1995).

This structural model for GPCRs is based on high and low resolution of crystal structures of rhodopsin (Schertler *et al.*, 1993), (Unger & Schertler, 1995), (Schertler & Hargrave, 1995), (Palczewski *et al.*, 2000) as well as models based on hydropathy analysis of the primary sequences of GPCRs (Strader *et al.*, 1989). Rhodopsin's N-terminus and the three loops between TM II-III, IV-V, and VI-VII are known to be on the outside surface of the plasma membrane as demonstrated by antibody labeling. The expected cytoplasmic location of the other loops has been demonstrated by their susceptibility to protease digestion and transglutaminase labeling (Hargrave & McDowell, 1992).

Most of the receptors known in this family have 1-9 consensus sites for Asn-linked glycosylation (Asn-X-Ser/Thr, X is any amino acid except Pro or Asp) in the N-terminus, with only a few exceptions. A number of receptors also have glycosylation sites in the first and/or second extracellular loops (Iismaa *et al.*, 1995). The rhodopsin and β-adrenergic receptors (**βAR**) (Hargrave & McDowell, 1992), (Strader *et al.*, 1989) as well as several others are known to be glycosylated (Iismaa *et al.*, 1995). The glycosylation does not appear to have a function in ligand binding or receptor activation, at least in the β2-adrenergic and m2 muscarinic acetylcholine receptors (Iismaa *et al.*, 1995). Glycosylation is important for trafficking and expressing the receptor at the cell surface, and may be necessary for proper protein folding and function in some cases. Insertion of the βAR in the membrane is dependent on glycosylation (Strader *et al.*, 1989). Removal of one of the N-terminus glycosylation sites in rhodopsin by point mutation (Thr_{17}→Met) is the cause of a degenerative disease in the human retina (Hargrave & McDowell, 1992).

Almost all GPCRs have a disulfide bond between two Cys residues on the first and second extracellular loops, respectively (Iismaa *et al.*, 1995). The presence of these two cysteine residues is invariant in all rhodopsins, and is necessary for the proper folding, glycosylation, and function of rhodopsin (Hargrave & McDowell, 1992). Replacement of either of these residues in the βAR or the muscarinic acetylcholine receptor results in altered function and binding characteristics (Strader *et al.*, 1989), (Iismaa *et al.*, 1995).

Palmitoylation of the C-terminus appears to be a common structural feature of Class A GPCRs, including rhodopsin and the adrenergic receptors. This is expected to occur at Cys residues in the C-terminus. Rhodopsin has two adjacent Cys residues that bear palmitate in a thioester linkage. The palmitates are expected to intercalate into the lipid bilayer, creating an additional loop in the C-terminus (Hargrave & McDowell, 1992). The mutation of the palmitoylation site in the βAR results in a dramatic reduction in G protein coupling (Iismaa *et al.*, 1995). Palmitoylation also appears to be important in the trafficking of GPCRs to the cell surface. Mutation of palmitoylation sites leads to the intracellular retention of several receptor types (Qanbar & Bouvier, 2003). For a complete review of palmitoylation in GPCRs, see Qanbar and Bouvier (2003).

Extended ternary complex model of receptor activation

The most widely accepted model of GPCR activation has been the ternary complex model, which was first proposed by De Lean *et al.* (1980). This model explains the cooperative interactions between agonists, receptors, and G proteins. The model was extended to include agonist-independent activation of G proteins by GPCRs (Samama *et al.*, 1993) and the effects of different classes of agonists (full, partial, neutral, inverse) (Gether & Kobilka, 1998). The model states that the receptor exists in an equilibrium between two states, the inactive (R) and the active (R*) states. The potency of a ligand is defined by its ability to move the receptor towards the active state, while receptor activity in the absence of ligands is defined as its equilibrium point between the states.

Inverse agonists move the receptor equilibrium towards the inactive state (Wess, 1997) (Gether & Kobilka, 1998). In the case of rhodopsin, the receptor is covalently bound to its ligand (retinal), which acts as an inverse agonist until light absorption changes its conformation and it becomes an agonist (Gether & Kobilka, 1998).

Despite the usefulness of this model, recent evidence suggests that it is not an accurate model of actual GPCR action. Gether and Kobilka (1998) have proposed a model that uses three receptor states to track receptor activation. In this model, the unliganded receptor exists in a unique state **R** that can transition to $\mathbf{R^0}$ and **R***. $\mathbf{R^0}$ is stabilized by inverse agonists and **R*** is stabilized by agonists. This model allows for the observation that both regular and inverse agonists can protect receptors from denaturation and proteolysis, while the **R** state is unprotected (Gether & Kobilka, 1998).

Ligand-binding by GPCRs

GPCRs do not have a single conserved ligand-binding domain; domains involved in ligand binding are quite diverse and vary among receptors (Gether & Kobilka, 1998). Binding of ligands typically involves interactions with specific amino acids in the extracellular domains and/or the hydrophobic transmembrane core. In receptors for smaller ligands, such as the biogenic amines, the ligand binding domain appears to lie within the hydrophobic core of the protein, while binding sites for larger ligands such as peptides and proteins, include the N-terminus and extracellular loops (Gether & Kobilka, 1998).

In the βAR, ligand binding occurs in the hydrophobic core with contributions from the side chains of residues from several TM domains. Deletion of most of the predicted extracellular and intracellular regions of the βAR by oligonucleotide-directed mutagenesis does not affect its binding characteristics (Strader *et al.*, 1989). Single amino acid substitutions of Asp^{79} in TM II and Asp^{113} in TM IIII of βAR resulted in a decrease of agonist binding. Ser residues in TM V and VII, two Phe residues in TM VI, and a Tyr in TM VII also appear to be critical for agonist binding (Strader *et al.*, 1989).

As far as we know, no studies of ligand binding sites have been done for arthropod neuropeptide receptors. Among mammalian neuropeptide receptors, the closest known analog to the insect kinin receptors are the neurokinin receptors. Site-directed mutagenesis of the neurokinin 2 (NK2) receptor revealed four residues in transmembrane domains that are critical for ligand binding. All of these residues are near the extracellular side of their respective transmembrane domains. Mutations of Gln109 (TM III), Ile202 (TM V), and Gly273 (TM VI) abolished the ability of NK2 receptor to bind neurokinin A, however the mutant receptors were still able to bind the antagonist SR48968. Mutation of His198 (TM V) abolished both neurokinin A and antagonist binding (Bhogal *et al.*, 1994).

The second extracellular loop appears to confer ligand selectivity in some Class A GPCRs. In mammalian systems, two receptors mediate the actions of cholecystokinin and gastrin. The cholecystokinin B receptor binds both gastrin and sulfated cholecystokinin peptides with high affinity, while the cholecystokinin A receptor (CCKAR) binds only the cholecystokinin peptides. Studies with chimeras of these two

receptors showed that replacement of the second extracellular loop resulted in the selective loss of gastrin affinity. Site-directed mutagenesis of this loop in the CCKBR indicated a sequence of 5 residues confers the majority of the selectivity for gastrin (Silvente-Poirot & Wank, 1996).

Conformational changes upon receptor activation

It is theorized that upon agonist binding, conformational changes in GPCRs result in the exposure of residues in the second and third intracellular loop that are critical for interaction with G proteins. Very little is known about the mechanisms by which agonist binding leads to the conformational changes necessary for an activated receptor (Gether & Kobilka, 1998). The nature of the activating ligand can affect the G protein coupling profile, suggesting that the precise structure of the activating receptor conformation may depend on the molecular properties of the ligand (Wess, 1997).

On the cytoplasmic side of TM III in all class A GPCRs is a highly conserved DRY motif that is key to the conformational changes involved in receptor activation. In some receptors this motif is present as ERY (Gether 2000) or ERH (Larhammar, 1996), but in every case the arginine is conserved (Iismaa *et al.*, 1995). It is believed that this motif is located in a hydrophilic pocket formed by polar residues from TM I, TM II, and TM VII, and that upon agonist binding protonation of the aspartic acid residue causes the arginine to shift out of the hydrophilic pocket (Gether & Kobilka, 1998). This shift is key to the exposure of residues in the intracellular loops that promote G protein activation. This hypothesis of receptor activation is supported by studies showing that

mutation of the aspartic acid residue to aspartate in the α_{1B} and the β_2 adrenergic receptor, as well mutation of the corresponding glutamic acid residue to glutamine in rhodopsin causes constitutive receptor activation (Arnis *et al.* 1994, Gether 2000).

Activation of rhodopsin has been analyzed by several spectroscopy methods, which have consistently shown conformational changes resulting in the movement of TM III and TM VI (Gether & Kobilka, 1998). TM III makes a relatively small movement, whereas TM VI rotates in a counter-clockwise direction (as viewed from the extracellular surface) , and the cytoplasmic end moved away from TM3 (Gether & Kobilka, 1998). Spectral analysis of mutant β_2 adrenergic receptors also indicates that TM3 and TM6 rotate in a counter-clockwise direction upon agonist binding (Gether & Kobilka, 1998).

Interactions with G proteins

Interactions between GPCRs and G proteins occur at the cytoplasmic surface of the receptors. In general, regions critical for these interactions have been localized to the second and third intracellular loops and the C-terminus (Beck-Sickinger, 1996) (Gether & Kobilka, 1998). In rhodopsin, proteolytic digestion of the third intracellular loop abolished its ability to activate transducin (Findlay & Pappin 1986), (Strader *et al.*, 1989). Deletion mutagenesis has also shown that the C- and N-terminal ends of intracellular loop 3 of βAR are critical for coupling to G_s and the activation of adenylyl cyclase, although deletions from the middle of this loop did not affect the ability of the receptor to stimulate adenylyl cyclase (Strader *et al.*, 1989). Additionally, single-residue

substitutions in intracellular loops 1 and 2 have been shown to attenuate adenylyl cyclase stimulation by βAR (Strader *et al.*, 1989).

The sequence of the intracellular regions of GPCRs that are expected to interact with G proteins are highly divergent, and no consensus sequence for G protein recognition has been identified. It is suspected that it is the secondary structure of the intracellular regions that is critical for G protein interactions; these domains are expected to form amphipathic alpha-helices with a conserved secondary structure that form G protein interaction sites (Albert & Robillard, 1993).

Each member of the Gα family of proteins has a single guanine-nucleotide binding site and intrinsic GTPase activity (Fields & Casey, 1997). There are multiple Gα subunits that can be grouped into 4 major groups, $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12/13}$ (Hamm, 1998), (Fields & Casey, 1997). Members of the $G\alpha_i$ subfamily generally inhibit adenylate cyclase, while $G\alpha_s$ stimulates it. Gαq activates phospholipase C-β, which converts phosphatidylinositol 4,5-diphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 causes the release of intracellular calcium, and DAG activates protein kinase C (Lewin, 1997). The functions of $G\alpha_{12/13}$ subunits remain mostly unknown, but they do appear to stimulate cell growth (Fields & Casey, 1997). In murine fibroblasts, $G\alpha_{12}$ activates the RhoA protein in a signal transduction pathway that leads to transcriptional activation and cellular transformation (Fromm *et al*., 1997).

Heterotrimeric G proteins are classified by the α subunit because of the historical view that this is the functional component, although it is now clear that the βγ heterodimer subunit also interacts with receptors and effectors (Fields & Casey, 1997).

Analysis of the human genome shows there are 27 α, 5 β, and 14 γ G protein subunits. Based on these numbers, 1890 possible combinations of heterotrimers are possible, although some combinations do not form *in vitro* (Albert & Robillard, 1993). However, the huge variety of G protein combinations available and receptor selectivity for them contributes to the complexity of signal transduction by GPCRs.

The first level of selectivity of GPCRs for G proteins is determined by the Gα subunit. Generally, each family of receptors will interact with only one Gα group, although there are some receptors that activate more than one (Albert & Robillard, 1993). In most cases, activated GPCRs interact with only a limited set of G proteins, as defined by the Gα subunit. The selectivity of receptors for G proteins seems to be the result of many factors, including receptor and G protein sequence, structure, density, localization of specific heterotrimers (Wess, 1997), and crosstalk from other receptors (Quitterer & Lohse, 1999) and the composition of the Gβγ subunit (Albert & Robillard, 1993). Studies of the $G\alpha_{i/0}$ family suggest that the ability of receptors to discriminate between the highly related members of this family is not due to the α subunit alone; the β and γ are much more divergent, indicating that it is the heterotrimer as a whole that confers specificity (Albert & Robillard, 1993).

Studies with hybrid GPCRs have indicated that G protein selectivity is primarily determined by residues in the 2nd intracellular loop and in the C- and N-terminal portions of intracellular loop 3, with contributions in some cases by the C-terminal tail (Wess, 1997). Biochemical studies using synthetic peptides corresponding to receptor segments generally agree well with the chimeric studies, and have shown that peptides

corresponding to intracellular loop 2 and the C/N terminal regions of intracellular loop 3 can mimic or inhibit receptor interactions with G proteins (Wess, 1997). In members of the muscarinic acetylcholine receptor family, a few hydrophobic residues at the N-terminus of intracellular loop 3 seem to play a key role in G protein selectivity. These residues are predicted to form part of the hydrophobic side of an amphiphilic α-helix along with an aromatic residue that is critical for selectivity of $G_{q/11}$ proteins (Wess, 1997).

Receptor conformation also seems, not surprisingly, to be important for G protein recognition. In receptors that couple more than one class of G protein, such as the α_{2A}-adrenergic and luteinizing hormone (LH) receptors, point mutations in regions other than the receptor/G protein interface have been shown to abolish coupling to one class of G protein (Wess, 1997).

The receptor-G protein interaction is not a simple case of the receptor affecting the G protein, as binding of the G protein also affects the receptor. Generally, dissociation of the G protein from the receptor decreases its affinity for the agonist (Fields & Casey, 1997). Wild-type βAR has a higher affinity for its agonist when G_s is bound than under conditions that disrupt G protein coupling (Strader *et al.*, 1989).

After a GPCRs activates a G proteins, the activity of the G protein may be modulated by a Regulator of G protein Signaling protein (RGS). These proteins bind directly to activated Gα subunits and stimulate their GTPase activity, leading to a rapid deactivation of their signal (Hollinger & Hepler, 2002).

Regulation/Desensitization of GPCRs

Regulation of GPCRs can occur at the transcriptional, translational, and receptor protein levels, with the latter being best understood. Upon prolonged exposure to agonists, GPCRs often show a diminishment of response, often known as desensitization or adaptation. This down-regulation can be the result of multiple independent processes such as desensitization, sequestration, and degradation. Desensitization and sequestration can occur in a matter of milliseconds to minutes, while down-regulation by degradation usually requires hours and results in the loss of receptors from cells. Desensitization can be due to regulation at the level of the receptor, G protein, and downstream effectors. However, most desensitization seems to result from the regulation of the receptor so as to impair its ability to activate G proteins (Pitcher *et al.*, 1998).

Desensitization is evident in diverse biological processes involving GPCRs including bacterial chemotaxis, mating responses in yeast, light perception in *Drosophila*, and neurotransmission (Pitcher *et al.*, 1998). Three families of proteins have been found that participate in receptor desensitization: G protein-coupled receptor kinases (GRKs), arrestins, and second messenger-regulated kinases such as PKA and PKC (Lefkowitz, 1998).

The major mechanism of rapid desensitization is receptor phoshorylation by a GRK followed by the binding of an arrestin (Lefkowitz, 1998). In mammals, seven specific GRKs have been found that primarily phosphorylate GPCRs, resulting in a profound impairment of receptor function (Pitcher *et al.*, 1998; Claing *et al*., 2002). In

rhodopsin, Ser residues are phosphorylated by rhodopsin kinase in a light-dependent manner. Phosphorylation allows the receptor to bind arrestin, which prevents interaction with transducin (Strader *et al.*, 1989). Several receptor kinases that are theorized to be involved in desensitization have been found, and most class A receptors have multiple Ser and Thr residues in the C-terminal tail that may serve as phosphorylation sites. However, phosphorylation is not always required for receptor desensitization by GRKs. In some cases, GRK binding alone is enough to inhibit receptor activity in a phosphorylation-independent mechanism (Dicker *et al*., 1999).

The study of constitutively active mutant GPCRs has also contributed to our understanding of receptor regulation. Several discrete mutations have been found in the C-terminus of the third intracellular loop of many receptors that result in constitutive activation (Wess, 1997; Gether & Kobilka, 1998). One explanation of this observation is the possibility that these mutations alter the normal conformation of the receptor in a way that mimics the opening of the intracellular surface normally associated with activation (Wess, 1997). Alternatively, important conformational constraints may maintain the receptor in an inactive state, and that these constraints are released upon activation allowing key residues to be exposed to the cytosolic G proteins (Gether & Kobilka, 1998).

The current model of receptor desensitization is that upon agonist exposure, GRKs phosphorylate the receptor, which allows it to bind a protein of the β-arrestin family (or for rhodopsin, arrestin). Binding of the β-arrestin protein prevents the receptor from interacting with and activating its corresponding G protein (Pitcher *et al.*,

1998). This method of receptor desensitization can reduce G protein activation by as much as 70-80% in the rhodopsin and β_2-adrenergic receptors. Additionally, binding of arrestin to phosphorylated receptors appears to initiate the process of endocytosis and sequestration into recycling endosomes (Pitcher *et al.*, 1998). There are at least six members of the arrestin family, some of which are found in many tissues and some only in the retina.

The binding affinity of the β_2-adrenergic receptor for β-arrestin is increased 10-30 times following phosphorylation by GRKs, whereas agonist binding alone has much less of an effect (Lefkowitz, 1998). GRK activity appears to be regulated by several processes, including binding by activated receptors and feedback from the βγ subunit of activated G proteins. Other factors that regulate activity include PKC, lipids, and the calcium–binding proteins recoverin and calmodulin (Lefkowitz, 1998). It appears that the free βγ subunit and phosphatidylinositol bisphosphate may interact with a pleckstrin homology domain in the C-terminus of GRK2 or GRK3, and translocate the GRK to the activated receptor. Different βγ isoforms have a preferential affinity for either of the GRKs, which may lend specificity to the receptor interaction (Lefkowitz, 1998).

Desensitization of GPCRs by their second-messenger regulated kinases provides a feedback mechanism to regulate receptor activity. Both PKA and PKC, activated by $G\alpha_s$ and $G\alpha_q$ respectively, can directly phosphorylate GPCRs. This phosphorylation interferes with G protein interactions by modifying the conformation of the receptor (Lefkowitz, 1998). This phosphorylation occurs at serine residues in the third intracellular loop and C-terminus of the β_2-adrenergic receptor (Lefkowitz, 1998), and

many receptors have multiple serines or threonines in these regions, as well as consensus sites for phosphorylation by PKA or PKC (Iismaa *et al.*, 1995). However, desensitization by second-messenger related kinases is not considered agonist specific, because other pathways may activate these proteins resulting in heterologous desensitization.

Sequestration of GPCRs by endocytosis may occur by the clathrin-coated vesicle pathway, or non-clathrin pathways (Lefkowitz, 1998). For at least some GPCRs, phosphorylation by a GRK and binding of arrestin are crucial to receptor sequestration. It has been demonstrated that β-arrestins 1 and 2 directly bind clathrin with high affinity, and most likely act as an adaptor in the clathrin-coated vesicle mediated endocytosis of GPCRs (Lefkowitz, 1998; Claing *et al*., 2002).

Oligomerization of GPCRs and Receptor Activity Modifying Proteins (RAMPS)

The classical view of GPCR function is that only receptor monomers are responsible for interacting with ligands and effecting receptor functions. However, recent evidence that GPCRs form dimers (the simplest form of oligomer), and that these dimers may serve important functions is challenging this view. Some of the strongest evidence for GPCR function as oligomers has come from trans-complementation studies, where a restoration of function is shown when two mutant or chimeric receptors are expressed together that have no function by themselves. For example, chimeric α2-adrenergic/M3 muscarinic receptors composed of the first five transmembrane domains of one receptor and the last two of the other showed no binding or function when

expressed alone. However, coexpression of the two chimeras restored binding and signalling in response to both adrenergic and muscarinic agonists (Bouvier, 2001). A similar restoration of function results from the co-expression of two binding defective angiotensin II receptor mutants (Bouvier, 2001).

Dimerization of receptors may explain the complex pharmacological profiles seen in some receptors that cannot be explained by the ternary complex model. For example, among opioid receptors there are many subtypes which have been identified pharmacologically, but for which no gene or cDNA has been found. These receptor subtypes may in fact be heterodimers of known opioid receptors for endogenous opioid peptides that have not yet been discovered (Bouvier, 2001).

In addition to the functional evidence of dimerization in mutant receptors, there is also biochemical evidence for oligomerization of wild-type receptors both *in vitro* and *in vivo* in intact cells. Solubilized β_2-adrenergic receptors differentially expressing Myc- or HA-tags may be co-immunoprecipated with either anti-HA or Myc antibodies, indicating intermolecular interaction between the receptors (Bouvier, 2001). This approach has also been used to demonstrate dimerization in $GABA_B$, mGluR5, δ-opoid, calcium, and M3 muscarinic receptors (Bouvier, 2001). Additionally, the β_2-adrenergic, δ-opoid, mGluR5, and calcium receptor all migrate as molecular species of twice the expected molecular mass during SDS-PAGE (Bouvier, 2001).

In living cells, fusion constructs between receptors and bioluminescent or fluorescent proteins have been used to show receptor homodimerization through fluorescent or bioluminescent resonance energy transfer (FRET or BRET, respectively).

Homodimers have been detected by BRET or FRET in the absence of agonist, indicating that some GPCRs can form constitutive dimers *in vivo* (Bouvier, 2001). These studies indicate that homodimers of GPCRs occur naturally in cells, however, there are conflicting results concerning heterodimers between different receptor types. Some research indicates that they are biochemical artifacts resulting from the handling of the cells, while other research has found evidence for functional heterodimers (Bouvier, 2001).

Studies on the regulation of receptor dimerization have highly variable results depending on the receptor type. Exposure to agonists has been shown to increase, decrease, or have no effect on dimerization in various GPCRs (Bouvier, 2001).

There is clear evidence that dimerization is critical for the proper folding and cellular transport of the $GABA_B$ receptor. The expression of two isoforms of this receptor, $GABA_BR1$ and $GABA_BR2$, is absolutely required for receptor function. When both are present, the proteins express at the cell surface and allow a functional response to GABA. When expressed alone, $GABA_BR1$ is retained intracellularly as an immature glycoprotein whereas $GABA_BR2$ reaches the cell surface but cannot bind GABA or effect signal transduction (Bouvier, 2001).

The issue of how receptor dimerization may effect signal transduction remains highly debated. The requirement of $GABA_BR1$ presence before $GABA_BR2$ can bind agonist strongly suggests the formation of a dimmer, although it may just be that $GABA_BR1$ is necessary for the proper folding of this receptor. A peptide derived from the proposed dimerization interface of the β_2-adrenergic receptor has been shown to

inhibit dimerization and the stimulation of adenylyl cyclase by this receptor. Additionally, FRET studies of the LH receptor have shown FRET between wild-type receptors but not between mutants that are capable of binding agonist but are unable to induce signal transduction. This may indicate that the mutants are unable to form dimmers, and thus are unable to transmit the signal.

Although current knowledge is too limited to propose a molecular model for the formation of receptor dimers, they clearly exist and may add a level of complexity to GPCR function that is currently beyond our understanding. What is clear is that as we gain more information, GPCR function appears to be more complex than previously imagined. Novel protein-protein interactions between GPCRs and receptor activity modifying proteins (RAMPS) have been shown to be involved in trafficking of the calcitonin receptor to the cell surface and its phenotypic expression. RAMPS can modify receptor phenotype by causing changes in receptor glycosylation and by interacting directly with the receptor to define the ligand-binding pocket (Sexton *et al.*, 2001). The recent identification of the RAMP family of proteins may cause speculation on the existence of other receptor modulating proteins.

CHAPTER II

CLONING AND TRANSCRIPTIONAL EXPRESSION OF THE FIRST ARACHNID NEUROPEPTIDE RECEPTOR FROM THE SOUTHERN CATTLE TICK, *Boophilus microplus* (ACARI: IXODIDAE)[*]

Introduction

The southern cattle tick or cattle fever tick, *Boophilus microplus*, is the most important tick-pest of cattle in tropical and sub-tropical regions of the world because of its ability to transmit *Babesia spp.*, the hemoparasites that cause cattle fever (Nunez *et al.,* 1985). Ticks cause greater economic losses in livestock production worldwide than any other group of external parasites (Bowman *et al.,* 1996). The compounded economic impact of cattle fever and tick parasitism prior to their eradication from the southern U. S. A. in 1960 was estimated at over one billion dollars annually (Graham & Hourrigan 1977). However, the ongoing detection of sporadic outbreaks of this tick in southern Texas is evidence that reintroduction of this pest to the U.S. presents a serious threat (Anonymous, 2000). Control of *B. microplus* has become increasingly difficult worldwide because it has rapidly developed resistance to pesticides. In Australia, where strains of the tick are resistant to all commonly used acaricides (Baxter & Barker, 1999), the loss to the cattle industry is one hundred million dollars annually (Angus, 1996). In

[*] Text and figures reprinted with permission from "Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae)" by Holmes *et al*., 2000. *Insect Molecular Biology*, 9, 457-465. Copyright 2000 by Blackwell Science Ltd.

Mexico, resistance to organophosphates (Rosario-Cruz *et al.,* 1997) and pyrethroids (He *et al.,* 1999) has been reported.

One effective control strategy for the southern cattle tick may be endocrine disruption. Mimetics of ecdysone and juvenile hormone, such as tebufenozide and methoprene, respectively, are effective insecticides (Wing *et al.,* 1988; Jones, 1995). However, a third group of currently unexploited hormones, the peptide hormones, may have a great potential for control because they are master regulators and affect a number of physiological processes (Keeley & Hayes, 1987). In order to develop neuropeptide mimetics useful in pest control, the chemical and conformational requirements of neuropeptide-receptor interactions must be understood (Nachman *et al.,* 1993). Although many neuropeptides have been isolated from insects (Nässel, 1996), very few of their receptors have been identified.

The myokinins or leucokinin-like peptides are a family of neuropeptides that have been found in several arthropod and invertebrate groups (Nässel, 1996). They have myotropic and diuretic activity in insects. Myokinins stimulate hindgut contractions in the cockroach, cricket, and locust (Holman *et al.,* 1987; Holman *et al.,* 1990; Schoofs *et al.,* 1992) and increase the rate of secretion in Malpighian tubules (Pannabecker *et al.,* 1993; Veenstra *et al.,* 1997; O'Donnell *et al.,* 1998; Cady & Hagedorn, 1999a; Holman *et al.,* 1999; Terhzaz *et al.,* 1999). Myokinins may also serve as neuromodulators of the central nervous system (CNS) (Nässel, 1996; Nässel 2002). Because of their multifunctional activities, myokinin receptors may represent an excellent target for the development of acaricidal and insecticidal mimetics. The first member of the myokinin

or leucokinin-like receptor subfamily is the lymnokinin receptor, cloned from a mollusc, the pond snail *Lymnaea stagnalis* (Cox *et al.* 1997). The first myokinin receptor known from an arthropod was cloned from the southern cattle tick *B. microplus*, and is the subject of this dissertation (Holmes *et al.*, 2000; Holmes *et al.*, 2003). Analysis of the *Drosophila* genome predicted that gene product CG10626 was a myokinin receptor (Holmes *et al.*, 2000; Hewes & Taghert, 2001). This prediction was shown to be correct by the functional expression of the receptor in S2 cells. Exposure to drosokinin induced a intracellular calcium response in transfected cells (Radford *et al.,* 2002). Additionally there is one report on the biochemical characterization of a 54 kDa leucokinin binding protein from the mosquito *Aedes aegypti* (Pietrantonio *et al.,* 2000), and a report on an achetakinin binding site in the Malpighian tubule of the cricket *Acheta domesticus* (Chung *et al.,* 1995).

Polymerase chain reaction (PCR) and other molecular techniques were used to obtain a cDNA of a novel myokinin receptor from the southern cattle tick, *B. microplus*. Here we report the characterization of this cDNA and show the developmental expression of this receptor by reverse transcription (RT)-PCR experiments. This work constitutes the first evidence of leucokinin-like regulated signal transduction in the Acari. The myokinin receptor is the first neuropeptide receptor to be cloned from the Acari, the second G-protein coupled receptor (GPCR) known from *B. microplus,* and the second member of the leucokinin-like receptor subfamily. In addition, based on the sequence similarity of the *Boophilus* receptor to the gene product CG10626 in the recently published *D. melanogaster* genome (Adams *et al.,* 2000), we predicted that

CG10626 may constitute the first identified insect myokinin receptor (Holmes *et al.,* 2000).

Methods

Ticks

Southern cattle ticks (*B. microplus*) were from the Gonzalez strain, a pesticide susceptible strain that is maintained at the Cattle Fever Tick Research Laboratory, USDA-ARS, Mission, TX. This strain was originally obtained from an outbreak in Zapata County, TX, in 1994. Eggs were collected after engorged females were allowed to oviposit in a humidified incubator at 24°C. Unfed larvae were collected after being raised in the incubator for ten days. Nymphs were obtained by placing larvae on cattle, then collecting them ten days after their nymphal molt. Adults were also raised on cattle and collected 15 days after their final molt. Ticks were frozen and stored at -80°C until use.

cDNA synthesis and cloning

Double-stranded cDNA was prepared by Dr. Haiqi He (USDA Southern Plains Agricultural Research Center, College Station, TX) according to the following methods. Total RNA was purified from tick larvae using TRIzol® Reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol. Poly-A^+ RNA was purified from the total RNA using an Oligotex™ mRNA Kit (Qiagen, Santa Clarita, CA).

Double-stranded cDNA was synthesized using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA).

PCR reactions were conducted in a PTC-200 Peltier Thermal Cycler or PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). Oligonucleotide primers were synthesized by Genosys Inc. (Houston, TX). All reactions were carried out in 50 µl volumes containing Clontech KlenTaq polymerase (1 µl), Clontech 10x cDNA PCR Reaction Buffer (5 µl), 0.2 mM each of four dNTPs and primers to final concentrations of 1 µM (degenerate) or 0.2 µM (specific primers) each. Marathon™ cDNA was used as the template for PCR with two degenerate oligonucleotide primers. Negative control reactions contained only one primer of the pair. These primers correspond to DNA sequences within the conserved regions of transmembrane regions III and VI of many G protein-coupled receptors (Cox *et al.* 1997): sense 5'-CCG GAT CCG (CT)(GC)A T(CT)(GA) (GC) (GC)I T(GT)G AC(CA) G(GC)T A-3' and antisense 5'-ACG AAT TCG G(GC) (CA) ICC A(GA)C AGA I(GC)(GA) (CT)(GA)A A-3'. PCR conditions were: initial denaturation at 94°C for 1 min followed by 8 cycles of 94°C for 20 sec, and an annealing/extension step of 72°C for 1.5 min that was decreased by 0.5°C per cycle, followed by 40 cycles of 94°C for 20 sec (denaturation), 68°C for 1.5 min (annealing/extension) then a final extension of 5 min at 68°C.

RACE PCR

Four sequence specific primers were designed to amplify the 5' and 3' ends of

the cDNA in RACE (rapid amplification of cDNA ends) PCR. Sense primer sequences were: 1F, (5'-AGT TCA TCA TCT GCG GTA TCT GGA C-3'); 2F, (5'-GTC TCA CGC GCT CAA CCT GAC TAA G-3'). Antisense primers: 3R, (5'-CAG ACA CAC GAG GAC GTG GTT ATA G-3'); 4R, (5'-GTA CAC GAA ACA GAT GGT GAG CAG C-3'). Primers 2F and 3R corresponded to sequence regions interior to those of primers 1F and 4R, respectively. During synthesis of Marathon™ cDNA, adaptors were ligated to the ends of all cDNAs. Primers complementary to the adaptor sequence AP1 (external) and AP2 (internal) are supplied with the Marathon kit. Primers 1F and AP1 were used in the initial 3' RACE PCR. Primers 4R and AP1 were used in 5' RACE.

Initial RACE products were diluted and amplified in a nested 3' RACE reaction with 2F and AP2 primers and a nested 5' RACE reaction with primers 3R and AP2. Final primer concentrations were 0.2 μM in 50 μl volumes. Cycling parameters for the initial RACE were: 94°C for 1 min, 5 cycles of 94°C for 20 sec and 72°C for 1.5 min, 5 cycles of 94°C for 20 sec and 70°C for 1.5 min, 26 cycles of 94°C for 20 sec and 68°C for 1.5 min, and a final extension step of 68°C for 5 min. Cycling parameters for the nested RACE reactions were: 94°C for 1 min, 25 cycles of 94°C for 20 sec and 70°C for 1.5 min, and a final extension step of 68°C for 5 min. Products were separated on an agarose gel and bands of the sizes 2.0 kb from the 3' reaction and 0.8 kb from the 5' reaction were cut. The DNA was extracted using the Qiagen Quickgel extraction kit. PCR products were cloned into pCR 2.1 or pCR-TOPO plasmid vectors (Invitrogen, Carlsbad, CA) and electroporated into Electrocomp™ Topo 10F' cells or heat-shocked

into Ultracomp™ Topo 10F' cells (Invitrogen), respectively. Positive colonies were selected with blue-white screening and plasmids were purified using Wizard™ Plus Minipreps (Promega Corp., Madison, WI).

Sequencing reactions were performed with AmpliTaq® DNA polymerase and fluorescent dideoxynucleotides according to manufacturer's protocols (PE Applied Biosystems 1998), and the reaction products were electrophoresed and analyzed on an automated DNA sequencer (Applied Biosystems model 373) by the Gene Technologies Laboratory at Texas A&M University. Sequences were analyzed using DNASTAR software (DNASTAR, Inc., Madison, WI).

Semi-quantitative RT-PCR

Methods used for semi-quantitative RT-PCR were similar to those described by Dozois *et al.* (1997). Whole tick samples (50 mg) of each life stage (eggs, larvae, nymphs, and adults of both sexes) were ground under liquid nitrogen with a mortar and pestle. Poly-A^+ RNA was purified from the ground samples using a Dynabeads® mRNA Direct kit (Dynal, Oslo, Norway). First strand cDNA was synthesized from one tenth of the Poly-A^+ RNA at 42°C for 50 min with oligo$(dT)_{12\text{-}18}$ primer using Superscript II Reverse transcriptase (Superscript Preamplification System, Life Technologies). PCR amplification of β-actin cDNA was performed using primers Act-3F, 5'-TCC TCG TCC CTG GAG AAG TCG TAC-3', and Act-4R, 5'-CCA CCG ATC CAG ACC GAG TAC TTC-3' specific to the *B. microplus* β-actin gene. The gene sequence was obtained by PCR using primers that correspond to conserved regions of

insect actin genes (H. He, unpublished). Reactions contained one-tenth (2 µl) of the synthesized cDNA, 200 µM each of four dNTPs, 0.2 µM of each primer, 1 µl Taq polymerase, and 1X reaction buffer (Boehringer Mannheim) in a final volume of 50 µl. Taq was added after the reaction was brought to 94°C. The following cycling parameters were used: 94°C for 1 min followed by 25 cycles of 94°C for 20 sec, 62°C for 30 sec, and 72°C for 45 sec. PCR amplification of receptor cDNA was performed using primers designated SC2-F, 5'-CTC CGG GAA GTT TTC CTA AAG A-3', and SC3-R, 5'-TGG TGG TTG GAC TCA AAT TAC AC-3'. PCR conditions were identical to the actin PCR amplification except that 35 cycles were used to amplify the receptor cDNA. Five microliters of each actin reaction and 20 µl of each receptor reaction were electrophoresed on 1% TBE agarose gels containing ethidium bromide. Gels were photographed with Polaroid film and the images were scanned with a Hewlett Packard ScanJet 3c. The intensity of the bands was determined using Kodak Digital Science 1D software (Kodak Scientific Imaging Systems, New Haven, CT). The relative level of receptor RNA in each tick stage was indirectly estimated by the ratio of the intensity of the receptor band to that of the actin band.

Southern blot

To assure that the RT-PCR products corresponded to the cloned receptor, each was diluted 1:500 and reamplified in a 100 µl reaction using the same reagents and concentrations as the original RT-PCRs. The following cycling parameters were used: 94°C for 1 min followed by 40 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for

30 sec. Twenty microliters of each reaction was run on a TBE 1% agarose gel that was photographed. DNA was transferred to S&S Nytran membrane (Schleicher and Schuell, Keene, NH) using standard upward blotting techniques following alkaline denaturation (Ausubel *et al.* 1995). After transfer, the blot was baked at 80°C for 30 min, then U.V. crosslinked for 3 min on a transilluminator.

In order to unequivocally confirm that the amplified products were receptor-specific, a receptor DNA fragment (base pairs 986-1304) (Fig. 2) was cloned and sequenced to serve as a template for a radiolabeled probe for Southern blots. For this, the Ambion DECAprime II™ Random Priming DNA Labeling kit (Ambion, Austin, TX) was used with α-^{32}P dCTP (NEN Life Science Products, Boston, MA). The blot was allowed to hybridize overnight at 42°C in ULTRAhyb™ (Ambion), then washed 2x 5 min in 1x SSC, 0.1% SDS at 42°C then 2x 15 min in 0.1x SSC, 0.1% SDS at 50°C. The blot was then exposed to Kodak Biomax™ ML film (Eastman Kodak, Rochester, NY).

Results

The PCR with degenerate primers corresponding to transmembrane (TM) regions III and VI, highly conserved in many GPCRs (Cox *et al.* 1997), amplified DNA products of about 450 bp and 600 bp. Comparisons to the GenBank database showed that the amplified sequence of 450 bp was most similar to those of the kinin receptor family. Nested gene-specific primers were then designed on the basis of the nucleotide sequence of the 450 bp PCR product and used in 5' and 3'(RACE) to amplify the full length

receptor cDNA. The primers were designed to encompass the majority of the 450 bp product in the 3' and 5' RACE reactions**,** respectively, to unequivocally obtain the corresponding cDNA ends. The 3' RACE product was 2 kb and the 5' RACE product was 850 bp. The sequences of the 3' and 5' RACE fragments were identical to each other in the expected region of overlap. The full-length cDNA was 2.7 kb, out of which 1541 bp are shown in Fig. 2. The complete sequence has been deposited in GenBank (AF228521).

The identified open reading frame (ORF) consists of 1194 bp and encodes a protein of 397 residues with a predicted molecular mass of 44.9 kDa (Fig. 2). Two possible start codons, beginning at positions 95 and 123, were identified within an ORF that terminates with a stop codon at position 1286. The first ATG at positions 95-97 was presumed to be the actual start codon because neighboring bases more closely matched the optimal sequence for translation initiation, GCC(A/G)CC**ATG**G (Lewin, 1997).

Blast P searches of GenBank showed that this sequence encoded a G protein-coupled receptor that was similar to the lymnokinin receptor from pond snail (40.3 % identity) (Cox *et al.,* 1997) and other invertebrate neuropeptide receptors (Fig. 3, Table 1). The predicted gene product from the *D. melanogaster* genomic sequence, CG10626, resulted **46.9 %** identical to the *B. microplus* receptor sequence. These sequences were analyzed for similarity with DNASTAR (Table 1, Fig. 3). Kyte and Doolittle hydrophilicity plots of the *B. microplus* receptor, lymnokinin receptor, and CG10626 (Fig. 4) reveal seven putative transmembrane regions. In the *B. microplus* receptor, two cysteine residues, Cys 128 and Cys 210, located in the first and second extracellular

loops, respectively, are expected to form a disulfide bond. The location of these residues is consistent in almost all GPCRs (Watson & Arkinstall, 1994; Schöneberg *et al.,* 1999). The N-terminal region contains two asparagine residues, Asp 30 and Asp 41, within a glycosylation consensus sequence N-X-S/T (Lewin, 1997). There is also a third glycosylation site, Asp-204, in the predicted second extracellular loop. Two cysteine residues in the intracellular C-terminus (Cys 348, Cys 350) represent likely sites for palmitoylation (Iismaa *et al.,* 1995).

The receptor sequence used for semi-quantitative RT-PCR experiments and synthesis of the probe for the Southern blot corresponds to the C-terminus, the region known to be the least similar among GPCRs from the same subfamilies and thus often used to identify specific receptors. In order to ensure specificity, the antisense primer was designed within the 3' untranslated region and the sense primer in a region of low similarity corresponding to the third extracellular loop (Figs. 2 and 5). Receptor messenger RNA (mRNA) was present in all life stages of the southern cattle tick as determined by semi-quantitative RT-PCR (Fig. 5A). These results suggested that receptor expression was highest in larvae and adult females. In these experiments the amount of amplified receptor cDNA produced by RT-PCR (318 bp, Fig. 5A) had been normalized by comparison with the amplification of tick β-actin (330 bp, Fig. 5A). A pixel-density analysis indicated that the maximal amplification of receptor PCR product was in females, followed by larvae, eggs, nymphs and males (Fig. 5A). The amplified products were confirmed to be identical to the cloned receptor by Southern blot (Fig.5B).

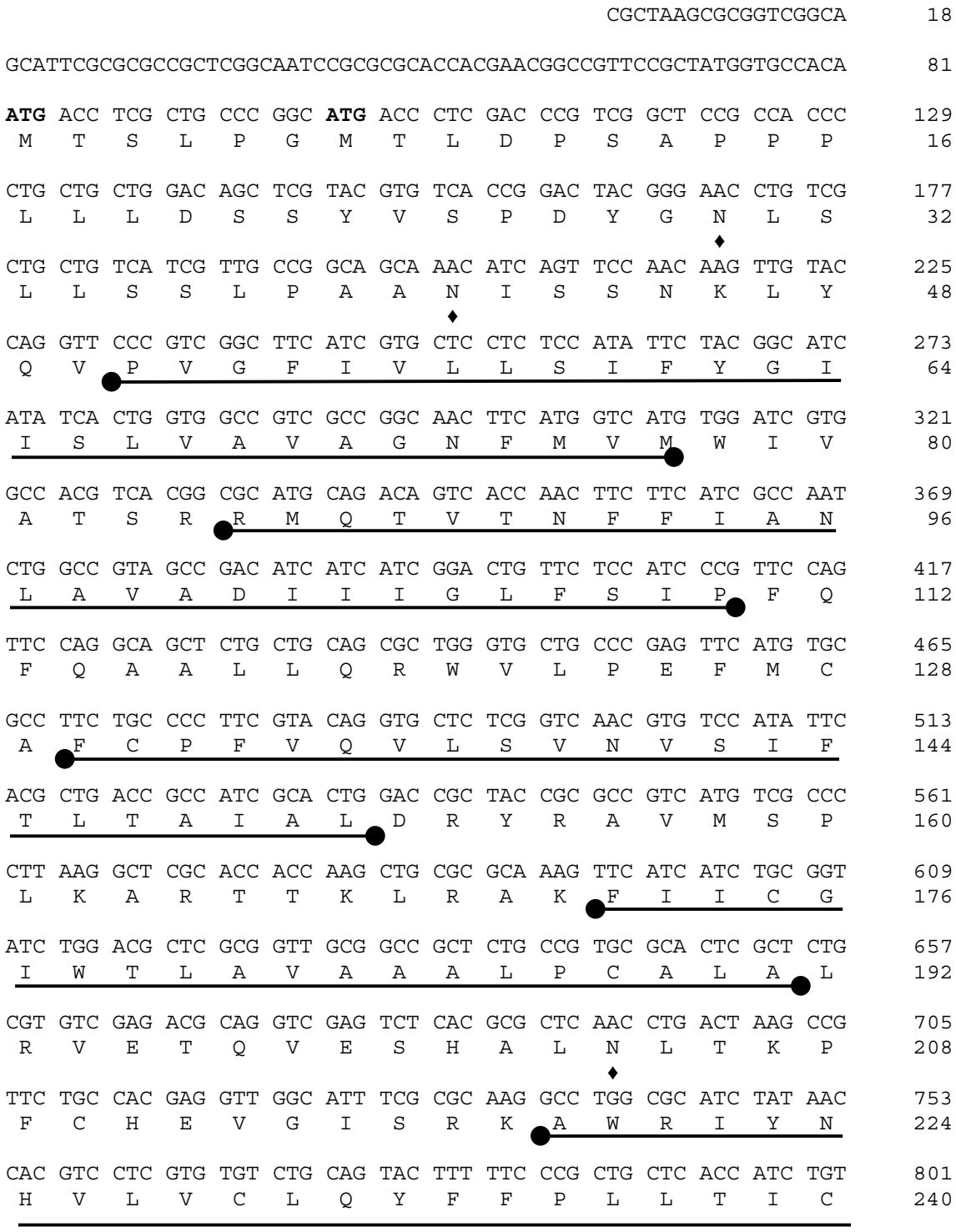


Figure 2. Nucleotide and deduced amino acid sequence of the myokinin receptor cDNA from the southern cattle tick, *B. microplus* (GenBank/EMBL accession number AF228521). Amino acids corresponding to predicted transmembrane regions have been underlined. Possible glycosylation sites are marked with a diamond.

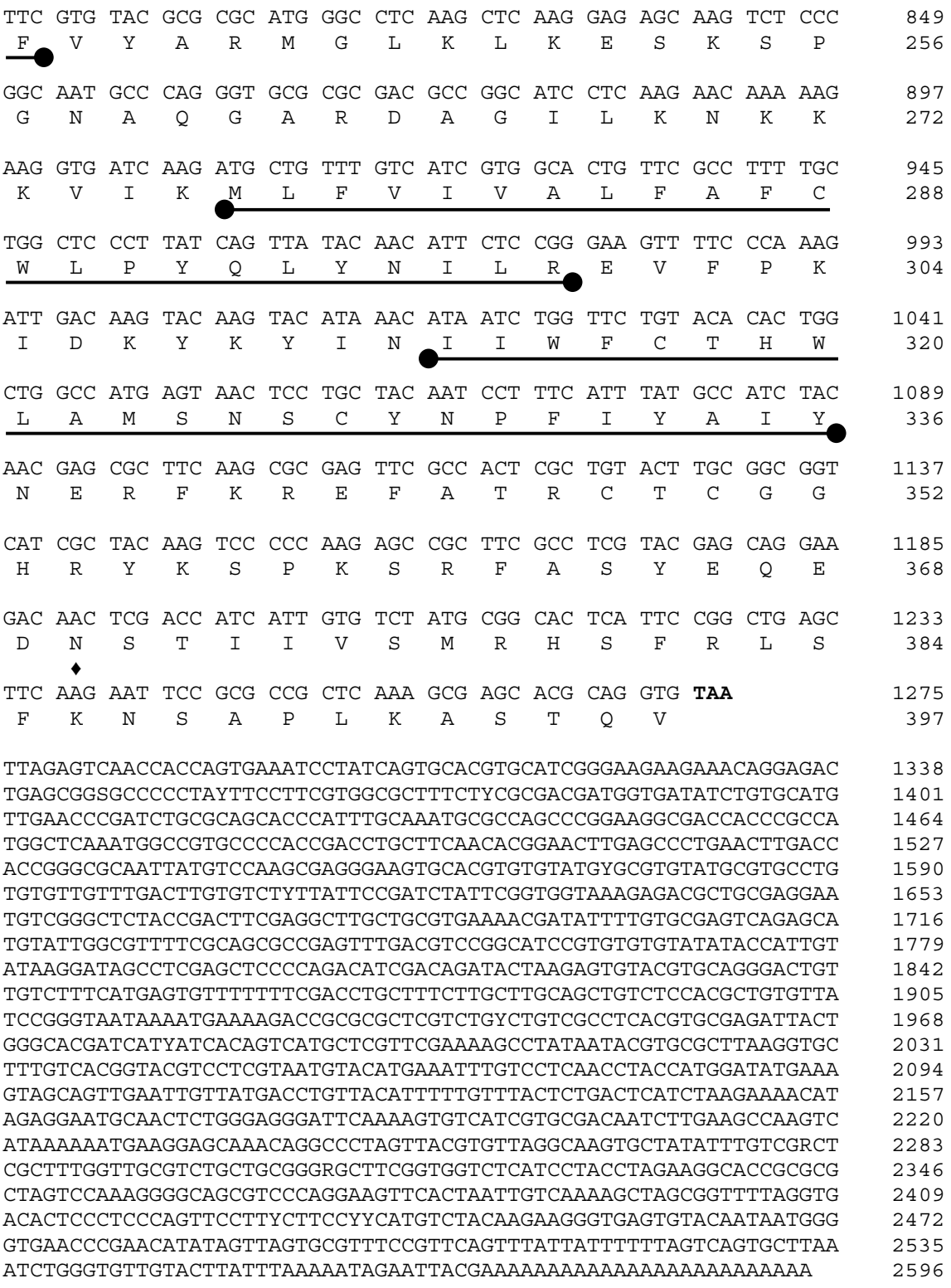


Figure 2. Continued.

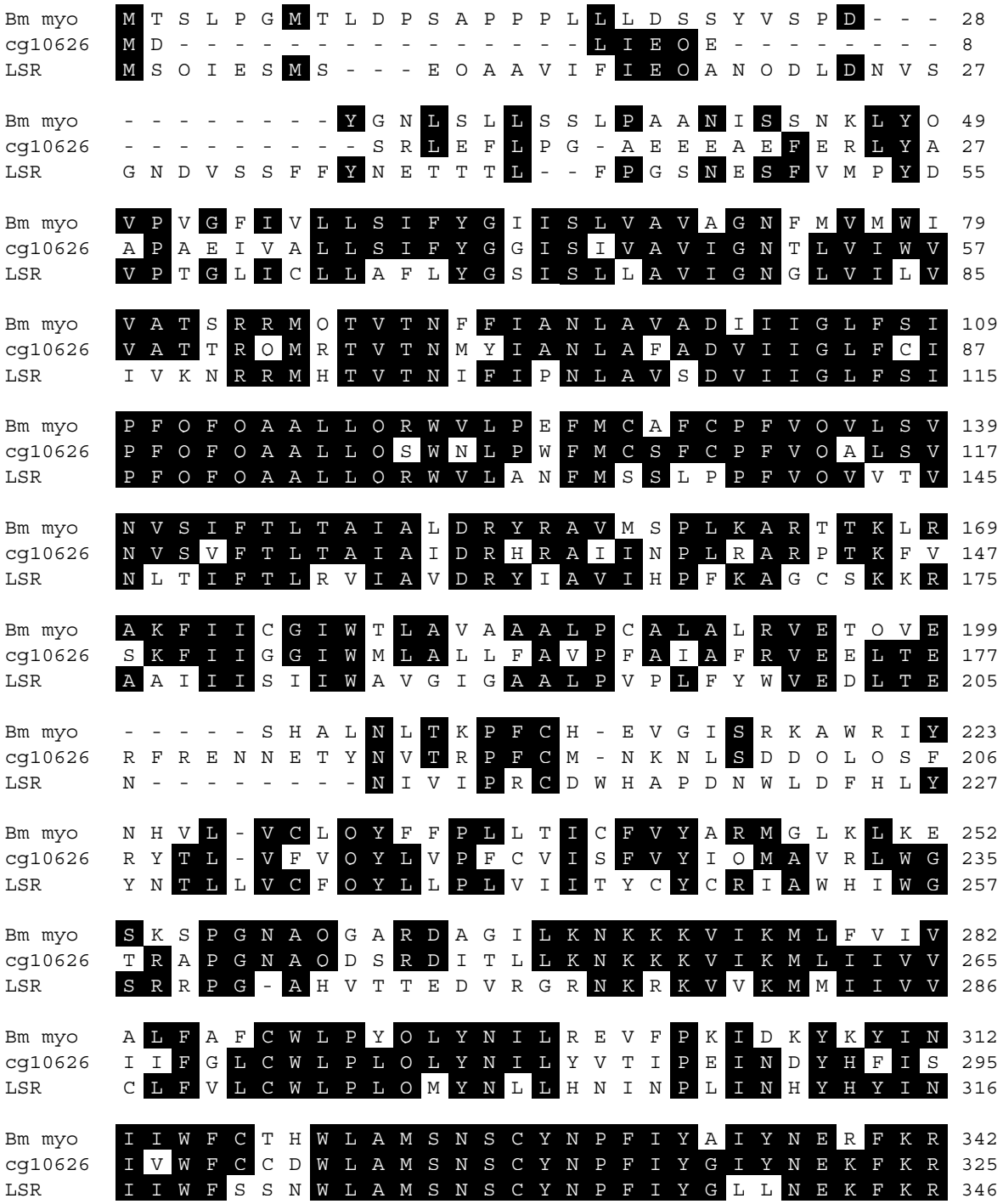


Figure 3. Amino acid alignment of the *B. microplus* myokinin receptor (**Bm myo**), with candidate invertebrate myokinin receptors. Identical residues are noted as white text on a black background. Aligned sequences are the **cg10626** gene product (*D. melanogaster* drosokinin receptor) and the *L. stagnalis* lymnokinin receptor (**LSR**) (U84499).

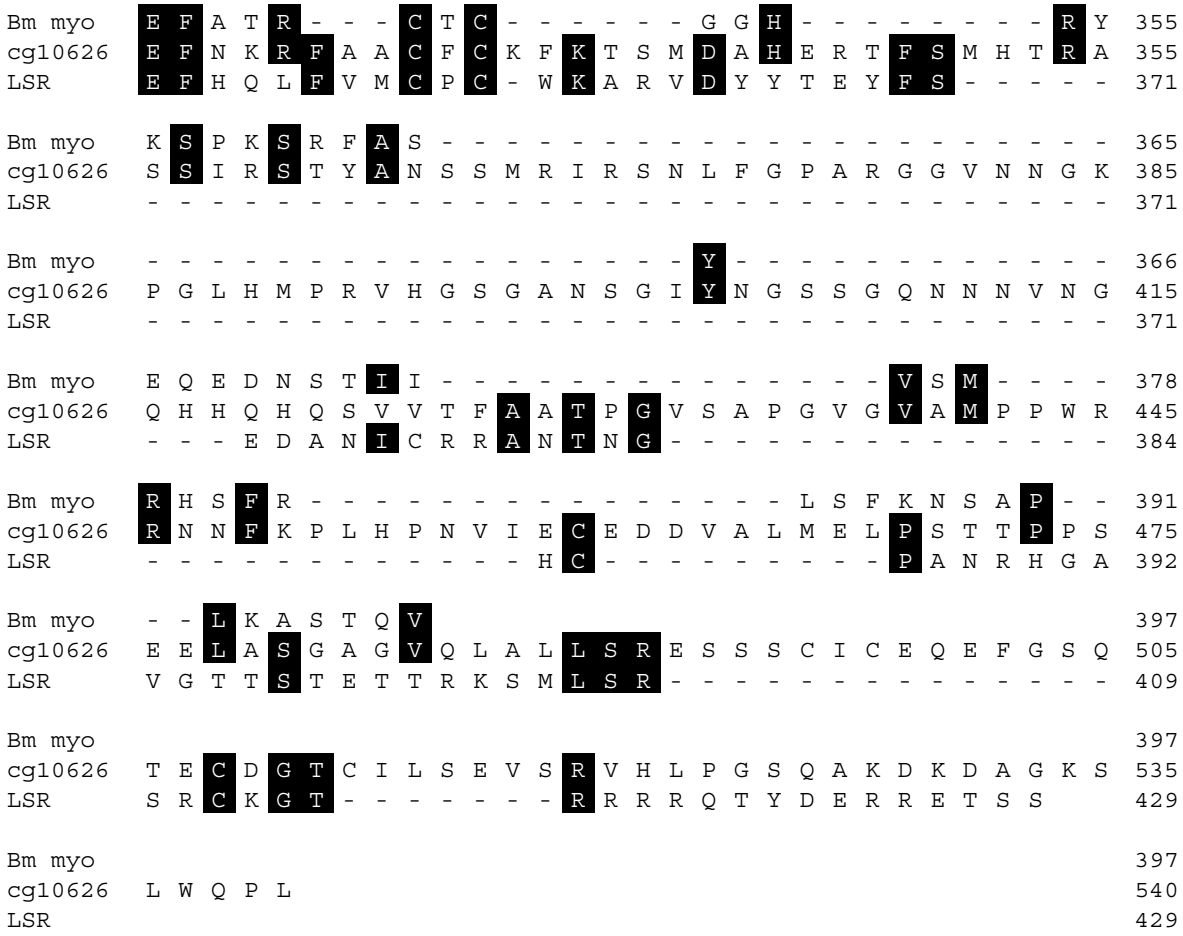


Figure 3. Continued.

Table 1. Amino acid identity of the *B. microplus* receptor to other invertebrate myokinin or related neuropeptide receptors and probability values from Blast searches.

Receptor	Identity	Blast P Probability
Drosokinin *Drosophila melanogaster* (fruit fly)	46.9 %	1e-100
Lymnokinin *Lymnaea stagnalis* (pond snail)	40.3 %	2e-79
Tachykinin-like *Stomoxys calcitrans* (stable fly)	29.0 %	1e-50
Neuropeptide y *Drosophila melanogaster*	28.0 %	2e-49

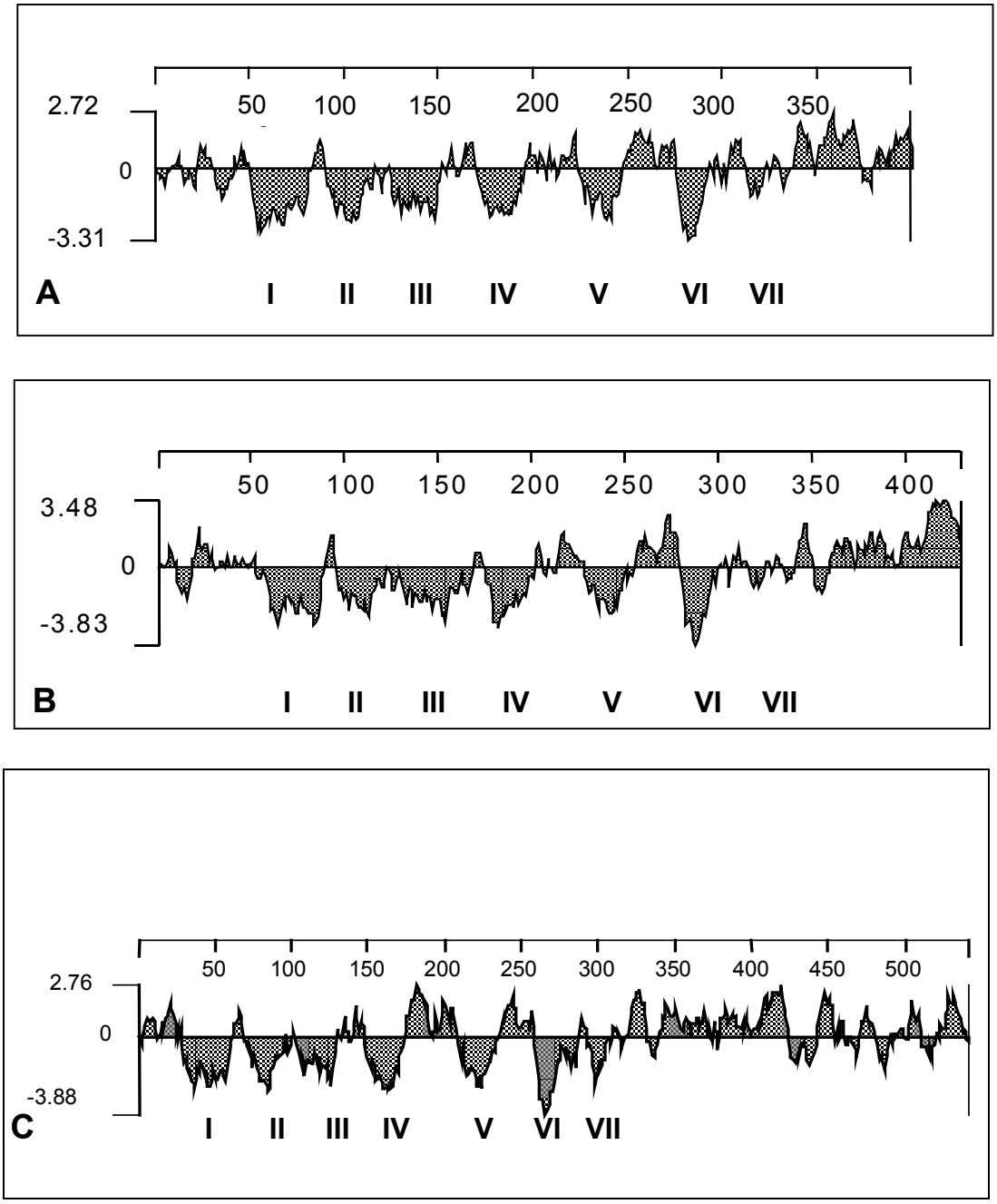
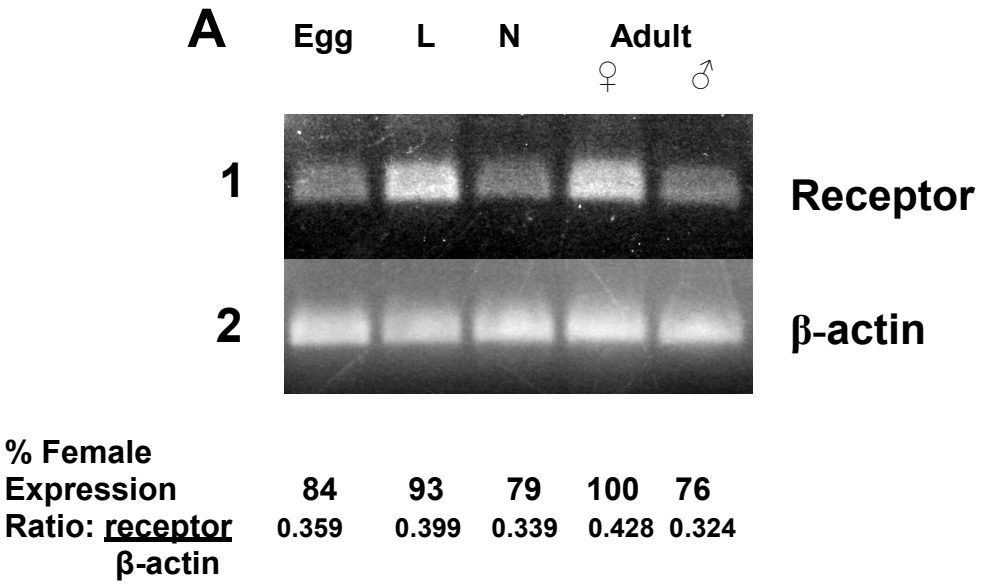


Figure 4. Kyte and Doolittle hydrophilicity plots of (A) the *B. microplus* myokinin receptor, (B) the *L. stagnalis* lymnokinin receptor, and (C) the *Drosophila* drosokinin receptor. Negative values indicate hydrophobic regions of the proteins. All receptors show a similar structure of seven putative transmembrane regions, characteristic of GPCRs. The figure was created with DNASTAR software.



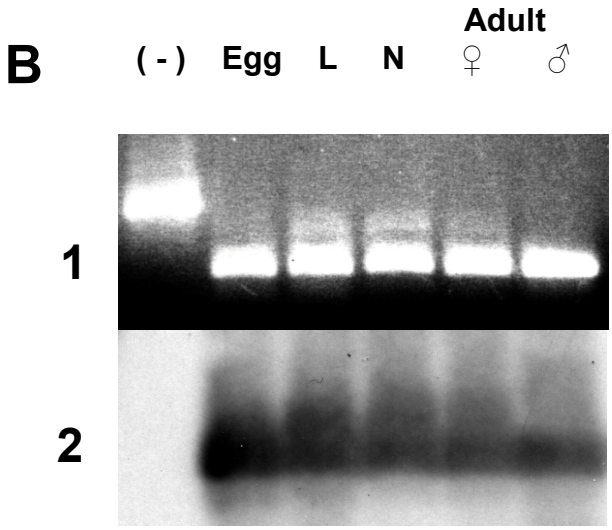


Figure 5. (A) Semi-quantitative RT-PCR of each life stage shows the relative amount of amplified receptor transcripts (Panel 1) in comparison to amplified tick β-actin transcripts (Panel 2). PCR products were obtained using single-stranded cDNA as the template. Note the higher intensity of receptor products in larvae and females. (B) Agarose electrophoresis of amplified receptor PCR products preparatory for Southern blot (Panel 1). Panel 2: Autoradiograph of the Southern blot of DNA in panel 1. Lane 1: negative control reaction (-) containing control cDNA not related to the receptor (Superscript™ preamplification system, Life Technologies). Lanes 2-5: PCR products corresponding to amplified receptor fragments from each life stage. N = nymphs, L = larvae.

Discussion

The myokinin receptor we have identified from the southern cattle tick *B. microplus* is the first neuropeptide receptor cloned from a tick and the second receptor identified in this species. Only six other receptors have been cloned from ticks. Three of these are GPCRs, an octopamine-like receptor (AJ0107043) (Baxter & Barker 1999) and two serotonin receptors cloned in our lab (Chen et al., 2003; S. Holmes, unpublished data) from *B. microplus*. There are also three nuclear receptors from *Amblyomma americanum,* an ecdysone receptor (AF020189) (Guo *et al.* 1997) and two retinoic x receptors (Palmer *et al.* 1999) (AF035577 and AF035578).

Knowledge of tick endocrinology is limited (Sonenshine, 1991; Lomas, *et al.* 1997). However, by analogy with insects, tissues expressing leucokinin receptors in ticks may be involved in water balance or neuromodulation, such as the Malpighian tubules and hindgut, or the CNS (synganglion), respectively. In insects, leucokinins act through increases in intracellular calcium in a cyclic nucleotide-independent mechanism to stimulate secretion in isolated Malpighian tubules (O'Donnell *et al.,* 1998; Cady & Hagedorn, 1999a; Terhzaz *et al.*, 1999). Leucokinins increase inositol triphosphate (IP_3) levels in isolated tubules and are thought to mediate the release of IP_3 sensitive calcium stores (Cady & Hagedorn 1999b). Receptor binding of lymnokinin results in an increase of intracellular calcium (Cox *et al.* 1997); it is therefore likely that the *B. microplus* receptor induces intracellular calcium release.

Prior to our report (Holmes *et al.,* 2000) no receptors for myokinins had been identified from arthropods. The characterization of myokinin receptors, such as the one

we have cloned from the tick, is key to further understanding of the function of these important myotropic and diuretic hormones.

The amino acid sequence of the *B. microplus* receptor is most similar to the drosokinin receptor (Radford *et al.*, 2002) and to the lymnokinin receptor from *L. stagnalis* (Table 1), which has been characterized as a leucokinin-like peptide receptor and is the first member of a new subfamily of GPCRs (Cox *et al.* 1997). It is also similar (28 % identity) to the neuropeptide Y receptor (NPY) from *Drosophila* (Li *et al.* 1992). Prior to the characterization of the CG10626 gene product, we presented evidence that it most likely was a myokinin receptor (Fig. 3). The CG10626 sequence showed higher homology to the *B. microplus* receptor than to the lymnokinin receptor, as expected due to the closer phylogenetic distances between Acari (ticks and mites) and insects than mollusks (Fig. 3). Therefore, we predicted that CG10626 was the first known insect myokinin receptor and that its endogenous ligand was most likely the *D. melanogaster* leucokinin (drosokinin) (Terhzaz *et al.,* 1999), which was later confirmed by its functional characterization (Radford *et al.,* 2002). This receptor-peptide pair may constitute a valuable system for further testing of structure-activity relationships for the myokinins.

The similarity between the *B. microplus* receptor and the drosokinin and lymnokinin receptors is greater than that found among the subtypes of the mammalian NPY receptor, Y1, Y2, and Y4, which are activated by the same ligand (Larhammar 1996). Thus, the ligand for the tick receptor is most likely closely related to droskinin (NSVVLGKKQRFHSWG-NH_2) or lymnokinin (PSFHSWS-NH_2), because GPCRs

which interact with closely related ligands have the greatest sequence homology and structural conservation (Iismaa *et al.* 1995; Cox *et al.* 1997; Terhzaz *et al.,* 1999). However, myokinins have not been identified in the Acari, but they have been isolated from insects (Nässel, 1996) and the pond snail (Cox *et al.,* 1997). There is immunological evidence for their presence in many invertebrates, including nematodes, molluscs and spiders (Smart *et al.* 1993; Elekes *et al.* 1994; Schmid & Becherer 1996). It appears that leucokinin-related peptides are important neuromodulators in the CNS of some invertebrates because of the abundance of leucokinin immunoreactive fibers in insect brain (Nässel 1996) and the presence of lymnokinin and its receptor in the CNS of the pond snail. Thus, it is not unexpected that myokinin-like peptides would also occur in ticks. Nevertheless, cloning of this *B. microplus* receptor constitutes the first indication for the presence of myokinin-type hormone signaling in the Acari (ticks and mites).

Little is known about developmental expression or regulation of neuropeptide receptors in arthropods. Our finding that the tick receptor mRNA is expressed throughout all life-stages suggests that the receptor protein may be required for critical functions, as leucokinin receptors mediate in other invertebrates.

The lymnokinin receptor was identified as a leucokin-like peptide receptor primarily by its mediation of leucokinin-induced intracellular calcium release (Cox *et al.* 1997). This receptor was the first member of the myokinin receptor subfamily, and thus it was not possible to identify conserved structural motifs that define this subfamily. However, with 3 myokinin receptors now known, it is possible to make some

comparisons. The C-terminal end of TM II (residues 103-110 in the *B. microplus* receptor) and the first extracellular loop (residues 111-123) are highly conserved between the three myokinin receptors, but not in the other neuropeptide receptor subfamilies (Fig. 3). Similarly, the first eight residues of the C-terminus (residues 337-344) are highly conserved among the three myokinin receptors, and less conserved among the other neuropeptide receptors. These conserved regions may be useful for the structural definition of this subfamily, or correspond to domains conferring ligand specificity to the receptors. It is interesting to note that TM VII is also highly conserved among all the compared neuropeptide receptors (Fig. 3).

The *B. microplus* receptor has two glycosylation sites in the N-terminus, Asn 30 and Asn 41 and one in the second extracellular loop Asn 204 (Fig. 2). Multiple glycosylation sites are found in the N-termini of several neuropeptide receptors (Cox *et al.* 1997) (Li *et al.* 1992) (Tensen *et al.* 1998a; Watson & Arkinstall 1994; Monnier *et al.* 1992; Tensen *et al.* 1998b). Two cysteines, Cys 348 and Cys 350 in the C-terminal region of the *B. microplus* receptor are potential sites for palmitoylation, which may be important for receptor function (Watson & Arkinstall 1994; Iismaa *et al.* 1995).

Receptor binding sites for peptides and protein agonists of GPCRs include the N-terminus and extracellular loops (Gether & Kobilka 1998). The *B. microplus* receptor has several lysine (5) and arginine (4) residues in the extracellular loops, similar to residues found in the Y1 receptors that are involved in ligand binding (Berthold & Bartfai 1997).

Among arthropods, ticks rank second only to mosquitoes as vectors of human disease (Bowman *et al.* 1996). The expression of tick receptor mRNA throughout all life stages indicates that the receptor protein may be required for critical functions. Our discovery presents a target for the development of novel specific acaricides. These may prove useful to the cattle industry and in the prevention of transmission of human diseases, such as Lyme disease. Lyme disease is the most common vector-borne disease in the United States, which is transmitted by closely related ixodids (Dolan *et al.* 1997). GPCRs have proven to be among the most successful drug targets, and orphan or novel receptors have great potential for drug discovery (Stadel *et al.* 1997). Over 30 % of clinically marketed drugs act on GPCRs (Wise *et al.,* 2002). Single treatments of myokinins elicit a fairly long response (30 min) in insect Malpighian tubules (Terhzaz *et al.* 1999). Therefore, leucokinin receptors may be good targets for novel acaricides because small doses could induce a prolonged physiological response. There is a precedent for the successful targeting of arthropod GPCRs. Formamidines, such as amitraz, are synthetic acaricides that act on the octopamine receptor, which is exclusively present in arthropods (Baxter & Barker, 1999). As all feeding stages of *B. microplus* remain on the host, our discovery also presents a target for the development of novel immunological approaches against this tick. Vaccines against gut antigens of *B. microplus* produce a protective immune response in cattle that reduces the number and the fecundity of ticks feeding on immunized cattle (De Rose *et al.,* 1999). Immunoglobins cross the midgut epithelium and enter the hemolymph of many blood-feeding arthropods without losing their immunological properties, so there is a

possibility that many cell membrane receptors could serve as targets for vaccines (Sauer *et al.,* 1994). The difficulty in exploiting this strategy has been the lack of identification of essential tick receptors (Sauer *et al.* 1994). The usefulness of the leucokinin-like receptor for preimmunizing cattle against *B. microplus* can now be explored.

CHAPTER III

FUNCTIONAL ANALYSIS OF THE *Boophilus microplus* NEUROPEPTIDE RECEPTOR IDENTIFIES IT AS THE FIRST ARTHROPOD MYOKININ RECEPTOR*

Introduction

In the last 16 years, knowledge about invertebrate neuropeptides has increased substantially, especially in insects; dozens have been isolated and sequenced (Nachman, 2001). However, very few peptide receptors from arthropods have been fully characterized. The sequencing of the *Drosophila melanogaster* genome (Adams *et al.,* 2000) revealed that approximately 200 genes code for G protein-coupled receptors (GPCRs), and about 100 of those code for putative neurotransmitter and hormone receptors (Brody & Cravchik, 2000). An analysis of the *Drosophila* genome has identified 44 genes that encode for peptide GPCRs (Hewes & Taghert, 2001). Although several insect GPCRs for neuropeptides have been identified, the majority of these are strictly orphan receptors for which endogenous ligands have not been unequivocally identified (Vanden Broeck, 2001). For reviews of insect GPCRs, see Vanden Broeck (2001) and Hewes & Taghert (2001).

At least 21 arthropod GPCRs for neuropeptides have been functionally expressed – three for allatostatin-like neuropeptides (Birgül *et al.*, 1999; Secher *et al.*, 2001;

* Text and figures reprinted with permission from "Functional analysis of a G protein-coupled receptor from the southern cattle tick *Boophilus microplus* (Acari: Ixodidae) identifies it as the first arthropod myokinin receptor" by Holmes *et al*., 2003. *Insect Molecular Biology*, 12, 27-38.

Auerswald *et al.*, 2001); receptors for sulfakinin (Kubiak *et al.*, 2002), corazonin (Hauser *et al*., 1998; Cazzamali *et al.*, 2002), proctolin (Johnson *et al.*, 2003; Egrod *et al.*, 2003), FMRFamide (Cazzamali & Grimmelikhuijzen, 2002), three receptors for neuropeptide F (DmNPF) (Garczynski *et al.*, 2002; Mertens *et al.*, 2002; Feng *et al.*, 2003), and two receptors for ecdysis triggering hormone (Iversen *et al.*, 2002), all from *D. melanogaster*; two for adipokinetic hormone from *D. melanogaster* and *Bombyx mori*, respectively (Staubli *et al.*, 2002); those for the corticotropin releasing factor (CRF)-related diuretic hormone of *Manduca sexta* (Reagan, 1995) and the cricket *Acheta domesticus* (Reagan, 1996); a receptor for tachykinin-like peptides from the stable fly *Stomoxys calcitrans* (Guerrero, 1997; Torfs *et al.*, 2000, 2001) and two from *D. melanogaster* (Li *et al.*, 1991; Monnier *et al.*, 1992); and the tick myokinin receptor, which is the subject of this dissertation. The fruit fly NPY receptor although expressed, was likely misidentified and continues to be an orphan receptor because no endogenous ligand has been identified (Li *et al.*, 1992; Hewes & Taghert, 2001).

Other insect receptors that have been cloned or characterized from *D. melanogaster* include one a second allatostatin receptor (Lenz *et al.*, 2000). The lack of more abundant information on neuropeptide receptors limits the understanding of the physiological processes needed for the development of new methods for control of arthropod pests.

The myokinins or leucokinin-like peptide family are multi-functional neuropeptides that have been found in several arthropod and invertebrate groups (Nässel, 1996). The arthropod myokinins share the evolutionarily conserved C-terminal

pentapeptide motif Phe-X_1-X_2-Trp-Gly-NH_2, where X_1 = His, Asn, Ser, or Tyr and X_2 = Ser, Pro, or Ala. This amidated pentapeptide is all that is required for biological activity (Coast *et al.*, 1990; Nachman & Holman 1991). Only one member of this peptide family does not adhere strictly to this pattern, the culekinin depolarizing peptide III in which X_1 = Phe (Clottens *et al.*, 1993). In the non-arthropod myokinins, the terminal Gly is substituted for the Ser in lymnokinin (Cox *et al.*, 1997) and for Ala in one of the two shrimp kinins (Pev-kinin 2) (Nieto *et al.*, 1998).

The myokinins have myotropic and diuretic activity in insects. Leucokinins stimulate hindgut contractions in the cockroach, cricket, and locust (Holman *et al.* 1987; Holman *et al.* 1990; Schoofs *et al.* 1992) and increase the rate of secretion in Malpighian tubules (Pannabecker *et al.* 1993; Veenstra *et al.* 1997; O'Donnell *et al.* 1998; Cady & Hagedorn 1999b; Holman *et al.* 1999), also interacting with CRF-related peptides, and serotonin in some species (O'Donnell & Spring, 2000; Pannabecker *et al.*, 1993; Veenstra *et al.*, 1997; O'Donnell *et al.*, 1998; Cady & Hagedorn 1999b; Holman *et al.* 1999). In insect tissues where leucokinin signaling has been studied, myokinins were found to act through increases in intracellular calcium (O'Donnell & Spring, 2000; Cady & Hagedorn 1999a; Yu & Beyenbach 2002).

In addition to their effects on isolated hind guts and Malpighian tubules, there is evidence that myokinins may have higher functions in the insect central nervous system (CNS) as neuromodulators or neurotransmitters (Clottens *et al.*, 1993; Nässel, 1996). Additionally, there is the possibility that they play a role in regulating food uptake, as

demonstrated by their ability to decrease weight gain in *H. virescens* larvae (Seinsche *et al.*, 2000).

Interestingly, although leucokinin-like peptides have been immunolocalized in the CNS and digestive system of the blood-feeding bug *Rhodnius prolixus*, they do not appear to have a direct effect on *Rhodnius* Malpighian tubules (Te Brugge *et al.*, 2001), reinforcing the possibility of alternate functions for myokinins beyond diuresis.

Although myokinin receptors mediate this variety of function, they were unknown in arthropods until recently. Binding assays with Malpighian tubule membranes from the house cricket, *A. domesticus*, have shown that the achetakinins (AK) specifically bind a single class of binding sites and competitively displace an iodinated bioactive AK-II analog (Chung *et al.*, 1995). Additionally, there has been one report of the biochemical characterization of a 54-kDa leucokinin receptor from the mosquito, *Aedes aegypti* (Pietrantonio *et al.*, 2000). The first known myokinin receptor was cloned from the pond snail, *Lymnaea stagnalis*, and its endogenous ligand, lymnokinin (PSFHSWS-NH_2), was isolated by a functional intracellular calcium assay (Cox *et al*., 1997). Two receptors with homology to the lymnokinin receptor have subsequently been identified, one which we cloned from the southern cattle tick, *Boophilus microplus* (Canestrini) (Holmes *et al.*, 2000), and one identified by the *Drosophila* genome project (CG10626) (Adams *et al.*, 2000; Radford *et al.*, 2002). There were no functional data available for these receptors prior to this study; however, a neighbor-joining phylogenetic tree analysis has placed these three receptors on a single

branch of a subgroup-specific tree of neuropeptide receptors (Hewes & Taghert, 2001). (See also Table 2).

The southern cattle tick or cattle fever tick, *Boophilus microplus* (Canestrini), is the most important tick-pest of cattle in tropical and sub-tropical regions of the world because of its ability to transmit *Babesia spp.*and *Anaplasma spp.*, the hemoparasites that cause cattle fever (Nuñez *et al.* 1985). Control of *B. microplus* has become increasingly difficult worldwide because it has rapidly developed resistance to pesticides (Baxter & Barker, 1999; Rosario-Cruz *et al.,* 1997; He *et al.*, 1999). One effective control strategy for the southern cattle tick may be endocrine disruption. Peptide hormones may have great potential for control strategies because they are master regulators and affect a number of physiological processes (Keeley & Hayes 1987; Nachman *et al.*, 2002). Therefore, myokinin receptors are potential targets for control of the cattle fever tick and other pest species. Our report on the cloning of the tick myokinin receptor is the first neuropeptide from a tick species (Holmes *et al.*, 2000). Myokinin peptides have found in several insects (Nässel, 1996), the pond snail (Cox *et al.*, 1997), and the white shrimp (Nieto *et al.*, 1998). Additionally there is evidence for their presence in a variety of invertebrates, including ascarid worms (Smart *et al.*, 1993), spiders (Schmid & Becherer, 1996), molluscs (Elekes *et al.*, 1994), and possibly crabs (Blitz *et al.*, 1995). Unfortunately, to our knowledge, no neuropeptides – including myokinins – have been isolated from ticks. However, it is expected that myokinins are also present in ticks.

In this chapter, functional expression of the *B. microplus* receptor in mammalian cells has shown that several myokinin peptides activate this receptor at nanomolar

Table 2. Comparison of selected neuropeptide receptors to the tick myokinin receptor.

Receptor and species	Percentage identity to tick myokinin receptor	E value (Blast P)	Endogenous ligand
Drosokinin receptor *D. melanogaster* (Radford *et al*., 2002)	46.9%	2×10^{-97}	*Drosophila* LK NSVVLGKKQRFHSWG-NH_2 (Radford *et al*., 2002)
Lymnokinin receptor *L. stagnalis* (Cox *et al*., 1997)	40.3%	2×10^{-72}	Lymnokinin PSFHSWS-NH_2 (Cox *et al*., 1997)
Neuropeptide Y receptor *D. melanogaster* (Li *et al.* 1992)	28.0%	5×10^{-45}	Unknown[1]
Tachykinin-like peptides receptor *D. melanogaster* (Li *et al.*, 1991)	27.2%	2×10^{-44}	Unknown
Cardioexcitatory receptor *L. stagnalis* (Tensen *et al*., 1998)	27.5%	2×10^{-43}	*Lymnaea* cardioexcitatory peptide (LyCEP) TPHWRPQGRF-NH_2 (Tensen *et al.,* 1998)
NKD tachykinin receptor *D. melanogaster* (Monnier *et al*., 1992)	27.0%	1×10^{-41}	Unknown[2]
Tachykinin-like receptor *Stomoxys calcitrans* (Guerrero 1997)	29.0%	5×10^{-41}	Potential ligand – Stc-TK APTGFFAVR-NH_2 (Torfs *et al.,* 2001)[3]
Neurokinin 2 receptor c *Homo sapiens* (Gerard *et al.,* 1990)	28.2%	6×10^{-41}	HKTDSFVGLM-NH_2 (Nawa *et al.,* 1984)

Percentage identity is based on a multiple sequence alignment of these neuropeptide receptors with the tick myokinin receptor protein sequence. Sequences were aligned using the Clustal method on DNASTAR software. E values from Blast P searches of GenBank were scored on the BLOSUM62 matrix and reflect the probability that sequence similarity is due to random variation rather than true homology.
1 = This receptor is activated by mammalian peptides PYY at > 30 nM and NPY at about > 90 nM when expressed in *Xenopus* oocytes (Li *et al.,* 1992) although no endogenous ligand has been identified.
2 = This receptor is activated by *Locusta migratoria* tachykinin although no endogenous ligand has been identified.
3 = The natural isolated peptide sequence is as in this table, however the C-terminus consensus sequence of the insectatachykinin group of peptides is Phe-Xxx-Gly-Xxx-Arg-NH_2 (Vanden Broeck *et al.,* 1999); the stomoxytachykinin analog was designed with a consensus sequence.

concentrations. The peptide-agonist induced calcium response in these cells originated from intracellular calcium stores. Taken together, the results indicate that the tick receptor is the first myokinin receptor to be cloned from an arthropod.

Methods

Expression construct

The cloning of the cDNA for the myokinin peptide receptor (AF228521) from larvae of the southern cattle tick, *B. microplus*, was described in the first chapter. The coding region of the receptor cDNA was amplified by PCR using primers modified to incorporate a 5' Kozak consensus sequence (GCC **A**/G CC **ATG** G) around the presumed start codon for optimal ribosomal binding in a mammalian system (Kozak, 1986) and restriction sites (5' primer XhoI, 3' primer BamHI) for unidirectional cloning. The primers were BM3.2 sense primer, 5'-TTC **CTC GAG** GGT GCC AC**C** ATG **G**CC T, and BM3.2 anti-sense primer, 5'-T A**GG ATC C**CA CTG GTG GTT GGA CTC (modified base pairs and restriction sites in bold). The modification of the first base pair following the start codon (**G** instead of A in the original sequence) is expected to change the second residue of the expressed protein from the threonine (T) of the native sequence to an alanine (A). The modified sequence was cloned into the XhoI/BamHI sites of mammalian expression vector pcDNA3.1(-) (Invitrogen, Carlsbad, CA), which includes the cytomegalovirus promoter and the neomycin resistance gene. The accuracy of the expression construct was verified by sequencing.

Cell culture and transfection

CHO-K1 cells were maintained in Kaighn's modification of Ham's F-12 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (EquiTech Bio, Kerrville, TX) in a humidified incubator with 5% CO_2 at 37° C as recommended by ATCC (ATCC Product Information Sheet, Manassas, VA). For transfection, CHO-K1 cells were seeded into 60-mm tissue culture plates and allowed to grow 24-48 h until 30-50% confluent. Cells were transfected in serum free Opti-MEM medium (Life Technologies) with the cationic lipid reagent Lipofectin® (Life Technologies) (6 µl) and 2 µg of the expression construct or the empty vector (for negative control) according to the manufacturer's protocol. A preliminary dose-response assay with untransfected cells against the antibiotic GENETICIN® G-418 (Life Technologies) was conducted to determine the concentration necessary to select for stably transfected cells. All untransfected cells died after ten days at a concentration of 700 µg/ml. Thus, 800 µg/ml GENETICIN® was used to generate stable transfectants by continuous selection for 14 days, which were thereafter maintained in media containing 400 µg/ml GENETICIN® (Anonymous 1999).

Isolation and screening of stably transfected clones

After antibiotic selection, four stably transfected clonal cell lines were isolated by serial dilution of cell suspensions across 96-well tissue culture plates beyond a limiting dilution (theoretically less than 1 cell per well). After 2-3 days of growth, the plates were examined and wells with a single colony that appeared to result from the

deposition of a single cell were selected. After single cell colonies had grown to 50-100 cells, they were trypsinized and replated in a 96-well plate and allowed to grow an additional 2-3 days until confluent. Trypsinization and growth was repeated with sequentially larger cell culture plates until there were enough cells to start T-25 cell culture flasks (approximately 200,000 cells). Cells were harvested from T-25 flasks for transcription analysis. Total RNA was isolated from transfected cell lines with TRIzol® Reagent (Life Technologies) according to the manufacturer's protocol. First strand cDNA was synthesized from approximately 1.3 µg of total RNA from each sample at 42° C for 50 min with a gene-specific anti-sense primer SC3-R (5'-TG GTG GTT GGA CTC AAA TTA CAC, AF228521 base pairs 1283-1304) using SuperScript II Reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Life Technologies). PCR amplification of the entire open-reading frame (ORF) of receptor cDNAs was performed with gene-specific primers (BM3.2 and SC3-R), corresponding to sequence flanking the 5' and 3' ends of the ORF, respectively, with an expected product size of 1227 bp. Reactions contained one-tenth (2µl) of the synthesized cDNA, 200 µM each of four dNTPs, 0.2 µM of each primer, 1 µl Taq polymerase, and 1X reaction buffer (Boehringer Mannheim) in a final volume of 50 µl. The following cycling parameters were used: 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. cDNA from control RNA (SuperScript™ First Strand Synthesis System for RT-PCR) was synthesized and amplified under identical conditions with gene-specific primers

provided by the manufacturer with an expected product size of 500 bp. Ten µl of each reaction was electrophoresed on a 1% TBE agarose gel containing ethidium bromide, then photographed. Cell lines positive for receptor transcript were challenged with a single dose (10 µM) of lymnokinin (PSFHSWS-NH_2) and intracellular calcium responses were monitored as described below. A single clonal cell line that responded to lymnokinin challenge with an increase in intracellular calcium was selected for the assays described in this chapter. Cells transfected with expression vector only were subjected to the same selection conditions until resistance to GENETICIN® was acquired.

Peptide synthesis and purification

Muscakinin (NTVVLGKKQRFHSWG-NH_2) was synthesized and purified as previously described (Holman *et al.*, 1999). Lymnokinin (PSFHSWS-NH_2), the stomoxytachykinin analog APTGFFGVR-NH_2 (Torfs *et al.*, 2001), and insect kinin analog FFFSWS-NH_2 were synthesized using solid phase FMOC protection chemistry on Rink Amide resin (0.4 meq/gm; Novabiochem, San Diego, CA) on an ABI 433A Peptide Synthesizer (PE Biosystems, Foster City, CA) according to previously described conditions (Nachman *et al.*, 1997). Side chain protection used was as follows: Arg(Pmc), His(Trt), Ser(tBu), Thr(tBu) and Trp(Boc). The peptide was cleaved from the resin complex with a mixture of trifluoroacetic acid (TFA)(90%), anisole (5%), thioanisole (4%), and ethanedithiol (1%) for 1.5 h at room temperature. The resin was filtered and volatile reagents were removed *in vacuo* on a Savant Speed Vac

concentrator at 40°C. The products were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 x 1-00 mm, 15 μm particle size, 100 A pore size) on a Waters Model 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous TFA; Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program 6 to 100% B over 40 min; flow rate, 2 ml/min. Delta Pak C_{18} retention times: lymnokinin, 7.5 min; stomoxytachykinin analog APTGFFGVR-NH_2, 9 min; insect kinin analog FFFSWS-NH_2, 15 min. The analogs were further purified on a Waters Protein Pak column (Milligen Corp., Milford, Ma). Conditions: Solvent A= 95% aqueous acetonitrile made to 0.01% TFA; Solvent B = 50% aqueous acetonitrile made to 0.01% TFA; 100% A isocratic for 4 min followed by a linear program to 100% B over 80 min. WatPro retention times: lymnokinin, 10.5 min; stomoxytachykinin analog APTGFFGVR-NH_2, 10.25 min; insect kinin analog FFFSWS-NH_2, 6.0 min. MALDI mass spectra were obtained on a Kratos Kompact probe MALDI instrument (Kratos, Manchester, UK) with alpha-cyano-4-hydroxycinnamic acid as a matrix. The structural identity was confirmed by the presence of the following molecular ions (MH^+): lymnokinin, 845.6 (Calc. MH^+: 845.38); stomoxytachykinin analog APTGFFGVR-NH_2, 949.6 (Calc. MH^+: 949.51); insect kinin analog FFFSWS-NH_2, 841.6 (Calc. MH^+: 841.37). The peptides were quantified via amino acid analysis as previously described (Nachman *et al.*, 1997). All other peptides (the insect kinin analogs FFFSWG-NH_2,

FFSWG-NH_2, and FYSWG-NH_2) were synthesized and purified as previously described (Pietrantonio *et al.*, 2000).

Peptides were solubilized in 80% acetonitrile with 0.01% trifluoroacetic acid (TFA), and aliquots for use were dried down then resuspended in Dulbecco's Modified Eagle's medium with F-12 salts without serum or phenol red (Life Technologies).

Analysis of peptide activity through intracellular Ca^{2+} measurement

Approximately 80,000 cells were seeded in 2-well Lab-Tek® Chambered Coverglass slides (Nalge Nunc, Naperville, IL) 48 hours prior to assays and grown to approximately 80% confluence. A stock solution of 1 mM fluo-4 AM (Molecular Probes, Eugene, OR), a Ca^{++}-sensitive fluorophore, was prepared in dimethyl sulfoxide (DMSO) and diluted with serum- and phenol red-free Dulbecco's Modified Eagle's medium with F-12 salts (DMEM F-12) to a 3 μM solution (0.3% final DMSO concentration) for loading cells. Cells were loaded for one hour with fluo-4 AM after which the monolayer was washed once with fresh medium prior to challenge with peptide agonists. The range of agonist concentration expected to be effective in assays was from 10^{-10} M to 10^{-5} M, the range that has been shown to effect changes by neuropeptides in isolated Malpighian tubules of the mosquito *Aedes aegypti* (Veenstra *et al*., 1997; Pietrantonio *et al.,* 2000) and the desert locust *Schistocerca gregaria* (Coast *et al.*, 1999). Therefore, kinin peptides were first tried at a high concentration of 10 μM followed by lower doses until a response was no longer observed. Negative control cells transfected with vector only were challenged with high concentrations (10 μM) of

lymnokinin under identical conditions. Agonist induced changes in intracellular Ca^{++} were monitored with a Meridian Ultima Confocal Microscope (Meridian Instruments, Okemos, MI) at the Image Analysis Laboratory, College of Veterinary Medicine, Texas A&M University. Cells were placed on the stage of the confocal microscope, and an area of the chamber slide was selected for analysis. For image collection, scan parameters were adjusted for maximum detection of fluorescence with minimum cellular photobleaching. Fluorescence was generated in the cells by excitation at 488 nm, and fluorescence emission of 530 nm was collected from individual cells by means of a photomultiplier tube. Initial basal fluo-4 fluorescence intensity was obtained from 5 image scans recorded from about 8-25 selected cells every 3 seconds. After the fifth scan, cells were exposed to peptide agonists resolubilized in DMEM F-12 media, and image scans were acquired at the same interval for a total time of 300 seconds. Negative control experiments were performed in an identical fashion with the addition of media containing no peptides.

Analysis of signal transduction

To analyze the mechanism of myokinin signal transduction it was necessary to establish a typical positive curve in CHO-BMLK3 cells. Thus, cells were assayed against 10 μM lymnokinin or 100 nM FFFSWG-NH_2, the latter chosen because of its high potency in the intracellular calcium assay described above and its strong depolarizing activity on mosquito Malpighian epithelium (Pietrantonio *et al.*, 2000). As

a negative control, CHO-BMLK3 cells were challenged with 100 nM of the unrelated peptide hormone oxytocin (Sigma) (Table 3).

To check the contribution of extracellular and intracellular sources of calcium to myokinin induced responses, CHO-BMLK3 cells were co-treated with thapsigargin, an inhibitor of the microsomal Ca^{2+}-ATPase that also produces an outward passive leak of Ca^{2+} from intracellular stores (Hofer, 1999), or EGTA, used as an efficient chelator for extracellular calcium ions (both obtained from Sigma, St. Louis, MO). Thapsigargin (1 μM final concentration; prepared in 0.1% DMSO) was added after scan number 14, about 30 s after the addition of lymnokinin and the occurrence of the expected peak in fluorescence. The respnse to thapsigargin would allow us to determine if lymnokinin produced a maximal cellular response, characterized by the complete depletion of intracellular calcium stores.

To determine if extracellular calcium is required for the initial intracellular fluorescence response to myokinins, EGTA (1mM) was added to media about 1 min prior to the first scan; the analog FFFSWG-NH_2 was added after the fifth scan.

To investigate the tick receptor interaction with G proteins, cells were pretreated with concentrations of pertussis toxin (Sigma) from 50 ng/ml to 1000 ng/ml for 16 hours prior to peptide analog challenge. For these assays, FFFSWG-NH_2 was added after the fifth scan (approximately 15-18 s).

Statistical analysis

Intracellular calcium concentration was expressed in relative fluorescence intensity units (FIU). Four samples per concentration were analyzed, each sample representing from 8-25 cells. For each cell, fluorescence data were normalized by dividing the value from each scan by the value from the first scan (basal fluorescence). For each cell, log transformation was done on normalized data (ratios) when used for statistical analysis. Traces correspond to either the raw or normalized FIU as indicated, and vertical lines correspond to the standard error of four samples. Student's T-test and the Dunnett's Multiple Comparison test were used to determine the level at which agonist induced fluorescence peak values were significant above controls.

Estimation of EC_{50} values and their respective 95% confidence intervals (Table 3) was done using nonlinear regression sigmoidal-dose response curve fitting (GraphPad Software, San Diego, CA). Briefly, the maximum normalized intensity values (Fig. 7, black bars in histograms) for the log of each concentration were used to fit a non-linear equation that calculated the corresponding log concentration on the *x* axis (log EC_{50}s) using the 50% maximal FIU. EC_{50}s and confidence intervals were converted from log values for presentation in Table 3. Analogues with overlapping confidence limits for the EC_{50} were considered of similar activity.

Results

Isolation of the clonal cell line CHO-BMLK3 expressing the B. microplus *receptor*

Four clonal cell lines transfected with the receptor construct were isolated following antibiotic selection. RT-PCR with primers corresponding to sequences flanking the coding region of the receptor revealed that only two of the cell lines, designated CHO-BMLK2 and CHO-BMLK3, were transcribing receptor mRNA (data not shown). These two cell lines and a cell line only transfected with expression vector were challenged with 10 μM lymnokinin. Only the cell line CHO-BMLK3 showed an increase in intracellular calcium upon challenge (Fig. 6), indicating that the other cell line was not properly expressing the receptor protein. Therefore, the CHO-BMLK3 cell line was chosen for further study. Figure 6 shows the raw fluorescence data (not normalized) of a typical assay. The top trace in Fig. 6 is typical of this cell line's response to challenge with high concentrations of myokinins. The application of lymnokinin to the cells after 5 scans (Fig. 6, arrow) resulted in a rapid rise in intracellular calcium that was maintained at a high level for longer than 5 min. As expected, cells transfected with vector only showed no response to lymnokinin.

In the following figures, except Fig. 8B, data were normalized to values obtained from the first scan (taken prior to agonist challenge) to account for differences in basal fluorescence between respective samples (see Methods).

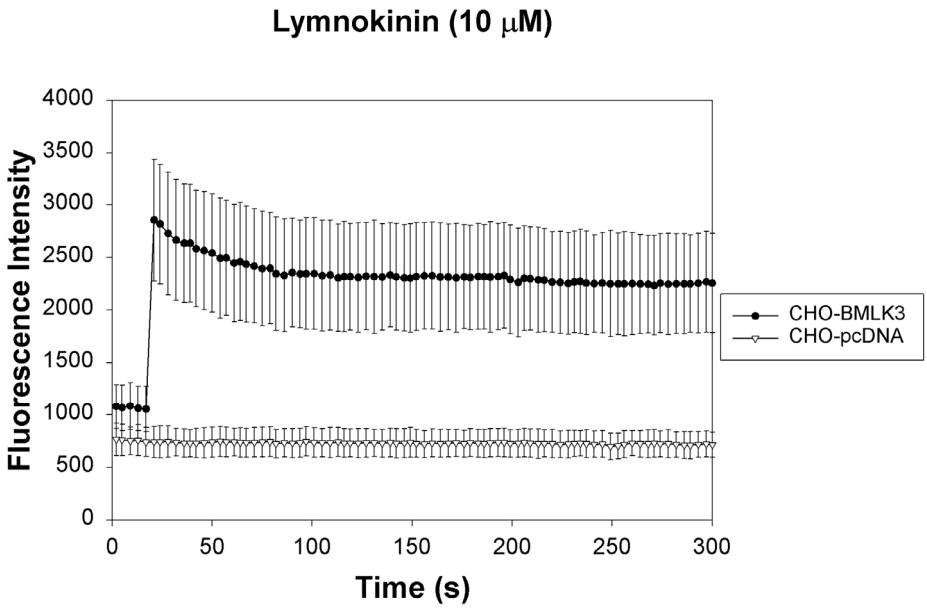


Figure 6. Typical response of CHO-BMLK3 (n=14) and CHO-pcDNA cells (n=12) to 10 μM lymnokinin (PSFHSWS-NH_2). Introduction of lymnokinin at 15-18 seconds (arrow) resulted in a rapid elevation of intracellular calcium as shown by fluo-4 AM fluorescence in CHO-BMLK3 only. Control cells CHO-pcDNA, transfected with vector only, showed no response to lymnokinin.

Receptor mediated response to myokinin peptides

The natural peptides lymnokinin and muscakinin were chosen for assays because of their efficacy in a functional calcium assay (Cox *et al.*, 1997) and in the house fly Malpighian tubule secretion assay (Holman *et al.*, 1999; Coast, 2001) respectively. The myokinin analogs FFFSWG-NH_2, FFSWG-NH_2, and FYSWG-NH_2 have previously been shown to cause depolarization and stimulate fluid secretion in the mosquito Malpighian tubule (Pietrantonio *et al.*, 2000; Hayes *et al.*, 1989). The analog FFFSWS-NH_2 was designed to investigate the effect of replacing the terminal Gly with Ser, a substitution in the myokinin peptide family unique to lymnokinin (Table 1 in Pietrantonio *et al.*, 2000). Each of the myokinin peptides tested in the functional calcium assay resulted in a dose-dependent rise in intracellular calcium (Figure 7A-F). High concentrations of peptide resulted in a rapid increase in intracellular calcium, which was then maintained for the duration of the assay. Lower concentrations induced peaks of varying intensity, which were not followed by sustained levels of fluorescence. An agonist for the related tachykinin receptor (STKR) from the stable fly (APTGFFAVR-NH_2, *Torfs et al.*, 2001) induced a weak response only at a 40 μM, the highest concentration tested. This concentration was approximately 1000-fold higher than concentrations of synthesized natural myokinins (lymnokinin, muscakinin) (Fig. 7G, Table 3) and approximately 4000-fold higher than concentrations of the least potent myokinin analog required to induce a similar response (Fig. 7G).

A curve-fitting analysis was used to estimate the EC_{50} for each myokinin peptide. The designed hexamers FFFSWS-NH_2 and FFFSWG-NH_2 were the most potent,

followed by the pentamers FFSWG-NH_2 and FYSWG-NH_2 (Table 3). Of the myokinins tested, muscakinin and lymnokinin were the least potent, with muscakinin nearly as potent as the artificial pentamers (EC_{50} = 17.02 nM) and lymnokinin about 43-fold less potent (EC_{50} = 566 nM) than the weakest pentamer. As expected the STKR agonist was much less potent than the myokinins, indicating the selectivity of this receptor for leucokinin-like peptides. Similarly, there was no response to 100 nM oxytocin (Fig. 8A), indicating that the response was specific to myokinins and could not be induced by an unrelated peptide hormone.

Signal transduction analysis

The absence of extracellular calcium ions did not have any detectable effect on the calcium response induced by myokinin agonists. In the presence of 1 mM EGTA, a chelator of calcium ions, the response of CHO-BMLK3 cells to 100 nM FFFSWG-NH_2 was unaffected, indicating that initial receptor-mediated increases in intracellular calcium are the result of release from intracellular stores (Fig. 8A). This was confirmed by an assay in which cells were first challenged with 1 μM lymnokinin 15-18 s after scans began and then 1.0 μM thapsigargin at 42-45 s (Fig. 8B). Treatment with thapsigargin, would have increased calcium levels by releasing any remaining calcium ions from a single pool of intracellular stores. Thapsigargin failed to further increase the calcium levels of transfected cells, indicating that internal stores the affected pool had already been depleted by treatment with lymnokinin and that at this concentration, lymnokinin produced a maximal response.

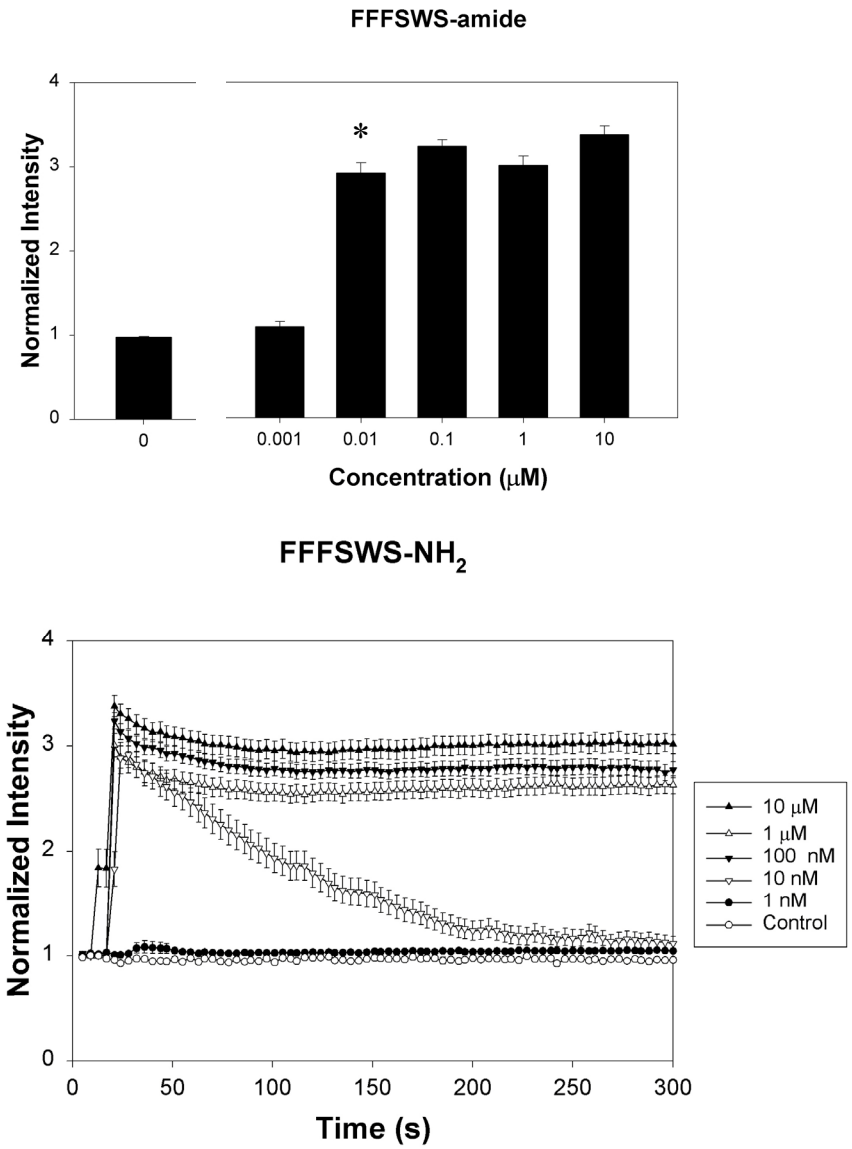
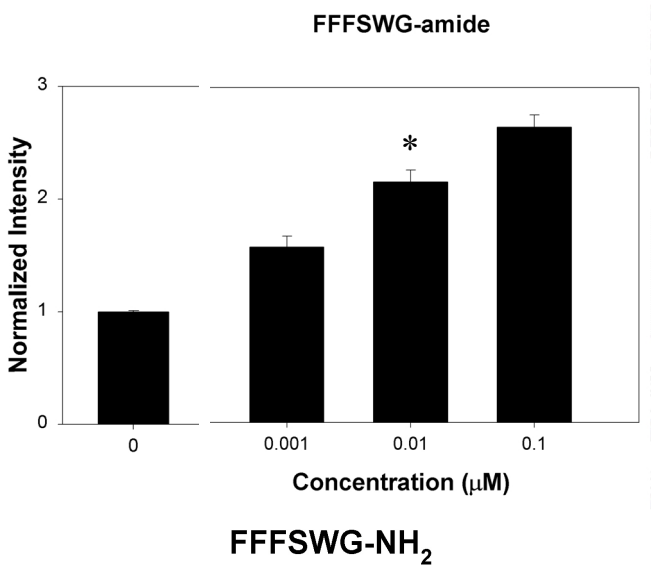
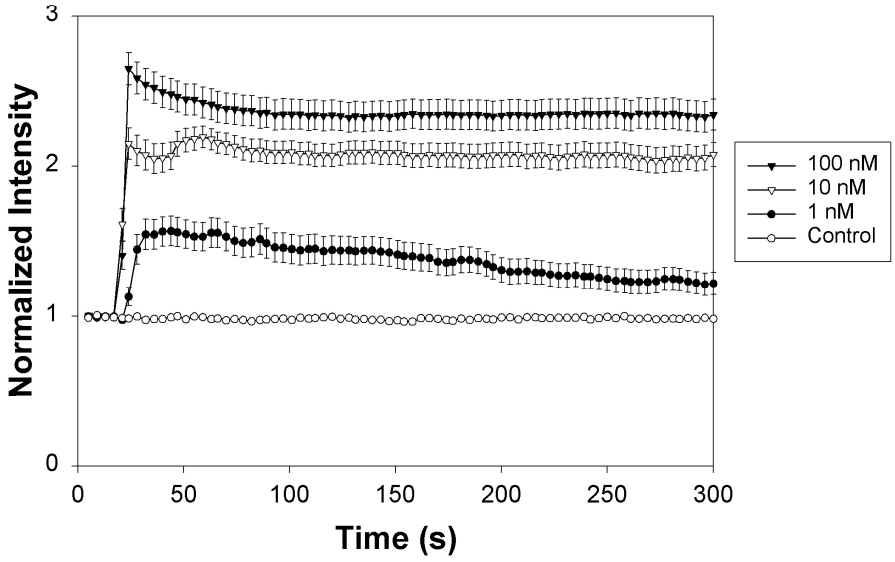


Figure 7. Response of CHO-BMLK3 cells to myokinins, myokinin analogs, and a tachykinin analog. DMEM F-12 media containing 10X final concentration of each peptide (100 µl) was added to cells for a final volume of 1 ml. Control was addition of 100 µl media without peptides. Fluo-4 AM fluorescence intensity was obtained from image scans recorded from about 8-25 cells every 3 seconds. After the fifth scan (15-18 seconds), cells were exposed to peptide agonists and image scans were acquired at the same interval for a total time of 300 seconds. Each concentration was replicated four times. Data presented is normalized fluorescence units. Histograms represent peak fluorescence levels of each concentration. The lowest concentration with a statistically significant ($p < 0.05$) peak over the corresponding control is marked with an asterisk (Student's T-test and Dunnett's Multiple Comparison test). Traces correspond to the mean fluorescence at each scan measured from the 8-25 individual cells of four replications. A) Response to FFFSWS-NH_2. B) Response to FFFSWG-NH_2. C) Response to FFSWG-NH_2. Trace for 0.0001 µM not shown. D) Response to FYSWG-NH_2. Trace for 0.00001 µM not shown. E) Response to muscakinin (NTVVLGKKQRFHSWG-NH_2). F) Response to lymnokinin (PSFHSWS-NH_2). Traces for 0.05 and 0.01 µM not shown. G) Response to *S. calcitrans* tachykinin-like peptide receptor (STKR) agonist (APTGFFGVR-NH_2). Traces for 10 and 20 µM not shown. In C, D, F and G, traces not shown were indistinguishable from the respective control trace.



FFFSWG-NH_2



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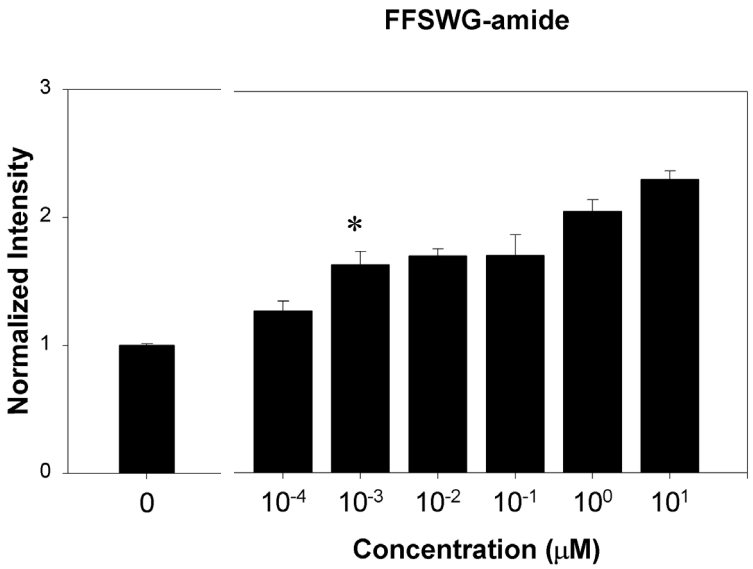
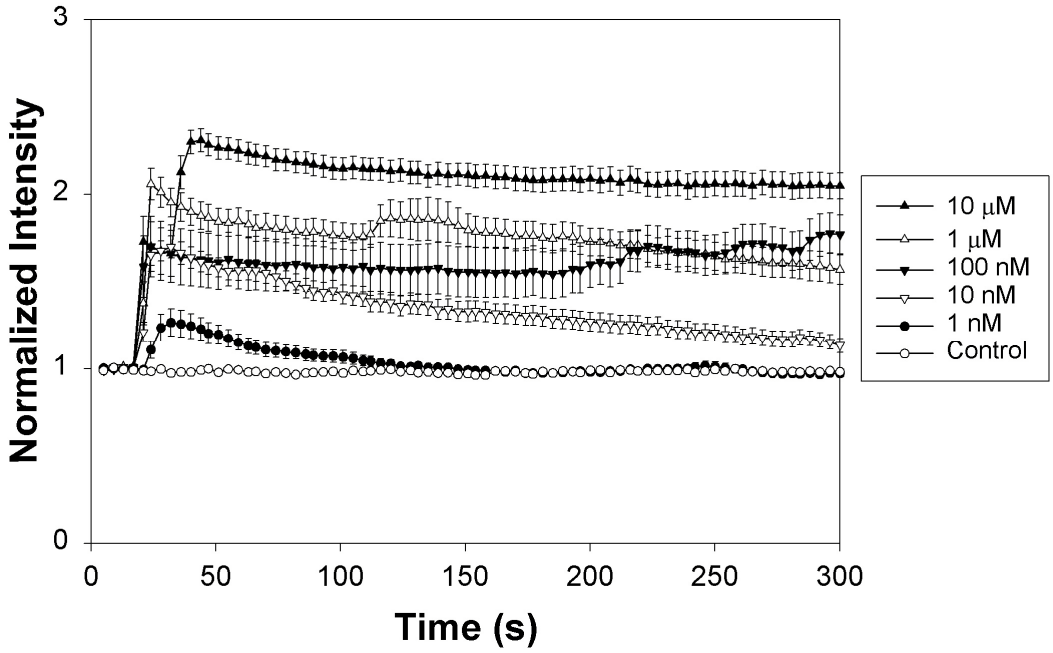


FFSWG-NH_2



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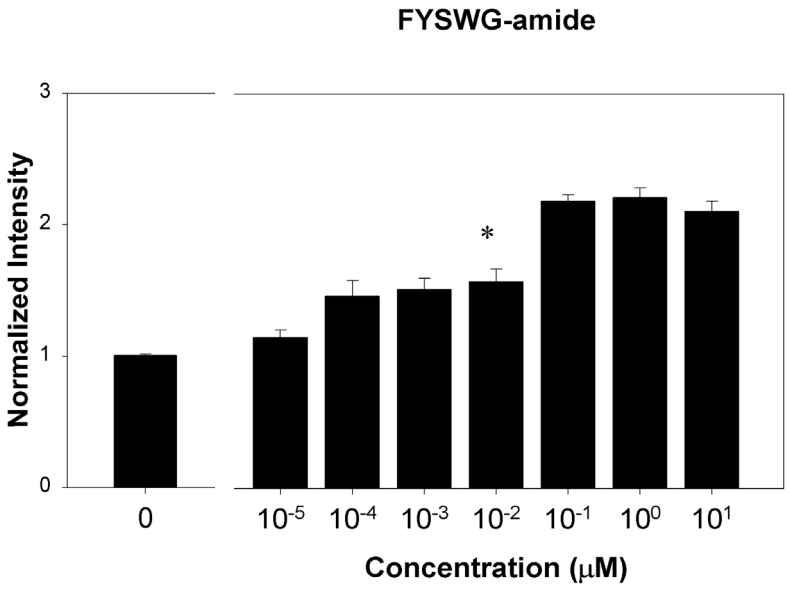

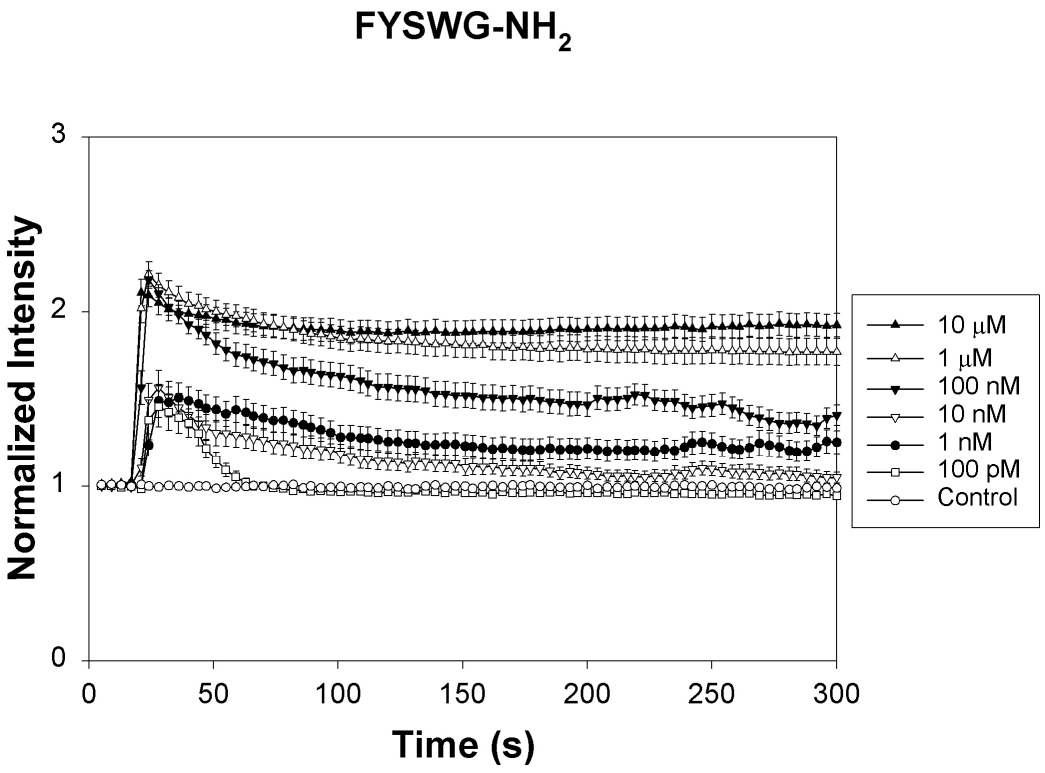

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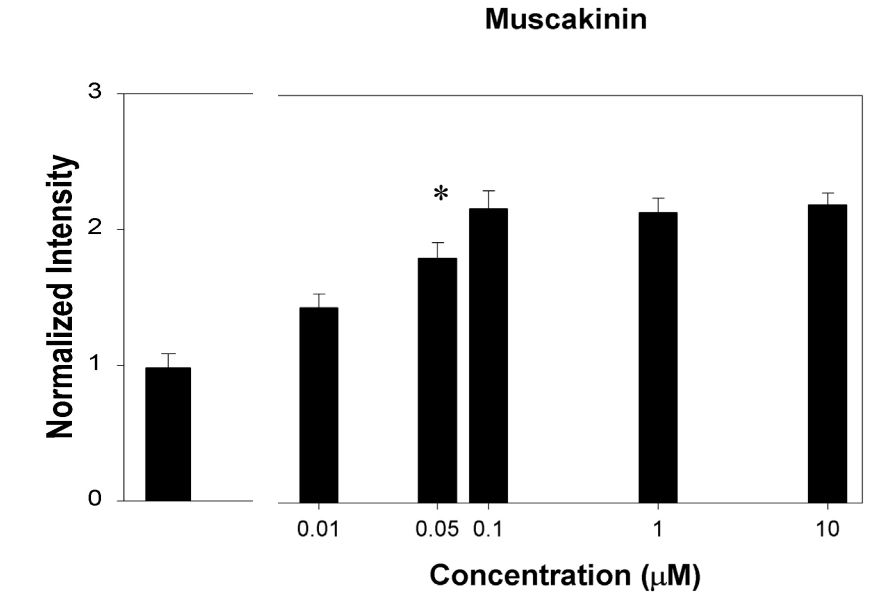

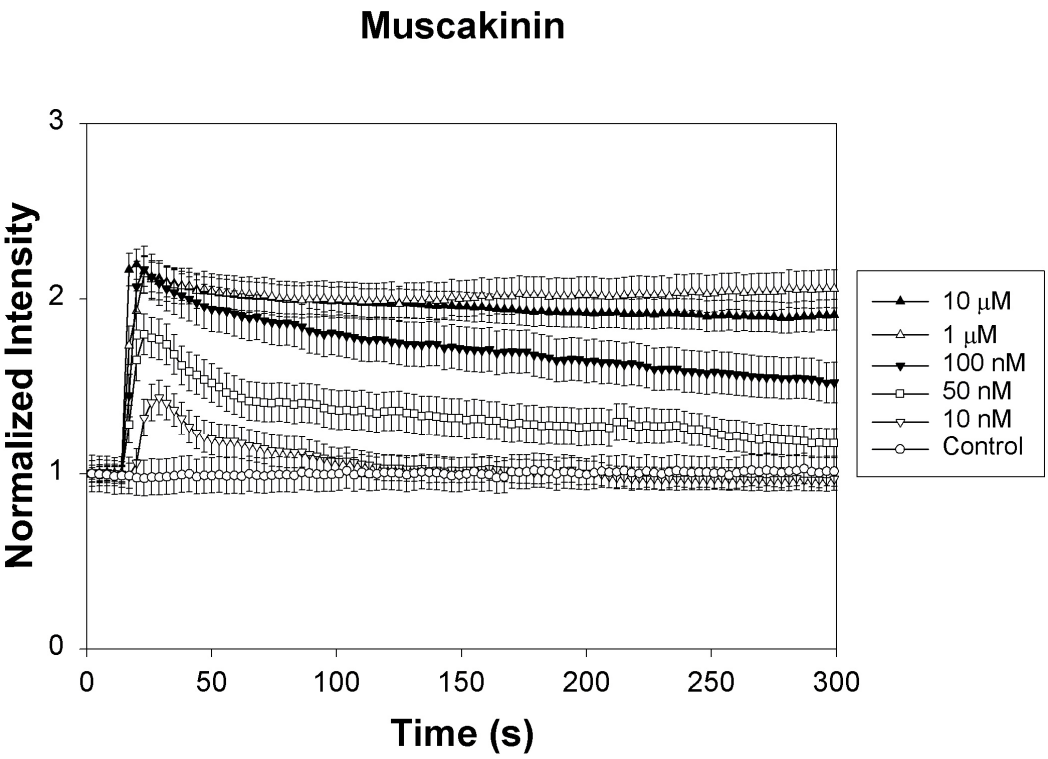

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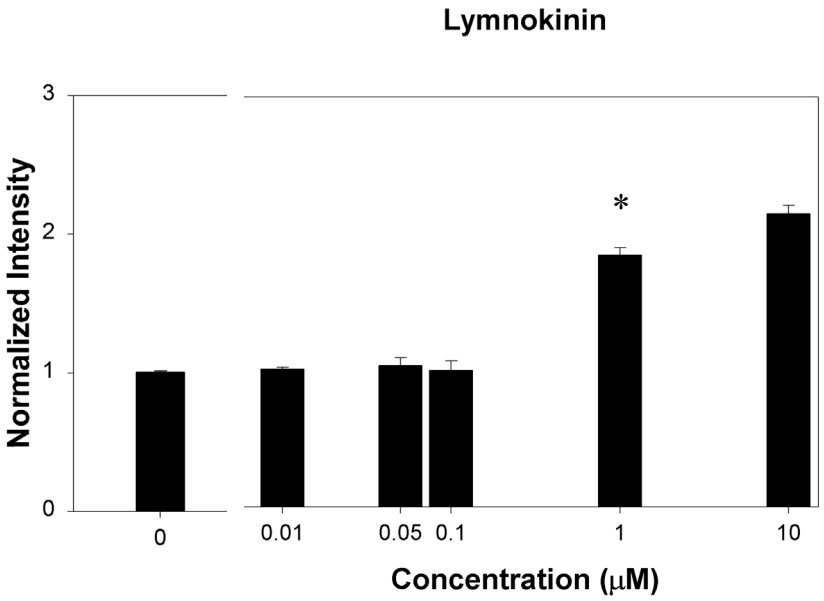

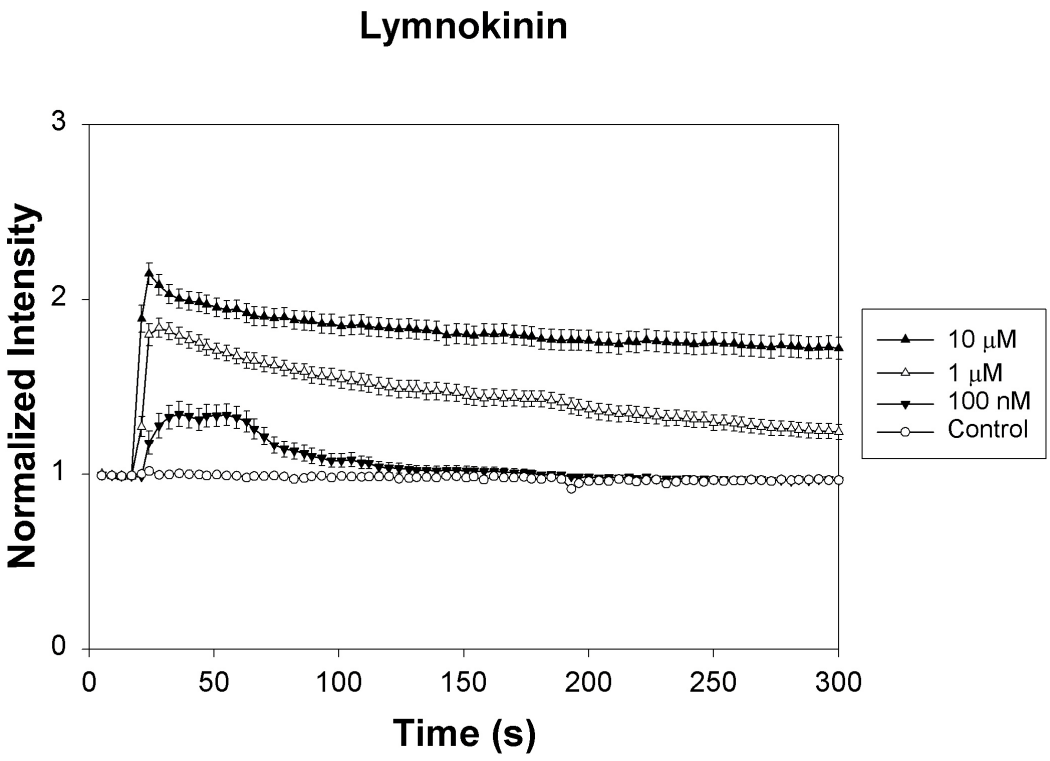

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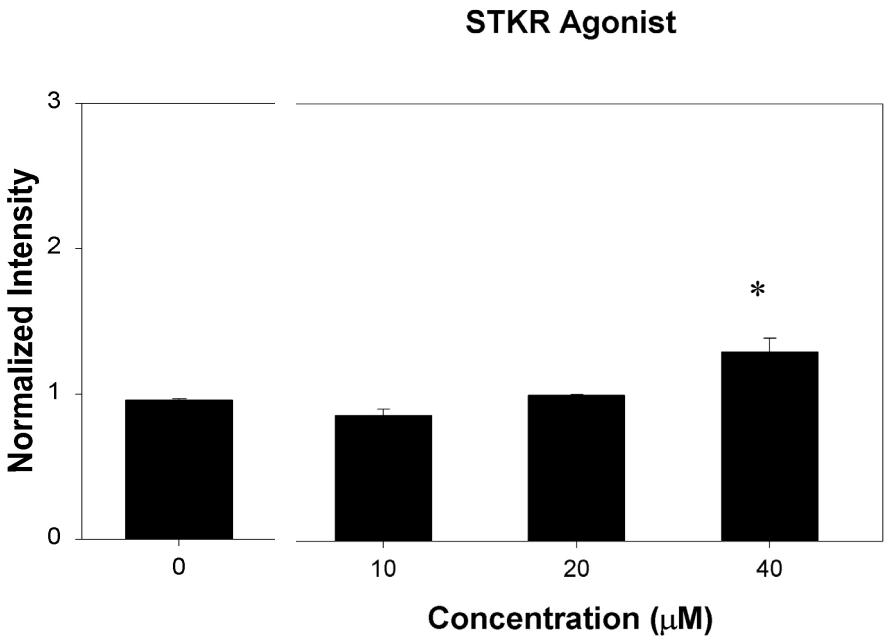

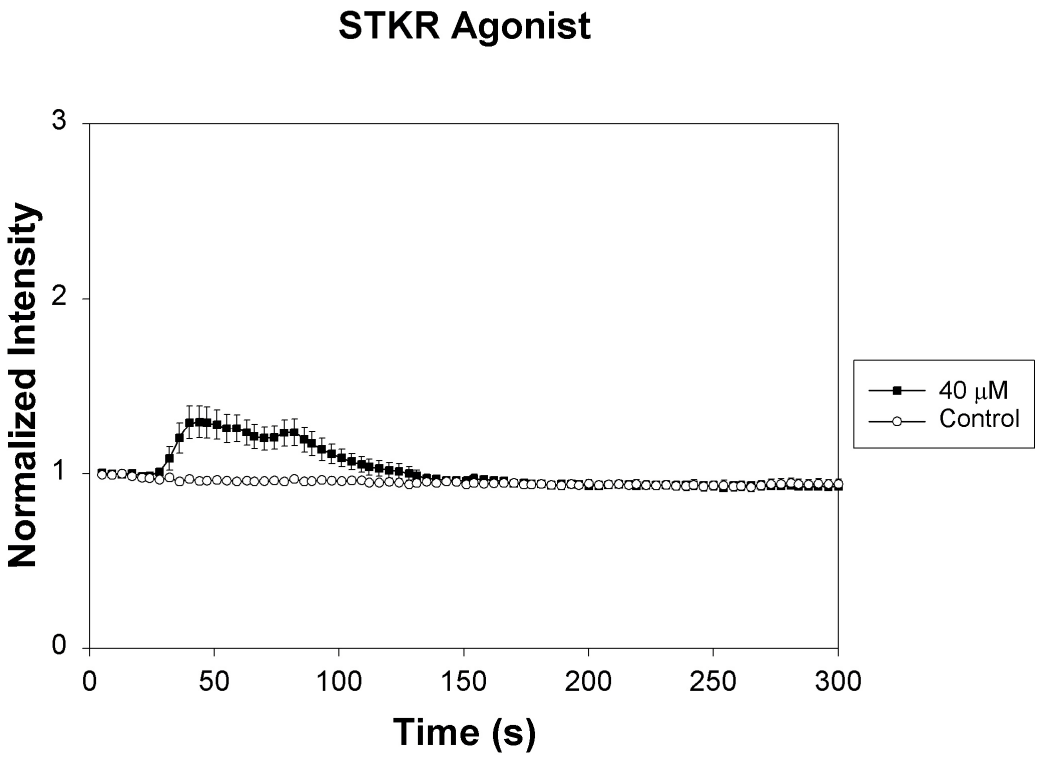

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Table 3. Amino acid sequence and estimated potencies (EC_{50}s ± 95 % confidence intervals) of myokinin peptides and designed analogs in inducing a calcium response in receptor transfected cell line CHO-BMLK3. EC_{50}s are an estimate of the concentration required to induce a half-maximal response. EC_{50}s followed by similar letters in superscript are not significantly different.

Peptide	EC_{50} ± C.I.
FFFSW**S**-NH_2	4.2 ± 1.7 nM^{A}
FFFSWG-NH_2	5.7 ± 2.1 nM^{AB}
FFSWG-NH_2	8.4 ± 2.4 nM^{B}
FYSWG-NH_2	13.1 ± 2.1 nM^{C}
Muscakinin NTVVLGKKQRFHSWG-NH_2	17.0 ± 1.6 nM^{D}
Lymnokinin PSFHSW**S**-NH_2	566 ± 1.5 nM^{E}
STKR agonist APTGFFGVR-NH_2	Not estimated (> 20 μM)
Oxytocin CYIQNCPLGG-NH_2	No response

Pertussis toxin is an AB5 protein homologous to cholera toxin that ADP-ribosylates a specific Cys residue of $G_{i\alpha}$. The modified G-protein does not exchange its bound GDP for GTP (Fields & Casey, 1997). Pretreatment of transfected cells with concentrations of pertussis toxin up to 1000 ng/ml for 16 h prior to agonist challenge did not inhibit the cellular response to FFFSWG-NH_2 (Fig. 8C), indicating that myokinin peptides exert their effects through a pertussis toxin-insensitive G protein mediated pathway in mammalian CHO-K1 cells.

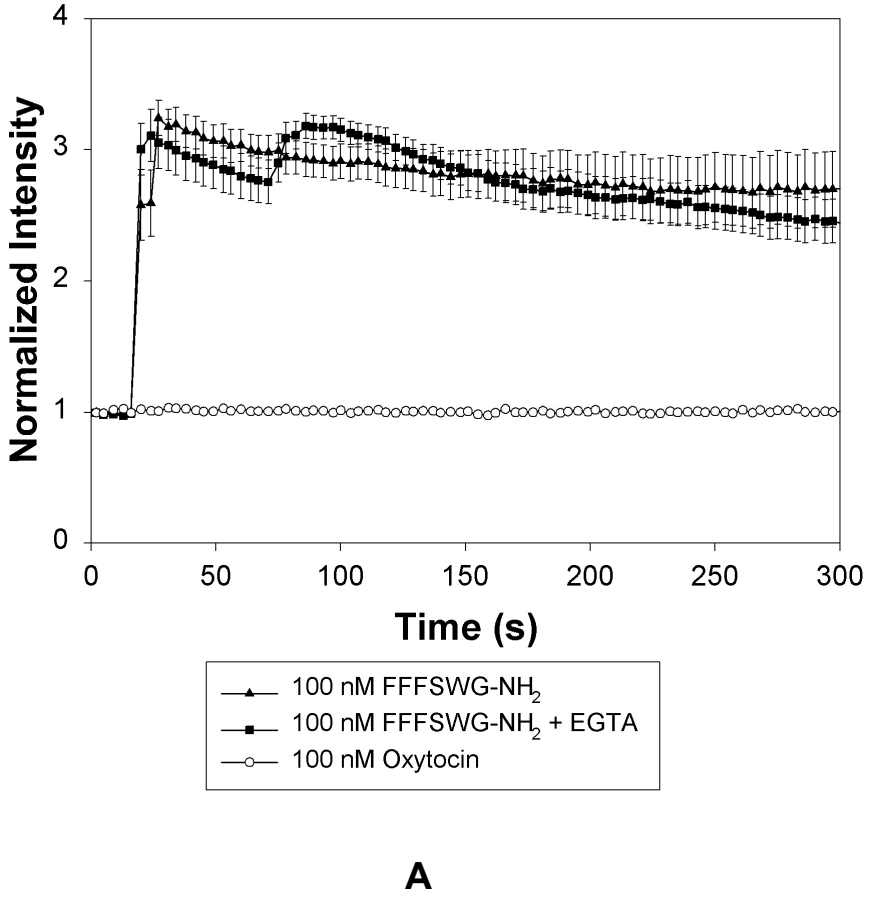
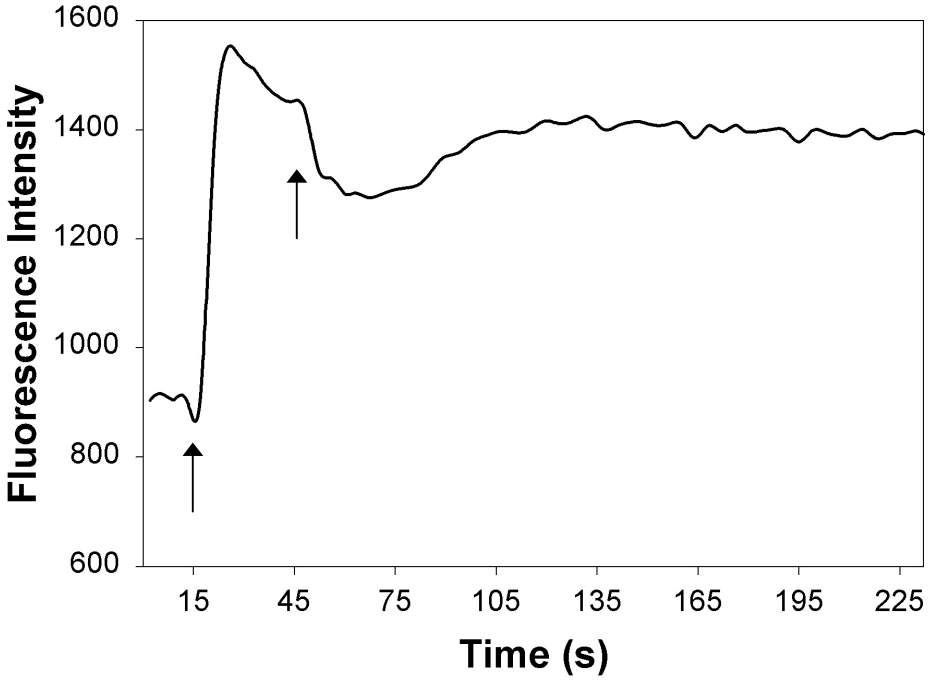


Figure 8. Analysis of signal transduction. A) Response of CHO-BMLK3 cells to 100 nM FFFSWG-NH_2 in comparison to cells co-treated with this peptide and 1 mM EGTA (EGTA added 1 min prior to peptide). EGTA had no significant effect. Cells did not respond to 100 nM of the unrelated mammalian peptide hormone oxytocin. B) Response of CHO-BMLK3 cells to 1 µM lymnokinin followed by 1 µM thapsigargin. Trace represents the raw fluorescence mean response of 16 cells. Lymnokinin was added at 15 seconds (first arrow) and thapsigargin at 42 seconds (second arrow). Thapsigargin treatment did not increase fluorescence indicating that lymnokinin treatment had depleted intracellular calcium stores. C) Response of CHO-BMLK3 cells to 1 µM thapsigargin followed by 1 µM lymnokinin. Thapsigargin was added at 15 seconds (first arrow) and lymnokinin at 90 seconds (second arrow). D) Response of CHO-BMLK3 cells to 100 nM FFFSWG-NH_2 in comparison to untreated cells (control) and cells pretreated with 50-1000 ng/ml pertussis toxin for 16 hours. Although only the traces for 50 and 500 ng/ml pertussis toxin are shown, this toxin in concentrations up to 1000 ng/ml did not significantly inhibit the calcium response.



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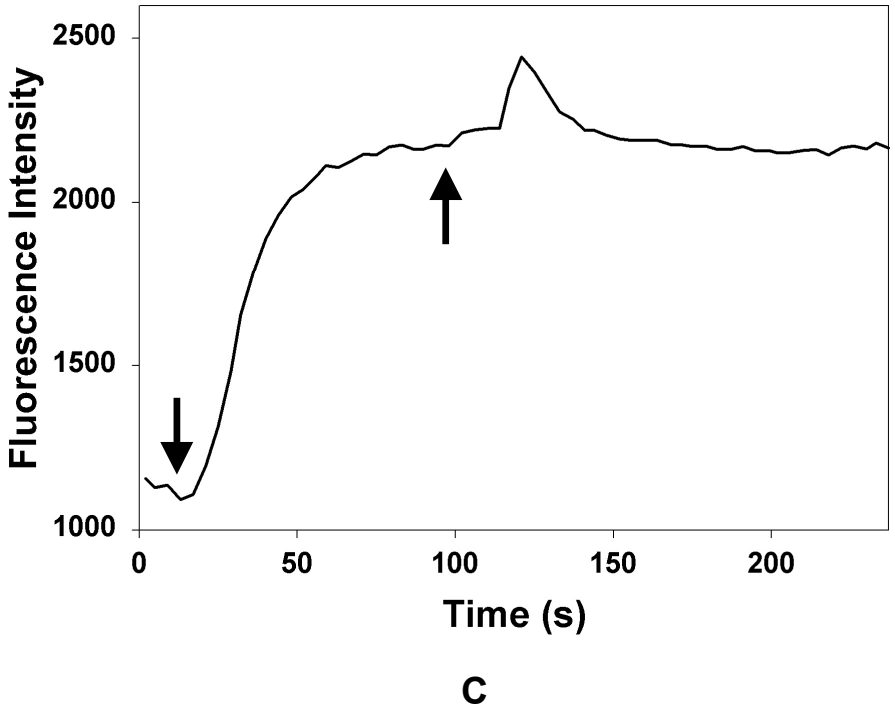

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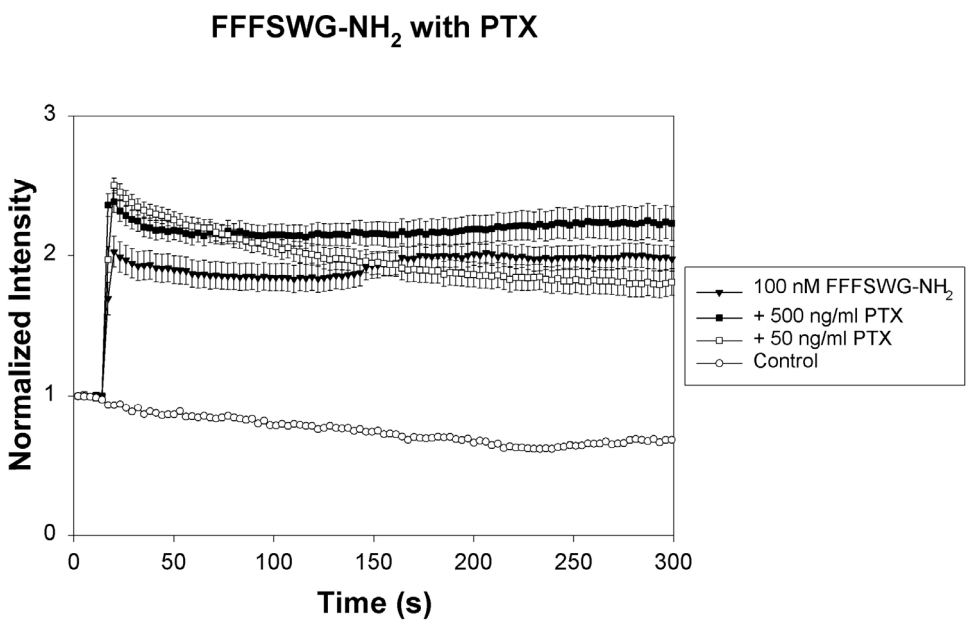

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Figure 8. Continued.

Discussion

Our study shows that the tick receptor, when stably expressed in a CHO-K1 cell line, mediates agonist-induced changes in intracellular calcium as a second messenger in response to nanomolar concentrations of myokinins. This study proves that the tick receptor (AF228521) is the first myokinin receptor to be cloned from an arthropod. The rank-order of potency for the peptides tested was FFFSWS-NH_2 ≥ FFFSWG-NH_2 ≥ FFSWG-NH_2 > FYSWG-NH_2 > muscakinin > lymnokinin. This corresponds to the ranking found with some of these peptides in a transepithelial voltage (T.V.) assay in *Aedes* Malpighian tubule, where FFFSWG-NH_2 > FFSWG-NH_2 > FYSWG-NH_2 (Pietrantonio *et al.*, 2000), although EC_{50}s for these three peptides were lower than we found in this study. Veenstra *et al.* (1997) also found that *Aedes* leucokinins 1-3 depolarized the *Aedes* Malpighian tubule at very low concentrations, with EC_{50}s ranging from 2.5 x 10^{-11} M to 3.9 x 10^{-10} M.

In contrast, higher concentrations of *Aedes* leucokinins 1 and 3 (10^{-8} to 10^{-6} M) were required to stimulate fluid secretion from the tubules, while *Aedes* leucokinin 2 had no effect on tubule secretion (Veenstra *et al.*, 1997). This difference between myokinin concentrations required for the observed effects, where approximately 100 times greater concentrations are required for secretion, may account for the wide range of effective concentrations reported by Pietrantonio *et al.* (2000), but not seen in our assay with the same myokinin analogs. The myokinin analog FYSWG-NH_2, which is identical to the terminal pentapeptide sequence of *Aedes* leucokinin 1, had an EC_{50} for fluorescence response on the order of 10^{-8} M and ranked fourth in our assays. This EC_{50} is closer to

the concentration of *Aedes* leucokinin 1 required to stimulate fluid secretion than that required to depolarize the T.V. in mosquito Malpighian tubules. In summary, our results appear to indicate that potencies estimated from T.V. assays may not be good indicators of myokinin physiological response – such as diuretic activity - because depolarization can be induced at much lower concentrations and is not always associated or causative of fluid secretion.

Although lymnokinin was not highly potent in our assay (EC_{50} = 566 nM), this was not unexpected because it is not known to be an endogenous tick peptide. In a similar assay with CHO-K1 cells expressing the lymnokinin receptor, lymnokinin and the synthetic peptide PSFHSWG-NH_2, identical to lymnokinin except for the Gly residue, induced increases in intracellular calcium. Both peptides had a similar potency (lymnokinin EC_{50} = 1.14 nM) (Cox *et al.*, 1997). Similarly we found that exchanging the terminal Ser for Gly did not significantly affect the potency on the tick receptor, since the peptides FFFSWG-NH_2 and FFFSWS-NH_2 had similar potencies. It appears that in the snail and tick, these two residues are interchangeable at the C-terminus. However the C-terminal Gly is conserved in all the known insect myokinins, suggesting that it is necessary for myokinin action in insects. In support of this, an alanine scan of the peptide FYSWG-NH2 revealed that replacement of the Tyr (Y) or Gly (G) residues made the peptide about 1/20 as effective in causing contractions of the roach hindgut (Nachman & Holman, 1991). The high conservation of the C-terminal Gly in the insect myokinins could thus indicate evolutionary differences in insect receptors. An interesting test would be to compare the effects of these two peptides in an insect

receptor expression system to determine if the results support those found with isolated tissues by Nachman & Holman (1991) mentioned above.

The natural myokinins have 1 to 10 variable N-terminal residues beyond the pentapeptide core which affect potency (Holman *et al.*, 1991; Coast *et al.*, 2002). Cox *et al.* (1997) also determined that leucokinins IV and VI (DASFHSWG-NH_2 and pESSFHSWG-NH_2, respectively) were about 1/100 as potent in eliciting a calcium response than lymnokinin, although their C-terminal pentapeptide sequence is identical to lymnokinin except that the C-terminus is Gly instead of Ser. However in our assay, muscakinin, which has a similar terminal pentapeptide sequence to lymnokinin, was approximately 30-fold more potent than lymnokinin. As discussed, the C-terminus substitution of Gly with Ser does not seem to significantly affect activity on the tick receptor, so the difference in activity between peptides with identical cores (except for G or S) and different N-terminal residues observed by us and by Cox *et al.* (1997), is most likely due to the effect of these variable N-terminal residues. In agreement with this, achetakinin-V (AFHSWG-NH_2) is 10-fold more potent than the core peptide FHSWG-NH_2 in inducing diuretic responses in isolated Malpighian tubules from the house cricket, *A. domesticus* (Coast *et al.*, 1990). The critical role of N-terminal residues in peptide activity was demonstrated by Coast *et al.*, (2002), who showed that the pentapeptide minimum active sequence of muscakinin was less potent than muscakinin by more than five orders of magnitude in the house fly Malpighian tubule secretion assay.

The response of CHO-BMLK3 cells to 100 nM FFFSWG-NH_2 was unaffected by the presence of 1 mM EGTA (Fig. 8A), indicating that calcium ions involved in receptor mediated signal transduction were released from intracellular stores, as was also found in the stellate cells from *Drosophila* Malpighian tubule (Rosay *et al.*, 1997). Treatment with thapsigargin following challenge with 1 μM lymnokinin failed to cause any further increase in intracellular calcium, indicating that intracellular stores were depleted by myokinin receptor maximal activation (Fig. 8B). Lack of inhibition of the myokinin-induced calcium response by pertussis toxin indicates that the signal transduction is effected through a pertussis toxin-insensitive G protein (Fields & Casey, 1997).

After the drosokinin and lymnokinin receptors, tachykinin receptors are the most similar in sequence to the tick receptor (Table 2). Therefore, observed reduced response elicited by high concentrations of a tachykinin-like peptide (Fig. 7G) demonstrates the high selectivity of the receptor for myokinins. The strong activity of muscakinin on the tick receptor (EC_{50} = 17 nM) indicates that its endogenous ligand may be a similar peptide. Muscakinin is identical - except for one amino acid substitution - to a myokinin peptide (drosokinin) isolated from *D. melanogaster* (Terhzaz *et al.*, 1999). Because the GPCRs that interact with closely related ligands also have the greatest sequence homology and structural conservation (Iismaa *et al.*, 1995), we predicted that CG10626, the *Drosophila* gene product now known as the drosokinin receptor, would have a closely related ligand that was most likely the drosokinin peptide (Holmes *et al.*, 2003).

Based on homology with other receptors, the tick receptor was expected to belong to the subfamily of myokinin receptors (Homes *et al.*, 2000; Hewes & Taghert, 2001). Our functional assays with the tick receptor have unequivocally established it as the first myokinin receptor identified from an arthropod, as well as the first neuropeptide receptor cloned from an arachnid. Although this receptor remains an orphan because no endogenous ligand has been isolated, its specificity for myokinins is clear. This receptor's reponse, the leucokinin-like immunoreactivity found in the spider (Arachnida) nervous system (Schmid & Becherer, 1996), and the detection of receptor mRNA in all life stages of the tick (Chapter II), indicate that myokinin peptides may play a critical role in the physiology of the tick. Myokinin receptors have been validated as novel targets for pest control in the cotton budworm *Heliothis virescens*. Larvae injected with helicokinins I increased mortality, as well as when co-injected with helicokinins II or III and an angiotensin-converting enzyme inhibitor (Seinsche *et al.*, 2000). The functional expression of the tick receptor makes available a system for screening compounds for agonist or antagonist activity.

CHAPTER IV

TISSUE DISTRIBUTION AND IMMUNOLOGICAL CHARACTERIZATION OF THE TICK MYOKININ RECEPTOR

Introduction

Relatively little is known of the expression and function of myokinin receptors in invertebrate organisms, despite their important roles in physiological processes of insects, including diuresis. This is not surprising considering that descriptions of only three myokinin receptors from the pond snail *Lymnaea stagnalis* (Cox *et al.*, 1997), cattle tick *Boophilus microplus* (Holmes *et al.*, 2000; Holmes *et al.*, 2002), and fruit fly *Drosophila melanogaster* (Radford *et al.*, 2002), respectively are recently available in the published literature. In contrast, much more is known of the myokinin neuropeptides that are the endogenous ligands. With the isolation of the *Drosophila* leucokinin, drosokinin (Terhzaz *et al.*, 1999), there are now 26 known members of the myokinin peptide family. The first members of this family, the leucokinins I and II, were isolated and described in 1986 (Holman *et al.*, 1986a).

In insects, the myokinin peptides increase the frequency of hindgut contractions and stimulate secretion from the Malpighian tubules. The leucokinins were initially isolated on the basis of their ability to stimulate hindgut contractions in the cockroach *Leucophaea maderae* (Holman *et al.*, 1986a; Holman *et al.*, 1986b; Holman *et al.*, 1987a; Holman *et al.*, 1987b). Related myokinins from the locust *Locusta migratoria* (Schoofs *et al.*, 1992), mosquito *Aedes aegypti* (Veenstra *et al.*, 1997), and the house fly

Musca domestica (Holman *et al.*, 1999) have been shown to stimulate hindgut contractions in those species. In the crab *Cancer borealis*, leucokinins excite the pyloric rhythm (Blitz *et al.*, 1995). In the Malpighian tubules, the myokinins act through increases in intracellular calcium to stimulate fluid secretion (Cady & Hagedorn, 1999b; O'Donnell & Spring, 2000; Yu & Beyenbach, 2000).

Both the lymnokinin receptor and peptide were isolated from the CNS of the pond snail *L. stagnalis* (Cox *et al.*, 1997), indicating expression in nervous tissue. Leucokinin-like immunoreactivity has consistently been found in the nervous system of several arthropod groups, including the brain and ventral ganglia of *L. maderae* (Nässel, 1992), abdominal ganglia of the tobacco hornworm, *Manduca sexta* (Chen *et al.*, 1994), brain and thoracic ganglia of the mosquito *Culex salinarius* (Clottens *et al.*, 1993), brain and fused thoracic-abdominal ganglion of *M. domestica* (Iaboni *et al.*, 1998), CNS and digestive system of the blood-feeding bug *Rhodnius prolixus* (Te Brugge *et al.*, 2001), the CNS and leg neuromeres of the spider *Cupiennius salei* (Schmid & Becherer, 1996), and the CNS of the flies *D. melanogaster*, *Calliphora vomitoria*, and *Phormia terraenovae* (Cantera & Nässel, 1992). Additionally, two myokinin-related peptides have been isolated from the brain of the white shrimp *Penaeus vannamei* (Nieto *et al.*, 1998).

The first localization of a myokinin receptor was of the *D. melanogaster* drosokinin receptor (Radford *et al.*, 2002), previously identified by the *Drosophila* genome project as gene product CG10626. Immunohistochemistry showed expression of this receptor in the stellate cells of the Malpighian tubules and pars intercebralis of the

adult CNS. Receptor transcript was additionally found by RT-PCR in the male and female gonads (Radford *et al.*, 2002). In the cricket *Acheta domesticus* and mosquito *A. aegypti*, myokinin binding sites have been biochemically characterized in the Malpighian tubules, and presumably correspond to myokinin receptors in those species (Chung *et al.*, 1995), (Pietrantonio *et al.*, 2000). Despite the strong possibility of myokinin receptor expression in the invertebrate brain, receptor localization efforts have focused mainly on the Malpighian tubules and digestive systems where their functions are best understood.

In this chapter, receptor transcripts were detected by RT-PCR in the dissected synganglion, gut, salivary glands, ovaries, and Malpighian tubules of partially engorged *B. microplus* adult females. This is the first evidence of myokinin receptor expression in the salivary glands and in non-nervous tissue in an arachnid. Rabbit anti-receptor antisera directed against a synthetic peptide corresponding to the predicted second extracellular loop of the *B. microplus* receptor was generated in two rabbits. Immunolocalization experiments were performed in order to determine if the antisera contained antibodies that would effectively bind receptor proteins and be useful for immunohistochemistry. Functional experiments with the anti-receptor antisera were performed to determine if antibody binding of the second extracellular loop would inhibit agonist induced activity. These experiments did not show that the antisera was able to specifically bind the receptor protein or inhibit function.

Methods

Production of antisera/antibodies

Both whole antisera and affinity-purified sera directed against the tick receptor were used in experiments. Receptor antisera were produced by Sigma Genosys (The Woodlands, TX) in two female New Zealand rabbits by a standard ten-week protocol. The synthetic peptide **RVETQVESHALNLTK**C was linked to keyhole limpet hemocyanin (KLH) and used to immunize the rabbits six times to boost antibody production. The first immunization contained 200 μg of the KLH-conjugate in Complete Freund's Adjuvant, and subsequent immunizations contained 100 μg in Incomplete Freund's Adjuvant. The synthetic peptide corresponds to the partial sequence from the predicted 2nd extracellular loop of the receptor, except for the terminal Cys which was added to facilitate C-terminal linking to KLH. Serum was collected in a preimmune bleed, four production bleeds, and a final exsanguination. Antisera were preserved with 0.1% sodium azide, and stored at -80˚C in 1 ml aliquots until use. Commercially prepared rabbit anti-integrin β5 subunit polyclonal antibody (Chemicon International, Temecula, CA) was used as a positive marker for the presence of plasma membrane proteins in western blots.

Affinity purification of antisera

To produce an affinity column for purification of anti-receptor peptide antibodies, approximately 6 mg of synthetic peptide was linked to Affi-Gel® 10

activated immunoaffinity support (Bio-Rad, Hercules, CA). Residual water was removed from the hygroscopic solvents DMSO and triethylamine by storing them over 4 Å molecular seives for a minimum of 48 h ("drying"). A 10 ml aliquot of immunoaffinity support was prepared by removing the storage solvent (isopropanol) and washing with "dry" DMSO. Solvents were removed by filtering with suction through a layer of Whatman 3M paper over a fritted funnel. The support was combined with synthetic peptide in 20 ml of dry DMSO, 100 µl of triethylamine was added, and the mixture was incubated overnight (16 h) at RT with gentle agitation. The mixture was incubated an additional hour at RT after the addition of 500 µl of ethanolamine, after which the solvent was removed and the linked immunoaffinity support was washed 3 times in 50 ml of DMSO, then twice with 1N acetic acid, then washed with MilliQ H_2O. Finally, the support was stored in MilliQ H_2O with 0.05% sodium azide at 4˚C until further use.

To prepare the immunoaffinity support for use, most of the water was removed and it was resuspended in 10X PBS and shaken by hand for 2 min. The pH was checked with test strips to ensure that it was near 7. The 10X PBS was then removed and the support was resuspended in 10 ml of 5X PBS. All further steps were done at 4˚C unless otherwise stated. To prepare the preimmune and antisera for affinity purification, 10 ml was thawed and Complete™ protease inhibitor cocktail (Roche) was added. The serum was then mixed with 10 ml of 10X PBS to bring it to a final concentration of 5X. The serum and support were mixed and allowed to incubate for 5 min then added to a glass column. The first flowthrough was collected and run through the column a second time.

The column was then washed with 100 ml of 5X PBS in 10 ml fractions, which were collected and measured for absorbance at 280 nM (A280) until absorbance decreased to values similar to a blank.

Before antibody elution, 5X PBS was drained from the column until it reached the top of the gel bed, then 5 ml of 100 mM NaCitrate pH 2.5 was added and allowed to stand for 2 min before the column was reopened. The eluant was collected in 1-1.5 ml aliquots into siliconized Eppendorf tubes already containing 200 µL of 1M Tris pH 8.8. Ten µl of normal goat serum (NGS) (Sigma) was then added to each tube, and the eluant fractions were combined and dialyzed against 1 L of 1X PBS overnight at 4˚C in Slide-A-Lyzer® dialysis cassettes (Pierce, Rockford, IL) with a 10,000 MW cutoff. After the sample was recovered, one tenth volume of 0.5% sodium azide was added and the sample was concentrated down to 1.5 to 4 ml using a Centricon Plus-20 centrifugal filter device (Millipore, Billerica, MA) with 100,000 MW cutoff. Samples were stored at 4ºC until use.

Functional assays with transfected CHO-K1 cells (CHO-BMLK3) were performed as described in Chapter III, except that treated cells were incubated in receptor antisera and preimmune sera for 4 hours at dilutions of 1:100 and 1:1000 in normal growth medium prior to fluorophore loading and agonist challenge.

Immunocytochemistry

Approximately 10,000 CHO-BMLK3 or CHO-pcDNA cells were seeded on glass slides using cytofuge concentrators (StatSpin Technologies, Norwood, MA) and

allowed to grow for 48 h in growth medium and conditions as described in Chapter III. Once cells reached 50-80% confluence, slides were cooled on ice and washed in 1X TBS (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.5). Cells were fixed for 30 min in 2% paraformaldehyde solution at 4˚C, then washed 2x 5 min in cold TBS. Cells were blocked in 2% normal goat serum (Sigma) for 30 min at RT, then incubated in 1:500 dilutions of preimmune or receptor antisera for 1 h at 4˚C. Cells were then washed 5x 5 min in cold TBS and incubated in 20 μg/ml Texas Red Anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for 1 h at 4˚C. Cells were washed again 5x 5 min in cold TBS, then mounted with Vectashield™ containing 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.). Microscopy was performed as previously described (Pietrantonio *et al.*, 2001).

Western blots with CHO-BMLK3 cells and purified plasma membranes

For blots with complete cell lysate, cells were grown in T-75 tissue culture flasks until monolayers reached confluence, then rinsed with PBS. Cells were dislodged with a rubber scraper into 3-5 ml of PBS per flask, then pelleted by centrifugation at 1000 xG for 3 min. Supernatant was discarded and cells were resuspended in SDS lysis buffer (5% SDS, 10 mM NaCl, 0.1 mM EDTA, 10 mM Tris) for complete cell lysis or Nonidet P-40 lysis buffer (1% Nonidet P-40, 50mM Tris, 150 mM NaCl), which does not solubilize cell membranes. Complete™ protease inhibitor cocktail (Roche) was added to lysis buffers shortly before use. Cell suspensions in lysis buffer were incubated at 37ºC for 10 minutes then centrifuged at 15,000 x *g* for for 10 min to pellet nuclei and debris.

The supernatant was retained and protein concentration was estimated by Bradford assay. From 20-40 μg of protein/lane from each sample and 10 μl of Rainbow™ coloured protein molecular weight markers (Amersham, Buckinghamshire, UK) was combined with an equal volume of 2X sample treatment buffer (0.125M Tris, 4% SDS, 20% glycerol, 0.1 mg/ml bromphenol blue, and 0.1M β-mercaptoethanol or 0.2M DTT) and heated in boiling water for 3 min. Samples and markers were loaded in a 10% polyacrylamide SDS-Tris precast minigel (BioRad) under SDS running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS) and electrophoresed at 100 volts for 75-120 min.

A piece of Immobilon PVDF membrane (Millipore) was soaked briefly in methanol then in transfer buffer (25mM Tris, 20mM glycine, 20% methanol) for 15 min. After electrophoresis, the gel was recovered and soaked in transfer buffer for 15 min. The gel and membrane were placed together between two pieces of Whatman 3M filter paper and a sponge on each side, then loaded in the transfer apparatus. The transfer apparatus was placed on ice, and transfer was at 100V for 75 min. After transfer, the membrane was rinsed briefly with 0.1% PBS-Tween (PBST) and blocked overnight (16 h) in 10% Carnation® instant nonfat dry milk (Nestle, Solon, OH) dissolved in PBST at RT with gentle agitation. The blot was washed briefly to remove blocking solution and incubated in primary antibody at various dilutions for 3 h at RT, then washed 5x10 min in PBST. The blot was then incubated in a 1:20,000 dilution of goat-antirabbit horseradish peroxidase conjugated antibody (Molecular Probes) for 1 h at RT, and washed 5x10 min in PBST. For detection, the blot was incubated in a 1:1 mixture of SuperSignal® West Pico Stable Peroxide Solution and SuperSignal® Luminol/Enhancer

Solution (Pierce) for 5 min, then placed between two pieces of Saran wrap and exposed to film (Fuji medical x-ray film, Fuji Photo Film Co., Tokyo, Japan).

For westerns using preabsorbed antisera, anti-receptor antisera (1:1000 dilution in 10 ml PBST with Complete protease inhibitor cocktail, Roche) were preabsorbed with either 500 μg of antigen-peptide or on a monolayer of prewashed CHO-pcDNA cells in a T-25 tissue culture flask for 7 h at RT with gentle agitation.

Purification of cell plasma membranes

Methods used for subcellular fractionation of cells by differential centrifugation in order to purify plasma membranes were adapted from protocols by Haga & Berstein (1997) and Graham & Higgins (1993). Cell monolayers from four T-75 tissue culture flasks were rinsed with ice cold homogenizing buffer (20 mM HEPES, 10 mM EDTA, protease inhibitor cocktail, pH 7.4), then dislodged with a rubber scraper into 4 ml of homogenizing buffer. Cell suspensions were centrifuged for 2 minutes at 1,000 RPM, and cell pellets were combined and resuspended in 3 ml of homogenizing buffer. Cells were homogenized for 30 strokes with a glass homogenizer, then sonicated for 10-15 seconds at power level 5 on a Fisher Scientific 50 sonic dismembranator. Homogenized samples were then centrifuged in several steps: 1) 10 min at 3,000 x *g*, 2) 10 min at 10,000 x *g*, 3) 20 min at 20,000 x *g*, and 4) 2 hours at 100,000 x *g*. After each centrifugation step the pellet was retained and the supernatant was subjected to further centrifugation. The final supernatant was retained as fraction 5. Each fraction was then

resuspended in 100 µl SDS lysis buffer, and protein concentration was estimated by Bradford assay.

RT-PCR

Five partially engorged female ticks were dissected under sterile PBS and guts, salivary gland, Malpighian tubules, synganglia, ovaries, and salivary glands were removed whole or in pieces and place directly into RNAlater solution (Ambion) and stored at -20°C. Poly-A+ RNA was extracted from the tissues using an mRNA Direct kit (Dynal), and eluted at 80°C in water containing RNAsecure solution (Ambion). First strand cDNA was synthesized from one half of the Poly-A^+ RNA at 42°C for 50 min with oligo$(dT)_{12\text{-}18}$ primer using Superscript II Reverse transcriptase (Superscript Preamplification System, Life Technologies). PCR amplification of receptor cDNA was performed using two separate sets of gene specific primers, 1F (5'-AGT TCA TCA TCT GCG GTA TCT GGA C) and 4R (5'-GTA CAC GAA ACA GAT GGT GAG CAG C), and SC1-F (5'-TTC CGC TAT GGT GCC ACA ATG A) and SC3-R (5'-TGG TGG TTG GAC TCA AAT TAC AC). Amplification of β-actin cDNA with primers Act-3F (5'-TCC TCG TCC CTG GAG AAG TCG TAC) and Act-4R (5'-CCA CCG ATC CAG ACC GAG TAC TTC) was also done as a positive control to show that sufficient quantity and quality of cDNA was present in each sample. Reactions contained synthesized cDNA approximately equivalent to the specific tissue from one half of a tick, 200 µM each of four dNTPs, 0.2 µM of each primer, 0.5 µl Advantage 2 polymerase mix and 5 µl 10X reaction buffer (ClonTech) in a final volume of 50 µl.

The following cycling parameters were used: 94°C for 1 min followed by 40 cycles of 94°C for 20 sec, 65°C for 30 sec, and 68°C for 45 sec. Final extension was at 68°C for 5 min. Products were electrophoresed on a 10% agarose-TBE gel containing ethidium bromide and photographed.

Results

In order to determine the presence of receptor transcript in specific tick tissues, cDNA was synthesized separately from dissected ovaries, synganglion, gut, salivary glands, and Malpighian tubules from partially engorged adult females. RT-PCR experiments to detect the presence of receptor transcript in specific tick tissues were repeated with two separate primer pairs to ensure that no false results could arise from PCR artifacts. Primer set 1 (1F & 4R) (Fig. 9B), and primer set 2 (SC1-F & SC3-R) (Fig. 9C) gave similar results. Amplified receptor cDNA bands of the expected sizes (330, 211, and 708 bp for Fig. 9A, B, C respectively) were visible in the corresponding lanes for each tissue tested. Although the PCR was not done in a quantitative manner, the band in the Malpighian tubule lane clearly contained the least amplified cDNA of all the samples while the corresponding control amplification of β-actin transcript was fairly high (Fig. 9A). These results likely indicate a low amount of receptor transcript in the Malpighian tubule relative to other tissues.

Antisera against the synthetic peptide **RVETQVESHALNLTK**C linked to keyhole limpet hemocyanin (KLH) was raised in two rabbits. The third production

bleed from rabbit GN-2489 had the highest antibody titer, which was 1:100,000 as determined by ELISA (Sigma Genosys), and was used in all further experiments. The synthetic peptide sequence corresponds to the N-terminal portion of the second extracellular loop, and was chosen because of its expected extracellular presentation, possible involvement in ligand-binding, and its predicted high antigenicity (Fig. 10). Other areas of the receptor with high antigenicity were intracellular, with the exception of the N-terminus.

In order to test if binding of receptors by antibody might have a blocking or other effect on live cells, CHO-BMLK3 cells were exposed to 100 nM of the peptide FFFSWG-NH2 after preincubation in media containing no additional sera, preimmune sera, or receptor antisera. Cells were incubated for four hours in antisera concentrations of 1:1000 and 1:100. None of the treatments had an observable effect on the intracellular calcium response (Fig. 11, only data for 1:100 concentrations shown). This lack of an effect from the antisera seemed to indicate that there were not enough antibodies specifically binding the tick myokinin receptor for it to be useful, although this result does not eliminate the possibility that antibodies were binding to the receptor without interfering with its function.

Additional experiments were conducted to determine if this antisera would interact specifically enough with the receptor to be useful for further immunolocalization studies in ticks. Immunocytochemistry was done with CHO-BMLK3 cells to see if the antisera could label plasma membranes expressing the receptor. Receptor antisera did

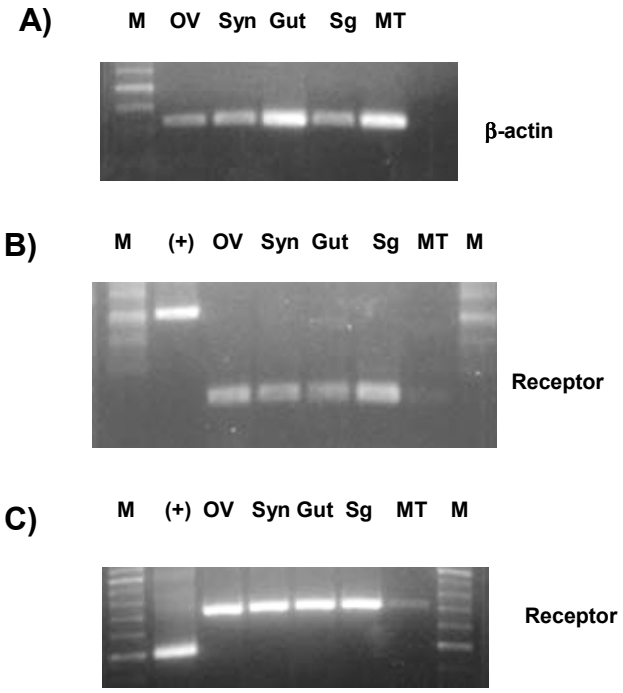

Figure 9. RT-PCR with isolated tick tissues. A) Control PCR with primers against β-actin shows presence of cDNA from each tissue sample. B) PCR with gene specific primers for tick kinin receptor 1F & 4R . C) PCR with gene specific primers (SC1-F & 3R) shows similar results to B). M = marker; (+) = positive control cDNA (Superscript preamplification system, Life Technologies); OV = ovary; Syn = synganglion; Sg = salivary gland; MT = Malpighian tubule.

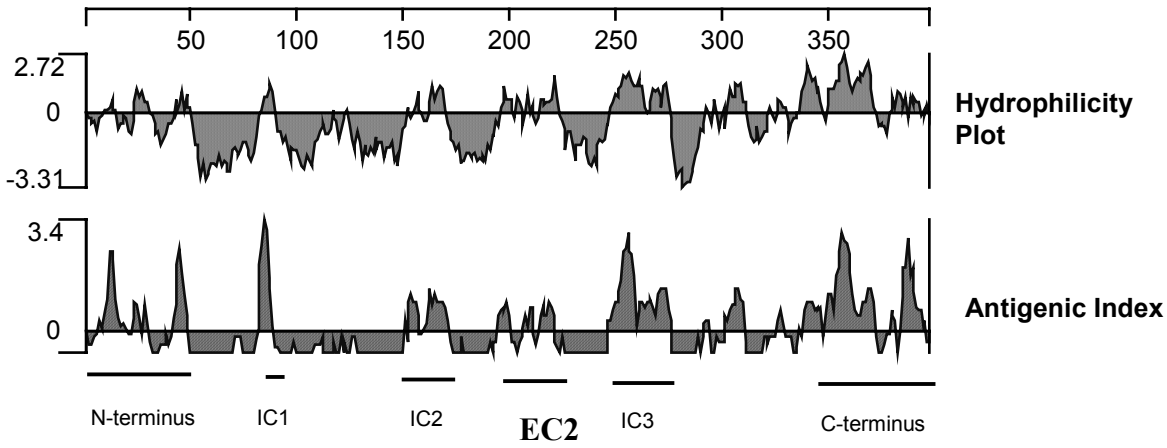

Figure 10. Kyte and Doolittle hydrophilicity plot and Jameson antigenic index of the tick myokinin receptor. Antisera was directed aginst the portion of the receptor labeled EC2. The figure was created with DNASTAR software (DNASTAR, Inc., Madison, WI). IC1 = intracellular loop 1, IC2 = intracellular loop2, EC2 = extracellular loop 2, IC3 = intracellular loop 3.

not generate any signal in plasma membranes above the background level observed in cells treated with preimmune sera (data not shown).

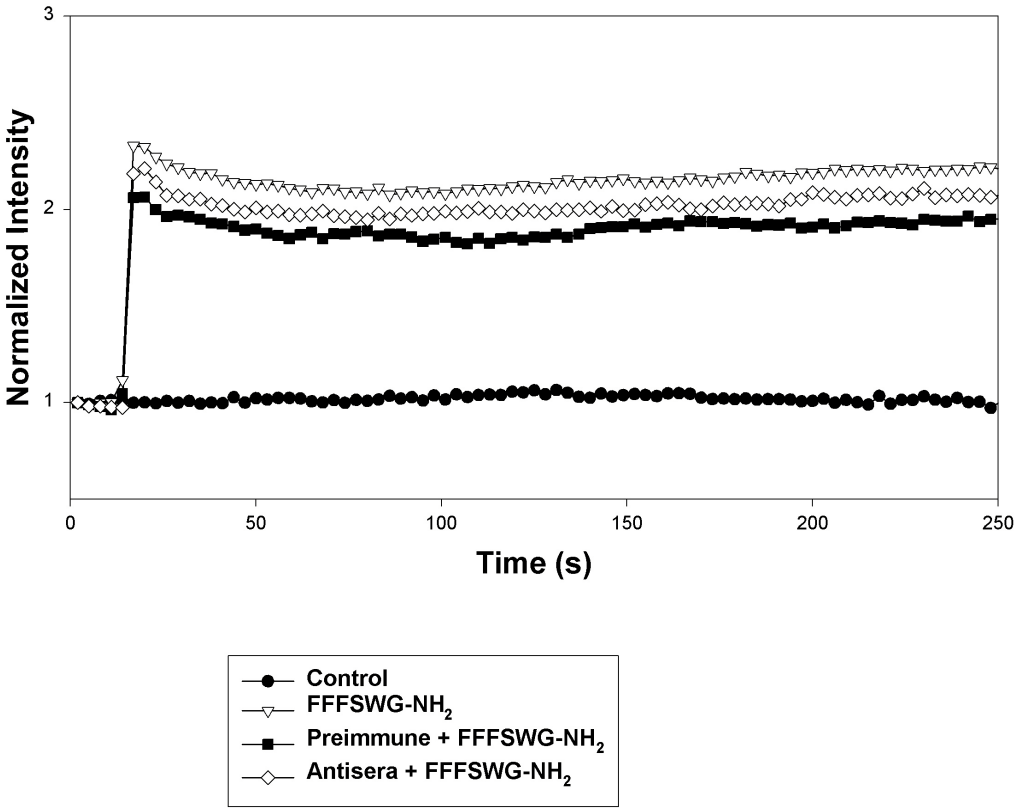

Figure 11. Response of CHO-BMLK3 cells to 100 nM FFFSWG-NH_2 following pretreatment with antisera. Control was addition of media with no peptide. The other traces represent a normal response with no pretreatment, and pretreatment for 4 h with 1:100 dilution of sera from preimmune and postimmune bleeds, respectively.

Several western blots were done in order to determine if the antisera contained antibodies that would recognize the denatured protein on a membrane. The receptor band was expected to be at 44.9 kDa or higher, based on its predicted molecular mass (Chapter II) and the possibility of glycosylation. The western blot technique is considered more sensitive than immunohistochemistry or immunocytochemistry. Protein lysates from both whole cells and purified plasma membranes of CHO-BMLK3 cells were used.

Western blots using entire cells prepared in Nonidet P-40 lysis buffer did not reveal any bands specific to the CHO-BMLK3 cells that were also present in the negative control cell line CHO-pcDNA (vector transfected only), although there was more unspecific signal in the receptor antisera blot than the preimmune blot (Fig. 12). Additionally, preabsorption of antibodies with the synthetic peptide **RVETQVESHALNLTK**C, did not result in the observable loss of any bands in the preabsorbed antisera in comparison with untreated antisera. Preabsorption of antisera with the negative control cell line did result in the loss of at least one background band approximately 70 kDa in size (Fig. 13).

Use of affinity purified antisera in western blots reduced much of the unspecific binding seen. Some bands were visible in these blots, but none were seen in the transfected cells that were not seen in the control lanes (Fig. 14).

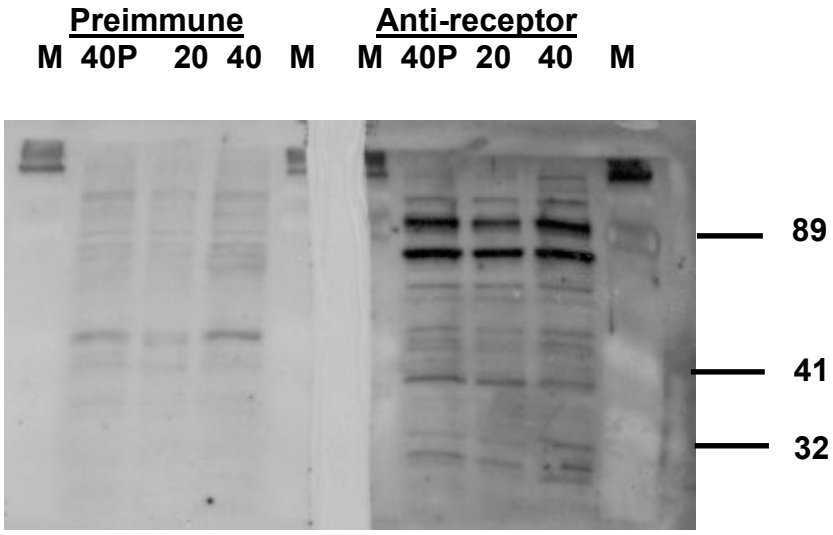

Figure 12. Western blot of CHO-BMLK3 and CHO-pcDNA cells with complete preimmune and anti-receptor antisera. Protein lysate was prepared in Nonidet P-40 buffer. Primary antibody concentration was 1:1000. M = marker, 40P = 40 µg total protein from CHO-pcDNA, 20 = 20 µg total protein from CHO-BMLK3, 40 = 40 µg total protein from CHO-BMLK3.

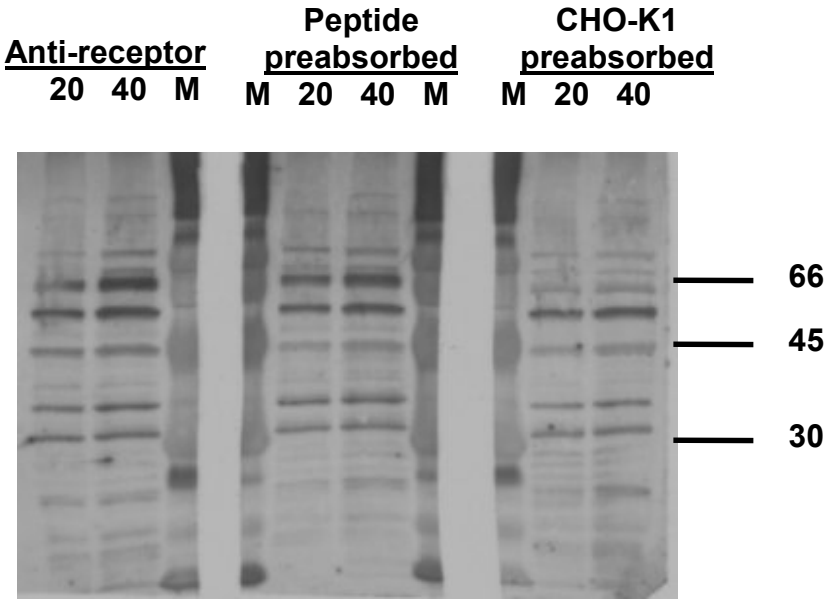

Figure 13. Western blot of CHO-BMLK3 cells with preabsorbed complete anti-receptor antisera. Protein lysate was prepared in Nonidet P-40 buffer. Primary antibody concentration was 1:1000. In anti-receptor lanes, antibodies were not preabsorbed. Peptide preabsorbed and CHO-K1 were preabsorbed for 7 h at RT in the presence of 50 μg/ml of peptide or intact untransfected CHO-K1 cells, respectively. M = marker, 20 = 20 μg total protein, 40 = 40 μg total protein.

In order to further reduce background interference and enhance any possible signal from specific bands, subcellular fractionation of CHO-BMLK3 and control cell lines was done by differential centrifugation to purify plasma membranes. A commercially prepared rabbit anti-integrin β5 subunit polyclonal antibody was used as a marker for the presence of membrane protein. This antibody labels a protein 100 kDa in size in humans (Ramaswamy & Hemler, 1990), although the expected size in CHO-K1

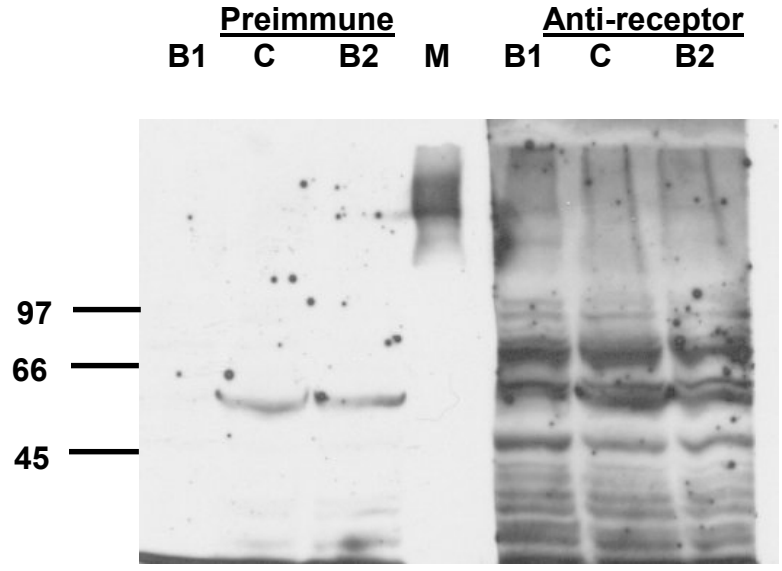

Figure 14. Western blot of CHO-BMLK3 and CHO-pcDNA cells with affinity purified preimmune and anti-receptor antisera. Primary antibody concentration was 1:50. M = marker, B1 = 200 µg total protein from CHO-BMLK3 (Nonidet P-40 lysate), C = 40 µg total protein from CHO-pcDNA (SDS lysate), B2 = 40 µg total protein from CHO-BMLK3 (SDS lysate).

cells is not known. In our Western blots using this antibody against subcellular fractions it labeled a protein approximately 70 kDa in size (Fig. 15). This band appeared to be

most purified in fraction #5 (the final supernatant) rather than in fraction #4 as expected (pelleted membranes and ribosomal material). Because of this discrepancy from the

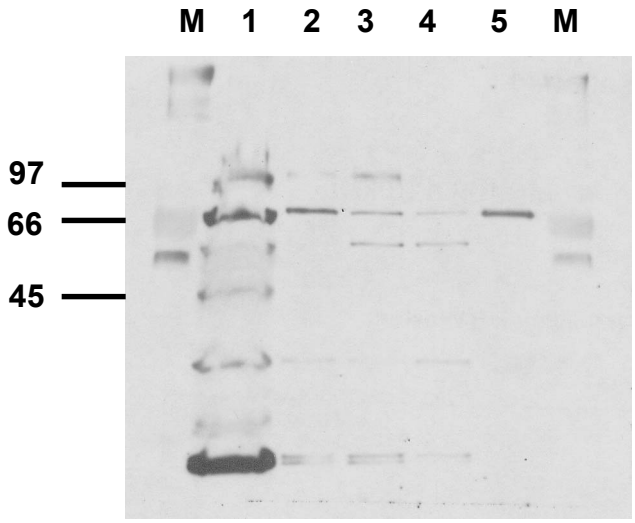

Figure 15. Western blot of subcellular fractions of CHO-BMLK3 cells with rabbit anti-integrin β5 subunit polyclonal antibody. Primary antibody concentration was 1:10,000. M = marker, 1-5 indicates fractions 1-5 respectively. Each sample lane contained 20 µg of total protein.

expected result, both fractions 4 and 5 were used in further Westerns using affinity purified receptor antisera. However, the use of affinity purified antisera and subcellular fractions did not result in the specific labeling of any protein bands in comparison to negative controls (Fig. 16).

In summary, no experiments conducted with rabbit anti-**RVETQVESHALNLTK**C peptide antisera produced any evidence for the presence of antibodies that would specifically label the tick receptor.

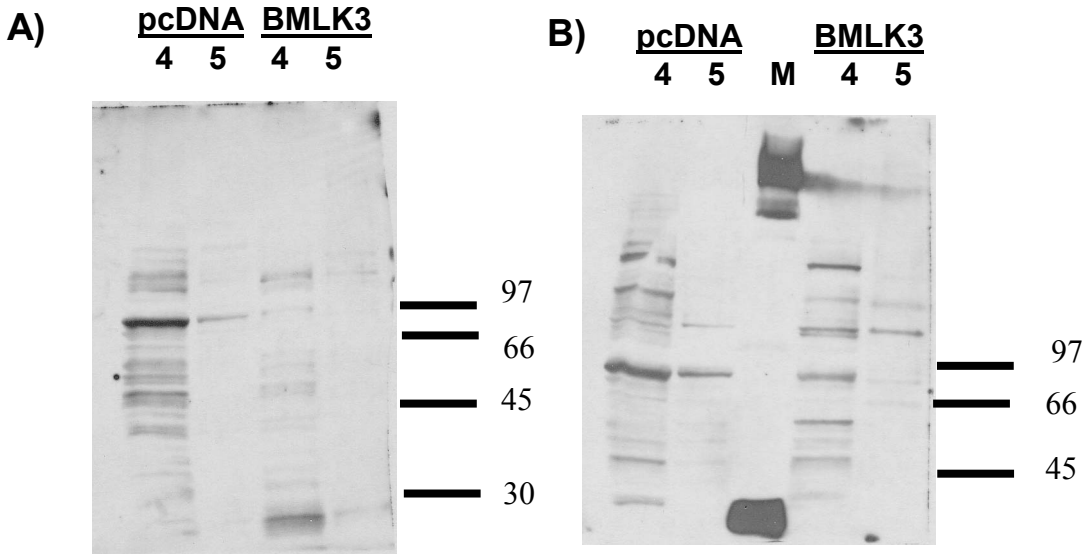

Figure 16. Western blot of subcellular fractions 4 & 5 of CHO-BMLK3 cells with affinity purified antireceptor antisera. Primary antibody concentration was 1:50. M = marker, 4 & 5 indicate fractions 4 & 5, respectively. Each sample lane contained 20 µg of total protein. A) Secondary antibody used was 1:20,000 dilution of goat-antirabbit horseradish peroxidase conjugated antibody (Molecular Probes). B) This blot was a replicate of the blot shown in A, except that the secondary antibody was used at 1:10,000 dilution.

Discussion

The presence of tick receptor transcript in multiple tissues most likely indicates that the myokinins have multiple or higher functions in ticks. Although the only established functions of myokins are limited to the insect hindgut and Malpighian tubules, it is not unexpected that the myokinins would also have functions in the brain or CNS of arthropods. It has previously been suggested that due to the distribution of leucokinin immunoreactive neurons, roles for myokinins as neuromodulators or neurotransmitters are possible in certain parts of the brain, visual system, and ventral ganglia of insects. Additionally, roles for myokinins in regulating feeding, heart function, and respiration have been suggested (Nässel *et al.*, 1992; Nässel, 2002). This first suggestion has been supported by a study showing that injection of *Heliothis virescens* larvae with myokinin peptides causes a significant reduction in weight gain (Seinsche *et al.*, 2000). Similarly, injection of another neuropeptide, sulfakinin, has been shown to significantly inhibit food uptake in nymphs of the desert locust, *Schistocerca gregaria* (Wei *et al.*, 2000).

Our finding that tick receptor transcript is present in the gut, synganglion, and Malpighian tubules of adult females was not unexpected due to similar results from other arthropods which have already been discussed. By analogy with insects, it is most likely that the tick myokinin receptor stimulates excretion in the digestive system (gut and Malpighian tubules), although this possibility has not been tested. It is possible that the

tick myokinin receptor has some function in the CNS, however there is as of yet no experimental data on this subject.

The tick myokinin receptor appears to be transcribed throughout all the life stages and in multiple tissues of the tick. The presence of the tick receptor transcript in the ovaries and salivary glands is similar to evidence of myokinin receptors or myokinin function that has only recently been demonstrated in *D. melanogaster* gonads (Radford *et al.*, 2002) and *Rhodnius* salivary glands (Orchard & Te Brugge, 2002). The drosokinin receptor transcript has been detected in multiple tissues including both the male and female gonads, leading to speculation that myokinins may be important in fertility or the peristaltic transfer of sperm or eggs (Radford *et al.*, 2002). It is equally possible that myokinins may effect fertility or egg transfer in ticks, however there are currently no experimental results to back up such speculation .

During blood feeding in the hard ticks, the salivary glands become more significant in maintaining osmotic balance and expelling excess water from the bloodmeal back into the host. Additionally, in blood feeding arthropods such as ticks and mosquitos the salivary glands are a key organ in the transmission of pathogens. Myokinins have an important role in regulating diuresis in insects, and if they have a simlar role in ticks it may be through regulation of salivary gland function. In insects, it has been demonstrated that in the blood-feeding bug *R. prolixus*, leucokinin I and a CRF-related peptide induce a dose-dependent increase in the basal tonus and phasic contractions of the salivary glands (Orchard & Te Brugge, 2002). Myokinins do not

seem to have a direct effect on *Rhodnius* Malpighian tubules (Te Brugge *et al.*, 2001), although they may increase hindgut contractions.

The possibility of myokinin regulation of function in the ovaries or salivary glands suggests that the tick myokinin receptor may indeed be an excellent target for novel acaricide development or protective vaccines. Perturbation of either of these critical functions could prove quite detrimental to tick populations, either through reduction in fertility or interference with normal feeding behavior. In addition to interfering with feeding, targeting a receptor in the salivary glands may also impact the vector competence of blood feeding arthropods, which may be an equally viable strategy to eradication for breaking the disease transmission cycle.

CHAPTER V

CONCLUSIONS

This dissertation provides significant novel contributions to the fields of tick physiology and myokinin signal transduction in arthropods.

The characterization of the *B. microplus* myokinin receptor cDNA described in this dissertation represents the cloning and identification of the first neuropeptide receptor from the Acari, as well as the first functional expression of a GPCR from the Acari. Additionally, the *B. microplus* receptor is the second G-protein coupled receptor (GPCR) known from any tick species.

The *B. microplus* receptor was the first myokinin receptor to be cloned from an arthropod, and is the second known member of the myokinin receptor subfamily after the lymnokinin receptor from the pond snail. While our report on the functional expression of the *B. microplus* receptor was in press (Holmes *et al*., 2003), a report on the functional expression of the drosokinin receptor was published online ahead of ours (Radford *et al.*, 2002). Thus, these two receptors became the first to be unequivocally identified as myokinin receptors from arthropods, and the first myokinin receptors to be functionally expressed from insects and ticks, respectively.

Evidence of the transcriptional expression of this receptor by RT-PCR experiments constitutes the first evidence of myokinin regulated signal transduction in the Acari. Based on the sequence similarity of the *B. microplus* receptor to gene product CG10626 in the *D. melanogaster* genome (Adams *et al.,* 2000), we were able to

correctly predict that this gene product was the *Drosophila* myokinin (drosokinin) receptor (Holmes *et al.,* 2000; Radford *et al.*, 2002).

When expressed in mammalian cells, the *B. microplus* receptor was activated by myokinin peptides at nanomolar concentrations, including the naturally occurring lymnokinin and muscakinin peptides as well as several designed myokinin analogs. Analysis of signal transduction in these cells showed that the peptide-agonist induced calcium response originated from intracellular calcium stores, most likely resulting from activation of pertussis toxin insensitive $G\alpha_q$ proteins. Expression of the *B. microplus* receptor in CHO-K1 cells makes available a system for screening compounds for agonist or antagonist activity, which has already been used for testing hypotheses of receptor-agonist interactions, and may be useful for identifying compounds with acaricidal activity.

The detection of receptor transcripts by RT-PCR in the dissected synganglion, gut, salivary glands, ovaries, and Malpighian tubules of partially engorged adult female ticks is the first indication of myokinin receptor expression in specific tick tissues. The distribution of myokinin receptor expression in the tick was more extensive than what we expected from what is known in insects. Until recently, myokinin function had only been demonstrated in the insect digestive system, and was suggested in insect brain and nervous system based on the high incidence of neurons with leucokinin-like immunoreactivity (Nässel, 1996; Nässel, 2002). Our report is the first evidence of myokinin receptor expression in the tissues of an arachnid outside of the nervous system. Consistent with our findings in the tick, evidence for myokinin receptor expression has

also been found in the reproductive organs of *Drosophila* (Radford *et al.*, 2002) and a myokinin-induced response has been demonstrated in the salivary glands of the blood-feeding bug *Rhodnius prolixus* (Orchard & Te Brugge, 2002). These new target tissues for myokinins support the possibility of multi-functional and complex signaling interactions in this peptide family.

The tick myokinin receptor responds to myokinin agonists, and appears to be present in multiple tissues, but no function in any tissue has been established. In *Drosophila*, the endogenous ligand of the drosokinin receptor and its function in the Malpighian tubules are known only due to research done prior to the identification of the receptor (Radford *et al.*, 2002).

Receptor localization in specific tissues is a critical first step for developing hypotheses regarding receptor function. Although the receptor transcript has been detected in multiple tick tissues, attempts to use specific antibodies in western blots or immunohistochemistry to unequivocally demonstrate the expression of the receptor protein were not successful. Development of a receptor antiserum that specifically binds a receptor antigen is critical for this effort.

In addition to immunolocalization, antireceptor antibodies could be used to identify functionally important regions of the receptor. For example, antibodies that block the agonist activated response or induce activity by themselves may identify the ligand binding domain(s) of the receptor. This knowledge could potentially be used to aid in the modeling and prediction of synthetic non-peptide agonists potentially useful as novel acaricides. Additionally, the identification of immunogenic antigens from the

receptor is a critical first step for the development of a vaccination strategy. Vaccination of cattle with "hidden"antigens, or those not normally presented to the immune system during the course of normal feeding, has already been demonstrated to be a valid control strategy for *B. microplus*. Inoculation of cattle with the tick gut antigen Bm86 gives cattle a protective response that is most effective against tick larvae (Willadsen *et al.*, 1995). If the tick myokinin receptor is present in salivary glands and the gut or associated muscles as predicted by RT-PCR results, host antibodies should have no trouble finding receptors to act on. Active antibodies from vertebrate hosts have been detected in the hemolymph of both *B. microplus* and the lone star tick *Amblyomma americanum* (Vaz *et al.*, 1996; Jasinskas *et al.*, 2000), so theoretically antibodies could interact with any target tissue in the tick.

The work reported in this dissertation includes the first evidence of myokinin-like signal transduction in ticks, and not suprisingly there is much basic information that remains unknown. Because we have shown that myokinin agonists induce the release of intracellular calcium in transfected cells, we have assumed that this second messenger is the result of IP_3 production by phospholipase C following activation by $G\alpha_q$. This is the most likely explanation, however this could be confirmed by testing receptor transfected cells for an agonist induced IP_3 response.

The nature of the endogenous ligand(s) of the tick myokinin receptor remains a critical and unanswered question. There is a possibility of additional myokinin receptors in the tick, and if they are present this would not be unexpected given the multifunctional nature of the myokinins in invertebrates. Currently it appears that there

is only a single myokinin receptor in the *Drosophila* and *Anopheles gambiae* genomes (Radford *et al.*, 2002; Hill *et al.*, 2002), and only single receptors have been isolated from *L. stagnalis* and *B. microplus* respectively (Cox *et al.*, 1997), (Holmes *et al.*, 2000). However, different responses to the three leucokinin peptides seen in the Malpighian tubule of the mosquito *Aedes aegypti* (Veenstra *et al.*, 1997) may indicate two or more myokinin receptors in this species. Because we expressed the *B. microplus* receptor in a heterologous system (CHO-K1 cells), it is also possible that the receptor may have different post-translational modifications in the tick than in our system, perhaps resulting in an altered response or pharmacological profile.

Unfortunately, no candidate myokinin-like peptides have been isolated from any tick. Clearly, the tick receptor responded specifically to peptides with the conserved C-terminal pentapeptide motif (FXXSWG-NH_2; see Chapter III) that defines the insect myokinins peptide family with the only exception that the Gly residue could be replaced with a Ser. Beyond the presence of this motif, it is difficult to speculate on the nature of an endogenous ligand with the data generated in this study. Endogenous ligands do not necessarily give the strongest response in functional assays in comparison to other synthetic agonists. Also there is a strong possibility that the tick has multiple endogenous ligands. Among insects from which myokinins have been isolated, only in the higher Diptera (*Musca domestica* and *D. melanogaster*) and in the locust *Locusta migratoria* have single ligands been found (Holman *et al.*, 1999; Terhzaz *et al.*, 1999). In other insects, from 3 to 8 myokinins have been isolated, including the mosquitoes

Aedes aegypti and *Culex salinarius*, which each have 3 peptides respectively (see Pietrantonio *et al.* 2000, Table 1).

Any discussion of myokinins and their potential functions of ticks must largely rely on analogy with insects, because knowledge of tick neuropeptides is non-existent and on GPCRs is extremely limited. No neuropeptides have been isolated from ticks, and only three tick GPCRs of any type have been described, all from *B. microplus* (Baxter & Barker, 1999; Holmes *et al.*, 2000; Chen *et al.*, 2003). Because the myokinins are widely distributed and conserved among invertebrate groups, our working hypothesis is that what is known in other arthropods (insects) will also hold true in ticks. In insects, myokinin-like immunoreactivity has been found in both neurosecretory cells and in interneurons (see Nässel 2002 for review) indicating function as both neurotransmitters or neuromodulators and as neurohormones. Although some insects have abdominal neurosecretory cells which are immunoreactive for myokinins, no innervation with myokinin-like immunoreactivity has been detected in Malpighian tubules or hindguts, except in the gut of *R. prolixus* (Te Brugge *et al.*, 2001; Nässel, 2002). The myokinins are almost certainly released into the hemolymph and act as neurohormones on target tissues such as the Malpighian tubules, hindgut, and other tissues that putatively express myokinin receptors.

This multifunctional aspect of the myokinins is most likely indicative of their involvement in many complex interactions, which may complicate studies involving whole organisms. However, this is not unexpected because peptide hormones in insects are generally master regulators and affect a number of physiological processes (Keeley

& Hayes, 1987). The multiple functions of myokinins and their receptors may make them attractive as targets for novel acaricides. Precedence for this approach has been set by the formamidines, a successful class of insecticides/acaricides that target the octopamine receptor. Although not a neuropeptide, the biogenic amine octopamine functions as a neurotransmitter, neuromodulator, and neurohormone in insects by acting through a GPCR in target tissues. The formamidines are receptor agonists that cause anorexia and death in insects (Ismail & Matsumura, 1991).

Further experiments are needed order to validate the myokinin receptor as a target for novel control strategies against the tick. One conceivable control strategy is the development of compounds that act as receptor agonists or antagonists. Although no insect or tick neuropeptide mimetics have been developed that have sufficient environmental persistence to be useful as pesticides, the transfected cell line CHO-BMLK3 or similar expression systems could easily be developed to screen candidate compounds for receptor activity. The most difficult aspect of this approach may be synthesizing compounds that have the required properties of peptide agonists but that are not easily degraded.

As molecular insect science moves into the post-genomic era, the now labor intensive efforts needed to clone genes by homology screening of libraries or degenerate RT-PCR will be facilitated by the information present in various insect genomes. GPCRs are notoriously difficult to characterize using classical molecular methods, and already the genomic information available from insects has made a large impact in this field. The genomes of the fruit fly *D. melanogaster* and the malaria mosquito *Anopheles*

gambiae have been completely sequenced (Adams *et al.*, 2000), (Holt *et al.*, 2002), and the sequence of the honey bee *Apis mellifera* genome is expected to be complete in December 2003 (http://www.hgsc.bcm.tmc.edu/projects/honeybee/). With the large sequencing capacity now available, genome projects of other economically important arthropods are sure to follow.

There are currently 30 identified genes that encode predicted neuropeptides and insulin-like peptides in *Drosophila* (Nässel, 2002). Additionally, about 200 GPCRs have been predicted (Brody & Cravchik, 2000), 44 of which are peptide receptors (Hewes & Taghert, 2001). Analysis of the malaria mosquito *Anopheles gambiae* genome has predicted 25 Class A peptide receptors, out of a total of 276 GPCRs (Hill *et al.*, 2002) and identified the genes of 35 putative regulatory peptides (hormones and neuropeptides) (Riehle *et al.*, 2002). Already the genomic information available has led to the expression and further characterization of multiple GPCRs. Based on information from the *Drosophila* genome, a novel family of over 100 genes that likely represent odorant receptors has been discovered (Vosshall *et al.*, 1999). Additionally, the first insect receptors for myokinin, ecdysis triggering hormone, FMRFamide, and proctolin were intitially predicted based on genomic information, then expressed and characterized (Holmes *et al.*, 2000; Radford *et al.*, 2002; Iversen *et al.*, 2002; Cazzamali & Grimmelikhuijzen, 2002; Johnson *et al.*, 2003; Egrod *et al.*, 2003).

The explosion of genomic information means that there will be more work to do in insect molecular science, not less. Although computer programs used for bioinformatics are very useful tools, they do not evaluate results within a biological

context or apply common sense, and are known to make errors. Additionally, the identification of a gene is just the first step that must be taken in the characterization of its final product and the role of that product in the cell, tissue, and whole organism. In some sense, genomics can be considered simply as a shortcut past the first step in molecular biology after which the hard work begins.

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