Orexin-A Depolarizes Nucleus Tractus Solitarius Neurons Through Effects on Nonselective Cationic and K⁺ Conductances

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Yang, Bo, and Alastair V. Ferguson. Orexin-A depolarizes nucleus tractus solitarius neurons through effects on nonselective cationic and K⁺ conductances, J Neurophysiol 89: 2167–2175, 2003. First published December 27, 2002; 10.1152/jn.01088.2002. The nucleus tractus solitarius (NTS) plays central roles in a number of autonomic functions including cardiovascular control. Orexin (ORX)-A is a 33-amino-acid peptide implicated in the central regulation of energy metabolism, sleep, and the cardiovascular system. Studies demonstrate the presence of ORX-immunoreactive axons and both ORX-A and ORX-B mRNA within NTS. In this study, whole cell patch-clamp recordings were obtained from NTS neurons in rat medullary slices. Current-clamp studies showed that bath application of various concentrations of ORX-A depolarized 90.7% (78 of 86) of neurons tested while the remaining cells were either unaffected or showed small hyperpolarizations in response to peptide administration. Depolarizing effects were maintained in the presence of 5 μM TTX, and were concentration dependent. Using voltage-clamp techniques, we also identified modulatory actions of ORX-A on specific ion channels. Our results demonstrate that not only does ORX-A inhibit a specific potassium conductance (the sustained K⁺ current) in NTS neurons, but it also activates a nonselective cationic conductance (NSCC). These data suggest that ORX-A effects on central cardiovascular control may result from direct actions on NTS neurons and also highlight the ability of this peptide to influence neuronal excitability as a consequence of concurrent modulation of multiple ion channels.

INTRODUCTION

Orexin (ORX)-A and -B (hypocretin-1 and 2) are two novel neuropeptides discovered in 1998 (de Lecea et al. 1998; Sakurai et al. 1998), proteolytically derived from the same precursor protein (Sakurai et al. 1998). ORX-producing neurons are almost exclusively distributed within and around the lateral hypothalamic area (LHA), the dorsomedial hypothalamic nucleus (DMH), and the perifornical nucleus (de Lecea et al. 1998), although ORX immunoreactivity is also reported in the testes (Sakurai et al. 1998). Central ORX administration stimulates feeding (Sakurai et al. 1998) and drinking (Kunii et al. 1999) and affects behavioral satiety (Rodgers et al. 2000). In contrast to the very specific distribution of ORX-producing neurons, ORX-IR axons show a widespread distribution throughout the adult rat brain (Date et al. 1999; Peyron et al. 1998). These results indicate that ORXergic neurons linking hypothalamic control regions to many other essential autonomic brain centers and play important roles in integrating the complex physiology underlying feeding behavior and other autonomic functions.

The biological actions of ORXs are transduced via two orexin receptors (OX₁R and OX₂R), which belong to the seven-transmembrane G-protein-coupled receptor family (Sakurai et al. 1998). OX₂R is considered to be nonselective because it binds ORX-A and -B with equal affinities. The OX₁R, however, shows a selective affinity (30–100 times greater) for ORX-A over ORX-B (Sakurai et al. 1998). The expression pattern of mRNA (Lu et al. 2000; Marcus et al. 2001; Sakurai et al. 1998; Trivedi et al. 1998) and protein (Cluderay et al. 2002; Hervieu et al. 2001) for OX₁R and OX₂R, although extensive, is not homogenous in different subregions of the CNS.

The nucleus tractus solitarius (NTS), located in the dorsomedial medulla oblongata is widely accepted as a pivotal brain region involved in the integration of cardiovascular, respiratory, gustatory, hepatic, and renal control mechanisms (Lawrence and Jarrott 1996). NTS receives afferent input from and sends efferent output to many CNS areas including essential autonomic control centers in the hypothalamus, midbrain and spinal cord (Andresen and Kunze 1994).

ORX-IR axons as well as ORX mRNA and protein have been reported within NTS. In addition, ICV ORX-A has been shown to induce fos activation of NTS neurons (Date et al. 1999; Qu et al. 1996), and we have recently reported that ORX-A acts in NTS to cause rapid reversible site-specific increases in blood pressure and heart rate (Smith et al. 2002). Collectively these observations support the hypothesis that NTS represents an important CNS site where ORX-A acts to influence central cardiovascular regulation as a consequence of direct modulation of the excitability of NTS neurons. The present electrophysiological study was designed to test the hypothesis that ORX-A exerts direct effects on the excitability of NTS neurons using a rat medullary slice preparation combined with whole cell patch-clamp recording techniques. Having identified such effects, our studies were extended to describe the modulatory roles of ORX-A on specific ion channels of NTS neurons that underlie such effects on single cell excitability.

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Medullary slice preparation

Male Sprague-Dawley rats (125–225 g, Charles River) were decapitated, the brain stem quickly removed from the skull and immersed in cold (0–2°C) artificial cerebrospinal fluid (ACSF). Medullary slices (400 μm) including NTS were cut using a vibratome and incubated in oxygenated ACSF (95% O2-5% CO2) for ≥90 min at room temperature. Prior to recording, slices were transferred into an interface-type recording chamber and continuously perfused with oxygenated ACSF. The flow was adjusted to ~1.5 ml/min and was maintained constant throughout the entire recording period. The recording chamber was maintained at room temperature (21–22°C) throughout all experiments. All procedures conformed to the standards outlined by the Canadian Council on Animal Care and protocols were approved by the Queen’s University Animal Care Committee.

Electrophysiological methods

Whole cell patch recordings were obtained using the whole cell configuration of the “blind” patch-clamp technique (see Li and Ferguson 1996) to record from NTS neurons, most of which are located in the commissural region of the nucleus. Electrodes of 4–7 MΩ were pulled from TW150F-6 glass (World Precision Instruments, Sarasota, FL) on a horizontal Flaming/Brown micropipette puller (Model P-97, Sutter Instrument, Novato, CA) and were filled with the appropriate filling solution (see Experimental solutions). After establishment of >1–2 GΩ seal, a brief suction pulse was applied to rupture the membrane and achieve whole cell configuration. Signals were amplified and processed using an AxoClamp 2B (Axon Instruments, Union City, CA) amplifier. A Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. After recording from each NTS neuron, the pipette was withdrawn from the cell membrane, the remaining junction potential was measured (3–8 mV), and the appropriate correction was applied to all data presented. Drugs were applied by switching perfusion from ACSF to a solution containing the desired drug. All signals were filtered at 3 kHz, digitized using the CED 1401 plus interface (Cambridge Electronic Design, Cambridge, UK) at 5 kHz, and stored on computer for off-line analysis. Data were collected using the Signal (episode based capture) or Spike2 (continuous recording) packages (Cambridge Electronic Design).

Cells were defined as neurons by the presence of ≥70 mV action potentials (current-clamp recordings) or by the presence of large rapid voltage-activated inward currents that were blocked by TTX (voltage-clamp recordings).

Experimental solutions

The standard internal pipette solution contained (in mM): 140 K-glucanate, 0.1 CaCl₂, 2 MgCl₂, 1.1 ethylene glycol-bis-(aminoethyl ether)-N,N',N",N"-tetraacetic acid (EGTA), 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 2 Na₂ATP and was adjusted to pH 7.25 with KOH. In experiments examining the role of Ca²⁺ in activating the nonselective cationic conductance (NSCC), the concentration of EGTA in the pipette solution was increased from 1.1 to 10 mM. The control bath solution consisted of ACSF (in mM) 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2.0 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. Osmolarity was maintained between 285 and 300 mosM and pH between 7.3 and 7.4.

Peptides and drugs

ORX-A (Phoenix Pharmaceuticals, Belmont, CA) was prepared fresh on the day of experiment by diluting 50 μl aliquots of 10⁻² M stock solution stored at ~70°C to concentrations ranging from 10⁻¹¹ to 10⁻⁷ M in ACSF. In experiments where synaptic transmission was blocked, tetrodotoxin (TTX; 5 μM) was added to external solutions, and blockade of Na⁺ channels was confirmed when either depolarizing current pulses to 0 mV failed to elicit fast spikes (current-clamp recordings) and/or the large rapid voltage-activated inward currents were abolished (voltage-clamp recordings). 4-aminopyridine (4-AP; 5 mM) was added to the bath solution to block the transient K⁺ current. Tetraethylammonium (TEA; 10 mM) was added to the external solution to block the sustained K⁺ current. All chemicals, unless otherwise stated, were obtained from Sigma Chemical (St. Louis, MO). All drugs were dissolved in ACSF and applied directly through the bath perfusion system.

Definition of response

A series of hyperpolarizing current pulses were applied to determine the identity of each neuron as a delayed excitation (DE), postinhibitory rebound (PIR), or neither DE nor PIR (NON) cell based on its electrophysiological fingerprint (Vincent and Tell 1997). Neurons were required to maintain a stable baseline for ≥2–3 min prior to application of test agents. The firing frequency of cells was measured in 20-s bins for 1 min prior to and ≤2 min after drug application. A response to ORX-A was arbitrarily defined as a sustained change in membrane potential of >3 mV.

Statistical analysis

For statistical analysis of effects of ORX-A on NTS neurons, means were calculated from cells that were determined to have been affected using the preceding criteria. Changes in input resistance, duration of action potentials, peak and steady-state K⁺ conductances and amplitude, and duration of afterhyperpolarizations in response to ORX-A were compared using the Student’s t-test. A minimum probability value of P < 0.05 was selected to determine significance. All values are plotted as means ± SE. The concentration-response curve was constructed from a sigmoidal function of nonlinear regression (Prism, GraphPad Software, San Diego, CA).

RESULTS

Whole cell recordings were obtained from a total of 180 NTS neurons. All of these cells demonstrated action potentials with amplitude of >70 mV (arbitrary minimum cut off for inclusion), they had a mean resting membrane potential (RMP) of −53.98 ± 0.25 mV and mean input resistance of 3.89 ± 0.13 GΩ.

ORX-A depolarizes NTS neurons

Current-clamp recordings from a total of 86 NTS cells showed that 94% (81 of 86) of this population responded to bath perfusion of ORX-A (see the criteria established in METHODS), whereas the remainder of neurons tested did not respond in a sustained manner and were therefore classified as non-responders (NON). Depolarization was the predominant effect caused by ORX-A exposure (78 of 86 cells, 90.7%). Similar proportions of DE, PIR, and NON cells were found to be responsive to ORX-A, and therefore these cell types were grouped together for all subsequent analysis.

Depolarizations usually occurred within 2 min of ORX-A reaching the slice and were usually accompanied by a rapid increase in firing frequency of action potentials. Effects of ORX-A lasted for 6–12 min and after washout of ORX-A membrane potential and action potential frequency returned to control levels as shown in Fig. 1A. In 49 cells exposed to 10⁻⁸ M ORX-A, the mean depolarization was 7.8 ± 0.2 mV. ORX-
A-induced depolarizations were accompanied by a significant decrease in IR as measured by the voltage responses to hyperpolarizing current pulses (control: 3.95 ± 0.36 GΩ vs. 10^{-8} M ORX-A: 2.71 ± 0.48 GΩ, P < 0.05, n = 10; Fig. 1, B and C), effects that were still observed when membrane potential was returned to baseline with hyperpolarizing current prior to assessment of input resistance.

In accordance with previous reports suggesting effects of ORX on voltage-gated potassium currents (Ivanov and Aston-Jones 2000), ORX-A also resulted in a significant broadening of action potentials [repolarization is slowed and action potential durations (APD) are prolonged; APD_{50} control: 1.3 ± 0.1 ms, P < 0.0001, n = 10; APD_{90} control: 2.0 ± 0.1 ms, P < 0.0001, n = 10], as illustrated in Fig. 2A and summarized in B. In contrast, we did not observe significant effect of ORX-A on afterhyperpolarizations (AHP) in those cells that expressed an

**FIG. 1.** A: whole cell current-clamp recording from a nucleus tractus solitarius (NTS) neuron demonstrates that bath administration of orexin (ORX)-A (represented in this and future figures by the horizontal bar above each trace) resulted in rapid sustained depolarization accompanied in most cases by a rapid increase in firing frequency of action potentials. After washout of ORX-A, the membrane potential and action potential frequency returned to control levels. Scale bars represent 60 s (horizontal) and 10 mV (vertical). - - -, baseline membrane potential. Bottom: expanded time scales from the same recording (before, during, and after bath application of ORX-A). The traces illustrate action potentials (truncated) and postsynaptic potentials (of ≤10 mV) with baseline noise ≤1 mV. Scale bars represent 1 s (horizontal) and 10 mV (vertical). B: ORX-A also caused consistent decreases in input resistance as shown in the current-voltage relationships obtained from this NTS neuron during application of control artificial cerebrospinal fluid (ACSF; ■) and 10^{-8} M ORX-A (○). Successive hyperpolarizing pulses (−1 to −6 pA) were delivered and the peak changes in membrane potential were measured. C: this bar chart shows summary data illustrating statistically significant effects of ORX-A on IR in NTS cells; control: 3.95 ± 0.36 GΩ vs. 10^{-8} M ORX-A: 2.71 ± 0.48 GΩ, P < 0.05, n = 10.

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**FIG. 2.** A: bath administration of 10^{-8} M ORX-A resulted in a significant broadening of action potentials while being without effect on afterhyperpolarization as illustrated in these waveforms recorded from an NTS neuron at the same membrane potential level before and during peptide administration. B: group data demonstrate that 10^{-8} M ORX-A results in a statistically significant increase in action potential duration measured at both the 50% (APD_{50}) and 90% (APD_{90}) repolarization level (P < 0.0001, n = 10). C: these current-clamp recordings illustrate the responses of a single NTS neuron to bath application of 10^{-8} M ORX-A in normal ACSF (top), and in ACSF containing TTX (bottom). As illustrated in this example, all cells tested in this way showed maintained depolarizations in response to ORX-A in TTX. Scale bars represent 60s (horizontal) and 10 mV (vertical); - - -, baseline membrane potential.
AHP (as seen in Fig. 2A; AHP amplitudes: control: $-5.98 \pm 0.37$ vs. $10^{-8}$ M ORX-A: $-6.02 \pm 0.33$ mV, $n = 10; P = 0.33$).

To determine if the observed actions of ORX-A were due to direct effects on NTS neurons, 14 neurons that responded to $10^{-8}$ M ORX-A, were tested with ORX-A during the blockade of action potentials by bath administration of TTX (5 μM; Fig. 2C). After treatment with TTX, bath administration of ORX-A elicited a similar depolarizing response in all 14 cells tested ($7.4 \pm 0.6$ vs. $7.8 \pm 0.2$ mV without TTX, $n = 49, P = 0.26$).

Similar reversible depolarizing responses, normally accompanied by increases in spike frequency were also recorded from NTS neurons in response to $10^{-8}$ and $10^{-10}$ ORX-A as illustrated in Fig. 3A. These effects of ORX-A were repeatable as a second bath application of the peptide resulted in similar changes in membrane potential. Analysis of group mean depolarization recorded from NTS neurons in response to ORX-A concentrations ranging from $10^{-11}$ to $10^{-7}$ M demonstrated these effects to be concentration dependent as illustrated in Fig. 3B ($EC_{50} = 3.7 \times 10^{-10}$ M). Although NTS neurons did not depolarize significantly ($<3$ mV, see the criteria established in METHODS) in response to $10^{-11}$ M ORX-A, all neurons tested with this concentration were included as a group to complete the concentration-response curve.

**ORX-A decreases net whole cell K$^+$ currents in NTS neurons**

The modulation of voltage-gated K$^+$ conductances has been shown to be important in the regulation of neuronal excitability. ORX-B has been reported to decrease K$^+$ conductances (Ivanov and Aston-Jones 2000) and reduce AHP (Horvath et al. 1999) in locus coeruleus neurons. In addition, ORX-A and -B have been shown to depolarize rat dorsal motor nucleus of the vagus neurons in vitro possibly by affecting a nonselective cationic conductance and a K$^+$ conductance (Hwang et al. 2001). Our own data showing that ORX-A broadens action potentials suggest modulatory effects of this peptide on K$^+$ channels in NTS neurons. We therefore used voltage-clamp techniques to examine the effects of ORX-A on peak whole cell K$^+$ currents evoked in response to 20-mV depolarizing voltage steps (0.5 s) applied from holding potentials of $-100$ to $+40$ mV (with 5 μM TTX in ACSF) before and after bath application of ORX-A (Li and Ferguson 1996). A typical response of an NTS neuron to ORX-A ($10^{-8}$ M) illustrated in Fig. 4A shows a partially reversible decrease of the net whole cell K$^+$ currents induced by ORX-A. The summary data presented in Fig. 4B support the conclusion that NTS neurons ($n = 16$) exhibit a decrease in whole cell K$^+$ currents during exposure to ORX-A ($10^{-8}$ M) followed by a return to control levels 5–12 min after washout of ORX-A.

**ORX-A decreases the sustained K$^+$ current in NTS neurons**

Vincent and Tell (1997) have shown that both transient and sustained outward potassium currents contribute to the whole cell K$^+$ currents in the NTS neurons. To determine the K$^+$ conductances affected by ORX-A, we used voltage-clamp techniques to examine the effects of ORX-A on pharmacologically isolated K$^+$ conductances (Vincent and Tell 1997). The sustained K$^+$ current was first isolated from NTS neurons recorded in 5 μM TTX to block Na$^+$ currents and 5 mM 4-AP to block the transient K$^+$ current and was evoked by 20-mV voltage steps (0.5 s) from $-100$ mV holding potential to $+40$ mV as shown in Fig. 5A, inset. Exposure to ORX-A ($10^{-8}$ M) resulted in statistically significant decreases in the sustained K$^+$ current (measured at the end of the pulse; Fig. 5A) in response to the larger depolarizing pulses as indicated by the summary data presented in Fig. 5A. After washout of ORX-A and replacement of the bath solution with control ACSF, the sustained K$^+$ current returned toward control levels.

To determine if ORX-A had similar inhibitory effects on the
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transient K⁺ current, the peak values of this current were measured in the absence of the sustained K⁺ current using ACSF containing 5 μM TTX and 10 mM TEA to block the sustained K⁺ current. The transient K⁺ current was evoked using similar pulse protocols to those described in the preceding text (Fig. 5B, inset) and as illustrated in the summary data presented in Fig. 5B, bath application of 10⁻⁸ M ORX-A had no significant effect on this current (n = 10).

ORX-A activates NSCC of NTS neurons

While the effects of ORX-A on K⁺ conductances described in the preceding text likely explain the influence of this peptide on spike broadening and possibly spike frequency, they do not provide a plausible explanation for either the effects of ORX-A on input resistance or for the depolarizing effects of this peptide in NTS neurons. In view of the considerable literature demonstrating peptidergic effects on neuronal excitability occurring as a consequence of the modulation of nonselective cationic conductances (NSCC) (Hiruma and Bourque 1995; Kirkpatrick and Bourque 1995) as well as recent studies identifying NSCC in area postrema (Yang and Ferguson 2002) and dorsal motor nucleus of vagus (Hwang et al. 2001) as potential mediators of ORX actions, we next used slow voltage ramps [−100 to 0 mV (10 s)] following a prepulse to −100 mV (0.5 s) to determine if ORX-A influenced NTS neurons as a consequence of activation of such conductances. These ramp experiments were not carried out in the presence of TEA (10 mM) because ORX-A’s inhibitory effects on Iₚ were only observed in voltage steps more positive than 0 mV (see Fig. 5A). The data presented in Fig. 6A show average currents recorded from a NTS neuron in response to such ramps (each trace is the mean of 5 ramps) recorded before, during, and after bath administration of ORX-A (10⁻⁸ M). Figure 6A, inset, shows the difference current (i.e., ORX-A-induced current) obtained by subtracting control ramps from those obtained during ORX-A. Application of ORX-A (10⁻⁸ M) caused a clear change in this ramp evoked current and ~5–10 min after replacement of ORX-A with ACSF, the current recovered toward control levels. Similar effects of ORX-A (10⁻⁸ M) were observed in 14/16 (87.5%) cells tested, a proportion which closely matches the proportion (90.7%) of NTS neurons depolarized by ORX-A in our current-clamp experiments. The mean ORX-A-evoked current for this group of responsive neurons is shown in Fig. 6B and was found to be linear throughout the voltage range tested (r² = 0.97), indicating a lack of voltage dependence. This conductance is voltage independent across the voltage scale of the slow ramp, which indicates that it is a NSCC (Bourque 1989; Hiruma and Bourque 1995; Kirkpatrick and Bourque 1995). The mean reversal potential of the ORX-A (10⁻⁸ M)-sensitive current was −43.8 ± 3.5 mV (n = 14), and the mean conductance of this NSCC is 0.34 ± 0.02 nS. We also examined the role of intracellular Ca²⁺ in activating this NSCC in experiments where we decreased the theoretical concentration of free intracellular Ca²⁺ to 10% of normal values by increasing EGTA in the pipette solution from 1.1 (standard) to 10 mM. Of five NTS

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neurons recorded with this high-EGTA pipette solution, four showed activation of the NSCC in response to ORX-A (mean reversal potential = −40.9 ± 5.1 mV and the mean conductance = 0.34 ± 0.03 nS), which was quite similar to that observed in cells recorded with standard internal solution. We usually held NTS neurons between 50 and 51 mV prior to ORX-A (10^−8 M) administration in our current-clamp studies. At these potentials, ORX-A would be expected to activate the NSCC as a 2.1- to 2.5-pA inward current (see Fig. 6B), which we calculate to evoke an 8.2- to 9.7-mV depolarization (average input resistance of NTS neurons is 3.89 GΩ), which is close to the average depolarization (7.8 ± 0.2 mV) caused by 10^−8 M ORX-A application in current-clamp recordings. To further test this idea, additional experiments were performed while the baseline membrane potentials of NTS neurons were held at −44 mV (close to the reversal potential of this NSCC) and −55 to −60 mV, respectively, before bath ORX-A application. Zero of five neurons held at −44 mV was depolarized by ORX-A (10^−8 M), and the depolarization of cells held at −55 to −60 mV was potentiated (12.3 ± 1.2 mV, n = 6).

DISCUSSION

Several lines of evidence have shown that ORX acts in the CNS to modulate feeding, sleep-wakefulness, neuroendocrine homeostasis, and autonomic regulation (Samson and Resch 2000; Samson and Taylor 2001; Sweet et al. 1999). The distribution of ORX-IR axons and both ORX receptor mRNA and protein within NTS, combined with ORX-A’s established involvement in the central modulation of feeding (Sakurai et al. 1998), central autonomic control, and cardiovascular function (Shirasaka et al. 1999), suggest that the NTS represents a significant site for potential neuroregulatory actions of this peptide. The data from this study are the first to demonstrate that ORX-A directly influences the excitability of NTS neurons and, in addition, identify modulation of the sustained K^+ current and a NSCC by this peptide as the likely membrane events underlying these effects.

Previous studies reporting electrophysiological properties and subtypes of NTS neurons in slice preparations have suggested lower input resistances for these cells (Vincent and Tell 1997) than we have recorded in the current study (3.89 ± 0.13 GΩ).
observed that the AHPs of those action potentials immediately by ORX-A. In two cells clearly showing this feature, we actions. Previous reports suggest that the activation of a Ca2+

![Fig. 2](image-url)

FIG. 6. A: mean whole cell currents (each trace is the mean of 5 ramps) were evoked from slow depolarizing (10 mV/s) voltage ramps before, during, and after exposure to ORX-A (10^{-8} M). Inset: the difference current was obtained by subtracting the control current from the current recorded during ORX-A application. This represents the ORX-A-evoked current. B: this graph illustrates the mean ± SE ORX-A (10^{-8} M)-evoked current for responsive neurons (n = 14). The mean reversal potential of the ORX-A (10^{-8} M)-sensitive current is −43.75 ± 3.5 mV (n = 14).

GΩ, n = 180). These differences are most likely the result of differences in the techniques for slice preparation (we recorded in room temperature (21–22°C) vs. 31–32°C) or the exceptionally high-resistance seals obtained in the present studies (4 GΩ).

Our current-clamp recordings clearly illustrate the ability of ORX-A to rapidly and reversibly influence the membrane potential of the majority (90.7%) of NTS neurons. The fact that these effects were observed in the presence of TTX suggests that they are the result of direct actions on each recorded NTS neuron. The present lack of specific ORX receptor antagonists precluded identification of the specific ORX receptor mediating these effects. However, the clear reversibility, and concentration dependence of these effects argue strongly that they are receptor mediated. Interestingly, some NTS neurons showed a sudden return from a depolarized state to baseline membrane potential after washout of ORX-A with ACSF (as illustrated in Fig. 2C and Fig. 3A, bottom). While this observation initially raised concerns about stability of recording conditions, the relative frequency (10^{-8} M ORX-A, 10/49) of this feature when combined with its repeatability in single neurons (see Fig. 2C) suggest it to be a direct consequence of ORX-A actions. Previous reports suggest that the activation of a Ca^{2+}-activated K^+ current is sufficient to account for the sudden ending of each burst cycle induced by NMDA in magnocellular neurosecretory cells of the rat supraoptic nucleus (Bourque et al. 1985; Hu and Bourque 1992). The membrane potential sudden return feature revealed in this study could very well be due to the activation of a Ca^{2+}-activated K^+ current induced by ORX-A. In two cells clearly showing this feature, we observed that the AHPs of those action potentials immediately before and after the sudden return had significantly longer durations (516.6 ± 68.5 vs. 320.0 ± 11.1 ms and 288.6 ± 31.4 vs. 165.1 ± 10.6 ms, respectively). This observation is consistent with the hypothesis that this sudden return feature could be due to the activation of a Ca^{2+}-activated K^+ current induced by ORX-A. Further experiments are needed to explore this possibility.

Potassium conductances are known to be important for the shaping of neuronal firing patterns (Hille 1992). ORX-B has been shown to both decrease K^+ conductances (Ivanov and Aston-Jones 2000) and reduce AHPs (Horvath et al. 1999) in locus coeruleus neurons, suggesting the possibility that ORX may influence the excitability of NTS neurons as a direct result of inhibition of voltage-gated K^+ channels to increase excitability and thus facilitate action potential firing. Our current-clamp studies show that ORX-A slows the repolarization that follows the action potentials significantly prolonging action potential duration of NTS neurons. We therefore examined the effects of ORX-A on voltage-gated K^+ currents of NTS neurons using voltage-clamp techniques. Our initial data demonstrated that ORX-A decreased whole cell voltage-dependent K^+ currents, and additional pharmacological dissection of these mixed whole cell currents allowed us to identify specific inhibitory effects of ORX-A on the sustained K^+ current while the transient K^+ current was not affected.

While the effects of ORX-A on K^+ conductances likely explain the influence of this peptide on spike broadening and possibly spike frequency, they do not explain the depolarizing effects of this peptide on NTS neurons. NSCCs are voltage-independent membrane channels which allow passage of cations (Na^+, K^+, or Ca^{2+}) in varying proportions

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(Kramer and Zucker 1985). These channels have been shown to participate in controlling neuronal excitability in many systems including generation of the depolarizing phase of bursting pace-maker activity in *Aplysia* burst-firing neurons (Kramer and Zucker 1985) and in the intrinsic activation of rat supraoptic neurons by hyperosmotic stimuli (Bourque 1989), neurotensin (Kirkpatrick and Bourque 1995) and P2 purinoceptor agonists (Hiruma and Bourque 1995). In addition, previous work from our laboratory has demonstrated that ORX-A depolarizes dissociated rat area postrema neurons through activation of NSCC (Yang and Ferguson 2002). The results from the current study illustrate direct reversible effects of ORX-A on a NSCC in a proportion of NTS neurons similar to that depolarized by the peptide. Such effects of ORX on this NSCC likely explain the depolarization of NTS neurons in response to the peptide, especially in view of the close correlation between the predicted (obtained by calculation using biophysical features of cells and conductance) and recorded potential changes. Our data suggest that this NSCC is not activated by cytoplasmic Ca$^{2+}$, a conclusion that is consistent with the current literature (Liu et al. 2002). These negative data suggest the involvement of alternative second messenger such as protein kinase A and C in mediating ORX-A effects as already demonstrated in other neuronal subpopulations (Korotkova et al. 2002; Samson and Taylor 2001; Uramura et al. 2001). This study and our own recent work demonstrating similar ORX-A effects on a NSCC in rat area postrema neurons (Yang and Ferguson 2002) and parvo cellular neurons in rat hypothalamic paraventricular nucleus (PVN) (Follwell and Ferguson 2003) as well as reports of ORX actions on an NSCC in serotonin neurons in the dorsal raphe nucleus (Liu et al. 2002) suggest that ORX receptor-mediated modulation of this conductance may represent a common mechanism through which ORX exerts control over neuronal excitability. The signal transduction mechanisms underlying this modulation of the NSCC have not been examined in the present study although previous work has shown that the OX1R couples to a Gq protein (Sakurai et al. 1998) the activation of which results in increases in phospholipase C (Exton 1994).

While the electrophysiological consequences of ORX-A actions on NTS neurons in increasing their excitability are clear, the question still arises as to the physiological implications of such actions of ORX-A on such a large proportion of NTS neurons. Studies have demonstrated important roles for these neurons in cardiovascular, respiratory, neuroendocrine, and gastrointestinal control. The homogeneity of the observed responses of NTS neurons to ORX-A suggests it unlikely that the physiological consequences of this peptide action in NTS would be limited to one or another of these specific autonomic outputs. A more plausible explanation is that these broad excitatory actions of ORX-A in NTS further contribute to the well-recognized general activational effects of this peptide (Bernard et al. 2002; Mieda and Yanagisawa 2002; Sato-Suzuki et al. 2002) as a result of the concurrent activation of diverse autonomic outputs.

In conclusion, this study provides the first evidence that ORX-A directly activates NTS neurons by modulating a NSCC and inhibiting the sustained K$^{+}$ current. These findings suggest that orexin may have a functional role in the central autonomic control at the NTS.

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**REFERENCES**


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