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Histamine operates Cl^--gated channels in crayfish neurosecretory cells

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Summary

We describe a histamine-activated Cl^- conductance in the X-organ neurons from crayfish *Cherax quadricarinatus*, which has comparable properties to the homomultimeric histamine-gated ion channels described in *Drosophila*. Topical application of histamine inhibited spontaneous neuronal firing in the X-organ sinus gland tract, concomitant with an increase in the membrane conductance. In X-organ neurons in culture and under voltage-clamp conditions, histamine evoked outward currents at –40 mV that reversed at the Cl^- equilibrium potential. Histamine sensitivity in these neurons had a half-maximal response (EC_{50})=3.3±1 μmol l^{-1}, with a Hill number of 2.6±0.4. The histamine-evoked current was blocked by tiotidine, cimetidine, ranitidine and mepyramine (IC_{50}=40±1, 98±3, 256±11 and 483±11 μmol l^{-1}, respectively) and *d*-tubocurarine (IC_{50}=21±2 μmol l^{-1}), but was insensitive to picrotoxin, bicuculline and strychnine. Neither GABA nor glutamate was capable of desensitizing the histamine response, indicating that histamine activates a particular Cl^- conductance. The presence of immunoreactive neurons to histamine in the medulla terminalis with axonal projections to the neuropile suggests a possible histaminergic modulation of the X-organ sinus gland system.

Key words: Cl^--gated channel, ionotropic histamine receptors, X-organ sinus gland system, peptidergic neuron.

Introduction

Histamine (HA) has been recognized as a transmitter or neuromodulator in crustaceans. The presence of the histidine decarboxylase in neural structures such as thoracic ganglia, brain ganglia and eyestalks of the crab was established by measuring the conversion of [^{14}C]histidine to radioactive HA in the presence of pyridoxal-5 phosphate (Arnould, 1987). Immunohistochemical studies demonstrated the presence of HA-like reactivity in lobster stomatogastric ganglion neurons (Dando and Selverston, 1972; Sigvart and Mulloney, 1982; Mulloney and Hall, 1991), thoracic and subesophageal ganglia (Mulloney and Hall, 1991), olfactory lobe (Orona et al., 1990), median protocerebrum, and deutocerebrum and tritocerebrum (Langworthy et al., 1997). By contrast, in the spiny lobster, 14 neurons in the stomatogastric ganglion responded to HA, increasing the Cl^- conductance (Claiborne and Selverston, 1984). Both *Panulirus argus* and *Homarus americanus* olfactory receptor neurons are inhibited by micromolar concentrations of HA acting directly on gating of a Cl^- channel (McClintock and Ache, 1989). In addition it has been demonstrated that HA mediates presynaptic inhibition in the escape reaction of the *Procambarus clarkii* crayfish (el Manira and Clarac, 1994), as well as presynaptic inhibition of primary olfactory afferents in lobster (Wachowiak and Cohen, 1999). It is known that HA injected into the circulation inhibits the black pigment dispersion that normally occurs in crabs transferred from a white to a black background in a dose-dependent manner (Hanumante and Fingerman, 1982). Recently we found that plasma levels of HA in the crayfish fluctuate during the day, reaching a maximum value at daybreak (Cebada et al., 2006). This evidence suggests that HA might be acting as a neuromodulator on the main neurosecretory structure of the crustacean, the X-organ sinus gland system (XO-SG). In agreement with our present findings, HA activates a Cl^- current in crayfish X-organ that has comparable properties to the homomultimeric HA-gated ion channels expressed in *Xenopus* oocytes injected with cRNAs that encode for HisCl-1 or HisCl-α2 (Zheng et al., 2002; Gisselmann et al., 2002).

The XO-SG system is formed by 120 monopolar neurons, whose axons converge within the medulla terminalis, forming a tract that runs distally to end in an neurohemal organ (SG), located between the medulla interna (MI) and the medulla externa (ME) of the eyestalk. This system participates in the control of different functions, such as molting, regulation of blood glucose levels, tegumentary and retinal pigment position, locomotion and neuronal activity (Fingerman, 1997; García and Aréchiga, 1998). Both spontaneous electrical activity and hormone release in X-organ neurons are regulated by environmental and endogenous influences, such as light and darkness, stress and circadian rhythms. These influences appear to be mediated by a host of neurotransmitters or modulators, most noticeably, GABA, glutamate (Glu) and serotonin (García and Aréchiga, 1998). GABA and Glu activate independent Cl^--gated channels in crab X-organ neurons (Duan and Cooke, 2000). In the crayfish X-organ neurons GABA induces a biphasic response, where the excitatory phase is due to the

activation of a Na^+-dependent inward current associated with the GABA uptake, that is followed by an inhibitory phase due to the activation of a Cl^- dependent outward current which is associated with the activation of $GABA_A$-like receptors (Garduño et al., 2002).

Materials and methods

Animals

Adult male and female specimens of *Cherax quadricarinatus* (von Martens 1868) of mass 60–80 g were obtained from a freshwater crayfish farm (Jojutla, Morelos, Mexico). Before experimentation, the animals were maintained in filtered water for at least 1 week in recirculating water tanks at 20°C under laboratory conditions, with *ad libitum* food and under 12 h:12 h light:dark cycles.

Dissection and culture

The method for isolating the eyestalk and intracellular recording was as described previously (Onetti et al., 1990). To obtain neuronal cultures, the animals were deeply anesthetized by burying them in triturate ice for 20 min, then the eyestalks were excised and placed in chilled crayfish saline solution, consisting of (in mmol l^{-1}): 205 NaCl, 5.4 KCl, 2.6 $MgCl_2$, 13.5 $CaCl_2$ and 10 Hepes adjusted to pH 7.4 with NaOH. The exoskeleton, muscles and connective tissue surrounding the neural structures were carefully removed under a dissecting microscope. Isolated X-organs were incubated with 200 μg ml^{-1} collagenase dispase (Roche, Mannheim, Germany) dissolved in modified Leibovitz L-15 (Gibco, Rockville, MD, USA) culture medium for 60 min. The enzyme was washed out, and the X-organ neurons were dissociated by gentle suction through fire-polished micropipettes, as described previously for the leech Retzius cells (García et al., 1990), and plated onto a 200 μl recording chamber, precoated with Concanavaline A (Type III, Sigma, St Louis, MO, USA). The ionic composition of the culture medium was adjusted to that of the crayfish saline solution. An additional 5.5 mmol l^{-1} glucose, 2 mmol l^{-1} L-glutamine, 16 μg ml^{-1} gentamycin (Schering-Plough, Mexico City, Mexico), 5 μg ml^{-1} streptomycin (Sigma) and 5 units ml^{-1} penicillin (Sigma) were added. Culture cells were kept in darkness for 24 h before the experiments were conducted.

Electrophysiology

Current- and voltage-clamp experiments in the standard whole-cell configuration were performed in X-organ neurons plated in the recording chambers, and mounted on the stage of an inverted microscope Diaphot (Nikon, Melville, NY, USA). The cells were continuously superfused with crayfish saline solution, but in some experiments, the bath solution was modified by reducing the NaCl concentration to 80 mmol l^{-1} and replaced equimolarly by sodium-methanesulfonate. Recordings were carried out using an Axopatch 200A amplifier (Axon Instruments, Sunnyvale, CA, USA) and then the low-pass filtered at 10 kHz with a four-pole Bessel filter and stored on a computer disk using commercially available hardware and software (Axon Instruments). To eliminate error signals caused by the pipette and holder capacitance, in cell-attached configuration, a –5 mV pulse was applied to cancel the fast transient. The same pulse was used in the whole-cell configuration to compensate both the cell membrane capacitive current and the series resistance, using the compensation circuits of the amplifier. The series resistance was estimated in the range 2.5–4.5 MΩ and reduced by 50–70%. Recording electrodes (2–3 MΩ) from borosilicate glass (Sutter Instruments, San Rafael, CA, USA) were filled with a solution consisting of (in mmol l^{-1}): 195 KCH_3SO_4, 12 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 EGTA-Na and 10 Hepes.

HA pulses were applied through a 'Y' tube placed 100 μm from the neurons, with continuous superfusion of crayfish saline solution. HA, mepyramine, tiotidine, cimetidine and ranitidine were purchased from RBI (Natick, MA, USA) and picrotoxin, bicuculline, strychnine and *d*-tubocurarine from Sigma. All solutions were prepared on the day of use.

Immunocytochemical staining

The eyestalks were fixed overnight in Bouin's solution at 4°C (10% formaldehyde, 5% glacial acetic acid and 75% picric acid, saturated aqueous solution), and then washed several times in sodium phosphate buffer (PBS) 0.1 mol l^{-1}, adjusted to pH 7.4. Then the eyestalks were permeabilized by incubation overnight with PBS solution containing 5% Triton X-100 (PBS-Triton). To prevent non-specific binding, the eyestalks were incubated for 4 h in a blocking solution consisting of 10% normal goat serum (Vector Lab., Burlingame, CA, USA) in PBS-Triton. After washing, the neural tissues were incubated in the primary antibody for 3 days at 4°C (rabbit anti-HA polyclonal antibody, Chemicon Int. Inc., Temecula, CA, USA), this antibody was diluted 1:500 in PBS-Triton. Then the eyestalks were rinsed three times and incubated in the secondary antiserum for 5 h at room temperature under darkness. Secondary antibody solution was goat anti-rabbit conjugated with fluorescein (FITC; 5 μg ml^{-1}; Vector Lab.) in the same buffer as the primary antibody. After washing, the eyestalks were dehydrated in a graded alcohol series from 50% to absolute ethanol (5 min each) and cleared with methylsalicylate (Merck, Darmstadt, Germany). The stained eyestalks were visualized using a confocal microscope TCS P2 (Leica, Heildelberg, Germany) equipped with an Argon-Crypton ion laser. Optical sections (1 μm) were collected in sequential mode and examined in stacks.

Results

Effects of HA on the X-organ neurons

To evaluate the HA effects on the XO-SG system, extracellular multiunitary and intracellular recordings were simultaneously obtained from XO-SG tract and X-organ cell bodies, respectively, in the isolated eyestalk preparation (Fig. 1A). In order to confirm that all the electrical activity recorded in the tract arose from the X-organ neurons, in some experiments (N=5) the X-organ somata were retrogradely stained by pressure injection of the tract with Calcium Green-dextran (10 kDa) dissolved in crayfish saline solution together with 0.01% Triton X-100 (Fig. 1B). In all cases, we confirmed by fluorescence microscopy that the X-organ cell bodies were stained exclusively. HA superfusion (50 μmol l^{-1}) notably reduced the spontaneous activity that propagates along the tract (Fig. 1C, upper trace), and elicited a sustained hyperpolarization

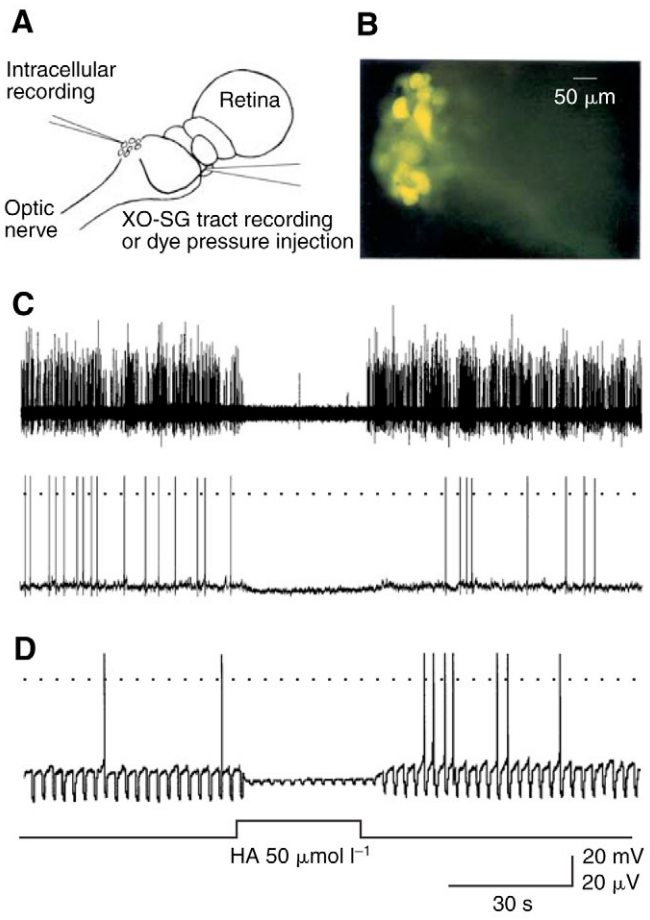

Fig. 1. HA inhibits the excitability of the XO-SG system in the isolated eyestalk preparation. (A) Schematic representation of the experimental array used to obtain simultaneous recordings from an X-organ cell body and the XO-SG tract. (B) Fluorescence micrography of the X-organ region obtained after retrograde staining of the tract OX-SG with Calcium Green-dextran. (C) Effect of HA on the spontaneous electrical activity propagated along the XO-SG tract (upper trace); note that during the HA superfusion (50 μmol l^{-1}) most of the electrical activity was inhibited. This effect is due to the hyperpolarization evoked by HA on the X-organ cells (intracellular recording, bottom trace). (D) Hyperpolarization evoked by HA is associated with an increase in membrane conductance, indicated by reduction of the input resistance. The dotted lines indicate zero membrane potential.

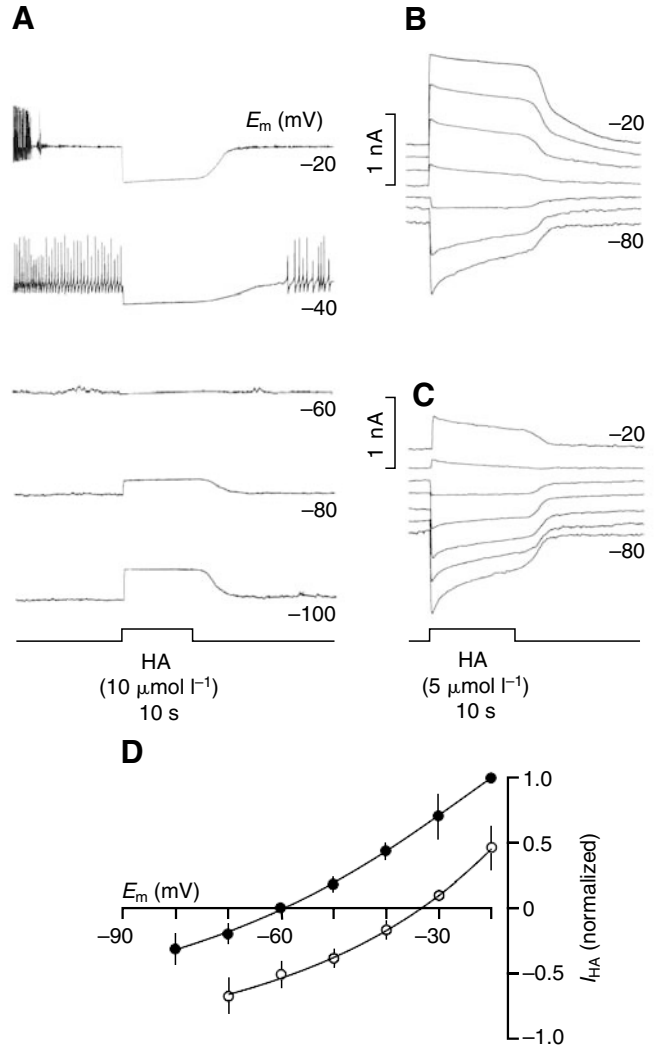

Fig. 2. HA activates a Cl^- conductance in X-organ neurons. (A) Current-clamp recordings from an X-organ neuron in culture. Note that during the application of HA, the membrane potential (E_m) reached –60 mV at all holding potentials explored. (B,C) HA-evoked currents (I_{HA}) obtained at different holding potentials from –80 to –20 mV; the interval between each HA pulse was 3 min. All the traces were obtained from the same neuron at two Cl^- equilibrium potentials (–62.5 mV for B and –32 mV for C). (D) Current–voltage relationship for the experimental conditions described in B and C. Both the solid circle and open circle curves correspond to the average ± s.e.m. of 12 cells, and the Cl^- equilibrium potential corresponded to –62.5 mV or –32 mV, respectively.

that suppressed neuronal firing in tonically active neurons (Fig. 1C, bottom trace). The hyperpolarization induced by HA (50 μmol l^{-1}) was accompanied by a significant decay in membrane input resistance, and was dependent on the concentration tested (Fig. 1D) in neurons where current pulses (–20 pA, 750 ms, 0.5 Hz) were applied to evaluate changes in input resistance, which were reduced drastically during the HA superfusion. On average, the reductions in input resistance were 8%, 45%, 72% and 88% for HA concentrations of 1, 5, 10 and 50 μmol l^{-1}, respectively. These results suggest that HA activates a Cl^- conductance, since the hyperpolarization is due to an increase in the membrane conductance.

To test whether HA activated a Cl^- current, X-organ neurons were dissociated and cultured for 24 h and the HA-evoked response was recorded in current-clamp conditions at membrane potentials from –100 to –20 mV using pipettes filled with the described internal solution. The voltage changes accompanying the HA response were dependent on the membrane potential; in all cases, voltage changes evoked by HA caused the membrane potential to decrease to –60 mV (Fig. 2A). Hyperpolarizing responses were observed at less polarized potentials and depolarizing responses at more polarized potentials. The reversal potential value suggested that the response is mediated by a Cl^- current. In addition, in voltage-clamp conditions HA-evoked currents reversed at the Cl^- equilibrium potentials (E_{Cl})

recorded. As illustrated in the Fig. 2B, HA-evoked currents reversed between –65 and –55 mV when the E_{Cl} corresponded to –62.5 mV and reversed between –40 and –30 mV when the extracellular solution was modifed to obtain a new E_{Cl} (–32 mV). The results of these experiments are summarized in Fig. 2D. The current–voltage curves obtained for both experimental conditions indicated that the zero current potential corresponded to –60±3 mV and –32±2 mV.

In agreement with previous results for some other HA-activated Cl^- channels (Hardie, 1989), the time course of the response to HA developed with latency <0.5 s, making it unlikely that a second messenger is involved. This conclusion is supported by the long-term stability (at least 1 h) of whole cell currents in the presence of only simple buffered saline.

HA activates receptors other than GABA or Glu in X-organ neurons

Previous work (Duan and Cooke, 2000) has shown that GABA and Glu activate different receptors and Cl^- conductances in crab X-organ neurons. To explore the time course of the currents elicited by HA, GABA or Glu, the cells were maintained at –40 mV holding potential using the standard pipette solution (calculated Nernst potential, E_{Cl} is approximately –62.5 mV), in order to evoke Cl^- outward currents in each case. For Glu responses, in 50% of the neurons tested (N=12), the Glu-evoked current reached its maximum value within the first 300 ms and then declined to 20% of the peak amplitude, generating a sustained current during the continued application of Glu (Fig. 3A). In the other 50% of the cells, Glu elicited a fully desensitizing current; similar results have been described previously (Duan and Cooke, 2000). By contrast, as previously reported in crayfish X-organ neurons (Garduño et al., 2002), GABA-evoked currents exhibited two components: an early transient inward current due to the electrogenic GABA uptake, followed by a sustained outward current generated through ligand-gated Cl^- channels (Fig. 3A, middle trace). Finally, the HA-evoked current declined slowly during the continued application (Fig. 3A, right trace). The time courses of the currents activated by Glu, GABA or HA suggest that different receptor types mediate each one of these responses. However, possible heterologous desensitization by GABA or Glu of the HA-evoked current was explored. Both Glu- and GABA-evoked Cl^- currents in X-organ neurons show significant desensitization when low concentrations of such substances are present in the bathing solution during the application of test pulses at concentrations close to the respective EC_{50} values (10 μmol l^{-1} for GABA and 32 μmol l^{-1} for Glu). Fig. 3B (upper traces) shows superimposed recordings obtained in response to Glu pulses applied before, during and after the superfusion of a low Glu concentration (3 μmol l^{-1}). The trace marked by the arrow was obtained under these conditions; note that the transient current was reduced notably but the amplitude of the sustained current remained the same. The other two traces correspond to the control condition and after the Glu washout. The recordings shown in Fig. 3B (bottom) were obtained from the same neuron but in response to HA, before, during and after the superfusion of Glu. Note that neither the amplitude nor the time course of the HA response was modified. Similar results were obtained when 1 μmol l^{-1} GABA was superfused during the HA pulse application (Fig. 3C).

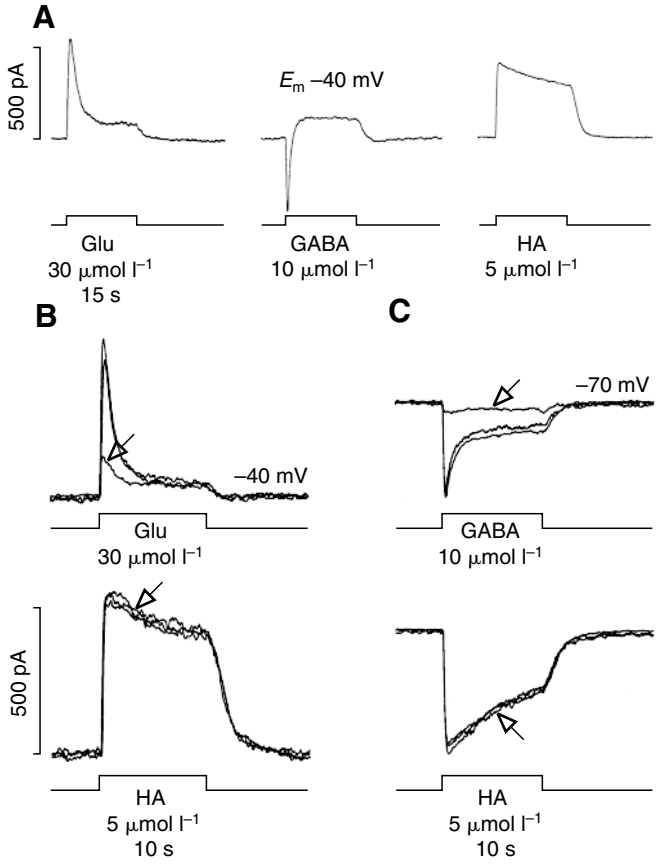

Fig. 3. Cl^- ligand-gated currents in X-organ neurons. (A) Representative current traces obtained from the same neuron in response to the EC_{50} concentrations for Glu, GABA and HA. (B) Glu-evoked currents obtained after, during and before the superfusion of a desensitizing Glu concentration (3 μmol l^{-1}, upper traces); note that the HA-evoked currents modified neither the amplitude nor the time course during the Glu superfusion (bottom traces). (C) As in B, GABA superfusion (1 μmol l^{-1}) did not modify the HA-evoked current (bottom traces), but desensitized the GABA response (upper traces). Arrows mark the current traces obtained during the superfusion of Glu or GABA.

HA dose–response relationship

The dose–response was explored by applying histamine pulses at concentrations between 0.01 to 100 μmol l^{-1}; at least five concentrations were tested in the same cell with washout intervals of 3 min. Low HA concentrations (0.01 to 0.1 μmol l^{-1}) failed to induce detectable currents, whereas concentrations between 1 and 2.5 μmol l^{-1} elicited sustained currents. Higher concentrations (5–100 μmol l^{-1}) induced a fast desensitizing component followed by a slower desensitizing component (Fig. 4A). The HA-induced current was measured at the peak and these values were plotted against the log of the HA concentration, giving a concentration inducing a half-maximal response (EC_{50}) of 3.28±1 μmol l^{-1} and Hill number of 2.6±0.4 (Fig. 4B). The EC_{50} value for HA differs considerably from those reported

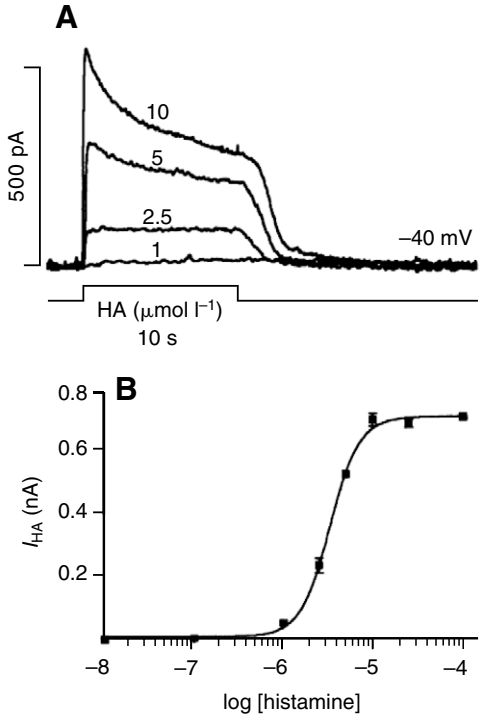

Fig. 4. X-organ neuron sensitivity to HA. (A) Representative HA-evoked currents obtained at –40 mV holding potential, during application of HA pulses at the indicated concentrations. (B) Peak currents (mean ± s.e.m.) *versus* HA concentration (3–5 observations per point). The solid line correspond to a non-linear regression using $I_{max}=1/[1+(EC_{50}/HA)^n]$, where HA=molar HA concentration, n=the Hill coefficient and EC_{50}=HA concentration giving half-maximal effect, being the free parameters. The fit yielded $EC_{50}=3.3\pm1$ μmol l^{-1} and $n=2.6\pm0.4$.

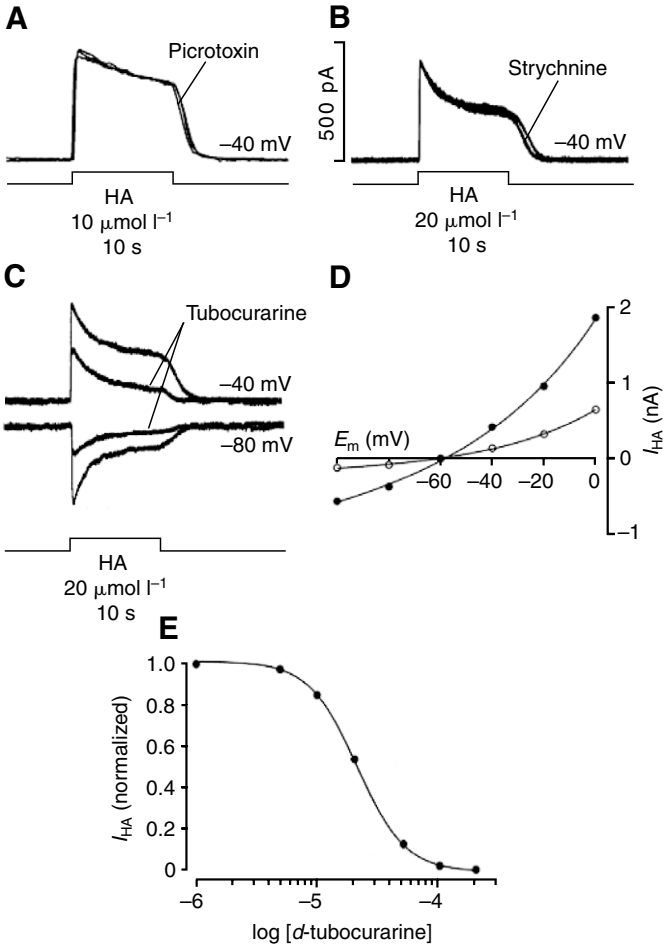

Fig. 5. Effects of Cl^- channel blockers on the HA response in X-organ neurons. (A,B) Superimposed current traces obtained after, during and before the superfusion of Cl^- channel blockers. Neither picrotoxin nor strychnine at 100 μmol l^{-1} modified the HA-evoked current. (C) The cholinergic antagonist, *d*-tubocurarine (dTC; 20 μmol l^{-1}) blocked the HA-evoked current reversibly. (D) Average current–voltage curves (5 observations per point); solid circles, control conditions; open circles, blockage exerted by dTC. (E) Inhibition curve. Each point represents the average value for five observations. The solid line corresponds to a non-linear regression giving an adjusted $IC_{50}=21\pm2$ μmol l^{-1}.

for Glu and GABA (Duan and Cooke, 2000; Garduño et al., 2002). Previous work has shown that the homomeric expression of the mRNAs encoding HA-gated Cl^- channel subunits from the fruit fly *Drosophila melanogaster* produced HA receptors with EC_{50} values of 166±12 μmol l^{-1} for DM-HisCl-α1 and 10.8±0.46 DM-HisCl-α2 (Gisselmann et al., 2002), whereas those for HisCl-1 and HisCl-2 were 4.2±1.3 and 14±2.5, respectively (Zheng et al., 2002). The HA receptor sensitivity in X-organ neurons was close to the homomultimeric HA-gated Cl^- channels HisCl-1 and DM-HisCl-α2. The pharmacological profile of the HA response in X-organ neurones cannot easily be classified within the pharmacological categories developed in mammals. The histamine receptors are a class of endogenous ligand. Activation of HA metabotropic receptors involves cytoplasmic second messengers, cofactors, coupling proteins and enzymes, and often could not occur in cells perfused internally by salts alone (Hille, 2001). Our results suggest that the HA response is mediated by ligand-gated anion channels.

Pharmacology

Cl^- channel blockers

Picrotoxin at concentrations between 1 and 100 μmol l^{-1} blocks most of the inhibitory Glu receptor, as well as $GABA_A$-like receptors from invertebrates (Lunt, 1991; Cleland, 1996). In contrast, the Cl^- currents evoked by HA in crustacean preparations are insensitive to picrotoxin (Claiborne and Selverston, 1984; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992; el Manira and Clarac, 1994). We found that the HA response in X-organ neurons was also insensitive to picrotoxin even at high concentrations (100 μmol l^{-1}). Fig. 5A illustrates representative current traces obtained before, during and after picrotoxin superfusion.

To date, glycine-gated Cl^- channels have not been identified in invertebrates (Roeder, 2003), but the HA-gated Cl^- channel subunits from *Drosophila melanogaster* show the highest degree of homology to human glycine receptors (Witte et al., 2002). By contrast, strychnine is a specific antagonist of these receptors that reversibly inhibited the glycine-induced currents. We did not detect glycine-evoked currents in X-organ neurons

(*N*=12) nor blockage of the HA-evoked currents with strychnine concentrations of 10–100 μmol l^{-1} (Fig. 5B).

The inhibitory effect of the competitive antagonist from the curare family, *d*-tubocurarine (dTC), on HA-activated Cl^- channels in crustacean preparations such as stomatogastric ganglion cells (Claiborne and Selverston, 1984), olfactory receptor neurons (Bayer et al., 1989) and cardiac ganglion motor neurons (Hashemzadeh-Gargari and Freschi, 1992), is well documented, as well as in oocytes that express HA-gated Cl^- channels (Gisselmann et al., 2002). In agreement with the above-mentioned reports, we found that dTC reversibly blocked the HA response in X-organ cells with an IC_{50} of 21±2 μmol l^{-1} (Fig. 5E). Fig. 5C shows current traces elicited by 20 μmol l^{-1} HA obtained after and during the superfusion of dTC at the indicated potentials. Note that the blockage exerted by the antagonist was dependent on the holding potential, resulting in more effective hyperpolarizing potentials (Fig. 5D). This result supports the notion that the HA Cl^{-gated} current in crayfish X-organ neurons is generated by native receptors, with similar characteristics to those described for DM-HisCl receptors.

HA antagonists

Fig. 6 illustrates the effect on the HA-evoked currents obtained at –40 mV from four different cells, before, during and after the superfusion of competitive antagonists type H1 and H2. In the presence of mepyramine (500 μmol l^{-1}), the residual current desensitized rapidly and almost completely (Fig. 6A), whereas in the presence of the H2 antagonists (ranitinine, tiotidine and cimetidine), the residual current did not showed desensitization during the continuous application of HA (Fig. 6B,C,D). The blockage exerted by all the antagonists was fully reversible. Mepyramine was the least potent, at concentrations of 1–10 μmol l^{-1}, and the amplitude of the HA-evoked current remained unchanged, reaching complete inhibition at concentrations close to 2 mmol l^{-1}, and with an IC_{50} of 483±11 μmol l^{-1} (Fig. 6E, triangles). The IC_{50} values for the H2 antagonists were 40±1.3 μmol l^{-1} for tiotidine, 98 ±2.6 μmol l^{-1} for cimetidine and 256±11 μmol l^{-1} for ranitidine (Fig. 6E, squares, diamonds and circles, respectively). These results are in agreement with previous reports where Cl^- conductance-gated by HA in *Musca domestica* and crustacean neurons was blocked predominantly by H2 antagonists (Hardie, 1989; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992). In addition, the pharmacological profile that we found is quite similar to those reported for the HA-gated Cl^- channel type DM-HisCl-α2 functional expressed in oocytes (Gisselman et al., 2002).

HA-like immunolabeling

To examine the distribution of HA immunoreactivity in the eyestalk, we carried out whole-mount immunocytochemistry followed by confocal microscopy. HA immunoreactivity in the eyestalk was concentrated in photoreceptors at the rhabdoms and under the basement membrane at the cartridges where the photoreceptor axons contact with monopolar neurons in the first synapse of the visual pathway (not shown). At the medulla terminalis we found consistently (*N*=8 preparations) one neuron located dorsally at the border of the

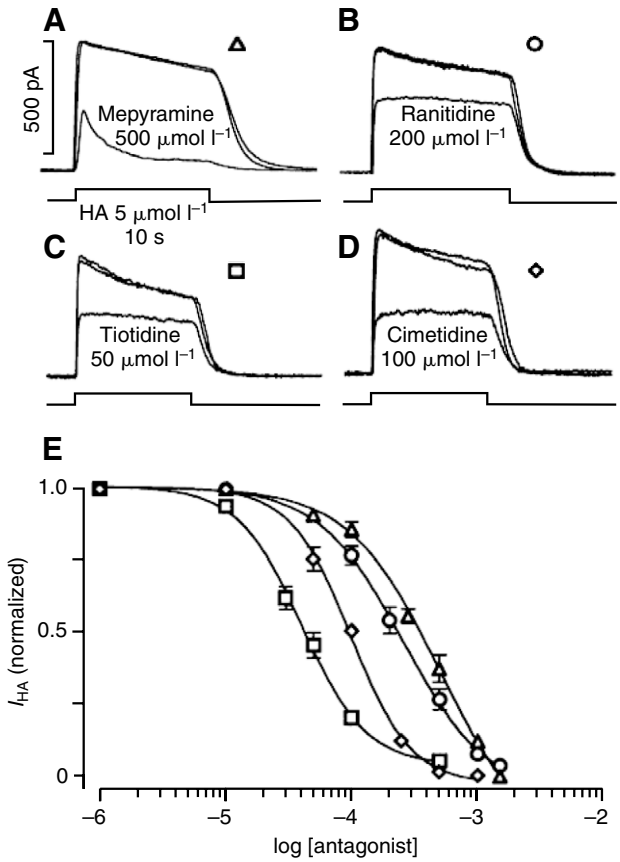

Fig. 6. Effects of HAergic antagonists on the HA evoked-current in X-organ neurons. (A–D) Current traces evoked by HA (5 μmol l^{-1}, 10 s) after, during and before the superfusion of H1 and H2 antagonists at the indicated concentrations; all the records were obtained at a holding potential of –40 mV, and the interval between pulses was 3 min. Note that in all cases the blockage was reversible. (E) Normalized peak currents (mean ± s.e.m.) *versus* log molar concentration of H1 and H2 antagonists (4–6 observations per point; see the IC_{50} values in the text). Squares, tiotidine; diamonds, cimetidine; circles, ranitidine; triangles, mepyramine.

X-organ nuclei; this cell projects its primary axon through the internal capsule and its secondary axons branched extensively in the neuropile of the medulla terminalis (Fig. 7B). Finally, two pairs of monopolar neurons were located dorsal and externally at the rim of the hemiellipsoidal body; the long primary axons of these neurons penetrate the inner capsule projecting in the direction of the X-organ, crossing the midline and branching too in the neuropile of medulla terminalis (Fig. 7C). In this region, the axons of the X-organ neurons bifurcate extensively and receive synaptic inputs (Iwasaki and Satow, 1971), so it is reasonable to suppose that the HAergic neurons contact with the neurosecretory system, however to date there are no reports to suggest that these presynaptic effects are evoked by the XO-SG system.

Discussion

In agreement with our previous observations, during the transition from day to night, HA concentration in crayfish hemolymph decays to the lowest levels, suggesting that the

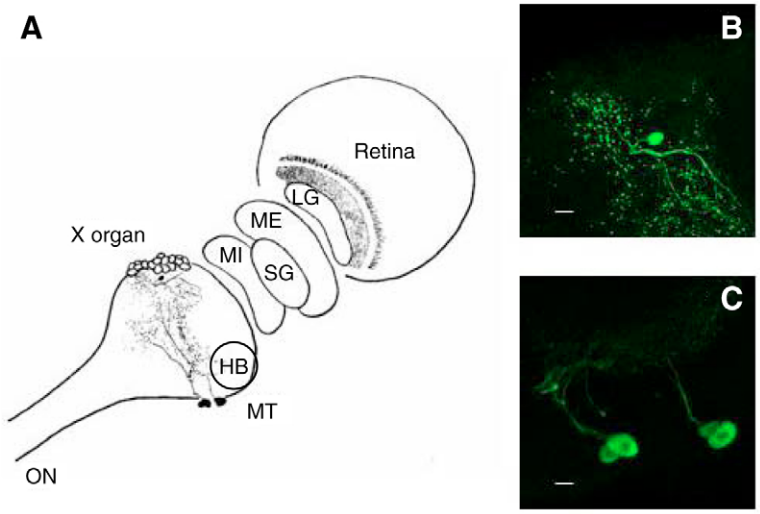

Fig. 7. Distribution of HA immunoreactivity in the crayfish eyestalk. (A) Schematic representation of a dorsal view of the eyestalk and the relative position of HAergic like single neurons. LG, lamina ganglionaris; ME, medulla externa; GS, sinus gland; MI, medulla interna; MT, medulla terminalis; HB, hemielpsoidal body; ON, optic nerve. (B,C) FITC fluorescence for HA immunoreactive neurons observed by confocal microscopy; image compositions were done by merging 200 slides (1 μm section thickness). Scale bars, 20 μm.

tonic inhibition of the X-organ neurons is removed, allowing recovery of the input resistance and consequently the capacity to generate burst firing (Cebada et al., 2006). Long-term intracellular recordings from X-organ neurons showed that the cells are silent or display a low-level of tonic activity during the morning and early afternoon hours, but change dramatically toward the late evening to a fully-fledged pattern of burst activity (García and Aréchiga, 1998). Overall these studies proved that HA is involved in the modulation of the X-organ excitability. In the present study, we have demonstrated that HA operates Cl^--gated channels in the X-organ neurons. These findings indicate that control of neural activity by HA could be a mechanism underlying the hormonal secretion from the XO-SG system to synchronize a variety of circadian physiological functions.

HA activates a Cl^- conductance

Four pharmacologically distinct metabotropic G-protein-coupled HA receptor subtypes have been described and cloned from mammals (Gantz el al., 1991; Yamashita et al., 1991; Lovenberg et al., 1999), and two ionotropic receptors predicted from *Drosophila melanogaster* genome coding for HA-gated Cl^- channels by functional expression in *Xenopus oocytes* have been described (Gisselmann et al., 2002; Zheng et al., 2002). HA directly activates a Cl^- conductance in a number of invertebrates, including mollusks (McCaman and Weinreich, 1985), insects (Hardie, 1989; Stuart, 1999) and crustaceans (Clairbone and Selverston, 1984; Prell and Green, 1986; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992). The receptor types mediating these effects are unknown, but could be related to the novel ligand-gated anion channel from the fruit fly. Our results indicate that exogenous HA elicits an inhibitory response in both *in situ* and cultured X-organ neurons; this response is mediated by an increase in membrane conductance that reversed at the expected Cl^- equilibrium potential. In addition the time course of the response to HA developed with short latency, and the long-term stability of whole cell currents in the presence of only simple saline support the notion that the Cl^- current in X-organ neurons is generated by ionotropic receptors.

Pharmacology of the HA receptor

In agreement with previous reports (Claiborne and Selverston, 1984; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992; el Manira and Clarac, 1994), we found that picrotoxin at concentrations between 10 and 100 μmol l^{-1} was incapable of blocking the Cl^- current evoked by HA. This is an advantage, making it easy to distinguish between the GABA- or the Glu-gated Cl^- currents that are present in X-organ neurons. In addition, the DM-HisCl receptors expressed in oocytes show a weak sensitivity to picrotoxin (Gisselmann et al., 2002; Zheng et al., 2002).

The HAergic antagonists H1 and H2 have been used to characterize native HA-invertebrate receptors and the HA evoked current is consistently more sensitive to H2 blockers (Haride, 1988; McClintock and Ache, 1989; Hashemzadeh-Gargari and Freschi, 1992; Gisselmann et al., 2002). In the X-organ neurons the HA response was more efficiently inhibited by H2 antagonists (tiotidine, cimetidine and ranitidine) than by the H1 antagonist (mepyramine). The pharmacological profile that we found is close to that reported for the homomeric DM-HisCl-α2 receptor (Gisselmann et al., 2002); however it could correspond to the expression of the homomeric channels HisCl-1 (Zheng et al., 2002). Further studies of molecular biology should help to confim the homology between fruit fly HA receptors and crustacean HA receptors.

List of abbreviations

DM-HisCl-α1	HA Cl^- subunit type 1 from *Drosophila melanogaster*
DM-HisCl-α2	HA Cl^- subunit type 2 from *Drosophila melanogaster*
dTC	*d*-tubocurarine
EC_{50}	half-maximal effective concentration
FITC	fluorescein isothiocyanate
$GABA_A$	γ-aminobutiric acid receptor type A
Glu	glutamate
H1	HA receptors type 1
H2	HA receptors type 2
HA	histamine
HB	hemielipsoidal body
HisCl-1	histamine subunit type 1

IC_{50}	half-maximal inhibitory concentration
LG	lamina ganglionaris
ME	medulla externa
MI	medulla interna
MT	medulla terminalis
ON	optic nerve
PBS	sodium phosphate buffer
XO-SG	X-organ sinus gland

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