Geometric considerations of nitric oxide–cyclic GMP signalling in the glomerular neuropil of the locust antennal lobe

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SUMMARY

Using NADPH-diaphorase staining as a marker for nitric oxide (NO) synthase and an antiserum against cyclic GMP, we recently reported the anatomical distribution of NO donor and target cells in the antennal lobe, the principal olfactory neuropil of the locust. The most striking NADPH-diaphorase activity in the olfactory pathway is concentrated in a cluster of intensely stained local interneurons innervating the glomeruli. After incubation of tissue in a NO donor and inhibition of phosphodiesterase activity, neurons of this cluster expressed cyclic GMP-immunoreactivity in the cell body and neurites. Here we examine the importance of the arrangement of NO donor and target cells for information processing in the glomeruli. The cellular organization of the NO–cyclic GMP system in olfactory interneurons, and the dendritic branching pattern, suggest that NO may not only act as intercellular, but also as intracellular messenger molecule in the glomerular neuropil of the antennal lobe.

1. INTRODUCTION

Nitric oxide (NO) serves as a transcellular signalling molecule which activates in adjacent cells its main target, the cGMP-synthesizing enzyme soluble guanylyl cyclase (sGC) (Bredt & Snyder 1992; Garthwaite & Boulton 1995). In the nervous system, NO is produced in an activity-dependent process by Ca²⁺–calmodulin-stimulated NO synthases (NOS). Since NO is not packaged in synaptic vesicles but diffuses from its site of production through cell membranes to its target receptor, it functions as a rather unconventional transmitter. NO-mediated signalling is not only found in the vertebrate, but also in invertebrate nervous systems (Elofsson *et al.* 1993). A summary of the anatomical distributions and biochemical properties of NOS and NOS-related markers has recently been compiled for insect nervous systems (Müller 1997).

The locust is a useful neurobiological preparation for cellular studies of information processing in an invertebrate nervous system (reviewed in Burrows 1996). In the locust brain, the presence of a Ca²⁺–calmodulin-stimulated NOS and a NO-activated guanylyl cyclase has been demonstrated (Elphick *et al.* 1993). Using NADPH-diaphorase (NADPHd) staining of fixed tissue as a marker for NOS, the distribution of NO synthesizing cell populations has been revealed in the brain and thoracic

ganglia (Müller & Bicker 1994; Bicker & Hähnelin 1995; Elphick *et al.* 1995, 1996; Seidel & Bicker 1997). The most striking neuronal NADPHd activity is concentrated in a cluster of intensely stained interneurons of the antennal lobe, the principal olfactory neuropil of the insect brain. Since the afferent antennal nerve and the output pathway of the antennal lobe, the antennoglomerular tract, are not labelled, the NADPHd-positive interneurons are local interneurons (Müller & Bicker 1994; Bicker & Hähnelin 1995; Elphick *et al.* 1995). The somata of these interneurons are immunoreactive to a polyclonal antiserum against rat cerebellar NOS (Elphick *et al.* 1995); however, NADPHd staining provides a more detailed histological resolution of the processes in the neuropil.

We have used NADPHd staining and an antiserum against cGMP (De Vente *et al.* 1987) to investigate the cellular organization of NO donor and potential target cells in the olfactory pathways of the locust brain (Bicker *et al.* 1996). Quantitative immunofluorescence demonstrated NO-induced cGMP-immunoreactivity (cGMP-IR) in the antennal lobe and in the mushroom bodies. In this paper, we focus on the co-localization of NADPHd staining and cGMP-IR in the antennal lobe to elaborate the physiological implications for transcellular and intracellular NO signalling. In addition, we compare the anatomical distribution of NO donor and target cells in the glomerular neuropil of the insect antennal lobe and vertebrate olfactory bulb.

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Figure 1. Cytochemical demonstration of NO-cGMP signalling in the antennal lobe. (a) cGMP-immunoreactivity in olfactory interneurons of the antennal lobe. The frozen section shows cGMP-IR neurites (arrowheads) in the neuropil, and labelled cell bodies (white asterisks) in the soma rind. Unstained cell bodies are indicated by black stars. (b)–(c) Double-labelling for NADPHd staining and cGMP-IR in a section cut through the soma layer of the antennal lobe. (b) NADPHd activity in the soma cluster of interneurons viewed under transillumination. (c) Immunofluorescence on the same section reveals co-localization of NADPHd-staining (b) and cGMP-IR (c) in the majority of stained somata. cGMP-immunofluorescence is also visible in neuronal nuclei, whereas blue NADPHd staining is only found in the cytosol. Because the blue formazan precipitate quenches fluorescence, care was taken to stain the sections only lightly for the diaphorase reaction. All scales: 50 μm .

2. MATERIALS AND METHODS

Locusts were chilled on ice and the cerebral ganglion was dissected out of the head capsule. The ganglionic sheath was completely removed under ice cold Leibovitz L-15 medium (Gibco). Then the tissue was incubated for 30 min in L-15 containing 1 mM 3-iso-butyl-1-methylxanthine (IBMX). After the addition of sodium nitroprusside (SNP) to a final concentration of 100 μM , the tissue was incubated for 15 min at room temperature. The tissue was fixed in 4% carbonate buffered formaline (pH 6.9) for 3 h at room temperature and sectioned in a cryostat. The sections were incubated with the cGMP-antiserum (diluted 1:1000 in PBS with 0.1% NGS). For the visualizing of cGMP-immunoreactivity (cGMP-IR), we used an ABC-kit (Vector Laboratories) according to the instructions of the manufacturer, using PBS as dilution buffer and DAB as chromogen.

For the simultaneous visualization of cGMP-IR and NADPH diaphorase (NADPHd) activity, the immunoreactivity was detected using a secondary FITC-coupled antirabbit IgG (Sigma, diluted 1:100). To detect NADPHd activity, the sections were treated as reported previously (Bicker & Hähnlein 1995).

Details of the generation and tests for specificity of

the employed anti-cGMP serum are given elsewhere (De Vente *et al.* 1987; De Vente & Steinbusch 1993; Bicker *et al.* 1996). Control sections of locust nervous tissue, incubated for immunocytochemistry, but in the absence of primary antiserum, showed no immunoreactivity. Preincubation of the antiserum with 10 μM cGMP for 2 h at room temperature completely blocked immunofluorescence on frozen sections, whereas preincubation with 100 μM cAMP or GMP did not affect immunoreactivity. The results of this paper are based on the evaluation of 22 cerebral ganglia.

3. RESULTS

The antennal lobe is the first-order olfactory neuropil of the insect brain receiving the afferent projections of the antennal chemosensory receptor neurones (Hildebrand 1995). In the locust, the sites of synaptic contacts between receptor terminals, local interneurons and projection neurones are arranged as approximately 1000 spherical glomeruli (Leitch & Laurent 1996). We used an antiserum against cGMP (De Vente *et al.* 1987) to stain NO-responsive cells in the antennal lobe. After incubation of intact brain

tissue in the NO donor SNP and inhibition of phosphodiesterase activity, cGMP-IR could be detected in cell bodies and in the neurites of the glomerular neuropil (figure 1a). The staining of the fine neurites, however, appears less distinct than the soma staining.

To visualize NADPHd staining and cGMP-IR simultaneously, sections were first processed for cGMP-immunofluorescence with subsequent NADPHd histochemistry. On average, about 45 NADPHd-expressing neurones were found in the antennal lobe, but in some preparations up to 60 stained somata could be detected. Because the blue formazan precipitate quenches fluorescence, care was taken to stain the sections only lightly for the diaphorase reaction. This resulted in a rather diffuse staining of the fine glomerular processes. However, previous cytochemical experiments using longer incubation times (Bicker & Hähnel 1995; Bicker *et al.* 1996) have clearly resolved NADPHd-positive arborizations on sections through the glomeruli. As shown in figures 1b,c, a large proportion (about 80%) of blue stained NADPHd-positive antennal lobe cells express cGMP-immunofluorescence, demonstrating cellular co-localization of both neurochemical markers. There are, however, some additional cGMP-IR local interneurons in the antennal lobe of the locust which do not stain for NADPHd. Moreover, we have previously demonstrated that the extracellular application of hemoglobin as NO scavenger reduces slightly the depolarization-induced cGMP-IR, indicating that endogenously released NO can diffuse in the extracellular space of the antennal lobe (Bicker *et al.* 1996).

Regarding the co-localization of NADPHd and cGMP-IR in the cell bodies as a model for the glomerular neurites, we like to consider the possibility that in the olfactory interneurons, NO can mediate intracellular signal transduction for the stimulation of sGC. One of the intriguing properties of the NO/cGMP system is that both release of NO and regulation of cGMP levels can occur in a Ca^{2+} -dependent fashion. Conceivably, inhibition of endogenous guanylyl cyclase (Knowles *et al.* 1989; Dizhoor *et al.* 1991; Lambrecht & Koch 1991), or alternatively, activation of Ca^{2+} -calmodulin-dependent phosphodiesterases (Mayer *et al.* 1992) will down regulate cGMP levels in Ca^{2+} -stimulated target cells of the NO/cGMP system. Therefore, NO is thought to act mainly as a transcellular messenger molecule raising cGMP concentrations in neighbouring cells (Bredt & Snyder 1992; Garthwaite & Boulton 1995). The Ca^{2+} dependence of the NO release and cGMP formation does, however, not preclude an intracellular NO/cGMP signalling system in neurones in which NOS and sGC are coexpressed. Presumably, dendritic Ca^{2+} elevations tend to be fairly localized signals, whereas the rapid three-dimensionally diffusing NO messenger can stimulate cGMP synthesis in adjacent neurites with low levels of Ca^{2+} .

The geometric considerations of this proposal are summarized in the diagram of figure 2 describing

Figure 2. Hypothetical illustration of NO as a transcellular and intracellular messenger in the neurites of antennal lobe interneurons. Two of the NO-releasing cells in the cluster of NADPHd-positive interneurons are schematically drawn. All cells contain Ca^{2+} -activated NOS and an NO-activated sGC. In addition, it is assumed that formation of cGMP is inhibited by Ca^{2+} at concentrations similar to those required for activating NOS. NO is synthesized in a Ca^{2+} -stimulated neurite of the lower interneurone. Transcellular diffusion of NO from its source (vertical arrow) leads to an increase of cGMP in the neurite of a neighbouring neurone which is not Ca^{2+} stimulated. Due to the geometry of the branching neurites in the glomeruli, NO generated in a Ca^{2+} -stimulated neurite can also diffuse into an adjacent neurite of the same cell (horizontal arrow). Under conditions where Ca^{2+} levels are low, this will lead to an increase of cGMP.

NO as a messenger molecule with both para- and autocrine properties that can diffuse freely and act within a restricted volume of glomerular neuropil. In essence, a release of NO from a synaptically depolarized dendrite may increase cGMP levels in neighbouring neurites of the same and of other adjacent neurones in a glomerulus. It should also be pointed out that para- and autocrine signalling strategies are by no means mutually exclusive. For example, the stimulation of a cellular autoreceptor such as sGC would be especially effective in an assembly of neurones which releases simultaneously the correspond-

ing transmitter molecule in close spacial vicinity.

4. DISCUSSION

Nitric oxide is released during nerve cell depolarization in a Ca^{2+} -dependent process by the activation of Ca^{2+} -calmodulin-stimulated NOS (Bredt & Snyder 1992; Garthwaite & Boulton 1995). The demonstration that dissociated cells from the antennal lobe generate NO after stimulation by agents, elevating cytoplasmic Ca^{2+} levels (Müller & Bicker 1994), and measurements of arginine to citrulline conversion in brain extracts (Elphick *et al.* 1995) have provided strong support for the presence of a NO signalling system in the antennal lobe. In this paper, we rely on NADPHd staining of fixed tissue as an indicator of NOS activity. Two caveats are, however, appropriate here. The first relates to the molecular identity of the NADPHd and NOS activity. In some tissues, such as the main olfactory epithelium of adult rodents, the NADPHd activity is caused by oxidative enzymes other than NOS (Kishimoto *et al.* 1993; Verma *et al.* 1993). In the nervous system of the locust, however, measurements of NOS activity in cell homogenates of the various neuropils did correlate well with the NADPHd activity and distribution patterns of NADPHd-positive cells (Müller & Bicker 1994; Elphick *et al.* 1995). Thus, we suggest that the NOS-expressing cells of the insect CNS can be identified by NADPHd histochemistry. Second, we cannot rule out that in the intact animal, other messenger molecules, such as carbon monoxide, CO (Verma *et al.* 1993), may contribute to the rise of cGMP levels in the cluster of local interneurons.

The reliable immunocytochemical detection of cGMP depends on the presence of the phosphodiesterase inhibitor and on the efficient diffusion of the fixative into the antennal lobe (Bicker *et al.* 1996). The faster diffusion of the fixative into the outer cell body layer as opposed to the more central neuropil may account for the pronounced soma staining in comparison to the less conspicuous cGMP-IR in the processes. In comparison to insects of similar body size, such as *Manduca sexta*, which has about 64 identifiable glomeruli ranging from 50 to 100 μm in diameter (Hildebrand 1995), the antennal lobe of the locust is comprised of 1000 small sized glomerular units (Leitch & Laurent 1996). The very condensed neuropilar structure of the rather small-sized locust glomeruli (25 μm diameter) tends to make the immunocytochemical staining of fine neuritic branches appear less distinct.

The strong cytoplasmic NADPHd reaction of the cell bodies implies that NO might be generated in the somata. Dissociated cells have been challenged with drugs elevating cytoplasmic Ca^{2+} levels, indicating that the somata are indeed capable of Ca^{2+} -regulated NO synthesis (Müller & Bicker 1994). In the intact antennal lobe, synaptic integration is mainly confined to the glomerular neuropil (reviewed in Hildebrand & Shepherd 1997). Thus, electrophysiological recordings will be necessary to

decide whether the somata of the NADPHd-positive interneurons express prominent Ca^{2+} conductances, as has been shown for locust thoracic neurosecretory cells (Goodman *et al.* 1980).

The olfactory systems of invertebrates and vertebrates share certain organizational and functional principles (Shepherd 1994; Hildebrand 1995; Hildebrand & Shepherd 1997). The antennal lobe of the insect brain appears as the functional equivalent of the vertebrate olfactory bulb because synaptic integration between sensory afferents, local interneurons and projection neurons takes place in a glomerular neuropil (Hildebrand & Shepherd 1997). Remarkably, the association of NOS with the glomeruli of the olfactory pathways is a common feature of the insect (Müller 1994, 1996, 1997; Müller & Bicker 1994; Bicker & Hähnel 1995; Elphick *et al.* 1995; Seidel & Bicker 1997) and vertebrate brain (Bredt & Snyder 1992; Vincent & Kimura 1992; Breer & Shepherd 1993; Kishimoto *et al.* 1993; Hopkins *et al.* 1995). Despite the common glomerular organization principle of the olfactory neuropil, the cellular distribution of NOS and sGC appears different in insects and vertebrates. Using similar cytochemical techniques, a differential distribution of cGMP and NOS has been detected in the layers of the rat olfactory bulb (Hopkins *et al.* 1995), supporting the proposal that NO is an intercellular messenger in the olfactory neuropil (Breer & Shepherd 1993). In contrast, the colocalization of NADPHd and cGMP in the cluster of antennal lobe interneurons allows for the possibility of intracellular NO-cGMP signal transduction. Therefore, we suggest that NO can act both as a transcellular and intracellular messenger in the antennal lobe of the insect brain.

The spherical compartments of the glomeruli provide the most efficient geometric solution for influencing a defined volume of neuropil by a diffusible membrane permeant compound like NO. An open issue is still the biological range of the NO action, since locust glomeruli (25 μm diameter) are smaller than the glomeruli of mammals (50–200 μm) (Hildebrand & Shepherd 1997). Model calculations of diffusional spread predicted a physiological sphere of influence of a point source of NO that emits for 1–10 s in the range of about 200 μm diameter (Wood & Garthwaite 1994). However, in the absence of any experimental data about the absolute concentration profile and time course of NO release in the neuropil, we can only surmise that a glomerulus corresponds to a functional domain of NO action. It is plausible though that the glomerular neuropil in the antennal lobe can facilitate lateral dendritic interactions via the NO-cGMP system irrespective of whether dendrites belong to the NO releasing or another cell. As a consequence, this could lead to a coordination of neural activity in the glomerulus, regardless of conventional synaptic connections.

The olfactory pathways of the locust exhibit an odour-evoked synchronization of neural activity (Laurent 1996), which can be abolished by blocking the GABAergic neurotransmission in the antennal

lobe (MacLeod & Laurent 1996). Using immunocytochemistry, we have demonstrated that all of the NADPHd positive local interneurons of the antennal lobe most likely employ GABA as conventional transmitter (Seidel & Bicker 1997). Thus, NO mediated signalling between the NADPHd-cGMP expressing local interneurons may operate in parallel to the conventional GABAergic transmission to synchronize neural activity in the antennal lobe. Such a coordination of neuronal assemblies by NO has been demonstrated in the olfactory system of a mollusc (Gelperin 1994).

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