Vasopressin/Oxytocin-Related Conopressin Induces Two Separate Pacemaker Currents in an Identified Central Neuron of *Lymnaea stagnalis*

PAUL F. VAN SOEST AND KAREL S. KITS
Faculty of Biology, Graduate School Neurosciences Amsterdam, Research Institute Neurosciences, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

van Soest, Paul F. and Karel S. Kits. Vasopressin/oxytocin-related conopressin induces two separate pacemaker currents in an identified central neuron of *Lymnaea stagnalis*. J. Neurophysiol. 78: 1384–1393, 1997. The molluscan vasopressin/oxytocin analogue Lys-conopressin excites neurons in the anterior lobe of the right cerebral ganglion of the snail *Lymnaea stagnalis*. Persistent inward currents that underlie the excitatory response were studied with the use of voltage-ramp protocols in the identified neuron RCB1 and other anterior lobe neurons. Under whole cell voltage-clamp conditions, two types of conopressin-activated current could be distinguished on the basis of their voltage dependence: 1) a pacemaker-like current that was activated at potentials above −40 mV (high-voltage-activated current, I<sub>HVA</sub>) and 2) an inward current that was activated at all potentials between −90 and +10 mV (low-voltage-activated current, I<sub>LVA</sub>). Ion substitution experiments indicate that sodium is the main charge carrier for I<sub>HVA</sub> and I<sub>LVA</sub>. Both currents are differentially affected by cadmium. I<sub>HVA</sub> and I<sub>LVA</sub> differ in dose dependence, with median effective concentration values of 7.7 × 10<sup>−6</sup> M and 2.2 × 10<sup>−7</sup> M, respectively. Vasopressin and oxytocin act as weak agonists for the conopressin responses. The kinetics of desensitization and washout of I<sub>HVA</sub> and I<sub>LVA</sub> are different. The HVA response shows little desensitization, whereas the LVA response desensitizes within minutes (time constant 80 ± 28 s, mean ± SD). The time constant of washout on removal of conopressin is 159 ± 63 s for I<sub>HVA</sub> and 36 ± 13 s for I<sub>LVA</sub>. These results suggest that two distinct conopressin receptors are involved in the activation of both currents. The conopressin-activated currents induce or enhance a region of negative slope resistance in the steady-state current-voltage relation. They differ from a third persistent inward current that is carried by calcium and completely blocked by cadmium. The presumed functional roles of these currents, possibly including autoregulation, are discussed.

INTRODUCTION

The neuropeptides vasopressin and oxytocin and their nonmammalian analogues appear to be ubiquitous neurotransmitters in various animal species. One of the molluscan analogues is Lys-conopressin, or conopressin G. Although originally discovered in the venom of the marine fish-hunting snail *Conus geographus* (Cruz et al. 1987), it was later shown to serve as a neurotransmitter (see, e.g., Martinez-Padrón et al. 1993). Conopressin is present in central neurons of various mollusks, including the pulmonate snail *Lymnaea stagnalis*. The gene for the *Lymnaea* conopressin prohormone was cloned and sequenced, and was found to be related to the vertebrate vasopressin and oxytocin prohormone genes. The most abundant expression of the conopressin gene occurs in the neurons of the anterior lobe of the right cerebral ganglion (Van Kesteren et al. 1995a). Van Kesteren et al. (1995b, 1996) cloned two endogenous *Lymnaea* conopressin receptors, at least one of which is also expressed in many cells of the anterior lobe. Interestingly, coexpression of conopressin and conopressin receptors occurs in several of these neurons, hinting at the possibility that autotransmission plays a role in the regulation of their activity (Van Kesteren et al. 1995b, 1996).

Most of the neurons of the anterior lobe of the right cerebral ganglion send projections into the penis nerve and are involved in the control of aspects of male copulatory behavior in *Lymnaea* (De Boer et al. 1996a,b). Eversion of the penile complex coincides with increased activity in these neurons (P.A.C.M. de Boer, unpublished results), and the neuropeptide Ala-Pro-Gly-Trp-NH<sub>2</sub>, which is also present in the anterior lobe neurons (De Lange et al. 1997), can evoke parts of the copulatory behavior when injected into the animal (De Boer et al. 1996b). Although the behavioral role of conopressin has not been fully clarified yet, the abundance of conopressin receptors within the anterior lobe suggests that modulation of the activity of these neurons may be one of its major functions. Furthermore, conopressin can modulate or induce contractions of the vas deferens and isolated muscles from the penile complex (Van Golen et al. 1995). Thus conopressin appears to play an important role in the regulation of male copulatory behavior in *Lymnaea*. Although the molecular biology of conopressin and some of its peripheral effects are well described, its physiological effects on the anterior lobe neurons themselves are still poorly understood. The related peptides vasopressin and oxytocin are known to be capable of modulating electrical activity in molluscan neurons. Vasopressin induced bursting properties in *Otala lactea* neurons (Barker and Gainer 1974, 1975), an effect that could be mimicked by an endogenous but unidentified vasopressin-like peptide (Ifshin et al. 1975). In an *Achatina fulica* neuron, pacemaker properties were induced by oxytocin, which activated a largely sodium-dependent but tetrodotoxin (TTX)-insensitive inward current (Funase 1990). This excitatory mechanism is apparently not confined to invertebrates, because similar persistent inward currents were activated by vasopressin and oxytocin in certain types of rat central neurons (Raggenbass and Dreifuss 1992; Raggenbass et al. 1991). Finally, conopressin induced bursting of R25/L25 neurons in *Aplysia californica* (Martí-
nez-Padrón and Lukowiak 1993) and excited several types of central neurons in Lymnaea (Van Kesteren et al. 1995b), but in neither case were the underlying membrane properties investigated.

These observations led us to investigate the effects of conopressin on slow excitatory currents in the anterior lobe neurons of Lymnaea. Here we report the presence of two distinct, persistent inward currents activated by conopressin in an identified neuron. Both currents differ in voltage dependence, agonist sensitivity, and rates of desensitization and washout. The results show that conopressinergic transmission onto these neurons may be an important factor in determining their activity and, thereby, the regulation of male reproductive behavior.

METHODS

Animals and preparations

In all experiments adult, laboratory-bred specimens of the pond snail L. stagnalis (L.) were used. The animals were kept under a 12:12-h light:dark regime in aerated, circulating water at a temperature of 20°C and were fed lettuce ad libitum. The CNS was dissected out and pinned down in a recording chamber filled with 0.2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered saline (HBS, see Peptides and solutions). The outer layers of connective tissue covering the anterior lobe were removed with the use of fine hooks, after which impalement with sharp microelectrodes was possible. Identification of the neurons in the anterior lobe was based on position, size, and color of the soma, as well as on firing pattern (see Khennak and McCrohan 1988).

Isolation of single neurons was performed with the use of a method adapted from Ridgway et al. (1991). Briefly, the CNS was incubated in an 0.2% solution of trypsin (Sigma type III) in HBS (see Peptides and solutions) at 37°C for 35 min. After the incubation period, the tissue was rinsed three times in HBS supplemented with 5 mM glucose. The nervous system was then pinned down in a small chamber and the sheet of connective tissue covering the cells of interest was removed with the use of a fine hook. Individual neurons could be isolated by means of a wide (inner diameter slightly larger than the soma diameter) glass pipette that was mounted on a micromanipulator and connected to an 0.2-ml micrometer syringe (Gilmont Instruments). The cells were plated in a 35-mm plastic culture dish. The cells were allowed to sit for ≥30 min and up to several hours before the dish was transferred to the experimental setup.

Recording techniques

Membrane potential recording of neurons in the isolated nervous system was performed with the use of sharp microelectrodes (resistance 20–60 MΩ) filled with 0.5 M KCl. The electrodes were mounted on micromanipulators and connected to custom-built amplifiers. A Unitrade (Philadelphia, PA) digital audio tape recorder was used to record the membrane potential; a Gould 2200 pen recorder was used for immediate hard copy output.

Voltage-clamp recording of isolated neurons was performed with the use of standard whole cell patch-clamp equipment, including an Axopatch IC patch-clamp amplifier (Axon Instruments, Burlingame, CA). All data were digitized with the use of a CED 1401 12-bit AD/DA converter (Cambridge Electronic Design, Cambridge, UK) and recorded on an IBM-compatible PC with the use of custom-written software. The incoming signal was filtered at 20 Hz and sampled at 40 Hz. Patch pipettes with a large tip opening were used to minimize series resistance. Series resistance normally amounted to 1.5–2 MΩ, 70–80% of which could be compensated for. Under these conditions, voltage errors due to series resistance did not exceed 5 mV, even at the largest current amplitude that could be recorded (10 nA), but were generally much smaller. This was verified in control experiments in which the actual membrane potential was recorded with the use of a separate microelectrode.

Pseudo-steady-state current-voltage (I-V) relations were obtained with the use of voltage-ramp protocols in which the command potential was swept from −90 to +10 mV at a rate of 5 mV/s. Ramps were applied at 2-min intervals. Experiments were started when the I-V relations had attained a stable shape (monitored by superimposing subsequent current traces), usually 10 min after entering the whole cell configuration. Because of the duration of the ramp protocols, time-dependent changes in current amplitude contribute to the shape of the I-V relation, thus altering the apparent voltage dependence. Accordingly, because ramps were started 30 s after the start of peptide application, before the low-voltage-activated (LVA) current fully reaches its peak, the amplitude of the current in the low-voltage range will probably be underestimated. Voltage ramps and peptide applications were exactly and reproducibly timed with respect to each other, so time-dependent changes due to current (in)activation are expected to contribute in a very similar manner to all experiments.

Membrane potential recording of isolated neurons was performed with the use of either a custom-built microelectrode amplifier (with the use of sharp microelectrodes) or the patch-clamp equipment described above (with the use of patch pipettes) with the amplifier in current-clamp mode. Membrane potential data were recorded on digital audio tape.

Data are presented as means ± SD and statistical significance is indicated by P values, obtained from Student’s t-tests, unless otherwise stated.

Peptides and solutions

Peptides were applied either by pressure ejection from glass pipettes (tip diameter 5–10 μm) placed 100–200 μm from the cell body or with the use of a Y tube system, which allowed rapid changing of the peptide solutions during an experiment. All peptides were dissolved in HBS, in some cases supplemented with 0.5 mM of the vital dye amaranth (Merck, Darmstadt, Germany) to enable visual control of the application. Control solutions were identical, except for the presence of the peptide, and were ineffective in inducing responses. Lys-conopressin, vasopressin, oxytocin, and the specific oxytocin receptor antagonist des-Gly-NH₂(CH₂)₅[O-Me-Tyr³, Thr⁶, Orm⁸]-vasotocin [d(CH₂)₅-OVT] (see Manning and Sawyer 1989) were obtained from Saxon Biochemicals (Hannover, Germany). In all cases, the recording chamber was continuously perfused with saline.

HBS was composed of (in mM) 30 NaCl, 1.7 KCl, 10 NaCHSO₄, 1.5 MgCl₂, 4 CaCl₂, 5 NaHCO₃, and 10 HEPES, pH set at 7.8 with NaOH. For the isolation procedure, normal saline was supplemented with 5 mM glucose. Calcium-selective saline consisted of (in mM) 40 tetraethylammonium (TEA) chloride, 1 MgCl₂, 4 CaCl₂, 10 HEPES, and 2 4-aminopyridine, pH set at 7.8 with TEA-OH. To test the effects of a reduction of the extracellular sodium concentration, we used normal sodium saline consisting of (in mM) 40 NaCl, 1.7 KCl, 1.5 MgCl₂, 4 CaCl₂, 10 HEPES, and 2 4-aminopyridine, pH set at 7.8 with TEA-OH. To test the effects of a reduction of the extracellular sodium concentration, we used normal sodium saline consisting of (in mM) 40 NaCl, 1.7 KCl, 1.5 MgCl₂, 4 CaCl₂, 10 HEPES, and low-sodium saline in which 20 mM of the total NaCl was replaced by 40 mM sucrose. Standard pipette solution contained (in mM) 2 NaCl, 64 KCl, 2.3 CaCl₂, 10 HEPES, 11 ethylene glycol-bis(β-aminomethyl ether)-N,N',N''-N'''-tetraacetic acid (EGTA), 2 MgATP, and 0.1 guanosine 5'-triphosphate (GTP)-tris(hydroxymethyl)aminomethane (Tris), pH set at 7.4 with KOH. Calcium-selective pipette solution consisted of (in mM) 64 CsCl, 2.3 CaCl₂, 10 HEPES, 11 EGTA, 2 MgATP, and 0.1 GTP-Tris, pH set at 7.4 with CsOH.
At resting membrane potential, some cells (n activity, whereas others did not show any response (0 were excited by conopressin and chosen for further characterization of peptide through connective tissue. That response recorded in situ lasts longer, probably because of slow diffusion of peptide through connective tissue. Whole cell voltage-clamp recording of isolated cell clamped at ±50 mV, with use of nonselective solutions in pipette and bath. Pressure application (20 s) of 1 μM conopressin (hatched bar) results in slow inward current that gradually subsides during washout of peptide.

RESULTS

Excitatory effect of conopressin

Neurons in the anterior lobe of the right cerebral ganglion of *L. stagnalis*, many of which express conopressin receptors (Van Kesteren et al. 1995b), were impaled in situ with microelectrodes and their membrane potential was recorded during pressure application of 10 μM conopressin (n = 24). At resting membrane potential, some cells (n = 9) responded to conopressin with a depolarization and enhanced spiking activity, whereas others did not show any response (n = 15). The identifiable neuron RCB1 was among the cells that were excited by conopressin and chosen for further characterization of these responses.

RCB1 is a large neuron from the B cluster on the dorsal surface of the anterior lobe of the right cerebral ganglion (see Khennak and McCrohan 1988). In the isolated nervous system, RCB1 has a resting membrane potential of around −50 mV and shows spontaneous firing activity. In all cells tested, pressure application of 1 μM (n = 2) or 10 μM (n = 11) conopressin depolarized the membrane. The depolarization was accompanied by a strong increase in spiking frequency and was followed by a period of bursting (Fig. 1A). The amplitude of the underlying depolarization could not be determined accurately because of the high spiking frequency. Spiking activity was enhanced for periods up to 20 min after peptide application.

To determine whether conopressin modulated RCB1 directly or via presynaptic neurons, single RCB1s were isolated and plated in a culture dish. Current-clamp recordings from isolated neurons with the use of microelectrodes revealed unambiguous responses to conopressin. Membrane potential recordings made in the whole cell configuration yielded similar results. Application of conopressin at either 1 μM (n = 8) or 10 μM (n = 3) resulted in membrane depolarization and either induction or enhancement of spiking activity (Fig. 1B). Application of a control solution failed to elicit these effects (n = 4, not shown). In contrast to neurons in situ, isolated neurons displayed a relatively short burst of action potentials, lasting 2 min at most. This may have been due to better perfusion. In all cases, the burst outlasted the actual peptide application. These data indicate that presynaptic neurons are not required for the conopressin-induced excitation of RCB1.

The conopressin-induced currents underlying the excitatory effects observed under current clamp were studied in whole cell voltage-clamp experiments. Application of 1 μM conopressin activated an inward current in single RCB1 neurons voltage-clamped at a holding potential of −50 mV (Fig. 1C). During long applications of the peptide, the inward current reached its maximal amplitude after ~30 s and subsequently subsided slowly (Fig. 7D). The average amplitude of the conopressin-induced inward current in RCB1 amounted to 0.83 ± 0.36 (SD) nA (n = 15).

Two types of response to conopressin under voltage clamp

To study the voltage dependence of the conopressin response in RCB1 and other anterior lobe neurons, a voltage-ramp protocol was used. Voltage ramps from −90 to +10 mV were applied at a rate of depolarization of 5 mV/s. In this way, pseudo-steady-state I-V relations were recorded before, during, and after application of 1 μM conopressin.

The voltage dependence of the conopressin-induced current was obtained by subtracting the I-V relation recorded under control conditions from that recorded in the presence of conopressin.

Under control conditions, a region of negative slope resistance (NSR) in the I-V relation was absent in 15 of 24 unidentified cells (Fig. 2B1) and present in the remaining 9 cells as well as in RCB1 (Fig. 2B1). The inward current underlying the NSR (I_{NSR}) was activated at potentials greater than −40 mV and reached its maximal amplitude around −10 mV. In 17 of the 24 unidentified neurons, conopressin unambiguously altered the pseudo-steady-state I-V relation, either enhancing or inducing a region of NSR. Two types of response could be distinguished. The first type of response, termed the high-voltage-activated (HVA) response, was characterized by an increase in net inward current at potentials greater than −40 mV (Fig. 2, A1 and A2). This type of response was observed in 11 cells. The second type of response, observed in the remaining six cells as well as in RCB1, appeared to be more complex. In these cells, conopressin activated a net inward current at all potentials from −90 to +10 mV (Fig. 2B). This caused a negative slope conductance to appear throughout the entire potential range. However, the amplitude of the conopressin-activated current in these cells increased sharply at higher potentials in a manner resembling the HVA response (Fig. 2B2). The I-V
investigated the ionic dependency of these currents with the use of channel blockers and ionic substitution experiments. Under conditions selective for calcium currents, i.e., in the absence of extracellular sodium and the presence of potassium channel blockers, $I_{NSR}$ was still observed (Fig. 3A). However, conopressin did not induce an inward current under these conditions (Fig. 3A). The maximal amplitude of the inward current amounted to $2.3 \pm 1.1$ nA in the absence of conopressin and to $2.1 \pm 0.7$ nA in the presence of $10 \mu M$ conopressin $(P = 0.26, n = 7)$. These observations suggest that $I_{NSR}$ is carried by calcium ions but that the conopressin-induced current is not carried by calcium ions.

Bath application of the calcium channel blocker cadmium

relation of the conopressin-activated current did not intersect the horizontal axis, indicating that the reversal potential exceeds $+10$ mV. Interestingly, this complex response to conopressin was only observed in cells displaying such a region of NSR. The isolated HVA response was mostly observed in cells without a region of NSR in the control $I$-$V$ relation.

These observations raised the question of whether the complex response to conopressin was due to activation of an LVA current in addition to the HVA response. Furthermore, the HVA component of the conopressin response might either be caused by an increase in $I_{NSR}$ by activation of another, novel conductance. Because the complex response was consistently observed in RCB1, further experiments to address these questions were performed on RCB1.

**Ionic dependency of conopressin-activated currents**

To distinguish between the presumed components underlying the persistent inward current in RCB1 (i.e., $I_{NSR}$ and the conopressin-activated LVA and HVA currents), we first

![Fig. 2. Pseudo-steady-state current-voltage ($I$-$V$) relations, measured during ramp depolarizations, in 2 anterior lobe neurons in absence and presence of conopressin (CP). Voltage ramps from $-90$ to $+10$ mV (at rate of depolarization of $5$ mV/s) were applied at 2-min intervals. Control $I$-$V$ relation was recorded before, and conopressin $I$-$V$ relation 30 s after, start of conopressin application. Washout $I$-$V$ relation was recorded after several minutes of washing out peptide. Identical voltage ramps were used in Figs. 3–8. A: conopressin-activated current in isolated, unidentified anterior lobe neuron. A1: application of 1 $\mu M$ conopressin activates inward current only at potentials above $-40$ mV. Response recovers completely within 5–10 min of washing. A2: difference plot of conopressin-activated current, obtained by subtracting control $I$-$V$ relation from $I$-$V$ relation recorded in presence of conopressin. Current does not reverse between $-90$ and $+10$ mV. B: conopressin-activated current in isolated RCB1. B1: under control conditions, $I$-$V$ relation shows region of negative slope resistance at potentials higher than $-35$ mV. Application of 1 $\mu M$ conopressin shifts entire $I$-$V$ relation in inward direction. This effect recovers almost completely after 10 min of washout. B2: difference plot showing voltage dependence of current activated by 1 $\mu M$ conopressin. As in A, no reversal occurred between $-90$ and $+10$ mV. (In this and following figures, CP indicates current in presence of conopressin.)

![Fig. 3. Effects of calcium-selective saline and cadmium on persistent inward currents in RCB1. A: in saline selective for recording calcium currents (i.e., no extracellular sodium; potassium currents pharmacologically blocked), conopressin fails to evoke response. B: addition of 0.1 mM cadmium to bathing medium completely blocks $I_{NSR}$, the inward current underlying negative slope resistance. Saline as in A. C1: cadmium (0.1 mM) reduces conopressin-induced inward current (compare Control & CP traces with Control + Cd$^{2+}$ and CP + Cd$^{2+}$ traces). Recordings were made in nonselective saline. C2: difference plots of conopressin-activated currents in absence and presence of cadmium, showing that cadmium differentially affects the low-voltage activated current $I_{LVA}$ (which is largely blocked) and the high-voltage activated current $I_{HVA}$ (which is only slightly affected). C3: difference plot of traces shown in C2, showing that conopressin-induced current that is blocked by cadmium is similar in its voltage dependence to $I_{LVA}$.}
(0.1 mM) caused a nearly complete block of $I_{\text{NSR}}$ recorded in calcium-selective saline (amplitude reduced from $5.2 \pm 1.1$ nA to $0.5 \pm 0.1$ nA, $P = 0.016$, $n = 3$, Fig. 3C1). Similar results were obtained in nonselective saline ($n = 7$, Fig. 3C). These observations are in line with the presumption that $I_{\text{NSR}}$ is a calcium current. In contrast, in the presence of 0.1 mM Cd$^{2+}$, conopressin still induced an increase in pseudo-steady-state inward current, but the amplitude was reduced as compared with controls (Fig. 3C1). Comparison of the difference plots of the conopressin-induced current in the absence and presence of Cd$^{2+}$ shows that cadmium had a differential effect on the LVA and HVA component of the conopressin response (Fig. 3C2). Although the HVA response was slightly affected by the addition of cadmium, the LVA response was largely suppressed. To quantify this, the LVA response was measured as the current amplitude at $-50$ mV, whereas the peak current amplitude was used as an estimate for the HVA response. Because the voltage dependencies of both responses partially overlap, the LVA response may contribute slightly to the thus obtained HVA current amplitude. On average, the HVA component was reduced by $22 \pm 19\%$, whereas the LVA component was reduced by $48 \pm 25\%$. Thus cadmium exerts a significantly larger effect on the latter ($P < 0.001$, $n = 7$). Figure 3C3 shows the difference plot of the conopressin responses with and without cadmium, which confirms that cadmium preferentially blocked the LVA response. The above data strongly suggest that the LVA and HVA components are distinct currents that are not (predominantly) carried by calcium ions and that are differentially affected by cadmium. In addition, they differ from $I_{\text{NSR}}$, which is a persistent calcium current.

To assess the involvement of extracellular sodium in the persistent inward currents, the effect of a $\sim 50\%$ reduction of the extracellular sodium concentration on the conopressin responses was studied. This treatment resulted in a significant reduction of the amplitude of the inward current induced by conopressin (Fig. 4A). The peak current was reduced by $41 \pm 13\%$ ($n = 5$, $P = 0.014$), whereas the current at $-50$ mV showed a $40 \pm 16\%$ reduction ($n = 5$, $P = 0.022$), demonstrating that the HVA and LVA currents $I_{\text{HVA}}$ and $I_{\text{LVA}}$ were affected equally. Accordingly, the voltage dependence of the conopressin-induced current appeared to be unchanged (Fig. 4A2). These observations indicate that the inward current activated by conopressin is carried by sodium ions. The fact that the reversal potential of the net conopressin-activated current exceeds $+10$ mV is in line with this notion. However, because the reversal potential could not be determined exactly, a contribution of other ions cannot be ruled out.

In agreement with its calcium dependence, $I_{\text{NSR}}$ was unaffected by the reduction of the extracellular sodium concentration (Fig. 4A1). The peak amplitude of $I_{\text{NSR}}$ amounted to $0.38 \pm 0.34$ under control conditions and to $0.38 \pm 0.24$ nA in the reduced-sodium saline ($n = 5$, $P = 0.89$). This result confirms the different nature of $I_{\text{NSR}}$ and the conopressin-induced currents.

Despite their sodium dependence, the conopressin-induced currents are quite insensitive to the sodium channel blocker TTX. The peak amplitude of the conopressin-activated current was $1.29 \pm 0.25$ nA in the absence and $1.18 \pm 0.23$ nA in the presence of $2 \mu M$ TTX ($n = 3$, $P = 0.13$, Fig. 4, B1 and B2).

The effect of conopressin could not be recorded under conditions selective for potassium currents, because the cells deteriorated rapidly in the appropriate salines. Therefore the contribution of potassium ions to the conopressin-activated current could not be assessed. However, because a qualitatively normal conopressin-induced current was observed in saline containing the potassium channel blockers 4-amino-pyridine and TEA ($n = 3$, not shown), it is unlikely that the apparent activation of inward current by conopressin was solely due to closure of potassium channels.

**Dose dependency of conopressin-activated currents**

Experiments on the concentration dependence of the conopressin-induced currents in RCB1 revealed further differences between the proposed LVA and HVA components. Figure 5A1 shows the pseudo-steady-state $I$-$V$ relations recorded in one cell in the presence of $100$ nM and $1 \mu M$ conopressin, respectively. The voltage dependence of the conopressin-activated current recorded in the presence of $100$ nM conopressin is markedly different from that recorded in the presence of $1 \mu M$ conopressin (Fig. 5A2). At the lower concentration, conopressin mainly activates current at potentials greater than $-40$ mV, whereas the higher dose induces the complete response. The ratio between the amplitudes of the HVA and LVA component is $8.6 \pm 2.4$ at 100
nM conopressin and 3.0 ± 0.6 at 1 μM conopressin (n = 6, P = 0.003). This observation supports the idea that the complex response induced by 1 μM conopressin is composed of an HVA current and an additional LVA component. Subtraction of the current activated by 100 nM conopressin from that activated by 1 μM conopressin reveals the voltage dependence of the proposed LVA current (Fig. 5A3).

To obtain dose-response relations for both currents, activation of \( I_{\text{LVA}} \) and \( I_{\text{HVA}} \) was measured as indicated above, utilizing the differences in voltage dependence. The responses to subsequent applications of increasing concentrations of conopressin were analyzed. Each concentration was tested only after washout of the previous application. In eight experiments, near-maximal responses for both currents were observed at a concentration of 10 μM. Fitting the Hill equation on the dose-response curves yielded an estimated median effective concentration (EC\(_{50}\)) of 7.7 × 10\(^{-8}\) M for the HVA current and 2.2 × 10\(^{-7}\) M for the LVA current (Fig. 5B). Most of the remaining experiments employed a 1 μM conopressin solution sufficient to activate both the LVA and the HVA current.

**Effects of oxytocin and vasopressin**

Oxytocin and, to a lesser extent, vasopressin partially mimicked the effect of conopressin. Application of 10 μM oxytocin activated the HVA current, but also weakly activated the LVA current (Fig. 6, A and B). On average, the ratio between the amplitude of the HVA and LVA current activated by 1 μM conopressin was 4.7 ± 2.9 (n = 6), whereas this ratio was 14.7 ± 11.5 for 10 μM oxytocin (n = 6) and 11.6 ± 6.4 for 50 μM vasopressin (n = 3). The latter ratios are similar to those obtained from responses to lower concentrations of conopressin (see preceding paragraphs and Fig. 5). It follows that both oxytocin and vasopressin appear to be weak agonists of the conopressin response in RCB1. Coapplication of 1 μM conopressin and 10 μM d(CH\(_2\))\(_5\)-OVT, a specific oxytocin receptor antagonist, resulted in a reduced amplitude of both the LVA and HVA current as compared with application of 1 μM conopressin alone. The HVA current showed a reduction from 2.1 ± 1.1 nA to 1.6 ± 1.0 nA (P = 0.033), whereas the LVA current was reduced from 0.9 ± 0.5 (SD) nA to 0.6 ± 0.4 (SD) nA (P = 0.046) in three cells (not shown), implying that d(CH\(_2\))\(_5\)-OVT acts as a weak antagonist to conopressin. These results seem to confirm the relationship between the Lymnaea conopressin receptor and the vertebrate receptors for oxytocin and vasopressin.

**Desensitization and washout**

In desensitization experiments, voltage ramps were applied every 2 min during 10-min conopressin applications. Figure 7A exemplifies the results of one such experiment. The first I-V curve recorded in the presence of conopressin [labeled CP(1) in Fig. 7] shows the expected activation of both the LVA and the HVA current. The I-V relation re-
recorded 8 min later [labeled CP(5)] shows a substantial reduction of the LVA current. The HVA current, on the other hand, did not show any desensitization. In Fig. 7B the averaged I-V relations of the current induced by conopressin during subsequent ramps are plotted \((n = 8)\). In Fig. 7C the normalized amplitudes of the HVA current (i.e., peak current amplitude) and LVA current (i.e., current amplitude at \(-50 \text{ mV}\)) are plotted against time. Fitting of a single exponential to the amplitude data of the LVA current yielded an estimated time constant of desensitization of \(80 \pm 28\) (SD) s \((n = 8)\). Similar rates of desensitization \((88 \pm 19\) s, \(n = 3)\) were observed when cells were continuously clamped at \(-50 \text{ mV}\) during peptide application (Fig. 7D). Because no substantial desensitization of the HVA current occurred within 10 min, it was not possible to estimate the rate of desensitization for this current.

Comparison of I-V relations recorded before, during, and immediately after a conopressin application revealed differences in rate of washout of the HVA and LVA components (Fig. 8A1). The LVA current disappeared rapidly after the end of the application, whereas the HVA current largely persisted (Fig. 8A2). To estimate the rate of washout of \(I_{HVA}\) and \(I_{LVA}\), we recorded the decay of both currents following a single conopressin application. The normalized amplitudes of \(I_{HVA}\) and \(I_{LVA}\) were obtained from a series of I-V relations, recorded at 2-min intervals, and plotted against time (Fig. 8B). An estimate of the time constant of washout for both currents was obtained by fitting a single exponential to these data. The estimated time constant of washout \((I_{HVA})\) amounted to \(159 \pm 63\) (SD) s compared with \(36 \pm 13\) s for \(I_{LVA}\) \((P = 0.002, n = 7)\). In cells continuously clamped at \(-50 \text{ mV}\), the estimated rate of washout for \(I_{LVA}\) was 23 \(\pm 5\) s \((n = 8)\), see Fig. 1C). Figure 8A3 shows the voltage dependence of the current that has washed out.

In conclusion, the conopressin-activated current in RCB1 is differentially affected by desensitization and washout, suggesting that different transduction mechanisms underlie the activation of the HVA and LVA current.

**Discussion**

The oxytocin/vasopressin analogue Lys-conopressin exerts a strong excitatory action on central neurons of the mollusk *L. stagnalis*. Among the neurons that responded to conopressin, two types of responses could be discerned. The first type was designated the HVA response, because it induces or amplifies a region of NSR at potentials greater than \(-40 \text{ mV}\). The second type, termed the LVA response, is relatively voltage independent and activated at potentials as low as \(-90 \text{ mV}\).

The presence of a region of NSR has been shown to underlie burst generation and other forms of spontaneous activity in several preparations (see, e.g., Adams and Benson...
1985; Smith et al. 1975; Wilson and Wachtel 1974, 1978). Furthermore, enhancement of bursting activity through agonist-induced increases in currents underlying the NSR, has been described in several studies (see, e.g., Barker and Gainer 1974; Barker et al. 1975; Funase et al. 1993). Presumably the HVA current would enhance bursting properties, whereas the LVA current would induce a regenerative depolarization toward spike threshold, regardless of the actual membrane potential at the moment of peptide application. Accordingly, we observed a depolarization and strong excitation under current-clamp conditions in RCB1, which has both the LVA and the HVA current. Conopressin alone need not necessarily modulate firing if it only amplifies or induces \( I_{\text{HVA}} \). It is to be expected that in cells lacking the LVA current, conopressin will only generate a detectable depolarization or enhancement of firing when the membrane potential is above approximately \( -40 \text{ mV} \). In this respect it is interesting that the type of response appeared to correlate to some extent with the electrophysiological properties of the cells under control conditions. Cells that did not show a region of NSR under control conditions only displayed the HVA response. On the other hand, most of the cells that displayed the LVA response already did show a pacemaker current in the absence of the peptide. Thus both conopressin responses may differentially modulate activity in the cells of the anterior lobe of Lymnaeidae. The observation that many of these neurons not only express conopressin receptors, but also the peptide itself (Van Kesteren et al. 1995b), suggests that autoexcitatory feedback within the anterior lobe might underlie modulation of the neurons’ activity.

The excitatory effect of conopressin under current-clamp conditions was preserved after isolation of the cell from the nervous system. The direct effects of conopressin seem adequate to explain the effects observed in situ. The apparent differences in duration of the excitation measured in situ and in vitro might be explained by delayed washout of the peptide from the connective tissue surrounding neurons in situ, and might also reflect effects of the dissociation procedure, e.g., effects of trypsin on the conopressin receptors.

Effects on rapidly inactivating currents, such as the fast sodium and calcium currents, were not investigated. Conopressin appears to modulate such currents in other cell types, however. For instance, the calcium current in isolated myocytes from the vas deferens of Lymnaea is enhanced by application of conopressin (Van Kesteren et al. 1995b). Conopressin exerted indirect effects on neurons involved in gill withdrawal reflex behavior in A. californica (Martinez-Padrón et al. 1992). Finally, conopressin may induce or enhance bursting properties in the R25/L25 network underlying respiratory pumping in Aplysia. (Martinez-Padrón and Lukowiak 1993). There, however, the underlying currents have not been characterized.

**Similarity to the effects of other related neuropeptides**

The conopressin-activated currents in anterior lobe neurons resemble peptide-induced slow inward currents in other preparations. Funase (1990) reported that oxytocin induces a slow inward current, mainly carried by sodium ions, in an identified Achatina fulica neuron. This current underlies a region of NSR in the steady-state \( I-V \) relation. The effect was mimicked by increasing adenosine 3',5'-cyclic monophosphate (cAMP) and blocked by inhibitors of cAMP-dependent protein kinase. Thus the response most likely involves cAMP-dependent phosphorylation of the channels (Funase 1990). Activation of slow sodium currents by intracellular cAMP has been reported in various molluscan preparations, including neurons from Lymnaea (McCrohan and Gillette 1988), Helix (Aldenhoff et al. 1983; Swandulla and Lux 1984), Helisoma (Price and Goldberg 1993), Pleurobranchaea (Green and Gillette 1983), Aplysia (Kehoe 1990), and Archidoris (Connor and Hockberger 1984).

A few reports on similar effects of oxytocin and vasopressin on vertebrate neurons have been published. Vasopressin activates a persistent inward current in motoneurons in the facial nucleus of newborn rats recorded in situ. This current is carried by sodium ions and is insensitive to TTX (Raggenbass et al. 1991). The amplitude of this current is reduced by increasing the extracellular concentrations of divalent cations to physiological concentrations (Alberi et al. 1993). Similarly, oxytocin activates a sustained sodium-dependent current in rat vagal neurons in situ. This current is also resistant to TTX and is modulated by extracellular divalent cations (Raggenbass and Dreifuss 1992).

Thus there is a striking similarity between the currents activated by vasopressin- and oxytocin-like peptides in preparations as diverse as molluscan and mammalian neurons. It appears that the capability to induce persistent inward currents in certain neurons is a common feature of these peptides conserved throughout several phyla.

**Divergence of the two responses to conopressin**

Results from several experiments indicate that a divergence occurs somewhere in the transduction of the conopressin signal toward both components of the response. First of all, differences in dose dependence of activation of the LVA and HVA currents point to differential stimulation of two signal transduction mechanisms. The preferential activation of the HVA current by vasopressin and oxytocin supports this notion. Second, desensitization differentially affects the LVA and HVA currents. The LVA current desensitizes rapidly, whereas the HVA current shows little desensitization. Finally, the LVA current disappears almost instantaneously during washout of the peptide, whereas the HVA current takes several minutes to decline. Taken together, these observations point to a divergence of the signal transduction mechanism somewhere between agonist binding and channel activation.

**INVolvement of two conopressin receptors.** Existing data indicate that the responses to conopressin may involve two different receptors. So far, Van Kesteren et al. found two conopressin receptors in Lymnaea, LSCPR1 and LSCPR2. In situ hybridization experiments on the distribution of LSCPR1 showed that it is expressed abundantly in the anterior lobe neurons. Furthermore, reconstitution experiments indicated that the receptors have a different affinity for conopressin and that only LSCPR1 can be activated by 1 \( \mu M \) oxytocin. The \( EC_{50} \) values for Lys-conopressin are 22 nM for LSCPR1 and 86 nM for LSCPR2 (Van Kesteren et al. 1995b, 1996). Interestingly, the ratio between these values is similar to our estimates of the \( EC_{50} \) values for activation.
of the HVA and LVA current, being 77 and 220 nM, respectively.

Thus the differences in dose dependency, desensitization, and recovery may be explained by assuming that two conopressin receptors, possibly LSCPR1 and LSCPR2, mediate the observed responses in RCB1. Each receptor would have a different affinity for the agonist. Differences in rates of desensitization and recovery might be explained by differences in agonist binding or differences in the signal transduction mechanisms between receptor and ion channel. Nevertheless, the possibility that a single type of conopressin receptor mediates the observed responses cannot be ruled out. In the latter case, divergence would occur in the signal transduction pathway or at the level of the ion channel.

**Characteristics of the LVA and HVA currents**

**Ionic dependence.** Classical blockers of potassium channels were ineffective in blocking the response to conopressin, indicating that the apparent activation of an inward current is not, in fact, caused by closure of a resting potassium conductance. However, this does not rule out the possibility that potassium ions contribute to the conopressin-activated current (see below).

Both the HVA and the LVA current are dependent on extracellular sodium, because 1) experimental manipulation of the extracellular sodium concentration affected the amplitude of both currents in a similar manner and 2) neither current is observed under conditions selective for calcium currents. It appears, however, that the reversal potential for $I_{LVA}$ is lower than that for $I_{HVA}$. This would suggest that, although sodium is the main charge carrier for both the LVA and the HVA current, other ions (e.g., potassium) may contribute to the LVA current.

The differential reduction of $I_{HVA}$ and $I_{LVA}$ by cadmium gives pharmacological support for the proposed distinction between both currents. Furthermore, although the voltage dependence of $I_{HVA}$ is similar to that of $I_{NSP}$, differences in cadmium sensitivity and calcium dependence indicate that both are carried through different channels.

**Voltage dependence.** Various experimental conditions either activate the HVA current relatively selectively (e.g., low doses of conopressin) or leave it intact while the LVA current has mostly disappeared (e.g., desensitization and washout). The voltage dependence of the HVA current in RCB1, obtained under those conditions, closely resembles that of the HVA current in other anterior lobe neurons that lack the LVA response. The HVA current activates at potentials above $-40$ mV and reaches its maximal amplitude around $-10$ to $-0$ mV (see Figs. 5A2, 7B, traces 2–5, and 8A2). The reversal potential could not be determined, but might be close to the calculated reversal potential for sodium, which equals $+60$ mV in standard solutions.

The voltage dependence of the LVA current was reconstructed by subtraction of the HVA current from the total conopressin-induced current. The resulting $I-V$ relations appeared to be similar in all cases, irrespective of whether they were obtained from desensitization or washout data or from the vasopressin/oxytocin experiments (see Figs. 3C3, 5A3, and 8A3). This suggests that it is the same current that is affected by all of these treatments. The LVA current is activated at all potentials tested and reaches its maximal amplitude around $-30$ to $-20$ mV. The current appears to reverse at a less depolarized potential than the HVA current.

In conclusion, although both currents share a dependence on extracellular sodium, they display a different sensitivity to cadmium and a striking difference in voltage dependence. On the basis of these results, we propose that the two responses in RCB1 involve two different channel populations giving rise to LVA and HVA currents, respectively. The observation that in other neurons in the anterior lobe the HVA part of the response occurred in isolation supports this conclusion.

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Address for reprint requests: K. S. Kits, Faculty of Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

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**References**


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