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NITRIC OXIDE MODULATES THE FIRING RATE OF THE RAT SUPRAOPTIC MAGNOCELLULAR NEURONS

R. R. VENTURA, J. F. AGUIAR, J. ANTUNES-RODRIGUES AND W. A. VARANDA*

Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, São Paulo, Brazil

Abstract—*In vitro*, nitric oxide (NO) inhibits the firing rate of magnocellular neurosecretory cells (MNCs) of hypothalamic supraoptic and paraventricular nuclei and this effect has been attributed to GABAergic activation. However, little is known about the direct effects of NO in MNCs. We used the patch-clamp technique to verify the effect of L-arginine, a precursor for NO synthesis, and N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of NOS, on spontaneous electrical activity of MNCs after glutamatergic and GABAergic blockade in Wistar rat brain slices. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) and DL-2-amino-5-phosphonovaleric acid (DL-AP5) (30 μM) were used to block postsynaptic glutamatergic currents, and picrotoxin (30 μM) and saclofen (30 μM) to block ionotropic and metabotropic postsynaptic GABAergic currents. Under these conditions, 500 μM L-arginine decreased the firing rate from 3.7±0.6 Hz to 1.3±0.3 Hz. Conversely, 100 μM L-NAME increased the firing rate from 3.0±0.3 Hz to 5.8±0.4 Hz. All points histogram analysis showed changes in resting potential from −58.1±0.8 mV to −62.2±1.1 mV in the presence of L-arginine and from −59.8±0.7 mV to −56.9±0.8 mV by L-NAME. Despite the nitrergic modulator effect on firing rate, some MNCs had no significant changes in their resting potential. In those neurons, hyperpolarizing after-potential (HAP) amplitude increased from 12.4±1.2 mV to 16.8±0.7 mV by L-arginine, but without significant changes by L-NAME treatment. To our knowledge, this is the first demonstration that NO can inhibit MNCs independent of GABAergic inputs. Further, our results point to HAP as a potential site for nitrergic modulation.

Key words: patch clamp, supraoptic neurons, firing rate, hyperpolarizing after-potential, nitric oxide.

The somata of magnocellular neurosecretory cells (MNCs) of the supraoptic (SON) and paraventricular (PVN) nuclei represent the main neuroendocrine pathway for vasopressin (AVP) and oxytocin (OT) synthesis (Brownstein et al., 1980; Swanson and Sawchenko, 1983). In the systemic circulation, AVP helps to maintain the set point of extracellular tonicity and OT induces sodium excretion and triggers the contraction of smooth muscle fibers of uterus and mammary glands. Because of the relevance of this system for the maintenance of body fluid homeostasis, as well as for reproductive functions, considerable efforts have been made in order to elucidate the mechanisms comprising the synthesis and release of these peptides.

As a genuine component of the neuroendocrine axis, hypothalamic MNCs are under control of several modulators, of which nitric oxide (NO) has gained considerable attention in recent years (Kadowaki et al., 1994; Srisawat et al., 2000; Ventura et al., 2002, 2005; Stern et al., 2003). NO is a free reactive gas, synthesized from L-arginine by the action of nitric oxide synthase (NOS) that acts as a neuromodulator (Moncada et al., 1991). The idea that NO could be involved in the control of AVP and OT release was reinforced by the identification of NOS in the hypothalamo-neurohypophysial system, as well as in other brain areas (Bredt et al., 1990). *In vivo* experiments have shown that NO has an inhibitory effect on OT release (Summy-Long et al., 1993; Kadekaro et al., 1998; Ventura et al., 2005), but for AVP the literature is controversial (Yasin et al., 1993; Ota et al., 1993; Liu et al., 1998; Yamaguchi and Hama, 2003; Ventura et al., 2005). Interestingly, electrophysiological studies have demonstrated that NO decreases the firing rate of the MNCs for both phenotypes, suggesting that NO is an inhibitor of AVP and OT release (Liu et al., 1997; Stern, 2004). In addition, it was shown that NO inhibitory effect on MNCs depends on the activation of GABAergic inputs (Stern and Ludwig, 2001). However, because of the presence of NOS in the MNCs and its short half-life, it would be reasonable to expect an additional direct effect of NO on the MNCs as well as an indirect effect.

In rat MNCs, each action potential is followed by a prominent hyperpolarizing after-potential (HAP), which has been demonstrated to be involved in the spike-frequency adaptation (Andrew and Dudek, 1984; Bourque et al., 1985). The HAP is drastically reduced in the presence of iberiotoxin, a potent and selective large conductance Ca^{2+}-activated K^+ channel (BK channel) blocker, indicating its dependency on transient increases in intracellular Ca^{2+} concentration (Greffrath et al., 2004). A recent study demonstrated that NO can directly affect the expression of the BK channels in the cell membrane of MNCs, suggesting a potential mechanism for nitrergic modulation of HAP in these cells (Kadekaro et al., 2006).

In this work, we used the patch clamp technique to evaluate the effects of NO on the spontaneous discharge

*Corresponding author. +55-16-36023024; fax: +55-16-36330017.
E-mail address: wvaranda@fmrp.usp.br (W. A. Varanda).
Abbreviations: AVP, vasopressin; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DL-AP5, DL-2-amino-5-phosphonovaleric acid; D-NAME, N_{ω}-Nitro-D-arginine methyl ester hydrochloride; gACSF, high glucose artificial cerebrospinal fluid; HAP, hyperpolarizing after-potential; L-NAME, N_{ω}-nitro-L-arginine methyl ester hydrochloride; mEPSC, spontaneous miniature excitatory post-synaptic current; mIPSC, spontaneous miniature inhibitory post-synaptic current; MNC, magnocellular neurosecretory cell; NO, nitric oxide; NOS, nitric oxide synthase; OT, oxytocin; SON, supraoptic nucleus.

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of MNCs after synaptic blockade. Our results confirm the *in vitro* observed inhibitory effect of NO on spike firing frequency of the MNCs and demonstrate that this effect does not depend on GABAergic inputs. Additionally, we suggest a nitrergic modulation of the HAP as a mechanism for controlling spike frequency in these neurons.

EXPERIMENTAL PROCEDURES

All experimental protocols used in this work were in accordance with the Institutional Ethical Committee for Animal Experimentation of the School of Medicine of Ribeirão Preto-University of São Paulo (032/2005) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Efforts were made to minimize the number of animals used and their suffering.

Preparation of brain slices

Wistar rats (30–35 days old) of both sexes were anesthetized with sodium thiopental (20 mg/kg, i.p.) and killed by decapitation. After craniotomy the brain was rapidly removed and submerged in ice-cold (1–3 °C) high glucose artificial cerebrospinal fluid (gACSF), pH 7.35–7.4, equilibrated with carbogen (95% O_2–5% CO_2). The gACSF contained (in mM): 120 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 25 $NaHCO_3$, 25 glucose, 1.25 NaH_2PO_4, and the osmolality was 308–313 mosm/kg H_2O (Fiske Mark 3 Osmometer, Norwood, MA, USA). A block of tissue containing the hypothalamic region was prepared and glued with cyanoacrylate glue to an agar block (4% agar in ACSF). Three coronal slices (300 μm) containing the supraoptic nuclei were obtained using a vibratome (model MA752, Campden Instruments, Loughborough, UK). After cutting, the slices were incubated for at least 60 min at 35 °C in ACSF constantly gassed with carbogen until the beginning of the recordings. A single slice was then transferred to the recording chamber placed on the stage of a microscope (E600, Nikon, Tokyo, Japan) and held in place with a nylon net mounted on a platinum wire. The slice was continuously superfused with ACSF, saturated with carbogen, at a rate of 2–3 ml/min. The composition of the recording ACSF was (in mM): 120 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, 1.25 NaH_2PO_4, pH 7.35–7.4 and osmolality 298–302 mosm/kg H_2O. Experiments were performed at room temperature (23–25 °C) and all drugs were applied at known concentrations by changing the perfusion line.

Electrophysiology

For intracellular recordings patch pipettes were pulled from borosilicate glass tubing (Sutter Instrument, Novato, CA, USA) on a P-97 puller (Sutter Instrument) and were fire polished on a microforge (MF-83, Narishige, Tokyo, Japan). For current- and voltage-clamp experiments the pipette solution was (in mM): 135 potassium gluconate, 10 KCl, 0.3 $CaCl_2$, 1.0 $MgCl_2$, 10 Hepes, 1.0 EGTA, 2.0 ATP Na-ATP, 0.25 Na-GTP, pH adjusted to 7.35 with KOH and osmolality of 285–295 mosm/kg H_2O. The pipettes had resistance between 4 and 8 MΩ. The junction potential between pipette and bath solution was calculated using the Clampex program (pClamp 8 software, Molecular Devices, Union City, CA, USA) and the value (14.8 mV) was subtracted from base line for data analysis. MNCs were visualized using a 60× DIC objective and the image was captured using a CCD digital camera (SensiCam qe, The Cooke Corporation, Romulus, MI, USA). Seal resistances up to 1 GΩ were accepted for recordings and access resistances were corrected between 40 and 60%. Whole cell voltages were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices), low-pass filtered at 5 kHz, digitized at 10 kHz by a computer driven A/D converter Digidata 1200B (Molecular Devices), and stored on hard disk. Data acquisition and storage were controlled with pClamp8 software. Data were analyzed off-line using the MiniAnalysis (Synaptosoft, Decatur, GA, USA), Clampfit (Molecular Devices) or Origin 6.0 (OriginLab Corporation, Northampton, MA, USA).

Drugs

L-Arginine monohydrochloride, D-arginine monohydrochloride, N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), N_ω-Nitro-D-arginine methyl ester hydrochloride (D-NAME) were purchased from Sigma Chemical (St. Louis, MO, USA). DL-2-Amino-5-phosphonovaleric acid (DL-AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), saclofen hydrochloride and picrotoxin were from Tocris Cookson (Ellisville, MO, USA). TTX was from Alomone Laboratories (Jerusalem, Israel). All other salts and reagents were from Sigma Chemical Co. Solutions were made with MilliQ Water (Simplicity, Millipore, Barueri, SP, Brazil) and filtered through a 0.22 μm diameter filter pore (Millipore).

Statistical analysis

Data reported are expressed as means±S.E.M. and a mean value of $P \leq 0.05$ was accepted as significant. A paired Student's *t*-test was used to compare the data before and after treatments.

RESULTS

The results described below were obtained in a total of 38 supraoptic MNCs recorded from rat brain slices. All the recordings obtained in our study were characterized by continuous or, in some cases, irregular firing pattern which are suggestive for OT neurons. The neurons analyzed had resting membrane potential ranging from −63 to −56 mV and amplitude of the action potential greater than 75 mV (measured from resting potential). Average membrane capacitance and series resistance were 25.6±1.7 pF and 15.2±1.2 MΩ, respectively.

NO affects the spontaneous firing rate of MNCs

Since the excitatory and inhibitory inputs could be intact in our brain slice preparation, we decided to test the hypothesis that NO could act on these cells by an independent pathway. Fig. 1 shows that spontaneous miniature excitatory (mEPSCs) and inhibitory (mIPSCs) post-synaptic currents are readily observed in MNCs. mEPSCs (Fig. 1A) were evident when the preparation was voltage clamped at −60 mV (close to the Nernst potential for Cl^- ions, $E_{Cl} = -56.4$ mV) in the presence of TTX. These spontaneous downward currents were totally blocked by the addition of 10 mM CNQX and 30 mM DL-AP-5 to the bath, indicating their glutamatergic origin (Fig. 1B). Changing the holding potential to −20 mV, in the presence of TTX, CNQX and DL-AP5, spontaneous mIPSCs were identified (Fig. 1C) and totally blocked by the application of 30 μM picrotoxin and 30 μM saclofen (Fig. 1D), blockers of the ionotropic and metabotropic GABAergic transmission, respectively. Therefore, the experiments that follow were performed with both glutamatergic and GABAergic blockers present in the perfusion solutions.

Under the conditions described above, addition of 500 μM L-arginine (a precursor for the synthesis of NO) to the bath drastically reduced the firing rate of MNCs from 3.7±0.6 Hz in the control to 1.3±0.3 Hz in the presence of