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Block by Amiloride Derivatives of Odor-Evoked Discharge in Lobster Olfactory Receptor Neurons through Action on a Presumptive TRP Channel

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Abstract

Amiloride and its derivatives inhibit a number of sensory transduction processes, including some types of chemosensory transduction. Here, we report that pyrazine derivatives of amiloride reversibly inhibit odorant-evoked activity in lobster olfactory receptor neurons. The potency sequence is as follows—(IC50, mM): 5-(*N*,*N*-hexamethylene)amiloride (0.015) ~ 5-(*N*-methyl-*N*-isobutyl)amiloride (0.02) ~ 5-(*N*-ethyl-*N*-isopropyl)amiloride (0.03) > 5-(*N*,*N*-dimethyl)amiloride (0.48); 3′,4′-dichlorobenzamil (0.4), phenamil (0.5), and amiloride itself (2) are ineffective. The same derivatives with the similar potency sequence also block a presumptive transient receptor potential (TRP) channel that is the likely downstream target of phosphoinositide signaling in these cells. Our results suggest that pyrazine derivatives of amiloride are useful probes to study more detailed mechanisms of chemosensory transduction in this system and possibly in other chemosensory systems in which TRP channels are the known or suspected downstream effector.

Key words: inhibition, invertebrates, olfaction, phospholipid signaling, pyrazinecarboxamides, sensory systems

Introduction

Phosphoinositide signaling plays an important role in several types of chemosensory transduction. Vertebrate taste receptor cells for bitter, sweet, and possibly amino acids signal through a common transient receptor potential (TRP) ion channel, TRPM5, and phospholipase C (PLCβ2) (Margolskee 2002; Liu and Liman 2003; Perez et al. 2003; Zhang et al. 2003; Clapp et al. 2004). The vomeronasal organ (VNO), that plays an essential role in the detection of pheromones (Dulac and Torello 2003; Trinh and Storm 2003) in vertebrates, likely relies on phosphoinositide signaling because the TRPC2 channel gene expressed in all VNO receptor cells (Liman et al. 1999) is essential for VNO function (Leypold et al. 2002; Stowers et al. 2002). Most members of the TRPC subfamily appear to be regulated via the canonical phosphatidylinositol turnover pathway, although details of VNO activation are still being resolved (Brann et al. 2002; Cinelli et al. 2002; Spehr et al. 2002; Liman 2003; Lucas et al. 2003). Whether phosphoinositide signaling plays a role in the main olfactory organ of vertebrates where cyclic nucleotide signaling mediates activation is less clear, although there is some evidence that it might (Spehr et al. 2002).

The involvement of phosphoinositide signaling in olfaction is better understood in invertebrate (lobster) olfactory receptor neurons (ORNs). In what could be an interesting parallel to invertebrate phototransduction (e.g., Ranganathan et al. 1995), activation of lobster ORNs is primarily mediated by phosphoinositide signaling (e.g., Fadool and Ache 1992). The outer dendrites of the cells express the major elements of the canonical turnover pathway, including a Gαq, PLCβ, and an inositol 1,4,5-trisphosphate receptor (IP_3R) (McClintock et al. 1997; Munger et al. 2000). An IP_3R and PLC activity can be functionally localized to the outer dendrites (Boekhoff et al. 1994; Hatt and Ache 1994), and PLCβ associates with G-proteins in response to odorants (Xu and McClintock 1999). There is also an emerging, but still to be understood, role of phosphatidylinositide 3-kinase (PI3K)–mediated signaling in these ORNs (Zhainazarov et al. 2001). A potential downstream target of phosphoinositide signaling in lobster ORNs is a sodium-gated nonselective cation (SGC) channel (McClintock and Ache 1990; Zhainazarov and Ache 1995, 1997) that contributes to the generation of a substantial part of the depolarizing receptor

potential (Zhainazarov et al. 1998). The channel, a presumptive member of the growing family of TRP channels, can be modulated by exogeneous phosphoinositides in cell-free patches (Zhainazarov and Ache 1999; Zhainazarov et al. 2001), but the channel has yet to be established as a target of phosphoinositide signaling in situ.

Toward that end, we screened compounds typically used to block TRP channel activity and found that the SGC channel is antagonized by 2-aminoethoxydiphenyl borate, SKF96365, Gd^{3+}, and La^{3+} and showed that these compounds also block odor-evoked activity in lobster ORNs in situ (Bobkov and Ache 2005). As these compounds can act nonspecifically, we continued our effort to identify potentially more specific blockers of the channel by testing the effects of amiloride and its derivatives. Although amiloride typically is not considered as a probe for TRP channels, it has been reported to block some members of the mammalian TRPC (Inoue et al. 2001; Maroto et al. 2005; Alexander et al. 2006), TRPML (Raychowdhury et al. 2004), TRPP (Gonzalez-Perrett et al. 2001; Delmas et al. 2004), and TRPA subfamilies (Nagata et al. 2005). Amiloride is one of a group of more than 1000 compounds collectively called pyrazinecarboxamides, some of which act with greater affinity (up to several orders of magnitude) and specificity than amiloride itself. Introduction of hydrophobic substituents on the terminal nitrogen of the guanidino moiety of amiloride enhances activity against epithelial sodium channels (ENaCs, Kleyman and Cragoe 1988), whereas addition of hydrophobic (or hydrophilic) groups on the 5-amino moiety of amiloride improves activity against the Na^+/H^+ exchanger (NHE, Kleyman and Cragoe 1988; Masereel et al. 2003), suggesting that the pyrazinecarboxamides are useful tools to characterize and discriminate different sodium and sodium-dependent ion transport systems, potentially including the lobster SGC channel.

Here, we report that some 5-amino substituents (pyrazine derivatives) of amiloride reversibly inhibit odorant-evoked activity in lobster ORNs although amiloride itself is ineffective and that the same derivatives with the similar potency sequence block the SGC channel. Our results suggest that pyrazine derivatives of amiloride are useful probes to study detailed mechanisms of chemosensory transduction in this system and possibly in other chemosensory systems in which TRP channels are the known or suspected downstream effector.

Materials and methods

Preparations and recording

We used 2 different preparations of spiny lobster (*Panulirus argus*) ORNs. The action potential discharge of intact ORNs in situ was studied using a modification of a preparation developed and described earlier (Doolin and Ache 2002). The modification allowed separate superperfusion streams to bathe the somata and the outer dendrites (transduction zone) of the ORNs. The somata were continuously bathed with *Panulirus* saline (PS, see Solutions and drugs). The outer dendrites of the ORNs were continuously bathed with either PS or PS containing a drug. The outer dendrites were incubated in a drug for a minimum of 1 min prior to testing the effect of the drug. The outer dendrites were transiently stimulated with odorant (see Solutions and drugs) pulses, the duration of which was controlled using a 9-channel rapid solution changer (RSC-100/160, Bio-Logic, Claix, France). The duration of the pulses varied from 40 to 800 ms, with pulse duration used to regulate odorant intensity. Patch pipettes were fabricated from borosilicate capillary glass (Sutter Instrument Co., Sunnyvale, CA, BF150-86-10) using a flaming-brown micropipette puller (P-87, Sutter Instrument Co.) and filled with PS. Extracellular currents were measured with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Novato, CA) through a digital interface (Digidata 1320A, Axon Instruments), low-pass filtered at 5 kHz, and sampled at 1–50 kHz. The discharge rates of individual cells in multicellular recordings were estimated using template search procedure provided by the pCLAMP 9.0 software. The intensity of the response of a cell to odor stimulation was quantified as the mean frequency of discharge during the 2-s interval following stimulus onset. All responses were normalized to the response intensity measured during the same interval in control conditions (no drug present) for the cell in question.

The effect of the drugs on the SGC channel was studied in cell-free membrane patches obtained from cultured lobster ORNs. The cultured cells were prepared as described previously (Fadool et al. 1991). Membrane patches containing SGC channels were excised from the cells and recorded in the inside-out configuration. Solutions were delivered to the inner face of the patch using a 9-channel RSC-100/160 (Bio-Logic). Patch pipettes were fabricated as for extracellular recording, only filled with low-calcium sodium solution (see Solutions and drugs). Currents were measured with an Axopatch 200B patch-clamp amplifier (Axon Instruments) through a digital interface (Digidata 1320A, Axon Instruments), low-pass filtered at 5 kHz, sampled at 20 kHz, and digitally filtered at 1–1.4 kHz. Channel activity was investigated in steady state conditions at a holding potential of −70 mV unless otherwise noted. The polarity of the currents is presented conventionally, that is, relative to intracellular membrane surface, in spite of the membrane patch configuration.

Data in both instances were collected and analyzed with pCLAMP 9.0 software (Axon Instruments) in combination with SigmaPlot 8.02 (SPSS, Inc., Chicago, IL). Two modifications of the Hill equation were used to fit the experimental data: 1) $F(x) = F_{max} * x^h / (x_{1/2}^h + x^h)$ for activation and 2) $F(x) = 1 - F_{max} * x^h / (x_{1/2}^h + x^h)$ for inhibition, where F is the open probability, normalized current, or frequency of action potentials, x is the agonist/antagonist concentration, $x_{1/2}$ is the half-effective agonist/antagonist concentration, and h is the Hill coefficient. An additional parameter reflecting the

basal level of F (Fb) was incorporated when necessary. The data are presented as the mean ± standard error of mean of n observations unless otherwise noted. All recordings were performed at room temperature (~21 °C).

Solutions and drugs

PS contained (mM) 486 NaCl, 13.4 KCl, 13.6 $CaCl_2$, 9.8 $MgCl_2$, 2 glucose, and 10 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.9–8.0. Low-calcium sodium solution contained (mM) 210 NaCl, 1 ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 0.1 $CaCl_2$, 696 glucose, and 10 HEPES, pH 7.9–8.0. Low-calcium lithium solution consisted of (mM) 210 LiCl, 1 EGTA, 0.1 $CaCl_2$, 696 glucose, and 10 HEPES, pH 7.9–8.0. The estimated free calcium concentration ($[Ca^{2+}]_{free}$) in low-calcium sodium/lithium solutions was ~1 nM. Solutions containing 100 μM Ca^{2+} were prepared without chelating agents. The pH was adjusted with Tris-base or NaOH. The odorant in all cases was an aqueous extract of Tetra-Marin (TET, Tetra Werke, Melle, Germany), a commercially available fish food, prepared as described earlier (Schmiedel-Jacob et al. 1990). The maximum concentration represented 0.1–0.5 mg of the dried powder dissolved in 1 ml PS. The following drugs were tested as potential blockers (Figure 1): amiloride hydrochloride hydrate (amiloride), 5-(*N*,*N*-dimethyl)amiloride hydrochloride (DMA), 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA), 5-(*N*,*N*-hexamethylene)amiloride (HMA), phenamil methanesulfonate salt (phenamil), and 3′,4′-dichlorobenzamil hydrochloride (DCBA). All drugs were obtained from Sigma-Aldrich, Inc., St. Louis, MO. Drugs were dissolved in dimethylsulphoxide (DMSO) to give 100–200 mM stock solutions.

Results

Effect of amiloride and its derivatives on ORNs in situ

All ORNs used in this study were tonically active and gradually increase their rate of discharge in a concentration-dependent manner to odorants (e.g., Figure 2A). Because all drugs were solubilized initially in DMSO, we first examined whether the solvent itself affected ORN activity. One

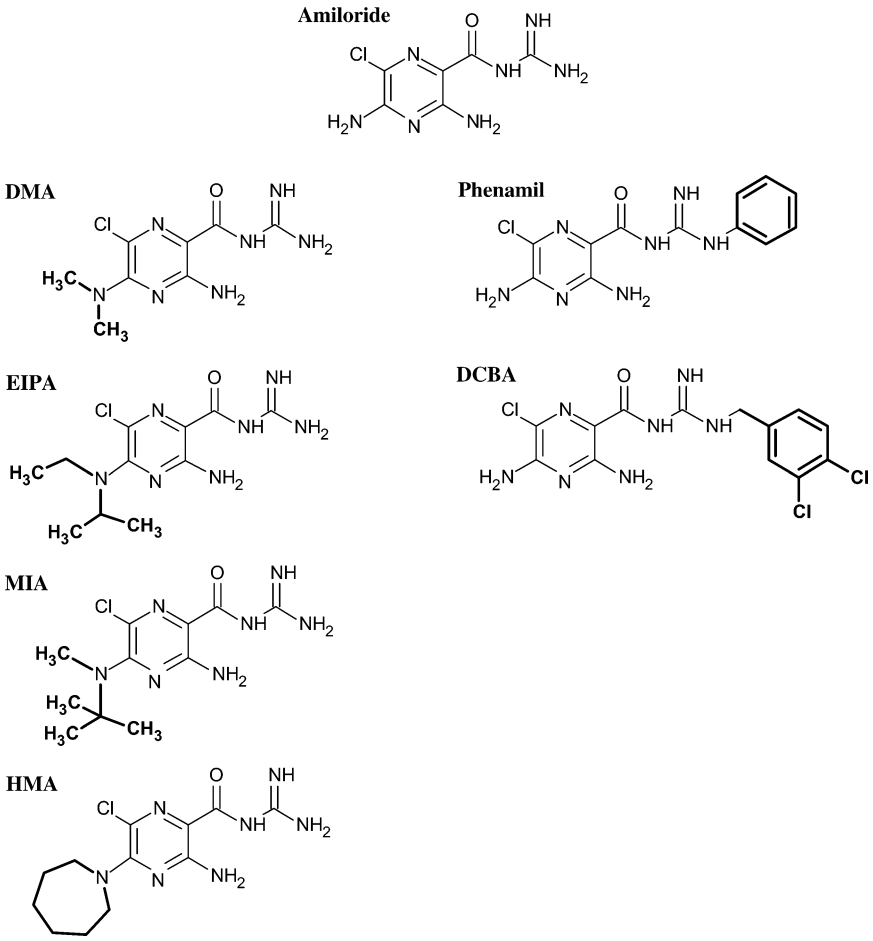

Figure 1 Chemical structures of amiloride and of its derivatives with modifications at the 5 position of the pyrazine ring (DMA, EIPA, MIA, and HMA) and the guanidinium group (phenamil, DCBA).

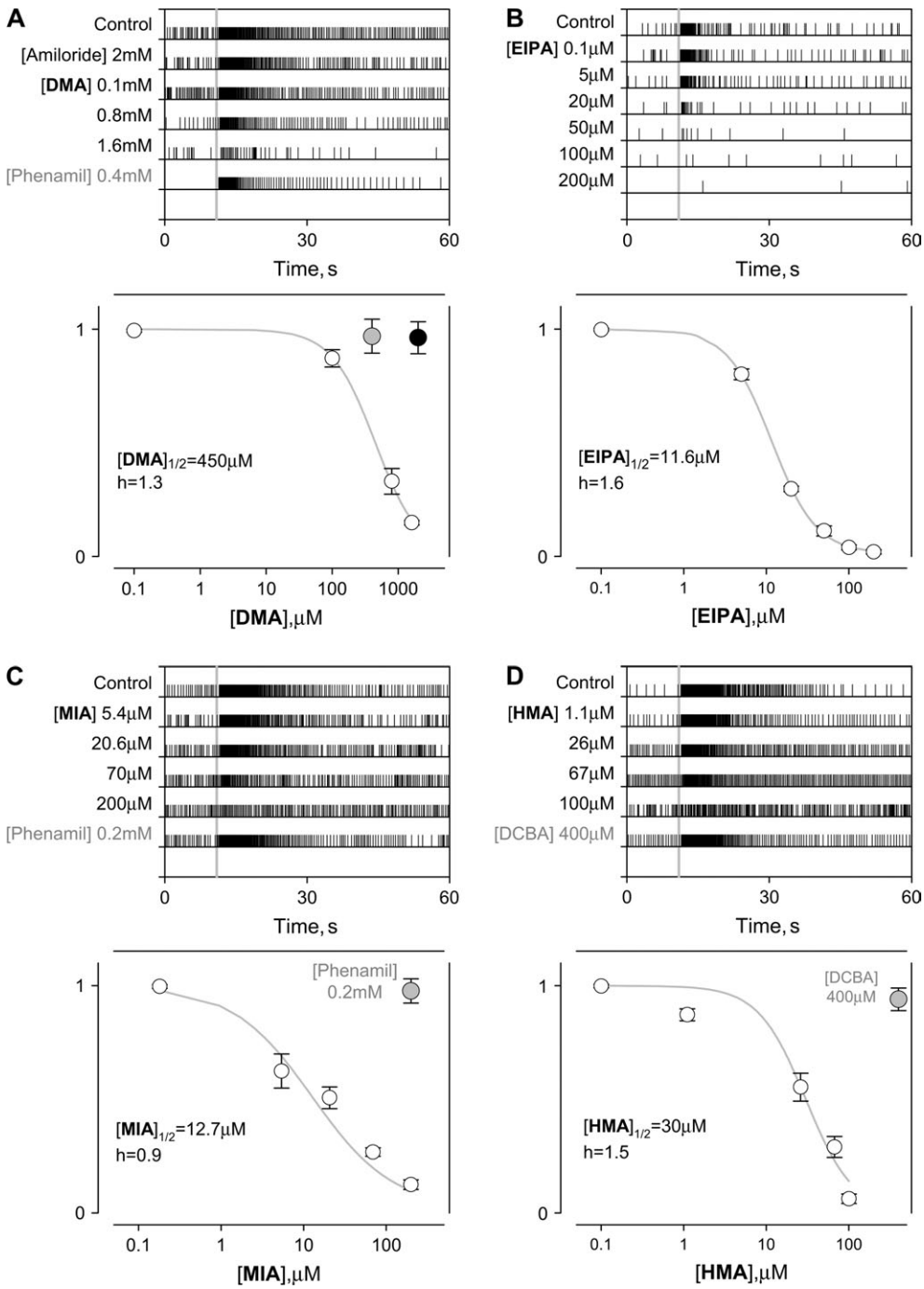

Figure 2 The effect of the amiloride derivatives on lobster ORNs in situ. **(A)** DMA, **(B)** EIPA, **(C)** MIA, and **(D)** HMA. The top panel in each part shows raster displays of individual action potentials from a single ORN in response to odorant pulses of the same intensity following exposure to the drug noted to the left of the sweeps. The lower panel in each part shows a plot of the normalized odor responses as a function of drug concentration. Each point represents the mean ± standard error of mean of 3–5 measurements. Solid line: Hill plot with the following constants: $[DMA]_{1/2}$ = 450 ± 71 μM, h = 1.3 ± 0.13; $[EIPA]_{1/2}$ = 11.6 ± 0.03 μM, h = 1.6 ± 0.05; $[MIA]_{1/2}$ = 12.7 ± 1.8 μM, h = 0.9 ± 0.1; $[HMA]_{1/2}$ = 30 ± 3.4 μM, h = 1.5 ± 2.3. Note: amiloride 2 mM (A, second trace; low panel, black circle), phenamil 0.4 mM (A, sixth trace; low panel, gray circle) and 0.2 mM (C, sixth trace; low panel, gray circle), and DCBA 0.4 mM (D, sixth trace; low panel, gray circle) do not change the odor-evoked activity of the ORNs.

percent DMSO, the maximum concentration used, applied to the outer dendrites as an ''odorant'' slightly increased the spontaneous discharge of 11 of 12 cells tested by 0.74 ± 0.24 Hz (n = 11). Given that neither did DMSO had noticeable effect on the spontaneous discharge of the cells at more dilute concentrations nor did it noticeably alter odor-evoked

ORN activity at any concentration (data not shown), we assumed any effects of the drugs were not solvent dependent.

Amiloride itself (0.05–2 mM) did not appreciably change the spontaneous activity or the odor-evoked response, as shown for one cell in Figure 2A (top panel, top 2 traces). For all cells tested, 2 mM amiloride, the highest concentration tested, changed the spontaneous activity from 2.09 ± 0.3 to 2.38 ± 0.48 Hz (n = 7), and the normalized intensity of the odor-evoked response to 0.96 ± 0.07 (n = 6) of control (Figure 2A, bottom panel, black circle), that is, had essentially no effect.

To investigate whether modification at the 5 position of the pyrazine ring changed the activity of amiloride, we first tested DMA (Figure 1). DMA at the highest concentration tested (1.6 mM) reduced both the spontaneous activity and the odor-evoked response, as shown for one cell in Figure 2A (top panel, third through fifth traces). DMA (1.6 mM) reduced the spontaneous activity in 5 of 6 cells from 2.65 ± 0.46 to 1.83 ± 0.53 Hz. The effect on the evoked response was concentration dependent and reversible, with an apparent inhibition constant, $[DMA]_{1/2}$, of 450 μM and a Hill coefficient, h, of 1.3 (Figure 2A, lower panel).

Analogs with a more complex alkyl (e.g., EIPA, MIA) or cycloalkyl (e.g., HMA) substitution on the 5-amino nitrogen atom (Figure 1) more effectively blocked ORN activity than did DMA. Like DMA, EIPA also suppressed both the spontaneous and odor-evoked activities, as shown for one cell in Figure 2B (top panel). EIPA at the highest concentration (200 μM) suppressed spontaneous activity in 7 of 8 cells tested from 2.07 ± 0.7 to 0.7 ± 0.3 Hz and also inhibited the odor-evoked response by 62–100%, with an average 84.2 ± 2.7% (n = 24). EIPA reversibly inhibited the odor-evoked response in a concentration-dependent manner, with an apparent inhibition constant, $[EIPA]_{1/2}$, of 11.6 μM and Hill coefficient, h, of 1.6 (Figure 2B, lower panel).

Like EIPA, MIA also suppressed both the spontaneous and odor-evoked activities, as shown for one cell in Figure 2C (top panel, second through fifth traces). MIA at the highest concentration tested (200 μM) suppressed spontaneous activity in 7 of 10 cells tested from 2.47 ± 0.46 to 1.18 ± 0.47 Hz and also inhibited the odor-evoked response by 64–100%, with an average 87.2 ± 1.8% (n = 26). MIA reversibly inhibited the odor-evoked response in a concentration-dependent manner, with an apparent inhibition constant, $[MIA]_{1/2}$, of 12.7 μM and Hill coefficient, h, of 0.9 (Figure 2C, low panel).

Like MIA, HMA also suppressed both the spontaneous and odor-evoked activities, as shown for one cell in Figure 2D (top panel, second through fifth traces). HMA at the highest concentration tested (200 μM) suppressed spontaneous activity in 8 of 11 cells tested from 2.61 ± 0.56 to 0.9 ± 0.41 Hz and also inhibited the odor-evoked response by 71–100%, with an average 86 ± 2.6% (n = 10). HMA reversibly inhibited the odor-evoked response in a concentration-dependent manner, with an apparent inhibition constant, $[HMA]_{1/2}$, of 30 μM and a Hill coefficient, h, of 1.5 (Figure 2D, lower panel).

To investigate the potential importance of the carbonyl-guanidino moiety in blocking activity, we tested 2 derivatives of amiloride in which terminal guanidino nitrogen atom is substituted with the hydrophobic phenyl (phenamil) and benzyl (e.g., DCBA) groups (Figure 1). Phenamil suppressed the spontaneous, but not the odor-evoked, activity as shown for one cell in Figure 2A (top panel, sixth trace). Phenamil at the highest concentration tested (400 μM) suppressed the spontaneous activity in 4 of 5 cells from 3.09 ± 1.08 to 1.3 ± 0.47 Hz. Phenamil, however, did not affect the odor-evoked response in any cell tested. The intensity of the normalized response was 0.97 ± 0.07 (n = 4) and 0.98 ± 0.05 (n = 4) following treatment with 0.4 and 0.2 mM phenamil, respectively, compared with control (Figure 2A,C, lower plots, gray circles).

DCBA, in contrast, slightly increased the spontaneous activity, although like phenamil it had no or at best a minimal effect on the odor-evoked response, as shown for one cell in Figure 2D (top panel, sixth trace). DCBA at the highest concentration tested (400 μM) increased the spontaneous activity from 2.5 ± 0.46 to 3.3 ± 0.7 Hz (n = 4). The intensity of the normalized response, however, was only 0.94 ± 0.05 (n = 4) of control following treatment with DCBA. (Figure 2D, lower plot, gray circle).

To summarize the relative effect of the different amiloride analogs tested on the odor-evoked activity of lobster ORNs, we compared data from 56 cells normalized to the maximum response intensity of the particular cell in control conditions, yielding data from 3–26 cells for any one point (Figure 3). Overall, the inhibition parameters of the analogs tested were $[DMA]_{1/2}$ = 478 ± 89 μM, h = 1.4 ± 0.17; $[EIPA]_{1/2}$ = 28 ± 18 μM, h = 1 ± 0.4; $[MIA]_{1/2}$ = 22 ± 18 μM, h = 0.8 ± 0.3; $[HMA]_{1/2}$ = 14.8 ± 6 μM, h = 1.1 ± 0.4 (Figure 3), indicating that activation is effectively blocked by amiloride derivatives with modifications at the 5 position of the pyrazine ring with a potency sequence of (IC50, μM) HMA(15) ~ MIA (22) ~ EIPA (28) > DMA (480) but is relatively insensitive to modification of the carbonilguanidino moiety.

Effect of amiloride and its derivatives on the SGC channel

To determine whether the SGC channel is a potential target of one or more of the derivatives that blocked odor-evoked activity in the cells in situ, we tested those compounds on cell-free membrane patches taken from cultured lobster ORNs containing SGC channels. Overall, we collected data from 36 patches and tested each derivative on 3–8 of the patches. We verified that all channels tested were calcium sensitive, which is the form of the channel that predominates in transduction zone of the cells in situ (Bobkov and Ache 2003). The channels were first activated by applying sodium (210 mM) from the intracellular side of the membrane, which reversibly activates the channel with $K_{1/2}$ = 113 mM for 10 nM Ca^{2+}

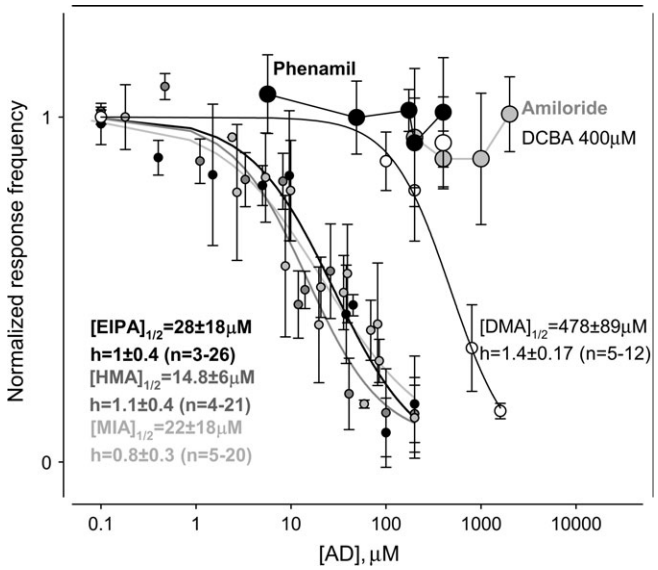

Figure 3 Summary plot of the effects of the amiloride derivatives on lobster ORNs in situ. Each data point is a mean ± standard deviation of the normalized responses of 3–26 cells. Total number of cells tested, 53. Solid lines, Hill plots equation with following constants: $[DMA]_{1/2}$ = 478 ± 89 μM, h = 1.4 ± 0.17, n = 5–12; $[EIPA]_{1/2}$ = 28 ± 18 μM, h = 1 ± 0.4, n = 3–26; $[MIA]_{1/2}$ = 22 ± 18 μM, h = 0.8 ± 0.3, n = 5–20; $[HMA]_{1/2}$ = 14.8 ± 6 μM, h = 1.1 ± 0.4, n = 4–21. Note: amiloride, phenamil, and DCBA do not have appreciable effects at the concentrations tested.

and with $K_{1/2}$ = 29 mM for 100 μM Ca^{2+} (Bobkov and Ache 2003, 2005), and then the drugs were tested for their ability to block SGC channel activity.

All four 5-amino substituents that reversibly suppressed activity in the cells in situ also reversibly blocked the channel in a concentration-dependent manner, DMA (Figure 4A), EIPA (Figure 4B), MIA (Figure 4C), and HMA (Figure 4D). In all cases, the drugs decreased the open probability of the channel without affecting the single channel conductance, as evidenced by the constant unitary current amplitude in the original traces. As in the intact cells, the binding affinity of DMA was relatively low (IC50 = 168 μM, Figure 4A) compared with that of EIPA, MIA, and HMA (IC50 = 45, 44, and 57 μM, respectively, Figure 4B,C,D). Also as in the intact cells, amiloride, phenamil, and DCBA (all, 200 μM) had no effect on the channel at normal membrane potential (−70 mv) (Figure 5A). However, at more depolarized potentials, both amiloride and phenamil blocked the channel, with amiloride being more effective at blocking the channel than phenamil (Figure 5B).

The inhibition parameters of the analogs tested determined from the concentration dependence of their effectiveness as approximated by their corresponding Hill equations (see Materials and methods) were $[DMA]_{1/2}$ = 199 ± 58 μM, h = 1.3 ± 0.4; $[EIPA]_{1/2}$ = 33 ± 5 M, h = 1.4 ± 0.2; $[MIA]_{1/2}$ = 50 ± 4 μM, h = 1.9 ± 0.3; $[HMA]_{1/2}$ = 34 ± 4.6 μM, h = 1.8 ± 0.3, with amiloride, phenamil, and DCBA being effectively inactive at physiologically relevant voltages (Figure 6). This is in agreement with the potency sequence of the same analogs when tested on the intact cell, that is, (IC50, μM) EIPA(33) ~ HMA(34) ~ MIA(50) > DMA(200).

Discussion

We show that pyrazine derivatives of amiloride reversibly inhibit odorant-evoked activity in lobster ORNs with a potency sequence of (IC50, mM) HMA (0.015) ~ MIA (0.02) ~ EIPA (0.03) > DMA (0.48), whereas 3′,4′-dichlorobenzamil (0.4), phenamil (0.5), and amiloride itself (2) are relatively ineffective. We also show that the same derivatives block the SGC channel, a presumptive TRP channel that is the likely downstream target of phosphoinositide signaling in these cells and that they do so with a similar potency sequence. These 2 findings taken together are consistent with the idea that the SGC channel plays a significant role in odor signal transduction in intact lobster ORNs.

The amiloride derivatives found to suppress ORN activity and block the SGC channels also block other ion-transporting proteins that potentially could contribute to the overall activation of the cell, allowing that the SGC channel may not be the sole target of pharmacological action. These include ion channels of the degenerin/epithelial Na^+ channels (DEG/ENaC) family thought to be involved in gustatory detection of salty and sour tastes in both vertebrates and invertebrates (Ugawa et al. 1998; Lin et al. 1999; Bigiani et al. 2003; Liu et al. 2003) and the perception of female pheromones by male *Drosophila* (Lin et al. 2005). However, the most specific inhibitors of DEG/ENaC channels are blocked by amiloride itself and amiloride analogs bearing hydrophobic substituents on the terminal nitrogen atom of the guanidino moiety (e.g., DCBA, benzamil, Phenamil), analogs we found to have little or no potency in the present study. For example, ENaCs are blocked with the potency sequence (IC50, μM): benzamil (structurally close to DCBA, 0.01–0.4) > phenamil (0.02) > amiloride (0.1–2.6) > DMA (8.5) ~ HMA (>8.5) > EIPA (400) (based on Kleyman and Cragoe 1988; Voilley et al. 1994; Kellenberger and Schild 2002), and different isoforms of acid-sensitive ion channel can be similarly blocked by low concentrations of amiloride (0.15 to >100 μM) and benzamil (1.6–16 μM; Waldmann et al. 1996, 1997; Kellenberger and Schild 2002).

The amiloride derivatives found to suppress ORN activity and block the SGC channels also block ubiquitously expressed transport proteins that maintain ionic homeostasis in cells, such as the NHE and the sodium–calcium exchanger (NCX). The potency sequence differs from what we found in the present study; NHE is blocked with a potency sequence of (IC50, μM) EIPA (0.01–2.4) ~ HMA (0.013–2.4) > DMA (0.23–14) > amiloride (1–100) > benzamil (>1000) > phenamil (>8000) (Kleyman and Cragoe 1988; Masereel et al. 2003), whereas NCX is blocked in a similar sequence but with a significantly lower inhibitory potency: EIPA (83) ~ MIA (84) > HMA (100) > phenamil (200) > DMA (550) > amiloride

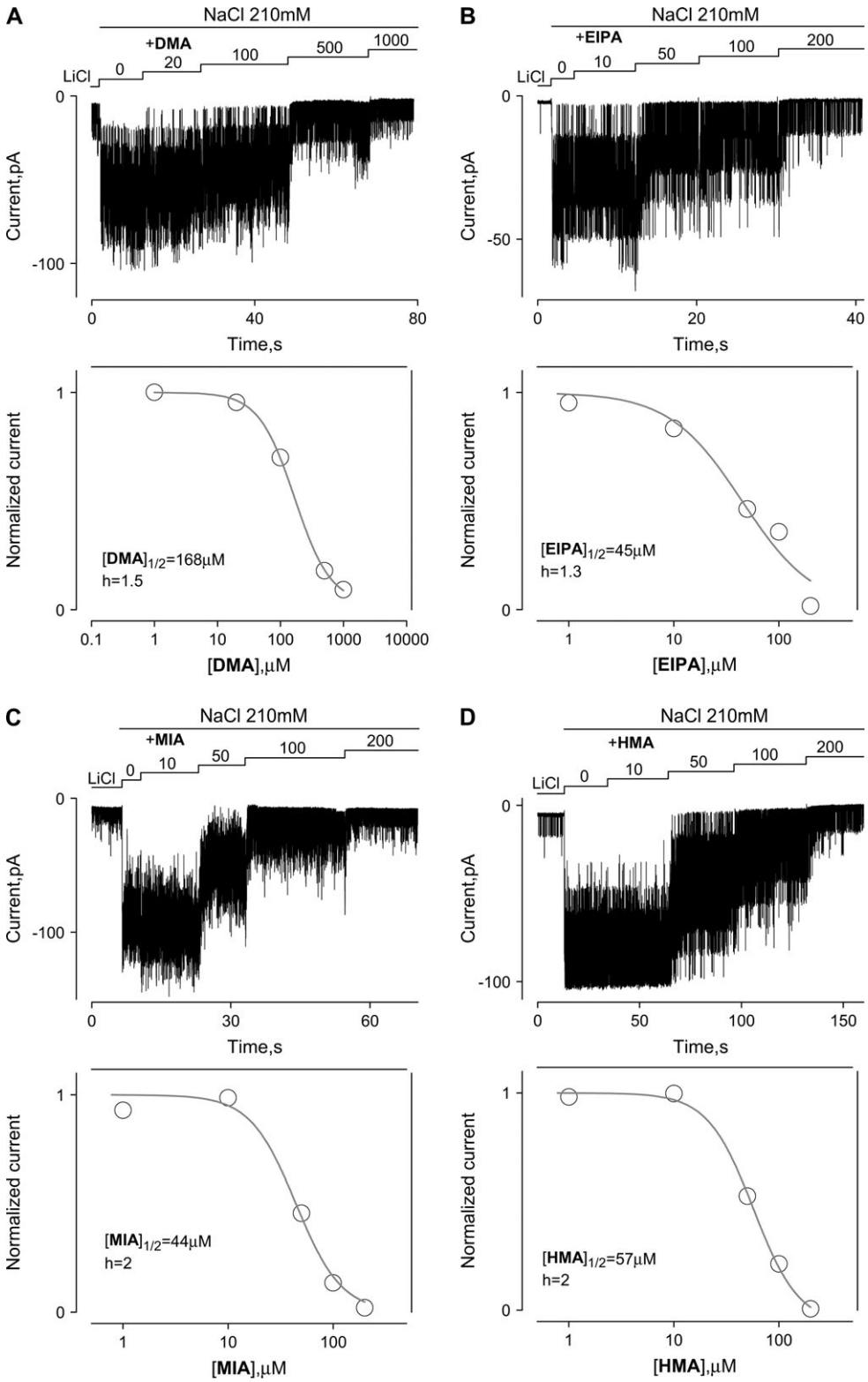

Figure 4 The effect of some amiloride derivatives on the lobster SGC channel. **(A)** DMA, **(B)** EIPA, **(C)** MIA, and **(D)** HMA. The top panel in each part shows current traces from a single patch containing SGC channels exposed to different concentrations of the drug (noted on line above traces) following activation with 210 mM NaCl. Note: the absence of any change in single channel amplitude. The lower panel in each part shows the Hill plot of the normalized mean current (ordinate) plotted as a function of drug concentration (abscissa) and fit with the Hill equation (solid line). Error bars represent standard error of mean. Inside-out patch recordings. Holding potential, −70 mV. Electrode solution, NaCl 210 mM + Ca^{2+} 10 nM. Current/time scales are different in all portions of the figure. **LiCl**—LiCl 210 mM + Ca^{2+} 10 nM; **NaCl**—NaCl 210 mM + Ca^{2+} 10 nM.

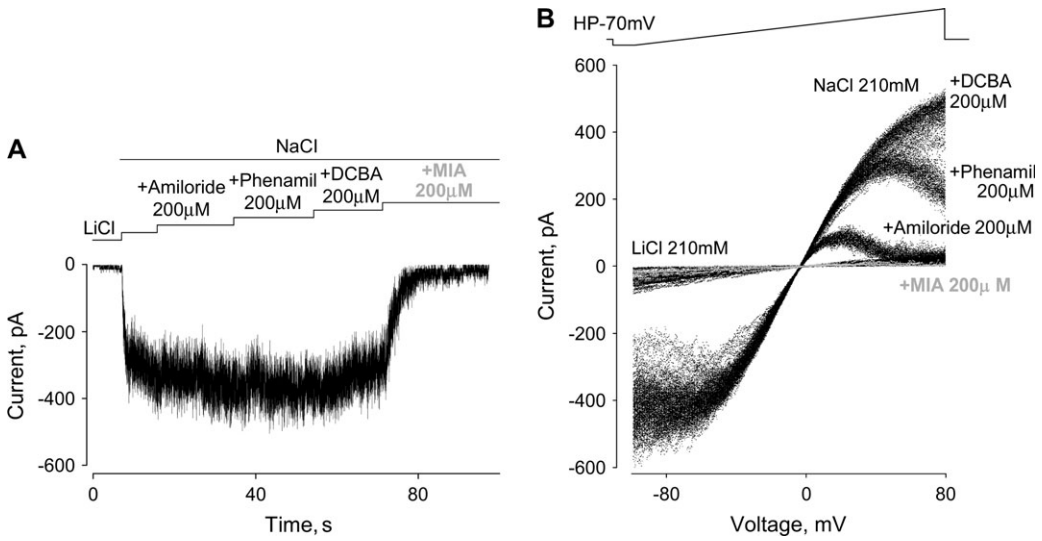

Figure 5 The effect of some amiloride derivatives on the lobster SGC channel. **(A)** Current traces from a single patch containing SGC channels exposed to different drugs (200 μM, noted on line above traces) following activation with 210 mM NaCl. Note: the channel appears to be insensitive to amiloride, phenamil, and DCBA but is blocked by MIA. Holding potential, −70 mV. **(B)** Current–voltage characteristics obtained in response to a voltage ramp from the same inside-out patch as in (A) in the presence of the derivatives noted (200 μM). Note: amiloride and phenamil inhibit the SGC channel at positive voltages. Forty ramps were generated in the presence of each drug and superposed on the graph. Every graph dot corresponds to single sampling value. Visible discrete current levels correspond to current through a number of simultaneously open SGC channels. Voltage protocol: 30 ms hyperpolarizing step to −100 mV preceding a linear 200 ms voltage changing (ramp). Electrode solution, NaCl 210 mM + Ca^{2+}_{free} 10 nM.

(700–1100) (Kleyman and Cragoe 1988; Murata et al. 1995; Rogister et al. 2001).

Although differences in pharmacological potency are not definitive by themselves because multiple factors can affect the apparent inhibition constants, including membrane potential, sodium concentration, pH, divalent cation concentration, and subunit composition (Kleyman and Cragoe 1988; Kellenberger and Schild 2002; Masereel et al. 2003; Ugawa et al. 2003; Linqueglia et al. 2006), it remains that the pyrazine derivatives of amiloride found to be most potent in the present study (EIPA, MIA, HMA, DMA) selectively target ion exchangers and transporters, including NHE and NCX. Thus, we cannot exclude the potential involvement of transporters based strictly on the pharmacology. One could envision, for example, that blockade of NHE would acidify the outer dendritic compartment of the cells and indirectly affect the SGC channel, which we reported earlier is pH sensitive (Bobkov and Ache 2003, 2005).

Amiloride and some of its derivatives interact with ion transport systems only in their protonated form (Kleyman and Cragoe 1988). Amiloride and its derivatives studied herein are weak bases, with pKa values 8.7 (amiloride); 8.8 (DMA); 8.5 (HMA); 7.8 (phenamil); 7.63 (DCBA). Based on Henderson–Hasselbach equation, the probabilities of these compounds being protonated at physiologically relevant pH for lobster ORNs (the pH of seawater, 8.0) would be 0.83, 0.86, 0.76, 0.4, 0.3, respectively. Lowering the pH to 7.2–7.4, the pH typically used in other studies, would significantly increase the probability of these compounds being protonated (particularly for phenamil 0.7 and DCBA 0.6)

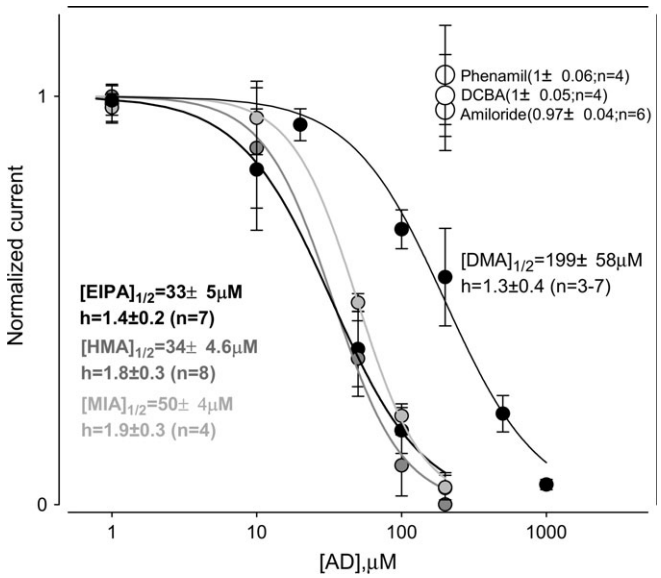

Figure 6 Summary plot of the effects of the amiloride derivatives on the SGC channel. Each data point is a mean ± standard deviation of normalized current obtained from 3–8 patches. Solid lines, Hill plots with following constants: $[DMA]_{1/2}$ = 199 ± 58 μM, h = 1.3 ± 0.4, n = 3–7; $[EIPA]_{1/2}$ = 33 ± 5 μM, h = 1.4 ± 0.2, n = 7; $[MIA]_{1/2}$ = 50 ± 4 μM, h = 1.9 ± 0.3, n = 4; $[HMA]_{1/2}$ = 34 ± 4.6 μM, h = 1.8 ± 0.3, n = 8. Note: amiloride, phenamil, and DCBA have only minimal effect.

and potentially change the potency profiles we measured. However, we were not able to test this possibility because the SGC channel itself is directly blocked by protons, both

extracellularly and intracellularly, with an IC50 close to pH 7.3–7.6 (Bobkov and Ache 2003, 2005).

Whereas the drugs acted on the intact cells and the SGC channel with the same potency sequence, they acted with lower apparent inhibition constants on the cells in situ than they did on the channels in cell-free membrane patches. This difference can be explained, however, by the difference in sodium and/or divalent cation concentrations in the 2 cases, as well as the fact that these compounds can accumulate within cells (Simchowitz et al. 1987; Kleyman and Cragoe 1988), leading to an underestimation of the apparent inhibition constant in the case of the ORNs in situ. Thus, the lower apparent inhibition constants we observed on the cells in situ compared with on the channels in cell-free membrane patches is probably not relevant.

The SGC channel occurs in both calcium-sensitive and calcium-insensitive forms (Bobkov and Ache 2003). Whereas the present study focused entirely on the calcium-sensitive form of the channel because that form is predominantly expressed in outer dendrites (transduction zone) of ORNs in situ (Bobkov and Ache 2003), the calcium-insensitive form of SGC channel appears to be equally sensitive to blockade by the same amiloride derivatives that affected the calcium-sensitive form of the channel. We did not study the calcium-insensitive form of the channel exhaustively, but HMA, MIA, EIPA (all at 200 μM), and DMA (1 mM) blocked the channel by reducing the channel-open probability to 0.002–0.01 in the presence of 210 mM NaCl (data not shown), suggesting that the amiloride derivatives are equally effective at blocking both forms of the channel.

Interestingly, the potency sequence of the amiloride analogs does not correlate with the lipophilicity of the compounds. The predicted lipophilicity sequence for the compounds (logD, pH 8.0) is DCBA (5.16), phenamil (3.21), EIPA (3.2), MIA (3.05), HMA (2.94), DMA (1.64), and amiloride (0.68). These values of LogD (octanol/water partition coefficients) were obtained using the ACD/I-Lab Web service (ACD/LogD [Toronto, Canada] 8.02) and are consistent with lipophilicity determined experimentally by measuring the distribution of amiloride and some of its derivatives between a buffered aqueous phase and octanol or chloroform (Kleyman and Cragoe 1988). Because DCBA, the most lipophilic compound, and amiloride, least lipophilic compound, were equally ineffective in blocking both the cells and the SGC channel, we assume that even though we did not study the mechanism of pharmacological blockade the drugs per se, that they exerted their effect on the cells and the channel through a mechanism more specific than one based strictly on their lipophilicity.

Amiloride and its derivatives interact in voltage-dependent manner on a variety of ion channels, including cyclic nucleotide-gated channels (Frings et al. 1992) and members of DEG/ENaC ion channel family (see references in Garty and Palmer 1997 and in Kellenberger and Schild 2002). Also in the present study, amiloride (200 μM) exhibited voltage-dependent block of the SGC channel when applied to the inside face of the channel (Figure 5B). However, further experiments using outside-out patch recording showed that amiloride (200 μM) applied extracellularly at equivalent transmembrane voltages failed to block the channel and that inhibition of the channel by amiloride could be observed only when the cytoplasmic surface of the patch was exposed to the drug and only at extreme levels of depolarization. As the voltage-dependent effect of amiloride would have little, if any, impact on channel function in physiologically relevant conditions, we did not pursue this observation further or attempt to reveal its underlying mechanism.

In summary, our finding that pyrazine derivatives of amiloride reversibly inhibit odorant-evoked activity in lobster ORNs and that the same derivatives act with the same potency sequence to block the SGC channel, a presumptive TRP channel that is the likely downstream target of phosphoinositide signaling in these cells, further implicate the SGC channel as a, if not the, major output channel in the olfactory transduction cascade. Our findings also suggest that the pyrazine derivatives of amiloride should be useful probes to study more detailed mechanisms of chemosensory transduction in this system and possibly in other chemosensory systems in which TRP channels are the known or suspected downstream effector.

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