Amiloride Derivatives Are Effective Blockers of Insect Odorant Receptors

Katharina Röllecke1, Markus Werner1, Paul M. Ziemba1, Eva M. Neuhaus2, Hanns Hatt1 and Günter Gisselmann1

1Lehrstuhl für Zellphysiologie, Ruhr-Universität Bochum, Universitätsstrasse 150, 44780 Bochum, Germany and 2NeuroScience Research Center, Charité, Charitéplatz 1, 10117 Berlin, Germany

Correspondence to be sent to: Günter Gisselmann, Lehrstuhl für Zellphysiologie, Ruhr-Universität Bochum, Universitätsstrasse 150, 44780 Bochum, Germany. e-mail: guenter.gisselmann@rub.de

Accepted December 15, 2012

Abstract

Heteromeric insect odorant receptors (ORs) form ligand-activated nonselective cation channels in recombinant expression systems. We performed a pharmacological characterization of Drosophila melanogaster and Bombyx mori ORs expressed in the Xenopus laevis oocyte expression system and characterized them using the 2-electrode voltage clamp. We identified amiloride derivatives as high-affinity blockers, which inhibit the ion current through the channel in a low micromolar range. For the heteromeric Drosophila Or47a + DmelOrco receptor, the potency sequence (IC50) is HMA [5-(N,N-hexamethylene)amiloride] (3.9 µM), MIA [5-(N-methyl-N-isobutyl)amiloride] (11.0 µM), and DMA [5-(N,N-dimethyl)amiloride] (113.3 µM). Amiloride itself is nearly ineffective. Other tested insect ORs (Drosophila Or49b + DmelOrco, B. mori BmorOr1 + BmorOrco) were blocked in a similar fashion suggesting that the amiloride derivatives were potential general blockers of all receptor combinations. Our results suggest that pyrazine derivatives of amiloride are useful probes to study the mechanism of chemosensory transduction in insects in more detail.

Key words: Bombyx mori, chemosensation, Drosophila melanogaster, olfaction, voltage clamp, Xenopus laevis oocytes

Introduction

Insect odorant receptors (ORs) comprise a large family of 7-transmembrane spanning receptors, which are characterized by extracellular C- and intracellular N-termini (Benton et al. 2006; Lundin et al. 2007; Smart et al. 2008). Sixty-two OR genes were shown to be expressed in the antennae, maxillary palps, and larvae in Drosophila melanogaster (Vosshall 2000). Unlike their mammalian counterparts, they form heteromeric ion channels that are opened by odorants (Larsson et al. 2004; Neuhaus et al. 2005; Benton et al. 2006; Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008). One key difference in the functional properties of vertebrate and invertebrate OR proteins is the ubiquitously expressed insect receptor Orco, which is strongly conserved across insect species (Krieger et al. 2003; Pitts et al. 2004; Jones et al. 2011). Orco interacts with all conventional ORs and is essential for transporting them to the sensory cilia (Larsson et al. 2004; Benton et al. 2006; Sato et al. 2008). Although rapid, solely ionotropic and G-protein-independent currents were described by Sato et al., nonselective cation currents activated by means of an ionotropic and a metabotropic pathway, with Dmel/Orco being directly activated by intracellular cAMP or cGMP, have been described in a study by Wicher et al. (2008). A dual function of insect ORs as ionotropic and G-protein-activating receptors is also possible (Deng et al. 2011).

The variable, non-Orco subunit determines the odor specificity of the OR complex, whereas Orco is required for cation influx (Elmore et al. 2003). Recently, an activator of Orco has been found, VUAA1, which is able to activate Orco when expressed alone or in combination with an OR subunit (Jones et al. 2011).

For the characterization of the receptor function, specific and potent pharmacological tools are valuable. However, pharmacology of these insect ORs is not well known and specific high-affinity blockers are lacking. In addition, such blockers could be of potential use as insect repellents in pest control as has been proposed for DEET (N,N-diethyl-m-toluamide) (Ditzen et al. 2008). Ruthenium Red (RR) is an unspecific cation channel blocker and can be used to inhibit the current elicited by insect ORs when expressed in a recombinant system (Nakagawa et al. 2005; Sato et al. 2008;
Jones et al. 2011; Nichols et al. 2011). Interestingly, the extent of this blocking is dependent on the subunit composition, suggesting that both subunits form the pore. Thus, we were looking for blockers for insect ORs beside DEET.

In lobster, another invertebrate system used for investigating olfactory signal transduction, a potential downstream target of phosphoinositide signaling in lobster olfactory receptor neurons (ORNs), is a sodium-gated nonselective cation (SGC) channel (McClintock and Ache 1990; Zhainazarov and Ache 1995, 1997). The channel can be modulated by exogenous phosphoinositides in cell-free patches (Zhainazarov and Ache 1999; Zhainazarov et al. 2001). This SGC channel is antagonized by 2-aminoethoxy-diphenyl borate, SKF96365, Gd

modulated by exogenous phosphoinositides in cell-free patches (Zhainazarov and Ache 1999; Zhainazarov et al. 2001). This SGC channel is antagonized by 2-aminoethoxy-diphenyl borate, SKF96365, Gd

in our screening on ORs of insects. In the recombinant Xenopus laevis oocyte expression system, we demonstrated that insect ORs are effectively blocked by amiloride derivatives, too.

Materials and methods

Functional expression of receptor cRNA in Xenopus oocytes

Essentially, expression of cRNA and electrophysiological experiments were performed as described (Saras et al. 2008). Briefly, the coding sequence for Or47a and Or49b was amplified by polymerase chain reaction from antennal Drosophila cDNA and cloned into the pSGEM vector. Plasmids containing BmorOr1, BmorOrco, and DmelOrco were a kind gift from K. Touhara (Nakagawa et al. 2005). cRNAs were synthesized using the AmpliCap T7/T3 high yield message maker kit (Epicerent), according to the manufacturer’s protocol, with linearized plasmids as templates. Xenopus laevis oocytes were prepared by collagenase digestion. After 24 h, stage V–VI oocytes were injected with cRNA (typically 5–25 ng/oocyte) and incubated at 16 °C in Barth’s solution. Two-electrode voltage clamp recording were generated after 2–4 days at room temperature. Agonists and antagonists were diluted to the concentrations indicated with Frog-Ringer’s solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2). Recording was done using a 2-electrode voltage clamp amplifier (TURBO TEC-03, npi) and pCLAMP software (Axon Instruments) with typical membrane potentials of −40 to −60 mV.

Results

Blocking of insect ORs by amiloride derivatives

The olfactory receptor Or47a in combination with DmelOrco was used to investigate the potential blocking of amiloride and various amiloride derivatives on insect olfactory receptors (Figure 1A). These derivatives are characterized by the 5-amino pyrazine substituents. Substances were screened and results were compared with the effect of DEET, a commonly known insect repellent, and SKF96365 (Figure 1B), which was used previously to block the odor-evoked activity in lobster ORNs (Bobkov and Ache 2007).

Heteromeric receptors were expressed in X. laevis oocytes, and electrophysiological recording performed using the 2-electrode voltage clamp method. Application of pentyl acetate evoked currents in Xenopus oocytes expressing Or47a + DmelOrco (Sato et al. 2008) with a calculated EC⁵₀ of pentyl acetate of 10.7 ± 2.0 µM, n = 7 (Figure 2A,B). For the screening of potential blockers, we used 30 µM pentyl acetate as agonist and observed that during a repetitive stimulation, the agonist-evoked amplitudes desensitized. This prevents the use of the typical standard application pattern in which the agonist is applied, followed, after a washout, by the agonist in combination with the blocker, and a subsequent final agonist application. Consequently, we used a modified application scheme: The application of the agonist was followed directly by an application of the agonist in combination with various concentrations of the blocker (Figure 2C,D). The analysis of the recordings was performed as follows: The difference between the current before the application of the agonist and the current just before the application of the antagonist was determined to be Iₘₐₓ, where I is the difference between the current before the application of the agonist and the current after the application of the antagonist (Figure 2C).

Several substances were tested on oocytes expressing Or47a + DmelOrco (Figure 2D,E). The IC₅₀ values for HMA [5-(N,N-hexamethylene)amiloride], DMA [5-(N,N-dimethyl)amiloride], DEET, SKF96365 (Figure 3), and MIA [5-(N-methyl-N-isobutyl)amiloride] (Figure 4) calculated from concentration–inhibition relationships varied over a wide range. HMA (IC₅₀ = 3.9 ± 1.0 µM, n = 6) was the most
Amiloride Derivatives Are Effective Blockers of Insect Odorant Receptors

Figure 1  (A) Structure of amiloride and its pyrazine derivatives: HMA [5-(N,N-hexamethylene)amiloride], MIA [5-(N-methyl-N-isobutyl)amiloride], DMA [5-(N,N-dimethyl)amiloride]. (B) Structure of DEET (N,N-diethyl-m-toluamide) and SKF96365.

Figure 2  Original 2-electrode voltage clamp recordings of *Xenopus laevis* oocytes injected with cRNA coding for Or47a + DmelOrco. (A,B) Application of pentyl acetate activates the receptor in a concentration-dependent manner. With an application scheme as depicted in (C), the current evoked by 30 µM pentyl acetate was blocked by various concentrations of DEET, HMA, DMA, and SKF96365, respectively (D).
potent blocker, followed by MIA (IC$_{50}$ = 11.0 ± 3.1 µM, n = 6), SKF96365 (IC$_{50}$ = 42.5 ± 8.3 µM, n = 6), DMA (IC$_{50}$ = 113.3 ± 20.6 µM, n = 6), and DEET (IC$_{50}$ = 929 ± 132 µM, n = 6). Amiloride itself was no effective blocker of Or47a + DmelOrco receptors (Supplementary Figure 1).

Subunit dependency of the blocking by MIA

Recently, it has been reported that the inhibition by RR depends on the subunit composition of the OR (Nichols et al. 2011). In order to test if the potency of blocking by MIA is also dependent on the subunit combination, we determined the IC$_{50}$ for ORs in various combinations: Like the previously tested blockers, MIA effectively blocks the pentyl acetate-induced current at the Or47a + DmelOrco combination (IC$_{50}$ = 11.0 ± 3.1 µM, n = 6). Substituting DmelOrco for BmorOrco did not lower the IC$_{50}$ significantly (P = 0.14) in the Or47a + BmorOrco combination (IC$_{50}$ = 6.0 ± 1.3 µM, n = 6). Next, we tested a different Drosophila OR. Receptors containing the OR49b subunit are sensitive to 2-methyl phenol (EC$_{50}$ = 239 µM ± 76, n = 5, data not shown). The current evoked by 300 µM agonist was blocked by MIA at the receptor combinations Or49b + DmelOrco (IC$_{50}$ = 7.2 ± 2.7 µM, n = 6) and Or49b + BmorOrco (IC$_{50}$ = 17.7 ± 3.0 µM, n = 6). Here, the exchange of the Orco subunit led to a significant rise in the IC$_{50}$ (P = 0.03). However, replacing OR47a for OR49b also led to a significant rise in receptors containing BmorOrco (P = 0.003) but not in those containing DmelOrco (P = 0.41).

In order to test whether the blocking is species-specific when the OR subunit is replaced, MIA was tested on oocytes coexpressing BmorOr1 + BmorOrco (Nakagawa et al. 2005). The receptor complex was fully activated by applying 30 µM Bombykol (EC$_{50}$ = 7.3 ± 1.4, n = 5, data not shown) and the response was inhibited by MIA, which is a potent blocker (IC$_{50}$ = 17.5 µM ± 3.7, n = 10, Figure 4).

Characterization of the blocking action of MIA

Next, we wanted to determine if the blocking by MIA prevents receptor activation by an agonist. We took advantage of the fact that applying higher agonist concentrations leads to current responses lasting much longer than the duration of the initial agonist application. Prolonged application of the agonist pentyl acetate leads to a persistent activation of Or47a + DmelOrco even after a long (>5 min) washout of the agonist. This fraction of open channels is blocked by MIA in the absence of any agonist as depicted by the apparent outward current induced by MIA application (Figure 5A). The IC$_{50}$ of 9.2 ± 3.4 µM (n = 3) in this experiment (Figure 5B) was comparable with the IC$_{50}$ of 11 µM in the presence of 30 µM pentyl acetate (Figure 4). The current blocked by 300 µM MIA absence of the agonist was 36% ± 8.8 (n = 4) of the current amplitude evoked by the prior agonist application.

Discussion

In our present study, we identified the amiloride derivatives MIA and HMA as potent blockers of insect ORs expressed in the recombinant Xenopus oocyte system. These substances have been used to block the odor-evoked activity in lobster ORNs (Bobkov and Ache 2007). Amiloride belongs to the chemical group of pyrazinecarboxamides and its derivatives block several members of the transient receptor potential (TRP channel) family, epithelial sodium channels, and
Amiloride Derivatives Are Effective Blockers of Insect Odorant Receptors

Na+/H+ exchangers (reviewed in Bobkov and Ache 2007). Amiloride itself does not block insect ORs; however, introduction of hydrophobic residues on the 5-amino group drastically improves the potency of inhibition. Same was found for the action on the Na+/H+ exchanger (Masereel et al. 2003). In terms of potency, our newly identified target for amilorides shares a similar pharmacology with the lobster sodium-gated nonselctive cation (SGC) channel (Bobkov and Ache 2007); however, as the SGC channel is not cloned, it is difficult to assess which membrane proteins they are related to. In contrast, the potency of the amiloride derivatives is about 100 times higher for the Na+/H+ exchanger block (Masereel et al. 2003). Furthermore, the pharmacological profile is also different to TRP and ENaC channels, which are blocked by amiloride itself in several cases (Sariban-Sohraby and Benos 1986; Kleyman and Cragoe 1988; Tytgat et al. 1990; Doi and Marunaka 1995; Murata et al. 1995; Stoner and Viggiano 2000; Hirsh 2002). SKF96365 is used as a blocker of transient receptor potential canonical (TRPC) channel and also acts against several other types of channels including voltage-gated T- and L-type Ca++ channels and voltage-gated Na+ channels (Singh et al. 2010).

We tested MIA on different receptor combinations of D. melanogaster and Bombyx mori. The potency of MIA is in the same range for all tested combinations, which leads us to suggest that it is a potential general receptor blocker and may act on the Orco subunit. However, the blocking mechanism and the subunit where MIA binds remain to be shown in future. MIA is an interesting tool for studying insect chemosensory signal transduction as it shows a broad spectrum of blocking across species. Such agents are also of potential interest for pest control because they affect chemosensory-driven behaviors as discussed for the DmelOrco activator VUAA1 (Jones et al. 2011). So far, MIA and HMA are the most potent known blockers for insect ORs; MIA is more potent than DEET, which acts as an insect repellent (Ditzen et al. 2008), and also RR, which was used for testing the subunit contribution of the channel block (Nichols et al. 2011).

Supplementary material
Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Funding
This work was supported by a grant of the Deutsche Forschungsgemeinschaft [NE 755/3-1 to E.M.N. and G.G.].

Acknowledgments
We thank H. Bartel, A. Stoeck, and J. Gerkrath for excellent technical assistance and S. Zbrolla for contributing data.

References


