Pharmacological Characterization of a Tyramine Receptor from the Southern Cattle Tick, Rhipicephalus (Boophilus) microplus

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Pharmacological characterization of a tyramine receptor from the southern cattle tick, *Rhipicephalus (Boophilus) microplus*

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**Abstract**

The southern cattle tick (*Rhipicephalus (Boophilus) microplus*) is a hematophagous external parasite that vectorsthe causative agents of bovine babesiosis or cattle tick fever, *Babesia bovis* and *B. bigemina*, and anaplasmosis, *Anaplasma marginale*. The southern cattle tick is a threat to the livestock industry in many locations throughout the world. Control methods include the use of chemical acaricides including amitraz, a formamidine insecticide, which is proposed to activate octopamine receptors. Previous studies have identified a putative octopamine receptor from the southern cattle tick in Australia and the Americas. Furthermore, this putative octopamine receptor could play a role in acaricide resistance to amitraz. Recently, sequence data indicated that this putative octopamine receptor is probably a type-1 tyramine receptor (TAR1). In this study, the putative TAR1 was heterologously expressed in Chinese hamster ovary (CHO-K1) cells, and the expressed receptor resulted in a 39-fold higher potency for tyramine compared to octopamine. Furthermore, the expressed receptor was strongly antagonized by yohimbine and cyproheptadine, and mildly antagonized by mianserin and phentolamine. Tolazoline and naphazoline had agonistic or modulatory activity against the expressed receptor, as did the amitraz metabolite, BTS-27271; however, this was only observed in the presence of tyramine. The southern cattle tick's tyramine receptor may serve as a target for the development of anti-parasitic compounds, in addition to being a likely target of formamidine insecticides.

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1. Introduction

The southern cattle tick (*Rhipicephalus (Boophilus) microplus*) is an ectoparasite that can have an economically devastating effect on the cattle industry worldwide (Grisi et al., 2014). *R. microplus*, in the United States, was estimated to have direct costs of more than $130 million to the cattle industry in 1906, which has been estimated to be over $3 billion today (USDA-APHIS August 2010). The significant economic impact of this tick is realized with the diseases that it is capable of vectoring. Specifically, the southern cattle tick is capable of vectoring the apicomplexan protozoa *Babesia bovis* and *B. bigemina*, which cause bovine babesiosis, and the rickettsia *Anaplasma marginale* that causes anaplasmosis (Pérez de León et al., 2012; Pérez de León et al., 2014).

The United States initiated the Cattle Fever Tick Eradication Program (CFTEP) in 1906, which eliminated the southern cattle tick (*R. microplus*) and the cattle fever tick (*R. annulatus*) from thirteen southern states by 1941 (Pérez de León et al., 2012). The mainstay of the CFTEP is the use of chemical acaricides; specifically, the organophosphate coumaphos. However, internationally several chemical acaricides, with different mechanisms of action, have been used to control the southern cattle tick, and has predictably resulted in acaricide resistance. Acaricide resistance, particularly with tick strains found in the Americas, has been reported for several chemical classes including organophosphates (Miller et al., 2005; Rodríguez-Vivas et al., 2007; Temeyer et al., 2007; Miller et al., 2008; Guerrero et al., 2012), synthetic pyrethroids (Li et al., 2007; Miller et al., 2007a;...
Cossio-Bayugar et al., 2008; Guerrero and Nene, 2008; Rosario-Cruz et al., 2009) and formamidines (Li et al., 2007; Miller et al., 2007b; Corley et al., 2013). Recently, a population of R. microplus of concern has been discovered in Mexico. This population is resistant to synthetic pyrethroids, organophosphates, amitraz (formamidine) and ivermectin (macrocyclic lactone family) (Fernandez-Salas et al., 2012). The permanent quarantine zone in south Texas is the portion of the region effort by the CFTEP to the U.S. free of cattle fever ticks and bovine babesiosis. As R. microplus is endemic to Mexico, there is the possibility of regular re-introduction of the southern cattle tick into the U.S. Re-introduction is possible with the movement of cattle into the U.S. from Mexico and the ability of the tick to be maintained on wild ungulates. For continued success of the CFTEP, it is imperative that integrative pest control measures are taken, that new biochemical targets for tick control are identified and a greater understanding of the mechanism(s) of acaricide resistance (Temeyer et al., 2012; Pérez de León et al., 2012; Guerrero et al., 2014).

Octopamine and tyramine are biogenic amines found in a many of invertebrates, including insects and ticks, and have been shown to be involved in different physiological functions of these species. Octopamine and tyramine are characterized as neurotransmitters, neurotransmitters and neuromodulators (Roeder et al., 2003; Roeder, 2005; Farooqui, 2012; Ohta and Ozoe, 2014). While the diverse physiological functions of octopamine have been realized for some time, we are only starting to understand the physiological relevance of tyramine; this is because, until recently, tyramine was thought to only be a synthetic precursor to octopamine (Guerrero and Dowd, 2010; Roeder et al., 2003; Roeder, 2005; Verllinden et al., 2010; Farooqui, 2012). Octopamine receptors are believed to be the target of formamidine insecticides/acaricides, which include chlordimeform (2,4-Dimethylphenyl)-N-methylformamidine, N-Methyl-N-2,4-xylylformamidine) Pestanal® (Fluka), naphazoline hydrochloride, 2-benzylhidazolinate (tolazoline).

2.2. Putative tyramine receptor

The wild-type putative tyramine receptor (EF490687.1) was isolated from the Gonzalez strain of tick (amitraz-susceptible). The Gonzalez strain of R. microplus was originally identified in Zapata County, Texas in 1984, and has been maintained in culture by the USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL), Kerrville, TX at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, TX (Chen et al., 2007). The receptor cDNA was synthesized by GeneScript USA Inc. (Piscataway, NJ) and cloned into a pCDNA3.1(−) expression vector (Life Technologies).

2.3. CHO cell culture, transfection and calcium mobilization assay

Cell culture materials were obtained from Life Technologies, unless otherwise stated. Chinese hamster ovary–K1 (CHO or CHO-K1) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). CHO cells were maintained in a NuAire humidified water jacket incubator (Plymouth, MN); the incubator was maintained at normal cell growth conditions of 37 °C, 90% humidity and 5% CO2. The normal cell growth medium consisted of 1X Ham’s F12K (Kaighn’s modification) medium supplement with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) plus 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO). CHO cells were subcultured when cell confluence reached 80%–90%. To subculture, cells were washed with Dulbecco’s Phosphate Buffered Saline (DPBS); then cells were detached from culturing flasks with 0.25% trypsin – 0.53 mM EDTA (trypsin-EDTA) solution. Cells were then centrifuged at 900 × g for 5 min at room temperature; cell pellet was resuspended in fresh normal cell growth media.

Transient transfection of CHO cells with the putative tyramine receptor was performed in 6-well cell culture plates (Corning). Cells were transfected using Lipofectamine 2000® in OPTI-MEM supplement with F12K + 10% FBS, according to the manufacturer’s protocols (Invitrogen). Cells were transfected with 2.5 μg of vector DNA, which allowed the examination of the putative tyramine receptor’s ability to link to the endogenous calcium pathway (Gαq). CHO cells were also transfected with individual G-protein chimeras (Gαq,Gβ, Gαq,Gβ, Gαq, or Gαq) to force receptor-mediated signaling down the calcium-signaling pathway as previously described (Conklin et al., 1998; Omar et al., 2007). CHO cells were incubated in the transfection medium for 12 h at normal cell growth conditions. After 12 h, cells were lifted from 6-well plate using trypsin-EDTA. Cells were washed with normal growth medium and moved to a
15-mL conical tube, and were centrifuged at 900 × g at room temperature for 5 min. The cell pellet was resuspended in 3–3.5 mL of normal cell growth medium. CHO cells were counted using a Countess® Automatic Cell Counter (Life Technologies), according to the manufacturer’s protocols. CHO cells were placed into black-wall clear-bottom 96-well plates (Corning) at a cell density of 27,500 cells per well, with a total volume of 100 μL. Cells were incubated at normal cell culture conditions for 24 h prior to performing the calcium mobilization assay. The calcium mobilization assay was performed using a Fluoro-4 NW Calcium Assay Kit (Life Technologies), according to the manufacturer’s protocol. Briefly, the cells were incubated in HBSS, 20 mM HEPES, a calcium-sensitive fluorescent dye (Fluo-4) and 2.5 mM probenecid; cells were incubated in this medium for 90 min at 37 °C prior to the start of the assay. Fluorescence was monitored using a FlexStation 1 (Molecular Devices, Sunnyvale, CA). Fluorescence changes were monitored with excitation at 494 nm and emission at 516 nm. Basal fluorescence was monitored for 20 s prior to the addition of screening compounds. The fluorescence readings were measured every 1.5 s for 120 s. Stable transfection was performed using the transient transfection procedure. However, instead of proceeding to the functional assay, cells were maintained in medium containing 100 μg/mL of Geneticin® (G418 sulfate; Corning). Single-cell colonies were selected in 96-well plates over four weeks. Successfully transfected cells, with both the putative tyramine receptor and G-protein chimera, were determined with a calcium mobilization assay and by RT-PCR.

Compounds were screened using an agonist and/or an antagonist screening method. The agonist screening method was utilized with the addition of the potential agonist added by the FlexStation after a 20-second background reading. Antagonist or modulator screening was performed by preincubating the putative antagonist or modulator with the cells in the black-walled clear-bottom 96-well plate for 30 min prior to the addition of tyramine (addition performed by the FlexStation).

2.4. Data analysis

A minimum of six wells per plate along with a minimum of four plates was analyzed for each treatment. Additionally, each plate contained several controls: a blank (calcium fluorescent indicator, probenecid (2.5 mM) and HBSS with 10 mM HEPES buffer), a vehicle control, along with an autofluorescent control (fluorescent indicator (Fluo-4), HBSS with 10 mM HEPES and the ligand). Additionally, non-receptor mediated effects were monitored for each treatment and consisted of untransfected cells not expressing the receptor (regular CHO cells). Concentration-response curves were analyzed in GraphPad Prism (La Jolla, CA). One-way ANOVA and a paired t-test were used to test the data for statistically significant differences of agonist and antagonist compounds using SAS 9.3 (Cary, NC). Student-Newman-Keuls (SNK) post-hoc analysis was used to determine significant differences between treatments with 95% confidence (α = 0.05). Antagonist and modulator screening results were normalized to 150 nM tyramine (100%), which is represented as a dashed line in the appropriate figures.

3. Results

The putative tyramine receptor was successfully expressed in Chinese Hamster Ovary (CHO) cells, and functional receptor activation was determined using a calcium-liberation assay. The calcium-liberation assay analyzes receptor activation via the Gq phospholipase-C (PLC) pathway. Receptor activation results in an increase of cytosolic calcium, which can be quantified using a calcium-sensitive fluorescent dye. However, not all receptors will signal using the Gq, PLC pathway; therefore, a set of Gq–protein chimera clones, which funnel receptor activation down the Gq PLC pathway resulting in an increase of intracellular calcium, were used (Fig. 1). These G-protein chimera were developed to have the receptor-interacting domain (5 amino acids) from different G-proteins (Gαs, Gαz, and Gαq), built on the Gq backbone to make a hybrid calcium-sensitive signaling molecule designated as Gq(5), Gq(5), Gq(5), and Gq(5). Therefore, heterologous expression of a receptor with the Gq, chimera allows for quantification of receptor activation using a fluorescence output (Fig. 1).

The R. microplus putative tyramine receptor was transiently expressed in CHO cells alone to see if the receptor activated the endogenous Gq, PLC pathway (Fig. 1). Expression of the receptor alone resulted in a weak fluorescent response to tyramine and octopamine at 1 μM (Fig. 1). Transient expression was performed with the putative tyramine receptor in combination with individual Gq chimeras (Gq(5), Gq(5), Gq(5), and Gq(5)), as shown in Fig. 1. Co-expression of the putative tyramine receptor resulted in the most significant response when co-expressed with the Gq(5) G-protein chimera (Fig. 1). Additionally, 1 μM tyramine had a stronger and more significant fluorescent response when compared to 1 μM octopamine, which indicated that tyramine preferentially activated the heterologously expressed receptor with the Gq(5) G-protein chimera. Tyramine and octopamine displayed concentration-dependent responses in CHO cells transiently expressing the receptor along with the Gq(5) chimeric protein (Fig. 2). Tyramine had an EC50 of 9 nM, whereas octopamine had an EC50 of 351 nM; therefore, tyramine is 39-fold more effective at activating the heterologously expressed receptor with the Gq(5) chimera, compared to octopamine (Fig. 2 and Table 1). Additional biogenic amines were screened at 1 μM to ensure that tyramine is the preferred ligand. Tyramine activated the receptor most significantly, followed by octopamine. Acetylcholine, along with the N-methylated analogue of octopamine, synephrine, were capable of mildly activating the expressed receptor and Gq(5) Chimeras, but not as significantly as tyramine or octopamine (Fig. 3). Dopamine, 5-HT (serotonin), norepinephrine, epinephrine and histamine had little effect against the expressed receptor and chimera at 1 μM (Fig. 3). These results indicated that the originally classified
different letters indicate a significant difference.

**Fig. 2.** Concentration-dependent response of transiently expressed RmTAR1 plus Gαq(5). Concentration-dependent response of the expressed coding sequence of the *R. microplus* tyramine receptor and the Gαq(5) G-protein chimera with several concentrations of tyramine (solid black line) and octopamine (solid gray line). These two biogenic amines were used to monitor the increase of intracellular calcium, using a calcium-sensitive fluorescent dye. Data analyzed using GraphPad Prism EC50 or EC90.

**Fig. 3.** Effect of biogenic amines against RmTAR1. Several biogenic amines were examined to determine if they activated the expressed receptor and increased the intracellular concentration of calcium by using a calcium-sensitive fluorescent dye that was measured in relative fluorescence units (RFU). Two-way ANOVA (α = 0.05) different letters indicate a significant difference.

**Table 1**

<table>
<thead>
<tr>
<th>Amine</th>
<th>EC50 ± SEM (nM)</th>
<th>95% CI</th>
</tr>
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<tbody>
<tr>
<td>Tyramine</td>
<td>9 ± 0.9 nM</td>
<td>(0.1 nM, 764 nM)</td>
</tr>
<tr>
<td>Octopamine</td>
<td>351 ± 2 nM</td>
<td>(302 nM, 1476 nM)</td>
</tr>
<tr>
<td>Tyramine (stable)</td>
<td>15 ± 7 nM</td>
<td>(5 nM, 60 nM)</td>
</tr>
</tbody>
</table>

**Fig. 4.** Tyramine concentration-dependent response of CHO cells transiently and stably expressing RmTAR1 plus Gαq(5). Concentration-dependent response of the expressed coding sequence of the *R. microplus* tyramine receptor and the Gαq(5) G-protein chimera. This concentration-dependent response compared CHO cells transiently expressing the Gαq(5) G-protein chimera (solid black), and CHO cells stably expressing the *R. microplus* tyramine receptor with the Gαq(5) G-protein chimera (dashed gray). CHO cells not transfected with the receptor were screened at various concentrations of tyramine (dashed black line). Data analyzed using GraphPad Prism EC50 or EC90.

putative octopamine receptor (referred to here as the putative tyramine receptor) aligns with the proliferation of tyramine receptor sequence data to show the receptor is a tyramine receptor. Specifically, this receptor appears to be a tyramine-1 receptor (RmTAR1) (Ohta and Ozoe, 2014).

A colonial cell line that stably expressed the cattle tick tyramine receptor and the Gαq(5) chimera was established and selected over several weeks. Efficiency of the stable transfection of both the tyramine receptor and the G-protein chimera was approximately 8% (n = 24). Tyramine resulted in a concentration-dependent response of CHO cells stably expressing the tyramine receptor and the Gαq(5) chimera, which had an EC50 of 15 nM. This is similar to CHO cells transiently expressing the tyramine receptor and the Gαq(5) chimera (Fig. 4 and Table 1).

The pharmacological toolbox to characterize octopamine receptors and tyramine receptors is limited. Adrenergic agonists and adrenergic antagonists are commonly used to determine the pharmacology of octopamine receptors and tyramine receptors. Six antagonists (propranolol, chlorpromazine, mianserin, phentolamine, cyproheptadine and yohimbine) were screened at three concentrations (0.1 μM, 1 μM and 10 μM) to determine if they were capable of antagonizing the receptor when the receptor was stimulated with 150 nM tyramine (Fig. 5). Propranolol enhanced the effect of 50 nM tyramine, but it was not statistically significant from tyramine alone (Fig. 5). Cyproheptadine and yohimbine were the only compounds capable of antagonizing 150 nM of tyramine significantly at all three concentrations (0.1 μM, 1 μM and 10 μM) tested (Fig. 5). Mianserin antagonized 150 nM of tyramine at 1 μM and 10 μM (Fig. 5). Phentolamine antagonized only at 10 μM. Yohimbine and phentolamine both antagonized the 1 μM tyramine response in a concentration-dependent manner; however, yohimbine was 18-fold more effective at antagonizing the RmTAR1 compared to phentolamine (Table 2). Compounds that were able to stimulate or act as agonists to the RmTAR1 were also examined. Agonists tested include clonidine, naphazoline and tolazoline, which were screened at 0.1 μM, 1 μM and 10 μM to see if they activated RmTAR1, compared to the same concentrations of tyramine (dashed gray line). Data analyzed using GraphPad Prism EC50.
concentrations of tyramine (Fig. 6). Clonidine, naphazoline and tolazoline did not significantly agonize RmTAR1 to the same level as tyramine (Fig. 6). Naphazoline and tolazoline were able to partially agonize RmTAR1 at 10 μM, just not as strongly as tyramine itself. Clonidine, naphazoline and tolazoline were also screened as antagonists or modulators of the tyramine receptor. This was performed by preincubating these compounds for 30 min prior to the addition of 150 nM tyramine. Tolazoline and naphazoline significantly enhanced the 150 nM response of tyramine at all three concentrations (0.1 μM, 1 μM and 10 μM), as shown in Fig. 7.

Previously, this putative tyramine receptor had been hypothesized to be involved in resistance to the chemical acaricide amitraz, which is a formamidine insecticide/acaricide. Amitraz’s metabolite, BTS-27271, was screened at 10 μM and 100 μM and significantly increased the 150 nM response of tyramine at all three concentrations (0.1 μM, 1 μM and 10 μM), as shown in Fig. 7.

Previously, this putative tyramine receptor had been hypothesized to be involved in resistance to the chemical acaricide amitraz, which is a formamidine insecticide/acaricide. Amitraz’s metabolite, BTS-27271, was screened at 10 μM and 100 μM and significantly increased the 150 nM tyramine response by 191 ± 16% and 150 ± 24%, respectively. This agonistic activity was only observed in the presence of 150 nM tyramine (Fig. 8).

4. Discussion

Reintroduction of R. microplus into the United States would have devastating consequences to the livestock industry. To aid in an integrative chemical approach to controlling the southern cattle

Table 2

<table>
<thead>
<tr>
<th>IC50 ± SEM (μM)</th>
<th>95% CI</th>
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<tr>
<td>Yohimbine</td>
<td>280 ± 151 (73 nM, 1070 nM)</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>5156 ± 969 (118 nM, 2.26 × 10^5 nM)</td>
</tr>
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</table>
Pharmacological characterization of tyramine receptors, like octopamine receptors, is performed with agonists and antagonists used for the mammalian adrenergic system. Yohimbine is a known \(\alpha_2\)-adrenergic antagonist, and has previously been shown to be effective at blocking the inhibitory effect of tyramine on forskolin-stimulated cAMP in heterologous expression systems (Ohta et al., 2003; Rotte et al., 2009; Wu et al., 2013). Rotte et al. (2009) found that yohimbine produced the strongest antagonistic effect, followed by chlorpromazine then cyproheptadine. However, we find that yohimbine and cyproheptadine produce the strongest antagonistic effect, at the three tested concentrations, followed by mianserin (significant antagonism at 1 \(\mu\)M and 10 \(\mu\)M), then followed by phentolamine (significant at 10 \(\mu\)M). Phentolamine is widely accepted as a more efficient antagonist for octopamine receptors than for tyramine receptors (Roeder, 2005), which is consistent with our results.

The tyramine receptor described here was also investigated for its potential role in amitraz resistance observed in southern cattle tick populations from Australia and the Americas (Baxter and Barker, 1999; Chen et al., 2007). The first identification of the tyramine receptor was in Australia, and no point mutations were discovered in the amitraz-resistant strain compared to the amitraz-susceptible strain (Baxter and Barker, 1999). However, the Santa Luiza and San Alfonso strains of southern cattle tick display two point mutations (T8P and L22S), and these ticks are resistant to amitraz (Chen et al., 2007). This was hypothesized to have a role in formamidine resistance, and this is currently the topic of ongoing studies in our laboratory. Recent studies have reinstalled the Australian *Rhipicephalus* tick as a separate species, *Rhipicephalus (Boophilus) australis* (Fuller, 1899) present in Australia, Southeast Asia and Indonesia (Estrada-Peña et al., 2012, Barker et al., 2014; Burger et al. 2014). Separation of the Australian and American ticks into separate species suggests potential differences in mechanisms for development of amitraz resistance between the two species as it has been documented for pyrethroid resistance (Guerrero et al., 2014).

Octopamine receptor(s) are believed to be the primary target of formamidine metabolites, including amitraz's metabolite BTS-27271. Demethylchlordeform (chlordeform's metabolite) did not have an effect on forskolin-stimulated cAMP on *B. mori*'s tyramine receptor when expressed in HEK-293 cells (Ozoe et al., 2004). We also did not see any effect of amitraz's metabolite alone (data not shown); however, significant receptor activation was observed when applied in combination with 150 nM tyramine (Fig. 7). This indicates that BTS-27271 can have an effect at the tyramine receptor but probably only when the tyramine receptor is activated. Given the emerging evidence that populations around the world may constitute a species complex, having an understanding of amitraz-resistance may aid in adapting strategies to control of southern cattle ticks. Recently, a mutation in a putative *R. microplus* \(\beta\)-adrenergic-like octopamine receptor has been hypothesized to have a role in amitraz-resistance (Corley et al., 2013). However, functional receptor and functional resistance assays have not been conducted against this octopamine receptor to date.

**Acknowledgment and Disclaimer**

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