The crustacean X-organ/sinus gland system (XO-SG) is a conglomerate of approximately 120 neurons in the eyestalk that are known to secrete a host of neuropeptide hormones (Beltz, 1988; Keller, 1992; García and Aréchiga, 1998). The neurons secreting some of these hormones have been identified by immunocytochemical studies and in situ hybridization. The biosynthesis and release of these hormones are regulated by a variety of environmental and physiological factors (see Keller, 1992; Aréchiga and Rodríguez-Sosa, 1997; García and Aréchiga, 1998). Light, darkness, stress, diurnal cycles and moulting are among the main influences that appear to control the secretion of XO neurohormones (Glantz et al., 1983; Aréchiga et al., 1993; Rodríguez-Sosa et al., 1994b). Pharmacological and electrophysiological studies have revealed a complex organization underlying the modulation of XO hormone secretion. Several putative neurotransmitters and modulators, which act in a variety of ways, have been proposed to mediate these regulatory influences (Fingerman and Nagabhushanam, 1992; García and Aréchiga, 1998) so that a given substance may facilitate the release of one hormone but inhibit that of another.

An additional dimension of complexity in the regulation of neuropeptide release in the crustacean eyestalk is the widespread distribution of cells containing a given peptide and the variety of physiological roles they appear to play. For instance, the neurons involved in the secretion of the octapeptide red pigment concentrating hormone (RPCH) in various crustacean species are distributed in different regions of the eyestalk and central ganglia (Bellon-Humbert et al., 1986; Mangerich et al., 1986; Rodríguez-Sosa et al., 1994a). In addition, a variety of roles have been proposed for RPCH. It is known to promote the retraction of the pigmentary matrix in tegumentary erythrophores, hence its name, but other physiological actions have also been documented, e.g. as a central neurotransmitter in the crab and lobster stomatogastric network (Nusbaum and Marder, 1988; Dickinson and Marder, 1989) and in the crayfish swimmeret system (Sherff and Mulloney, 1991). More recently, it has been shown to promote the retraction of pigment in accessory retinal shielding cells and to act as a neuromodulator on retinal photoreceptors (Garfias et al., 1995).

It has been suggested that serotonin (5-HT) is a neurotransmitter/modulator in the crustacean XO-SG system. Its presence has been quantitatively determined and its cellular localization has been established. Of particular relevance was the demonstration of 5-HT-immunopositive efferent axons to the medulla terminalis and of a profuse mesh of 5-HT-immunoreactive fibres in the neuropile (Rodríguez-Sosa et al.,...
1997), suggesting its role in the regulation of XO hormone secretion. The excitatory role of 5-HT on unidentified XO neurons has been documented (Sáenz et al., 1997). For instance, 5-HT mimics the action of crustacean hyperglycaemic hormone (CHH), elevating haemolymph sugar levels in several crustacean species. Evidence has been obtained to support the idea that this hyperglycaemic action of 5-HT is due to a direct stimulation of CHH release (Keller and Beyer, 1968; Fingerman and Nagabhushanam, 1992). In contrast, 5-HT suppresses neuronal firing in CHH-immunoreactive neurons in culture (Glówik et al., 1997).

No direct evidence is available about the ionic mechanisms underlying the effects of 5-HT on crayfish neurosecretory cells. In the present study, we demonstrate an inhibitory effect of 5-HT on the electrical activity of RPCH-containing neurons that can be ascribed to an increase in a Ca$^{2+}$-dependent K$^+$ conductance.

**Materials and methods**

Adult crayfish Procambarus clarkii (Girard) of either sex and in intermoult were collected from Rio Conchos, Chihuahua, México, and acclimated to laboratory conditions for 2 weeks under a 12 h:12 h L:D photoperiod at room temperature (20–26 °C).

**Dissection and culture conditions**

Eyestalks were excised and placed in chilled crayfish saline solution (in mmol l$^{-1}$): 205 NaCl, 5.4 KCl, 13.5 CaCl$_2$, 2.6 MgCl$_2$ and 10 Heps, adjusted to pH7.4 with NaOH. The exoskeleton, muscles and connective tissue surrounding the neural structures were carefully removed under a dissecting microscope. Isolated XOes were incubated in collagenase-dispase (200 μg ml$^{-1}$, Boehringer Mannheim) dissolved in modified Leibovitz L-15 (Gibco) culture medium for 60 min. The enzyme was washed out, and the XO neurons were dissociated by gentle suction through fire-polished micropipettes, as described previously (García et al., 1990), and plated onto a 200 μl recording chamber, precoated with Concanavalin A (Type III, Sigma). 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 (Shering Plough), 5 μg ml$^{-1}$ streptomycin (Sigma) and 5 units ml$^{-1}$ penicillin (Sigma) were added.

To isolate tegumentary erythrophores, exoskeleton fragments were obtained from the cephalothorax region. The non-pigmentary epithelium was then removed, and the pigmentary epithelium attached to the exoskeleton was incubated in filtered crayfish saline solution plus RPCH (1 pmol l$^{-1}$) for 30 min (Peninsula Laboratories). After washing, the fragments were incubated in modified Leibovitz’s L-15 medium plus protease type I at 1 mg ml$^{-1}$ (Sigma) for 35–45 min. The pigmentary epithelium was removed from the exoskeleton and mechanically dispersed to obtain a cell suspension that was centrifuged over Lymphoprep (Nycoderm Pharma AS, Oslo, Norway) at 1000 revs min$^{-1}$ for 30 min. The red band containing the erythrophores was collected and added to the recording chamber, where XO neurons had been plated 1 h previously. The identification of RPCH-containing neurons was made according to the procedure described by Alvarado-Alvarez et al. (1999). Briefly, presumptive RPCH-containing neurons were readily recognized by their ability to maintain local retraction of the pigmented matrix in filipodia from neighbouring co-cultured erythrophores. The co-cultures were kept in darkness for 24–48 h before the experiments were conducted.

**Electrophysiology**

Only XO cells that induced retraction of the pigmented matrix in neighbouring erythrophores were used for this study. Current- and voltage-clamp recordings were monitored in the whole-cell mode using the gramicidin-perforated patch-clamp technique (Horn and Marty, 1988). Pipettes were pulled from borosilicate capillaries (Sutter Instruments) and used without flame polishing. The pipettes were filled with a solution consisting of (in mmol l$^{-1}$): 195 KCH$_3$SO$_4$, 12 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 5 EGTA-Na and 10 Heps, plus 200 μg ml$^{-1}$ gramicidin, connected via a Ag/AgCl wire to the input stage of an Axopatch 200A amplifier (Axon Instruments). The output of the amplifier was filtered at 5 kHz and stored on computer disk using commercially available hardware and software (Axon Instruments). Series resistance averaged 6.5±2.2 MΩ (mean ± S.D.) and was 80–85 % compensated to minimize voltage error. All experiments were conducted at room temperature (20–26 °C).

**Application of 5-HT**

Serotonin (5-HT) was delivered either by pressure ejection from pipettes (tip diameters 2–4 μm) placed 50 μm from the neurons or by superfusion (2 ml min$^{-1}$). Pressure ejection of 5-HT was accomplished using a Picospritzer (General Valve). Serotonin, tetraethylammonium (TEA$^+$) and methysergide were purchased from Sigma Chemical Co. Charybdotoxin (ChTX) and apamin were purchased from Alomone Laboratories. Drugs were prepared on the day of use.

**Results**

A group of 3–7 neurons has been identified, in a superficial position at the distal rim of the XO, that show a positive reaction to an antiserum against RPCH. Under co-culture conditions, this subset of neurons corresponds to the cells that induce pigmentary matrix aggregation in neighbouring erythrophores (Alvarado-Alvarez et al., 1999). Firing of these neurons induced by depolarizing current injection resulted in progressive retraction of the pigment within the filopodia of neighbouring erythrophores, as is illustrated in Fig. 1A–C. In all these neurons (N=47), 5-HT induced a long-lasting hyperpolarization capable of suppressing ongoing tonic activity (Fig. 1D).

The hyperpolarization evoked by a pressure pulse of 5-HT under current-clamp conditions (Fig. 2A) was concurrent with
Fig. 1. Co-culture of a red pigment concentrating hormone (RPCH)-containing X organ neuron with tegumentary erythrophores 48 h after plating. (A) A photomicrograph taken before electrical stimulation of the neuron. Note that the pole of the erythrophore adjacent to the neuron is retracted. Electrical activity evoked by intracellular current injection into the neuron for 5 min induced a progressive aggregation of the pigmentary matrix. (B) A photomicrograph taken at the end of neuronal stimulation. (C) The pigment in the erythrophores was fully aggregated after a further 15 min. Scale bar, 50 μm. (D) Neuronal firing was suppressed by topical application of serotonin (5-HT; 100 μmol l⁻¹) onto the RPCH-containing neuron.

Fig. 2. Hyperpolarization induced by serotonin (5-HT) (A) coincided with an increase in membrane conductance (B). Under voltage-clamp conditions, 5-HT elicited an outward current at a holding potential of −60 mV (C). Black bars under each recording indicate 5-HT application (100 μmol l⁻¹, 3 s and 69 kPa). Membrane conductance was determined in the same cell by applying hyperpolarizing current pulses (0.33 Hz, 300 ms). \( I_m \), membrane current.
a significant increase in membrane conductance (of 48.2±12.2 %; mean ± s.d.) in all eight neurons tested (Fig. 2B). Under voltage-clamp conditions at a holding potential of −60 mV, 5-HT elicited an outward current with a time course corresponding to the changes observed in the current-clamp mode (Fig. 2C). These results suggest that the ionic mechanism of the response to 5-HT involves an increase in K⁺ conductance. Given that the Cl⁻ equilibrium potential (E_cl) predicted by the Nernst equation was close to −60 mV, no Cl⁻ current component could be expected. In addition, the increase in membrane conductance associated with the hyperpolarization rules out a possible blockage of inward currents.

The dose-dependence of the 5-HT response was explored under voltage-clamp conditions by superfusing concentrations of 0.1–500 μmol l⁻¹. The interval between each application was 10 min, during which time the cultured neurons were washed with crayfish saline solution. All these experiments were conducted at a holding membrane potential of −50 mV. The lowest concentration tested, 0.1 μmol l⁻¹, failed to induce detectable currents, whereas concentrations between 1 and 10 μmol l⁻¹ evoked a sustained current (Fig. 3A, upper trace). Higher concentrations (20–500 μmol l⁻¹) induced a fast-desensitizing component followed by a slower component (Fig. 3A, bottom trace). The 5-HT-induced current was normalized for each experiment to the maximum current obtained for the highest concentration tested. The experimental values were fitted with the Michaelis–Menten equation, giving a concentration inducing a half-maximal response (K_m) of 10 μmol l⁻¹ (Fig. 3B).

To demonstrate the ionic nature of the 5-HT-evoked current, the reversal potential of the response to 5-HT was explored at two extracellular K⁺ concentrations ([K⁺]_o). In normal saline (5.4 mmol l⁻¹), 5-HT induced a long-lasting outward current that decreased gradually at negative potentials, reaching a value of zero close to the predicted equilibrium constant for K⁺ (E_K) value of −90 mV (Fig. 4A). In this condition, at holding potentials below −90 mV, 5-HT-evoked currents were difficult to measure because they showed rapid fluctuations (Fig. 4A, bottom trace). When [K⁺]_o was increased to 19 mmol l⁻¹, the reversal potential shifted from −90 to −60 mV (Fig. 4B). In this case, at holding potentials below −60 mV, the inward currents evoked by 5-HT in the five cells tested showed no such fluctuations and they were easily measured. The results are summarized in the current/voltage (I/V) relationship (Fig. 4C) and suggest that a K⁺ current is mediating the effect of 5-HT.

To elucidate the K⁺ channel type involved in the response to 5-HT, various possibilities were explored. In six experiments, the co-cultures were superfused for 5 min with ChTX (100 nmol l⁻¹), a selective blocker of the Ca²⁺-activated K⁺ channel (Miller et al., 1985). This toxin blocks large-conductance BK channels in crayfish XO neurons (Murbartián et al., 1998; Lara et al., 1999). Superfusion with 5-HT induced a hyperpolarizing response, and subsequent superfusion of ChTX onto RPCH-containing neurons resulted in a slight depolarization of the membrane potential (by approximately 5 mV) that induced spontaneous firing in silent cells. Under this condition, the inhibitory effect of 5-HT was abolished (Fig. 5B), suggesting that a Ca²⁺-activated K⁺ current underlies the inhibition induced by 5-HT. To test this possibility further, we explored the effect of TEA⁺ on the response to 5-HT. TEA⁺ acts as a selective blocker of BK-type Ca²⁺-activated K⁺ channels when applied from either side of membrane, but is more effective at submillimolar concentrations on the extracellular side (Yellen, 1984). As shown in Fig. 6, when we superfused TEA⁺ (500 μmol l⁻¹) for 5 min, the hyperpolarization induced by 5-HT was reversibly blocked. The presence of a ChTX- and TEA⁺-sensitive Ca²⁺-activated K⁺ current has been described previously in unidentified XO cells in culture (Murbartián et al., 1998). In addition, in excised inside-out patches from XO neurons, large-conductance (223 pS) BK channels have been recorded in symmetrical [K⁺] solutions (Lara et al., 1999). Our results suggest that BK channels are involved in the response to 5-HT.
Since a wide variety of effects of 5-HT on K⁺ currents have been described in invertebrate neurons (Gerschenfeld and Paupardin-Tritsch, 1974; Klein et al., 1982; Kaczmarek and Levitan, 1987; Zhang and Harris-Warrick, 1994; Achee and Zoran, 1997), other possibilities besides a Ca²⁺-activated K⁺ current were explored. One of them was a possible inward rectifying current. To explore this, long command (1 s) pulses from -120 to -20 mV were applied from a holding potential of -50 mV. The control traces obtained before 5-HT superfusion indicated an absence of inward rectification, and neither the magnitude nor the time course of the evoked currents was modified by 5-HT (data not shown). Apamin, a selective blocker of the low-conductance Ca²⁺-activated K⁺ channel (Hugues et al., 1982), was not effective in modifying the response to 5-HT (data not shown). In other experiments, Na⁺ and Ca²⁺ currents were blocked to isolate voltage-dependent delayed rectifier (I_K) and transient (I_A) K⁺ currents. Neither the amplitude nor the time course of either current was modified during 5-HT superfusion (data not shown).

The presence of 5-HT receptors mediating the inhibitory action on RPCH-containing neurons was explored during the superfusion of co-cultures with methysergide (100 μmol l⁻¹), a...
non-selective 5-HT receptor blocker. Methysergide has been shown to suppress the excitatory effects of 5-HT on other XO neurosecretory cells (Sáenz et al., 1997), the effects of 5-HT on other crustacean neurons (Zhang and Harris-Warrick, 1994) and the inhibitory effects of 5-HT on Retzius cells from the leech (Smith and Walker, 1975). Fig. 7A illustrates the hyperpolarization induced by 5-HT. This effect was blocked after 10 min of methysergide superfusion (Fig. 7B) and reversed after 10 min of washout (Fig. 7C). In vertebrates, methysergide appears to block the 5-HT1C and 5-HT2B receptors (Peroutka, 1991); however, there is evidence that 5-HT receptors in invertebrate nervous system are markedly different from the well-characterized 5-HT receptor subtypes of vertebrates (Zhang and Harris-Warrick, 1994).

**Discussion**

The inhibitory effects of 5-HT on the electrical activity of RPCH-containing neurons has not been described previously. It is worth mentioning that all the neurons tested in our experiments were inhibited by 5-HT. This is in contrast to the facilitatory influences previously described for this amine on the release of crustacean hyperglycaemic hormone (CHH) (Keller and Beyer, 1968) and pigment dispersing hormone (PDH) from neurons (Hanumante et al., 1984) and the excitatory effects of 5-HT on XO neurons (Sáenz at al., 1997). Given the opposite physiological functions of RPCH and PDH, it is plausible that the same substance might mediate the release of one neuropeptide but inhibit that of its physiological antagonist.

The physiological role of 5-HT in the control of RPCH release is supported by the widespread distribution of 5-HT-immunopositive cell bodies and fibres in the crustacean eyestalk (Elofsson, 1983; Sandeman et al., 1988) and particularly in the neuropile of the medulla terminalis (Rodríguez-Sosa et al., 1997), where the XO neurons receive their synaptic inputs.
Although most of the effects of 5-HT on crustacean neurons so far described are excitatory, there are known instances of 5-HT-induced inhibition. For example, in lobster superficial abdominal flexor muscles, 5-HT, while facilitating the excitatory motoneurons, hyperpolarizes the inhibitory motoneurons (Kravitz et al., 1983). More recently, a 5-HT-induced hyperpolarizing effect was reported in CHH-containing cells of the crab *Cancer borealis* (Glowik et al., 1997). The wide repertoire of cellular responses to 5-HT could be explained by at least two mechanisms: target neurons might possess different 5-HT receptors that evoke different electrophysiological responses, or there could be differences in intrinsic cellular response properties through the activation of different G-protein subtypes or coupled second-messenger mechanisms (Zhang and Harris-Warrick, 1994).

There is evidence for at least six different types of response to 5-HT application in invertebrate neurons (*Helix* sp. and *Aplysia* sp.). Three of these responses are excitatory and three are inhibitory (Gerschenfeld and Paupardin-Tritsch, 1974). Activation of K+ currents has been proposed as the underlying mechanism of the inhibitory effects of 5-HT (Kaczmareck and Strumwasser, 1984; Strong and Kaczmareck, 1986; Shuster et al., 1991). In these instances, 5-HT-induced hyperpolarization was associated with an increase in the inward rectifying K+ current (Lotshaw and Levitan, 1987) mediated by cyclic AMP (Drummond et al., 1980). Our observations suggest the absence of an inward rectifying K+ current in RPCH-containing neurons, as has been described in arthropods (Hille, 1992).

BK channels are Ca2+- and voltage-dependent, and they are generally believed to be insensitive to physiological elevations of [Ca2+]i at the resting membrane potential. This assumption arises from experiments in which the intracellular factors that may influence the activity of Ca2+-activated K+ channels have been removed (excised membrane patches and whole-cell recordings). Microfluorometric measurements using Fura-2 indicate that, at the resting membrane potential, the [Ca2+]i of unidentified XO neurons in culture is 100 nmol l−1. In these cells, BK channel blockers, ChTX, iberiotoxin (100 nmol l−1) and TEA+ (500 µmol l−1) induce a depolarization associated

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Fig. 7. Preincubation with methysergide (100 µmol l−1) reversibly blocks the effect of serotonin (5-HT) on an identified X-organ neuron in culture. (A) A typical response to superfusion of 5-HT (100 µmol l−1) onto a red pigment concentrating hormone (RPCH)-containing neuron. (B) The effects of 5-HT (black bar) were blocked by superfusion with methysergide (white bar). (C) The response recovered 10 min after washing out the methysergide.
with an increase in membrane resistance, suggesting that a subpopulation of BK channels is active at this membrane potential (Murbartíán et al., 1998).

In the present study, given the blockage that ChTX and TEA⁺ exerted on the response to 5-HT, we conclude that the inhibitory effects of 5-HT in RPCH-containing neurons is due to an increased K⁺ conductance through BK channels. The strong rectification of the IV relationship for BK channels at values more negative than -90 mV has been explained by Yellen (1984), who showed that millimolar concentrations of internal Na⁺ induced a voltage-dependent flickery block that could be removed when the external K⁺ concentration was increased. This suggests that K⁺ entering from the outside can expel the blocking Na⁺. In our experiments, to change the $E_K$ from -90 to -60 mV, we increased [K⁺]₀ from 5.4 to 19 mmol/l⁻¹; this manoeuvre probably removed the channel blockage caused by Na⁺ and allowed the generation of inward currents, even at values more negative than -60 mV.

Given the long duration of the 5-HT-induced inhibition in RPCH neurons, it is plausible to suggest that metabotropic 5-HT receptors may be involved. These may activate an intracellular mechanism that modulates the conductance through BK channels. This speculation is based on the fact that a fast run-down of the 5-HT response occurs in the standard whole-cell configuration. Further studies will be necessary to explore this possibility.

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References


