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MODULATION OF *APLYSIA CALIFORNICA* SIPHON SENSORY NEURONS BY CONOPRESSIN G

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Summary

Conopressin G, a molluscan vasopressin-like peptide, when superfused over the abdominal ganglion suppressed gill withdrawal reflex behavior patterns. The effects of conopressin G on *Aplysia californica* central sensory neurons and on the sensory neuron to motor neuron synapse were inconsistent with its behavioral effects. That is, superfusion of the peptide facilitated synaptic transmission at this synapse and reversed low-frequency homosynaptic depression. Further, conopressin G potentiated frequency-dependent spike broadening, reduced spike threshold and reduced accommodation. A voltage-dependent outward K^+ current was suppressed by the peptide. This current was also suppressed by Co^{2+} and Ba^{2+} and was relatively resistant to tetraethylammonium and 4-aminopyridine. The effects produced by conopressin G on the sensory neurons were not observed when Ca^{2+} was removed from the saline, when a low-Ca^{2+}, high-Mg^{2+} saline was used or when other procedures that impair synaptic transmission were used. These results suggest that the effects of conopressin G were mediated by a polysynaptic pathway acting on the sensory neurons.

Introduction

A novel molluscan vasopressin analogue, conopressin G, extracted from the venom of fish-hunting snails of the genus *Conus* (Cruz *et al.* 1987), has been shown to suppress the siphon-evoked gill withdrawal reflex (GWR) and to increase the rate of spontaneous gill movements (SGMs) in semi-intact *Aplysia californica* preparations (Martínez-Padrón *et al.* 1992). Furthermore, conopressin G or a closely related peptide might be endogenous to *Aplysia californica* (McMaster *et al.* 1989*a*,*b*). The experiments described here explore the possibility that conopressin G suppresses the GWR at the level of the sensory neuron to motor neuron synapse.

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The siphon sensory neuron to motor neuron synapse located in the abdominal ganglia of *Aplysia californica* has traditionally been considered to be an important neural correlate of the GWR and its plasticity. Behavioral sensitization has been shown to be caused, at least in part, by presynaptic facilitation of this synapse (Castellucci and Kandel, 1976; Klein and Kandel, 1978). For the most part, changes in the efficacy of this synapse have been attributed to changes in the duration of the action potential of the sensory neuron and in the presynaptic calcium current, which alter the number of transmitter quanta released by the sensory cell during synaptic transmission (Castellucci and Kandel, 1976).

Considerable information has been gathered on the ability of various neuroactive agents to influence the electrical properties and synaptic efficacy of siphon sensory cells. Both serotonin and small cardioactive peptide (SCP_b) broaden the action potential of the sensory neuron, facilitate synaptic transmission (Abrams *et al.* 1984; Brunelli *et al.* 1976; Klein and Kandel, 1978) and enhance the siphon-stimulated GWR (Abrams *et al.* 1984). Conversely, arginine vasotocin (AVT) and FMRFamide cause narrowing of the action potential, synaptic depression (Goldberg *et al.* 1987; Mackey *et al.* 1987) and suppression of the GWR (Thornhill *et al.* 1981).

A common target for the action of endogenous modulatory neuroactive agents seems to be a specific, relatively voltage-independent potassium current in the sensory neurons termed the S current because of its sensitivity to serotonin (Klein *et al.* 1982). For example, serotonin both depolarized and caused an increase in the input resistance of *Aplysia californica* sensory neurons by closing the S channel (Pollock *et al.* 1985). Similar increases in the input resistance of these cells and closure of the S channel are caused by SCP_b (Abrams *et al.* 1984). In constrast, both FMRFamide (Belardetti *et al.* 1987; Brezina *et al.* 1987) and acetylcholine (Ichinose *et al.* 1989) open the S channel. Modulation of the S channel is thought to be an important process in synaptic plasticity. Since the S current contributes to the repolarization of the neuron during an action potential, its closure by serotonin or SCP_b will increase the duration of the action potential in sensory neurons so that more calcium enters the presynaptic terminal, allowing more transmitter quanta to be released (Klein and Kandel, 1980; Pollock *et al.* 1985). Opening of the S channel by FMRFamide results in narrowing of the action potential with the opposite consequences (Mackey *et al.* 1987). From these studies it seemed reasonable to hypothesize that conopressin G suppressed the GWR in semi-intact preparations (Martínez-Padrón *et al.* 1992) by narrowing the sensory neuron action potential in a similar manner.

However, the experiments described here show that conopressin G depresses a voltage-dependent potassium current in the sensory neurons and increases the efficacy of the sensory neuron to motor neuron synapse. These effects resemble those produced by serotonin on these cells. Such effects at the level of the single neuron are contrary to what was expected from the behavioral effects of conopressin G and are inconsistent with the prominent role given to these elements as neuronal correlates of the GWR.

Materials and methods

Aplysia californica ranging in mass from 100 to 250 g were obtained from Alacrity Marine Biological Services (Redondo Beach, California). Experiments were performed on the isolated abdominal ganglion or on a preparation consisting of the abdominal ganglion attached to the siphon. In the latter, the abdominal ganglion was isolated from the bath by means of a surrounding plastic ring sealed with Vaseline. This made it possible to apply chemicals specifically to either part of the preparation. To improve space-clamp conditions, in some experiments clusters of siphon sensory neurons were isolated surgically and secured by suction to the tip of a glass pipette to facilitate penetration.

Animals were anesthetized (by injection of one-third of their body weight of isotonic $MgCl_2$), after which the abdominal ganglion was removed and pinned down, ventral side up, on a Sylgard (Dow-Corning) coated culture dish. Desheathing of the left ventral side, to gain access to the sensory neurons, was performed after bathing the ganglion in hypertonic artificial sea water (2 mol l^{-1} sucrose in ASW 1:1) for 15 min. In some cases, the ganglia had previously been bathed for 1 h in ASW containing 2 % protease type IX (Sigma P-6141) to facilitate desheathing. No differences in the effects of conopressin G were observed as a result of the use of protease.

Siphon sensory neurons were identified by their morphology, their position in the ganglion and their firing properties as described in the literature (Byrne *et al.* 1974; Koester and Kandel, 1977). A group of small siphon motor neurons (LFS cells, Frost *et al.* 1988) located in close proximity to the sensory cells at the entrance of the siphon nerve, as well as L7 and LBS motor neurons, were used as follower cells.

Single-barrelled micropipettes were pulled from thin-walled borosilicate glass (1.5 mm outer diameter) to a final resistance of 5–15 MΩ when filled with 0.8 mol l^{-1} KCl solution. A Wheatstone bridge circuit (Getting M-5 or Axoclamp 2-A) was used for membrane potential recordings and/or current injection into the cells. In order to achieve proper voltage control of the postsynaptic membrane potential, large cells such as L7 and LBS were impaled with two electrodes, one for passing current and the other for voltage recording. Moderate-intensity (1 g, 50–250 ms) tactile stimulation was delivered to the siphon by means of a mechanical tapper, as described by Peretz and Lukowiak (1975). Neuronal activity was displayed on digital (Nicolet 2090-III) and analog (Tektronix) storage oscilloscopes and on a four-channel Gould recorder.

The effect of conopressin G on the duration of the sensory neuron action potential was assessed during frequency-dependent spike broadening (FDSB, Edstrom and Lukowiak, 1985). Trains of action potentials were produced by injecting short (5 ms) depolarizing current pulses (3–7 nA) into the cell at a frequency of 6 Hz for 5 s. The short duration of the pulses ensured that the action potential was triggered after the current break, preventing possible distortions in shape due to interaction between electrode and membrane currents. The trains were acquired with pCLAMP software running on a COMPAQ Deskpro

computer and the action potentials were aligned using the maximum rate of change of voltage for comparison.

Neuronal excitability was quantified as the number of spikes triggered by a 500 ms depolarizing pulse (0.75–1.5 nA) injected into the cell. During both FDSB and excitability experiments, the sensory cells were stimulated once every 5 min. At least three control measurements were taken before application of any drug to ensure that the response of the cell was reproducible. In experiments examining the effects of conopressin G on homosynaptic depression, the sensory neurons were stimulated once every 50 s. Conopressin G was added to the bath following the tenth stimulus.

Single action potentials were evoked in the sensory cell by injecting short (5 ms) depolarizing current pulses (3–5 nA). This produced a complex mono- and polysynaptic excitatory postsynaptic potential (EPSP) in the follower cells. To avoid low-frequency homosynaptic depression, the sensory cell was stimulated once every 15–20 min. To improve the resolution of individual components within the EPSP, the excitability of polysynaptic elements of the circuit was reduced by increasing the concentration of extracellular Mg^{2+} to 80 mmol l^{-1}. On several occasions during conopressin G application, the amplitude of the first component of the EPSP could not be resolved, being masked by the appearance of an early second component. In such cases all EPSP amplitudes were taken at the time when the second component started.

Cells were voltage-clamped using an Axoclamp-2A amplifier (Axon Instruments) in the discontinuous single-electrode voltage-clamp configuration. The electrodes were shielded to within 100 mm of the tip with silver conductive paint connected to the driven shield and insulated with a coating of polystyrene Q-Dope to reduce the capacitance of the electrodes and to allow the use of high switching rates (15–20 kHz).

The cells were voltage-clamped at −40 mV and membrane currents were activated by 400 ms pulses to hyperpolarized and depolarized subthreshold potentials (usually up to −20 mV). Pulses were elicited at 5 s intervals, and no cumulative inactivation was observed with this protocol. Voltage command generation and data acquisition were managed by pCLAMP software (clampex, 5.5). Up to three control trials were taken prior to the initiation of the experiment to ensure that the recording was stable. Conopressin G was applied once to verify the responsiveness of the cell prior to any attempt to block its effect. Passive membrane leakage was calculated by measuring current flow in response to a 5 mV voltage command in the hyperpolarizing direction. The leakage current was assumed to be a linear function of membrane potential and it was subtracted, after scaling, from the current traces in order to isolate the time- and voltage-dependent components of the total membrane current.

To perform tail-current analysis, the membrane currents were activated by depolarizing voltage steps to −20 mV for 270 ms followed by a second pulse to a variety of membrane potentials in the range of the expected reversal potential (see Fig. 7A). Strong hyperpolarizing command pulses, however, produced an acti-

Table 1. *All solutions were adjusted to pH 7.78 at room temperature and contained 26 mmol l^{-1} $MgSO_4$ and 5 mmol l^{-1} Hepes buffer, and the following concentrations (mmol l^{-1}) of other salts*

Solutions	NaCl	KCl	Ca_2Cl	$MgCl_2$	Other
ASW	425	10	10	22	
100 K^+ ASW	335	100	10	22	
0 K^+ ASW	435	0	10	22	
Low-Na^+ ASW	5	10	10	22	420 mmol l^{-1} *N*-methyl glucamine*
TEA^+ ASW	225	10	10	22	200 mmol l^{-1} TEA^+
4-AP ASW	0	10	10	22	20 mmol l^{-1} 4-AP
Low-Cl^- ASW	0	10	10	22	425 mmol l^{-1} sodium methane sulfonate
0 Ca^{2+} ASW	425	10	0	22	10 mmol l^{-1} Ba^{2+} or Co^{2+}
High-Ca^{2+}, high-Mg^{2+} ASW	221	10	50	118	
Low-Ca^{2+}, high-Mg^{2+} ASW	297	10	0.5	118	
80 Mg^{2+} ASW	377	10	10	54	

TEA^+, tetraethylammonium; 4-AP, 4-aminopyridine; ASW, artificial sea water.
*Choline chloride was used in some cases instead of *N*-methyl glucamine.

vation of other current(s), which contaminated the recordings, and the analysis had to be restricted to membrane potentials more positive than −75 mV. Each family of tail currents thus obtained was fitted by a series of single-exponential equations with the aid of a computer program (CLAMPFIT, pCLAMP), and the amplitudes of the tail currents were calculated by extrapolating the exponential values to the time of the beginning of the second pulse. By plotting the tail current amplitude *versus* holding membrane potential the data points can be fitted by a straight line whose intersection with the zero point on the *y*-axis gives an estimate of the reversal potential. This series of experiments was performed on isolated abdominal sensory clusters, because inadequate space-clamp of the cell tended to distort measurements of the reversal potential (see Jack *et al.* 1975).

The chamber containing the abdominal ganglia was superfused at a constant rate of 1–3 ml min^{-1} with one of the various solutions listed in Table 1. Intermediate concentrations of tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP) were obtained by mixing their respective solutions with ASW in the proper proportions. Likewise, intermediate concentrations of extracellular potassium were obtained by mixing specific proportions of 0 mmol l^{-1} K^+ ASW with 100 mmol l^{-1} K^+ ASW. In some experiments the recording electrode contained 2 mol l^{-1} CsCl solution. CsCl was usually allowed to leak passively or was injected into the cell by applying depolarizing direct current.

Drugs were applied to the preparation after being dissolved in the superfusate or were added directly into the chamber to the desired final concentration. Conopressin G was used at the same concentration (1 μmol l^{-1}) as that required to produce behavioral changes of the GWR and the respiratory rate (Martínez-Padrón *et al.* 1992). Other vasopressin analogues used in these studies (usually at

10 μmol l^{-1}) included conopressin S, arginine vasopressin, lysine vasopressin, arginine vasotocin, lysine vasotocin, oxytocin and mesotocin. These were purchased from Bachem Inc. (Torrance, CA). Serotonin and SCP_b were purchased from Sigma Ltd (St Louis, MO). Conopressin G and S were a gift from Dr W. Gray (Utah).

Results

Conopressin G enhances frequency-dependent spike broadening

The normal broadening of a sensory neuron action potential during a repetitive train of activity is shown in Fig. 1A, where alternate action potentials in a train of 30 are shown superimposed. The duration of the action potential increases during the train, with the growth of a shoulder on the repolarization phase. The last action potential in the train is much longer than the first. In the presence of conopressin G, the shoulder grows wider more rapidly (Fig. 1B). This effect is reversible within 10 min (not shown) and is not mimicked by any other vasopressin analogue.

Differences in FDSB in two trains can be quantified by subtracting each action potential in one train from its counterpart in another, to obtain a family of curves showing the difference in voltage throughout each action potential (Fig. 1C). The phenomenon can be summarized by taking the peak amplitude of each curve and using it as an index of the degree to which broadening was increased between trains. This is defined as the maximum amplitude increase (MAI, Edstrom and Lukowiak, 1985) and is shown in Fig. 1D, where the enhanced FDSB in 1 μmol l^{-1} conopressin G is depicted by filled squares. The filled triangles show the MAI for two control trains (C1 and C2), demonstrating the reproducibility of the phenomenon under these conditions. The difference between the two traces is statistically significant (Wilcoxon signed-rank test, $P<0.01$). Statistically similar results to those shown in Fig. 1 were obtained in all other siphon sensory cells analyzed ($N=10$) in this study. The maximum amplitude increase for the last action potential of the trains ranged between 9 and 40 mV. The effect of conopressin G was usually not apparent when the first action potentials of each train were compared or when the peptide was tested on single action potentials elicited at 5 min intervals.

This observed enhancement of FDSB by conopressin G was not due to its effect on the membrane potential, since it was not inhibited by injecting hyperpolarizing current to hold the resting potential constant and it could not be induced by a comparable depolarization with depolarizing current in the absence of conopressin G.

Sensory neuron excitability

The ability of conopressin G to increase the excitability of sensory neurons, defined here as the ability of depolarizing current injected at the soma to induce action potentials, is shown in Fig. 2. Under normal conditions a 500 ms pulse of

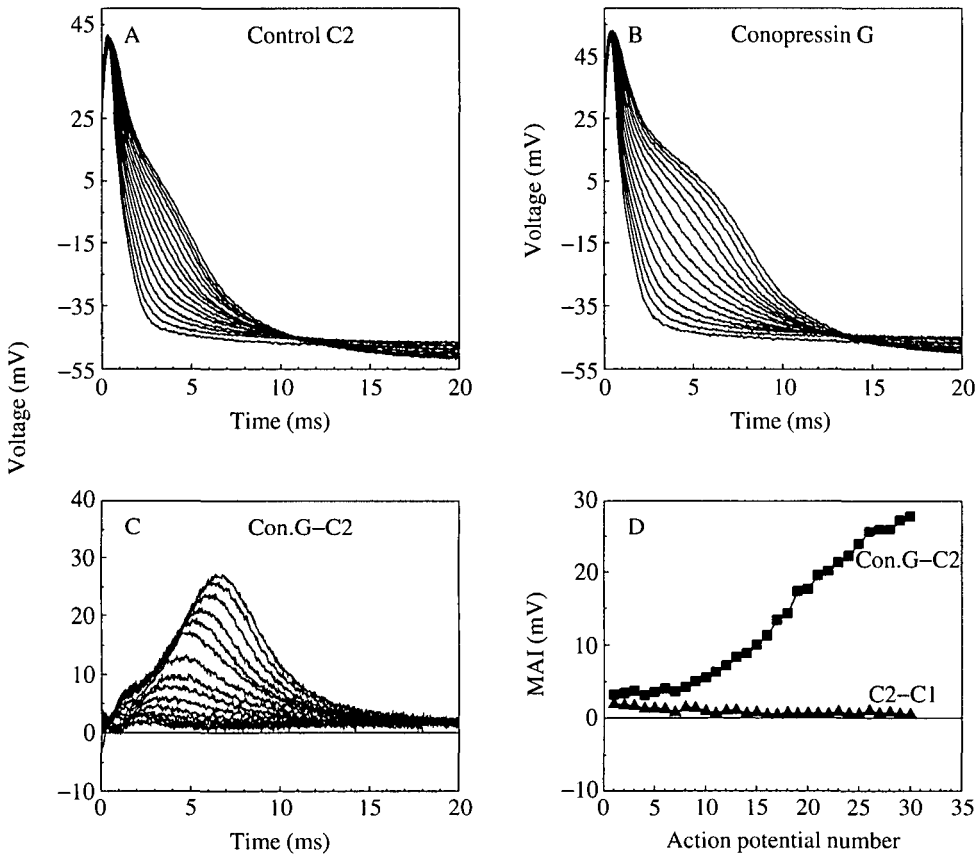


Fig. 1. Representative data showing the effect of conopressin G on frequency-dependent spike broadening in siphon sensory neurons. (A) A train of 30 action potentials was produced by injecting short depolarizing pulses (3–5 nA, 5 ms) at a frequency of 6 Hz for 5 s in normal ASW. Alternate action potentials are displayed after alignment on the maximum rate of voltage change. (B) Bath application of conopressin G (1 μmol l^{-1}) results in a delayed repolarization of the membrane with respect to the control. (C) Computer subtraction of the train of action potentials in ASW from that produced during superfusion of conopressin G (Con.G). The progressive increase in the plateau caused by the peptide appears as a series of positive curves showing a maximum between 4 and 7 ms. (D) Plot of the maximum amplitude increase for each action potential of the train. The filled squares represent the change in voltage produced by conopressin G with respect to the control at the time of the peak effect (from C). For comparison, the triangles display the voltage change between the control (C2) and a previous control (C1, not shown) taken 5 min earlier.

current triggers no more than 2–3 action potentials at the beginning of the pulse (Fig. 2A). In the presence of conopressin G, the threshold current for the first action potential is reduced, and action potentials can occur throughout the pulse (Fig. 2B) and, in some cases, outlast the pulse (not shown) so that the number of

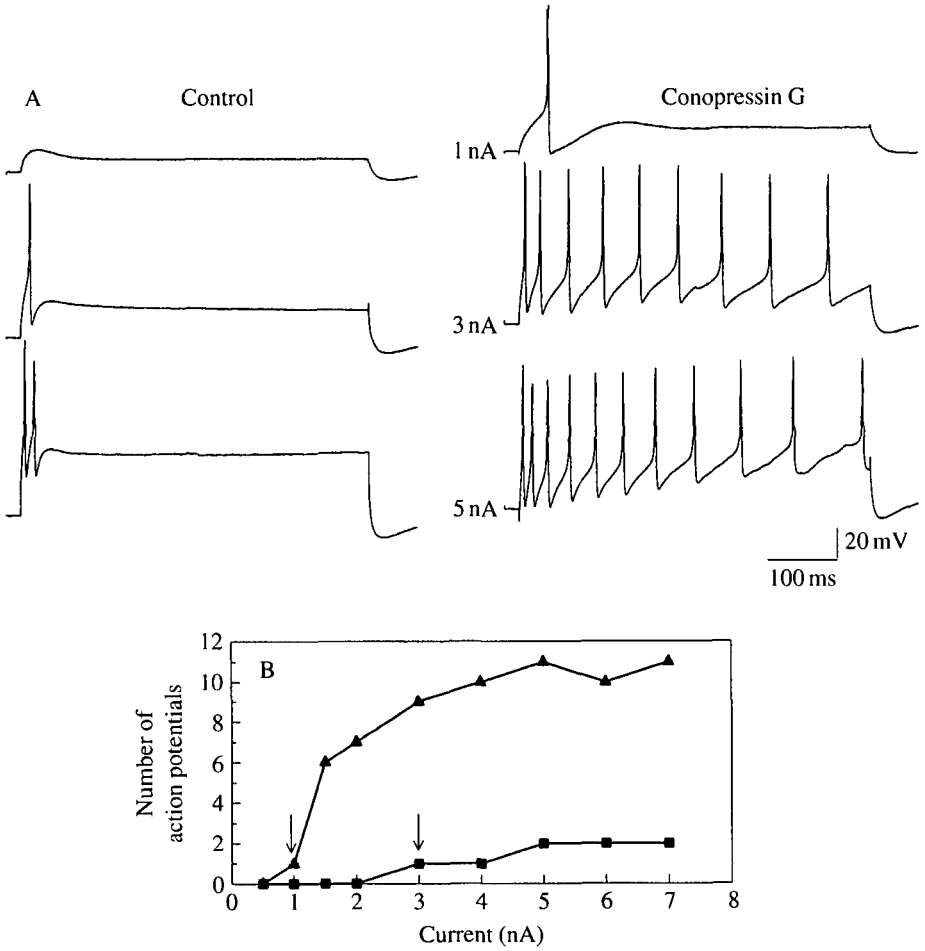


Fig. 2. An example of the effect of conopressin G on siphon sensory neuron excitability. (A) Long depolarizing current injection (500 ms) produces a characteristic accommodating firing pattern in *Aplysia californica* sensory cells (left-hand traces). Note that, in ASW, increasing the current from 3 to 5 nA produces only one additional action potential and the cell accommodates strongly. 1 μmol l^{-1} conopressin G applied to the bath greatly decreases accommodation, allowing the cell to fire during the entire duration of the pulse (right-hand traces). (B) Plot of the number of spikes triggered by the cell represented in A *versus* current injected in normal ASW (squares) and after bath application of conopressin G (triangles). The firing threshold for both situations is indicated by the arrows, and it was lowered by conopressin G, in this example from 3 to 1 nA. Note that the sensory cell did not fire more than two action potentials in ASW when injected current was 5 or 7 nA.

action potentials for a given current is increased. This effect is reversible within 10 min and is not mimicked by the other vasopressin analogues tested.

The average number of spikes in 17 cells in response to a constant pulse of current was 1.88±0.26 (mean±S.E.M.) under control conditions, 7.76±0.75 in the

presence of conopressin G and 2.00±0.24 after washout; the difference between the control and the conopressin G treatment was statistically significant ($t=-9.71$, d.f.=15, $P<0.01$). In five other experiments similar to that depicted in Fig. 2, several levels of current injection were applied to the cells. In all cases the peptide not only increased the number of spikes at all current levels, but also caused a reduction in the firing threshold.

Abdominal sensory neurons are naturally activated by mechanical stimulation of the siphon and mantle skin rather than by synaptic input to the soma or dendrites (Byrne *et al.* 1974). It is possible that these changes in excitability seen in the soma may affect the volley of action potentials coming from the siphon. To test this hypothesis the siphon-attached preparation was used (see Materials and methods). Mechanical stimulation of the siphon (1 g, 50–250 ms) produced a train of action potentials in the sensory cell soma rising suddenly from the baseline and reflecting propagation of spikes into the soma from the axon. These trains retained a relatively constant pattern when the siphon was stimulated at 5 min intervals. In these preparations the application of conopressin G to the abdominal ganglion increased somatic excitability as expected, but did not have any noticeable effect on the siphon-evoked sensory discharge. The average number of action potentials following siphon stimulation in six experiments was 4.66±1.63 (mean±S.E.M.) before and 4.33±1.75 after conopressin G ($t=1.58$, d.f.=4, $P>0.05$).

Like conopressin G, serotonin and SCP_b have also been shown to produce an increase in somatic excitability (Klein *et al.* 1986) and action potential duration (Abrams *et al.* 1984; Klein and Kandel, 1978; Pollock *et al.* 1985). These agents appear to act directly on the sensory neurons, since they are active on isolated cells and/or in the presence of high Ca^{2+} and high Mg^{2+} concentrations (Abrams *et al.* 1984; Baxter and Byrne, 1990). Whether conopressin G acted directly on siphon sensory neurons or through interneurons was tested by applying the peptide after blocking synaptic transmission with low-Ca^{2+}, high-Mg^{2+} ASW. Under these conditions, conopressin G did not increase sensory neuron excitability ($N=5$), whereas serotonin (Fig. 3) and SCP_b continued to increase neuronal excitability to the same extent as they did in normal saline. In addition, although conopressin G very often produced a slight depolarization of the membrane, this effect was slow, more than 1 min was needed to see an effect, compared with the effects of serotonin and SCP_b, which appeared within a few seconds.

Siphon sensory neuron to motor neuron synaptic transmission

The application of 1 $\mu mol\,l^{-1}$ conopressin G produced a reversible increase in the amplitude of the initial component of the motor neuron's EPSP, which presumably corresponds to the monosynaptic pathway (Fig. 4). In these experiments ($N=9$), conopressin G significantly increased the peak amplitude of the initial component of the EPSP to 229 % ($t=3.7$, d.f.=7, $P<0.02$). This increment in amplitude sometimes occurred without a change in the rate of rise, resulting in an increased time-to-peak of the synaptic potentials. In other cases, conopressin G produced an EPSP with a faster rising rate (see Fig. 5B).

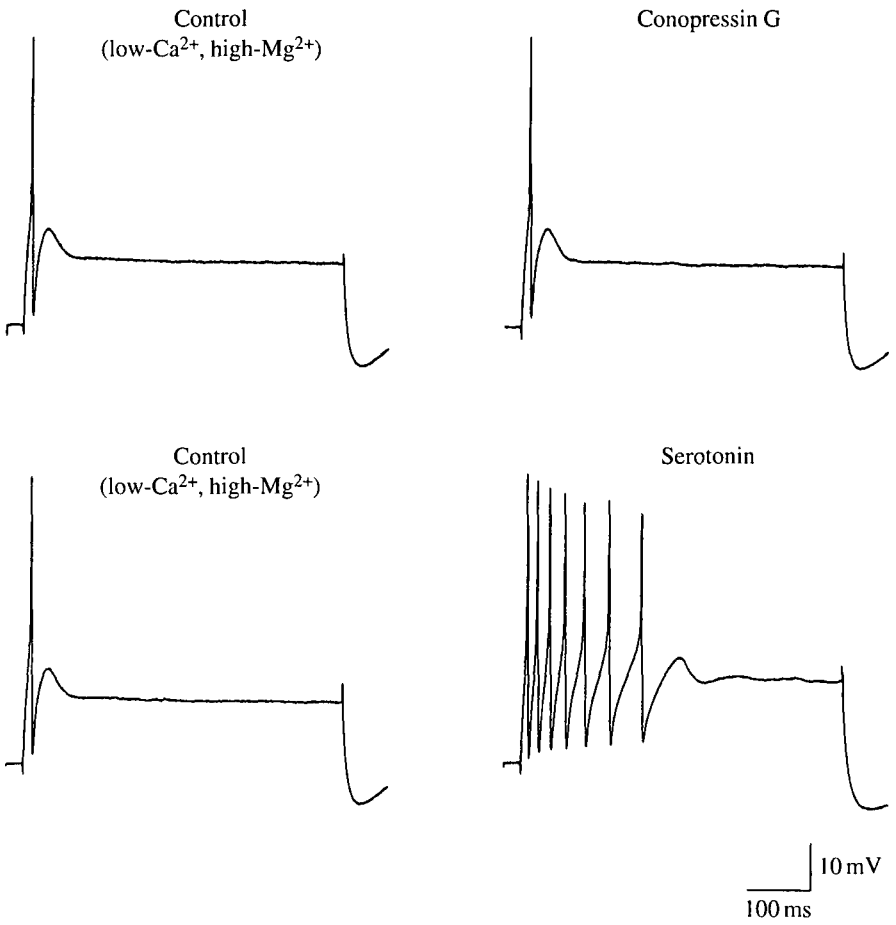
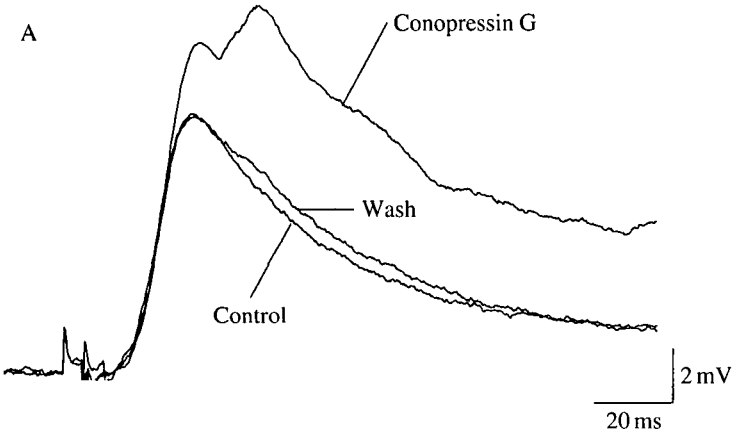


Fig. 3. Blocking synaptic transmission abolishes the effect of conopressin G on sensory neuron accommodation. Application of 1 μmol l^{-1} conopressin G in low-Ca^{2+}, high-Mg^{2+} solution, a treatment that prevents synaptic transmission, did not result in increased excitability (upper traces), although the same cell was responsive in ASW (not shown). The same treatment did not prevent the effect of 50 μmol l^{-1} serotonin, which acts directly on the sensory cell (lower traces).

Conopressin G counteracted homosynaptic depression produced by stimulating the sensory neuron every 50 s (Fig. 5). Conopressin G (1 μmol l^{-1}) was introduced into the bath in a single application immediately after trial 10. By trial 13 the initial component of the EPSP in the postsynaptic cell had recovered to control levels; it remained elevated for several more trials. The EPSPs from trials 1, 10 and 13 have been aligned in Fig. 5B to compare their rates of rise. Between trials 1 and 10 there was a progressive decrement in the rate of rise that accompanied the reduction in amplitude. Conopressin G restored the rate of rise to the initial control level and produced an additional increase in amplitude. Reversal of homosynaptic depression by conopressin G only became apparent 2 min after the



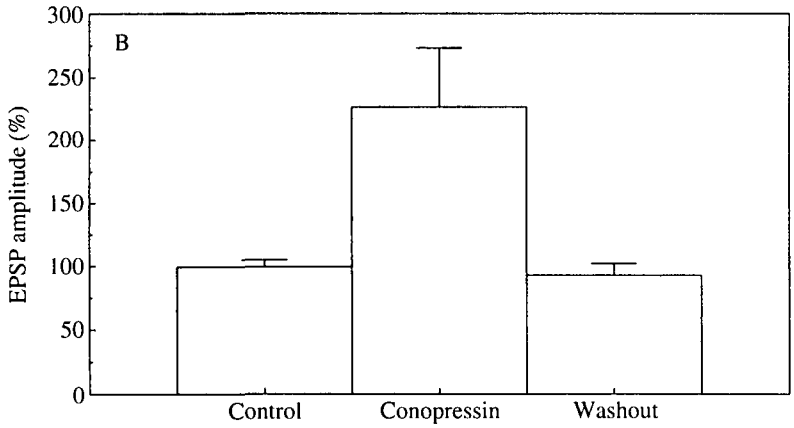


Fig. 4. (A) Data from a single experiment showing the synaptic facilitation induced by 1 μmol l^{-1} conopressin G applied to the bath. EPSPs were evoked in an LFS motor neuron by firing the sensory neuron every 15 min. At this frequency the amplitude of the EPSP was stable. The peptide was applied to the bath 5 min before the experimental trial. As can be seen, conopressin G increased the amplitude of the monosynaptic EPSP. The amplitude of the EPSP returned to control levels following washout of the peptide. (B) Normalized data from nine similar experiments. Conopressin G caused a greater than 100 % increase in the average amplitude of the EPSP. Values are mean+S.D.

application of the peptide. This is in contrast to the more rapid effects of serotonin and SCP_b observed within a few seconds in 10 other experiments (not shown).

To test whether the action of conopressin G was directly mediated, the effect of

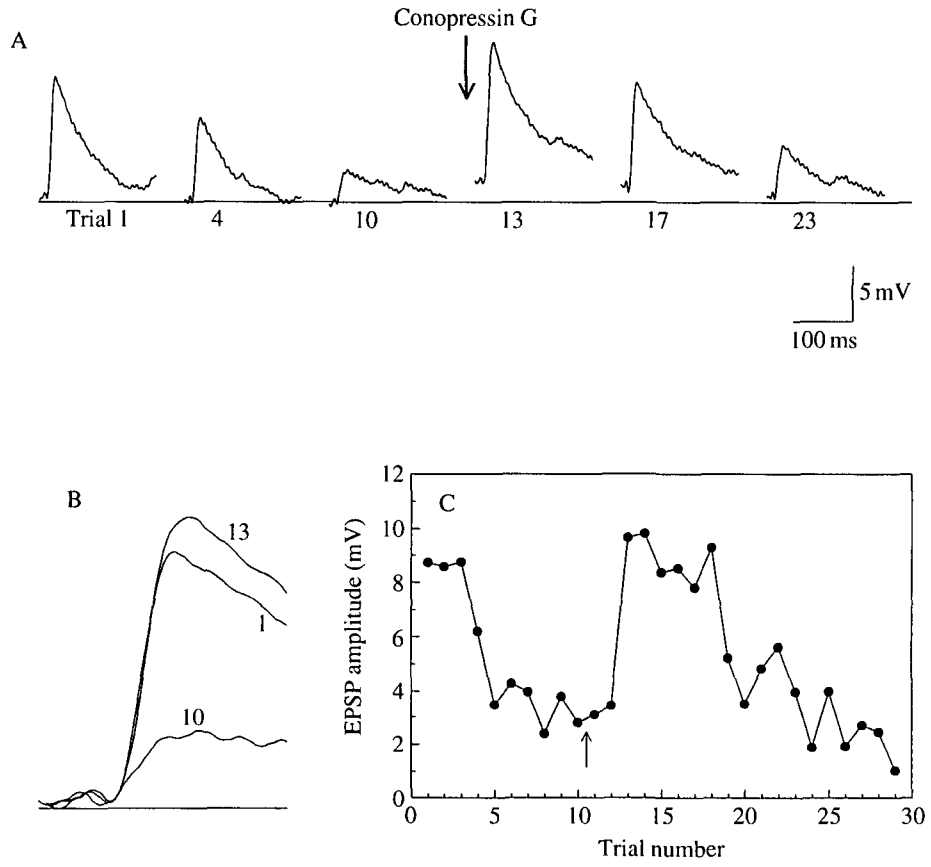


Fig. 5. Conopressin G counteracts homosynaptic depression. In A and C a sensory neuron was stimulated by current injection to produce one action potential every 50 s, which resulted in homosynaptic depression of the EPSP recorded in a follower LFS motor neuron. A few selected traces are shown in A. (B) Traces 1, 10 and 13 from A aligned to compare their amplitudes and rising rate. Note that homosynaptic depression resulted in smaller amplitudes and slower rising rates of the EPSP and that $1\,\mu mol\,l^{-1}$ conopressin G was able to restore the rising rate and slightly increase the amplitude of the EPSP with respect to control levels. (C) The EPSP amplitude for all trials in this experiment are plotted. The addition of conopressin G (arrow) produces a marked increase in the EPSP amplitude after a delay of 100 s.

the peptide was tested after the ganglion had been bathed in high-Ca^{2+}, high-Mg^{2+} solution. This procedure abolished the facilitatory effect of conopressin G on the initial component of the EPSP (N=5) and also abolished the ability of this peptide to reverse homosynaptic depression (not shown).

Conopressin G suppresses an outward potassium current

Typically *Aplysia californica* sensory neurons have a resting membrane potential close to −40 mV and show no synaptic input or spontaneous activity. When

they are voltage-clamped close to their resting membrane potential, step command pulses to subthreshold depolarized potentials elicit a relatively slow, non-inactivating outward current followed by a large tail current upon return to the holding potential.

Bath application of 1 μmol l^{-1} conopressin G had a variety of effects on the total membrane current (Fig. 6). First, there was a shift of the holding current in the inward direction, paralleled by a slight depolarization of the resting potential under current-clamp conditions (not shown). Second, there was a reduction in the magnitude of the leakage current. Third, conopressin G produced a significant reduction in the total outward membrane current activated by pulses in the depolarizing direction (Fig. 6A). These effects were completely reversible. Usually, the more depolarized voltage commands (to −20 mV) caused the cells to fire uncontrolled action potentials when conopressin G was present in the bath (not shown). The threshold concentration for the action of conopressin G was about 1 nmol l^{-1}. This concentration caused a small decrease in the outward current in two out of four cells tested, whereas 50 % suppression of the outward current occurred in the range 0.1–1 μmol l^{-1}.

The current–voltage relationship of the total membrane current in ASW and conopressin G is shown in Fig. 6B (same cell as in A), where the current observed at the end of the step is plotted against the voltage step. Conopressin G decreased membrane current at all potentials. The current–voltage relationship for the conopressin-G-sensitive current was obtained by subtracting the plots in the presence of conopressin G from those in the presence of ASW (Fig. 6C) and it usually consisted of two components; an outward current that is approximately linear at potentials below about −50 mV and an outward rectifying component above −45 mV. The voltage-sensitive component of the conopressin G effect was isolated by subtracting a straight line fitted to the lower portion of the current–voltage relationship from the total net current (Fig. 6C).

A tail current analysis was performed (Fig. 7), as described in the Materials and methods section, after the extracellular concentrations of a number of ions had been varied in order to determine their ability to affect the reversal potential of the outward rectifying current. Almost complete substitution of extracellular sodium with *N*-methyl glucamine (three cells, not shown) or partial substitution of extracellular chloride with methane sulfonate (three cells, not shown) had little or no effect on the extrapolated reversal potential. In contrast, increasing the concentration of extracellular potassium (three cells) produced a progressive reduction in the tail current amplitudes for a given membrane potential and a shift in the reversal potential in the positive direction. As shown in Fig. 7B, a slope of about 45–50 mV in the reversal potential occurred per 10-fold change in $[K^+]_o$; this is close to that predicted by the Nernst equation for a K^+-selective conductance, strongly suggesting that the slow, non-inactivating outward current produced by depolarizing command steps to subthreshold membrane potentials is carried mainly by potassium.

Reduction of the outward potassium current by conopressin G could be due

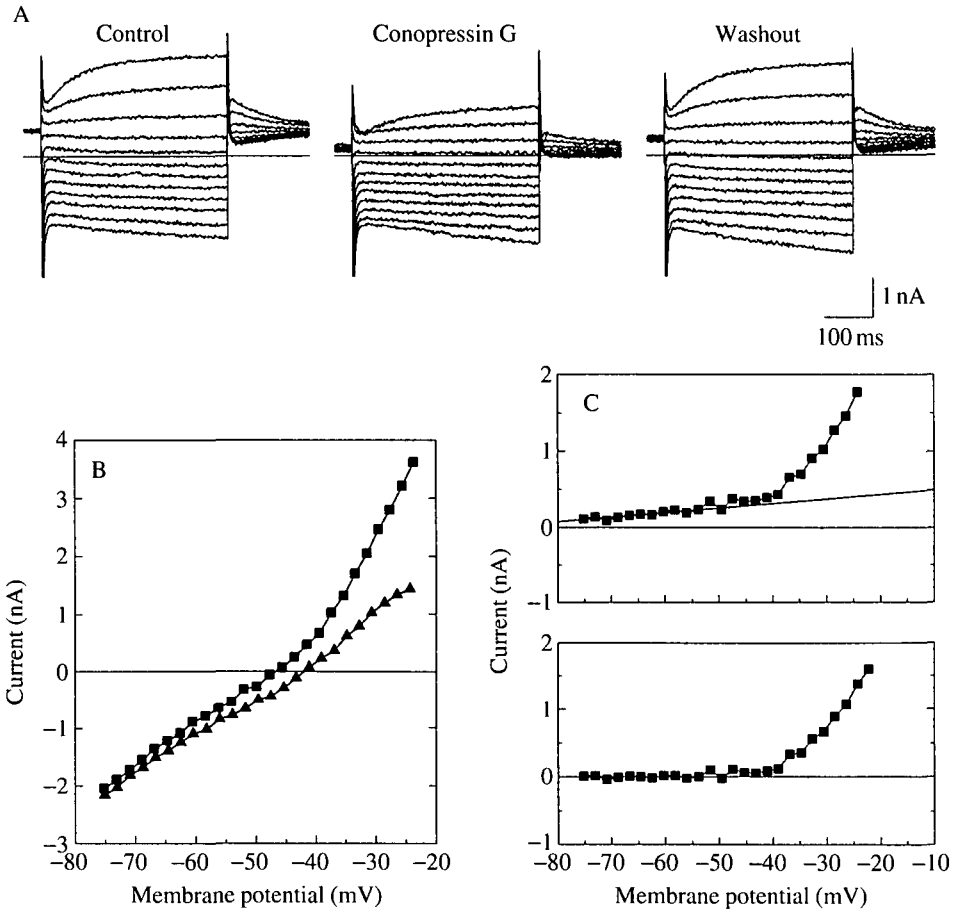


Fig. 6. Effect of conopressin G on sensory neuron outward membrane current. (A) Membrane current produced by 400 ms square command voltages (3 mV steps) from a holding potential of −40 mV in the negative and positive directions; before, during and after bath application of 1 μmol l^{-1} conopressin G. The horizontal line represents the zero current level. Conopressin G produced a reversible reduction of the total outward membrane current and an inward shift of the holding current. (B) Quasi steady-state current–voltage plot of the cell represented in A, at the end of the depolarizing pulse (no leakage subtraction applied). Control, filled squares; conopressin G, filled triangles. (C) Quasi steady-state current–voltage plot of the conopressin-G-sensitive current obtained by subtracting the total current in conopressin G from that in ASW (top). The voltage-dependent component of the conopressin G effect was isolated by subtracting its effect on the linear leakage current (bottom).

either to a suppression of the outward potassium current or to an increase in an antagonistic inward current. To investigate this, conopressin G was tested after the outward potassium current had been blocked by internal perfusion of cesium (2 mol l^{-1} CsCl in the electrode, Fig. 8). In the first part of the experiment, diffusion of cesium into the cell was reduced by injecting repeated hyperpolarizing

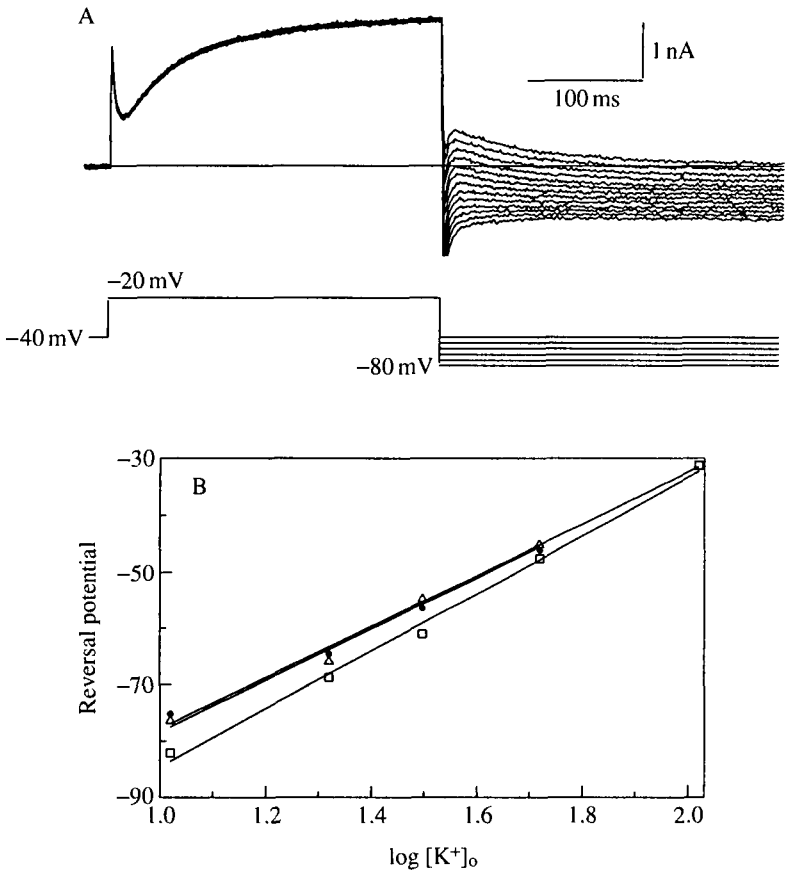


Fig. 7. Tail current analysis of the outward membrane current produced by subthreshold depolarizing voltage commands. (A) From a holding potential of −40 mV, a 270 ms prepulse to −20 mV was applied to activate the current; this was followed by hyperpolarizing pulses between −40 and −80 mV. Tail currents obtained in this manner were fitted by exponential equations and the tail amplitude was extrapolated to the beginning of the pulse (see Materials and methods for details). (B) The extrapolated reversal potential plotted against the logarithm of extracellular potassium concentration (in mmol l^{-1}). The slopes of three individual experiments shown in the graph were 45.6, 45.8 and 51.3 mV per 10-fold increased in $[K^+]_o$. This strongly suggests that under these conditions the outward membrane current is largely carried by potassium.

current pulses to retard the leakage of Cs^+ from the electrode into the cell long enough to ascertain that the cell was responsive to the peptide. Although some leakage of cesium may have taken place, the top tracings in Fig. 8 show that conopressin G was still effective and able to reduce the outward current. The current could be partially restored after washing (not shown). Next, cesium was allowed to diffuse or was actively injected into the cell until the reduced magnitude of the outward current reached a constant level (10–25 % after approximately

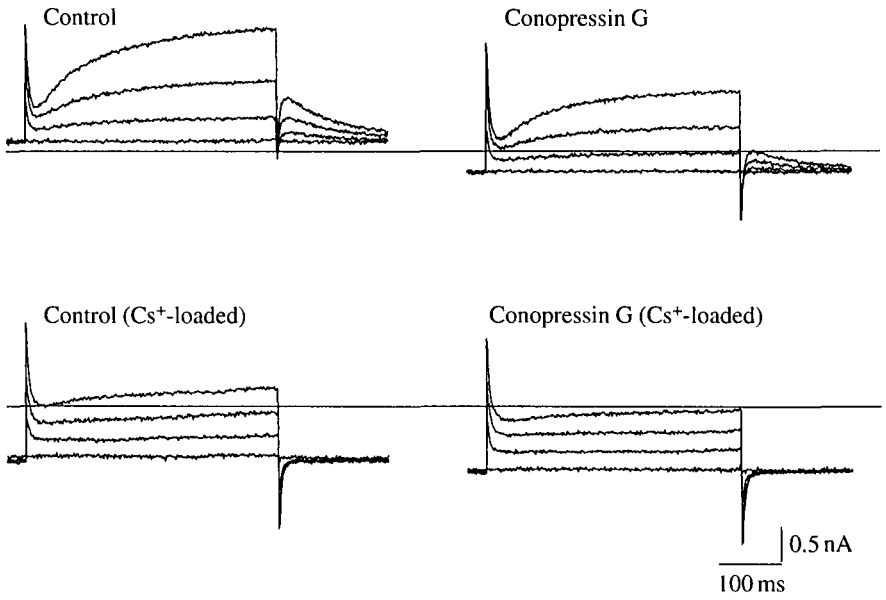


Fig. 8. Representative recordings showing that ionophoretic injection of CsCl blocks the conopressin-G-sensitive K^+ current. Cells were impaled with electrodes containing 2 mol l^{-1} CsCl. From a holding potential of −40 mV, membrane currents were activated by command pulses up to −20 mV in 5 mV steps. In the first part of the experiment, the cell was kept hyperpolarized between trials to prevent leakage of Cs^+ from the electrode in order to obtain a control for the effect of conopressin G (upper traces). Next, Cs^+ was injected into the cell by applying 2–3 nA depolarizing direct current. Conopressin G had little or no effect on the remaining outward current after most of the membrane current had been blocked by Cs^+ (lower traces).

30 min). Under these conditions, the effect of conopressin G was greatly reduced or practically absent, as shown in the bottom tracings in Fig. 8 (representative of five cells).

Characterization of the conopressin-G-sensitive potassium current

Known potassium currents in molluscs can be characterized by their voltage sensitivity and kinetics as well as by their sensitivity to TEA^+ and 4-AP. Both TEA^+ and 4-AP reduced the outward potassium current in response to voltage steps from −40 to −20 mV (not shown). The conopressin-G-sensitive current is highly resistant to TEA^+ and moderately resistant to 4-AP. The dose–response curve (Fig. 9) shows an apparent dissociation constant (K_d) of about 235 mmol l^{-1} for TEA^+ (filled triangles, four cells) and 11 mmol l^{-1} for 4-AP (filled squares, three cells).

The similarities between the effects of conopressin G and serotonin on the electrical properties of the sensory neurons suggested that they could be acting through common mechanisms. This possibility was explored by applying them alone and together. The effects on the outward potassium current of serotonin and

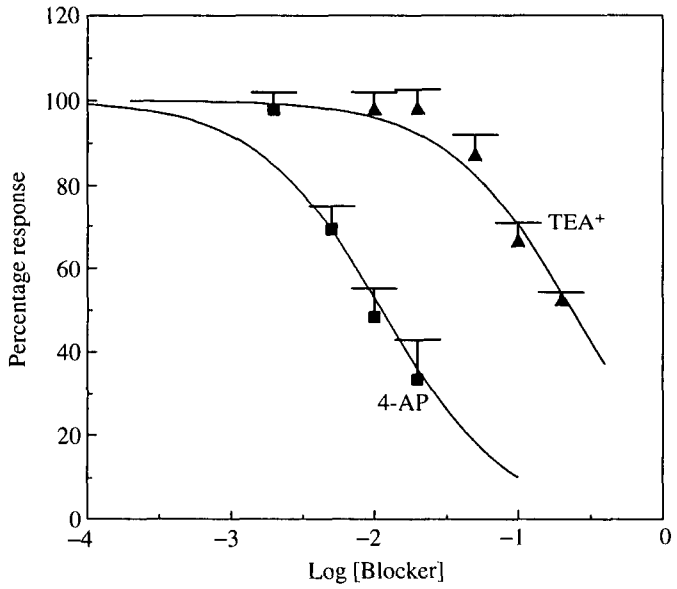


Fig. 9. Block of the outward K^+ current by 4-AP (three cells) and TEA^+ (four cells). Membrane currents were activated by voltage commands from −40 to −20 mV in ASW, followed by exposure to increasing concentrations of both blockers, and are given as a percentage of the control current. Continuous lines were obtained using the expression: percentage current=100/(1+[blocker]K_d) for the binding of a single molecule. The apparent dissociation constant values (K_d) was 11 mmol l^{-1} for 4-AP and 235 mmol l^{-1} for TEA^+. Values are mean+S.D.

conopressin G were not additive (Fig. 10). In six cells in which the effects of both agents were tested, the outward potassium current produced by depolarization steps to −20 mV was reduced by 53 % by 1 μmol l^{-1} conopressin G and by 46 % by 50 μmol l^{-1} serotonin. The combined action of conopressin G and serotonin, however, caused only 48 % suppression of the current. If conopressin G and serotonin were acting *via* a serotoninergic interneuron then conopressin superfusion should have no effect when serotonin was used. Morever, two serotonin antagonists, ritanserine and spiperone, tested in this study (N=3) failed to block the action of serotonin and were not tested against conopressin G.

The dependence of the outward potassium current on Ca^{2+} was tested by replacing Ca^{2+} in the extracellular solution with Co^{2+} or Ba^{2+}. Exposure of the sensory neurons to Ca^{2+}-substituted ASW caused a slight membrane depolarization, reduction of firing threshold (not shown) and increased excitability during long depolarizing current injections (N=3, Fig. 11A). The outward potassium current was also inhibited by replacing Ca^{2+} with Ba^{2+} (three cells, not shown) or Co^{2+} (four cells, Fig. 11B). The effect of Co^{2+} on the quasi-steady current–voltage relationship is shown in Fig. 11C. As can be seen, there was a reduction in the outward potassium current.

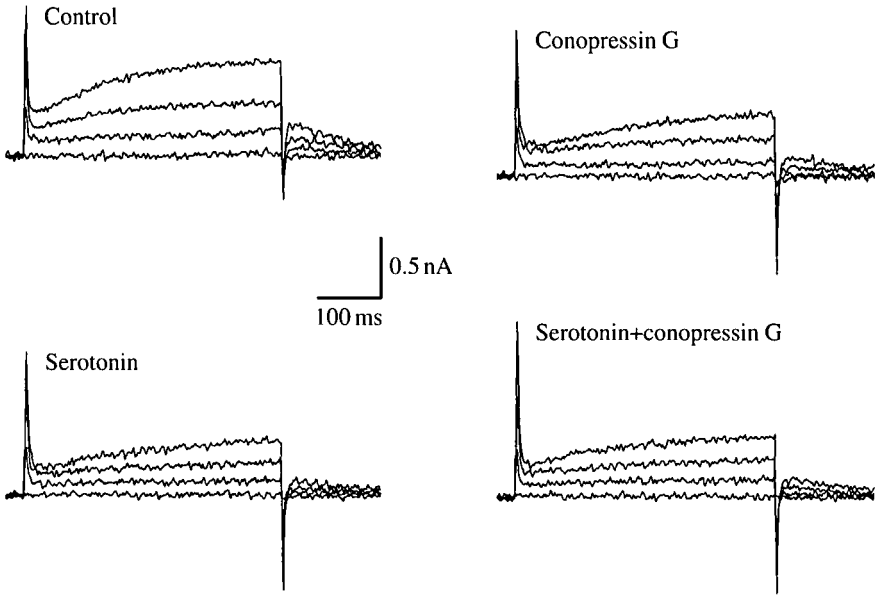


Fig. 10. Co-perfusion of serotonin and conopressin G produces non-additive suppression of the K^+ current. Representative data from a single experiment showing the current suppression caused by conopressin G ($1\,\mu mol\,l^{-1}$) and serotonin ($50\,\mu mol\,l^{-1}$) when applied separately or in combination. Notice that the current reduction resulting from co-application of both substances was not larger than that produced by either substance alone. Voltage pulses from −40 mV in 5 mV steps.

Discussion

Conopressin G was the only peptide of the vasopressin family tested that was able to increase the excitability and prolong the duration of the action potentials in *Aplysia californica* siphon sensory neurons. These included conopressin S, which is derived from a different species, *Conus striatus*. The effects of other vasopressin analogues on synaptic transmission were not systematically investigated. Arginine vasotocin, however, has been reported to narrow the sensory neuron action potential slightly during FDSB and to depress synaptic transmission (Goldberg *et al.* 1987). In our experiments, arginine vasotocin was as likely to produce a slight potentiation as a slight reduction of FDSB, although the number of experiments was insufficient to determine whether there is a trend in either direction. Its effects, in any case, were considerably smaller than those of conopressin G, even though 10 times higher concentrations were used.

The effects of conopressin G on siphon sensory neurons more closely resemble those of serotonin and SCP_b than those of arginine vasotocin. First, like serotonin and SCP_b (Klein *et al.* 1986), conopressin G increased the somatic excitability of sensory neurons by decreasing the threshold current needed to elicit action potentials in the soma and by reducing accommodation. This strong effect did not alter the number of action potentials appearing in the sensory cell soma in

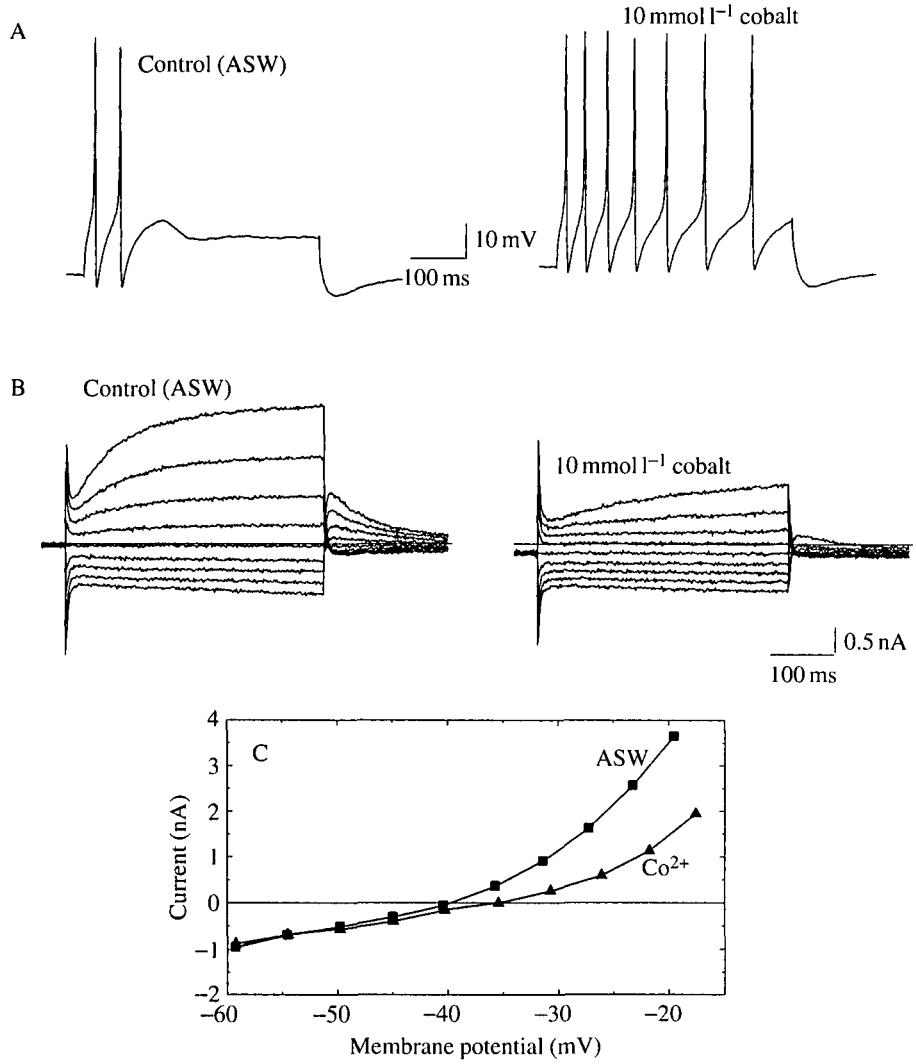


Fig. 11. Substitution of Ca^{2+} with Co^{2+} mimics the effect of conopressin G on *Aplysia californica* sensory neurons. Replacement of Ca^{2+} with Co^{2+} (10 mmol l^{-1}) caused (A) a membrane depolarization and a large decrement in spike frequency adaptation and (B) a substantial suppression of the outward membrane current in response to depolarizing voltage steps from a holding potential of −40 mV. (C) Quasi steady-state current–voltage plot of the cell represented in B.

response to mechanical stimulation of its receptive field in the siphon, again like serotonin (Klein *et al.* 1986). These studies employed short, moderately intense mechanical stimulation of the siphon and it remains to be determined whether the observed increase in the soma excitability is effective during persistent or noxious (e.g. an intense stimulus such as a pinch) stimulation.

Second, conopressin G, like serotonin, enhanced frequency-dependent broadening of the action potential but, unlike serotonin, broadening of single action potentials by conopressin G was not usually apparent in recordings from the cell body when single action potentials were elicited at 5 min intervals. It is not known whether the peptide increases the duration of the action potentials at the terminal and, if so, whether this effect is related to its ability to facilitate synaptic transmission.

Third, conopressin G, like serotonin, facilitated synaptic transmission between sensory neurons and motor neurons. Further studies, including quantal analysis, are necessary to determine whether the enhancement of the monosynaptic EPSP is presynaptic. However, potentiation of the EPSP would be expected on the basis of the ability of the peptide to reduce the potassium current and, consequently, to prolong the action potential. During synaptic facilitation due to action potential broadening, which is an important contributor to serotonin-induced synaptic facilitation, there is no change in the rate of rise of the EPSP (Hochner *et al.* 1986*a*). Synaptic facilitation without an increased rate of rise of the EPSP was usually observed in this study (Fig. 4A), but synaptic facilitation induced by conopressin G was sometimes accompanied by an increase in the rate of rise of the EPSP. This, and the fact that conopressin G was able to facilitate the EPSP after homosynaptic depression (Fig. 5), a condition under which broadening of the action potential has little effect (Hochner *et al.* 1986*b*), suggests that, like serotonin (Dale and Kandel, 1990), conopressin G may affect additional processes contributing to facilitation of this synapse.

Fourth, the effects of serotonin and conopressin G on membrane currents in *Aplysia californica* sensory cells are very similar with respect to their magnitude, kinetics and sensitivity to pharmacological agents. Under voltage-clamp conditions, nanomolar concentrations of conopressin G, like serotonin, suppress a voltage-dependent outward membrane current that is activated by depolarizing voltage steps to −20 mV and which tail current analysis indicates is carried primarily by potassium and shows little or no inactivation over time. This potassium current is not the early potassium current (I_A) because steady-state inactivation of I_A is almost complete at −40 mV (Connor and Stevens, 1971; Neher, 1971), the holding potential used in these experiments. This potassium current is not likely to be the delayed potassium current (I_{KV}) either. This current is activated by depolarizing steps above −30 mV, with maximum currents occurring between 0 and 10 mV (Aldrich *et al.* 1979*a*; Thompson, 1977). The delayed potassium current takes about 100 ms to reach its peak, after which it deactivates to a non-zero steady-state value (Aldrich *et al.* 1979*a*,*b*). This is different from the conopressin-G- and serotonin-sensitive currents, which show no apparent inactivation over time. Also, Baxter and Byrne (1989) have shown that depolarizing pulses to −20 mV do not significantly activate I_{KV} in *Aplysia californica* sensory neurons. The relative insensitivity of the conopressin-G-sensitive potassium current to blockade by either TEA^+ or 4-AP also supports the conclusion that it is not I_{KV}. Externally applied TEA^+ blocks I_{KV} with a K_d of

about 6–8 mmol l^{-1} (Baxter and Byrne, 1989; Hermann and Gorman, 1981*b*) and externally applied 4-AP blocks I_{KV} at a concentration of around 2 mmol l^{-1} (Hermann and Gorman, 1981*a*). These values are much less than the K_d for blockade by TEA^+ (235 mmol l^{-1}) or 4-AP (11 mmol l^{-1}) reported here and are close to the concentrations of these drugs necessary to block I_S, the serotonin-sensitive potassium current (K_d for TEA^+ is about 100 mmol l^{-1}, Baxter and Byrne, 1989; Brezina *et al.* 1987; Shuster and Siegelbaum, 1987; K_d for 4-AP is about 7 mmol l^{-1}, Brezina *et al.* 1987; Brezina, 1988).

It is difficult to interpret the observation that the conopressin-G-sensitive outward potassium current was greatly suppressed when extracellular calcium was replaced by divalent cations (Ba^{2+} and Co^{2+}) that prevent calcium entry into the cell. First, it is not possible to assess the extent to which calcium replacement interferes with the conopressin-G-induced effects since calcium replacement impairs synaptic transmission. Conopressin G had no effect when synaptic transmission was impaired. Nevertheless, the similarity of the actions of these divalent cations suggests that they suppress the same potassium current as conopressin G. Like exposure to conopressin G, exposure of the cell to Ca^{2+}-substituted ASW caused slight membrane depolarization and a large increase in sensory neuron excitability (Fig. 11). An alternative explanation, that barium and cobalt may induce activation of an inward current, seems unlikely. Although calcium channels are more permeable to Ba^{2+} than to Ca^{2+} (Hagiwara *et al.* 1974), the observation that similar effects were obtained with cobalt, which does not permeate the channel, argues against this possibility. Thus, it appears that the conopressin-G-sensitive current is a calcium-sensitive potassium current.

Since the effects of conopressin G and serotonin on membrane currents are almost indistinguishable and since serotonin can occlude the effect of conopressin G on membrane current; it is necessary to consider the possibility that they both suppress the same current. Consequently, it is necessary to compare the observed effects of conopressin G with published descriptions of I_S.

Although the voltage- and time-dependence of the conopressin-G-sensitive potassium current do not conform to the theoretically expected behavior of I_S, based on single-channel analysis of the S current, they do compare favorably with actual descriptions of macroscopic whole-cell I_S in the literature. Patch-clamp studies show that the serotonin-sensitive S channel remains open most of the time over the entire physiological range of membrane potentials (Siegelbaum, 1987; Camardo and Siegelbaum, 1983) and is only weakly affected by membrane potential (Brezina *et al.* 1987; Siegelbaum *et al.* 1982). This suggests that, during a depolarizing voltage step, current will flow instantaneously through the majority of the S channels, which are open at the holding potential, and only a small proportion of the remaining closed channels will subsequently open at the new voltage. This predicted time-independent, outward-rectifying leakage is in stark contrast to the slow activation and marked voltage-dependence of the serotonin-sensitive current observed below −20 mV in *Aplysia californica* pleural and abdominal sensory neurons, which are called I_S and attributed to the S channel in

the literature (Baxter and Byrne, 1989; Klein and Kandel, 1980; Klein *et al.* 1982), but it is very similar to the properties of the current we describe here.

The literature concerning the sensitivity of serotonin-sensitive potassium currents to calcium is less than clear. Suppression of I_C by serotonin in molluscan neurons has been reported (Paupardin-Tritsch *et al.* 1981), and even in *Aplysia californica* pleural sensory neurons, where serotonin modulates a steady-state Ca^{2+}-activated potassium current (Walsh and Byrne, 1989). This form of I_C is sensitive to 5 mmol l^{-1} extracellular TEA^+, whereas the conopressin-G-sensitive current in abdominal sensory cells is highly resistant to TEA^+. Given the similar electrical properties of pleural and siphon sensory neurons, it may seem unlikely that this discrepancy could be due to differences in cell types but the possibility cannot be dismissed since it has been reported that serotonin reduces the outward current produced by Ca^{2+} injections into pleural (Walsh and Byrne, 1989) but not abdominal sensory neurons (Klein *et al.* 1982).

Initial reports on the responses of siphon sensory neurons to serotonin emphasized that replacement of Ca^{2+} with Ba^{2+} did not affect serotonin suppression of the membrane current activated by 25 ms depolarizing command pulses from −50 mV (Klein *et al.* 1982; Pollock *et al.* 1985), a protocol unsuitable for detecting a slowly activating current. These same authors mention in passing that calcium channel blockers, such as cobalt and cadmium, attenuate the serotonin response but they do not specify the conditions under which this significant observation was made. The slowly activating serotonin- and conopressin-G-sensitive current we describe here would have contributed to the serotonin effect observed during the long step pulses used by Klein *et al.* (1982) and the ramps used by Pollock *et al.* (1985). The reduction of this current following calcium substitution would have been significant and might account for their incompletely described results.

Taken together, the results presented here indicate that conopressin G and serotonin suppress a slowly activating, non-inactivating outward rectifying potassium current that is present at the resting membrane potential. This current might be a species of I_C, which, unlike other Ca^{2+}-dependent potassium currents, is insensitive to TEA^+. Alternatively, suppression of the current by a Ba^{2+}- and Co^{2+}-substituted solution might be due to a requirement for Ca^{2+} on the extracellular side or to a more effective screening of membrane charges by Co^{2+} and Ba^{2+}, resulting in a voltage shift of channel activation towards more positive potentials. Further experimentation is needed to investigate this. Given these assumptions, the voltage-independent, linear component of the effect of conopressin G may represent suppression of the true I_S.

Several lines of evidence suggest that the action of conopressin G on siphon sensory neurons is not directly mediated, but that it requires one or more intermediate neurons. First, the effects of conopressin G require more than 1 min to develop. This is in contrast to the rapid onset of the effects of serotonin and SCP_b, which are known to act directly on the sensory cells (Abrams *et al.* 1984; Baxter and Byrne, 1990). In addition, blocking synaptic transmission in the

ganglion with a low-Ca^{2+}, high-Mg^{2+} solution prevents the effect of conopressin G on accommodation, whereas it does not prevent the effect of serotonin and SCP_b. In a similar manner, synaptic facilitation produced by conopressin G was blocked by bathing the abdominal ganglion in high-Ca^{2+}, high-Mg^{2+} solutions, a procedure that would reduce the excitability of putative interneurons whose activation by conopressin G might ultimately lead to the effect of the peptide on synaptic transmission. If the effects of conopressin G are indeed mediated through an intermediate cell as suggested, there is the possibility that this cell releases serotonin or another facilitatory substance such as SCP_b. Unfortunately, two serotonin inhibitors, ritanserine and spiperone, did not block the effects of serotonin itself and, therefore, it was not possible to investigate this further.

Regardless of its exact identity, because of its slow activation kinetics the voltage-dependent component may not contribute to the broadening of the action potential, which occurs within 10 ms. The activation kinetics of this current, however, may be much faster at more depolarized potentials. Suppression of this current would be expected to contribute to the increased excitability and, because it is active at rest, to the membrane depolarization caused by conopressin G in current-clamp conditions.

The effects of conopressin G and serotonin are different in one very important respect: despite its facilitatory effect on the sensory neuron to motor neuron synapse, conopressin G suppresses the gill withdrawal reflex (Martínez-Padrón *et al.* 1992). In semi-intact preparations, the superfusion of conopressin G over the abdominal ganglion suppresses gill withdrawal reflex behavior. That is, not only does the observed synaptic facilitation fail to enhance the reflex but also the reflex is suppressed in spite of it. This clearly indicates that facilitation of the sensory neuron to motor neuron synapse is not sufficient to mediate behavioral sensitization. Since the central sensory neuron to motor neuron synapse is assumed to be a neural correlate of the gill withdrawal reflex, we tested the hypothesis that behavioral suppression may be accompanied by synaptic depression at this synapse. This proved not to be the case, since conopressin G caused potentiation of FDSB, facilitation of the sensory neuron to motor neuron synapse, and dramatically increased the excitability of the sensory neurons. Therefore, it appears that, in this case, the behavioral and synaptic changes caused by conopressin G are not correlated. A similar lack of correlation between events at these two levels of organization has been reported during associative learning (Colebrook and Lukowiak, 1988).

The function of FDSB is not known, but its effects on the action potential are likely to be substantial during persistent or noxious stimulation when the cell fires prolonged discharges. Obvious broadening of the action potential is observed in the presence of conopressin G when neurons fire long, self-regenerating trains that outlast the stimulation. The potentiation of FDSB by conopressin G may have an even more dramatic effect on the synaptic output of the sensory cells than the one described here for single action potentials. It is relevant to note that a nociceptive function has been attributed to pleural and, presumably, to siphon sensory

neurons (Walters *et al.* 1983) and site-specific sensitization of the tail withdrawal reflex using noxious electrical stimulation of the skin causes activity-dependent changes in pleural sensory neurons similar to those described here for siphon mechanoreceptors (Walters, 1987). These changes include lowering of the spike threshold, enhanced synaptic transmission and, in some cases, the appearance of regenerative bursts during short-duration stimulation. This last characteristic was occasionally observed in the presence of conopressin G.

Interestingly, there is evidence that the noxious or aversive stimulation traditionally used to induce sensitization of the *Aplysia californica* GWR also causes behavioral inhibition (Mackey *et al.* 1987; Marcus *et al.* 1988). Both neurally mediated processes are triggered simultaneously by tail shock but the rapidly decaying inhibition is initially strong enough to mask sensitization so that inhibition is only apparent briefly before sensitization predominates (Mackey *et al.* 1987). In addition, Krontiris-Litowitz *et al.* (1989) found that the GWR can be suppressed by hemolymph from animals subjected to intense electrical or mechanical stimulation and by unidentified factors released from traumatized body wall. Therefore, noxious stimulation seems to generate both neural and humoral signals that suppress defensive reflexes.

In this context, it may be possible to suggest a role for conopressin G in aversive stimulus-induced behavioral inhibition and subsequent sensitization. Conopressin G might cause suppression at some sites in the network (Martínez-Padrón *et al.* 1992) and facilitation at others (perhaps by causing release of serotonin), with the suppressive process being stronger, but shorter-lived. These effects include a suppression of the ability of the gill motor neurons to elicit gill contractions and a decrease in the excitability of the motor neuron itself. Termination of such suppression would allow sensitization to be expressed. Detailed experimentation on the parametric characteristics of the behavioral effects of conopressin G will be necessary to address this possibility. Contrary to this idea, however, is the observation that tail shock produces narrowing of the sensory neuron action potential and heterosynaptic depression (Mackey *et al.* 1987). However, this latter effect could be produced by noxious stimulation through a conopressin-independent mechanism that helps to ensure the initial pre-eminence of behavioral inhibition. Therefore, it would be interesting to see whether FMRFamide, which has been linked to synaptic depression during behavioral inhibition (Abrams *et al.* 1984; Mackey *et al.* 1987), can counteract the effects of conopressin G.

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