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Analysis of C-terminally substituted tachykinin-like peptide agonists by means of aequorin-based luminescent assays for human and insect neurokinin receptors

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Abstract

Aequorin-based assays for stable fly, *Stomoxys calcitrans*, (STKR) and human (neurokinin receptor 1 (NK1), neurokinin receptor 2 (NK2)) neurokinin-like receptors were employed to investigate the impact of a C-terminal amino acid exchange in synthetic vertebrate ('FXGLMa') and invertebrate ('FX_1GX_2Ra') tachykinin-like peptides. C-terminally (Arg to Met) substituted analogs of the insect tachykinin-related peptide, *Lom*-TK I, displayed increased agonistic potencies in luminescent assays for human NK1 and NK2 receptors, whereas they showed reduced potencies in the STKR-assay. The opposite effects were observed when C-terminally (Met to Arg) substituted analogs of substance P were analysed. These substance P analogs proved to be very potent STKR-agonists, being more potent than *Lom*-TK I. On the other hand, *Lom*-TK-LMa, was shown to be a very potent NK1-agonist and was suggested to have more substance-P-mimetic than neurokinin-A-mimetic properties. NK1 and NK2 receptor agonists appeared to be more sensitive to changes at the penultimate amino acid position than STKR-agonists. This is also reflected in the sequence conservation that is observed in the naturally occurring tachykinin subgroups ('FXG**LM**a' *vs.* 'FX_1G**X_2R**a'). The differential Arg–Met preference appears to be a major coevolutionary change between insect and human peptide-receptor couples. With regard to the peptide agonists, this change can theoretically be based on a single point mutation.

Keywords: Aequorin; Agonist; Calcium; Evolution; Neuropeptide; Receptor

1. Introduction

Neurokinins or tachykinins belong to an evolutionary conserved family of multifunctional brain/gut peptides identified in vertebrate and invertebrate species. They play an important neuromodulatory role in the central nervous system and exhibit a broad range of peripheral activities [1–3]. Based on sequence data, these peptides can be classified into two distinct subfamilies. All known vertebrate tachykinins have a characteristic C-terminal FXGLMa sequence. The tachykinin-related peptides (also referred to as ''insectatachykinins''), which have been identified in protostomian invertebrates (Arthropods, Mollusks, Echiuroid worms), display structural similarities to the first group [4] and possess a C-terminal FX_1GX_2Ra consensus sequence [5,6].

Tachykinin receptors belong to the large superfamily of serpentine or G protein-coupled receptors (GPCR). In mammals, there is strong evidence for the existence of at least three distinct, tachykinin or neurokinin (NK) receptor subtypes, which have been named NK1, NK2 and NK3 receptors [7]. Mammalian tachykinins, such as

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Abbreviations: CHO, Chinese hamster ovary cell line; GPCR, G protein-coupled receptor; *Lom*-TK I, *Locusta migratoria* tachykinin-related peptide 1 or locustatachykinin-1; NK1, neurokinin receptor 1; NK2, neurokinin receptor 2; NK3, neurokinin receptor 3; PLC, phospholipase C; RLU, relative light units; S2, Schneider 2 cell line; SD, standard deviation; STKR, *Stomoxys calcitrans* tachykinin receptor.

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substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), all appear to interact to varying degrees with each of these subtypes. The three receptors display pharmacological differences and have different binding affinities for the naturally occurring agonists: NK1 shows highest affinity for substance P, NK2 for NKA and NK3 for NKB. Agonist-activated NK receptors induce an activation of phospholipase C (PLC) leading to increased IP_3 production and an intracellular Ca^{2+} response. Similarly, insect GPCR which display pronounced sequence homology with mammalian neurokinin receptors (NK1–3) have been shown to activate PLC in response to insect tachykinin-related peptide agonists [8,9]. Previously, we reported on the functional expression [9] and pharmacological characterisation [10] of the stable fly (*Stomoxys calcitrans*) tachykinin-like receptor, STKR, in permanently transfected *Drosophila* S2 cells. In the present paper, we analyse the effect of C-terminal amino acid substitutions in insect and mammalian tachykinin-like peptide agonists by means of aequorin-based bioluminescent assays for human (NK1 and NK2) and insect (STKR) neurokinin receptors. Synthetic analogs with hybrid insect-mammalian sequence characteristics are utilised to study the possible impact of structural and evolutionary changes in the C-terminal motif of these peptides.

2. Experimental procedures

2.1. Peptides

The insect tachykinin-related peptide, locustatachykinin-1 (*Lom*-TK I), as well as C-terminally substituted analogs of substance P and of *Lom*-TK I were synthesised by means of Fmoc polyamide chemistry. The purity (>95%) of these peptides was determined by MALDI-TOF-MS. The vertebrate neurokinins, substance P and neurokinin A, were purchased from Sigma. The sequences of the tachykinin-like analogs that were employed in this study are displayed in Table 1.

Table 1
Amino acid sequences of the peptides that were utilised in this study

Peptide name	Amino acid sequence
Lom-TK I	Gly–Pro–Ser–Gly–Phe–Tyr–Gly–Val–Arg–NH_2
Lom-TK-Ma	Gly–Pro–Ser–Gly–Phe–Tyr–Gly–Val–**Met**–NH_2
Lom-TK-LMa	Gly–Pro–Ser–Gly–Phe–Tyr–Gly–**Leu**–**Met**–NH_2
Substance P	Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2
Substance P-Ra	Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–**Arg**–NH_2
Substance P-VRa	Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–**Val**–**Arg**–NH_2
Neurokinin A	His–Lys–Thr–Asp–Ser–Phe–Val–Gly–Leu–Met–NH_2

Locustatachykinin-1 (*Lom*-TK I), neurokinin A and substance P and derived, synthetic analogs. The substituted amino acid residues are shown in bold and underlined.

2.2. Cell lines

The *Drosophila* S2 cell line, S2-STKR-Aeq, expressing both apoaequorin and STKR, the *Stomoxys calcitrans* tachykinin-like peptide receptor, was produced as follows. The mitochondria-targeted apoaequorin-encoding cDNA (Molecular Probes) was placed under control of a constitutive insect actin promoter in pAc5.1V5His (Invitrogen). A second vector, pFJ*iegprom* [9], contained the STKR-encoding region controlled by another constitutive insect promoter, *Bm*NPVieg [11,12]. The hygromycin B selection construct, pUC*hshyg*, was kindly provided by J. Carlson (Colorado State University, Fort Collins, CO, USA). S2 cells [13] were transfected with the plasmids by employing the lipofection agent, Cellfectin™ (Gibco BRL, Life Technologies). For selection, 400 μg/mL hygromycin B (Duchefa) was added to the culture medium. After 4 weeks of continuous selection, the resulting hygromycin-resistant cell population was diluted and subdivided in six-well plates to obtain clonal cell lines. Stable clones were examined by means of the aequorin-based luminescence assay. The cell clone, S2-STKR-Aeq, displaying the highest maximal response and the lowest EC_{50} was selected for further experiments. Non-transfected S2 cells didn't display any detectable calcium responses to the peptides that were utilised in this study.

The Chinese Hamster Ovarian cells (CHO) cell lines PAM28-NK1 and PAM28-NK2, permanently expressing the human NK1 and NK2 receptor, respectively, were prepared as follows. CHO cells were transfected with an expression plasmid encoding mitochondria-targeted apoaequorin (Molecular Probes) and stable cells were selected for resistance against 25 μg/mL puromycin. The resulting population of puromycin-resistant CHO cells was diluted and cloned. Fifty clones were obtained and further examined with 1 μM ATP and 0.1% Triton X-100 in a bioluminescence assay, as described previously [14]. The clone with the highest signal-to-background ratio was selected and further transfected with a bicistronic plasmid containing the NK1, or NK2, receptor cDNA and an antibiotic resistance gene. The transfected cells were selected with an additional antibiotic (400 μg/mL neomycin) and the resulting cell populations were cloned. Screening of the resulting clones was performed with substance P and the clones with the highest signal-to-background ratio showing the lowest EC_{50}-values were selected for pharmacological characterisation. Non-transfected CHO cells didn't show any detectable calcium responses to peptides employed in this study. The cell clones PAM28-NK1 and PAM28-NK2 are now commercially available from Euroscreen.

2.3. Cell culture conditions

S2-STKR-Aeq cells were grown in Schneider's medium (Serva) supplemented with $CaCl_2$ (0.6 g/L), $NaHCO_3$

(0.4 g/L), 10% heat-inactivated foetal calf serum (Gibco BRL, Life Technologies) and antibiotics (25 U/mL penicillin G and 25 μg/mL streptomycin, Sigma). The pH of the prepared medium was adjusted to 6.45 by adding NaOH. Cells were grown in monolayers at 23°.

The CHO cell clones were cultured in sterile MEM ALPHA (Gibco BRL) medium (w/o ribonucleotides and deoxyribonucleotides) supplemented with 2.2 g $NaHCO_3$/L, 10% heat-inactivated foetal bovine serum (Gibco BRL), 400 μg/mL Neomycin (G418, Sigma) and 5 μg/mL Puromycin (Sigma). The cells were grown as monolayers at 37° in an atmosphere of 100% humidity and 5% CO_2.

2.4. Aequorin charging protocols

S2-STKR-Aeq cells, expressing apoaequorin and STKR, were examined for viability and counted by Trypan Blue exclusion. Cells were spun down (5–8 min at 900 rpm) and resuspended in basal Schneider's medium (Serva) [pH 6.45; supplemented with $CaCl_2$ (0.6 g/L), $NaHCO_3$ (0.4 g/L)], at a density of 1×10^7 cells/mL. "Coelenterazine h" (Molecular Probes) was added to a concentration of 5 μM and the cells were incubated in the dark at room temperature during 2–4 hr. These coelenterazine-loaded cells were diluted 5 times in basal Schneider's medium immediately before starting the experiments.

The CHO cells were grown until 90% confluent monolayers were obtained. Cells were detached by changing the growth medium for 1 × PBS buffer supplemented with 100 μM EDTA (pH 8). The cells were spun down and resuspended at 5×10^6 cells/mL in D-MEM/F12 medium without phenol red (BioWhittaker) supplemented with 0.1% BSA. Coelenterazine h (Molecular Probes) was added to reach a concentration of 5 μM. This mixture was incubated for at least 3–4 hr at room temperature in a slowly stirred flask. After coelenterazine loading, the cells were 10× diluted in the same medium and incubated for minimum 30 min.

2.5. Aequorin luminescence assay

Dilution series of peptides were prepared in 96-well plates. A 10 μL of each dilution was added to 90 μL basal Schneider's medium (for the insect cells) or D-MEM/F12 medium (BioWhittaker) without phenol red (for the mammalian cells). In all experiments, one positive (0.1% Triton X-100) and one negative (only medium) control sample were included in each row of the microplate. The other wells in the row contained the serially diluted experimental samples. The well plate was then loaded in a "Microlumat plus, LB96V" microplate luminometer (EG&G Berthold, Perkin-Elmer™ Life sciences). The wells were screened one by one and each measurement started at the moment of injection. Into each well, a 100 μL suspension containing 1–2.5 × 10^5 S2-STKR-Aeq, or 5×10^4 PAM28-NK1 or -NK2, cells was injected, by means of the automated injector of the instrument. Light emission was recorded over a period of 30 s per well.

2.6. Data analysis

Luminescence data (peak integration) were calculated by means of Winglow™ software (Perkin-Elmer) which is linked to the Microsoft Excel program. The resulting data were then transferred to and processed by SigmaPlot 4.0 (SPSS Inc.) software. This program was employed to analyse the results by means of a three-parameter sigmoidal curve-fitting algorithm and to calculate EC_{50}-values.

3. Results

3.1. Arg (R) to Met (M) replacement

Vertebrate neurokinins and insectatachykinins differ in their amidated C-terminal amino acid residue which is Met–NH_2 and Arg–NH_2, respectively. By changing the C-terminal Arg–NH_2 of *Lom*-TK I into Met–NH_2, a peptide is formed with hybrid sequence properties (*Lom*-TK-Ma, see Table 1). Interestingly, this single amino acid substitution results in a spectacular increase in the peptide's potency to induce an intracellular rise of calcium ions in NK1-expressing CHO cells (PAM28-NK1). Whereas *Lom*-TK I appeared to be an extremely weak NK1-agonist, showing only a very small response at a concentration as high as 10 μM, the C-terminally substituted *Lom*-TK-Ma already elicits detectable bioluminescent responses in these cells at concentrations that are in the nM range. The dose-response curve for this hybrid peptide is shifted to the left by at least 3–4 orders of magnitude (i.e. 10^3–10^4 more potent) when compared to the one of locustatachykinin-1 (Fig. 1). An additional substitution of the penultimate amino acid residue (–Val– into –Leu–) leads to a second chimeric peptide, *Lom*-TK-LMa, which has increased 'mammalian tachykinin-like' sequence characteristics. Compared to *Lom*-TK-Ma, this peptide shows an additional gain of potency in NK1-expressing CHO cells of about three orders of magnitude. Surprisingly, *Lom*-TK-LMa even seems to be somewhat more potent than substance P, the natural NK1-agonist, itself. These observations are also reflected in the EC_{50}-values that were calculated for the NK1-mediated bioluminescence of PAM28-NK1 cells (Table 2).

Substance P, Neurokinin A, *Lom*-TK I and both *Lom*-TK-I-analogs, *Lom*-TK-Ma and *Lom*-TK-LMa, were also tested in a functional assay using another vertebrate neurokinin receptor. When CHO cells co-expressing the human NK2 receptor and apoaequorin were challenged with the Met–NH_2-containing peptides, bioluminescent responses were recorded (Fig. 2). The rank order of potencies for these agonists was: Neurokinin A (=substance K, the natural NK2-agonist) >> substance P > *Lom*-TK-LMa >> *Lom*-

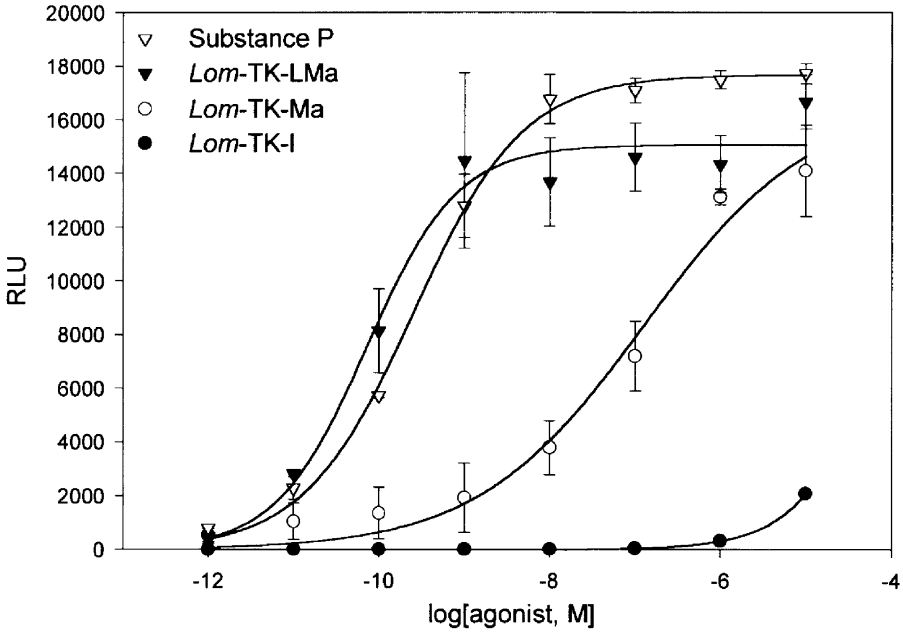

Fig. 1. Dose-response curves of bioluminescent responses induced in CHO-PAM28-NK1 cells by *Lom*-TK-LMa, substance P, *Lom*-TK-Ma and *Lom*-TK I. Data are the average ± SD of N measurements (N = 9 for each point, obtained from three experiments done in triplicate). They are shown in RLU (relative light units).

TK-Ma. This clearly differed from the one obtained for the human NK1 receptor (*Lom*-TK-LMa ≥ substance P >> *Lom*-TK-Ma >>> *Lom*-TK I). No responses were detected when locustatachykinin-1 was applied at concentrations as high as 10^{-5} M to PAM28-NK2 cells (Fig. 2). In addition, both substance P and *Lom*-TK-LMa seemed to behave as partial agonists for the NK2-mediated response. The results shown in Figs. 1 and 2 indicate that the hybrid peptides are better agonists for NK1 than for NK2 receptors, suggesting that they have substance-P-mimetic properties.

For both human NK1 and NK2 receptors, *Lom*-TK-Ma appeared to be more potent than *Lom*-TK I and *Lom*-TK-LMa was still a more potent agonist than *Lom*-TK-Ma. In the functional insect cell-based assay for STKR, this situation is reversed for *Lom*-TK-Ma and *Lom*-TK I. The single amino acid substitution (Arg to Met) resulted in a decrease in agonistic potency of at least 100-fold. The additional replacement of Val into Leu did not lead to a supplementary drop in activity. Interestingly, when substance P was applied at a concentration of 10 μM, a small bioluminescent response was detected in S2-STKR-Aeq cells (Fig. 3). This stimulation could not be observed with Fura-2 fluorescence measurements [9].

3.2. Met (M) to Arg (R) replacement

Substitution of the C-terminal Met–NH_2 (Ma) in substance P by Arg–NH_2 (Ra), gave rise to a peptide with mixed, mammalian and insect tachykinin-like sequence properties (substance P-Ra, see Table 1). Surprisingly, this

Table 2
This table summarises maximum response values (in RLU) and EC_{50} (in nM) values ± SD of natural and synthetic peptides tested on three different tachykinin receptors

Peptides	STKR		NK1		NK2	
	Max. ($\times 10^4$ RLU)	EC_{50} (nM)	Max. ($\times 10^3$ RLU)	EC_{50} (nM)	Max. ($\times 10^3$ RLU)	EC_{50} (nM)
Lom-TK-I	12.2 ± 2	181 ± 32	N.D.	N.D.	N.D.	N.D.
Lom-TK-Ma	N.D.	N.D.	14.1 ± 1.6	110 ± 39	N.D.	N.D.
Lom-TK-LMa	N.D.	N.D.	14.6 ± 1.1	0.091 ± 0.002	7.8 ± 2.8	283 ± 17
Substance P	N.D.	N.D.	17.7 ± 0.4	0.25 ± 0.05	8.2 ± 1.2	25 ± 2
Neurokinin A	N.D.	N.D.	16.5 ± 1.4	2.0 ± 0.1	13.1 ± 1.4	1.1 ± 0.1
Substance P-Ra	9.2 ± 0.5	13 ± 1	15.4 ± 1.0	21 ± 3	N.D.	N.D.
Substance P-VRa	13.3 ± 0.3	4.8 ± 0.1	N.D.	N.D.	N.D.	N.D.

STKR is expressed in S2 cells, NK1 and NK2 are both expressed in CHO cells. All cells coexpress the calcium-sensitive photoprotein apoaequorin and the calcium responses of these peptides were recorded by means of the aequorin assay. RLU: relative light units; N.D.: not determined; Max.: maximal response level.

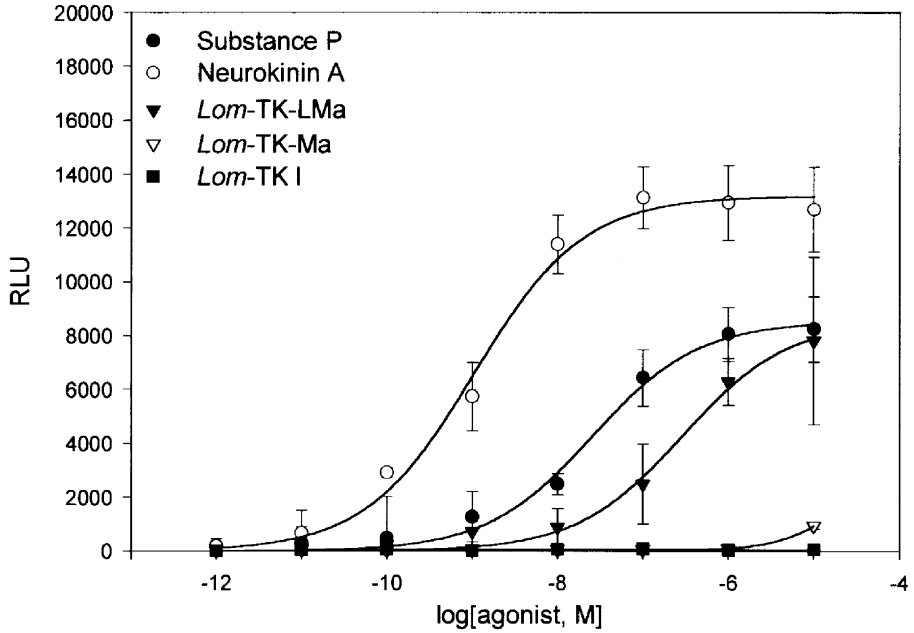

Fig. 2. Dose-response curves of bioluminescent responses induced in CHO-PAM28-NK2 cells by neurokinin A, substance P, *Lom*-TK-LMa and *Lom*-TK-Ma. *Lom*-TK I did not elicit any detectable responses in this functional NK2 receptor assay. Data are the average ± SD of N measurements (N = 9 for each point, obtained from three experiments done in triplicate). They are shown in RLU (relative light units).

hybrid peptide proved to be a more potent agonist for STKR than the insect-derived *Lom*-TK I (Fig. 4). The results indicate that this single amino acid change (Met to Arg) is responsible for a 1,000–10,000-fold increase in potency compared to substance P itself. A supplementary increase in potency, as well as the maximal response, was obtained with substance P-VRa, a peptide that has an additional replacement of the penultimate amino acid residue (Leu to Val). The C-terminal pentapeptide sequence of substance P-VRa is similar to the one of *Lom*-TK I. Nevertheless, substance P-VRa is still about 50-fold more potent than *Lom*-TK I as an STKR-agonist. This is also reflected in

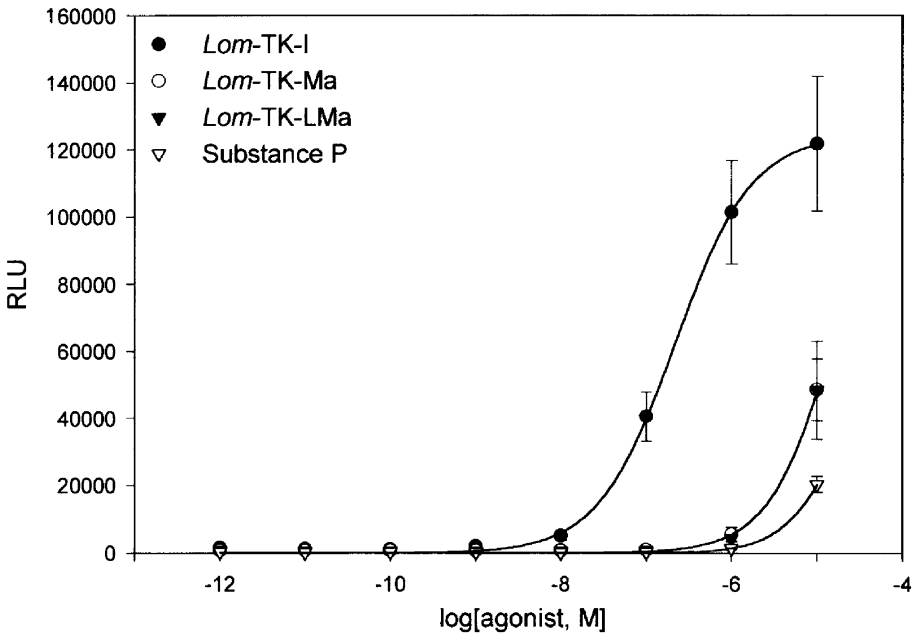

Fig. 3. Dose-response curves of bioluminescent responses induced in S2-STKR-Aeq cells by *Lom*-TK I, *Lom*-TK-Ma, *Lom*-TK-LMa and substance P. Data are the average ± SD of N measurements (N = 10 for each point, obtained from five experiments done in duplicate). They are shown in RLU (relative light units).

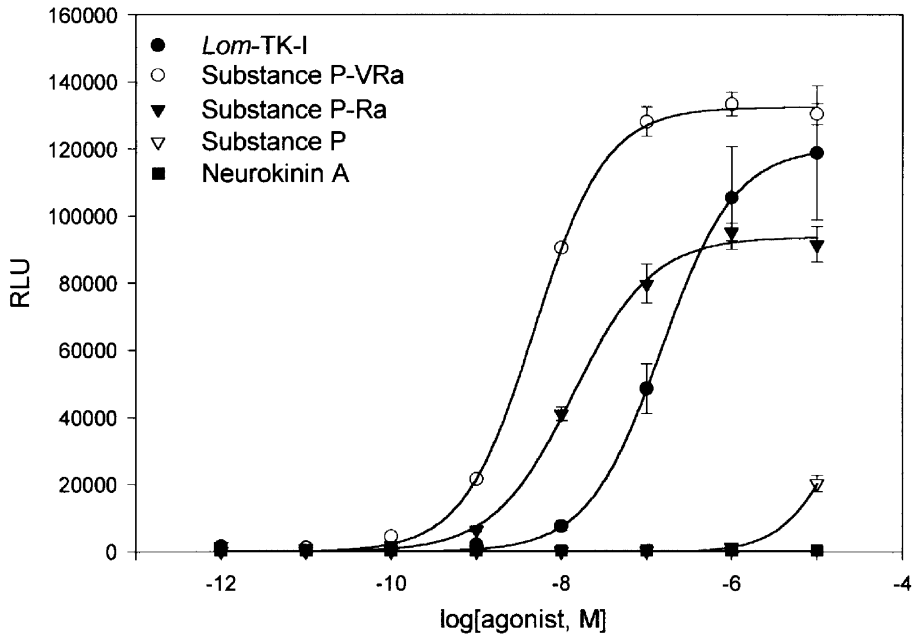

Fig. 4. Dose-response curves of bioluminescent responses induced in S2-STKR-Aeq cells by substance P-VRa, substance P-Ra, *Lom*-TK I and substance P. Neurokinin A did not produce any detectable responses in these cells at concentrations up to 10 μM. Data are the average ± SD of N measurements (N = 10 for each point, obtained from five experiments done in duplicate). They are shown in RLU (relative light units).

the EC_{50}-values of these ligands for the luminescent response of S2-STKR-Aeq cells (see Table 2).

The Met–Arg-substituted analogs of substance P did not show any activity in CHO-PAM28-NK2 cells (Fig. 5). In addition, compared with substance P, these analogs showed severely reduced potencies in the functional assay based on CHO-PAM28-NK1 cells (Fig. 6). Nevertheless, these chimeric peptides are much more potent NK1-agonists than the insectatachykinin *Lom*-TK I. Compared to substance P, the dose-response curve of substance P-Ra displays a shift to the right of about two orders of magnitude (see also EC_{50}-values in Table 2).

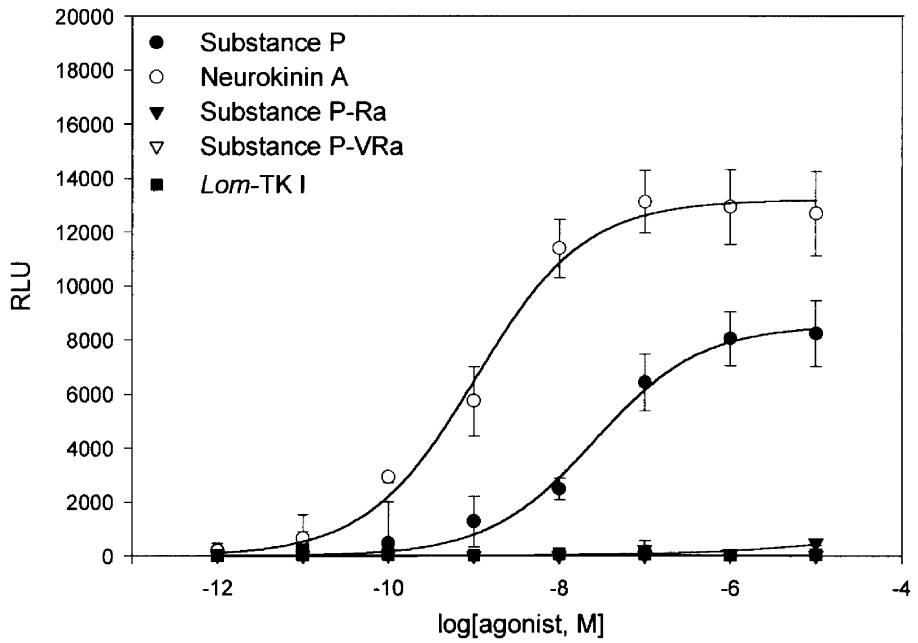

Fig. 5. Dose-response curves of bioluminescent responses induced in CHO-PAM28-NK2 cells by neurokinin A and substance P. Substance P-Ra, substance P-VRa and *Lom*-TK I did not produce any detectable responses in these cells at concentrations up to 10 μM. Data are the average ± SD of N measurements (N = 9 for each point, obtained from three experiments done in triplicate). They are shown in RLU (relative light units).

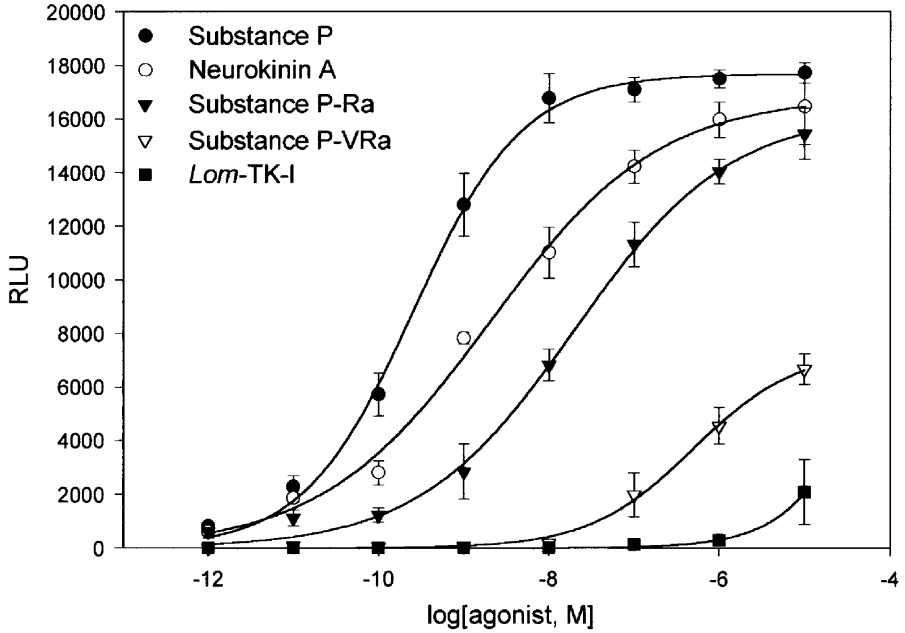

Fig. 6. Dose-response curves of bioluminescent responses induced in CHO-PAM28-NK1 cells by substance P, neurokinin A, substance P-Ra, substance P-VRa and *Lom*-TK I. Data are the average ± SD of N measurements (N = 9 for each point, obtained from three experiments done in triplicate). They are shown in RLU (relative light units).

4. Discussion

The development of fast and sensitive assays that are based on the bioluminescent Ca^{2+}-sensitive reporter protein aequorin [15], provides us with powerful tools to detect receptor-mediated intracellular calcium changes, to perform pharmacological profiling studies [14] and to investigate agonist-dependent structure-activity relationships. Aequorin is a bioluminescent complex of apoaequorin with the luminophore cofactor, coelenterazine. Recombinant expression of apoaequorin in a broad range of cell types greatly expands its use as a sensitive calcium indicator [14–18]. In these cells, reconstitution of aequorin can be obtained by simple addition of coelenterazine to the medium. The cofactor permeates into the cells. In addition, mitochondrially targeted aequorin was shown to generate the most robust receptor-mediated responses [19,20]. The present paper extends the use of aequorin as a reporter for calcium changes towards genetically engineered insect cells (S2-STKR-Aeq). The bioluminescence assay for the *Stomoxys calcitrans* tachykinin-like peptide receptor STKR appears to be more sensitive than the Fura-2-based analysis which was reported earlier [9,10]. Small, but significant, flash light responses were still detectable by means of the aequorin assay at agonist concentrations that did not induce significant changes in Fura-2 fluorescence. For example, the mammalian tachykinin substance P produced a small bioluminescent response in S2-STKR-Aeq cells at 10 μM (Fig. 3), whereas no response could be measured by means of Fura-2. The improved sensitivity of the aequorin assay, compared to Fura-2, is probably based on its high signal-to-noise ratio and/or its lower Ca^{2+}-buffering capacity.

These cell-based aequorin assays for human NK1, human NK2 and insect STKR receptors were employed to study the impact of amino acid changes at the C-terminus of tachykinin-related peptides. The results clearly show that peptide agonists for NK1 and NK2 appeared to be very sensitive to both changes that were introduced in their C-terminus (LMa to LRa, LRa to VRa). None of the peptides carrying an Arg–NH_2 (Ra) end produced any detectable responses in CHO-PAM28-NK2 cells. Nevertheless, these hybrid substance P analogs still displayed considerable, but reduced activity in CHO-PAM28-NK1 cells. In this NK1-assay, the LRa-peptide was much more potent than the VRa-peptide, despite the fact that both Leu and Val are hydrophobic amino acids with similar physicochemical properties. The observation that both amino acid positions are extremely important for agonist activity is consistent with the fact that in vertebrate tachykinins these amino acid residues have been strictly conserved, suggesting the existence of a strong selective pressure on vertebrate preprotachykinin genes to preserve this active ('FXG**LM**a') configuration during evolution. Therefore, it is not surprising that the insectatachykinin analogs *Lom*-TK-Ma and, especially, *Lom*-TK-LMa showed increased activity in the NK1 and NK2 receptor assays. Interestingly, *Lom*-TK-LMa appeared to be a very potent NK1-agonist. It was even somewhat more potent than the physiological NK1-agonist, substance P, itself. In addition, both substance P and *Lom*-TK-LMa displayed partial agonism in CHO-PAM28-NK2 cells. These results suggest that *Lom*-TK-LMa is more a substance-P-mimetic than a neurokinin-A-mimetic.

The presence of the C-terminal Arg–NH_2 (Ra) in insectatachykinin agonists, such as *Lom*-TK I, appeared to be

very important for their activity in S2-STKR cells. For STKR-agonism, the impact of a conservative substitution of the penultimate residue (LRa to VRa or VMa to LMa) appeared to be less drastic than for NK1- and NK2-agonism. The observation that the penultimate amino acid position is less sensitive to changes in insectatachykinin agonists is fully in line with the fact that in the naturally occurring peptides this residue is not highly conserved ('$FX_1G\underline{\mathbf{X_2R}}a$', X_2 = V, M, T, L, S or A). Interestingly, substance P analogs containing a C-terminal Arg–NH_2 residue clearly displayed highly increased potencies in S2-STKR-Aeq cells. As insectatachykinin-mimetics, they were even more potent than *Lom*-TK I.

The observed Arg–Met preference is a major pharmacological and evolutionary difference between insect (STKR) and human (NK1, NK2) neurokinin receptors. This study also shows that a single amino acid substitution can turn an STKR-agonist into an NK-agonist and *vice versa*, even though the rest of the molecule remains the same. Interestingly, this Arg–Met change can theoretically be obtained by means of a single point mutation in the nucleotide sequence. Together with the structural information obtained from restricted-conformation analogs which indicates a similar active conformation of insect and mammalian tachykinin-like peptides [4], these data are consistent with a distant evolutionary relationship between FXGLMa and FX_1GX_2Ra peptides, as well as between their respective receptors.

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References

[1] Erspamer V. The tachykinin peptide family. Trends Neurosci 1981;4:267–73.
[2] Maggio JE. Tachykinins. Ann Rev Neurosci 1988;11:13–28.
[3] Khawaja AM, Rogers DF. Tachykinins: receptor to effector. Int J Biochem Cell Biol 1996;28:721–38.
[4] Nachman RJ, Moyna G, Williams HJ, Zabrocki J, Zadina JE, Coast GM, Vanden Broeck J. Comparison of active conformations of the insectatachykinin/tachykinin and insect kinin/Tyr-W-MIF-1 neuropeptide family pairs. Ann NY Acad Sci 1999;897:388–400.
[5] Nässel DR. Tachykinin-related peptides in invertebrates: a review. Peptides 1999;20:141–58.
[6] Vanden Broeck J, Torfs H, Poels J, Van Poyer W, Swinnen E, Ferket K, De Loof A. Tachykinin-like peptides and their receptors a review. Ann NY Acad Sci 1999;897:374–87.
[7] Nakanishi S. Mammalian tachykinin receptors. Annu Rev Neurosci 1991;14:123–36.
[8] Monnier D, Colas JF, Rosay P, Hen R, Borrelli E, Maroteaux L. NKD a developmentally regulated tachykinin receptor in *Drosophila*. J Biol Chem 1992;267:1298–302.
[9] Torfs H, Shariatmadari R, Guerrero F, Parmentier M, Poels J, Van Poyer W, Swinnen E, De Loof A, Åkerman K, Vanden Broeck J. Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line. J Neurochem 2000;74:2182–9.
[10] Torfs H, Oonk HB, Vanden Broeck J, Poels J, Van Poyer W, De Loof A, Guerrero F, Meloen RH, Åkerman K, Nachman RJ. Pharmacological characterization of STKR an insect G protein-coupled receptor for tachykinin-like peptides. Arch Insect Biochem Physiol 2001;48:39–49.
[11] Vulsteke V, Janssen I, Vanden Broeck J, De Loof A, Huybrechts R. Baculovirus immediate early gene promoter based expression vectors for transient and stable transformation of insect cells. Insect Mol Biol 1993;2:195–204.
[12] Vanden Broeck J, Vulsteke V, Huybrechts R, De Loof A. Characterisation of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. J Neurochem 1995;64:2387–95.
[13] Schneider I. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. J Embryol Exp Morphol 1972;27:353–65.
[14] Detheux M, Standker L, Vakili J, Munch J, Forssmann U, Adermann K, Vassart G, Kirchhoff F, Parmentier M, Forssmann WG. Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor CCR1 and CCR5 agonist with anti-HIV properties. J Exp Med 2000;192:1501–8.
[15] Prasher D, McCann RO, Cormier MJ. Cloning and expression of the cDNA coding for aequorin. Proc Natl Acad Sci USA 1985;82:3154–8.
[16] Tanahashi H, Takashi I, Inouye S, Tsuji FI, Sakaki Y. Photoprotein aequorin: use as a reporter enzyme in studying gene expression in mammalian cells. Gene 1990;96:249–55.
[17] Button D, Brownstein M. Aequorin-expressing mammalian cell lines used to report Ca^{2+} mobilization. Cell Calcium 1993;14:663–71.
[18] Ungrin MD, Singh LMR, Stocco R, Sas DE, Abramovitz M. An automated aequorin luminescence-based functional calcium assay for G protein-coupled receptors. Anal Biochem 1999;272:34–42.
[19] Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M, Rees S. A bioluminescent assay for agonist activity at potentially any G protein-coupled receptor. Anal Biochem 1997;252:115–26.
[20] Stables J, Mattheakis LC, Chang R, Rees S. Recombinant aequorin as reporter of changes in intracellular calcium in mammalian cells. Meth Enzymol 2000;327:456–71.