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Intrinsic Nitric Oxide Regulates the Taste Response of the Sugar Receptor Cell in the Blowfly, *Phormia regina*

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Abstract

The taste organ in insects is a hair-shaped taste sensory unit having four functionally differentiated contact chemoreceptor cells. In the blowfly, *Phormia regina*, cGMP has been suggested to be a second messenger for the sugar receptor cell. Generally, cGMP is produced by membranous or soluble guanylyl cyclase (sGC), which can be activated by nitric oxide (NO). In the present paper, we electrophysiologically showed that an NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO), an NO donor, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC 7) or an NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) specifically affected the response in the sugar receptor cell, but not in other receptor cells. PTIO, when introduced into the receptor cells in a sensillum aided by sodium deoxycholate (DOC, pH 7.2), depressed the response of sugar receptor cells to sucrose but did not affect those of the salt or water receptor cells. NOC 7, given extracellularly, latently induced the response of sugar receptor cells; and L-NAME, when introduced into the receptor cells, depressed the response of sugar receptor cells. The results clearly suggest that NO, which may be produced by intrinsic NOS in sugar receptor cells, participates in the transduction cascade of these cells in blowfly.

Key words: fly, nitric oxide, nitric oxide synthase, sugar receptor cell, taste, transduction cascade

Introduction

Nitric oxide (NO) is known as an intercellular and/or an intracellular messenger in nervous systems of vertebrates (Garthwaite and Boulton, 1995; Esplugues, 2002) and invertebrates (Davies, 2000; Bicker, 2001). It is well known that NO is produced by NO synthase (NOS) in postsynaptic nerves and diffuses into presynaptic nerves through membranes; where NO activates sGC to produce cGMP, resulting in the feedback control of presynaptic nerve activity. In such NO signaling systems, it is conventionally accepted that the target cells of NO are separated from NO-donor cells having NOS activity.

However, taste sensory systems might act differently. Immunohistochemical studies have demonstrated that NOS is expressed in apical parts as well as cell bodies of the taste receptor cells in rat (Kretz *et al.*, 1998) and frog (Zaccone *et al.*, 2002). NADPH-diaphorase activity that indicates an NO production site has been shown in taste buds of rat (Kretz *et al.*, 1998). Electrophysiological studies suggested that taste receptor cells use cGMP as a second messenger for taste transduction in rat (Okada *et al.*, 1987), mouse (Tonosaki and Funakoshi, 1988) and frog (Krizhanovsky *et al.*, 2000). Some pharmacological experiments in circumvallate and foliate papillae of mouse showed that an increase of cGMP level induced by bitter stimuli was suppressed by sGC inhibitor and boosted by NO donor (Rosenzweig *et al.*, 1999). In those vertebrate taste receptor cells, therefore, NO produced by NOS could function for taste transduction.

In the blowfly, *Phormia regina*, Amakawa *et al.* (1990) observed that membrane-permeable cGMP analogs induced the response of sugar receptor cells, suggesting that sugar receptor cells use cGMP as a second messenger for taste transduction. Wieczorek and Schweikl (1985) reported a

high concentration of cGMP and high activity of GC in the labellum, which is the taste sensillum-rich region of the proboscis. Thus, we expected that NO could increase intracellular cGMP levels via activation of sGC in sugar receptor cells of *P. regina*; as indeed the fly is shown to be a suitable organism for electrophysiological investigation on the function of NO. Tip-recording, a simple extracellular recording method established by Hodgson *et al.* (1955) is applicable to recording responses of a single taste receptor cell in a chemosensillum. Moreover, through the tip opening of the sensillum, various reagents such as inhibitors or activators can be extracellularly applied, or even introduced into the taste receptor cells, by the DOC method (Ozaki and Amakawa, 1992).

In the present paper, we show electrophysiologically that in successive transduction cascades of the sugar receptor cell, NOS is activated to produce NO, which in turn activates sGC to produce cGMP within the same cell.

Material and methods

Flies

Blowflies, *P. regina*, were reared in an environmental chamber with 12 h light/12 h dark cycles in a laboratory at 24 ± 1°C, on chicken liver and yeast bait at the larval stage and on 100 mM sucrose at the adult stage. Experiments were performed with animals aged 5–7 days after emergence.

Recording procedure

A tip-recording method (Hodgson *et al.*, 1955) was applied to record responses of the taste receptor cells. The largest (LL-type; Wilczek, 1967) chemosensilla were stimulated with a stimulating solution in a glass capillary electrode (50–100 μm diameter). Impulses were recorded using a tip recording set that consisted of an AC amplifier, an AD/DA converter (PCI-3523A; Interface Co., Tokyo, Japan) and a home-made program, 'TipRecUEC' written with Microsoft Visual Basic v.6 (Redmond, WA), on a personal computer (Deskpro Workstation 300; Compaq, Tokyo, Japan). For the analysis of data, Origin v.5 (OriginLab Co., Northampton, MA) was used. Impulses during the initial 150 ms after the beginning of stimulation were ignored according to previous reports (e.g. Morita, 1969, 1972; Ozaki and Amakawa, 1992) so that the impulse frequency, an index for the response magnitude, is proportional to the receptor potential. Solutions of 100 mM sucrose, 500 mM NaCl, 10 mM NaCl and 250 mM CsCl were used to independently stimulate the sugar, salt and water and the so called 'fifth' receptor cells, respectively according to previous reports (e.g. Ozaki *et al.*, 2003). The sucrose solution contained 10 mM NaCl to give electrical conductance at the electrode. All experiments were performed at room temperature (24 ± 1°C), and relative humidity was kept >70% to ensure correct stimulus concentration.

Applications of pharmacological reagents to chemosensilla

To examine the activity of NO in the taste transduction cascade, we introduced pharmacological reagents into the taste receptor cells. The generally used method of introduction, microinjection, was unsuitable for the preparations in our experiments because the dendrites were thin and enclosed with a rigid cuticle sheath in the chemosensillum. An NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO), or an NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), was introduced into the cells by the 'DOC method' (Ozaki and Amakawa, 1992): 10 mM PTIO was dispersed in 67 mM phosphate buffer (pH 7.2) with the aid of 0.5% dimethyl sulfoxide (DMSO) and 250 μM L-NAME or its inactive enantiomer, N^G-nitro-D-arginine methyl ester (D-NAME), was dissolved in the same buffer. We added 0.03% sodium deoxycholate (DOC) to these solutions. The chemosensillum tip was incubated at room temperature for 2 min with these solutions in the glass capillaries. During incubation, 0.03% DOC, three times lower than the critical micelle concentration, hardly lyses receptor membranes of taste cells but temporarily makes the receptor membranes permeable to the reagents. The chemosensillum tip was then kept in air for 5 min so that the membrane structure recovered. To examine the effects of the introduced reagents, responses to stimuli recorded before and 5 min after incubation were compared. In the experiment with the NO donor, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC 7), we examined if the NOC 7 solution induced impulses from a taste receptor cell. We expected that, during the stimulation, NOC 7-released NO in the electrode capillary would pass through the tip membrane into the cells to discharge impulses. NOC 7 was dissolved in 1 N NaOH aq. for stock, and was diluted with 20 mM MOPS buffer (pH 6.6) to prepare 8 mM solution immediately before use, in which NOC 7 began to and kept on releasing NO for some minutes.

Statistical analyses

The data obtained in the experiments with PTIO or L-NAME/D-NAME application were statistically analyzed with Kruskal–Wallis test and multiple pairwise comparisons (Wilcoxon rank sum test modified by Bonferroni–Holm) or with Wilcoxon rank sum test, respectively. These analyses were performed with R v.1.6.2 (Free Software Foundation Inc., Boston, MA) software.

Chemicals

Reagents used in our experiments were purchased as follows: sucrose and DOC from Kanto Chemical Co. Inc (Tokyo, Japan); NOC 7 and L-NAME from Dojindo Laboratories (Kumamoto, Japan); D-NAME from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA); and the other chemicals from Wako Pure Chemical Industries Ltd (Saitama, Japan).

Results

NO scavenger depressed sugar response

We examined intracellular effects of an NO scavenger, PTIO, on responses of sugar, salt or water receptor cells. To introduce PTIO into taste receptor cells in labellar chemosensilla, we mixed DOC in a treatment solution of PTIO (see Materials and methods). We confirmed that DOC alone did not affect responses of sugar, salt nor water receptor cells as shown by Ozaki and Amakawa (1992).

When the sensillum tip was incubated with 10 mM PTIO plus 0.03% DOC to introduce PTIO into the cells, the impulse frequency of the sugar receptor cell decreased. Figure 1A shows a representative set of impulses induced by 100 mM sucrose before (a) and after (b) the incubation. As previous studies revealed that initial (0–150 ms) and later (350 ms) parts were disturbed by capacitive noise and an adaptation phenomenon, we analyzed the frequency of impulses during the 150–350 ms period that has been reported to be proportional to the stimulation intensity (e.g. Morita, 1969, 1972; Ozaki and Amakawa, 1992). Here, the number of impulses during 150–350 ms after the beginning of the stimulation was decreased from 28 to 14 by the incubation with PTIO. Figure 1B summarizes such relative responses, after incubation to that before, to 100 mM sucrose, 500 mM NaCl and 10 mM NaCl, respectively. The relative responses to 100 mM sucrose after incubation with mixtures of PTIO and DOC, PTIO alone and DOC alone were 0.46 ± 0.09, 0.81 ± 0.08 and 0.93 ± 0.07. By statistical multiple pairwise comparisons, we found that incubation with PTIO plus DOC significantly reduced the response to 100 mM sucrose, compared to incubation with either PTIO ($P = 0.035$) or DOC ($P = 0.014$). PTIO scarcely affected the response without the help of DOC, and DOC incubation itself did not affect the response to sucrose. This clearly implies that NO is involved as an activating factor in the transduction cascade of the sugar receptor cell. In salt or water receptor cells, incubation with PTIO plus DOC hardly affected responses.

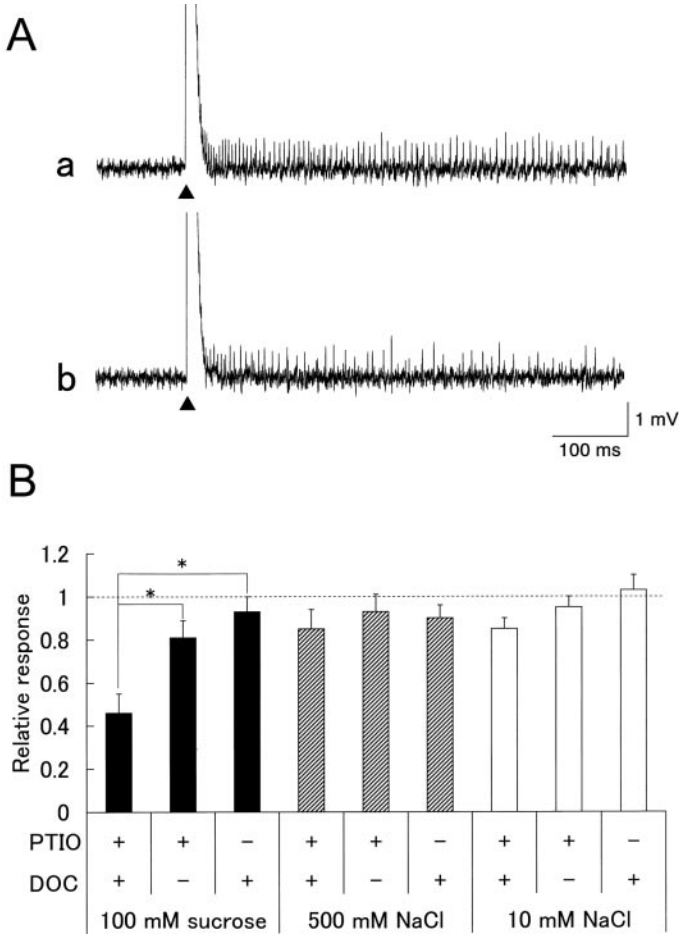

Figure 1 Effects of PTIO on taste responses of blowfly chemosensilla. **(A)** Representative impulses generated by a labellar LL-type chemosensillum of *Phormia regina* in response to 100 mM sucrose: before (a) and after (b) application of a mixture of 10 mM PTIO and 0.03% DOC for 2 min. Triangles indicate the beginning of sucrose stimulation. **(B)** Relative responses to 100 mM sucrose, 500 mM NaCl and 10 mM NaCl are measured when 10 mM PTIO and 0.03% DOC were applied together (++) or separately (+) to chemosensilla for 2 min. Values are means ± SEM. Each number in parentheses in a column of the graph indicates experimental trail times. *Significant at $P < 0.05$.

NO donor induced sugar response

When we applied NOC 7 to a sensillum, two kinds of impulses with different amplitudes were observed (Figure 2D). Following generation of small impulses, large impulses started to appear with ~15 s of latency. To identify which taste receptor cells in a sensillum discharged the small or large impulses, we compared their amplitudes with those of the sugar, salt, water and 'fifth' receptor cells recorded by standard stimulations according to Ozaki *et al.* (2003). The amplitude of the impulses of the sugar, salt, water and 'fifth' receptor cells were different from one another, as shown in Figure 3. The small impulses that appeared at the beginning of the NOC 7 application were observed even when a control MOPS buffer or 10 mM NaCl was applied to the sensillum (data not shown). Presumably, these impulses were derived from the water receptor cells. The amplitude of the larger impulses induced by NOC 7 (0.84 ± 0.01 mV) was the same as those of sugar receptor cells (0.87 ± 0.02 mV; Figure 3), indicating that the larger impulses induced by NOC 7 were derived from sugar receptor cells.

Responses to binary mixtures of NOC 7 plus sucrose or NaCl also support that larger impulses induced by NOC 7 came from sugar receptor cells. When 30 mM sucrose alone was applied to chemosensilla, two kinds of impulses differentiated in amplitude were generated, which were identified as sugar and water responses (Figure 4A). No other impulses differentiated in amplitude from the two kinds of impulses were observed when a binary mixture of 8 mM NOC 7 plus 30 mM sucrose was applied to the chemosensilla. On the contrary, in case of NOC 7 plus NaCl, the

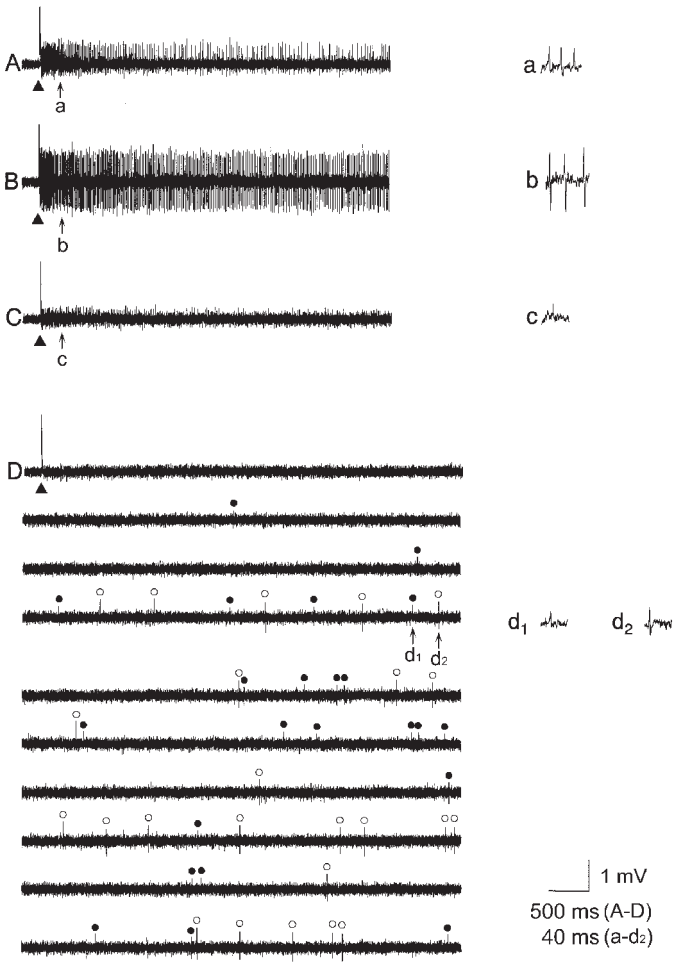

Figure 2 Representative impulses generated by one labellar LL-type chemosensillum of Phormia regina in response to **(A)** 100 mM sucrose, **(B)** 500 mM NaCl, **(C)** 10 mM NaCl and **(D)** 8 mM NOC 7 (time scale bar: 500 ms). Triangles in the record indicate the beginning of stimulation. Open or filled circles in (D) indicate impulses expected to be derived from sugar or water receptor cells, respectively. Impulses indicated with arrows were redisplayed in expanded time scale on the right side (a, b, c, d_1 and d_2; time scale bar: 40 ms).

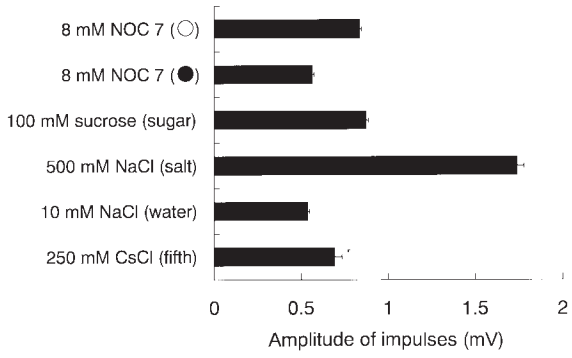

Figure 3 Amplitude of impulses induced by test solutions from labellar LL-type chemosensilla. Values are means ± SEM (n = 20). The amplitudes of the impulses induced by 8 mM NOC 7, 100 mM sucrose, 500 mM NaCl and 10 mM NaCl belonged to one fly and those induced by 250 mM CsCl to another. An open or filled circle in 8 mM NOC 7 corresponds to the notations in Figure 2D. The amplitude of impulses from 250 mM CsCl was corrected using the amplitude of impulses from water receptor cells as a standard measure.

binary mixture evoked another kind of impulses differentiated in amplitude from the impulses induced by 150 mM NaCl alone. When 150 mM NaCl alone was applied to the chemosensillum, two kinds of impulses identified as salt and water responses were generated (Figure 4B); the binary mixture of 8 mM NOC 7 plus 150 mM NaCl induced three kinds of impulses differentiated in amplitude (Figure 4D). The additional kind of impulses induced by the binary mixture corresponded in amplitude to impulses from sugar receptor cells induced by 30 mM sucrose or the larger impulses induced by 8 mM NOC 7 alone (Figure 4A,C).

NOS inhibitor depressed sugar response

The experiments above using PTIO and NOC 7 suggested that NO functions in sugar receptor cells. However, where NO is produced was still unclear; thus, we tested the effects of NOS inhibitor. Figure 5A shows a representative set of impulses induced by 100 mM sucrose before (a) and after (b) a 2 min incubation of a chemosensillum tip with a mixture of 250 μM L-NAME and 0.03% DOC. The incubation caused the impulse frequency to decrease from 30 to 18/200 ms. Figure 5B shows means of the relative responses to 100 mM sucrose, 500 mM NaCl and 10 mM NaCl (n = 7). Average relative response to 100 mM sucrose after incubation with L-NAME plus DOC (n = 7) was 0.66. To evaluate the specific inhibitory effect of L-NAME on NOS activity, we also tested 250 μM D-NAME as a control for the L-NAME experiment. Unlike L-NAME, incubation with 250 μM D-NAME plus DOC did not decrease the impulse frequency of sugar receptor cells: the average value of the relative response was 0.94 (n = 7). Statistical analysis with Wilcoxon rank sum test revealed significant differences between treatments with L-NAME and D-NAME (P = 0.002), suggesting that the inhibitory effect of L-NAME was due to the specific effects on NO synthesis in sugar receptor cells. L-NAME did not affect the response of salt receptor cells to 500 mM NaCl or of water receptor cells to 10 mM NaCl.

Discussion

NO works as a second messenger where there is intrinsic NOS in blowfly sugar receptor cells

Our results strongly suggest (i) that cytosolic NO is involved in the transduction cascade of blowfly's sugar receptor cells, probably by activating sGC and, to our surprise, (ii) that NO is produced by NOS and functions in the same sugar receptor cells.

Application of the NO scavenger, PTIO, and the NO donor, NOC 7, to taste receptor cells of the blowfly revealed that intrinsic NO contributes to generating responses of

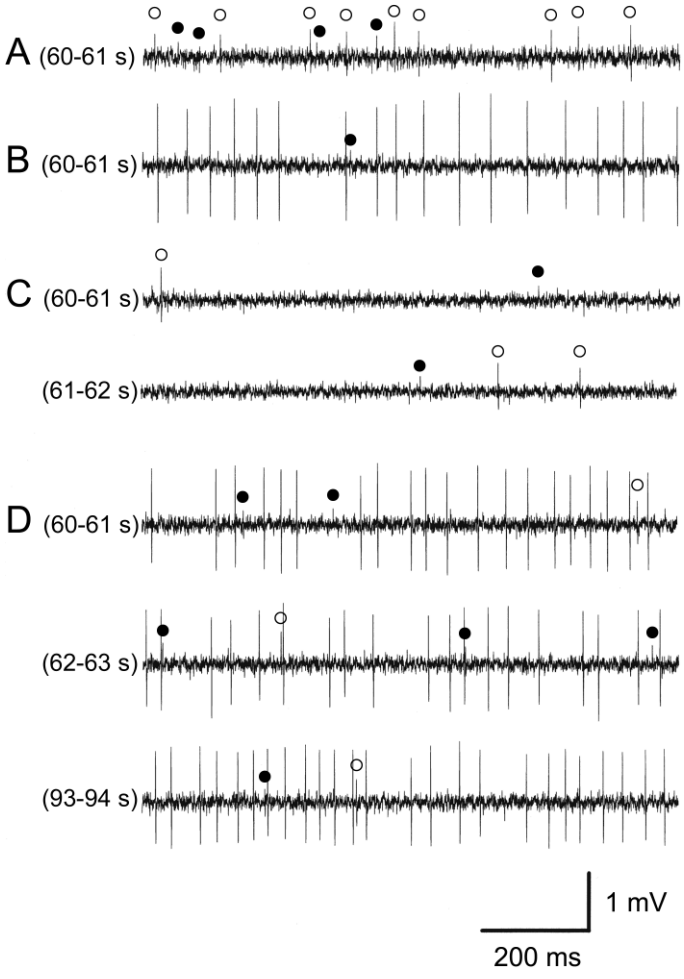

Figure 4 Representative impulses generated by one labellar LL-type chemosensillum of *Phormia regina* in response to **(A)** 30 mM sucrose, **(B)** 150 mM NaCl, **(C)** 8 mM NOC 7 and **(D)** 150 mM NaCl plus 8 mM NOC 7. Time after beginning of stimulation is shown in parentheses at the top of each record. Open or filled circles indicate impulses expected to be derived from sugar or water receptor cells, respectively.

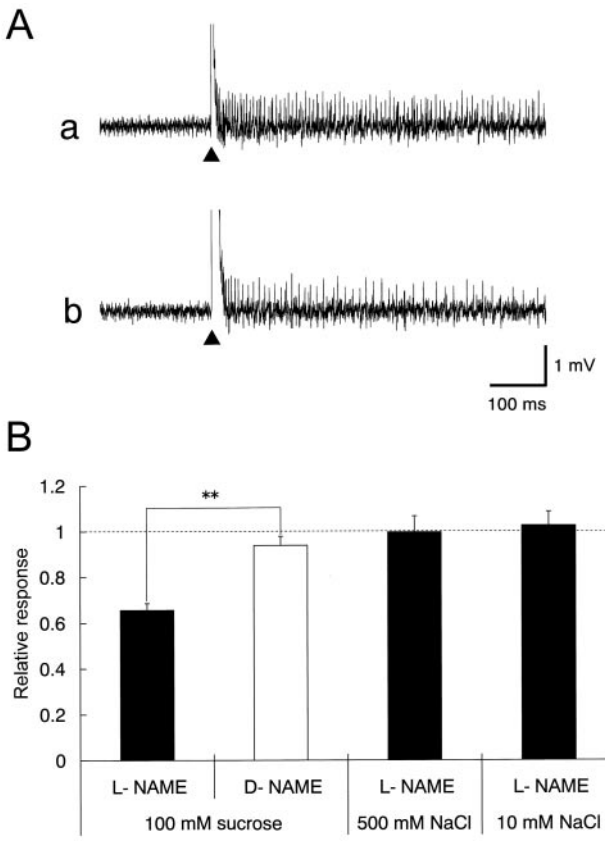

Figure 5 Effects of L-NAME and D-NAME on taste responses of blowfly chemosensilla. **(A)** Representative impulses generated by a labellar LL-type chemosensillum of *Phormia regina* in response to 100 mM sucrose: (a) before and (b) after application of a mixture of 250 μM L-NAME and 0.03% DOC for 2 min. Triangles in the records indicate the beginning of stimulation. **(B)** Relative responses to 100 mM sucrose, 500 mM NaCl and 10 mM NaCl measured when 250 μM L-NAME or D-NAME plus 0.03% DOC was applied to chemosensilla for 2 min. Values are means ± SEM ($n = 7$). **Significant at $P < 0.01$.

sugar receptor cells. When we applied PTIO to the sensillum, the coexistence of DOC in the incubation solution was essential for PTIO to depress the response of sugar receptor cells. The application of PTIO alone scarcely depressed the response, indicating that extracellular PTIO did not affected sugar receptor cells. DOC by itself similarly did not affect the response of sugar, salt or water receptor cells (Figure 1B). Probably, 'DOC treatment' of a sensillum transiently made the receptive membranes fragile at the tip of the four taste receptor cells, so that PTIO could penetrate into these four cell types. Nevertheless, only the sugar receptor cell response was depressed. In contrast, NOC 7 was effective even when extracellularly applied to a sensillum. The buffer solution containing NOC 7 induced impulses from sugar and water receptor cells (Figures 2–4), while the buffer solution without NOC 7 induced impulses only from water receptor cells. Therefore, we conclude that NOC 7, in particular, induces sugar receptor impulses; and that the induced impulses appear with a long latency of ~15 s, which might be the time necessary for NO to penetrate the membrane and to be concentrated until at a sufficient level to activate sGC in sugar receptor cells.

Application of the NOS inhibitor, L-NAME, to taste receptor cells of the blowfly revealed that intrinsic NOS also contributes to regulating responses of sugar receptor cells where NO functions as a second messenger. The application of L-NAME depressed the response of sugar receptor cells (Figure 5B), indicating that L-NAME affected them after penetrating the receptive membrane. However, the application of D-NAME, the inactive enantiomer of L-NAME, did not change the response. These results indicate that the depression of the sugar receptor cell response by L-NAME was caused by the specific inhibition of NO synthesis in the cells. Thus, we concluded that NOS might be involved in the

transduction cascade of sugar receptor cells. It is well known that NO-producing and NO-dependent cascades are separately located in different cells, and that NO is regarded as an intercellular messenger. Here our electrophysiological study indicates an unconventional case where NO-producing and NO-dependent cascades coexist and function in the same nerve cells. A coexistent system has only been reported in mitochondrial biogenesis of primary cultures of mouse adipocyte precursors (Nicoli *et al.*, 2003). Such a system might also be employed in the olfactory receptor cells of the fleshfly, *Neobellieria bullata* (Wasserman and Itagaki, 2003), in which L-NAME inhibited electrophysiological responses to odorants.

Unfortunately, we could not examine the effects of PTIO and L-NAME on the response of 'fifth' receptor cells, because no adequate stimulant is available to induce a sufficient high frequency of impulses in these cells. However, we observed that the NO donor did not induce any impulses in 'fifth' receptor cells.

The NO signaling system supports the cGMP-dependent cascade as a transduction system in sugar receptor cells in the blowfly

Our findings have important implications for understanding the cGMP-dependent cascade that has previously been suggested as a transduction system in fly sugar receptor cells; and for which three hypotheses have been raised. Cyclic GMP in *P. regina* (Amakawa *et al.*, 1990) and IP_3 in the fleshfly, *Boettcherisca peregrina* (Koganezawa and Shimada, 2002) were proposed to be second messengers, while a channel directly gated by sugar itself was reported in *B. peregrina* (Murakami and Kijima, 2000). The cGMP-dependent cascade was hypothesized from an electrophysiological study in which membrane-permeable cGMP analogs induced impulses from sugar receptor cells (Amakawa *et al.*, 1990). Cyclic GMP has been demonstrated to induce impulses from sugar receptor cells, though it is unresolved how cGMP is produced in these cells. If the NO signaling system functions, cGMP is most likely to be produced by the activation of sGC through the NO signaling system. We predict that the cGMP-dependent cascade is combined with the NO signaling system to produce cGMP in fly sugar receptor cells. The present data also raise the possibility that NO directly regulates ion channels to excite sugar receptor cells. The existence of channels regulated by NO has been shown in olfactory receptor cells, the cyclic nucleotide-gated (CNG) channel in tiger salamander (Broillet and Firestein, 1996) and the potassium channel in toad and rat (Schmachtenberg and Bacigalupo, 1999), which were activated by NO, while the CNG channel in rat (Lynch, 1998) was inhibited by NO. Further studies should clarify details of the NO signaling system such as the NOS activating factor and the NO target. Such studies could solve the interesting question emerging from this work, as to the advantage of the NO signaling system in transduction of fly sugar receptor cells.

We should notice that the responses in sugar receptor cells were not perfectly depressed by the introduction of PTIO or L-NAME into the cells (Figures 1 and 5). The NO signaling system could collaborate with a metabotropic system related to IP_3 production (Koganezawa and Shimada, 2002) and/or an ionotropic system (Murakami and Kijima, 2000), suggested in *B. peregrina*, to activate sugar receptor cells. Additionally, IP_3 has been suggested to work in an adaptation cascade of sugar receptor cells in *P. regina* (Ozaki and Amakawa, 1992). Further studies will clarify the roles of NO in the combinational control of the cascades in sugar receptor cells of the fly.

The NO signaling system we suggest here will provide a powerful platform to help decode the complete transduction from receptors to ion channels that activates taste receptor cells.

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