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D-GLUCOSE-SENSITIVE NEUROSECRETORY CELLS OF THE CRAB *CANCER BOREALIS* AND NEGATIVE FEEDBACK REGULATION OF BLOOD GLUCOSE LEVEL

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Summary

We studied the effects of glucose on cultured X-organ neurons of the crab *Cancer borealis* using single-electrode current- and voltage-clamp techniques. A subpopulation of the cells responded to D-glucose with a hyperpolarization. These cells, but not glucose-insensitive cells, showed immunoreactivity to crustacean hyperglycemic hormone (CHH), the hormone responsible for the elevation of blood glucose levels in crustaceans. Glucose-sensitive cells were also inhibited by serotonin and γ-aminobutyric acid but were not affected by dopamine and Leu-enkephalin. The response was specific for D-glucose, with an EC_{50} of 0.25 mmol l^{-1}. No response was seen to L-glucose, sucrose, galactose, mannose or fructose. The glucose response persisted in the absence of extracellular Na^+ and in low-Ca^{2+}/Mn^{2+} saline. In voltage-clamp experiments, D-glucose evoked a small current with a reversal potential close to that of voltage-dependent K^+ currents. We conclude that D-glucose activates a K^+ current in CHH-immunoreactive cells that, in normal saline, induces a hyperpolarization. We propose that this enables glucose to regulate directly the release of CHH into the hemolymph, thus constituting a negative feedback mechanism regulating hemolymph glucose concentration.

Key words: X-organ, glucose, CHH, *Cancer borealis*, K^+ current, neurosecretion, crab

Introduction

The X-organ/sinus gland (XO-SG) system is one of the major peptidergic neurosecretory systems of decapod crustaceans. It consists of a cluster of somata located on the proximal surface of the medulla terminalis in the eyestalk, whose axons project to a neurohemal organ, the sinus gland, where they arborize into numerous terminals (for a review, see Cooke and Sullivan, 1982). The X-organ (XO) contains a heterogeneous population of neurosecretory cells that produce several neuropeptides involved in the regulation and homeostasis of a variety of physiological processes (for a review, see Keller, 1992). Among these neuropeptides is crustacean hyperglycemic hormone (CHH), which plays a crucial role in the regulation of hemolymph glucose levels *via* mobilization of glucose from the hepatopancreas and muscle glycogen stores. Secretion of CHH has been demonstrated using isolated XO-SG preparations *in vitro* (Stuenkel and Cooke, 1988; Keller *et al.* 1994), but very little is known of the regulation of XO neurons *in vivo*. Indirect evidence from *in vivo* experiments suggests the involvement of serotonin and dopamine (Keller and Beyer, 1968; Lüschen *et al.* 1993; Sarojini *et al.* 1995) and enkephalins (Jaros, 1990; Rothe *et al.* 1991; Sarojini *et al.* 1995) in the regulation of CHH secretion. In all these experiments, the substances were injected into the bloodstream of intact or eyestalkless animals and the glucose level was measured. The reported results vary for different species, and the cellular pathways mediating these responses remain unclear. In this study, we have examined the direct effects of glucose and some putative neurotransmitters on the electrical activity of isolated, cultured XO neurons from *Cancer borealis*.

The XO-SG system is analogous to the vertebrate hypothalamic–neurohypophyseal system in that both produce and release neuropeptides that participate in vegetative and homeostatic processes. Glucose-sensitive neurons have been found in different regions of the vertebrate brain. Glucose decreased the electrical excitability of cells in the lateral hypothalamic nucleus (Karádi *et al.* 1992) and in the substantia nigra (Saller and Chiodo, 1980; Watts *et al.* 1995), while excitatory effects were found in the lateral and ventromedial hypothalamus, the substantia nigra (Mizuno and Oomura, 1984; for a review, see Edwards and Weston, 1993) and the hippocampus (Tromba *et al.* 1994).

In vertebrates, almost all known effects of glucose, both in peripheral excitable tissue and in neurons, are mediated

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by ATP-sensitive K^+ channels (for reviews, see Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993). Briefly, glucose is transported into the cell, leading to a metabolic increase in the ATP level which, in turn, closes ATP-dependent K^+ channels. These channels are prime candidates to link cellular metabolic and electrical activity and are also associated with the regulation of neurotransmitter release (Saller and Chiodo, 1980; for a review, see Edwards and Weston, 1993).

In invertebrates, very little is known about the cellular responses of neurons to glucose. Excitatory effects have been described in neurosecretory cells of the pond snail *Lymnaea stagnalis* (Kits *et al.* 1991) and of the crayfish *Procambarus clarkii* (García *et al.* 1993; Onetti *et al.* 1996). To explain the mechanisms of action in these preparations, an electrogenic Na^+-coupled glucose transport system has been suggested for the snail, whereas for the crayfish, recent evidence indicates the operation of an ATP-sensitive K^+ channel (Onetti *et al.* 1996).

In the present study, we show that of all the XO neurons tested only CHH-immunoreactive (CHH^+) neurons responded to glucose. This response was always hyperpolarizing in normal saline, highly selective for D-glucose in the concentration range found in the hemolymph of living animals and was carried by K^+. However, the glucose-regulated K^+ conductance does not appear to be an ATP-sensitive K^+ channel and thus may constitute either a new kind of sugar-activated channel or receptor, or a channel-coupled glucose transport system. Some of these results have been reported in abstract form (Glowik *et al.* 1996).

Materials and methods

Adult male rock crabs, *Cancer borealis* Stimpson, were obtained from local fishermen in Boston, MA, USA, and were maintained at 15 °C in artificial seawater tanks until used. We used 112 animals, taking one eye from each. They were returned to the tanks for later use in other projects.

Dissection and culture conditions

We cultured XO neurons under sterile conditions using a modified protocol from Cooke *et al.* (1989) and Grau and Cooke (1992). Dissections were performed in a laminar-flow hood. Crabs were anesthetized by chilling them in ice for 30–60 min. Eyes were removed and rinsed in 70 % ethanol. The X-organs were dissected in cold *Cancer borealis* saline containing PSF (100 i.u. ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 1.25 mg ml^{-1} fungizone). They were placed in Ca^{2+}/Mg^{2+}-free saline containing PSF, and enzymatically treated with 2 % dispase in Ca^{2+}/Mg^{2+}-free saline for 90 min at room temperature (19–21 °C). After this, they were washed thoroughly with cold (4 °C) Ca^{2+}/Mg^{2+}-free saline containing PSF and transferred into defined culture medium. The culture medium was Leibovitz's L-15 medium diluted with an ion-balanced supplement solution (see below) and freshly added gentamicin (0.1 mg ml^{-1}), fungizone (0.25 mg ml^{-1}) and glutamine (final concentration 2 mmol l^{-1}), pH 7.4, 960–980 mosmol l^{-1}. Single neurons were removed from the X-organ by aspiration into fire-polished micropipettes and plated in uncoated 35 mm Nunclon plastic culture dishes (Delta, Denmark) in a drop of culture medium. The dishes were left undisturbed for at least 1 h to permit the cells to adhere to the dish. Finally, the volume of the medium was increased to 2.0 ml, and the dishes were sealed with Parafilm and kept in the dark at 15±1 °C until used. The culture medium was not changed during this time.

The cells were examined and recorded from using an inverted microscope (Nikon, Diaphot-TMD) with Hoffman modulation-contrast optics. Photographs of cells were taken with a Nikon camera using Kodak Ektachrome 160T film.

Saline composition

Cancer borealis saline (in mmol l^{-1}): 440 NaCl, 11 KCl, 26 $MgCl_2{\cdot}6H_2O$, 13 $CaCl_2{\cdot}2H_2O$, 12 Trizma base, 5 maleic acid. *Ca^{2+}/Mg^{2+}-free saline*: 476 NaCl, 11 KCl, 12 Trizma base, 5 maleic acid. *Ion-balanced supplement solution*: 740.4 NaCl, 16.2 KCl, 50.2 $MgCl_2{\cdot}6H_2O$, 24.7 $CaCl_2{\cdot}2H_2O$, 14 Hepes. *Na^+-free saline*: 440 *N*-methyl-D-glucamine, 11 KCl, 26 $MgCl_2{\cdot}6H_2O$, 13 $CaCl_2{\cdot}2H_2O$, 12 Trizma base, 5 maleic acid, 423 sucrose. *Low-Ca^{2+}/Mn^{2+} saline*: 440 NaCl, 11 KCl, 26 $MgCl_2{\cdot}6H_2O$, 0.1 $CaCl_2{\cdot}2H_2O$, 12.9 $MnCl_2$, 12 Trizma base, 5 maleic acid, 58 sucrose. All solutions were adjusted to 960–980 mosmol l^{-1} and to pH 7.4. High-K^+ solutions were prepared by replacing equimolar amounts of NaCl with KCl.

Chemicals

Leibovitz's L-15 medium, penicillin, streptomycin, fungizone and gentamycin were purchased from GIBCO. Dispase was purchased from Boehringer-Mannheim. D-Glucose and sucrose were obtained from Fisher Chemical. All other chemicals were obtained from Sigma.

Electrophysiology

Intracellular recordings were made using microelectrodes pulled from borosilicate glass (1.0 mm o.d. by 0.75 mm i.d.) on a Sutter P-87 puller and filled with 0.6 mol l^{-1} K_2SO_4 and 20 mmol l^{-1} KCl (15–35 MΩ), and an Axoclamp-2A amplifier. Cells were continuously superfused with *C. borealis* saline. All substances and salines were applied in the bath at the indicated concentrations. Sugars were dissolved in distilled water at 0.5 mol l^{-1}, portioned and frozen. Serotonin (5-HT), dopamine, γ-aminobutyric acid (GABA) and Leu-enkephalin were dissolved in distilled water at 10^{-3} mol l^{-1}, portioned and frozen. Ouabain and 4-aminopyridine (4-AP) were dissolved in *C. borealis* saline before use. Tetrodotoxin (TTX, 10^{-4} mol l^{-1} stock solution), $CdCl_2$ (1 mol l^{-1} stock solution) and tetraethylammonium (TEA^+, 1 mol l^{-1} stock solution) were kept at 4 °C in the dark. Samples were thawed and/or diluted into saline immediately before use. Tolbutamide was prepared as a 1 mol l^{-1} stock solution in 1 mol l^{-1} NaOH and diluted with

saline before use. Cs^+ was kept as a 1 mol l^{-1} stock solution and diluted in saline before use.

Voltage recordings were made in discontinuous single-electrode current-clamp mode using electrodes with resistances 20–35 MΩ. Current recordings were made in discontinuous single-electrode voltage-clamp mode with electrodes of slightly lower resistance (15–20 MΩ). The sampling rate in both cases was between 4 and 5 kHz. For voltage-clamp experiments, the gain of the amplifier was routinely increased to the instrument's maximum. Pulse-driven current measurements and analysis were carried out using pClamp software (Axon Instruments, CA, USA). All experiments were also directly recorded on a Gould 2400 chart recorder.

Immunocytochemistry

We used a three-step peroxidase–antiperoxidase (PAP) method to stain the cells for CHH-like immunoreactivity. Cultured cells were fixed in Stefanini's fixative (2 % paraformaldehyde, 15 % picric acid, 24 mmol l^{-1} NaH_2PO_4, 158 mmol l^{-1} Na_2HPO_4, pH 7.4) with 1 % sucrose, at 980 mosmol l^{-1}. Cells were permeabilized with 0.1 mol l^{-1} phosphate-buffered saline (PBS) containing 0.01 % saponin and 20 % sucrose, pH 7.4, 980 mosmol l^{-1}. The subsequent washes were performed with PBS. To prevent nonspecific binding, the neurons were incubated in 0.5 % normal goat serum (NGS) for 30 min, followed by an incubation with the primary rabbit anti-crustacean hyperglycemic hormone antiserum (α-CHH, Dircksen *et al.* 1988) diluted 1:19 000 in PBS for 1–2 h at room temperature (19–21 °C). A secondary goat anti-rabbit antibody (Nordic Immunology, Tilburg, Netherlands) diluted 1:200 in PBS was used. The third step included an incubation for 30–60 min at room temperature with a rabbit antibody–PAP complex diluted 1:1200 in PBS (DAKO, Denmark), followed by the staining reaction with 0.008 % diaminobenzidine in 0.1 mol l^{-1} phosphate buffer (PB) and 0.125 μl ml^{-1} H_2O_2 (30 %).

Hemolymph glucose concentrations were measured using the glucose oxidase method on whole unprocessed hemolymph (Glucose-Kit, Sigma, using method indicated for 'plasma or serum directly'). Two samples of 50 μl of hemolymph were drawn from a leg joint of each animal. Each sample was diluted 1:10 in distilled water and then processed according to the kit's instructions.

Values are expressed as the mean ± standard deviation of the mean.

Results

Cell identification and immunocytochemistry

Low-density cultures of XO cells produced a neuronal population with heterogeneous morphologies and dimensions. Cell body diameters ranged between 10 and 60 μm. In agreement with previous studies (Cooke *et al.* 1989; Grau and Cooke, 1992), we could distinguish two basic morphologies: cells with lamellipodial outgrowths and cells with filopodial outgrowths. A significant fraction of the neurons showed outgrowths with both features. We observed all possible combinations of outgrowths on cultured cells either having or lacking a neurite segment. The cells started growing immediately after plating and continued to do so for 4–5 days, but their initial morphology was not essentially modified during this time. Most cells survived for 7–10 days.

We stained a total of 1842 neurons, ranging in age between 2 and 6 days in culture (Fig. 1). 8.0 % of them showed α-CHH immunoreactivity (N=147). There were three groups among these α-CHH-immunoreactive cells, distinguishable by the diameter of the soma: cells with somata 35–45 μm in diameter (36.7 %), cells 45–55 μm in diameter, representing the majority of cells (59.5 %), and cells 55–60 μm in diameter (3.8 %). On the basis of their outgrowth characteristics, 38.9 % of the total number of CHH^+ cells did not extend any outgrowths, 36.8 % grew lamellipodia, 13 % grew filopodia exclusively and 11.3 % extended both types of outgrowths. The intensity of the α-CHH immunoreactivity varied over different parts of the cell. We observed staining either in the whole cell or sometimes only in the distal parts of the outgrowth. Fig. 1A shows an example of an α-CHH-immunoreactive (CHH^+) cell and Fig. 1B shows a non-CHH-immunoreactive (CHH^-) cell.

For the electrophysiological recordings, we preferentially selected cells with soma diameters in the range 45–55 μm to increase the chances of recording from a CHH^+ cell. We recorded electrophysiologically from 211 neurons that had been in culture for 2–6 days. 105 neurons responded to D-glucose with a hyperpolarization in normal saline (Fig. 1A). We were able to process 53 of these for α-CHH immunoreactivity, and 96 % were CHH^+. 106 cells did not respond to glucose. We processed 47 for α-CHH immunoreactivity, and none of them was α-CHH^+. Thus, we conclude that only CHH^+ neurons show a response to glucose while CHH^- neurons fail to do so. There was no significant difference in input resistance (as measured under resting conditions) between glucose-insensitive cells (493±281 MΩ, N=89) and glucose-sensitive cells (564±259 MΩ, N=66). There was a significant decrease in input resistance (P<0.02, paired Student's t-test) between cells before (451±154 MΩ) and during glucose application (354±128 MΩ, N=14). Both cell types showed variable intrinsic activity, being silent, or showing tonic action potential firing, or bursting activity or plateau activity. The cells often changed their activity pattern during a recording session.

Selectivity of the response

Fig. 2 shows the responses of one typical glucose-sensitive cell to the application of a variety of different sugars. As can be seen, the cell responds exclusively to D-glucose, while L-glucose, sucrose, galactose, mannose and fructose failed to elicit responses. This experiment was repeated in eight other cells with the same results, indicating a high level of selectivity of the response to D-glucose.

We also measured the concentration-dependence of the

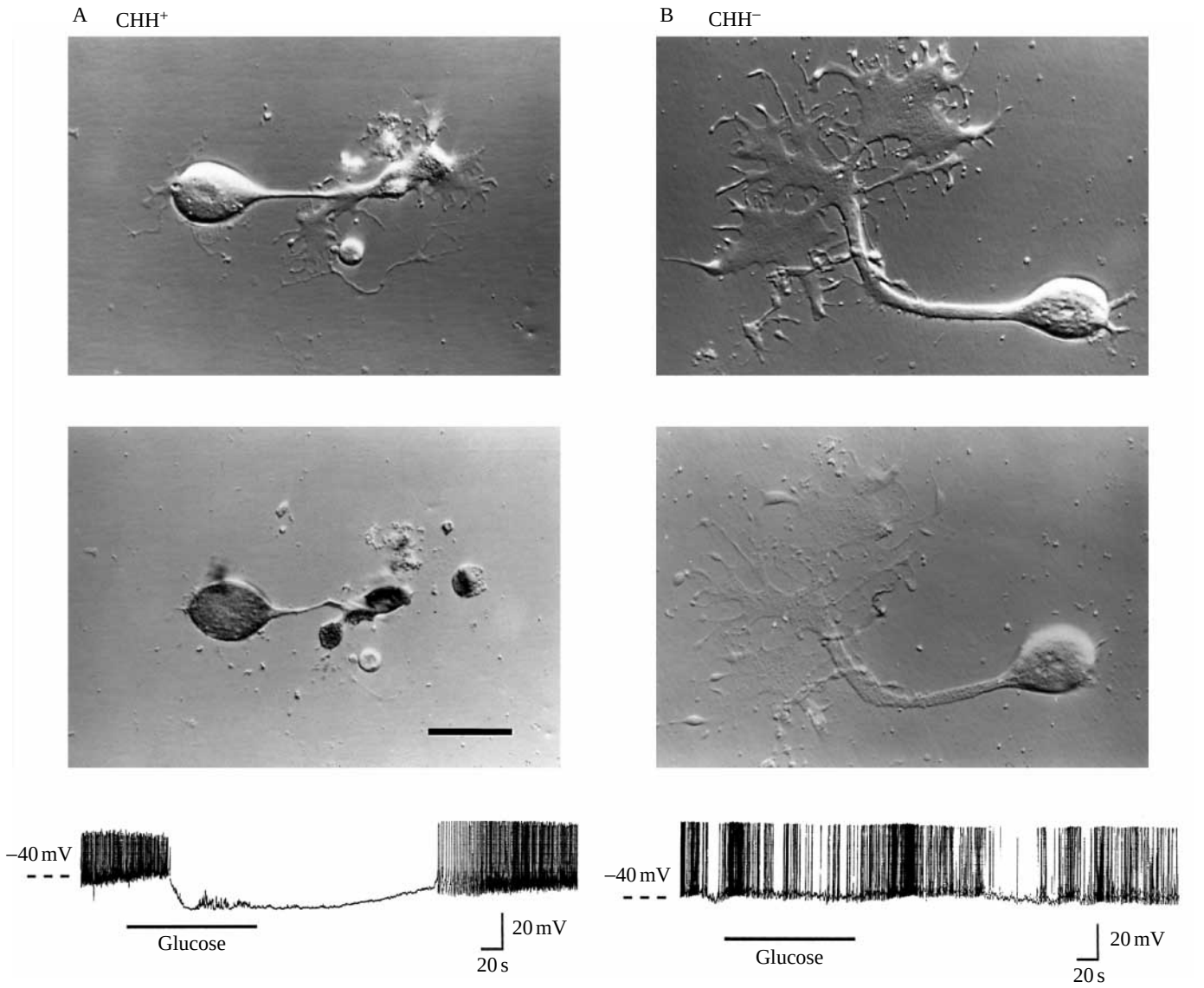


Fig. 1. Glucose-sensitive neurons are α-CHH-immunoreactive (CHH$^+$). (A) CHH$^+$ cell viewed using Hoffmann modulation-contrast optics before recording (top panel) and after recording and staining for CHH (middle panel). The bottom panel shows a characteristic response of one such cell to 5 mmol l^{-1} D-glucose (black bar). Same cell as shown in Fig. 4. (B) Non-CHH-immunoreactive (CHH$^-$) cell. Same conditions as in A: before recording (top panel), and after recording and staining (middle panel). The bottom trace shows no effect of 5 mmol l^{-1} D-glucose on a different CHH$^-$ neuron. Notice that the morphology of the cells has changed somewhat after recording (middle panel photographs). Scale bar, 50 μm.

response to D-glucose (Fig. 3). The dose–response curve to D-glucose shown in Fig. 3 exhibits saturation binding with an EC_{50} of 0.25 mmol l^{-1}. The smooth line is a fit to the mean responses of six cells with the Michaelis–Menten equation:

$$V = \frac{V_{max} \times [\text{glucose}]}{K_m + [\text{glucose}]},$$

where V_{max} is 105 % and K_m is 0.25 mmol l^{-1}.

Hemolymph glucose levels

In other crustacean species, various hemolymph glucose levels have been reported: 0.9±0.2 mmol l^{-1} in *Procambarus clarkii* (García *et al.* 1993), 0.03–0.19 mmol l^{-1} in *Orconectes limosus* (Keller and Orth, 1990), 0.1–0.3 mmol l^{-1} in *Carcinus maenas* (Lüschen *et al.* 1993) and 0.8 mmol l^{-1} (mean value) in *Cancer pagurus* (Webster, 1996). In *Cancer borealis*, we measured values in hemolymph samples from 10 animals in the range 0.05–0.49 mmol l^{-1} (0.23±0.11 mmol l^{-1}).

Mechanism of action

Using current- as well as voltage-clamp measurements, we performed a series of experiments aimed at identifying the mechanism of action of D-glucose on CHH$^+$ cells.

Fig. 4 shows the effects of eliminating extracellular Na^+ and

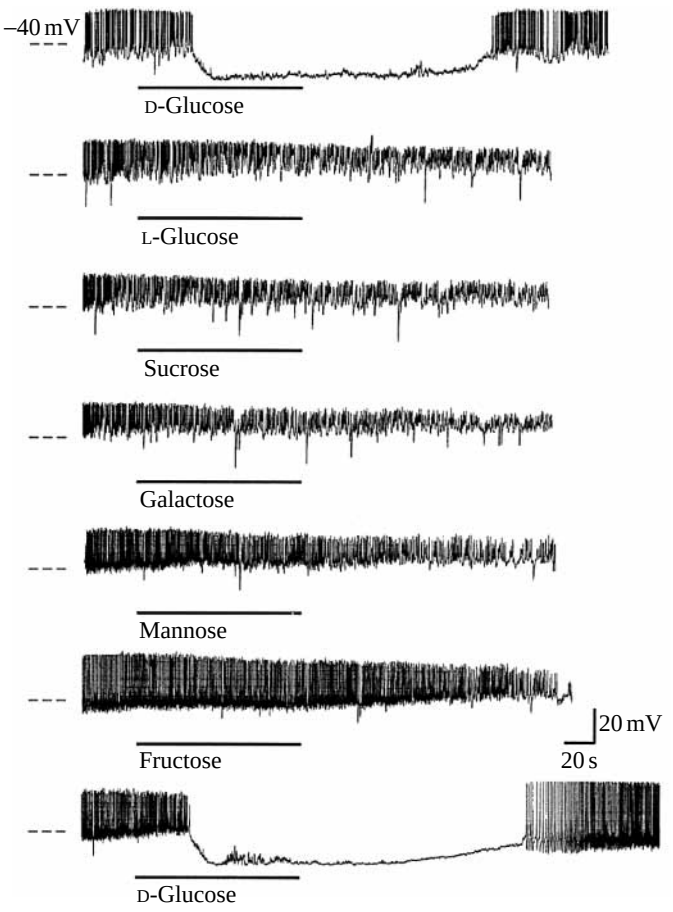


Fig. 2. Sugar selectivity of the response. Response of a CHH^+ cell to applications of various sugars. Each sugar was applied for 2 min at a concentration of 5 mmol l^{-1} in normal *Cancer borealis* saline (black bars). The sugars were applied in the order shown, including the last control application of D-glucose. The traces were brought to similar baseline membrane potentials with constant depolarizing current injections approximately 2 min before the beginning of the trace shown. The decrease in action potential amplitude in the middle traces is due to action potential adaptation after current injection onset, not to the effects of the sugars. The delay here and in subsequent figures corresponds to the dead volume of the superfusion system.

Ca^{2+}. When the external Na^+ was replaced with *N*-methyl-D-glucamine, the cells usually hyperpolarized slightly. However, the glucose response persisted (Fig. 4, middle trace, *N*=6). Action potential generation was largely unaffected, although the amplitude as well as the firing frequency were slightly decreased (Fig. 4, compare top and middle traces). We obtained similar results when we used 10 μmol l^{-1} TTX to block TTX-sensitive Na^+ channels (*N*=2, results not shown). The glucose response was not blocked by 0.5 mmol l^{-1} ouabain (*N*=3, results not shown). Replacement of Ca^{2+} with Mn^{2+} (leaving 0.1 mmol l^{-1} Ca^{2+} in the saline to prevent the deterioration of the cells) caused a rapid block of action potential generation. However, the response to D-glucose was unchanged (Fig. 4, bottom trace; *N*=5). Cd^{2+} is known to block Ca^{2+} currents in the *Cardisoma carnifex* neurosecretory XO neurons (Meyers *et al.* 1992; Richmond *et al.* 1995). We found that Cd^{2+} (200 μmol l^{-1}) did not block the glucose response (*N*=3, results not shown). Cd^{2+} proved toxic to the cells and in subsequent experiments, where blocking of action potential generation was necessary, we used Mn^{2+} in exchange for equimolar amounts of Ca^{2+}.

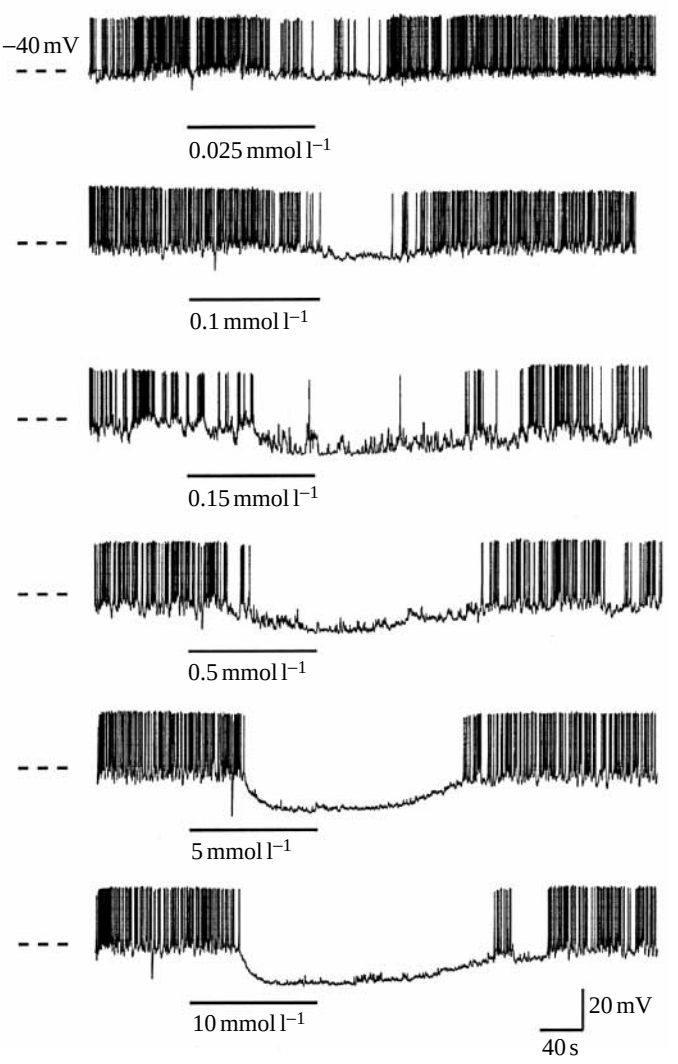
In current-clamp recordings, the glucose response did not reverse at voltages between −10 and −80 mV in normal saline (*N*=5, results not shown). Since −80 mV is the lowest value to which we were able to bring these cells stably, we examined the response in voltage-clamp mode in saline with different K^+ concentrations. Fig. 5A shows an example of glucose-evoked currents at different membrane potentials in normal saline. We observed only outward currents at all membrane potentials tested (*N*=4). When the external K^+ concentration was raised to 10 times the normal concentration (10×[K^+]), we obtained a reversal potential of approximately −35 mV, as shown in Fig. 5B (*N*=12). Fig. 5C shows the mean current–voltage (*I*–*V*) curves for the glucose-evoked current in normal external [K^+] (1×[K^+]), 5×[K^+] and 10×[K^+]. The reversal potentials obtained from these curves are −35 mV in 10×[K^+], −50 mV in 5×[K^+] and approximately −70 mV in 1×[K^+].

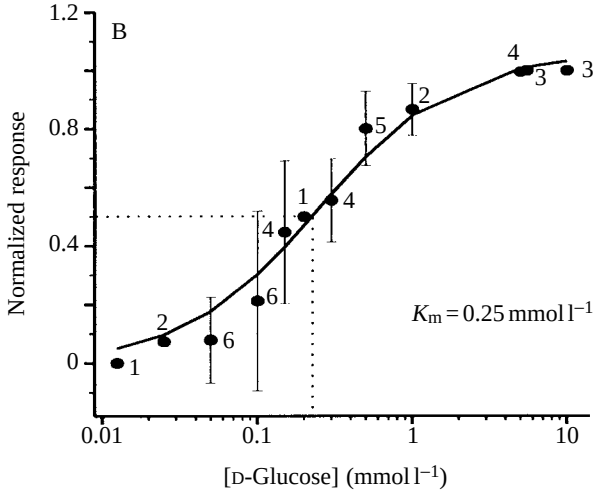
To determine whether these reversal potentials correspond to the K^+ equilibrium potential, we measured the reversal potentials of the combined voltage-dependent outward currents generated by these same cells using a tail current protocol. These currents are known to be carried mostly by K^+ and include a delayed rectifier, a Ca^{2+}-dependent K^+ current and the A-type K^+ current (Meyers *et al.* 1992). Fig. 6 shows these results under the same three external K^+ concentrations: 1×[K^+], 5×[K^+] and 10×[K^+]. We repeated this experiment in four glucose-sensitive cells. The reversal potentials of the tail currents for the three different K^+ concentrations (Fig. 6) are very similar to those of the glucose-evoked currents (Fig. 5). At normal external K^+ concentration, we obtained a mean reversal potential of −76±5 mV (Fig. 6A), in 5×[K^+], we measured a reversal potential of −48±3 mV, and in 10×[K^+] the outward current's reversal potential was −33±5 mV (Fig. 6C). The high level of correspondence between the reversal potentials of K^+-current tails and the reversal potentials of the glucose-evoked currents led us to conclude that the glucose-activated current is mostly carried by K^+.

Pharmacology of the glucose response

To identify the nature of the K^+ current activated by D-glucose, we carried out a series of experiments using known pharmacological agents to block K^+ currents (Fig. 7). We observed a partial block of the glucose response in TEA^+ (Fig. 7A). In five cells, the inhibitory effect of 10 mmol l^{-1} TEA^+ was 44.5±35.0 %. 4-AP (3 mmol l^{-1}), a known blocker of the A-type K^+ current, had mixed effects. In 38 % of the cells (*N*=3/8) tested, we observed a total block of the glucose response, in 38 % of the cells (*N*=3/8) we observed a partial block, while in 25 % of the cells (*N*=2/8) we saw no effect (results not shown). Tolbutamide (1 mmol l^{-1}), a specific

blocker of ATP-sensitive K^+-currents had no effect (Fig. 7B, N=3). Finally, Cs^+ (5 mmol l^{-1}), a known blocker of a number of K^+ currents (Hille, 1992) also had no measurable effect on the glucose response (N=3, results not shown).





Pharmacological profile of glucose-sensitive cells

A number of substances have been implicated in the control of hemolymph glucose levels (Keller and Beyer, 1968; Lüschen *et al.* 1993; Sarojini *et al.* 1995; Jaros, 1990; Rothe *et al.* 1991). For this reason, we studied the effects of 5-HT, dopamine, GABA and Leu-enkephalin on glucose-sensitive cells.

Fig. 8 shows responses of an identified CHH^+ cell. This cell showed a pronounced hyperpolarization in response to glucose, 5-HT and GABA, but did not respond to dopamine or Leu-enkephalin. 5-HT (10 μmol l^{-1}) elicited a hyperpolarization in 61 % of the cells we tested (N=11/18). GABA (10 μmol l^{-1}) elicited a hyperpolarization in 55 % of the tested cells (N=6/11). Of the six glucose-sensitive cells on which both 5-HT and GABA were also tested, 66 % (N=4/6) responded to all three substances with a hyperpolarization, one hyperpolarized in response to 5-HT but did not respond to GABA, and one responded only to glucose. Finally, dopamine (10 μmol l^{-1}) elicited no response in 100 % of the cells tested (N=9). Likewise, Leu-enkephalin (10 μmol l^{-1}) showed no effect on six cells tested. The apparent inhibition seen in response to dopamine and Leu-enkephalin in Fig. 8 corresponds to spike-rate adaptation, not to an effect of these substances. The same spike-rate adaptation phenomenon can be observed in the other traces where D-glucose, 5-HT and GABA also have a hyperpolarizing effect on the cell.

Discussion

Animals need to regulate their blood glucose concentration to ensure that adequate glucose levels are available for all cells and that excess glucose can be stored. In the present study, we provide, for the first time, direct evidence that the neurons that secrete a major glucose-regulating hormone, CHH, in crustaceans are directly sensitive to circulating glucose levels in a way that would provide a negative feedback regulatory mechanism of glucose levels.

We have studied the morphology and the responses to D-glucose of cultured neurosecretory cells from the XO of the crab *Cancer borealis*. We found that only α-CHH-immunoreactive neurons responded to glucose and that the responses were always hyperpolarizing in normal saline. It has been shown that CHH is released from the terminals in the sinus gland as the XO neurons depolarize and fire action

Fig. 3. Glucose dose–response curve. (A) Responses of a glucose-sensitive and α-CHH-immunoreactive neuron to 2 min applications of D-glucose (black bars) at the indicated concentrations. Same cell as shown in Fig. 8. (B) Normalized peak hyperpolarizations recorded in response to applications of different concentrations of D-glucose plotted as a function of the sugar concentration. Hyperpolarization was measured from the trough of the interspike interval. Responses were normalized to the amplitude of the response at 5 mmol l^{-1}. Numbers of cells tested are indicated next to each point. The curve is a fitted to the mean experimental values using the Michaelis–Menten equation (see Results for further details).

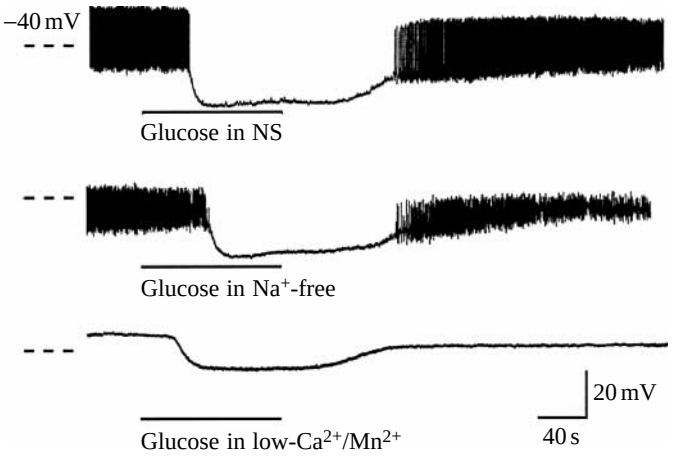


Fig. 4. Effect of external Na^+ and Ca^{2+} on the glucose response. Discontinuous single-electrode current-clamp recordings of the response of a single cell to 2 min bath applications (black bars) of 5 mmol l^{-1} D-glucose in normal *Cancer borealis* saline (NS) (top trace), Na^+-free solution (Na^+ replaced with *N*-methyl-D-glucamine, middle trace) and low-Ca^{2+}/Mn^{2+} solution (12.9 mmol l^{-1} Ca^{2+} replaced with an equimolar amount of Mn^{2+}).

potentials (Stuenkel and Cooke, 1988; Keller *et al.* 1994). In our study, whenever glucose-sensitive cells fired spontaneous action potentials, D-glucose always inhibited spiking. Because most, if not all, glucose-sensitive cells in culture were also CHH^+, the effect of D-glucose on these cells indicates that D-glucose itself plays a crucial role in the regulation of glycemia in crustaceans. We suggest that this occurs as a result of an inhibition of CHH release and consequently a lowering of the glucose level in the hemolymph when the glucose concentration in the blood is high (Fig. 9). Indeed, we have shown that CHH^+ cells appear to be sensitive to D-glucose at around the concentrations to which they are exposed under normal conditions *in vivo*.

In vertebrates, glucose evokes both depolarizing and hyperpolarizing responses in several regions of the brain. Most of these responses are mediated by ATP-sensitive K^+ channels (for reviews, see Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993). In an invertebrate, the crayfish *Procambarus clarkii*, Onetti *et al.* (1996) recently reported the existence of ATP-sensitive K^+ channels in XO neurons, which are affected by glucose through a second messenger system, probably ATP itself. We have shown that a K^+ current is involved in the response of XO neurons to D-glucose in *C. borealis*. In contrast to what has been described in vertebrates, however, this current is insensitive to the specific ATP-sensitive K^+ channel blocker tolbutamide, but is slightly sensitive to other known K^+ channel blockers such as 4-AP and TEA^+. In XO cells, the current activated by D-glucose appears to be voltage-independent, which suggests that it may be part of the leak current of these cells. This current therefore differs from vertebrate ATP-sensitive K^+ currents. This raises the possibility that there may be an alternative mechanism coupling extracellular D-glucose to a K^+ channel in CHH^+ cells. Such a mechanism could involve a glucose receptor (Fig. 9). Other monosaccharide receptors have been described in bacteria, where they are involved in chemotaxis and sugar transport (for a review, see Macnab, 1987). However, no examples of glucose receptors other than transporters are known in invertebrates or vertebrates. Alternatively, a specific glucose transport system coupled to the K^+ electrochemical gradient that differs from the known Na^+-coupled glucose transport system could be at work in these cells. However, known passive glucose transport systems show different

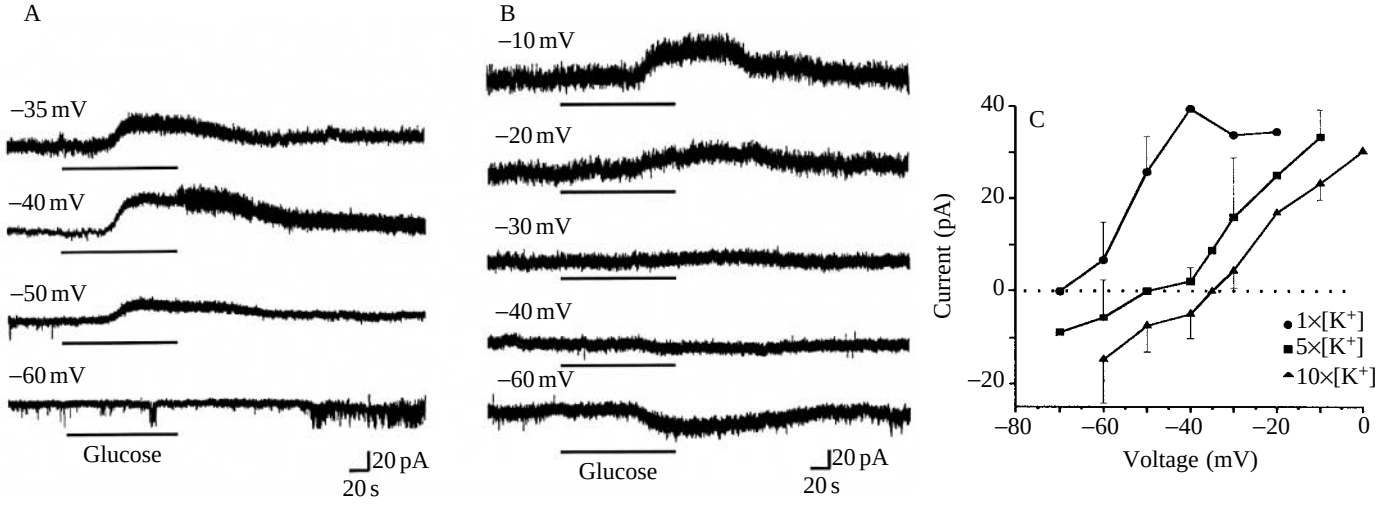


Fig. 5. Effect of external K^+ concentration on the glucose-evoked current. Discontinuous single-electrode voltage-clamp recordings of glucose-evoked currents in response to 2 min bath applications (black bars) of 5 mmol l^{-1} D-glucose at different holding potentials. (A) Currents measured in normal *Cancer borealis* saline at the holding potentials indicated above each trace. (B) Currents measured in 110 mmol l^{-1} external [K^+] (10×[K^+]) and low-Ca^{2+}/Mn^{2+} solution at the holding potentials indicated. (C) Mean glucose-evoked current plotted against the holding potential in three different external K^+ concentrations: 11 mmol l^{-1} (1×[K^+]), 55 mmol l^{-1} (5×[K^+]) and 110 mmol l^{-1} (10×[K^+]). Measurements obtained in normal-Ca^{2+} saline and in low-Ca^{2+}/Mn^{2+} saline were pooled.

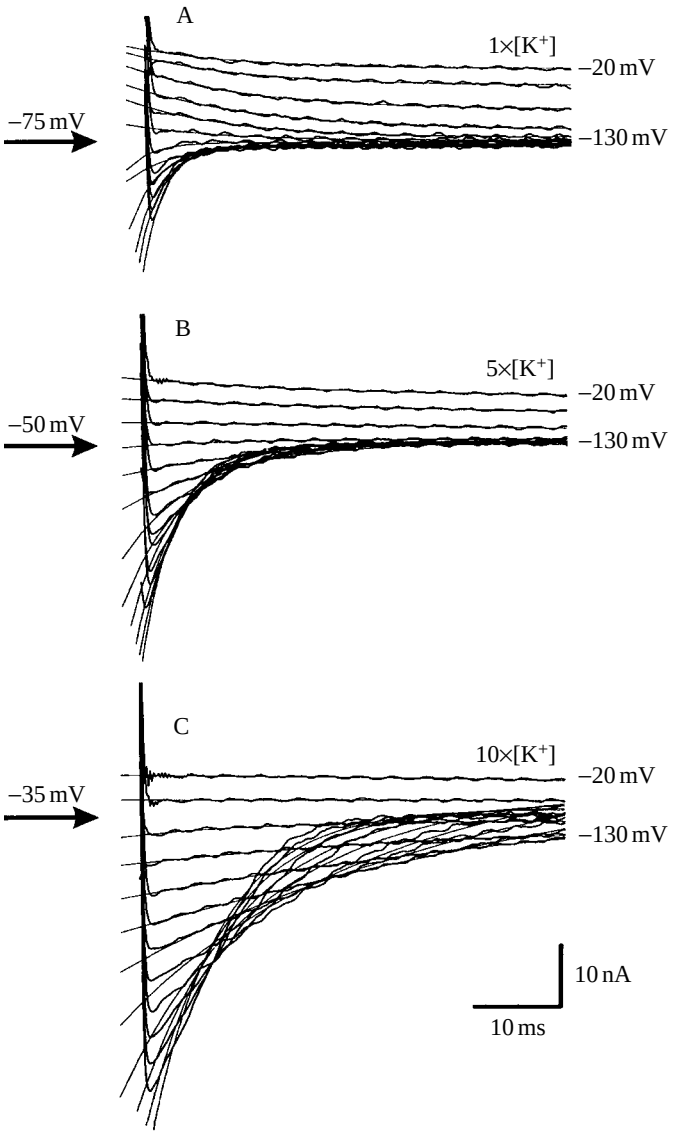


Fig. 6. K^+ current tail current measurements. Outward currents were activated at +10 mV for 20 ms after holding the cell at −120 mV for 480 ms in order to activate all voltage-dependent outward currents and maximize the tail currents. Tail currents were measured at 10 mV intervals from −130 to −20 mV and fitted with single exponentials (smooth lines) starting 3 ms after the beginning of the voltage transition in order to avoid contamination from the capacitance artifact. The currents were also leak-subtracted using the p/n protocol (N=6) of the pClamp acquisition software and filtered at 2 kHz. Three different external K^+ concentrations were used: 11 mmol l^{-1} (1×[K^+]), 55 mmol l^{-1} (5×[K^+]) and 110 mmol l^{-1} (10×[K^+]). The reversal potentials are indicated to the left of the traces.

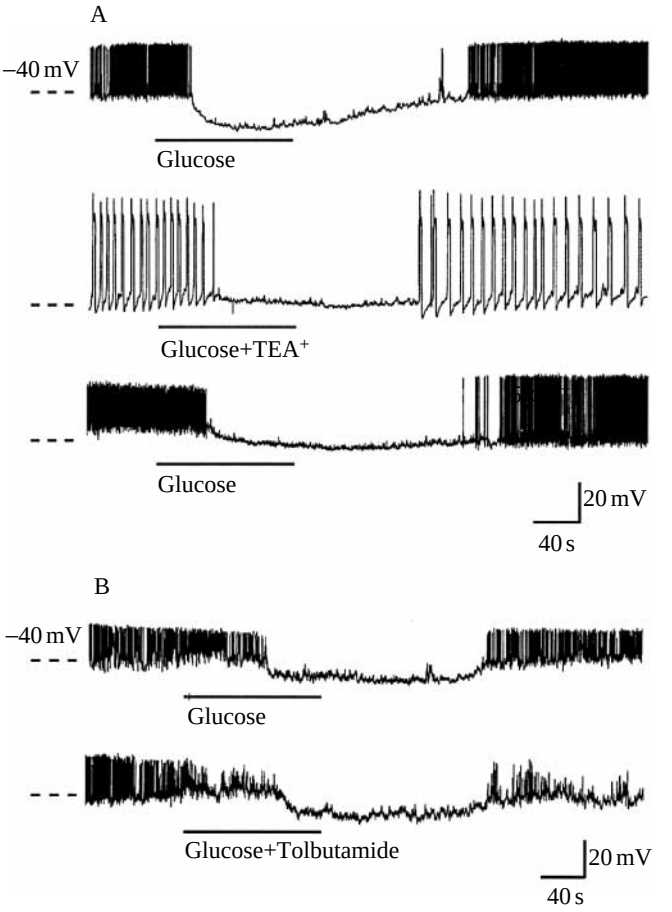


Fig. 7. Pharmacology of the glucose response. (A) Effect of TEA^+. Response to 5 mmol l^{-1} D-glucose measured in normal saline (top trace), in the presence of 10 mmol l^{-1} TEA^+ (middle trace) and in normal saline after washing out TEA^+ for 10 min. (B) Effect of tolbutamide. Response to 5 mmol l^{-1} D-glucose in normal saline (top trace) and in the presence of 1 mmol l^{-1} tolbutamide (bottom trace). Results are from a different cell from the one shown in A.

sensitivities and less specificity for glucose (Carruthers, 1990) than those we have observed here. Finally, a metabolically driven activation of K^+ channels different from known ATP-sensitive K^+ channels might be involved.

Several *in vivo* studies suggest that 5-HT, dopamine, GABA and Leu-enkephalin are involved in the regulation of hemolymph glucose concentration. However, the evidence comes from several different species, the results are inconsistent and they do not directly address the mechanisms involved (Keller and Beyer, 1968; Jaros, 1990; Rothe *et al.* 1991; Lüschen *et al.* 1993; Sarojini *et al.* 1995). Our present results support a possible role for 5-HT and GABA in the regulation of CHH^+ cell activity, as has been suggested by *in vitro* experiments on unidentified XO neurons (Nagano, 1986; García *et al.* 1994). Since none of the glucose-sensitive cells tested showed any response to dopamine or Leu-enkephalin, our results also indicate that neither substance regulates glycemia directly by action on CHH^+ cells, as had originally been suggested by Jaros (1990), Lüschen *et al.* (1993) and Sarojini *et al.* (1995). However, we cannot eliminate the possibility that axonal collaterals or dendrites projecting into the medulla terminalis neuropil and the sinus gland, eliminated during the culturing procedure, normally express dopamine and/or Leu-enkephalin receptors. Our results indicate that subpopulations of CHH-containing neurons may exist in the

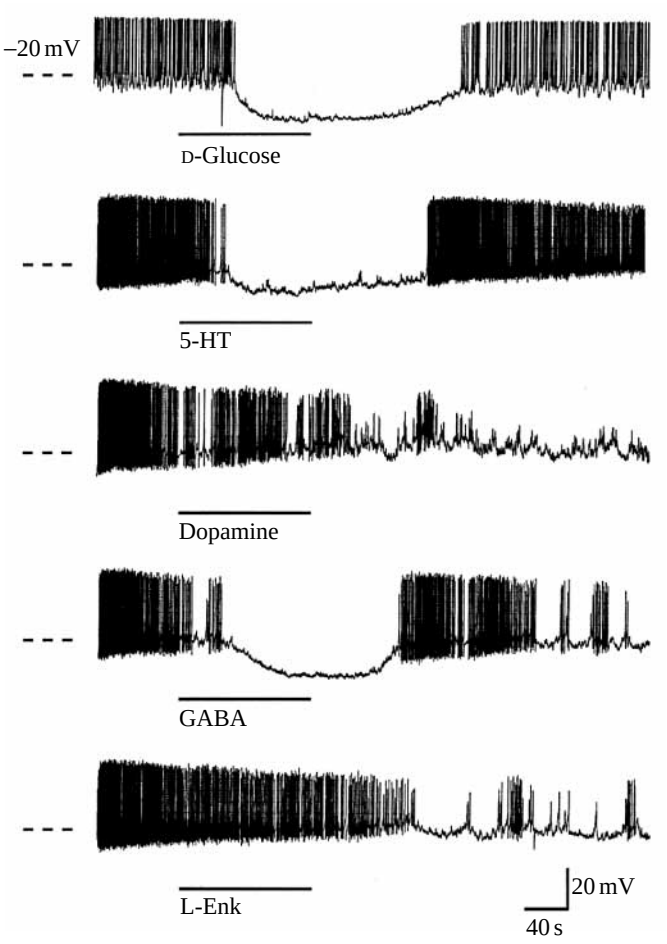


Fig. 8. Pharmacological profile of a glucose-sensitive cell that subsequently stained positively for CHH. Responses to 2 min applications of 5 mmol l^{-1} D-glucose in normal saline (top trace), 10^{-5} mol l^{-1} serotonin (5-HT) (second trace), 10^{-5} mol l^{-1} dopamine (third trace), 10^{-5} mol l^{-1} GABA (fourth trace) and 10^{-5} mol l^{-1} Leu-enkephalin (L-Enk) (bottom trace). Bars indicate the time of agonist application. Dashed line indicates –40 mV in all traces. Depolarizing current was injected into the cell a few seconds before the recordings to bring it to action potential threshold.

X-organ because both 5-HT and GABA inhibited different subsets of these cells, and these subsets do not completely overlap. This is also suggested by the observation of CHH^+ cells of varying size and different patterns of immunoreactivity, some cells staining only in the soma, some staining only in the outgrowths or some staining in both. A segregation of CHH^+ cells into subpopulations might also be related to the finding that CHH exists in multiple isoforms (for a review, see Keller, 1992; Soyez *et al.* 1994; Yasuda *et al.* 1994). These subpopulations could be modulated differentially by different substances.

To our knowledge, the present report is the first to indicate that CHH-immunoreactive cells are directly inhibited by D-glucose. Our results contrast sharply with those of García *et al.* (1993) and Onetti *et al.* (1996). These authors reported that XO neurons *in situ* respond to D-glucose exclusively with depolarizations. Surprisingly, we have never recorded a depolarization in any cultured neuron in response to D-glucose. However, they recorded from unidentified neurons of the crayfish *P. clarkii* XO *in situ* and it is not known whether these recordings were from CHH^+ cells. The most likely explanation

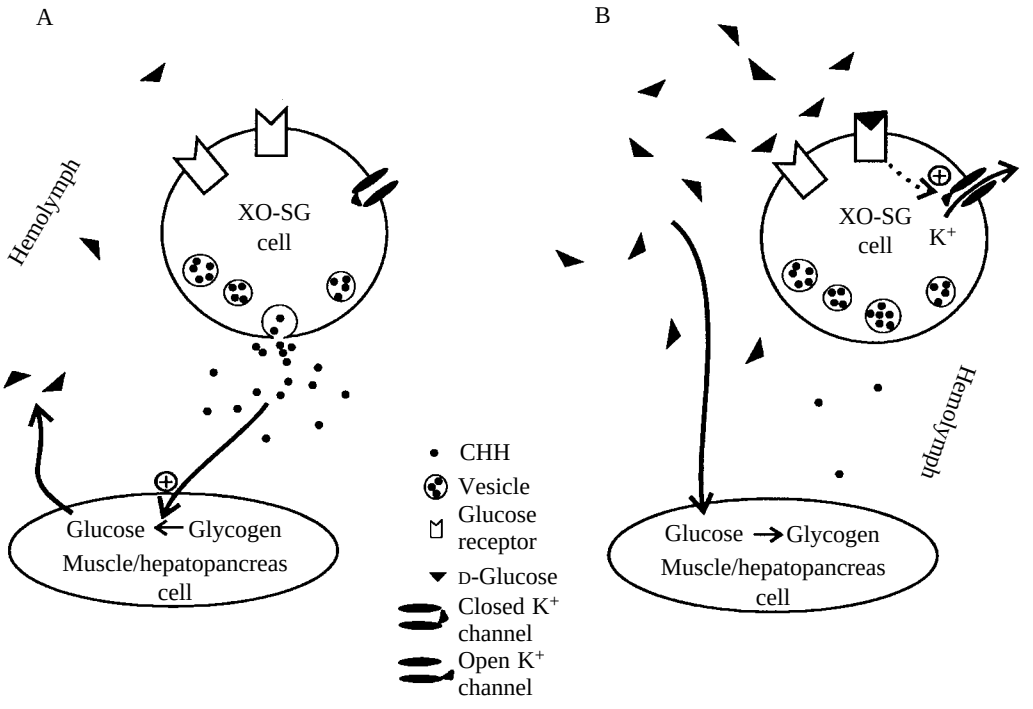


Fig. 9. Proposed model of D-glucose action on CHH-containing neurons of the X-organ/sinus gland (XO-SG). (A) When D-glucose levels are low in the hemolymph, the glucose-sensitive K^+ current decreases, CHH^+ cells depolarize, they release CHH and this induces the hydrolysis of glycogen, with a consequent increase in D-glucose concentration in the blood. (B) When D-glucose concentration in the hemolymph increases, putative glucose receptors on CHH^+ cells are activated, K^+ channels open, the cells hyperpolarize and CHH release is inhibited, thus reversing the process of glucose production in the target tissues (muscle and hepatopancreas). The putative glucose-sensing element (receptor or transporter) and the K^+ channel are drawn here as separate molecules, but this is speculative because we have no direct evidence that they are either separate or the same entity.

for this discrepancy is species differences, or that these researchers recorded polysynaptic effects of a D-glucose-evoked inhibition of CHH^+ cells, or that our culturing protocol selected against the cells that depolarize in response to D-glucose. Our results are, however, in agreement with those of Santos and Keller (1993), who found that glucose injected into the hemolymph of *Carcinus maenas* caused a significant decrease in circulating levels of CHH. Interestingly, in *Lymnaea stagnalis*, an excitatory effect of glucose has been reported on identified neurosecretory light green cells (Kits *et al.* 1991). These cells produce insulin-related peptides which, in contrast to CHH, are likely to cause a decrease in hemolymph glucose levels. Therefore, the excitatory action of glucose on these cells would have, as in pancreatic β-cells, a clear role in regulating blood glucose levels.

Cooke *et al.* (1989) and Keller *et al.* (1995) have reported a strong correlation between lamellipodial outgrowths and CHH-immunoreactivity and CHH content of crab *Cardisoma carnifex* cultured neurosecretory cells. In contrast with these observations, we found no clear relationship between CHH immunoreactivity and cell morphology. These discrepancies may be related to species differences as well as to differences in the culturing protocol.

In summary, we report here, for the first time to our knowledge, a response to glucose by identified (CHH-immunoreactive) cells. We conclude that D-glucose activates a K^+ current in CHH^+ cells that, in normal saline, induces a hyperpolarization (Fig. 9B). This would inhibit the release of CHH, thus inhibiting the mobilization of glucose from glycogen stores in the muscle and hepatopancreas cells. When hemolymph glucose level is low, CHH-containing cells depolarize, inducing the release of CHH and the mobilization of glucose from glycogen (Fig. 9A). Because D-glucose inhibits α-CHH-immunoreactive cells exclusively, CHH neurons appear to act as glucose sensors and this enables glucose to play an important role in regulating the release of CHH into the hemolymph and therefore in regulating hemolymph glucose concentrations through a negative feedback mechanism.

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