Activation of Protein Kinase C by Oxytocin-Related Conopressin Underlies Pacemaker Current in *Lymnaea* Central Neurons

PAUL F. VAN SOEST, JOHANNES C. LODDER, AND KAREL S. KITS

Department of Neurophysiology, Research Institute Neurosciences, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands

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**INTRODUCTION**

The molluscan neuropeptide Lys-conopressin, a vasopressin/oxytocin analogue, is expressed abundantly by neurons in the anterior lobe of the right cerebral ganglion of *Lymnaea stagnalis* (Van Kesteren et al. 1995a). Most of these neurons also express a G protein–coupled conopressin receptor. So far, two receptor subtypes have been identified, both of which are related to mammalian vasopressin V1 and oxytocin receptors (Van Kesteren et al. 1995b, 1996). The abundance of both conopressin and its receptors makes the anterior lobe of *Lymnaea* an advantageous system to study the effects of this neuropeptide.

Previously, we have studied the responses of isolated anterior lobe neurons to conopressin application under voltage-clamp conditions (Van Soest and Kits 1997, 1998). In most of the anterior lobe neurons, conopressin activates either one or both of two distinct persistent inward currents. The first type, a highly voltage-dependent current that is activated at potentials above $-40 \text{ mV} (I_{\text{HVA}})$, occurs in most of the cells. The second type, a weakly voltage-dependent current that is activated at all potentials between $-90$ and $+10 \text{ mV} (I_{\text{LVA}})$, occurs in a minority of neurons, and only in combination with $I_{\text{HVA}}$. Both currents are carried mainly by sodium ions.

The HVA and LVA currents differ in some important aspects. Desensitization experiments revealed that the amplitude of the LVA current decreases strongly within a few minutes in the continuous presence of the peptide, while the HVA current showed hardly any desensitization. Similarly, the LVA current declined rapidly after wash out of conopressin, while the HVA current takes several minutes to disappear. Thus a single peptide can modulate various physiological parameters at different time scales. The observations suggested that different signal transduction mechanisms, and possibly different conopressin receptors underlie activation of the HVA and LVA currents (Van Soest and Kits 1997).

So far, the identity of the second-messenger systems involved in the conopressin responses has remained unknown. Insight in this matter will shed light on the complex mechanisms through which conopressin controls the activity of cells by differentially modulating various ion channels. This seems especially interesting in the case of the HVA current, whose activation substantially outlasts the actual conopressin application.

The responses of molluscan neurons to vasopressin, oxytocin, and related peptides have previously been attributed to increases in intracellular cAMP. A vasopressin-related peptide factor extracted from molluscan ganglia enhanced bursting pacemaker activity in an identified *Otala* neuron (Ifshin et al. 1975). A peptide extract with similar properties was shown to increase levels of cAMP in *Helix* and *Aplysia* neurons, and the burst-inducing effects of these peptides were mimicked by application of cAMP and treatment with the phosphodiesterase-inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Levitan et al. 1979; Treistman and Levitan 1976). Similarly, the persistent sodium current induced by oxytocin in an identified *Achatina* neuron was mimicked by injection of cAMP, augmented by...
IBMX, and partially blocked by cAMP-dependent protein kinase (PKA) inhibitors, indicating that the response is mediated by PKA-dependent protein phosphorylation (Funase 1990).

Moreover, cAMP has been shown to induce or to mediate agonist-induced activation of sodium currents in many molluscan preparations (see, e.g., Aldenhoff et al. 1983; Connor and Hockberger 1984; Deterre and Pia 1981), and to enhance the opening frequency of single channels carrying inward current in Pleurobranchaea neurons (Green and Gillette 1983). Although many reports indicate that cAMP exerts its action on ion channels through PKA-mediated phosphorylation, a sodium channel that is directly gated by cAMP was identified in Pleurobranchaea neurons (Sudlow et al. 1993). In Lymnaea, intracellular injection of cAMP induces a slow sodium current in neurons involved in feeding behavior (McCrohan and Gillette 1988). Thus it seems reasonable to propose that a cAMP-dependent pathway might underlie the conopressin-induced persistent sodium LVA and HVA currents.

On the other hand, the homology of the Lymnaea conopressin receptors to vertebrate vasopressin V1 and oxytocin receptors, both of which interact with G proteins of the Q type, suggests that they are likely to couple to phospholipase C (Van Kesteren et al. 1995b, 1996). Thus conopressin might activate protein kinase C (PKC) through production of 1,2-diacylglycerol (DAG) (for reviews see Nishizuka 1984, 1995). Further support for this scheme was supplied by in vitro reconstitution experiments, both of which interact with G proteins of the Q type, and to enhance the opening frequency of single channels carrying inward current in Pleurobranchaea neurons (Green and Gillette 1983). Although many reports indicate that cAMP exerts its action on ion channels through PKA-mediated phosphorylation, a sodium channel that is directly gated by cAMP was identified in Pleurobranchaea neurons (Sudlow et al. 1993). In Lymnaea, intracellular injection of cAMP induces a slow sodium current in neurons involved in feeding behavior (McCrohan and Gillette 1988). Thus it seems reasonable to propose that a cAMP-dependent pathway might underlie the conopressin-induced persistent sodium LVA and HVA currents.

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Various studies have suggested a role for PKC in molluscan neurons. An endogenous PKC was identified in the CNS of Aplysia (de Riemer et al. 1985a), and PKC activity was subsequently characterized in further studies (e.g. Pepio et al. 1998; Sossin and Schwartz 1994; Sossin et al. 1993). Activation of PKC was shown to stimulate high-voltage-activated calcium currents in neuroendocrine cells in Aplysia (de Riemer et al. 1985b) and in Lymnaea (Dreijer and Kits 1995). Similarly, a number of papers addressed PKC modulation of potassium currents (e.g. Critz and Byrne 1992; Sugita et al. 1994, 1997). However, there is little evidence for the activation of slow inward currents by PKC. A notable exception is found in the bag cells of Aplysia, where a nonselective cation channel is up-regulated by PKC and presumably underlies the prolonged firing pattern, known as afterdischarge (Wilson et al. 1996, 1998). Treatment with a phorbol ester or a DAG analogue induced a slow and weakly voltage-dependent inward current in Aplysia motor neurons, but this current was not further characterized (Sawada et al. 1989).

Thus the present study was undertaken to elucidate the signal transduction pathway underlying activation of the conopressin induced currents, focusing on the possible involvement of cAMP-activated PKA and DAG-activated PKC.

METHODS

Animals and preparations

All experiments were performed on isolated anterior lobe neurons from adult, laboratory-bred specimens of the pond snail Lymnaea stagnalis (L.). 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. To obtain isolated neurons, the CNS was dissected out and incubated in an 0.2% solution of trypsin (Sigma type III) in HEPES-buffered saline (HBS) at 37°C for 35 min. After the incubation period, the CNS was rinsed in HBS and pinned down in a dish. The outer layers of connective tissue covering the anterior lobe of the right cerebral ganglion were removed, and the anterior lobe was severed from the CNS. Single anterior lobes were transferred to 35-mm plastic culture dishes (Corning Costar, Cambridge, MA) and mechanically dissociated. After dissociation, the cells were allowed to sit for at least 30 min before the dish was transferred to the experimental setup.

FIG. 1. Reproducibility of the conopressin response. A: isolated high-voltage–activated (HVA) current induced by 4 subsequent applications of 1 μM conopressin, separated by 20-min periods of washing. Pseudo steady-state current-voltage (I-V) relations were obtained using voltage-ramp protocols. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation. The voltage dependence of the HVA current was obtained by subtraction of the control I-V relation from that recorded in the presence of conopressin. Traces were taken from a cell that displayed only the HVA current in isolation.
**Recording technique**

Whole cell voltage-clamp recording of isolated neurons was performed in a standard patch-clamp setup, containing an Axopatch 1C or 200B (Axon Instruments, Burlingame, CA) patch-clamp amplifier. Current and voltage traces were digitized using CED 1401 (Cambridge Electronic Design, Cambridge, UK) or Digidata 1200 (Axon Instruments) AD/DA convertors and recorded on an IBM-compatible PC using custom software (developed by P. F. van Soest) or p-Clamp (Axon Instruments). Series resistance normally amounted to 1–3 MΩ, approximately 75% of which could be compensated for.

Pseudo steady-state current-voltage (I-V) relations were obtained by applying voltage ramp protocols in which the command potential was swept from −90 to +10 mV at a rate of 5 mV/s. Ramps were generally applied at 2-min intervals. Conopressin-induced current profiles were obtained by subtracting the control I-V relation from that obtained in the presence of cAMP. The latter I-V relation was obtained between 30 and 50 s after injection of dibutyryl-cAMP when no time-dependent changes in the current response occur, as shown in C. D1: pseudo steady-state I-V relations obtained under control conditions and in the presence of dibutyryl-cAMP. The latter I-V relation was obtained between 30 and 50 s after injection of dibutyryl-cAMP when no time-dependent changes in the current response occur, as shown in C. D2: time course of the current response to injection of dibutyryl-cAMP (20 ms).

**Drugs and solutions**

Standard HBS was composed of (in mM) 30 NaCl, 1.7 KCl, 10 NaCH₃SO₄, 1.5 MgCl₂, 4 CaCl₂, 5 NaHCO₃, and 10 HEPES, pH set at 7.8 with NaOH. The standard pipette solution consisted of (in mM)...

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**FIG. 2.** Current induced by injection of cyclic AMP (cAMP) induces a pacemaker current. A: current induced by injection of 20 mM cAMP (20 ms) in a neuron clamped at −60 mV. Two traces are superimposed, indicating that the current response is highly reproducible. B1: pseudo steady-state I-V relations of an isolated anterior lobe neuron, obtained by applying a voltage ramp (shown here from −80 to 0 mV) at 5 mV/s under control conditions (“Control”) and after intracellular injection of 1 mM cAMP for 5 s (“cAMP”). Injection of cAMP shifts the entire I-V relation in the inward direction. B2: subtraction of the control I-V relation from that obtained in the presence of cAMP yields the voltage dependence of the cAMP-activated current (I_{cAMP}). I_{cAMP} is only weakly voltage dependent and is activated at all potentials between −90 and 0 mV. C and D: experiments as in A and B, but now using nonhydrolyzable dibutyryl-cAMP, to prevent that time-dependent changes in cAMP levels influence the current responses and I-V relations. C: time course of the current response to injection of 20 mM dibutyryl-cAMP (20 ms). D1: pseudo steady-state I-V relations obtained under control conditions and in the presence of dibutyryl-cAMP. The latter I-V relation was obtained between 30 and 50 s after injection of dibutyryl-cAMP when no time-dependent changes in the current response occur, as shown in C. D2: I-V relation of the dibutyryl-cAMP induced current obtained by subtraction of the traces shown in D1. The voltage dependence of this current is similar to that of the cAMP-activated current shown in B2.
29 KCl, 2 NaCl, 10 HEPES, 11 ethylene glycol-bis (β-aminoethyl ether)-N,N',N'',N'''-tetraacetic acid (EGTA), 2.3 CaCl₂, 2 Mg-ATP, and 0.1 GTP-Tris, pH set at 7.4 with KOH (~35 mM). The calculated concentration of free Ca²⁺ in the pipette medium was 10 nM (cf. Stockbridge 1987). Cesium pipette medium had the same composition, except for the KCl, which was replaced by 29 mM of CsCl, and had its pH set with CsOH. 1,2-bis(2-aminophenoxy)ethane-N,N',N'',N'''-tetraacetic acid (BAPTA) pipette medium was identical to cesium pipette medium except for EGTA, which was replaced by 11 mM BAPTA cesium salt.

Adenosine 3',5'-cyclic monophosphate (cAMP) was dissolved in water supplemented with an equimolar amount of KOH, 50 mM KCl, and 10 mM HEPES, buffered at pH ~7.4. Stock solutions ranging from 1 to 20 mM were injected intracellularly by means of pressure ejection from blunt microelectrodes. Injection pressure and duration were adjusted to induce stable responses as required in most experiments. To obtain desensitizing responses (in the cross-desensitization experiments) cAMP was injected at 20 mM.

The PKC-activating phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA, also known as phorbol 12-myristate 13-acetate or PMA) and the inactive phorbol ester 4-α-phorbol 12-myristate 13-acetate (4-α-PMA) were obtained from Research Biochemicals International (Natick, MA). The phorbol esters were dissolved in dimethylsulfoxide (DMSO) and diluted to a final concentration of 1 mM in HBS (final concentration of DMSO amounted to 0.1%). No effects of DMSO on the anterior lobe neurons were observed.

The protein kinase inhibitors 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) and staurosporine and GF-109203X were obtained from Research Biochemicals International (Natick, MA). The phosphodiesterase-inhibitor IBMX was obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). H7, staurosporine, GF-109203X, and IBMX were dissolved in DMSO and diluted to a final concentration of 10, 1, 1, and 100 μM, respectively (final concentration of DMSO amounted to 0.1% in all cases). All agents were applied extracellularly by pressure ejection from a glass pipette.

Synthetic Lys-conopressin G (Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-NH₂) was obtained from Saxon Biochemicals (Hannover, Germany) and was applied by pressure ejection from a glass pipette. In all experiments, the recording chamber was continuously perfused with HBS.

RESULTS

Stability of conopressin responses

Responses of individual anterior lobe neurons to conopressin (applied at 1 μM) were characterized by testing the effect of the peptide on their pseudo steady-state I-V relation, obtained with the use of voltage-ramp protocols. Most of the anterior lobe neurons (~60% of the cells) had I_HVA, which is activated by conopressin at potentials positive to ~240 mV. The isolated HVA current manifested itself as a downward deflection of the steady-state I-V relation at potentials positive to ~240 mV (Fig. 1). Only some anterior lobe neurons (~10%) had I_LVA, which is activated by conopressin at all potentials between ~90 and ~110 mV. The latter cells always displayed combined activation of the LVA and HVA current by conopressin, resulting in...
an inward shift of the entire I-V relation between −90 and +10 mV (Fig. 3B).

As most of the experiments involved comparing responses to conopressin before and after pharmacological treatments, the ability to evoke reproducible responses was essential. Accordingly, the reproducibility of the conopressin-induced currents was investigated by repeatedly applying conopressin to the same cell. The applications were separated by approximately 20 min of washing, to allow the current to recover. Figure 1 shows the normalized amplitude of $I_{\text{HVA}}$ at each application (mean ± SD, number of cells indicated above each bar), indicating that no significant changes occur over a period of 1 h.

**Involvement of cyclic AMP and PKA**

Persistent inward currents in molluscan neurons have often been attributed to cAMP-dependent phosphorylation processes. To investigate whether the conopressin-induced persistent inward currents in Lymnaea anterior lobe neurons are also mediated by cAMP, several experimental approaches were followed.

Intracellular application of cAMP, by means of pressure ejection from an intracellular microelectrode, induced an inward current similar to that observed in other mollusks ($I_{\text{cAMP}}$; $n = 5$; Fig. 2A). Voltage ramps following long-lasting cAMP injections revealed that the voltage dependence of $I_{\text{cAMP}}$ showed large overlap with that of the currents induced by conopressin ($n = 5$; Fig. 2B). However, because it is uncertain whether the intracellular cAMP concentration was constant in time, the apparent voltage dependence of $I_{\text{cAMP}}$ may be distorted. Therefore similar experiments were performed using the nonhydrolysable cAMP analogue dibutyryl-cAMP. First we measured the time course of the current response on injection of dibutyryl-cAMP at a constant holding potential of −60 mV. We found that the response lasted for several minutes and that the current remained at a constant amplitude for at least 60 s after injection (Fig. 2C; $n = 9$). We then measured the pseudo steady-state I-V relation, using the voltage-ramp protocol between 30 and 50 s after injection. These I-V relations were very similar to the ones obtained on injection of cAMP (Fig. 2D; $n = 5$). In summary, the experiments demonstrate that $I_{\text{cAMP}}$ is activated at a wide range of potentials (from −90 to 0 mV). In this respect, it mostly resembles the LVA current.

Since intracellular injection of cAMP could mimic at least part of the conopressin response, we investigated whether the response required cAMP. To this end, anterior lobe neurons were treated with the phosphodiesterase-inhibitor IBMX. The resulting inhibition of cAMP breakdown is expected to aid the presumed increase in cAMP levels, thus potentiating the conopressin response if it were cAMP dependent. The current induced by direct cAMP injection was potentiated by application of 100 µM IBMX (Fig. 3A; $n = 5$), indicating that the IBMX treatment was effective. However, application of 100 µM IBMX had no effect on the conopressin responses in five cells (Fig. 3B). The amplitude of the HVA current (measured as the peak inward current, around −10 mV) amounted to 1.09 ± 0.26 nA under control conditions, and to 0.99 ± 0.19 nA after 15 min in the presence of IBMX (mean ± SD, $P = 0.430$). The LVA current was not affected either; its amplitude (measured as the inward current at −50 mV) was 0.41 ± 0.08 nA under control conditions and 0.37 ± 0.14 nA in the presence of IBMX ($n = 4$, $P = 0.537$). These data suggest that the conopressin-induced responses are not mediated by cAMP. The implication that also $I_{\text{LVA}}$ (which resembles $I_{\text{cAMP}}$) is not activated by cAMP is in line with the observation that $I_{\text{LVA}}$ still could be evoked after complete desensitization of $I_{\text{LVA}}$ ($n = 5$; data not shown).

As an additional control, we examined whether the response to conopressin could still be evoked after desensitization of the response to injection of cAMP. Desensitization of $I_{\text{cAMP}}$ was induced by repeatedly injecting a high concentration of cAMP (20 mM) into the cell until the current amplitude approached

![Fig. 4. Lack of cross-desensitization between currents induced by cAMP injection and conopressin. Left: pseudo steady-state I-V relation of the conopressin-induced current (obtained by subtraction) in an anterior lobe neuron, showing a prominent HVA current at potentials above −40 mV. Middle: the current induced by cAMP-injection ("$I_{\text{cAMP}}$") nearly completely desensitizes during a series of injections. The last injection hardly evokes any current ("$I_{\text{cAMP}}$ desensitized"). Arrow indicates onset of cAMP injection. Right: after desensitization of $I_{\text{cAMP}}$, application of conopressin still activates the low-voltage-activated (LVA) and HVA currents.](https://www.jn.physiology.org/article/b10.220.3.5/10.220.33.5/10.220.35.7/pdf)
zero (Fig. 4, middle). The conopressin-induced currents, characterized using voltage ramps, were recorded before and after desensitization of the cAMP response (Fig. 4, left and right). Even after the response to injection of cAMP had fully desensitized, conopressin could still activate the HVA and LVA current. The amplitude of the HVA current amounted to 0.92 ± 0.22 nA before and to 0.74 ± 0.38 nA after desensitization of $I_{cAMP}$ ($n = 4$; $P = 0.436$). For the LVA current (measured at −50 mV), the values were 0.20 ± 0.20 nA before, and 0.26 ± 0.31 nA after the treatment ($n = 4$; $P = 0.439$).

These observations, together with the lack of effects of IBMX, suggest that cAMP elevation does not mediate conopressin-induced activation of neither the LVA current nor the HVA current. It may be, however, that cAMP and conopressin activate the same population of LVA channels in anterior lobe neurons, be it through different pathways.

### Involvement of PKC

The sequence homology of the *Lymnaea* conopressin receptors to mammalian vasopressin V1 and oxytocin receptors suggested that the former might also couple to G proteins of the Q type (Van Kesteren et al. 1995b, 1996). Therefore the involvement of PKC in the conopressin response was tested by applying a phorbol ester that activates PKC in the absence of DAG.

In 11 cells exhibiting the HVA current on application of 1 μM conopressin, extracellular application of the phorbol ester TPA (1 μM) induced a persistent inward current at voltages above −40 mV, thus mimicking the activation of the HVA current by conopressin (Fig. 5). Furthermore, conopressin could not induce additional inward current during the response to TPA ($n = 8$). The occlusion of the conopressin-induced HVA current by TPA suggests that both conopressin and TPA activate the same population of ion channels.

**FIG. 5.** The PKC-activating phorbol ester TPA activates the HVA current. **A1**: pseudo steady-state $I$-$V$ relations recorded under control conditions, in the presence of 1 μM conopressin, after application of 1 μM TPA, and in the presence of conopressin after application of TPA. **A2**: currents induced by TPA and by conopressin before and after application of TPA (obtained by subtraction). TPA mimics the conopressin-induced activation of the HVA current and occludes subsequent effects of conopressin. **B**: peak amplitude of HVA inward current (measured during voltage ramps at potentials around −10 mV) plotted vs. time. Both conopressin and TPA strongly enhance the peak current amplitude, but after TPA application, conopressin cannot induce additional inward current. Shaded boxes indicate times of application; labels indicate measurements corresponding to traces in A.
Application of 1 μM of the inactive phorbol ester 4-α-PMA, which cannot activate PKC, did not induce current by itself, nor did it prevent conopressin from activating the HVA current (n = 3, not shown). Subsequent application of TPA, however, affected the cells as described above. These observations confirm that the observed effect of TPA involves activation of PKC and is not due to nonspecific effects on membrane components (see Hockberger et al. 1989). In none of our experiments we observed a mimic of the LVA current by TPA. Therefore the following pharmacological experiments on the involvement of PKC in the conopressin response were restricted to the HVA current.

**PKC inhibitors**

To demonstrate that activation of PKC is not only sufficient, but also necessary to activate the HVA current, we tested the effect of protein kinase inhibitors on the response induced by conopressin. The nonspecific protein kinase inhibitor H7 attenuated the conopressin response (not shown). After a 10-min application of 10 μM H7, the amplitude of the conopressin-induced HVA current was reduced by 25 ± 20% (from 0.76 ± 0.65 nA to 0.64 ± 0.72 nA; mean ± SD, 9, mean = 0.012). A 20-min application of 100 μM H7 caused a similar reduction of the current amplitude by 32 ± 18% (from 0.73 ± 0.61 nA to 0.52 ± 0.56 nA; n = 5, P = 0.037), The current amplitude partially recovered after a 20-min wash out. This confirms that a protein kinase is involved in the conopressin response.

Subsequently, the effects of the membrane-permeable protein kinase inhibitor staurosporine were tested, both on the TPA-induced current and on the conopressin response. Staurosporine blocked the activation of the HVA current by 1 μM TPA (Fig. 6A). In five cells, the amplitude of the TPA-induced current was reduced by 76 ± 34% (from 1.43 ± 0.20 nA to

**FIG. 6.** Amplitude of conopressin-activated HVA current and TPA-induced current is reduced by the protein kinase C inhibitors staurosporine and GF-109203X. A: pseudo steady-state I-V relation of the TPA-induced current in the absence and in the presence of staurosporine (1 μM; current traces in A–C obtained by subtraction; original traces not shown). B: pseudo steady-state I-V relation of the conopressin-activated HVA current in the absence and presence of staurosporine (1 μM). C: pseudo steady-state I-V relation of the conopressin-activated HVA current under control conditions and in the presence of the specific protein kinase C inhibitor GF-109203X (1 μM). D: average peak amplitude of HVA current induced by conopressin or TPA application, under control conditions, and in the presence of protein kinase blockers (bars indicate means ± SE).
0.43 ± 0.33 nA; mean ± SD; Wilcoxon nonparametric test: 
\( P = 0.009 \). In addition, staurosporine largely blocked the 
HVA current response to conopressin. Figure 6B shows the 
response to 1 μM conopressin before and after application of 
1 μM staurosporine. Comparable results were obtained with 1 
and 10 μM staurosporine (n = 3 each); both treatments re-
duced the amplitude of the HVA current by 40–80% within 20 
min. On average, the amplitude was reduced from 0.93 ± 0.82 
nA to 0.30 ± 0.25 nA (n = 6, P = 0.028). However, because 
stauroporine (and H7) only have a limited specificity for PKC 
and also affect other kinases, we also tested the specific PKC 
inhibitor GF-109203X. At 1 μM, this inhibitor blocked the 
conopressin-evoked HVA current by 58 ± 7% after 15 min, 
and by 82 ± 4% after 35 min, reducing the HVA current 
amplitude from 0.87 ± 0.25 nA to 0.15 ± 0.39 nA in the latter 
case (Fig. 6C; n = 5, P = 0.015).

These results, summarized in Fig. 6D, indicate that cono-
pressin activates the HVA current through a PKC-dependent 
mechanism, probably phosphorylation of the ion channel or 
associated proteins.

**Involvement of intracellular calcium**

All experiments performed so far employed a pipette solu-
tion containing 10 mM of the calcium chelator EGTA, buffered 
with calcium to yield a final [Ca\(^{2+}\)\(_{\text{in}}\)] of approximately 10 nM. 
The sole fact that the conopressin-induced currents are ob-
erved under these conditions (see also Van Soest and Kits 
1997) argues against a strong involvement of changes in the 
intracellular free calcium concentration. To substantiate this 
collection, we investigated the responses to conopressin using 
pipette solutions containing either 0.1 mM EGTA, or 10 mM of 
the fast calcium chelator BAPTA.

Due to its stabilizing effect on [Ca\(^{2+}\)\(_{\text{in}}\)], the presence of 10 
mM EGTA might have obscured parts of the conopressin 
response that were dependent on changes in [Ca\(^{2+}\)\(_{\text{in}}\)] (e.g., due 
to release of calcium from intracellular stores). Therefore the 
response to conopressin was recorded with the use of a pipette 
solution containing only 0.1 mM EGTA, but having the same 
concentration of free calcium as the normal solution. With this 
pipette solution, [Ca\(^{2+}\)\(_{\text{in}}\)] will be able to vary substantially 
more. Under these circumstances, a strongly enhanced and 
presumably calcium-dependent outward current was ob-
served at depolarized voltages, interfering with the conopres-
sin-induced pacemaker currents. However, at voltages less than 
or equal to −20 mV, conopressin still induced apparently 
normal LVA and HVA currents, similar to the responses ob-
served in standard saline (Fig. 7A; n = 7). In another series of 
experiments, the low-EGTA pipette solution was supple-
mented with cesium to block the outward current and resolve 
better the conopressin- induced HVA current (Fig. 7B; n = 7). 
Again, apparently normal LVA and HVA currents were ob-
erved.

Subsequently, the response to conopressin was tested with a 
pipette solution containing 10 mM of the fast calcium chelator 
BAPTA, yielding a free calcium concentration of ~10 nM. 
Due to the fast calcium binding rate of BAPTA, transient 
variations in free calcium will be much less than with 10 mM 
EGTA. Figure 7C shows the responses to conopressin in a cell 
dialyzed with the BAPTA pipette solution. It is clear that 
conopressin is still capable of activating the HVA current, 
indicating that substantial changes in the intracellular calcium 
concentration are not necessary for the HVA current to occur. 
In the course of several tens of minutes, however, the ampi-
tude of the current becomes progressively smaller (Fig. 7B), 
possibly suggesting that a calcium-dependent process is re-
quired for the long-term maintenance of (parts of) the signal 
transduction system.

Taken together, these experiments indicate that changes in 
the concentration of free calcium are not required for the 
conopressin-induced current responses. Apparently, synthesis 
of DAG suffices to activate PKC at this concentration of free 
calcium (around 10 nM; see also DISCUSSION), and thus to 
induce phosphorylation of the HVA channels or their associ-
ated proteins.

**DISCUSSION**

**Activation of PKC underlies HVA current**

The present study demonstrates that activation of the HVA 
current by conopressin is mediated by PKC. First of all, the 
HVA current response is mimicked by application of the PKC-
activating phorbol ester TPA. During current activation by 
TPA, conopressin cannot activate additional HVA current, 
indicating that both treatments affect the same population of 
ion channels. These effects cannot be attributed to nonspecific 
effects of TPA on, e.g., the ion channel itself (see Hockberger 
et al. 1989), since the inactive phorbol ester 4α-PMA neither 
immimics nor occludes the HVA current response to conopres-

Second, activation of the HVA current is inhibited by stau-
roporine and H7, general protein kinase inhibitors with limited 
preference for PKC, and by the specific PKC-inhibitor inhibi-
itor GF-109203X. As would be expected, the effect of TPA is 
also reversed by application of staurosporine. On the basis of
these results, we conclude that activation of the HVA current is mediated by PKC. It might be argued that this conclusion needs to be considered as tentative, because our inferences on the specificity of the various pharmacological agents are based on biochemical data obtained in vertebrates and *Aplysia*. Notably, between vertebrates and invertebrates, this specificity may differ. For this reason we have used several agents, either blocking, stimulating, or mimicking, the effects of which all support the above stated conclusion.

The lack of effects of changing the calcium-buffering capacity in the intracellular medium suggests that calcium dynamics do not play a pivotal role in the signal transduction underlying the HVA current. Apparently, PKC can be activated by DAG alone at the low level of calcium (≥10 nM) imposed by 10 mM BAPTA. At a free calcium concentration of 100 nM, DAG is known to suffice for activation of mammalian PKC (Nishizuka 1984).

**Activation of LVA and HVA currents by conopressin involves different second messengers**

The inward current that is activated in *Lymnaea* anterior lobe neurons by direct injection of cAMP ($I_{cAMP}$) mostly resembles the conopressin-induced LVA current: it is activated at potentials below −40 mV and its amplitude shows little dependence on the membrane potential. In this respect, it bears resemblance to the cAMP-induced current in *Lymnaea* buccal neurons (McCrohan and Gillette 1988).

However, the present results suggest that cAMP is not involved in activation of the LVA current by conopressin. The amplitude of the LVA current was unaffected by treatment with the phosphodiesterase-inhibitor IBMX. In contrast, $I_{cAMP}$ was clearly augmented by IBMX. In itself, these observations may not be entirely conclusive, as it is possible that receptor activation would generate a saturating concentration of cAMP, and further increases due to reduced breakdown could not augment the response. However, the lack of cross-desensitization between the LVA current and the cAMP-induced current clearly confirms that they arise through different mechanisms.

The present results are compatible with the idea that cAMP may induce activation of the same population of LVA channels as conopressin, be it through a parallel and independent pathway.

Our experiments argue against an effect of cAMP on the HVA current. There is no cross desensitization between $I_{HVA}$ and $I_{cAMP}$, there is no effect of IBMX on $I_{HVA}$, and cAMP does not mimic the HVA response. Furthermore, because only activation of $I_{HVA}$ involves PKC, whereas $I_{LVA}$ is mimicked by cAMP, we assume that conopressin activates $I_{LVA}$ and $I_{HVA}$ through different signaling pathways. This conclusion is in line with previous observations, that the dose dependence and the rates of desensitization and wash out differ for the LVA and HVA current (Van Soest and Kits 1997).

**Pacemaker currents in other mollusks**

Our observation that activation of the HVA current is mediated by PKC and not by PKA is not in line with previous studies on the actions of vasopressin/oxytocin-related peptides in mollusks, that point to a role of cAMP (e.g., Ishin et al. 1975; Levitan et al. 1979; Treistman and Levitan 1976). For example, the oxytocin-induced pacemaker current in an identified *Achatina* neuron was concluded to be mediated by cAMP-dependent phosphorylation, as the PKA inhibitors H8 and PKI partially blocked the oxytocin response, while intracellular injection of cAMP mimicked the response and IBMX augmented it (Funase 1990). Interestingly, the oxytocin-induced current in the *Achatina* neuron appears to be a composite of two currents, similar to the LVA and HVA currents in *Lymnaea* anterior lobe neurons. Although the author does not make this distinction, his results seem in line with the idea that PKA mainly underlies the LVA part of the response.

Many other agonist-induced slow inward currents in molluscan neurons have also been attributed to cAMP-dependent phosphorylation processes. Still, the evidence regarding the transduction mechanisms underlying many of these responses is inconclusive or even contradictory. For instance, serotonin-induced currents have been attributed to direct effects of cAMP (Price and Goldberg 1993), phosphorylation by cAMP-dependent protein kinase (Funase et al. 1993), an unknown cAMP-related mechanism (Kirk et al. 1988), but also to a system completely independent of cAMP (Kudo et al. 1991).

The PKC-dependent HVA current in *Lymnaea* resembles to some extent the nonselective cation current in bag cells, which is also activated by PKC (Wilson et al. 1998), and displays a clear Ca dependence (Knox et al. 1996; Wilson et al. 1996). Interestingly, this channel seems to cluster with two different kinases (PKA and a tyrosine kinase) as well as a phosphatase, indicating multiple phosphorylation mechanisms to act on the channel (Wilson et al. 1998).

Apparently, different signal transduction mechanisms may underlie similar responses in different cells or species. This may imply entirely different routes, involving different protein kinases such as PKA and PKC, toward the same type of channel, thus activating identical currents. Alternatively, if multiple types of ion channel with semblant biophysical properties are present, different signal transduction routes may activate distinct ion channels giving rise to similar currents.

Present address of P. F. van Soest: Dept. of Developmental Neurobiology, Faculty of Biology, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands.

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