The Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor, KB-R7943, blocks a nonselective cation channel implicated in chemosensory transduction

Pezier A.\(^1\), Bobkov Y.V.\(^1\) and Ache B.W.\(^{1,2}\)

\(^1\)Whitney Laboratory for Marine Bioscience, Center for Smell and Taste, McKnight Brain Institute, and \(^2\)Depts. of Zoology and Neuroscience, University of Florida, Gainesville, FL 32610

Correspondence to be sent to:
Dr. Adeline Pezier
Whitney Laboratory for Marine Bioscience
University of Florida
9505 Ocean Shore Blvd
St. Augustine FL 32080-8610
USA
Phone: (904) 461-4035
Fax: (904) 461-4008
pezier@whitney.ufl.edu

Running title:
KB-R7943 inhibits a TRP-related channel

Abbreviations:
ORN: olfactory receptor neuron; SGC channel: non-selective sodium-gated cation channel; TRP: transient receptor potential; TRPC: canonical transient receptor potential; NCX: sodium/calcium exchanger; PS: Panulirus saline.

Key Words:
KB-R7943, TRP channels, calcium-activated nonselective cation channel, sensory transduction, invertebrates, olfaction.
ABSTRACT

The mechanism(s) of olfactory transduction in invertebrates remains to be fully understood. In lobster olfactory receptor neurons (ORNs), a non-selective sodium-gated cation (SGC) channel, a presumptive transient receptor potential (TRP)-C channel homolog, plays a crucial role in olfactory transduction, at least in part by amplifying the primary transduction current. In order to better determine the functional role of the channel it is important to selectively block the channel independently of other elements of the transduction cascade, causing us to search for specific pharmacological blockers of the SGC channel. Given evidence that the Na\(^+\)/Ca\(^{2+}\) exchange inhibitor, KB-R7943, blocks mammalian TRPC channels, we investigated this probe as a potential blocker of the lobster SGC channel. KB-R7943 reversibly blocked the SGC current in both inside- and outside-out patch recordings in a dose- and voltage-dependent manner. KB-R7943 decreased the channel open probability without changing single channel amplitude. KB-R7943 also reversibly and in a dose-dependent manner inhibited both the odorant-evoked discharge of lobster ORNs and the odorant-evoked whole-cell current. Our findings strongly imply that KB-R7943 potently blocks the lobster SGC channel and likely does so directly, not through its ability to block the Na\(^+\)/Ca\(^{2+}\) exchanger.
INTRODUCTION

The transient receptor potential (TRP) superfamily of ion channels includes a growing number of non-selective cation channels in organisms from yeast to mammals (Nilius 2007; Venkatachalam and Montell 2007). TRP channels play a major role in sensory signaling, including light, taste, sound, temperature and touch stimuli, i.e., most major sensory modalities. A notable exception has been the potential involvement of TRP channels in sensory signaling in olfaction. Recently, however, TRPM5 has been suggested to play a functional role in mammalian olfactory receptor neurons (ORNs) (Lin et al. 2007, 2008). Based on its biophysical and pharmacological properties, the lobster olfactory non-selective sodium-gated cation (SGC) channel (McClintock and Ache 1990; Zhainazarov and Ache 1997; Bobkov and Ache 2005) also appears to be a TRP channel involved in olfactory transduction, with potential homology to the mammalian canonical TRP (TRPC) family (Urban et al. 2005). The lobster SGC channel has the interesting property of being both activated by and permeant to Na\(^+\) and Ca\(^{2+}\), allowing it to contribute to the generation of a substantial part of the depolarizing receptor potential through regenerative activation (Zhainazarov et al. 1998; Bobkov and Ache 2003). As is true for many TRP channels (e.g., Hardie 2003), the lobster SGC channel is the downstream target of phosphoinositide signaling (Zhainazarov et al. 2001). In order to better determine the role of this and potentially other TRP channels in olfactory transduction, and to understand the mechanism(s) through which phosphoinositides target TRP channels involved in sensory transduction, we sought to specifically block the channel independently of other elements of the transduction cascade (Bobkov and Ache 2005).

Since its introduction as the first specific Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) inhibitor (Iwamoto et al. 1996) and its emergence as a leading member of a class of synthetic NCX oriented drugs, KB-R7943 has been widely used to study the physiological and/or pathophysiological roles of the NCX exchanger at both the cellular and organismal levels of organization (IC\(_{50}\) = 1 to 30 \(\mu\)M for NCX, Amran et al. 2003). As research on KB-R7943 expanded, so did the understanding of its action, showing that the drug also blocks transporters (Pintado et al. 2000; Santo-Domingo et al. 2007) as well as voltage- and ligand-gated cation channels (Birinyi et al. 2005; Ouardouz et al. 2005; Sobolevsky and Khodorov 1999), including canonical TRPC channels (Kraft 2007). Given that the functional properties of the lobster SGC channel are consistent with TRPC subfamily assignment (Urban et al.
2005), together with the channel’s Na$^+$ sensitivity, we tested the possibility that KB-R7943 could specifically block the lobster SGC channel. Given that Na$^+$ entry and modulation of Na$^+$/Ca$^{2+}$ exchange has been proposed to be a key mechanism in TRPC signaling (Eder et al. 2005), we also considered whether action of KB-R7943 on the lobster SGC channel would be direct or mediated via an NCX.

We show that KB-R7943 is a potent inhibitor of the lobster SGC channel, that the drug inhibits the odorant-evoked response of individual ORNs in situ in a manner consistent with the blocking action of the drug on the channel itself, and that the drug affects the channel directly and not via action on an NCX. Our findings extend the pharmacological profile of the lobster SGC channel and more further implicate the channel in lobster olfactory transduction.

MATERIAL AND METHODS

Preparations

The olfactory organ, the lateral filament of the antennule, was excised from the spiny lobster, Panulirus argus, and used for two different preparations. Single-channel inside-out and outside-out recordings were performed on excised patch from cell bodies of cultured ORNs. Clusters of ORN somata were scraped from the lumen of the organ, bathed in Ca$^{2+}$- and Mg$^{2+}$-free Panulirus saline (PS, see Solutions) with trypsin (1 mg/mL, Sigma) for 15 min, and mechanically dissociated by trituration in PS. Dispersed cells were plated on 35-mm glass bottom Petri dishes filled with PS supplemented with gentamicin solution (4 µL/mL, Sigma). The cultured ORNs were kept at 21°C and used for cell-free patch-clamp recording at least 6h after dissociation.

In situ recordings were performed on individual sections (annuli) of the olfactory organ prepared for patch-clamp recording. The somata of the ORNs were cleaned by bathing the annuli in Ca$^{2+}$- and Mg$^{2+}$-free PS containing trypsin (1 mg/mL, Sigma) and then rinsing with PS. Most of the cuticle not supporting the olfactory sensilla (aesthetascs) was removed to provide access to the ORNs and the resulting hemi-annulus was mounted in the bottom of a 35-mm Petri dish filled with PS. The hair-like aesthetascs containing the outer dendrites of the ORNs, were superfused with either PS, PS + drug of interest or PS + odorant. The somata of
the ORNs were continuously superfused with PS to restrict the application of the drug and/or the odorant to the hairs.

**Electrophysiological recording and analysis**

Patch electrodes for single-channel, extracellular (spikes) and whole-cell recordings were pulled from borosilicate capillary glass (Sutter Instrument, BF150-86-10) using a Flaming-Brown micropipette puller (P-87, Sutter Instruments). All currents were recorded using an Axopatch 200A or 200B amplifiers controlled by a Digidata 1322A and pClamp 9.2 and data sampled at 5 kHz. Unitary currents were recorded in inside-out or outside-out configuration. In order to obtain current-voltage characteristics, in inside-out experiments, series of 50-ms step at -100 mV followed by 150-1000 ms voltage ramp from -100 mV to +100 mV were applied from a holding potential of -60 to -70 mV. In outside-out experiments, series of 15-ms step at -120 mV followed by a 150-ms voltage ramp from -120 mV to +100 mV were applied from a holding potential of -60 to -70 mV. Action potentials (APs, spikes) were recorded in track mode using cell-attached loose-patch configuration as described previously (Bobkov and Ache 2005). Whole-cell currents were recorded in voltage clamp mode at a holding potential of -70 mV. Bath solution change for single-channel recordings or odor delivery for *in situ* recordings were performed with a stepper motor (Haydon Switch and Instruments Inc., Waterbury, CT, USA) controlled by a fast-step SF-77B perfusion system (Warner Instruments Inc., Hamden, CT, USA) and pClamp 9.2 software (Molecular Devices, CA, USA) or rapid solution changer, RSC-160 (Bio-Logic - Science Instruments, Claix, France). Experiments were carried out at ~21°C.

Data were analyzed with Clampfit 9.0 (Molecular Devices) in combination with SigmaPlot 10.0 (SPSS Inc., Chicago, IL, USA). All-points current amplitude histograms were generated to estimate the SGC current amplitude in different conditions. The amplitude of AP discharge frequency was taken as the mean spike frequency within the first 2 s after the beginning of the stimulation and normalized to the maximal response frequency in control conditions. After low-pass filtering, amplitudes of the odorant-evoked whole-cell currents were measured and normalized to that before application of KB-R7943. Two modifications of the Hill equation were used to fit the experimental data: 1) $F(x) = F_{\text{max}} \times x^h / (x_{1/2}^h + x^h)$ for activation and 2) $F(x) = 1 - F_{\text{max}} \times x^h / (x_{1/2}^h + x^h)$ for inhibition, where $F$ is the normalized current or frequency of APs, $x$ is the odor or KB-R7943 concentration, $x_{1/2}$ is the half-maximum
odor/KB-R7943 concentration, and h is the Hill coefficient. An additional parameter reflecting the basal level of F (Fb) was incorporated when necessary. All results are expressed as means ± s.e.m. of n observations.

Solutions and chemicals

For single-channel experiments, three solutions were used: the Na⁺-based solution (in mM): 210 NaCl, 1 ethyleneglycolbis(amo) etylether)-tetraacetic acid (EGTA), 0.1 CaCl₂, 696 glucose, and 10 HEPES; the high-Ca²⁺/Na⁺-based solution (in mM): 210 NaCl, 0.1 CaCl₂, 696 glucose, and 10 HEPES and the Li⁺-based solution (in mM): 210 LiCl, 1 EGTA, 0.1 CaCl₂, 696 glucose, and 10 HEPES. The pH of solutions was adjusted with NaOH or Trizma base (Sigma) to 7.9. The free Ca²⁺ concentration was <10 nM for the Li⁺-based and Na⁺-based solutions as calculated with WebmaxC v.2.20 (http://www.stanford.edu/~cpatton/webmaxcS.htm).

To characterize the effects of KB-R7943 on the SGC channel, the pipette was filled with Na⁺-based solution corresponding to the extracellular medium for inside-out experiments and cytoplasmic medium for outside-out experiments and the bath consisted of high-Ca²⁺/Na⁺-based solution corresponding to the cytoplasmic medium for inside-out experiments and extracellular medium for outside-out experiments. Solutions contained different calcium concentrations are specified in the text.

For APs and whole-cell in situ recordings, Panulirus saline (PS) containing (in mM): 486 NaCl, 5 KCl, 13.6 CaCl₂, 9.8 MgCl₂, 10 HEPES was used to bath the ORNs. PS also fill the pipette electrode for APs recordings. The whole-cell pipette solution contained (in mM): 210 KCl, 0.1 CaCl₂, 0-1 EGTA, 570 Glucose, 10 HEPES. The free Ca²⁺ concentration of the pipette solution was from <10 nM to 100 µM as calculated with WebmaxC v.2.20. At these concentrations of Ca²⁺ and in absence of Na⁺ ions, no current is generated in absence of odorant stimulation. The pH of solutions was adjusted with NaOH or Trizma base (Sigma) to 7.7-7.9.

The odorant consisted of an aqueous extract of a commercial marine aquarium food, TetraMarine, TET, (Tetra Werke, Melle, Germany). Prior each experiment aliquots of stock solution were diluted in PS to a final concentration of 0.5 mg/mL.
KB-R7943, 2-[2-(4-(4-nitrobenzoyloxy)phenyl)ethyl]isothiourea mesylate and SN-6 (2-[2-[(4-Nitrophenyl)methoxy]phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester (Tocris Biosciences), were prepared as stock solution of 100 mM and 50 mM respectively in dimethylsulfoxide (DMSO) and diluted either in PS or solutions used for single-channel recordings. The final DMSO concentration was always at or below 0.1%, a concentration without any effect on the lobster SGC channel. The acidic dissociation constant (pKa) value for the isothiourea group of KB-R7943 is about 10 (Iwamoto et al., 1996). Thus, under our experimental conditions (pH 7.9) KB-R7943 was almost completely protonated and carried the charge of +1, which could potentially explain the utility of the drug in our system. Verapamil was prepared as stock solution of 100 mM in water prior its dissolution in PS or high-Ca²⁺/Na⁺ solution.

RESULTS

**KB-R7943 blocks the lobster SGC channel from the intracellular side**

The SGC channel is expressed in lobster ORNs in both a Ca²⁺-sensitive and a Ca²⁺-insensitive form (Bobkov and Ache 2003). Due to the predominance of the Ca²⁺-sensitive form in the outer dendrites (transduction zone), we focused on the Ca²⁺-sensitive form of the channel. The first series of experiments were performed in inside-out patch recording on excised patches taken from the soma of cultured ORNs. In these experiments, a solution rich in Na⁺ filled the pipette (extracellular medium). In absence of Na⁺ and Ca²⁺ ions (Li⁺-based solution) on the cytoplasmic side of the membrane, the SGC channel was almost totally inactive. To activate the channel the bath was switched from Li⁺-based solution to Na⁺-based solution followed by high-Ca²⁺/Na⁺-based bath solution to determine the Ca²⁺-sensitive form of the SGC channel. In this last condition referred also as control in the text, the concentration of Na⁺ was symmetrical across the membrane. The only difference between the extracellular and intracellular mediums was the Ca²⁺ concentration. Fig. 1A shows a representative recording of the Ca²⁺-sensitive form of the SGC channel. In this example five SGC channels could be seen in predominantly open state when the solution was switched to the control high-Ca²⁺/Na⁺-based bath condition at -60 mV. KB-R7943 applied to the intracellular side of the membrane patch produced a reversible blockage of the SGC current in a voltage-dependent manner (Fig. 1B-D). At -60 mV, even a high concentration of the drug (50-100 µM) applied
for 15 s failed to block the SGC current (Fig. 1B, upper panel). In contrast, holding the same patch at +60 mV application of 50 µM of KB-R7943 rapidly and totally blocked the SGC current (Fig. 1B, middle panel). The drug decreased the channel open probability but not the single-channel amplitude as seen at higher time resolution and according to parameters obtained from Gaussian distribution approximation of all-points current amplitude histograms in Fig. 1B, lower panel. The value of the unitary amplitude estimated at +75 mV was 12.20 ± 0.21 pA (n=30) in control high-Ca²⁺/Na⁺ solution and 12.02 ± 0.14 pA in the presence of 50 µM of KB-R7943 (n=43). The channel activity typically recovered within <20 s (Fig. 1B, middle panel).

To better characterize the voltage-sensitivity of the drug blockage of the SGC current we applied ramp protocols (linear change in membrane voltage) to membrane patches from a holding potential of -60 mV (Fig. 2A). In control high-Ca²⁺/Na⁺ bath solution, the current-voltage (I-V) relationship of the SGC current generated by ramp protocols had a characteristic double rectification in the presence of divalent cations (in given case 100 µM intracellular Ca²⁺) with a reversal potential close to 0 mV (Fig. 2A, grey trace top graph). Application of 50 µM KB-R7943 on the intracellular side of the patch mainly blocked the outward current component at voltages higher than +40 mV confirming that the channel blockage was voltage dependent (Fig. 2A, black trace top graph).

Given that inhibitory effect of KB-R7943 on the NCX was shown to be Ca²⁺ dependent (Iwamoto et al. 1996), we next examined the possible Ca²⁺-dependence of the blocking effect of KB-R7943 on SGC current. KB-R7943 (50 µM) was applied on the intracellular face after activation of the SGC channel recorded in different combinations of external and internal Ca²⁺ concentrations chosen among 10 nM, 100 µM or 13.6 mM. As seen in Fig. 2A, Ca²⁺ did not appear to be an important factor of KB-R7943 mediated inhibition of the SGC channel. With PS outside, the increase in Ca²⁺/divalent cations concentration reduced the single SGC channel amplitude, shifted the reversal potential from 0 mV (Fig. 2A, top graphs) to ~+35 mV (Fig. 2A, bottom graph) and changed the voltage dependence of the SGC currents. However, the pattern of KB-R7943 mediated inhibition is still similar at all Ca²⁺ concentrations tested (compare I-Vs in Fig. 2A). Overall, saturating concentration of KB-R7943 inhibited the SGC channel activity by 93.11 ± 1.98% ([Ca²⁺]i/[Ca²⁺]o = 100µM/10nM, 10nM/10nM and 10nM/100µM) and by 69.38 ± 4.37% when PS was an extracellular solution ([Ca²⁺]i/[Ca²⁺]o = 10nM/13.6mM; Fig. 2B).
KB-R7943 blocked the SGC channel in a dose-dependent manner (Fig. 2C-D). Fig. 2C shows the inhibition of the SGC channel activity recorded from the same patch at increasing concentrations of KB-R7943 within positive voltage range. For this example, 85.8 ± 1.8% of the SGC channel activity was inhibited at +100 mV with 50 µM KB-R7943 in high-Ca²⁺/Na⁺ bath solution ([Ca²⁺]i/[Ca²⁺]o = 10nM/100µM). A concentration-inhibition curve of KB-R7943 (Fig. 2D) was generated with eight concentrations of the drug at +100 mV. Three to 11 patches were tested for each KB-R7943 concentration. Fitting of the curve with the Hill equation yielded a half maximal inhibitory concentration (IC₅₀) of 11.74 µM and a corresponding Hill coefficient of 2.92. Since KB-R7943 is complexed with mesylate (CH₃SO₃H) we had to test that the inhibition we observed was due to KB-R7943 and not mesylate. Mesylate alone even at high concentration (200 µM, n=2; Fig. 2D, histogram) did not block the SGC current. Additionally, SN-6, another blocker with a greater selectivity for the NCX (Niu et al. 2007), tested at the maximum concentration permitted by the solubility properties of the compound in water at pH = 8 (5 µM, calculated using Advanced Chemistry Development – ACD/Labs – Software V8.14 for Solaris), had no effect on the SGC current (n=7, Fig. 2D, histogram).

**KB-R7943 blocks the lobster SGC channel from the extracellular side**

The effect of the drug was also investigated by recording in the outside-out configuration. In these experiments, a solution rich in Na⁺ filled the pipette (intracellular medium) to activate the SGC channel. In control conditions, the patch was perfused in high-Ca²⁺/Na⁺-based bath solution (extracellular medium). KB-R7943 applied to extracellular side of the membrane patch reversibly blocked the SGC channel in a dose-dependent manner (Fig. 3). Application of 50 µM of KB-R7943 potently inhibited the SGC current at a holding potential of -70 mV (Fig. 3A). The drug decreased the channel open probability without changing single-channel amplitude as seen at higher time resolution and, according to amplitude histograms of the top panel Fig. 3A. The value of the unitary amplitude estimated at -70 mV was 11.20 ± 0.18 pA (n=12) in control high-Ca²⁺/Na⁺ solution and 11.32 ± 0.11 pA (n=16) in presence of 50 µM of KB-R7943. To test if the inhibition of the channel was sensitive to membrane potential we applied ramp protocols to membrane patches (Fig. 3B). In control high-Ca²⁺/Na⁺ solution, the I-V relationship of the SGC current generated by ramp
protocols had a characteristic double rectification with a reversal potential close to 0 mV. Application of 50 µM KB-R7943 equally blocked both the inward and outward current components suggesting that the channel blockage was not voltage dependent (Fig. 3B).

A concentration-inhibition curve was generated with six concentrations of KB-R7943 using steady-state averaged current at -70 mV (Fig. 3C). Fitting the data with the Hill equation yielded an IC₅₀ of 13.82 µM and a corresponding Hill coefficient 1.32. The channel activity totally recovered within 5-20 s after drug removal. As a control, mesylate alone even at high concentration (200 µM, n=5) did not block the SGC channel (Fig. 3C, histogram).

**KB-R7943 blocks the odorant-evoked discharge in lobster ORNs in situ**

Previous studies identified at least two different patterns of odorant-evoked discharge in lobster ORNs, tonically discharging cells and bursting cells (Bobkov and Ache 2007). We tested the effect of KB-R7943 on the tonic cells since they represent the predominant ORN subpopulation. Toniaclly active ORNs gradually increase their ongoing rate of odorant-evoked discharge in response to increasing stimulus intensities (Fig. 4A, B). KB-R7943 (50 µM) decreased the spontaneous discharge of the cells by 81% in the presence of 50 µM of the drug in 7 cells out of 10, almost completely inhibited the odorant-evoked discharge at low odorant concentrations and strongly decreased the response to high odorant concentrations, as shown for one cell in Fig. 4A, B. The inhibition by KB-R7943 could not be reversed for at least 5 min after washout. Importantly, KB-R7943 was not observed to change either shape or spike amplitude (Fig. 4B insets). This would suggest the drug did not change the activity of ion channels directly involved in AP generation.

Changing the concentration of the drug altered the response to a fixed odorant intensity in a concentration-dependent manner (Fig. 4C, D). Increasing the inhibitor concentration decreased the stimulus evoked discharge as shown for one cell in Fig. 4C and collectively for 4-8 cells in Fig. 4D. The concentration-dependent inhibition of the discharge by KB-R7943 fit with a Hill equation gave an IC₅₀ of 16.57 µM and a Hill coefficient of 1.74. Inhibition was almost total between 50-100 µM KB-R7943. The blockade was relatively slow; KB-R7943 blocked the odorant-evoked discharge in 91% of the cells tested (21 out of 23) after 5-10 min. Washout was also relatively slow, with, in some cases, only partial
recovery after 5-10 min of washout (Fig. 4A). Additionally, verapamil (100 µM), another NCX blocker (Amran et al. 2003), had no effect on the odorant-evoked discharge tested on 4 tonically discharging cells (AP discharge frequency in presence of verapamil/AP frequency in control = 1.03 ± 0.05). Finally, SN-6 (5 µM), had no effect on the odorant-evoked discharge tested on 3 tonically discharging cells (AP discharge frequency in presence of SN-6/AP frequency in control = 1.00 ± 0.07).

**KB-R7943 blocks the whole-cell current in lobster ORNs in situ**

As mentioned earlier, the parameters of spikes (spike positive/negative peak amplitudes and temporal characteristics) did not noticeably change in the presence of KB-R7943. This would suggest that the drug did not affect the activity of ion channels directly involved in AP generation. On the other hand, given the reduction in ORN spontaneous spike discharge frequencies, there is a possibility that KB-R7943 does not actually block the stimulus activated channels but rather affects ion transport systems leading to ORN hyperpolarization significant enough to dramatically decrease odorant evoked ORN activity. To examine the possibility we investigated the effects of the drug on the odorant-evoked ORN responses using the whole-cell voltage clamp recordings (Fig. 5). Typically the lobster ORNs recorded in voltage clamp mode generated inward currents in response to stimulus application in a dose dependent manner (Fig. 5A). As in many other primary chemosensory neurons from a variety of different species, the lobster ORN odorant evoked currents exhibited rundown when recorded in standard whole cell mode, although the response rundown kinetics was relatively slow. KB-R7943 (50 µM) applied focally to outer dendrites area (transduction zone) of the ORNs did not considerably change resting whole cell currents (e.g. Fig. 5B) whereas almost completely and reversibly inhibited the odorant-evoked currents (Fig. 5B). Overall, KB-R7943-dependent inhibition of the normalized peak amplitude of the whole-cell current was 69 ± 22% (n=7, Fig. 5C). These results indicate that the drug dependent ORN hyperpolarization appears to have little if any effect on odor evoked ORN discharge and KB-R7943 targeted primarily ion channels involved in the lobster chemosensory transduction cascade.
DISCUSSION

We show that KB-R7943 blocks the SGC channel. The lobster SGC channel occurs in both calcium-sensitive and calcium-insensitive forms (Bobkov and Ache 2003). The data we reported were obtained exclusively from the calcium sensitive form of the channel since that form is predominantly expressed in the outer dendrites (transduction zone) of the ORNs. However, preliminary data suggest that the calcium-insensitive form of SGC channel appears to be equally sensitive to the effects of KB-R7943 (50-100 µM, n=6). We also show that KB-R7943 blocks odorant-evoked activity in tonically active ORNs. These cells represent the major subpopulation of ORNs in the lobster olfactory organ (Bobkov and Ache 2007). However, preliminary data suggest that a subpopulation of rhythmically active ORNs in the olfactory organ are also blocked by KB-R7943 (n=6), suggesting the SGC channel occurs and subserves a common transduction and/or amplification mechanism in all ORNs.

While our results are qualitatively consistent, showing similar voltage dependence and comparable half maximal concentrations of inhibition across the three electrophysiological approaches (IC50 = 12, 14 and 17 µM in inside-out, outside-out, and in situ recordings, respectively), the Hill coefficients show considerable variability (h = 2.92, 1.32 and 1.74 in inside-out, outside-out, and in situ recordings, respectively), suggesting different degrees of cooperativity and therefore different numbers of inhibitor molecules (3 and 1, respectively, between inside-out and outside-out recording) are required to block the channel from the intracellular and or extracellular face. However, this difference could result from the different voltages (-60 mV vs. +100 mV) and/or the different experimental protocols (gap free recording vs. ramp) used to collect the data. Adequately interpreting this potentially interesting finding would demand detailed biophysical analysis and be well beyond the scope of the current report.

Our finding that KB-R7943 (50 µM) decreased the spontaneous discharge frequency of 7 of 10 tonically-active ORNs suggests that a fraction of SGC channels are constitutively active and may contribute to the resting state of the cell, as known to occur for transduction channels in vertebrate ORNs (e.g. Kleene 2000; Pun and Kleene 2003). The extent to which the channel contributes to the resting state of the cells, however, presumably would be subject
to agonist concentration, e.g., intracellular sodium, intracellular calcium, and phosphoinositides, but that question was not pursued as part of the present study.

One of the interesting properties of the lobster SGC channel is its complex interaction with $\text{Na}^+$ and $\text{Ca}^{2+}$ ions. Opening of the channel evokes $\text{Na}^+$ and $\text{Ca}^{2+}$ influx into the cell that, in turn, potentially can serve as positive or negative (high intracellular $\text{Ca}^{2+}$) feedback for channel gating. It has been proposed that $\text{Na}^+$ and $\text{Ca}^{2+}$ transport systems, including nonselective cation channels, must closely communicate with NCXs in order to integrate $\text{Ca}^{2+}$ signaling in cells and/or control intracellular $\text{Na}^+$/Ca$^{2+}$ homeostasis (Rosker et al. 2004; Eder et al. 2005; Eder et al. 2007; Algara-Suarez et al. 2007). Indeed, using a glutathione S-transferase pull-down assay, Rosker and others (2004) found that NCX1 associates with the cytosolic C-terminus of TRPC3, suggesting such a functional interaction could be mediated by physical interaction between the TRPC channel and NCX proteins. The functional consequences of having an intimate linkage between the TRPC channel and NCX is not completely understood. It is still unclear, for instance, whether the linkage reflects direct interaction or interaction mediated through scaffolding proteins and, related to that, whether pharmacological intervention targeting one element of such a signaling complex could somehow disrupt the structural and/or more importantly the functional integrity of the whole complex. Evidence that KB-R7943 is a potent blocker of TRPC3, TRPC5 and TRPC6 channels (Kraft 2007), suggests that co-assembly with exchangers could be a general property of TRPC channels, possibly including the lobster SGC channel.

Yet, our data herein and elsewhere strongly suggest the inhibitor acts directly on the SGC channel and not via an NCX. First, of the broad spectrum of drugs known or suspected to modify NCX activity in other systems (Blaustein and Lederer 1999; Amran et al. 2003) - verapamil ($\text{IC}_{50} = 0.1-10 \ \mu\text{M}$, Erdreich et al. 1983), amiloride ($\text{IC}_{50} \sim 1 \ \text{mM}$), benzamil and 3',4'-dichloro-benzamil (DCBA) in a variety of animals including crustaceans ($\text{IC}_{50} \sim 3-30 \ \mu\text{M}$, Niggli and Lederer 1991; Danaceau and Lucero 2000; Wheatly et al. 2002), and diltiazem in crustaceans per se (Zhuang and Ahearn, 1998), none of the ones we tested - verapamil (10-200 $\mu\text{M}$, see also Zhainazarov et al. 1998), amiloride (2 $\text{mM}$, Bobkov and Ache 2007), DCBA (400 $\mu\text{M}$, Bobkov and Ache 2007) and diltiazem (400 $\mu\text{M}$, n=6 in situ and n=4 in inside-out) - noticeably changed the gating of the SGC channel and/or ORN activity in situ. Second, the inhibitory effect of KB-R7943 on the NCX was shown to be NCX substrate-dependent, especially $\text{Ca}^{2+}$ dependent (Iwamoto et al. 1996), suggesting that KB-
R7943 may compete with Ca$^{2+}$ to bind to the exchanger. In our experiments, however, neither changing extracellular (10 nM-13.6 mM) nor intracellular (10 nM-100 µM) Ca$^{2+}$ appeared to change the blocking effect of KB-R7943 (50-100 µM). Third, complete or partial replacement of monovalent ions did not significantly alter the effects of KB-R7943 blockade, excluding the possibility that an NCX is likely involved in mediating SGC channel activity. Finally, preliminary experiments using a newer blocker with greater selectivity for NCX, SN-6 (IC$_{50}$ = ~2 µM, Niu et al. 2007), tested at 5 µM had no effect on either the odorant-evoked discharge of the ORNs nor on the SGC channel recorded in inside-out patches. We conclude, therefore, that KB-R7943 acts directly on the channel and not by blocking an odor-induced NCX current.

An NCX has yet to be identified in lobster ORNs, although NCXs have been identified in ORNs of several phylogenetically diverse animals, including *Xenopus* (Jung et al. 1994), squid (Lucero et al. 2000), and rodents (Noé et al. 1997; Schulze et al. 2002; Pyrski et al. 2007). As the precise role of the exchanger in ORNs is unclear, however, they may not be expressed in all ORNs. Nor would they necessarily have the same function if they were. Whereas the Na$^+$/Ca$^{2+}$ exchange is proposed to be responsible for ~26% of the odor-induced current in squid (Danaceau and Lucero 2000), for example, it is reportedly involved in termination of the receptor current response in frog ORNs by returning the concentration of intracellular Ca$^{2+}$ to its basal level following odor stimulation (Reisert and Matthews 1998). Thus, while we cannot eliminate the possibility that lobster ORNs express an NCX, and indeed they may well, it does not appear that KB-R7943 acts through or in association with it.

In summary, we show that KB-R7943 is a potent inhibitor of the lobster SGC channel, and that it inhibits the odorant-evoked response of individual ORNs *in situ* in a manner consistent with its action on blocking the SGC channel. These findings extend the pharmacological profile of the lobster SGC channel and more specifically implicate the channel in lobster olfactory transduction. They also raise the possibility that KB-R7943 can be a useful tool to study chemosensory transduction in other systems where TRP channels, at least TRPC channels, have been implicated in creating or shaping the output of the receptor cells.
ACKNOWLEDGEMENTS

We thank Ms. Anna Mistretta-Bradley for technical assistance. This work was supported by the National Institute on Deafness and Other Communication Disorders through grant DC001655.
REFERENCES


Bobkov YV and Ache BW. Block by amiloride derivatives of odor-evoked discharge in lobster olfactory receptor neurons through action on a presumptive TRP channel. Chem Senses 32: 149-159, 2007a.


Sobolevsky AI and Khodorov BI. Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the Na\(^{+}\)/Ca\(^{2+}\) exchange inhibitor KB-R7943. *Neuropsychopharmacol* 38: 1235-1242, 1999.


Fig.1 KB-R7943 blocks the lobster SGC channel recorded in inside-out configuration
A. Representative inside-out single-channel recordings from a patch taken from the soma of a cultured lobster ORN showing the activation of Ca\(^{2+}\)-sensitive sodium-gated cation (SGC) channels. The patch contained 5 SGC channels. The pipette was filled with Na\(^+\)-based solution. In Li\(^+\)-based bath solution the SGC channel was almost totally inactive (Po ~ 0.03). To activate the channel the bath was switched to Na\(^+\)-based solution.
B. Inside-out recording from a different patch than A containing 7 SGC channels simultaneously opened in high Ca\(^{2+}\)/Na\(^+\) bath condition (referred as control in the figures) at -60 or +60 mV. At +60 mV the SGC current activity was inhibited by KB-R7943 but not at -60 mV. Current traces in higher time resolution show single-channel currents in presence of 50 \(\mu\)M of KB-R7943. The amplitude histogram corresponding to currents recorded in control and in the presence of KB-R7943 (50 \(\mu\)M) provided on the bottom of the panel indicates a decrease in channel activity but not in single channel current amplitude (12.20 ± 0.21 pA, n=30 in control solution and 12.02 ± 0.14 pA, n=46 in presence of KB-R7943). The inhibition was reversible with a time constant close to 5 s for this patch.

Fig.2 KB-R7943 blocks the lobster SGC channel in a dose- and voltage-dependent manner
A. Current-voltage (I-V) relationships determined from different patches recorded in control condition (grey trace) and in presence of 50 \(\mu\)M KB-R7943 (black trace) in different conditions of Ca\(^{2+}\) concentrations across the membrane. To generate I-V relationships, series of ramp protocols from -100 to +100 mV were applied. Whatever the internal and external Ca\(^{2+}\) concentrations, KB-R7943 appeared to strongly block the SGC current only at positive voltages. Slight reduction of the current seen at negative voltages in the presence of the drug appears to result from short interval between the ramps and incomplete recovery of the channel activity after preceding inhibition. Note, when pipette was filled with PS the reversal potential of SGC current shifted by ~+35 mV; corresponding corrections need to be done to directly compare voltage dependence of SGC channel conductance in different ion conditions.
B. Histogram of the averaged amplitude of the SGC current in presence of KB-R7943 normalized to the SGC current in control at +100 mV from several patches in the same internal and external Ca\(^{2+}\) concentrations as in Fig. 2A. The blockage of the SGC current at positive voltages by KB-R7943 was strongly present in all Ca\(^{2+}\) conditions across the membrane tested.
C. Concentration-dependence and voltage-dependence of the blocking effect of KB-R7943 on the SGC channel. The percent inhibition of the averaged I-V curves for different concentrations of KB-R7943 was plotted as a function of the positive voltages. The percent inhibition was calculated as \((\text{mean current in control} - \text{mean current in presence of KB-R7943})/\text{(current at +100 mV)}\)*100.
D. Concentration-inhibition curve for KB-R7943 on the mean amplitude of the SGC current at +100 mV. Averaged currents were normalized to the averaged current in absence of KB-R7943. Data points represent means ± s.e.m. of n=3-11 patches and were fit to a Hill equation providing an IC\(_{50}\) of 11.74 \(\mu\)M and a Hill coefficient 2.92. KB-R7943 blockage was reversible (n=13, histogram). Mesylate (\(\text{CH}_3\text{SO}_3\text{H}, 200 \, \mu\text{M}\)) alone did not block the SGC channel (n=2 both shown on the histogram). SN-6, a potent Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor, did not block the SGC current.


Fig. 3 KB-R7943 reversibly blocks the lobster SGC channel recorded in outside-out configuration

A. Outside-out patch recording from a cultured lobster ORN showing the inhibition of the lobster SGC channel activity by KB-R7943 in concentration dependent manner. The patch contained at least 11 SGC channels. Holding potential was -70 mV. The white dotted lines indicate the averaged current amplitude for each concentration of KB-R7943 tested for this particular example. The percentage of inhibition of the averaged current by KB-R7943 is shown above the dotted line. Current traces in higher time resolution allow observing single-channel currents in control condition and in presence of 50 µM of KB-R7943 (grey plots). Current traces and corresponding amplitude histogram provided on the right of the panel indicate a decrease in channel activity but not in single channel current amplitude (11.20 ± 0.18 pA, n=12 in control and 11.32 ± 0.11 pA, n=16 in presence of KB-R7943).

B. I-V relationships of the SGC current recorded before, during and after application of 50 µM KB-R7943. To generate I-V relationships, series of 150-ms ramp protocols from -120 to +100 mV were applied every 2 s. Two ramp currents are presented in the graph for each condition. The I-V relationships show the reversible blockage of the SGC current by KB-R7943.

C. Concentration-dependence of KB-R7943 effects. Averaged steady-state currents were normalized to the averaged current in absence of KB-R7943 (holding potential: -70 mV). Data points represent means ± s.e.m. of n=7-9 patches and were fit to a Hill equation providing an IC50 of 13.82 µM and a Hill coefficient of 1.32. KB-R7943 blockage was reversible (n=4; histogram). Mesylate (CH3SO3H, 200 µM) alone did not block the SGC channel (n=5; histogram).

Fig. 4 KB-R7943 reversibly blocks the odorant-evoked discharge of lobster ORNs in a dose-dependent manner

A. Representative raster displays of action potentials (APs) from a single lobster ORN recorded in the loose-patch configuration in response to increasing odor stimulus intensity (shortest duration at the top of each block) before (top), during (middle) and after (bottom) application of 50 µM of KB-R7943.

B. Normalized peak AP discharge frequency of the same ORN as in A plotted as a function of odor stimulus duration before, during and after application of 50 µM of KB-R7943. Data points were fitted to a Hill equation. KB-R7943 reversibly blocked the output of the cell. KB-R7943 did not change either spike shape or the spike peak-to-peak amplitude (212.7 pA in control vs 215.1 pA in presence of 50 µM KB-R7943, insets).

C. Representative raster displays of APs from a single lobster ORN in response to identical odor stimulus intensity in control condition (top), during applications of increasing concentrations of KB-R7943 (middle) and wash-out (bottom). KB-R7943 reversibly blocked the output of the cell in a dose dependent manner.

D. Concentration dependence of KB-R7943 effects on the odorant-evoked ORN discharge. Data points were fitted to a Hill equation providing an IC50 of 16.57 µM and a Hill coefficient of 1.74. Data points represent means ± s.e.m. of n=4-8 cells. SN-6, a potent Na+/Ca2+ exchanger inhibitor, did not block the odorant-evoked AP discharge.

Fig. 5 KB-R7943 reversibly blocks the odorant-evoked whole-cell current of lobster ORNs
A-B. Whole-cell recordings from lobster ORNs in situ held at -70 mV and stimulated by odorant pulse. A and B – different ORNs.
A. Representative odorant-induced whole-cell currents in response to increasing stimulus intensity (160 to 800-ms odorant pulse) in control and the corresponding peak current amplitude plotted in function of odorant stimulus.
B. Representative odorant-induced whole-cell currents in response to constant 200-ms odorant pulse recorded before, during and after application of 50 µM of KB-R7943.
C. Histograms showing the averaged peaks of whole-cell currents measured before, during and after application of 50 µM of KB-R7943. Averaged peak currents were normalized to the averaged peak current in absence of KB-R7943. Data points represent means ± s.e.m. KB-R7943 greatly reduced the whole-cell peak current compared to control (paired t-test, P<0.001).
Fig. 4

A  odor stimulus
(from 60 to 420 ms)

B  Normalized AP frequency
vs. Stimulation duration (ms)

C  odor stimulus
(200 ms)

D  Normalized AP frequency
vs. KB-R7943 (µM)