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Imaging of nitric oxide in the retina ☆

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Abstract

Nitric oxide (NO) is the most widespread signaling molecule found in the retina in that it can be made by every retinal cell type. NO is able to influence a wide variety of synaptic mechanisms ranging from increasing or decreasing neurotransmitter release to the modulation of gap junction conductivity. Although biochemical methods can analyze overall levels of NO, such methods cannot indicate the specific cell types involved. In the last few years, fluorescent imaging methods utilizing diaminofluorescein have allowed the real-time visualization of neurochemically or light stimulated NO-induced fluorescence (NO-IF) in specific retinal cells. Recent experiments have shown that this NO-IF can be stabilized using paraformaldehyde fixation. This aldehyde stabilization has allowed the imaging of NO production in the dark and in response to light, as well as the neurochemical modulation of light stimulated NO production. The results of these studies indicate that NO is not always freely diffusible and that NO is largely retained in many cells which make it. The NO production in retina is highly damped in that in the absence of stimulation, the endogenous levels of NO production are extremely low. Finally, different neurochemical or light stimulation protocols activate NO production in specific cells and subcellular compartments. Therefore, although the NO signaling is widespread in retina, it is very selectively activated and has different functions in specific retinal cell types. The use of NO imaging will continue to play a critical role in future studies of the function of NO in retina and other neural systems.
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1. Introduction

The retina is a massively parallel analog visual processing computer that uses a wide variety of neurotransmitters and millions of synaptic connections. A large percentage of these connections involve conventional neurotransmitter release at anatomically defined synaptic connections onto well characterized and localized receptors to produce postsynaptic changes in electrical activity at the millisecond time scale. However, many of these same transmitters and receptors can also activate biochemical signal transduction systems which process this same information at the millisecond to minutes time scales. In recent years, it has become apparent that the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway is one of the most widespread in the retina (Eldred, 2000). This pathway is distinguished by having NO produced on demand, that NO is not released using synaptic vesicles, and that NO does not bind to specific receptors on the postsynaptic membrane. Basically, any synaptic mechanism that can increase intracellular calcium either directly through receptor channels or by release from intracellular stores can potentially activate this pathway. This increased calcium can activate calmodulin which in turn activates either endothelial nitric oxide synthase (eNOS) or neuronal nitric oxide synthase (nNOS) to synthesize NO.

NO has been shown to influence the physiology of all neuronal types in the retina. The most clearly

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characterized downstream signaling pathway for NO has been the activation of soluble guanylate cyclase (sGC) to synthesize cGMP. For instance, NO has been shown to increase the gain and extend the voltage range of exocytosis in cone photoreceptors (Rieke & Schwartz, 1994; Savchenko, Barnes, & Kramer, 1997). In bipolar cells, NO donor produces an inward current accompanied by a rise in dim and bright flash response amplitudes, and an increase in membrane conductance (Shiells & Falk, 1992). Cyclic GMP has recently been shown to selectively enhance responses to dim, but not bright, stimuli through a purely postsynaptic mechanism that is blocked by inhibitors of cGMP-dependent kinase (Snellman & Nawy, 2004). These last authors propose that cGMP-dependent kinase decreases coupling of the ON-bipolar cell metabotropic glutamate receptors to their downstream signaling cascade, which has the effect of amplifying small decreases in photoreceptor transmitter release.

Perhaps the most comprehensive studies have examined the role of NO in horizontal cells. Miyachi, Murakami, and Nakaki (1990), report that injection of the NO precursor, L-arginine, into H1 luminosity-type horizontal cells in turtle retina reduces their light responses, dramatically increases their input resistance, decreases their response to a surround, and increases their response to stimulation of their receptive field center. McMahon and Ponomareva (1996) report that bath application of L-arginine also decreases the kainate responses in H1 cells in a manner similar to cGMP and NO. Thus, it is likely that the H1 horizontal cells can serve as their own source and target of NO to negatively modulate the gain at photoreceptor-horizontal cell synapses. Finally, Yu and Eldred (2003) have shown that $GABA_A$ and $GABA_C$ receptor antagonists increase retinal cGMP levels through the activation of nitric oxide synthase (NOS) and that NO stimulates GABA release and inhibits glycine release in retina (Yu & Eldred, 2005). The NO stimulated GABA release from horizontal cells was shown to be due to the reversal of the GABA uptake transporter.

In amacrine cells, Mills and Massey (1995) conclude that by working through cGMP, the NO released by light stimulation (Koistinaho, Swanson, de Vente, & Sagar, 1993) decreases the rod input and increases the cone input during light adaptation by uncoupling the AII amacrine cells from the cone bipolars. Wexler, Stanton, and Nawy (1998) demonstrate that NO can depress $GABA_A$ receptor function on amacrine cells. They conclude that NO stimulates sGC to increase cGMP levels, which then increases phosphorylation through protein kinase G to depress GABA currents. The increased cGMP also stimulates a cGMP-activated phosphodiesterase to decrease cAMP levels and phosphorylation through protein kinase A. Wexler et al. (1998) conclude that activators of adenylate cyclase, like dopamine, enhance $GABA_A$ currents, while activators of guanylate cyclase, like NO, do the opposite, and therefore cAMP and NO/cGMP function in a push-pull mechanism in GABAergic transmission in these amacrine cells. NO also modulates cyclic nucleotide gated channels by activating a NO-sensitive sGC to increase levels of cGMP in photoreceptors (Savchenko et al., 1997), bipolar cells (Shiells & Falk, 1992), and ganglion cells (Ahmad et al., 1994). Finally, Wang, Liets, and Chalupa (2003) report that bath application of L-arginine or NO donor usually reduces the peak discharge rates of ON responses in ganglion cells by about 40%, and completely blocks the OFF responses in most ganglion cells.

Clearly, the NO/cGMP signaling system is critical for many aspects of retinal function and it will be important to understand its role in specific retinal cell types. In particular, it will be important to analyze which cells contain specific NOS isoforms, what stimuli can activate NO production in identified cells, and what downstream signaling pathways are activated by the NO that is produced. Answers to these questions have been facilitated through the use of isoform specific nNOS antisera, fluorescent imaging methods to image NO in retina (Blute, Lee, & Eldred, 2000), and the use of antiserum directed against cyclic guanosine monophosphate (cGMP). This report will describe the methods used for NO imaging and how they can be combined with the study of specific NOS isoforms and the cGMP signaling systems downstream from NO. It will also provide the light and electron microscopic localizations of nNOS in the retina that are necessary to put the NO imaging in perspective.

2. Methods

2.1. Imaging hardware and software

The images in this report were captured using one of three camera/imaging software packages. The first was an Apogee AP-7 cooled, thinned, back-illuminated CCD camera with 512×512 pixel resolution (Apogee Instruments, Auburn, CA) controlled with MaxIm DL CCD imaging software (Cyanogen Productions, Ottawa, Ont.). This camera was attached to an Olympus BH-2 epifluorescence microscope with the normal filters for fluorescein. This camera system has very high sensitivity but a slow frame acquisition rate (one 16 bit frame/10 s). The second and third systems were attached to a conventional Olympus BX-51WI fluorescence microscope with a 75 xenon arc lamp (Oriel lamp housing, Oriel, Irvine, CA; Opti Quip model 1600 power supply, Opti Quip; Highlands Mills, NY). The filters used for NO-IF are an Omega 475 nm $\pm$40 nm excitation filter (XF1073), the appropriate dichroic mirror (XF 2010), and a 515–575 nm bandpass emission filter (XF3010) (Omega Optical Brattleboro, VT). The second

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