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Myotropic effect of helicokinins, tachykinin-related peptides and *Manduca sexta* allatotropin on the gut of *Heliothis virescens* (Lepidoptera: Noctuidae)

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Abstract

Different insect neuropeptides (helicokinins, tachykinin-related and allatoregulating peptides) were investigated with regard to their myostimulatory effects using whole-gut preparations isolated from fifth instar *Heliothis virescens* larvae. The experiments demonstrated that representatives of all three peptide families are able to induce and amplify gut contractions in this species in a dose-dependent manner. Structure–activity studies (alanine scan, D-amino acid scan and truncated analogues) with the helicokinin Hez-K1 supported the finding, that the core sequence for biological activity of kinins is the amidated C-terminal pentapeptide (FSPWG-amide). Similar investigations with insect tachykinin isolated from *Leucophaea madera* (Lem-TRP1) revealed that the minimum sequence evoking a physiological gut response in *H. virescens* is the amidated hexapeptide (GFLGVR-amide), which represents the conserved amino acid sequence for *Leucophaea* TRPs in general. The peptide concentration causing a half-maximal gut contraction (EC_{50}) for Lem-TRP1 was about 26 nM. Although the potency of Lem-TRP1 was 9-fold lower compared with Hez-KI (EC_{50}: 3 nM), the maximal tension of the gut obtained with Lem-TRP1 was 1.7-fold higher compared with Hez-KI. The EC_{50} of *Manduca sexta* allatotropin (Mas-AT; 79 nM) was of lowest potency among all three peptides tested. In a pharmacological study, co-incubation experiments with Lem-TRP1, Hez-KI or Mas-AT and compounds interfering with signal transduction pathways were employed to investigate the mode of action of the myotropic effects of these peptides. Cadmium and the protein kinase C (PKC) inhibitor tamoxifen attenuated the contractile effects of all three peptides tested. The data suggest that in the gut muscle of *H. virescens* the myotropic peptides bind to G-protein-coupled receptors that cause contraction by promoting the entry of extracellular calcium mediated by a PKC involved pathway.

Keywords: Myotropic neuropeptides; Gut bioassay; Helicokinin; Tachykinin; Allatotropin

1. Introduction

Insect hormones can be divided into four different classes: amines, steroids, juvenoids and peptides. Nearly all peptidergic hormones, which have been identified so far are secreted from the insect nervous system and therefore called neuropeptides. To date, plenty of different neuropeptides have been isolated from insects. Interestingly, a majority of them exhibit myotropic activities, which means they either stimulate or inhibit the muscle activity (Schoofs et al., 2001). From neurohaemal organs of the cockroach *Periplaneta americana* alone more than 20 myostimulatory peptides could be purified (Predel et al., 2001). In contrast, for the economically important tobacco budworm, *Heliothis virescens*, little is known about specific myotropic peptides and the humoral control of gut peristaltic. For that reason, three specific peptide families (helicokinins, tachykinin-related peptides (TRPs), allatoregulating peptides) were tested and characterized for their influence on gut motility in *H. virescens*.

Kinins are peptides which share a common feature, the highly conserved C-terminal pentapeptide sequence Phe-Xaa^1-Xaa^2-Trp-Gly-NH_2, where Xaa^1 is either Asn, His, Ser or Tyr and Xaa^2 is Ser, Pro or Ala. This core

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sequence is assumed to be responsible for the biological activity (Coast et al., 1990; Nachman et al., 1986). The helicokinins (Hez-KI, Hez-KII, Hez-KIII) were the first members of the kinin family which could be isolated from lepidopteran species and showed both diuretic and myotropic activity in *Manduca sexta* and *Spodoptera frugiperda*, respectively (Blackburn et al., 1995; Howarth et al., 2002b). The effects of helicokinins on water balance were examined by Seinsche et al. (2000) in *H. virescens* using a specific in vitro assay. This so-called Ramsay assay is based on assessing increased droplet secretion from single Malphigian tubules which have been stimulated by neuropeptides compared to untreated controls (Chung and Keeley, 1989). Furthermore, the myotropic actions of helicokinins were investigated by Howarth et al. (2002b) with isolated whole-gut preparations from *S. frugiperda* larvae. Such an isotonic gut contraction assay was originally used to identify and isolate myotropic fractions from tissue extracts (Predel et al., 2001; Schoofs et al., 2001). Howarth et al. (2002b) found that Hez-KI, Hez-KII and Hez-KIII stimulate spontaneous contractions of whole-gut preparations from *S. frugiperda*. In the current study a similar test system, an isometric gut contraction assay was established to investigate the effects of helicokinins on the gut motility in *H. virescens*.

Another class of myostimulatory neuropeptides is the TRPs. The TRPs show sequence similarity to vertebrate tachykinins and are characterized by a carboxyterminus pentapeptide FX_1GX_2R-amide, where X_1 and X_2 are variable amino acid residues. TRPs were first isolated from *Locusta migratoria* (Schoofs et al., 1990) and later nine analogues were found in the cockroach *Leucophaea maderae* (Muren and Nässel, 1996, 1997). Meanwhile, additional TRPs could be identified in other insect orders as well as in worms, crabs and molluscs (Nässel, 1999). For *L. maderae* it is known that the TRPs are located in the nervous system and the midgut (Muren et al., 1995). The exact physiological function of the TRPs is still obscure but in vitro assays indicated that these peptides may have a multifunctional role. They acted as myotropic peptides of visceral and skeletal muscles, induced the release of adipokinetic hormone from locust *corpora cardiaca* and stimulated pheromone biosynthesis in *Bombyx mori* (Nässel, 1999; Schoofs et al., 1993; Vanden Broeck et al., 1999).

Finally, the allatoregulating peptides, *M. sexta* allatotropin (Mas-AT) and *M. sexta* allatostatin (Mas-AS) were tested. The peptides were first isolated from *M. sexta* (Kataoka et al., 1989; Kramer et al., 1991), where Mas-AT and Mas-AS stimulated and inhibited juvenile hormone (JH) biosynthesis, respectively. Recently, Mas-AT could also be identified in the moth *S. frugiperda* and the authors found that JH biosynthesis seems to be regulated by an interaction between Mas-AT and Mas-AS (Oeh et al., 2000). Additionally, when injected into *S. frugiperda* Mas-AT led to increased mortality in this insect (Oeh et al., 2001). Possible explanations may be a myotropic effect of Mas-AT on the gut reported for several insect species (Duve et al., 1999, 2000).

In order to investigate the contractile effects of the three peptide families on *H. virescens* gut, structure–activity studies were carried out with selected representatives of these neuropeptides. Additionally pharmacological experiments were undertaken to elucidate the mode of action of the peptide-induced stimulatory effects on the gut muscle with focus on Lem-TRP1.

2. Material and methods

2.1. Insects

The gut contraction experiments were carried out using *H. virescens* guts. The insects were reared on artificial diet based on bean meal and maintained at 27 °C and ca. 70% relative humidity under a L16:D8 photoperiod. Due to the sensitive nature of the lepidopteran guts, whole guts (oesophagus to rectum) from 2- to 4-day old fifth instar larvae were used for the in vitro assay.

2.2. Isometric gut contraction assay

The gut was dissected out of the larvae and preincubated in a lepidopteran saline (slightly modified according to Howarth et al. (2002b); 12 mM NaCl, 32 mM KCl, 3 mM $NaHCO_3$, 18 mM $MgCl_2$, 9 mM $CaCl_2$, 165 mM sucrose, 10 mM HEPES, pH 7.5) at room temperature for 20 min prior to the addition of the test compounds. The organ bath itself consisted of a 5 ml plastic syringe filled with saline and gassed with a constant airflow. Saline was exchanged rapidly via a three-way valve at the base of the syringe. In order to record the effects of peptides on gut motility, the organ was fixed in the saline bath with ligatures; the oesophagus was attached to a hook at the bottom of the bath and the rectal region was connected with a piece of yarn to an isometric transducer detector (World Precision Instruments, USA). The isometric transducer measured changes in tension of the attached tissue with its rigid torsion sensing element (which provides a constant length of the gut) and the force generated corresponded with the amplitude of the contraction response. The output was amplified and plotted on a thermal array recorder (Graphtec Corp., Japan). Dose–response curves for the test compounds were obtained by using an 8-min cycle with three washes and a recording period of 2 min, respectively.

During the pharmacological experiments the contractile effects of the test peptides were measured in the absence and presence of second messenger pathway sub-

stances. Therefore, the gut was preincubated in saline containing the drug for 8 min before the test peptide was added. The results are given as a percentage of the maximal response of the peptide without supplement. The contractile effects of the drugs alone were also considered by measuring their maximal contraction during the 8-min time of preincubation and the results were compared to the values obtained by application of the current solvent alone. All peptides and drugs were dissolved in water or dimethyl sulphoxide (DMSO).

2.3. Test substances

The helicokinins (Hez-KI: YFSPWG-amide; Hez-KII: VRFSPWG-amide; Hez-KIII: KVKFSAWG-amide), the TRPs (Lem-TRP1: APSGFLGVR-amide; Lem-TRP5 (5–10) residue: GFQGVR-amide; Lem-TRP9 (5–10) residue: GFQGMR-amide; Lom-TKII (4–10) residue: SGFYGVR-amide) and their truncated analogues were obtained from BioGenes GmbH (Berlin, Germany). The alanine and D-amino acid substituted analogues of these peptides as well as Mas-AS were synthesized by Hubert Dyker, Bayer AG (Leverkusen, Germany). Mas-AT was obtained from Bachem (Bubendorf, Switzerland). All other chemical substances were obtained from Sigma (Darmstadt, Germany).

2.4. Data interpretation

The response curves obtained upon stimulating the whole-gut preparation allow several possibilities for data interpretation. A typical agonist-induced response (Fig. 1) can be characterized by the period of latency (*L*), the amplitude of the phasic contraction (PC) and the amplitude of the tonic contraction (TC). One of the most reliable and reproducible parameters is the total contractile response (TCR) represented by the curve integral (area covered by the curve and the baseline) (Howarth et al., 2002b). Due to the recording method used, this integral could not be easily measured here and was therefore not chosen for data analysis. Howarth et al. (2002b) showed that there was no significant difference between the results for the total contractile response and the amplitude of the TC. Furthermore the amplitude of TC is very easy to determine, so this parameter was chosen for data interpretation in this study. To construct the dose–response curve, in each single experiment the maximum amplitudes of TC were determined and expressed as a percentage of the highest value in that run. This strategy compensates the methodical problem that the absolute values for the maximum amplitudes of TC varied in different experiments with different tissues.

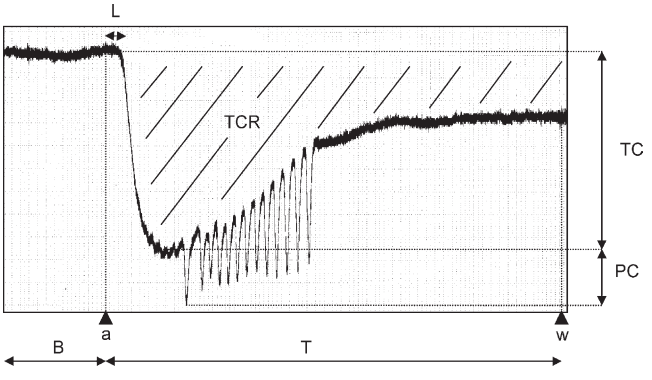

Fig. 1. Typical agonist-induced response obtained from the gut contraction assay. Effects of 10^{-7} M Hez-KI on a whole-gut preparation from a *H. virescens* larva. B: baseline, T: duration of contractile response (2 min), L: latency, a: peptide addition, w: saline wash, TC: tonic contraction, PC: phasic contraction, TCR: total contractile response.

The EC_{50} value is the concentration of peptide causing 50% of the contractile response compared with the reference peptide. The reference peptide is the first (non-modified) peptide tested in each experiment (Hez-K1, Lem-TRP1 or Mas-AT, respectively). The EC_{50} values were utilized to calculate the potency of each compound. The potency was expressed as the quotient (EC_{50} of the reference peptide)/(EC_{50} of the test peptide) in per cent. On the other hand the intrinsic activity represents a value, which rates the contractile powerfulness of a substance. It was calculated as (maximal contraction of the test peptide)/(maximal contraction of the reference peptide) in per cent.

Statistic treatment of the data was performed with the non-parametric tests, Mann–Whitney *U*-test or Kruskal–Wallis *H*-test, followed by a Tukey–Kramer post test as needed (Sachs, 1992). A value of $P < 0.05$ was considered to be significant. The results are presented as means ± standard error (SE).

3. Results

3.1. Evaluation of the in vitro bioassay

Fig. 2 shows the data output from a typical isometric contraction run with Hez-K1. The amplitude of the maximal TC from each single experiment was used to construct the dose–response curve for the whole run. With increasing peptide concentrations the contractile response increased in a dose-dependent manner and slightly decreased when a specific concentration was exceeded. In addition to the TC, phasic contractile movements of the gut were sometimes observed. These phasic effects did not show a reliable and reproducible dose-dependent response, so this parameter was not included in further evaluations.

Due to the fact that the gut was dissected out of the larva and although the tissue was incubated in an appropriate and well aerated insect saline, the question was how long the gut activity could be kept in a constant

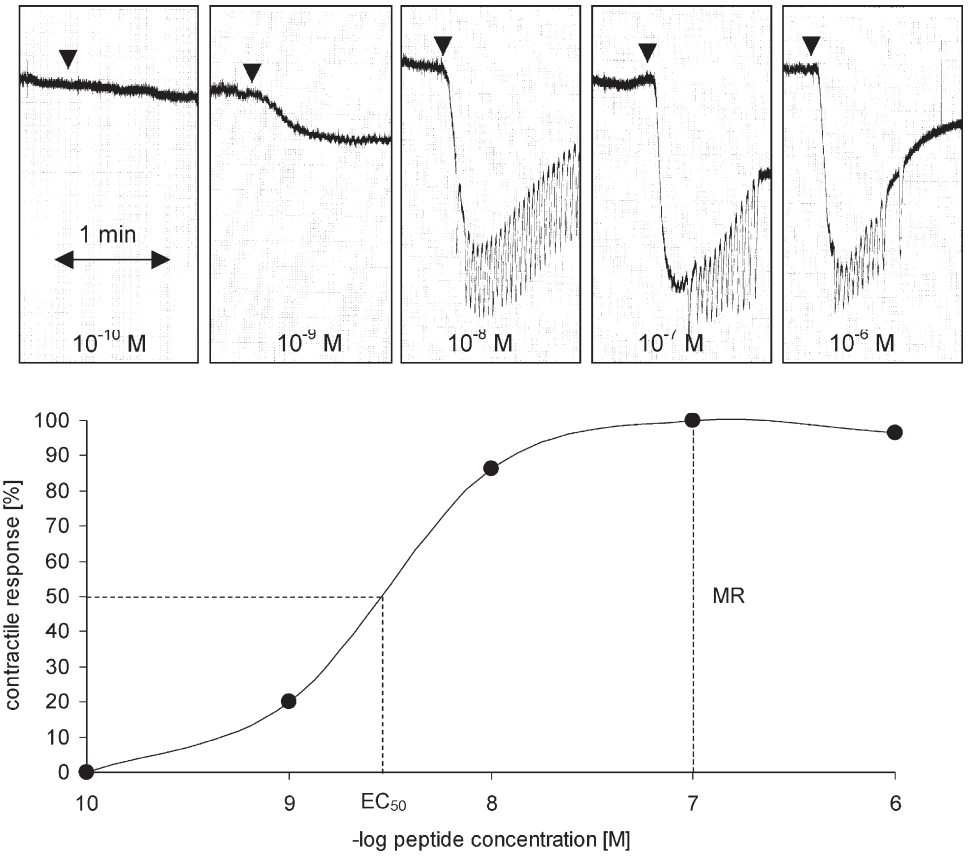

Fig. 2. Effect of different concentrations of Hez-KI on the contraction of a typical whole-gut preparation from a *H. virescens* larva (upper figures). Arrows indicate Hez-KI addition to the organ bath. The results can be expressed in a dose–response curve (lower graph) by considering the extents of the TCs. EC_{50} represents the dose of peptide causing a half-maximal contraction. MR represents the maximal response.

range. In initial experiments the response curves of tissues incubated in saline for different periods of time prior to stimulation were investigated. The dose–response curve with Hez-K1 and the corresponding EC_{50} value for freshly dissected organs (1.5 ± 0.2 nM) were not significantly different from the results obtained with 1-h old guts (1.9 ± 0.5 nM) (Mann–Whitney *U*-test, $P < 0.05$). With organs preincubated for 4 h prior to the test, the EC_{50} (13.4 ± 4.4 nM) significantly increased, so that the tissue seemed to lose some sensitivity or intrinsic activity. Therefore, all tests were recorded not longer than 1 h.

3.2. Structure–activity studies with helicokinins

First, the helicokinins Hez-KI, Hez-KII, Hez-KIII and truncated or substituted analogues of Hez-KI were tested with respect to their effects on the gut motility of *H. virescens* larvae in isolated whole-gut preparations (Fig. 3). All three peptides Hez-KI, Hez-KII and Hez-KIII elicited a contractile response in a dose-dependent manner (Fig. 3A). The response curve obtained with Hez-KI showed a significantly lower EC_{50} value (2.0 nM) than with Hez-KII (8.4 nM) and Hez-KIII (12.7 nM) (Table 1). Comparing the maximum amplitudes of the contractile response, Hez-KI showed a higher intrinsic activity (100%) than Hez-KII (90%) and Hez-KIII (88%). Thus, by comparing all three helicokinins, Hez-KI was the most potent and most active peptide and therefore used for further investigations.

Fig. 3B shows the results from the structure–activity experiments with truncated Hez-KI analogues and the non-amidated Hez-KI analogue. The truncation of only one amino acid from the N-terminal end of Hez-KI led to a significant decrease in contractile activity. The potency dropped to 0.9% and the intrinsic activity was only 61% of that from the original peptide (Table 1). Further truncation of Hez-KI did not cause a significant contraction at all. Using non-amidated Hez-KI analogues the same result was obtained, i.e. no contractile effects were observed.

In the next experiment an alanine scan was performed in order to investigate the significance of the amino acid side chains (Fig. 3C). Substitution of the amino acids tyrosine, serine and proline with alanine, respectively, in order to replace the side chain by the methyl group from alanine resulted in agonists which showed similar contractile response curves as the reference peptide, however, the EC_{50} values did not significantly differ (Table 1). When glycine was replaced by alanine the affinity

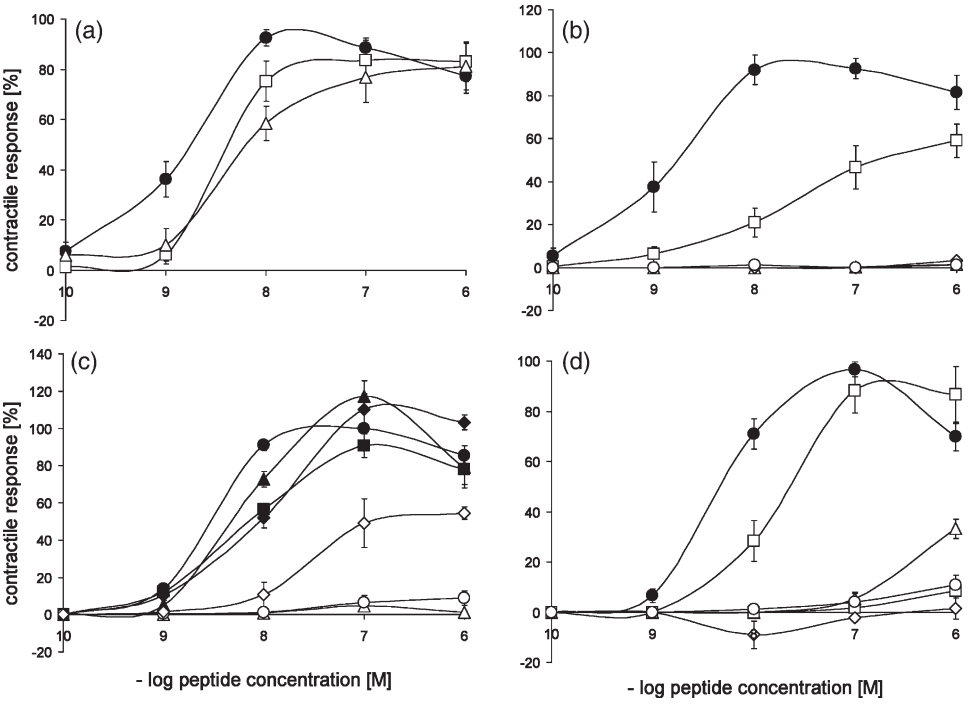

Fig. 3. Dose–response relationship for gut contraction with isolated whole-gut preparations of *H. virescens* larvae after treatment with helicokinins and truncated or substituted analogues. Mean values ± SE of five determinations as a percentage of the maximum response of this experiment. Statistical significance in terms of EC_{50} is signified by using black or white symbols (Tukey–Kramer post test after Kruskal–Wallis *H*-test, $P < 0.05$) A: Hez-KI (●), Hez-KII (□), Hez-KIII (△). B: Hez-KI amidated, not amidated and truncated analogues: YFSPWG-amide (●), FSPWG-amide (□), SPWG-amide (△), PWG-amide (◇), YFSPWG (○). C: Hez-KI and Ala substituted analogues: YFSPWG-amide (●), AFSPWG-amide (■), YASPWG-amide (△), YFAPWG-amide (▲), YFSAWG-amide (◆), YFSPAG-amide (○), YFSPWA-amide (◇). D: Hez-KI and D-amino acid substituted analogues: YFSPWG-amide (●), (D-Y)FSPWG-amide (□), Y(D-F)SPWG-amide (△), YF(D-S)PWG-amide (□), YFS(D-P)WG-amide (◇), YFSP(D-W)G-amide (○).

decreased significantly with an EC_{50} value 100-fold higher than that of the parent peptide. Substitution of phenylalanine or tryptophan with alanine, respectively, resulted in analogues which did not induce any significant contraction. In this case no EC_{50} values could be determined.

In order to evaluate the significance of the three-dimensional structure of Hez-KI for receptor interaction, a D-amino acid scan was performed, where each amino acid was replaced by its D-isomer. Fig. 3D shows that only the N-terminal tyrosine could be substituted by its D-isomer to keep a contractile action, which is however significantly reduced. The potency was 16% and the intrinsic activity 91% of the values from the reference peptide. Substitution of the other amino acids with their D-isomer resulted in agonists which did not show any significant in vitro action (Table 1).

3.3. Structure–activity studies with tachykinin-related peptides

In the following experiments the effects of TRPs on the gut motility of *H. virescens* were investigated. Three of the substances tested were TRPs originally isolated from the cockroach *L. maderae* (Lem-TRP) and one from the locust *L. migratoria* (Lom-TK). Lem-TRP1 (APSGFLGVR-amide) was the only peptide with the complete amino acid sequence, whereas the other TRPs were used with six or seven amino acid residues from the C-terminal end (Lem-TRP5 (5–10) residue: GFQGVR-amide, Lem-TRP9 (5–10) residue: GFQGMR-amide, Lom-TKII (4–10) residue: SGFYGVR-amide), since the conserved C-terminal hexapeptide was considered to be the bioactive core sequence (Nässel, 1999). Table 1 shows the results of the in vitro assay. All TRPs increased the contractile response of isolated guts from *H. virescens* in a dose-dependent manner but with great differences in the level of potency. With EC_{50} values of 25.0 and 71.3 nM, respectively, Lem-TRP1 and the Lom-TKII (4–10) residues were the most potent contractants. In contrast, by looking at the EC_{50} values of the Lem-TRP5 (5–10) and the Lem-TRP9 (5–10) peptides (2.9 and 6.7 μM, respectively) the potency of these analogues was drastically reduced. Hence, Lem-TRP1 was chosen for further investigations.

In order to examine the structure–activity relationships, truncated Lem-TRP1 analogues were tested in the gut contraction assay (Fig. 4). When the N-terminal alanine was cut off, the potency of the contractile action (40% of the reference) was significantly reduced,

Table 1
Summary of contractile effects of different insect neuropeptides on the isolated gut of *H. virescens*

Peptide/analogue	EC_{50} ± SE	Potency (%)	Intrinsic activity (%)
Helicokinins			
Hez-KI: YFSPWG-amide	2.0 ± 0.6 nM (a)	100	100
Hez-KII: VRFSPWG-amide	8.4 ± 4.4 nM (b)	24	90
Hez-KIII: KVKFSAWG-amide	12.7 ± 4.7 nM (b)	16	88
Truncated Hez-KI analogues			
Hez-KI: YFSPWG-amide	1.9 ± 0.9 nM (a)	100	100
FSPWG-amide	219.7 ± 142.1 nM (b)	0.9	61
SPWG-amide	n.f.	–	7
PWG-amide	n.f.	–	4
Not amidated: YFSPWG	n.f.	–	1
Alanine substituted Hez-KI analogues			
Hez-KI: YFSPWG-amide	3.2 ± 0.2 nM (a)	100	100
AFSPWG-amide	8.2 ± 0.9 nM (a)	39	98
YASPWG-amide	n.f.	–	5
YFAPWG-amide	5.9 ± 1.2 nM (a)	54	127
YFSAWG-amide	9.7 ± 2.8 nM (a)	33	119
YFSPAG-amide	n.f.	–	10
YFSPWA-amide	330.0 ± 160.4 nM (b)	1	59
D-Amino acid substituted Hez-KI analogues			
Hez-KI: YFSPWG-amide	4.7 ± 1.1 nM (a)	100	100
(D-Y)FSPWG-amide	30.1 ± 13.2 nM (b)	16	91
Y(D-F)SPWG-amide	n.f.	–	56
YF(D-S)PWG-amide	n.f.	–	42
YFS(D-P)WG-amide	n.f.	–	1
YFSP(D-W)G-amide	n.f.	–	18
Tachykinins			
Lem-TRP1: APSGFLGVR-amide	25.0 ± 4.2 nM (a)	100	100
Lom-TKII (4–10) residue: SGFYGVR-amide	71.3 ± 24.7 nM (b)	35	93
Lem-TRP5 (5–10) residue: GFQGVR-amide	2.9 ± 0.6 μM (c)	0.8	89
Lem-TRP9 (5–10) residue: GFQGMR-amide	6.7 ± 2.8 μM (c)	0.4	81
Truncated Lem-TRP1 analogues			
Lem-TRP1: APSGFLGVR-amide	28.3 ± 5.8 nM (a)	100	100
PSGFLGVR-amide	73.3 ± 12.7 nM (b)	40	104
SGFLGVR-amide	770.0 ± 124.9 nM (c)	4	83
GFLGVR-amide	692.5 ± 51.2 nM (c)	4	87
FLGVR-amide	n.f.	–	12
Not amidated: APSGFLGVR	n.f.	–	1
M. sexta allatoregulating peptides			
Mas-AT	78.7 ± 23.4 nM (a)	100	100
Mas-AS	n.f.	–	1
Mas-AT + Mas-AS	51.3 ± 22.9 nM (a)	153	98

EC_{50} represents the dose of peptide causing 50% of the contractile response from the reference peptide (i.e. the first peptide tested in each experiment: Hez-KI, Lem-TRP1 or Mas-AT, respectively). Where 50% of the maximal reference contraction was not achieved, the destination of the EC_{50} was not feasible (n.f.). Means ± SE of five determinations. Within each experiment significantly different values are denoted by different letters in parentheses (Tukey–Kramer post test after Kruskal–Wallis *H*-test, $P < 0.05$). The potency was calculated as (EC_{50} of the reference peptide)/(EC_{50} of the test peptide) in per cent. The intrinsic activity was calculated as (maximal contraction of the test peptide)/(maximal contraction of the reference peptide) in per cent.

whereas the intrinsic activity (104% of the reference) remained constant (Table 1). The truncation of two more amino acids from the N-terminal residue led to an intensifying decrease in potency and intrinsic activity (for details see Table 1). Cutting off four N-terminal amino acids or applying the non-amidated Lem-TRP1 analogue did not evoke a significant contractile response.

3.4. Contractile effects of Mas-AT and Mas-AS

In the next part of the study the influence of *M. sexta* allatotropin (Mas-AT) and allatostatin (Mas-AS) on gut motility was examined (Fig. 5). Mas-AT showed a contractile response in a dose-dependent manner with an EC_{50} of 78.7 nM (Table 1). In contrast, application of

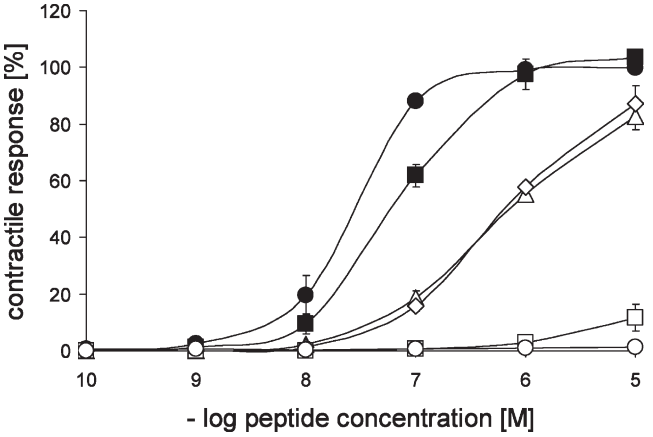

Fig. 4. Dose–response relationship for gut contraction with isolated whole-gut preparations of *H. virescens* larvae after treatment with Lem-TRP1 amidated, not amidated and truncated analogues: APSGFLGVR-amide (●), PSGFLGVR-amide (■), SGFLGVR-amide (△), GFLGVR-amide (◇), FLGVR-amide (□), APSGFLGVR (○). Mean values ± SE of five determinations as a percentage of the maximum response of this experiment.

Mas-AS to the in vitro assay neither affected the gut contractility, nor did Mas-AS alter the effects of Mas-AT when both peptides were mixed together and applied to the test system.

3.5. Comparison of contractile effects of Hez-KI, Lem-TRP1 and Mas-AT

Table 2 shows a summary of the comparison of Hez-KI, Lem-TRP1 and Mas-AT. By performing a test run with all three peptides, a relation could be set up with Hez-KI being the reference. Hez-KI was the most potent peptide with an EC_{50} of 3.0 nM, followed by Lem-TRP1 with an EC_{50} of 26.3 nM and Mas-AT with an EC_{50} of 78.7 nM. Although being a less potent contractant, Lem-TRP1 was able to induce a more powerful maximal contraction with an intrinsic activity of 168% compared to Hez-KI. A similar result could be observed with Mas-AT, here the resulting intrinsic activity was 131% of the reference value.

3.6. Mode of action of Lem-TRP1

In pharmacological experiments the mode of action of the contractile action caused by Lem-TRP1 was evaluated. Consequently, tissues were incubated in second messenger manipulating substances in order to investigate the link between Lem-TRP1 receptors and the signal transduction pathway. Table 3 exhibits the summary of the results from all chemical substances applied in the test. The data output consists of two values for every single drug. The first result represents the exclusive action of the drug on the gut and the second value indicates the impact of the drug on Lem-TRP1-induced contraction. At the concentrations tested no substance showed a significant influence on the gut motility alone, except for caffeine and to some extent thapsigargin also. Addition of caffeine and thapsigargin to the saline, respectively, stimulated gut contraction in a dose-dependent manner. On the other hand, no second messenger manipulating drug was able to significantly alter the Lem-TRP1 stimulated gut motility, except for cadmium, chelerythrine and tamoxifen. These substances inhibited

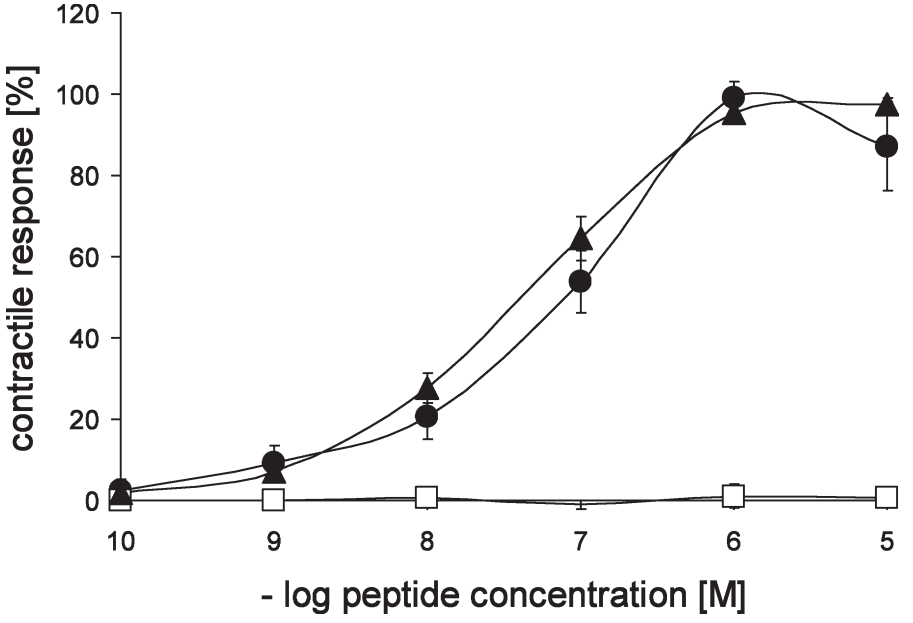

Fig. 5. Dose–response curves for gut contraction with isolated whole-gut preparations of *H. virescens* larvae after Mas-AT and Mas-AS treatment. Mas-AT (●), Mas-AS (□), Mas-AT + Mas-AS (▲). Mean values ± SE of five determinations as a percentage of the maximum response of this experiment.

Table 2
Comparison of contractile effects of Hez-K1, Lem-TRP1 and Mas-AT on the isolated gut of *H. virescens*

Peptide/analogue	EC_{50} ± SE	*n*	Potency (%)	Intrinsic activity (%)
Hez-KI	3.0 ± 0.5 nM (a)	20	100	100
Lem-TRP1	26.3 ± 3.2 nM (b)	10	11	168
Mas-AT	78.7 ± 23.4 nM (c)	5	4	131

For further explanations see legend of Table 1. Means ± SE of 5–20 determinations. Significantly different values are denoted by different letters in parentheses (Kruskal–Wallis *H*-test, $P < 0.05$).

Table 3
Summary of the effects of second messenger manipulating substances on Lem-TRP1-induced contraction of the isolated gut of *H. virescens*

Test substance	Contractile response (%)		*n*
	Alone	Lem-TRP1 (10 nM)	
Alone (reference)	–	100	
Caffeine (10 μM)	3.1 ± 1.2	91.4 ± 8.5	3
Caffeine (100 μM)	27.1 ± 1.7∗	95.3 ± 2.0	3
Caffeine (1 mM)	130.2 ± 5.0∗	103.1 ± 4.2	3
Forskolin (10 μM)	3.3 ± 1.9	97.8 ± 7.5	3
RP-8-Br-cAMPS (10 μM)	4.3 ± 1.5	93.9 ± 2.6	3
Dibutyryl-cAMP (10 μM)	1.7 ± 0.8	106.2 ± 8.3	3
Dibutyryl-cAMP (100 μM)	2.2 ± 1.2	97.7 ± 5.4	3
8-Br-cAMP (10 μM)	0.4 ± 1.4	111.1 ± 7.9	3
8-Br-cAMP (100 μM)	4.1 ± 2.5	108.4 ± 9.1	3
Cadmium (1 mM)	4.2 ± 2.4	94.4 ± 5.0	3
Cadmium (2 mM)	8.3 ± 4.2	66.7 ± 4.8∗	3
Cadmium (4 mM)	6.9 ± 1.4	19.4 ± 2.8∗	3
Cadmium (6 mM)	−1.4 ± 3.7	2.1 ± 1.2∗	3
Cadmium (10 mM)	4.5 ± 1.3	105.2 ± 3.4	3
Nifedipine (10 μM)	3.0 ± 0.7	94.0 ± 5.6	3
Verapamil (10 μM)	−4.5 ± 3.9	88.8 ± 5.8	3
Nimodipin (10 μM)	0.7 ± 2.7	109.7 ± 6.5	3
Tetrandrin (10 μM)	−2.2 ± 1.3	86.6 ± 3.9	3
Diltiazem (10 μM)	4.5 ± 1.3	105.2 ± 3.4	3
Dantrolene (10 μM)	9.9 ± 2.1	94.9 ± 5.3	3
Xestospongin (1 μM)	6.6 ± 1.8	100.9 ± 9.0	3
Dantrolene (10 μM) + xestospongin (1 μM)	5.7 ± 1.9	85.2 ± 8.6	3
Ryanodine (10 μM)	1.3 ± 0.9	98.4 ± 5.4	3
Thapsigargin (100 nM)	1.4 ± 0.6∗	105.0 ± 3.9	3
Thapsigargin (1 μM)	8.3 ± 2.6	91.9 ± 5.0	3
Thapsigargin (10 μM)	38.0 ± 5.7∗	106.4 ± 8.8	3
Chelerythrine (10 μM)	5.8 ± 0.8	42.8 ± 3.4∗∗	5
Tamoxifen (10 μM)	3.4 ± 1.5	9.4 ± 1.9∗∗	7
Tamoxifen (100 μM)	0.3 ± 1.9	11.0 ± 4.2∗∗	6

First the contractile activity of the drug alone was measured, then 10 nM Lem-TRP1 was applied to the gut preincubated with the drug for 8 min and the resulting contraction was recorded. The values are given as a percentage of the maximal response of Lem-TRP1 without drug. Means ± SE of 3–7 determinations. Within each experiment values being significantly different from the results achieved with only solvent or with only 10 nM Lem-TRP1, respectively, are marked with asterisks; $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann–Whitney *U*-test).

the contractile response usually evoked by Lem-TRP1 (for details see Table 3).

In order to verify the results, a control was integrated in the experiment where Lem-TRP1 or caffeine was applied to tamoxifen or ryanodine incubated guts, respectively (Fig. 6). The antioestrogen drug, tamoxifen, is known to inhibit protein kinase C (PKC) activity (Issandou et al., 1990). Ryanodine prevents the release of intracellular calcium by blocking the ryanodine receptor at the endoplasmic or sarcoplasmic reticulum

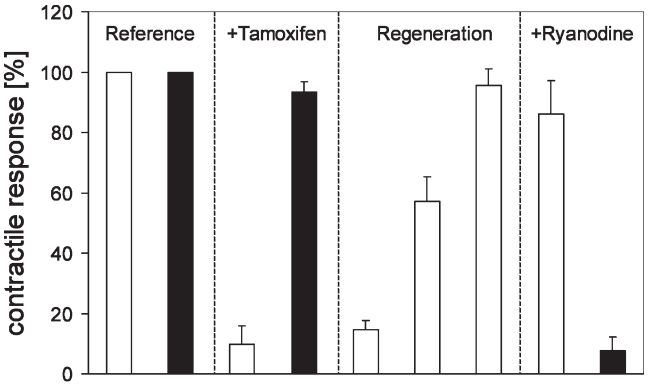

Fig. 6. Effects of second messenger manipulating substances on Lem-TRP1 and caffeine-induced contraction of the isolated gut of *H. virescens*. The values are given as a percentage of the maximal response of 10 nM Lem-TRP1 (grey bars) or 1 mM caffeine (black bars), respectively. 10 μM tamoxifen and 10 μM ryanodine were added to the saline 8 min prior to the addition of Lem-TRP1 or caffeine, respectively. In between gut was regenerated and contractile response of Lem-TRP1 was retested after rinsing with saline by using an 8-min cycle. Means ± SE of four determinations.

(Ogawa, 1994). In this context the application of caffeine served as a control, since caffeine at high concentrations (mM) represents a pharmacological agonist of the ryanodine receptor (Zuchhi and Ronca-Testoni, 1997) leading to calcium release from intracellular stores and hence to muscle contractions (see Table 3). As expected, this caffeine-induced reaction was not significantly influenced when the gut had been incubated with 10 μM tamoxifen (Fig. 6). In contrast, after addition of 10 μM Tamoxifen to the saline the contractile response of 10 nM Lem-TRP1 displayed only about 10% of the normal answer when tested without drug. Rinsing the gut in saline up to 24 min (3 × 8 min cycles), the Lem-TRP1-induced contractions could be steadily reestablished, so that the inhibiting impact of tamoxifen on the Lem-TRP1-induced contractile effect is considered to be reversible. Finally, the application of 10 μM ryanodine did not alter the muscle response of Lem-TRP1, but significantly inhibited that of caffeine.

3.7. Mode of action of Hez-KI

In preliminary experiments the mode of action of the contractile effects of Hez-KI was also examined. Therefore, only the second messenger manipulating substances RP-8-Br-cAMPS, cadmium, dantrolene, xestospongin C and tamoxifen were applied (Table 4). Again no drugs showed significant impact on the gut motility when added alone to the saline. However, 10 μM of either cadmium or tamoxifen was able to inhibit Hez-KI-induced contractile effects to a significant extent. Cadmium nearly abolished the gut contraction completely (4% of the reference response), whereas tamoxifen led to a significant reduction, i.e. 63% of the control answer.

3.8. Mode of action of Mas-AT

When Mas-AT was tested with respect to its second messenger system for contractile action, similar results to those obtained with Hez-KI were observed (Table 5). Cadmium (10 μM) almost prevented gut contractions induced by 10 nM Mas-AT completely and 10 μM tamoxifen reduced the intensity of the contraction by about two-third. In addition to the observations with Hez-KI, application of RP-8-Br-cAMPS also resulted in a reduced contractile response being about 47% of that from the reference without drug.

4. Discussion

Investigations of helicokinins with respect to their influence on the gut from *H. virescens* were carried out using an isometric contraction assay system. The study demonstrated that the gut motility could easily and reliably be measured with the equipment used. Application of Hez-KI led to a significant contractile response. With growing peptide concentrations the amplitude of the TC increased in a concentration-dependent manner. The reason for the slight decrease at higher concentrations is probably the tachyphylaxis caused by desensitizing effects of the receptors at very high peptide concentrations (Howarth et al., 2002b). Similar dose–response curves were obtained with Hez-KII and Hez-KIII. These results demonstrated for the first time, that helicokinins also play a role in the control of myotropic activity in the economically important pest species, *H. virescens*.

Howarth et al. (2002b) already observed similar effects for helicokinins on the gut motility in larvae of *S. frugiperda* by using an isotonic contraction assay. The authors found EC_{50} values for Hez-KI, Hez-KII and Hez-KIII in a similar range as shown in this study. But the order of potency and also the order of intrinsic activity were different from the results reported here. In *S. frugiperda* Hez-KII was found to be the most potent contractant of the gut followed by Hez-KIII and Hez-KI, this is in contrast to *H. virescens* where Hez-KI was the peptide with the highest potency followed by Hez-KII and Hez-KIII. The main conclusion from both studies is, on the one hand, that helicokinins which were originally isolated from *Helicoverpa zea* (Blackburn et al., 1995) act as myotropic hormones in several related lepidopteran species (Noctuidae). The reason for that is probably the conserved sequence and structure of the peptide receptor in related insect species. On the other hand there are also differences in potency and intrinsic activity of the peptides, which might indicate species-specific variations of the kinin binding sites.

Helicokinins do not only affect the insect muscular system. Other investigations in terms of their hormonal

Table 4
Summary of the effects of second messenger manipulating substances on Hez-K1-induced contraction of the isolated gut of *H. virescens*

Test substance	Contractile response (%)		*n*
	Alone	Hez-K1 (10 nM)	
Alone (reference)	–	100	
RP-8-Br-cAMPS (10 μM)	14.3 ± 7.0	94.5 ± 10.9	3
Cadmium (10 μM)	−0.6 ± 2.8	4.0 ± 2.1∗	3
Dantrolene (10 μM)	5.1 ± 1.2	101.7 ± 17.6	3
Xestospongin (1 μM)	16.7 ± 6.1	96.7 ± 14.8	3
Tamoxifen (10 μM)	4.4 ± 2.0	62.5 ± 9.6∗	5

First the contractile activity of the drug alone was measured, then 10 nM Hez-K1 was added to the gut preincubated with the drug for 8 min and the resulting contraction was recorded. The values are given as a percentage of the maximal response of Hez-K1 without drug. Mean ± SE of three determinations. Within each experiment values being significantly different from the results achieved with only solvent or with only 10 nM Hez-KI, respectively, are marked with asterisks; $P < 0.05$ (Mann–Whitney *U*-test).

Table 5
Summary of the effects of second messenger manipulating substances on Mas-AT-induced contraction of the isolated gut of *H. virescens*

Test substance	Contractile response (%)		*n*
	Alone	Mas-AT (10 nM)	
Alone (reference)	–	100	
RP-8-Br-cAMPS (10 μM)	4.5 ± 1.0	47.4 ± 6.7∗	4
Cadmium (10 μM)	−3.4 ± 5.5	3.3 ± 0.3∗	3
Dantrolene (10 μM)	5.4 ± 1.3	91.4 ± 5.5	
Xestospongin (1 μM)	4.0 ± 1.1	86.2 ± 6.7	
Tamoxifen (10 μM)	3.1 ± 1.1	33.2 ± 9.9∗	5
Tamoxifen (100 μM)	2.9 ± 2.7	27.4 ± 14.7∗	6

First the contractile activity of the drug alone was measured, then 100 nM Mas-AT was added to the gut preincubated with the drug for 8 min and the resulting contraction was recorded. The values are given as a percentage of the maximal response of Mas-AT alone. Mean ± SE of 3–6 determinations. Within each experiment values being significantly different from the results achieved with only solvent or with only 10 nM Mas-AT, respectively, are marked with asterisks; $P < 0.05$ (Mann–Whitney *U*-test).

impact on insect tissues focused on the diuretic effects of these neuropeptides. Blackburn et al. (1995) found that the helicokinins stimulated fluid secretion in Malphigian tubules isolated from *M. sexta* at doses in the range of 10^{-10} M. Thereby Hez-KIII was more potent than either Hez-KII or Hez-KI. With Malphigian tubules isolated from *H. virescens* larvae Seinsche et al. (2000) demonstrated similar effects but in this case Hez-KI showed the highest potency followed by Hez-KII and Hez-KIII. Thus, the order of potency and activity for the diuretic effects are identical to that obtained for the myotropic action in this study. This supports the hypothesis that the peptide receptors triggering the kinin effects in different tissues are at least very similar. If both in vitro assays, the Ramsay assay for diuretic activity and the isometric gut contraction assay for myotropic activity, are in principle comparable, then the contraction assay might be useful to identify or characterize new diuretic compounds. This would be of great benefit since the Ramsay assay is very difficult to handle, particularly with Malphigian tubules from lepidopteran larvae.

In order to investigate the structural motifs essential for the binding to the helicokinin receptor, more detailed, structure–activity experiments were done. The tests indicated that successive removal of N-terminal amino acid residues resulted in a loss of peptide activity. Already the truncation of the first tyrosine led to a 100-fold fall in potency. Smaller analogues than the pentapeptide FSPWG-amide or a non-amidated Hez-KI analogue did not stimulate any significant gut contraction. The results are in good conformity with those of Howarth et al. (2002b) where successive truncation led to a resembling decrease in contractile response. In contrast to that, Seinsche et al. (2000) found that for diuretic activity in Malphigian tubules from *H. virescens* the removal of the tyrosine had no significant influence on the physiological in vitro effect. However, the levels of intrinsic activity of the pentapeptide FSPWG-amide were in all three studies more or less as high as the values for the hexapeptide.

The results from the alanine scan demonstrated that substitution of tyrosine, serine and proline (position 1, 3, and 4) by alanine had no significant impact on the gut

contractility compared with Hez-KI. Replacement of the C-terminal glycine, however, led to a significant decrease in activity. The potency was only 1% of that from the parent peptide. Furthermore, when phenylalanine or tryptophan was exchanged, the resulting analogues were not able to stimulate gut contraction any more. Comparable observations were made by Howarth et al. (2002b) in the gut contraction assay with *S. frugiperda* and by Seinsche et al. (2000) for diuretic activity in Malphigian tubules from *H. virescens*. This confirms the presumption that the aromatic groups in positions 1 and 4 of the core pentapeptide are of great importance for the receptor binding which was already supposed earlier by Nachman and Holman (1991).

Summarizing these findings, the Hez-KI receptor mediating the contractile effects in the gut muscular system seems to be quite conservative in related insect species but apparently has different structural requirements from that initiating fluid secretion in Malphigian tubules. To complete the investigations of the peptide-receptor interaction, a D-amino acid scan of Hez-KI was undertaken. Only the N-terminal tyrosine could be replaced by the amino acid alanine to sustain a contractile effect which was, however, significantly attenuated. This corresponds very well with the results from our experiments with truncated Hez-KI analogues.

Taking all structure–activity experiments together, the pentapeptide fragment FSPWG-amide seems to be the core sequence for activating the physiological effects. The same finding was achieved for diuretic activity with helicokinins (Seinsche et al., 2000) and achetakinins (Coast et al., 1990) and for myotropic activity with leucokinins (Nachman and Holman, 1991). However, since in our experiments the truncation of the N-terminal tyrosine led to a reduced activity, this residue may also play a role for receptor binding. Consistently, when tyrosine was truncated from Hez-KI the resulting pentapeptide lost its diuretic properties in in vivo experiments (Seinsche et al., 2000) indicating the significance for that N-terminal residue in this physiological aspect. Further on we demonstrated that the amidation of the peptide is of high importance for the core sequence, since a non-amidated Hez-KI analogue did not show any myotropic activity. The amino acids at position 3 and 4 (serine, proline) are not relevant for biological activity so that these residues probably function as a spacer to keep the correct distances between the side chains or as a secondary structure element (Seinsche et al., 2000).

Analysis of the effects of insect tachykinins was performed with various representatives or truncated analogues of TRPs from different insect species (see Table 1). For the first time it could be shown that TRPs isolated from non-lepidopteran species do act as myotropic hormones on the gut from *H. virescens* larvae and also on the gut of the closely related noctuid *S. frugiperda* (additional observations not shown here). Moths are sparsely investigated in terms of TRPs. One reason is that no TRP has so far been isolated from this insect order. With the help of antiserum raised to one of the locust TRPs, however, TRP-immunoreactive neurons could be found in *Spodoptera litura* (Kim et al., 1998). But the physiological functions of these peptides in vivo are still unknown, even in the well examined cockroach species. Only in in vitro assays some actions of the peptides have been demonstrated. TRPs stimulated contractions of visceral and skeletal muscle in locusts and cockroaches (Evans, 1994; Nässel et al., 1998; Schoofs et al., 1993; Winther et al., 1998) and can induce release of adipokinetic hormone in the locust *corpora cardiaca* (Nässel et al., 1995). The only effects of TRPs in lepidoptera were investigated by Fonagy et al. (1992). TRPs were able to induce pheromone biosynthesis in the moth, *B. mori*. In this context the results of the present study may help to supplement this state of knowledge.

The contractile response evoked by the insect tachykinins on *H. virescens* guts was dose-dependent with Lem-TRP1 exhibiting the highest potency and intrinsic activity so that this peptide was used for further evaluation. Structure–activity experiments revealed that the minimal core sequence for biological activity is at least the amidated hexapeptide GFLGVR-amide, whereas the next two N-terminal amino acids, proline and serine, seem to be important in order to reach the maximal potency of Lem-TRP1 in the in vitro assay. Looking at the conserved pentapeptide FX_1GX_2R-amide, it is obvious that the minimal core sequence for biological activity is definitively longer than the C-terminal pentapeptide generally characterizing all TRPs. This is in contrast to the structure–activity tests achieved with helicokinins where the peptide family-specific pentapeptide seems to represent the core sequence for biological activity too.

In order to extend the knowledge of the myotropic gut control in lepidoptera, further neuropeptides, the lepidopteran allatotropin Mas-AT and allatostatin Mas-AS, were examined with the muscle contraction assay. Similar to helicokinins and TRPs, Mas-AT stimulated the gut contraction in a dose-dependent manner. Mas-AS showed no effect on the gut. It was neither able to influence the tonic response of the organs itself nor could the effects caused by Mas-AT be significantly abolished or inhibited. The fact that Mas-AT plays a role in controlling the gut contraction in *H. virescens* is an interesting finding since the peptide was originally considered to affect the *corpora allata* by regulating the JH biosynthesis. But already Duve et al. (1999) showed that Mas-AT stimulated the foregut muscle contraction in *Heliothis armigera*. The same authors found that in the closely related moth *Lacanobia oleracea* allatotropin also led to increased gut motility which interestingly could be abolished or inhibited by Mas-AS. The conclusion one could draw is that the allatoregulating pep-

tides may control the gut peristaltic by antagonistic up- and downward regulation of the spontaneous contractions (Duve et al., 2000). However, these findings could not be confirmed with *H. virescens* in this study. Mas-AT did stimulate gut motility, but it could not be influenced by Mas-AS. Mas-AT and Mas-AS are the only functional allatotropin and allatostatin, respectively, which were isolated from lepidopteran species and therefore are known to control JH biosynthesis in moths. The results about their myotropic activity indicate that neuropeptides certainly have more than one physiological function in insects.

To compare the dose–response curves of helicokinins, TRPs and Mas-AT, representatives of all three peptide families were tested together in one experiment. The most striking point is the clear difference between the EC_{50} values (potency) and the values for intrinsic activity. On the one hand, Hez-KI showed a higher potency than Lem-TRP1 and Mas-AT. On the other hand, tachykinin exhibited a greater intrinsic activity than allatotropin followed by helicokinin. This suggests that there are clearly distinct systems for the tested peptide families controlling gut contraction. One point being surely involved is the fact that the hormone receptors are obviously different for the three peptide groups, each with agonist-specific properties. It should also be taken into account, that the tissue utilized in the in vitro assay was a whole-gut preparation. But myotropic peptides are known to act only on certain parts of the gut. For instance Lom-TKI exclusively stimulates the hindgut of *L. maderae* (Schoofs et al., 1993; Winther et al., 1998). Other TRPs can also affect the foregut of cockroaches but this seems to be substance and species-specific, which is correlated with the innervation of the foregut by specific TRP-immunoreactive interneurons (Nässel et al., 1998). Thus, the different myotropic peptides may affect distinct regions and muscular systems of the gut which may lead to the different contractile responses obtained.

It is commonly known that neuropeptides act via G-protein-coupled receptors (GPCRs) with a seven hydrophobic α-helical domain structure spanning the plasma membrane (Darlison and Richter, 1999; Vanden Broeck, 1996). The GPCRs represent a large diversity and information of the exact signalling pathways for triggering the physiological action is scarce. In order to investigate the second messenger systems involved in controlling gut peristaltic movement in *H. virescens*, pharmacological experiments were carried out. First, the focus was on Lem-TRP1. Generally, application of caffeine led to a contraction of the insect gut. Caffeine is known to prolong the effects of intracellular cAMP by inhibiting the phosphodiesterase, which degrades this second messenger molecule. Since caffeine did not potentiate the contractile effects initiated by Lem-TRP1, the physiological activity of caffeine seems to come from its agonistic action on the ryanodine receptor (Zuchhi and Ronca-Testoni, 1997) leading to the release of intracellular calcium. On the other hand, the result indicates that Lem-TRP1 may not act via a cAMP linked second messenger system. These findings are in contrast to those of Howarth et al. (2002b), who reported that the phosphodiesterase inhibitor theophylline caused significant potentiation of the response to applied Hez-KI, indicating that cAMP is involved in the signalling pathway of the Hez-KI receptor. Similarly Lundquist and Nässel (1997) postulated that locustatachykinins may trigger their physiological effects via cAMP. But in our experiments even the application of membrane-permeable cAMP analogues 8-Br-cAMP and dibutyryl-cAMP or forskolin, an activator of adenylate cyclase (de Souza, 1983), did not show any significant effects. The drugs were neither able to stimulate gut contraction alone nor could they alter the Lem-TRP1-induced response. Furthermore, RP-8-Br-cAMPS which represents a more effective RP-cAMPS analogue, known to inhibit basal cAMP-kinase activity (Gjertsen et al., 1995) also did not attenuate the gut contractions triggered by the tachykinin.

Subsequently, other second messenger pathways, the inositol trisphosphate (IP_3) and the diacylglycerol (DAG) system, were investigated by means of specific effectors interfering with these pathways. IP_3 is generated by a G-protein activated phospholipase C (PLC) and acts as second messenger by releasing calcium from intracellular stores. If Lem-TRP1-induced gut contraction is triggered by opening such calcium channels, more or less specific blockers such as xestospongin C, dantrolene or ryanodine (Haskó et al., 1998; Miyamoto et al., 2000; Ogawa, 1994) should inhibit the physiological response. But in our experiments these drugs did not show any significant effects, so that intracellular calcium stores may not play a role for signal transduction. Additional experiments with thapsigargin supported the conclusion. Application of thapsigargin known to inhibit sarco-endoplasmic reticulum Ca^{2+}-ATPases (Treiman et al., 1998) did not show any influence on Lem-TRP1 triggered gut contraction. On the other hand, thapsigargin alone was able to stimulate a slight contractile response. This can be explained by the fact that after an inhibition of the Ca^{2+}-ATPases, which are normally responsible for the retransport of intracellular calcium into sarcoplasmic or endoplasmic stores, the calcium titre in the cytosol rises which again could initiate a muscle contraction.

When intracellular calcium does not play a role for the signal transduction, the extracellular availability of calcium should be examined. Cadmium at high concentrations is an antagonistic blocker of calcium channels located in the cell membrane. The addition of cadmium ions to the medium in our experiment abolished almost totally the contractile effects of Lem-TRP1. This indicates the requirement for extracellular calcium. The

result was moreover confirmed by the observation that addition of EGTA to the medium in order to obtain a calcium-free test fluid also inhibited gut contractility (not shown here). Several L-type calcium channel blockers (nifedipine, verapamil, nimodipin, tetrandrin, diltiazem) had no effect in our experiments suggesting that the relevant ion channel here belongs to another type or that these calcium channel blockers acting on human channels are very weak or inactive on *Heliothis* calcium channels.

O'Brian et al. (1985) and Issandou et al. (1990) found that the antioestrogen drug, tamoxifen, inhibits the activity of PKC in vitro. Similarly, chelerythrine is a potent and specific inhibitor of PKC (Herbert et al., 1990). We could show in our experiments that application of tamoxifen and chelerythrine, respectively, resulted in a distinct and significant reduction of Lem-TRP1-induced contractile response. The effects were reversible and may not come from unspecific artefacts (see Fig. 6). This finding highlights that PKC might be involved in the signalling pathway of Lem-TRP1. Hinton et al. (1998) explored the connection between PKC and a verapamil-sensitive calcium channel in the foregut of the locust *Schistocerca gregaria*. Activation of PKC promoted calcium entry via the opening of the ion channel. A similar mechanism could also be considered for the Lem-TRP1 signalling pathway. The tachykinin binds to the GPCR, activating PLC which catalyses the synthesis of DAG. The second messenger, DAG, again activates the membrane-bound PKC which finally opens a calcium channel in the plasma membrane leading to influx of extracellular calcium and thereby to muscle contraction. A similar transductional pathway was also claimed by Howarth et al. (2002a) for 5-HT receptors in the isolated gut of *S. frugiperda*. The calcium channel blocker lithium (Howarth et al., 2002a) and the PKC inhibitor H7 (Hidaka et al., 1984) both markedly attenuated the effects of applied 5-HT whereby the authors suggested a link from the phosphatidylinositol second messenger system to the extracellular calcium influx. On the other hand, these results are at odds with the findings of Torfs et al. (2001). In *Drosophila melanogaster* Schneider 2 cells, which were transfected with the tachykinin-like receptor S2-STKR, a variety of natural insect tachykinin analogues produced a significant dose-dependent calcium rise. Further on, this response did not depend on the presence of extracellular Ca^{2+} ions, indicating a different transduction mechanism for insect tachykinins than it is concluded in the current study.

Similar to our pharmacological experiments with Lem-TRP1, preliminary tests with the myotropic neuropeptides Hez-KI and Mas-AT also suggest a PKC involved transduction. Again the in vitro tests revealed that cadmium almost abolished and tamoxifen significantly inhibited the contractile effects caused by the peptides. Moreover, application of RP-8-Br-cAMPS, an inhibitor of cAMP-kinase, attenuated significantly the myotropic effects triggered by Mas-AT. This indicates that the second messenger cAMP may also be involved in signal transduction for that peptide. Since the number of repeated experiments was quite low and some drugs have been only tested at very limited concentrations, much more work has to be done in order to learn more about myotropic peptide action.

To conclude, both isometric (current study) and isotonic (Howarth et al., 2002a,b) gut contraction assays provide an interesting tool for examining neuropeptides or other compounds with myotropic activity in insects. Under specific circumstances this assay can also be a good alternative to the Ramsay assay used recently to examine and characterize helicokinins or related substances. The gut contraction assay is easy to handle and rapidly performed. But for screening purposes one disadvantage might be the unspecificity of the assay, so that novel specific targets and their agonists will be difficult to identify. A great number of neuropeptides are still known to affect the muscle contraction in insects (Evans, 1994; Nässel, 1999; Predel et al., 2001; Schoofs et al., 1993). In the locusts, *L. migratoria* and *S. gregaria*, more than 60 neuropeptides have so far been identified. The majority indeed stimulates the contraction of the gut, the oviduct or the heart of the insects in vitro (Schoofs et al., 2001). This does not necessarily mean, however, that all these peptides act as real hormones and are involved in the regulation of muscle contractility in vivo. But to learn more about the function of neuropeptides and their involvement in physiological processes, in vitro tests like the gut contraction assay are the appropriate and sometimes the only instruments available to reliably assess their potential.

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