Nitric oxide in the amphibian (*Ambystoma tigrinum*) lateral line

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Abstract

Nicotinamide adenine dinucleotide phosphate reduced-diaphorase (NADPH-d) histochemistry was investigated in the axolotl (*Ambystoma tigrinum*) lateral line. Hair cells of neuromast organs of the head skin and neurons of the postotic ganglia showed a significant NADPH-d reaction. Multiunit recording of neuromast afferent activity was also performed. Nitric oxide synthase inhibitor N omega-nitro-L-arginine methyl ester (L-NAME) produced an initial slight excitation followed by a significant inhibition of the resting discharge of neuromast afferent neurons. In contrast, N G-nitro-L-arginine (L-NOARG) produced non-significant actions on the afferent neurons discharge. These findings suggest that afferent neurons and hair cells of the lateral line produce nitric oxide that plays an active role in the mechanisms sustaining basal spike discharge in afferent neurons.

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In the lateral line of the amphibians, the mechanoreceptor organs are mainly concentrated in the skin of the head around the orbit and nostrils. Tree types of lateral line organs have been described: neuromasts, pit and ampullary receptors [8,15]. The lateral line has been used as an experimental model to study hair cell systems function because it is easy to access for electrophysiological recording and isolated skin preparations provides a very good control of experimental variables.

Previous studies reported the presence of nitric oxide synthase (NOS) and the effects of NO in the cochlea [5,12,20,21,26], the vestibular system [6,7,9,13], the cephalopod statocyst [22] and the electrosensory system of teleosts [23]. Particularly in the lateral line system, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity was determined in electrosensory and electromotor systems of the weakly electric gymnotiform teleost *Apteronotus leptorhynchus* as an indicator of putative nitric oxide synthase-containing cells. NADPH-d activity was detected in electroreceptors and in afferent nerves of both ampullary and type I and type II tuberous organs. All cell bodies within the anterior lateral line nerve ganglion were positive for NADPH-d activity, as were the primary afferent axons and their terminal fields in the medullary electrosensory lateral line lobe [23].

Studies from our laboratory have shown that in the axolotl (*Ambystoma tigrinum*), vestibular system hair cells and afferent neurons are NADPH-d positive, and that basal and evoked electrical activity in the afferent nerve from the semicircular canals are sensitive to NOS inhibitors [6,7]. Thus, we decided to search for NOS activity in the axolotl lateral line system in order to define whether NO production is a general property of hair cell systems. In this work, we determined whether there is a synthetic machinery for NO in the amphibian lateral line, and performed physiological experiments to determine NO functional significance in the coding of afferent information.

Seven larval axolotls (*Ambystoma tigrinum*) were deeply anesthetized by immersion in a 0.1% solution of 3-aminobenzoic acid ethyl ester and perfused with paraformaldehyde 4% in 0.1 M phosphate buffer (pH 7.4). The right atrium was opened to enable venous outflow. The rostral part of the cranium skin and the postotic ganglia were removed. Tissues were postfixed for 2 h in paraformaldehyde solution. Tissue pieces were immersed in sucrose 15% for 24 h, cut serially with a cryostat (Leica CM 3050 S) into 20 μm slices, mounted in chromo-alum gellatinized slides and stored for 4 h (4 °C). Each slide included tissue from two or three animals. Slides were covered with coverslips to ensure a homogenous distribution of reagents. The reaction for NADPH-d was done according to the procedure described by Vincent and Kimura [24]. Sections were incubated for 1 h at 37 °C in a solution containing 0.1 M phosphate buffer, 0.3% Triton X-100, 0.1 mg/ml nitrifluoromethylketone, 1 mg/ml...
β-NADPH. For control, β-NADPH was removed from the reaction solution. Slides were assigned randomly to test or control group. Tissue sections were rinsed, dried overnight and dehydrated in an ethanol series, some slides were counterstained with safranine (0.01%). Safranine is a cationic colorant that basically stains nucleic acids and acid polysaccharides. Finally, slices were dried and mounted with synthetic resin. The sections were analyzed in a light microscope (Zeiss Axioplan) and photographed with Kodak Ektachrome 100 film. For the purpose of publication, photographic slides were digitized using a 36 bit Epson scanner (Expression 836 XL) and converted to gray scale using Adobe Photoshop™. Brightness and contrast were adjusted and no other digital processing was performed (color pictures are added as supplementary material available online).

Electrophysiological experiments were performed in an isolated preparation of the skin of the cranium. Briefly, the animals were decapitated and the skin of the rostral part of the cranium, including the eye, subdermic tissue and the nerves arising from it were isolated and mounted on a recording chamber. Multiunit electrical discharge from afferent fibers originating from neuromasts innervated by the buccal ramus of anterodorsal lateral line nerve was recorded by means of a suction electrode (AM Systems). The electrical signal was led to an AC amplifier (P-16 Grass Inst.) to a window discriminator (WPI), and to a computer for spike counting [19]. The preparation was continuously perfused by gravity flow with amphibian Ringer solution (in mM): NaCl 111, KCl 2.5, CaCl2 1.8, MgCl2 1, HEPES 5, glucose 10, pH adjusted to 7.4 with NaOH. In some of the experiments, a low Ca2+ (0.01 mM) high Mg2+ (10 mM) Ringer solution was used in order to block the synaptic neurotransmission and to study purely postsynaptic actions of drugs being applied.

The excitatory amino acid agonist quisqualic acid (QA) and α-amino-3-hydroxy-5-methyl-isoxasole-4-propionic acid (AMPA) and the NOS inhibitor N omega-nitro-L-arginine methyl ester hydrochloride (l-NAME) (Sigma Chemicals) and Nω-nitro-L-arginine (l-NOARG) (RBI) were applied by microperfusion or by bath perfusion as indicated. The NOS inhibitors were systematically protected from light exposure. For microperfusion, the tip of a pipette was placed in the vicinity (±0.5 mm) of the fibers origin in the neuromast and 20 μl pressure ejected in 2 s. Microperfusion experiments allowed to produce a concentration peak of the drug in the vicinity of the tip of the perfusion pipette. In contrast, bath application produced a slowly raising sustained change in the concentration of the drug. The reason to use microperfusion is because it allows to study receptors activation avoiding desensitization phenomena that would otherwise be produced by bath application. Although microperfusion was performed from the sub-epidermic side of the skin, thus no mechanical stimulus was directed to the neuromast hair bundles, controls indicating that extracellular Ringer solution ejection did not produce a mechanical response were systematically performed.

The procedures concerning animal experiments in this study complied with the Declaration of Helsinki. NADPH-d histochemistry produced an intense reaction in hair cells from both neuromasts and pit receptors (Fig. 1). Hair cells showed a very intense reaction particularly in their basal pole. NADPH-d reaction stained neither the mantle nor the supporting cells. NADPH-d reaction was also clearly identifiable in the apical part of neuromast cells. At the level of the postotic ganglia, cell bodies were ordered in rows separated by fiber bundles. Nerve cell bodies were intensely stained with the NADPH-d reaction, the nuclei remained distinguishable because they were not stained (Fig. 2). Thin fibers arising from the ganglia were also well defined by the nitroblue tetrazolium reaction. Unstained cells, visible in the preparation thanks to the safranine counterstaining, were of much smaller size than neuronal cell bodies. In control experiments conducted without β-NADPH, no staining was observed in the hair cells or the neuronal cell bodies.

Basal activity of neuromast nerves was recorded in control conditions and after bath perfusion with the selected drugs. In all the experiments a basal discharge in the range of 3–20 spikes per second was found. Microperfusion of 10 μM QA (n = 3) and of 10 μM AMPA (n = 6) increased the resting discharge of the afferent neurons in 265 ± 127% and 203 ± 102% respectively. Bath perfusion of the preparation with a low Ca2+, high Mg2+ solution produced a rapid decrease of the basal discharge (n = 5, Fig. 3). The sensitivity of the basal discharge to the low Ca2+, high Mg2+ solution was much lower than that observed in the vestibular afferent discharge in the axolotl [17]. The most regular the spike discharge of the afferent neurons, the most resistant was the basal discharge to the low Ca2+, high Mg2+ solution. In fact, in about 30% of the experiments the basal discharge was not significantly modified by the low Ca2+, high Mg2+ solution: in these cases no further experimental maneuver were performed. Control experiments in which low Ca2+ high Mg2+ solution was applied by microperfusion evoked no response indicating that afferent neurotransmitter release is completely blocked and effects thus observed are postsynaptic. While perfusing with low Ca2+, high Mg2+ solution QA 100 μM (n = 3) application produced a significant excitatory action similar to the one evoked in normal Ringer solution (Fig. 3).
Fig. 2. Afferent neurons located at the postotic lateral line nerve ganglia. (A) Neuronal cell bodies are arranged in rows within which there are numerous thin fibers. Some of these cells (asterisk) show a very intense NADPH-d reactivity. (B) Higher magnification of a group of cells displaying NADPH-d reactivity. Blue stain, due to NADPH-d activity, is located exclusively to the cytoplasm of ganglion neurons. Thin fibers running through the nerve cell bodies were also positively stained.

Fig. 3. Plot of the discharge frequency vs. time of the neuromast discharge rate. After about 3 min of bath including the preparation with a low Ca<sup>2+</sup> (0.1 mM), high Mg<sup>2+</sup> (10 mM) solution, the resting discharge fades to zero. The microperfusion of 100 µM QA in this condition produced a significant excitatory response of pure postsynaptic origin. Microperfusion of Ringer solution produced no significant mechanical artifact (Mech). After about 5 min washing the preparation with normal saline solution, the resting discharge tend to return to control value.

Bath perfusion of the NOS inhibitor L-NAME in concentration 0.1 mM (n = 5) produced a non-significant 12 ± 6% inhibition of the afferent resting discharge (paired Student’s t-test, P < 0.05). However, 1 mM L-NAME (n = 5) produced an initial slight excitation followed by an inhibition of the resting discharge of neuromast afferent neurons (Fig. 4). This effect was significant (paired Student’s t-test, P < 0.05) only after 20 min perfusion with the NOS inhibitor and was only partially reversible, reaching a maximum inhibition of 26 ± 4% of the resting electrical discharge. Bath perfusion of L-NOARG 0.1 mM (n = 4) and 1 mM (n = 3) induced a maximal 12 ± 7% non-significant inhibition of the basal discharge of the nerve fibers after 20 min of its application (data not shown).

These results reveal the presence of NADPH-d staining in the lateral line organ hair cells and afferent neurons. The NADPH-diaphorase intense reactivity of lateral line afferent neurons in the octavolateralis ganglion, and the reaction of hair cell basal pole support the hypothesis that NO signaling mechanisms are common to hair cell systems in amphibians. There have been some controversy about NOS activity and NADPH-d staining relationship [25]; however, a very high correlation between NADPH-d positive neurons and NOS immunoreactivity has been described in various sites, including the axolotl olfactory epithelium where staining intensity were similar with both techniques [18].

Previous results in the vestibular system [4,6,7,13] and in the cephalopod statocyst [22] have shown that NO plays a
significant role in the sensory coding of afferent information, contributing to the basal and mechanically evoked discharge of hair cell synapsing afferent neurons. The lack of effect of l-NOARG (a more specific neuronal NOS blocker) and the significant effect of l-NAME (a non-specific NOS blocker) perfusion indicate that most probably an endothelial NOS is involved in the NO production in lateral line system in the axolotl. However, in other hair cell systems in mammals and amphibia both endothelial and neuronal isoforms of NOS have been found [10,11]. Since labeling with NADPH-diaphorase did not distinguish between NOS isoforms, the physiological data are not sufficient to exclude the expression of nNOS, particularly given the small effects exerted by both l-NAME and l-NOARG.

The low inhibitory potency of l-NAME in our experimental conditions suggests that NO in lateral line exert a minor influence in the mechanisms sustaining basal discharge of afferent neurons. It seems plausible that afferent neurons produce NO under the influence of the neurotransmitter released by hair cells. It is worth to note that in some of the experiments the basal discharge showed a Ca\(^{2+}\) independent component since the high Mg\(^{2+}\) and low Ca\(^{2+}\) bath solution did not produce a significant modification of the resting discharge of the neuromast afferents. Excitatory amino acids (EAA) have been shown to mediate modification of the resting discharge of the neuromast afferents. NOARG.
