Pharmacology of stomoxytachykinin receptor depends on second messenger system

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\textbf{Abstract}

STKR is a neurokinin receptor derived from the stable fly, \textit{Stomoxys calcitrans}. Insect tachykinin-related peptides, also referred to as “insecttachykinins”, produce dose-dependent calcium and cyclic AMP responses in cultured \textit{Drosophila melanogaster} Schneider 2 (S2) cells that were stably transfected with the cloned STKR cDNA. Pronounced differences in pharmacology were observed between agonist-induced calcium and cyclic AMP responses. The results indicate that the pharmacological properties of STKR depend on its coupling to a unique second messenger system. Therefore, a model postulating the existence of multiple active receptor conformations is proposed. This article presents the first evidence that an insect peptide receptor with dual coupling properties to second messenger systems can display agonist-dependent functional differences.

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1. Introduction

Neurokinins or tachykinins are multifunctional brain/gut peptides. They play an important neuromodulatory role in the central nervous system and exhibit a broad range of peripheral activities [7,17,21]. Tachykinin-like peptides have been discovered in both vertebrate and invertebrate species [24,36,37]. Based on their respective sequence characteristics, these peptides can be separated into two distinct groups. All known vertebrate, and a few invertebrate tachykinins share a common C-terminal sequence motif, -FXGLMa. The “insecttachykinins” (also referred to as “tachykinin-related peptides, TRP”), which have now been identified in a large number of protostomian invertebrates (arthropods, mollusks, echiurid worms), display structural similarity to the first group and possess a C-terminal—GFX\textsubscript{1}GX\textsubscript{2}Ra consensus sequence [23,24,40,46]. Peptides belonging to this group have not been found in vertebrates.

Insect \textit{G} protein-coupled receptors, which display pronounced sequence homology with mammalian neurokinin receptors (NK-1-3) have been shown to be activated by tachykinin-like peptide agonists [19,22,44]. One of these receptors, STKR, which was cloned from the stable fly, \textit{Stomoxys calcitrans} [13], is the subject of our present study. Previously, we reported on STKR’s functional expression in a stably transfected insect cell clone (S2-STKR cells) and showed that insect tachykinin-related peptides (or

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"insectatachyklinins"), such as *Lom-TK* III, produced dose-dependent calcium and cyclic AMP responses in these cells [44]. In this paper, we provide the first evidence showing that the pharmacological properties of this insect peptide G protein-coupled receptor differ depending on the effector system that is monitored in response to agonist stimulation.

2. Methods

2.1. Cell culture

Insect S2-cells (Schneider 2 cells derived from *Drosophila melanogaster* embryos) [34] and stably transfected S2-STKR cells (S2 cells that permanently express STKR; [44]) were maintained in Schneider’s medium (Serva) with addition of CaCl$_2$ (0.6 g/l) and NaHCO$_3$ (0.4 g/l), 10% heat-inactivated fetal calf serum (Gibco-BRL Life Technologies) and antibiotics (50 U/ml penicillin G and 50 µg/ml streptomycin, Sigma). The pH of the medium was adjusted to 6.45 by adding NaOH. The cells were grown in monolayers at 22 °C.

2.2. Peptides

The insectatachyklinin agonists (*Lom-TK* II, *Lom-TK* III, *Anc-TK* and *Stc-TK*) were synthesized using Fmoc polyamide chemistry. The purity of the synthetic peptide preparations was monitored by MALDI-TOF-MS. *Stc-TK* is a peptide that was recently purified and sequenced from the stable fly, *Stomoxys calcitrans* [23]. This peptide contains an Ala residue instead of the conserved Gly residue in the C-terminal consensus sequence. The sequences of the peptides that were employed in this paper are displayed in Table 1.

2.3. Loading of cells with fura-2

The fluorescent Ca$^{2+}$-indicator fura-2-AM was employed to monitor changes in intracellular calcium concentration [11]. Cells were resuspended in Elliot insect buffer (129.7 mM NaCl, 5.44 mM KCl, 1.2 mM MgCl$_2$, 6H$_2$O, 4.2 mM NaHCO$_3$, 7.3 mM Na$_2$HPO$_4$, 20 mM Hepes, 63 mM saccharose; pH 6.2) supplemented with 1 mM CaCl$_2$, 4 µg/ml fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA), 10 mM glucose and 1 mM probenecid and incubated in the dark for 30 min at room temperature (22 °C). After this treatment, the cells loaded with fura-2 were rinsed twice in Elliot insect buffer containing 1 mM CaCl$_2$, 10 mM glucose and 1 mM probenecid, and then transferred into a stirred quartz cuvette. This was put in the thermoregulated cuvette-holder (at 22 °C) of a spectrophotometer (Hitachi F4000, Hitachi F2000 and PTI were used). Fluorescence was monitored at 505 nm (emission) using an excitation wavelength of 340 nm. No absorbance or fluorescence artefacts were observed with any of the compounds used. Each experiment was calibrated by treatment of the cells with 60 µg/ml digitonin (Merck, Darmstadt, Germany) to determine the maximum fluorescence value ($F_{\text{max}}$), followed by 10 mM EGTA (ethylene glycol-bis N,N,N′,N′-tetra-acetic acid; Sigma, St. Louis, MO, USA) to measure the minimum fluorescence value ($F_{\text{min}}$). The free calcium concentration was calculated from the fluorescence ($F$) data by means of the equation, $[\text{Ca}^{2+}] = (F - F_{\text{min}}) / (F_{\text{max}} - F) \times K_d$, in which the extracellular fura-2 fluorescence was subtracted from the $F$ values [15]. The $K_d$ was calculated according to Shuttleworth and Thompson [39]. Data for the dose–response curves ($n \geq 8$ for each data point) were processed with the Excel (Microsoft) computer program.

2.5. Cyclic AMP assay

To monitor cyclic AMP levels, receptor-expressing S2-STKR and control S2 cells were incubated for 30 min in Elliot insect buffer containing 200 µM 3-isobutyl-1-methylxanthine (IBMX) and the peptide agonist. The reaction was stopped by adding two volumes of ice-cold 100% ethanol. The supernatants were collected in polypropylene tubes, centrifuged to remove cell debris and evaporated by vacuum centrifugation. Each sample was redissolved in 250 µl of assay buffer and cyclic AMP concentrations were determined according to the SPA (scintillation proximity assay) protocol provided by the manufacturer (Amersham Pharmacia biotech, UK). Data ($n \geq 3$) were processed using the Excel (Microsoft) program.

3. Results

3.1. Calcium measurements and dose–response curves

The dose–response curves for the calcium signals that were generated in receptor-expressing S2-STKR cells by four
different insect tachykinin-related peptide agonists; Lam-TK II, Lam-TK III, Ste-TK, and Anc-TK, are displayed in Fig. 1. The sequences of these four peptides are compared in Table 1. The average Ca^{2+}-concentration in resting cells prior to addition of agonist was 81 ± 3 nM. An increase in intracellular calcium was obtained with each of the four peptides (in the range of 10^{-8} to 10^{-5} M). At 1 μM Lam-TK II or Lam-TK III, a maximal increase of intracellular calcium ion concentration (142 ± 13 nM above the basal level) was obtained. However, when S2-STKR cells were challenged with Stomoxys calcitrans tachykinin-like peptide, Ste-TK, or with the molluscan peptide, Anc-TK, the maximal Ca^{2+} stimulation (63 ± 12 nM) reached only half the level found with Lam-TK II and Lam-TK III. These results indicate that Ste-TK and Anc-TK behave as partial agonists for this receptor. Despite the lower maximal response, Ste-TK and Anc-TK activity was already detectable at concentrations of 10 nM. Non-transfected Schneider 2 cells (S2) did not show any calcium responses to insect tachykinin-like peptides.

3.2. Cyclic AMP measurements and dose–response curves

In addition to the calcium response, Lam-TK III [44] also induces a dose-dependent increase in the accumulation of cyclic AMP in IBMX-treated S2-STKR cells (IBMX: 3-isobutyl-1-methylxanthine). When compared to the increase in intracellular Ca^{2+}, the rise in cyclic AMP required a 10-fold higher concentration of peptide. The dose–response curves for cyclic AMP accumulation induced in S2-STKR cells by four insect tachykinin agonists are shown in Fig. 2. Lam-TK III and Ste-TK behave as full agonists for the cyclic AMP effect (Fig. 3A and B). S2-STKR cells that were challenged with Lam-TK II and Anc-TK did not attain the maximum level of cyclic AMP production obtained with these agonists. Non-transfected S2 cells showed no significant change in cyclic AMP accumulation. The average basal level of cyclic AMP production in S2 cells and in non-induced S2-STKR cells was 5.1 ± 0.3 pmol/10^6 cells.

4. Discussion

The present study shows that S2-STKR, a clonal insect cell line expressing a cloned insect neurokinin receptor (STKR; [44]), differentially responds to four agonists belonging to the insect tachykinin group of peptides. The pronounced differences in pharmacology that were observed between STKR-induced calcium and cyclic AMP responses suggest that receptor properties strongly depend on the cellular effector system to which this receptor is coupled. The fact that these agonist-induced responses of STKR were monitored in an insect-derived cell line increases the probability that the natural coupling mechanisms of this insect receptor are mimicked. In this respect, the obtained results may be relevant to in vivo situations. In addition, both coupling efficacy and selectivity are affected by the nature of the agonist bound to this receptor. These results can be explained by a hypothetical model that proposes the existence of multiple receptor conformations (Fig. 4).

This model more generally applies to other G protein-coupled receptors (GPCRs). Despite the enormous diversity of ligands and receptor types, all G protein-coupled receptors are believed to share a remarkably uniform, heptahelical (or 7 TM) structure [3,8,29,45]. Agonist binding is thought to stabilize an active receptor conformation that selectively interacts with a given heterotrimeric G protein, consisting of specific α-, β- and γ-subunits, to form a ternary complex (of agonist, receptor and G-protein). This promotes the
exchange of GTP for GDP on the Gα-subunit [2,4,42], which results in dissociation of Gα-GTP and Gβγ from the receptor. Both subunits then regulate the activity of cellular effector proteins, such as enzymes or ion channels, until they reassociate upon hydrolysis of GTP to GDP by Gα (GTPase activity). Currently, high-resolution structural data of active GPCR conformations are not available. Therefore, the exact molecular mechanisms of receptor activation and G protein coupling remain poorly defined [12,18,35,47,48]. It is very well documented, however, that limited changes in the primary structure of a receptor, even at positions that are not directly involved in G protein interaction, can have a drastic impact on the selectivity and efficacy of the coupling process [1,5,6,10,26,27,32,41,43]. Moreover, various data from the literature support the notion that G protein-coupled receptors can have different active conformations. This hypothesis is particularly well documented for receptors displaying dual coupling characteristics. Several GPCRs, mainly for amine agonists, have previously been shown to couple to distinct second messenger systems in an agonist-dependent manner [14,30,31]. Moreover, there are also several studies that suggest the occurrence of different mammalian neurokinin receptor states. Competitive binding and functional analyses have already led to a ‘dual state’ model for the NK1 receptor [20]. The existence of at least two distinct pharmacological states of the NK1 receptor has been further substantiated by

Fig. 3. Superimposed dose–response curves for the cyclic AMP (cAMP, dashed lines, right Y-axis scale) and calcium effects (Ca, full lines, left Y-axis scale) generated in S2-STKR cells by each of the tachykinin-related peptides. Each peptide analog has a different concentration–activity profile. (A) Lom-TK II is a full agonist for both responses, but it preferentially activates the PLC/Ca2+ pathway. (B) Ste-TK is a partial agonist for the PLC/Ca2+ pathway and it preferentially activates the AC/cAMP pathway. In addition, (C) Lom-TK II is a partial agonist for the AC/cAMP pathway, whereas (D) Anc-TK is a partial agonist for both pathways. This study demonstrates that differences in the primary structure of peptide analogs can cause important changes in the signaling properties of an insect G proteina-coupled receptor (STKR).

Fig. 4. This figure illustrates the hypothetical model explaining how a single receptor (R) can adopt multiple active conformations (R1, R2) depending on its interaction with distinct G proteins (G1, G2), each coupled to a different effector system (E1, E2). The activation of a receptor by different agonists (A1, A2) can thus lead to the generation of distinct agonist-specific responses in the same cell clone. The model presented here shows a simplified situation (of dual coupling) in which two ‘active’ receptor conformations, two G proteins and two effectors are involved.
mutant analysis and G protein fusion experiments [16]. Interestingly, two distinct molecular phenotypes of the NK.1 receptor, each displaying mono-component agonist binding curves, were obtained by forcing this receptor to interact with either Go<sub>a</sub> or Go<sub>q</sub> in C-terminally adapted fusion constructs. Therefore, the heterogenous nature of the wildtype NK1 receptor is probably based on the existence of at least two active conformations or molecular phenotypes representing functionally different complexes including distinct G proteins. The NK.1 receptor indeed displays different pharmacological properties depending on the second messenger pathway. As a consequence, some full agonists of the NK.1-induced phospholipase C (PLC) pathway can serve as partial agonists or even antagonists for the adenyl cyclase pathway which is affected by the same receptor, or vice versa [33]. The existence of multiple conformational states, with distinct pharmacological and signaling properties, has also been reported for the NK2 receptor [25]. Our present study with the stromatoxytachykinin receptor is in line with these reports on mammalian neurokinin receptors that also appear to display dual coupling characteristics resulting in different pharmacological phenotypes. This evolutionary conserved principle may refer to a very fundamental aspect of tachykinin-related peptide signaling.

The structural requirements for an agonist to elicit the PLC-dependent calcium rise in S2-STKR cells differ from the ones needed to induce cyclic AMP stimulation in the same clonal cell line. Interestingly, Stc-TK and Anc-TK, the peptides that show partial agonism for the calcium response, both contain an Ala-residue instead of the Gly-residue that is conserved in all other tachykinin-like peptides. They are the only natural tachykinin-related peptides that are known to have this characteristic. By analyzing synthetic analogs via a very sensitive aequorin-based Ca<sup>2+</sup>-assay, we have recently shown that this Gly/Ala exchange is fully responsible for partial STKR-agonism [28]. However, this amino acid change appears to be much less critical for the cyclic AMP response, indicating that the pharmacology of both responses is certainly not identical. Stc-TK was recently purified from *Stomoxys calcitrans* [23], the insect species from which STKR was cloned. This does not necessarily mean that it is the only possible natural agonist for STKR. Most insect species appear to contain multiple tachykinin-related peptides [24,38,46]. The *Drosophila* genome contains at least five different insect-tachykinins [40], whereas only two possible receptors were described [19,22]. Does this multiplicity of peptide variants lead only to an increased functional redundancy? Based on the proposed multiple conformation model, a single receptor can mediate different in vivo effects via distinct signaling pathways, not only depending on the cell-type or stage in which it is expressed, but also depending on the natural peptide isoform(s) that is (are) available for binding. Therefore, the natural, intra-species diversity of the insect-tachykinins may have a physiological basis. In general, such a receptor model may also have important therapeutic consequences (e.g. many human GPCRs are important drug targets), since it should be possible to find receptor ligands that act more specifically upon a given signaling pathway.

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