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Detection of Nitric Oxide Release from Single Neurons in the Pond Snail, *Lymnaea stagnalis*

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Multiple film-coated nitric oxide sensors have been fabricated using Nafion and electropolymerized polyeugenol or *o*-phenylenediamine on 30-μm carbon fiber disk electrodes. This is a rare study that utilizes disk electrodes rather than the widely used protruding tip microelectrodes in order to measure from a biological environment. These electrodes have been used to evaluate the differences in nitric oxide release between two different identified neurons in the pond snail, *Lymnaea stagnalis*. These results show the first direct measurements of nitric oxide release from individual neurons. The electrodes are very sensitive to nitric oxide with a detection limit of 2.8 nM and a sensitivity of 9.46 nA μM^{-1}. The sensor was very selective against a variety of neurochemical interferences such as ascorbic acid, uric acid, and catecholamines and secondary oxidation products such as nitrite. Nitric oxide release was measured from the cell bodies of two neurons, the cerebral giant cell (CGC) and the B2 buccal motor neuron, in the intact but isolated CNS. A high-Ca^{2+}/high-K^{+} stimulus was capable of evoking reproducible release. For a given stimulus, the B2 neuron released more nitric oxide than the CGC neuron; however, both cells were equally suppressed by the NOS inhibitor L-NAME.

Nitric oxide has a role as a neurotransmitter/neuromodulator (a compound that has no effect on its own but can alter response to a classical transmitter) in both mammalian[1] and nonmammalian species. Specifically, nitric oxide has been shown to be involved in regulating neuronal excitability, synaptic transmission, the functioning of neuronal networks, arousal, and learning and memory mechanisms.[1,2]

While its role as a central nervous system (CNS) neurotransmitter/modulator has been demonstrated using a variety of electrophysiological and pharmacological approaches, relatively few studies have measured nitric oxide release from neuronal tissue and none have examined release from single neurons and compared how release differs between neurons.

The use of microelectrodes for electrochemical detection of analytes in neurons and other cells makes real-time measurements of nitric oxide possible. The small size of these microelectrodes (0.5−35-μm diameter) allows good spatial resolution and makes them excellent sensors for direct placement into biological preparations without causing extensive damage to tissues.[3−5]

Single-cell recordings have been widely used in Wightman's and Ewing's groups[6−9] to study the transmission process and mechanism. They have used chromaffin and PC12 cells to measure release of adrenaline and dopamine, respectively. There are no published studies measuring the release of nitric oxide from single neurons or intact neuronal systems, and this information would provide valuable information on the role of nitric oxide in the CNS.

Nitric oxide sensors for in vivo measurements can conveniently be divided into two classes: (i) chemically modified electrodes, such as porphyrin-based sensors[3] or (ii) film-coated electrodes using one or more polymeric coatings such as Nafion,[10] *o*-phenylenediamine,[4] and polyeugenol.[11,12]

Recently, film-coated sensors have been widely used to measure nitric oxide levels from brain tissue. Nafion/*o*-phenylenediamine (*o*-PD) microelectrodes have been used to measure nitric oxide release from brain slices. These electrodes showed good sensitivity (954 ± 217 pA μM^{-1}) and linearity to nitric oxide concentrations in the range of 100−1000 nM. The limit of detection was 6 ± 2 nM with a 50% response time of 1 s.[13]

In this study, we have looked at measuring the levels of nitric oxide released from the cell body of two different identified neurons from the pond snail, *Lymanea stagnalis*. This study shows for the first time real-time determination of nitric oxide release from individual neurons in intact CNS. We have used a new multiple membrane film coating based upon Nafion and polyeu-

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genol (4-allyl-2-methoxyphenol).[11,12] Specifically, we have used a multiple film coating using a layer of Nafion and adapted the electropolyermization parameters of the eugenol film coating to carbon fiber electrodes rather than platinum wires used by Ciszewski and Milczarek[11,12] The Nafion improves adhesion, uniformity, and stability of the polyeugenol layer compared with direct coating of polyeugenol on the electrode surface as a single film.

The neurons we have chosen to examine are the buccal motorneuron B2 and the large cerebral modulatory neuron, the CGC, in the intact CNS. Both neurons have been previously shown to contain the enzyme nitric oxide synthase (NOS)[14,15] and, therefore, have the potential to synthesize and release nitric oxide. Both neurons can be reproducibly identified in the relatively simple CNS of *Lymnaea*, providing a unique opportunity to compare nitric oxide release from single neurons in an intact but isolated CNS preparation.

The main reason for using the CNS of these invertebrates over mammalian systems is due to the ease of reproducible analysis and interpretation. The brain of *Lymnaea* is relatively simple compared to that of a mammal and consists of 11 ganglia and a total of ~25 000 neurons. The CNS of the pond snail has been extensively studied as the neurons are large (~100 μm) and pigmented,[16] aiding reproducible analysis.

EXPERIMENTAL SECTION

Chemicals. Fresh aqueous stock solutions of nitric oxide were prepared using nitric oxide gas (Sigma-Aldrich). Measurements were carried out in either phosphate-buffered saline (PBS) or *N*-(2-hydroxyethyl)piperazine-*N*′(ethanesulfonic acid) (HEPES)-buffered saline (consisting of 10 mM HEPES, 50 mM NaCl, 1.7 mM KCl, 2 mM $MgCl_2 \cdot 6H_2O$, and 4.0 mM $CaCl_2 \cdot 2H_2O$, buffered to pH 7.9). Chemicals for making these buffers were obtained from Sigma-Aldrich.

Sodium nitrite, uric acid, ascorbic acid, 3-hydroxytyramine (dopamine), 5-hydroxytrptamine (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and L-arginine were obtained from Sigma-Aldrich. These chemicals were used to assess the selectivity of the sensor and made freshly on the day of use in PBS buffer.

Preparation of Nitric Oxide Solution. Preparation of the stock nitric oxide solution was based on the method of Feelisch.[17] Briefly, the method utilized three Dreschel bottles and one gas collection jar, which has a side arm and a PTFE-coated septum. The nitric oxide was introduced via a two-way gas valve. All the cylinders were made from borosilicate glass, and connections were made using stainless steel tubing and PVC tubing. Prior to assembly all the glassware was rinsed in 5 M nitric acid and deionized water.

The setup is as follows: (i) The first Dreschel was used as an oxygen scrubber made using 2% ammonium metavandate in 1 M sulfuric acid with the presence of a mercury/zinc amalgam;[18] (ii) directly after the two-way valve for the introduction of nitric oxide gas were two bottles in sequence containing solid potassium hydroxide and 10% w/v potassium hydroxide solution, and (iii) the custom-built gas collection bottle (containing a frit and a screw cap to seal in the PTFE-coated septum) containing double-deionized water (~20 mL) which had also been filtered using a 0.2-μm pore filter.

Initially, a stream of nitrogen gas (White Spot, BOC) was purified through the oxygen scrubber, to deoxygenate the whole setup for 30 min. The valves were then switched, and nitric oxide was then delivered for 30 min to obtain a saturated stock solution. After removal of the sampling bottle, the seals were covered with Parafilm and the glass bottle was wrapped in aluminum foil to protect it from light. The final concentration of this solution was 1.93 mM based upon Henry's law.[17,19] The stock solution was stored at 4 °C. Samples were obtained using glass syringes via the septum and under positive pressure of nitrogen.

Electrodes. Electrode fabrication was based upon the work done by Miller and Pelling.[20] The 30-μm carbon fibers (World Precision Instruments, Inc., Sarasota, FL) were cut to the desired length. Carbon fibers were then sonicated in acetone for 15 min, cleaned in methanol and deionized water, and dried using nitrogen.

Glass capillaries (0.69-mm i.d., Clark Electromedical Instruments, Reading, UK) were pulled using a double pull on the pipet puller (model PP-830, Narishige, Tokyo, Japan), and then the tips were polished down on emery paper to facilitate the insertion of the carbon fiber. The capillaries where then rinsed with ethanol and deionized water and dried in nitrogen. The carbon fiber was then threaded through the pulled end of the glass capillaries.

Once the carbon fiber was threaded through, it was sealed within the capillary using epoxy resin (CY1301 epoxy resin and HY1300 hardener; Robnor Resins Ltd., Swindon, Wilts, UK). Drying and curing took 72 h at room temperature. Connection was achieved by contacting 0.25-mm silver wire via Wood's metal or conductive silver epoxy (Circuit Works).

Disk electrodes were prepared by insulating the shaft of the fiber with nail polish. The carbon fiber was then cut using a scalpel to expose a carbon fiber disk, which was coated with selective polymeric membranes.

Electrodes were initially coated with a Nafion film layer. The electrode was dip coated three times in a 5% v/v Nafion in ethanol solution and after each coating was left to air-dry. After the third coating, the electrode was heat cured at 200 °C for 4 min. The electrode was then placed in 0.1 M sodium hydroxide containing 10 mM eugenol (Fluka). The electrode was cycled at potentials between 0 and 1.25 V at a scan rate of 100 mV s^{-1} for 25 cycles (Figure 1) in order to electropolymerize the eugenol to the electrode surface. The 30-μm platinum electrodes were film coated using the same protocol used for carbon fiber electrodes, though without the use of Nafion.

o-PD nitric oxide electrodes were prepared initially with an additonal Nafion coating. Nafion was dip coated three times in a 5% v/v solution and after each coating was left to air-dry. After the third coating, the electrode was heat cured at 200 °C for 4 min. The *o*-PD was electropolymerized through the Nafion layer.

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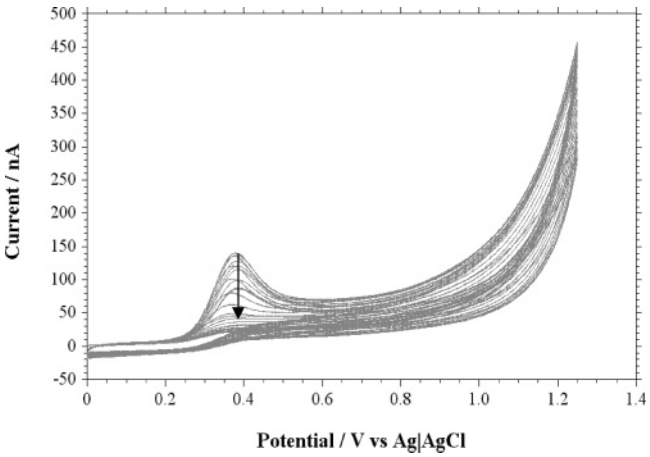

Figure 1. Cyclic voltammogram showing the polymerization of 10 mM eugenol on a 30-μm carbon fiber microelectrode. Arrow indicates increasing scan time.

The electrode was placed in a solution consisting of 5 mM *o*-PD (Sigma-Aldrich) and 100 μM ascorbic acid in PBS buffer. Coating was carried out at + 900 mV versus Ag|AgCl for 15 min. After modification, the electrodes were rinsed in deionized water and tested.

Data Acquisition. All electrode characterization measurements were carried out on a CHI 650A potentiostat (CH Instruments, Austin, TX). Differential pulse voltammetry (DPV) was employed to measure nitric oxide responses. Data collection and analysis were carried out on CH Instruments model 650A Electrochemical Analyzer software.

All DPV measurements were carried out between the potential range of 0.2 and 1.0 V versus Ag|AgCl (KCl (3 M)) reference electrode where the pulse width was 50 ms and pulse amplitude was 50 mV.

Sensor Characterization Methods. All in vitro experiments were steady-state measurements with the electrode potential held at +0.7 V. In order to test whether the electrode was responding to dissolved nitric oxide, differential pulse voltammograms were recorded while using nitrogen to sparge the nitric oxide from the solution. Any voltammetric features would be due to nitrite rather than nitric oxide.

The microelectrodes were assessed for selectivity and sensitivity. For sensitivity measurements, fixed aliquots (from 1 to 250 μL) of stock nitric oxide solution were added to 20 mL of PBS buffer (which had previously been deoxygenated under nitrogen for 30 min). The calibration plots were obtained using DPV. During all calibration experiments, the headspace of the electrochemical cell was nitrogen blanketed.

The selectivity of the electrodes was assessed using typical concentrations of a number of redox-active components found in the neurochemical matrix that can interfere with the measurement of nitric oxide.(4 μM serotonin, 4 μM dopamine, 4 μM DOPAC, 4 μM 5-HIAA, 100 μM ascorbic acid, 100 μM uric acid, 100 μM nitrite. 1 mM arginine).

Biological Preparation. *L. stagnalis* were bred in-house at the University of Brighton. Animals were kept in large tanks at 18−20 °C on a 12-h light/dark cycle in copper-free tap water. They were fed on alternate days with either lettuce or fish flakes (Tetra U.K. Ltd.). Animals were kept in groups of up to 600 in large circulating tanks at a stocking density of ~1 snail/L.

The isolated CNS preparation consisting of the main ganglionic ring, including the cerebral ganglia and a pair of buccal ganglia with a small piece of the pro-oesophagus still attached was removed from *Lymnaea* and then mounted in a Sylgard-lined chamber that was continuously perfused with HEPES-buffered saline, concentration (in mM) NaCl 50, KCl 1.7, $CaCl_2$ 4, $MgCl_2$ 2, HEPES 5, pH 7.8, at a rate of ~1 mL min^{-1}. The buccal and cerebral ganglia were desheathed using fine forceps to allow recordings from CGCs and the buccal motor neurons B2. The remaining connective tissue sheath covering the ganglia was enzymatically treated with 0.1% protease (Sigma type XIV).

Biological Experiments. For in vitro biological measurements, the electrode was placed on the soma of either the CGC or B2 motor neuron under microscopic observation with the aid of a micromanipulator. The Nafion/polyeugenol-coated 30-μm disk electrode was held at +850 mV using an Axoclamp 200B amplifier (Axon Instruments), which was sufficient for the oxidation of nitric oxide to be diffusion limited. The data were sampled at 100 Hz and digitized using a Micro 1401 A/D converter (CED, Cambridge, UK). Data storage and analysis was carried out using Spike 2 software (CED).

Various methods were used to evoke nitric oxide release: (i) 5-s depolarizing current pulses using an intracellular microelectrode, (ii) chemically using 3 mM arginine, and (iii) using a high-Ca^{2+}/high-K^+ buffer (consisting of 12.2 mM NaCl, 30 mM KCl, 10 mM $CaCl_2$, 2.0 mM $MgCl_2$, 10.0 mM HEPES, pH 7.9) to depolarize the cell. The superfusion pipet containing the high-Ca^{2+}/high-K^+ buffer was placed within 100 μm of the cell body, and cells were perfused with the buffer for 100 s at a rate of 0.2 mL min^{-1} to evoke nitric oxide release.

The ability of the competitive NOS inhibitor L-NAME to inhibit high-Ca^{2+}/K^+ evoked NO release was determined by perfusing the neuron with 5 mM L-NAME for 100 s prior to high-Ca^{2+}/K^+ stimulus. As a control for the mechanical stimulation potentially caused by superperfusion, neurons were superperfused with a normal HEPES-buffered Ringers and their effect on nitric oxide release was determined.

Data Analysis. Preliminary data showed that the response to nitric oxide was complex. Traces were recorded and viewed using Spike 2 software (CED), and Igor Pro software (WaveMetrics, Portland, WA) was used to analyze the peak area from each response. Mean peak areas of responses from both the CGC and B2 neurons were analyzed using a two-way ANOVA followed by post hoc Tukey tests (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Sensitivity and Selectivity. Square wave voltammetry of nitric oxide solutions in the range 0.1−10 μM showed a single, well-defined peak at 0.68 V. To confirm this peak was due to nitric oxide and not oxidation products, a stream of nitrogen was passed through the solution to sparge the nitric oxide (Figure 2). After 30 min of bubbling, there were no faradic features confirming the stability of the calibration protocol, consistent with the peak at 0.68 V being due to nitric oxide and confirming stability of the saturated solution, with respect to oxidation.

The linear range over which the Nafion/polyeugenol microelectrode could detect nitric oxide ranged from 100 nM to 10 μM; thus, two different calibration curves were generated to deal with the change in order of magnitude of response. The limit of

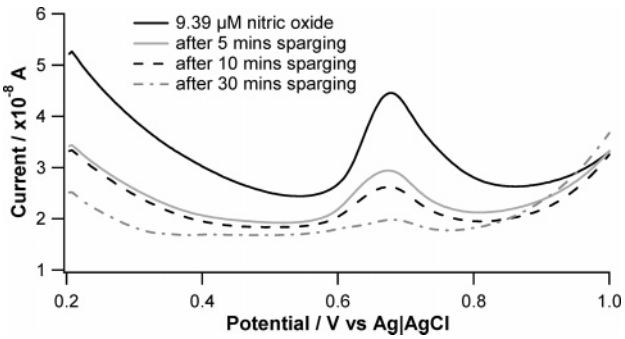

Figure 2. Square wave voltammograms showing a clear nitric oxide peak at +680 mV, which shows decreases following sparging with nitrogen for the times shown.

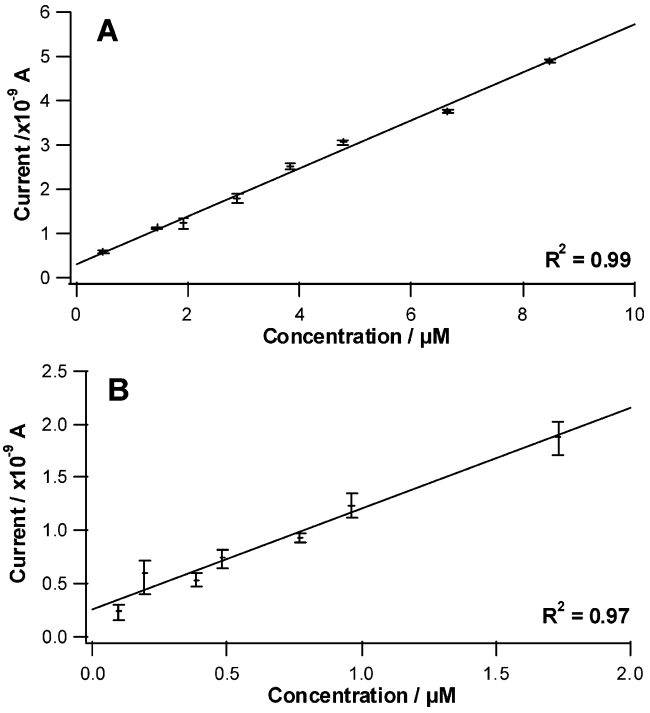

Figure 3. Calibration working curve of nitric oxide using Nafion/polyeugenol carbon fiber disk microelectrode. (A) shows the response of the Nafion/poly enugenol microelectrode between 10 and 1 μM (n = 3, mean ± range), and (B) shows responses in the concentration range of 75 nM to 2 μM (n = 3, mean ± range). Experiments carried out in deoxygenated PBS buffer.

detection (defined as 3 times the signal-to-noise ratio) based upon the response from Figure 3A is 11.3 nM with a sensitivity of 5.39 nA μM^{-1}, while for the calibration curve dealing with lower concentrations (Figure 3B), the detection limit was 2.8 nM with a sensitivity of 9.46 nA μM^{-1}. This was far better than other electrodes tested in the same disk electrode geometry. The poly-*p*-eugenol-coated platinum electrodes had a limit of detection around 420 nM while the Nafion/*o*-phenylenediamine electrode was slightly better, with a limit of detection around 51 nM. The Nafion/polyeugenol electrode coating used provides a more stable background and allows for a lower limit of detection compared to these previously reported and well-characterized electrodes.[4,11−13]

These results are similar to those reported by other groups for other major selective coated electrodes used for the detection of nitric oxide; however, they are based upon using protruding tip-based microelectrodes. Protruding tip geometry presents two major problems: (i) in biological systems, analyte concentration is highly inhomogeneous on the length scale of the fiber and thus current traces cannot be interpreted quantitatively, and (ii) the shanks of carbon fibers show electrocatalytic properties different from the exposed cross section.[21] Nafion/*o*-phenylenediamine electrode has a limit of detection of 35 ± 7 nM.[4,13] The sensitivity of the electrode is also far better than chemically modified nitric oxide electrodes that incorporate a electropolymeried nickel porphyrin film coating for which a linear response up to 300 μM nitric oxide and a detection limit of 10 nM have been claimed.[3]

Table 1. Response of Interference Relative to Nitric Oxide Concentrations

compound	% response (relative to equiv concns)
4 μM 5-HIAA	21.10
4 μM DOPAC	8.70
4 μM serotonin	54.52
4 μM dopamine	49.20
100 μM Ascorbic acid	0.62
100 μM uric acid	0.09
100 μM nitrite	0.47
1 mM arginine	0

The selectivity of the electrode was assessed against known matrix interferents. The responses are shown in Table 1. The sensor shows excellent selectivity against the matrix components ascorbic acid and uric acid, but also against a major oxidation product of nitric oxide which is nitrite. The electrode, however, is less selective against monoamines or their metabolites. However, in a previous publication,[22] we have shown the concentrations of the monoamine metabolites to be 1 order of magnitude lower than the monoamines and they should therefore present no problem in this preparation.

Selectivity over dopamine and serotonin was less good. However, for measurements in the pond snail, both dopamine and serotonin are released vesicularly in events typically lasting 1−5 ms. This major difference in time scales allows nitric oxide to be followed in the snail without distortion by dopamine and serotonin by the use of appropriate data acquisition rates and filtering.

There have been limited investigations on the selectivity of nitric oxide electrodes; however, the selectivity of our electrode is similar to other published methods, where reported.[4,10−12]

Methods To Evoke Nitric Oxide Release. In a series of experiments, we examined whether we could record nitric oxide release from identified CNS neurons from the pond snail *L. stagnalis*. Measurements were carried out on B2, which has been shown indirectly by Moroz[23] to have the greatest amount of NOS activity in *Lymnaea* and therefore was expected to produce the greatest flux of nitric oxide.

A nonphysiological chemical stimulation using high-Ca^{2+}/high-K^+ HEPES-buffered saline was used to increase cystolic calcium

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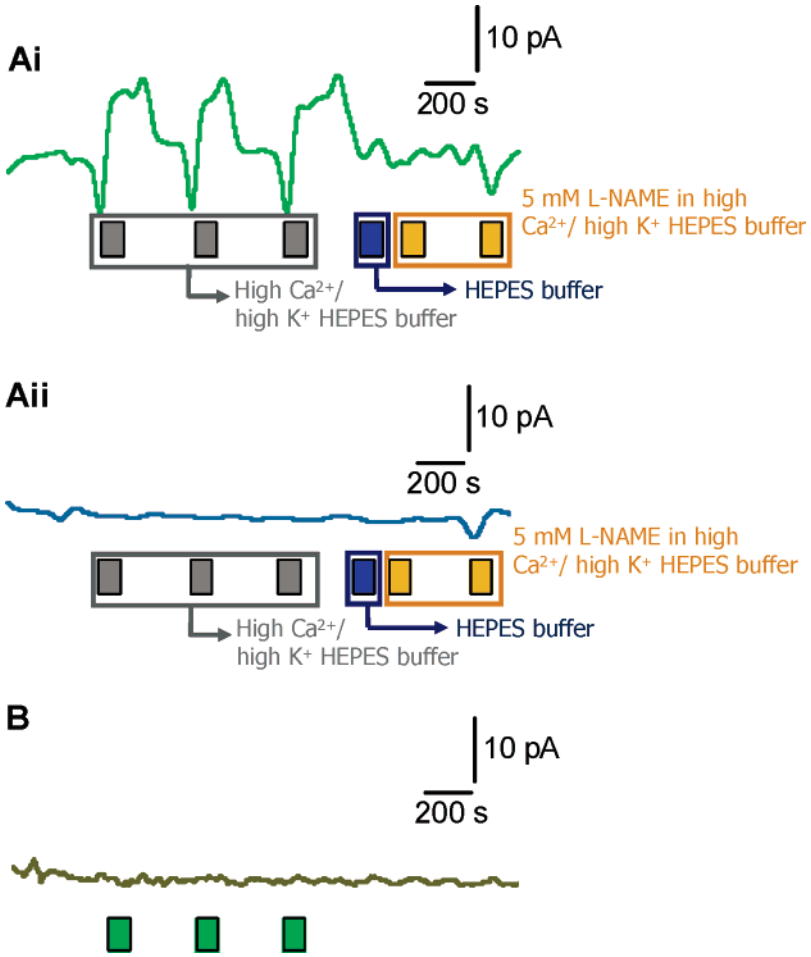

Figure 4. Nitric oxide release from neurons following chemical stimulation. (A) shows chemical stimulation using high-K^+/high-Ca^{2+} HEPES-buffered saline evoked a series of consistent responses that were inhibited using 5 mM L-NAME. (Ai) shows response from B2 cell from a young animal, and (Aii) is a control blank trace. In (B), no clear responses were evoked by 1 mM arginine to the B2 soma. All measurements carried out at +850 mV vs Ag|AgCl using a 30-μm Nafion/polyeugenol carbon fiber disk microelectrode. Application of high-K^+/high-Ca^{2+} HEPES in gray, the blank response in blue, and the application of the competitive inhibitor L-NAME shown in orange.

concentration, thus increasing nitric oxide release. Release was also attempted using arginine, which is converted to citrulline and nitric oxide by NOS. Increasing levels of arginine should increase the output of the enzymatic reaction thus increasing nitric oxide levels. Figure 4 shows a typical series of responses recorded from a young B2 neuron following chemical stimulation.

High-Ca^{2+}/high-K^+ buffer was applied by superperfusion on the neuronal preparation. On occasions, the jet would impinge on the sensor surface causing a transient drop in anodic current due to double layer effects. The phenomenon is observed in buffer as well as on the snail neurons (Figure 4).

The results showed that the high-Ca^{2+}/high-K^+ buffer was capable of reproducibly inducing reproducible responses that were completely blocked following the perfusion of 5 mM L-NAME, confirming that the recorded signal was NO. These results support the observations of Moroz,[15,23] who showed B2 to contain NOS using NADPH-daphorase staining and immunohistochemistry. Release from the B2 cell was completely inhibited using the NOS inhibitor L-NAME in high-Ca^{2+}/high-K^+ buffer. Application of arginine to the same cell failed to evoke measurable responses, which may be due to the high levels of arginine (6 mM) present within the neuron.[24]

Attempts were also made to evoke nitric oxide release using electrical stimulation on the B2 neuron by depolarizing the neuron cell body using an intracellular probe but were unsuccessful. Measurable release could not be evoked by electrical stimulation. The most likely explanation for this is that the levels of nitric oxide released are too low to detect after electrical stimulation. No other investigators have detected nitric oxide release following electrical stimulation. However, we cannot rule out that release is occurring at some other site in the neuron since we measured only at the cell body.

Responses from CGC and B2 Neuron. Figure 5 shows high-Ca^{2+}/K^+ evoked responses from both the B2 motor neuron and CGC. The high-Ca^{2+}/high-K^+ stimulus evoked a greater release (area under response) in the B2 cell than the CGC (Figure 5Ai, Tukey test; $p < 0.01$). Moroz et al.[23] showed, using high-performance liquid chromatography, that the activity of NOS in the B2 cell was greater than that of the CGC; thus, more nitric oxide release is expected from the B2 neuron. The NADPH-d staining shows a greater intensity of the NOS protein in the B2 compared to the CGC,[15] which may explain the higher amount of

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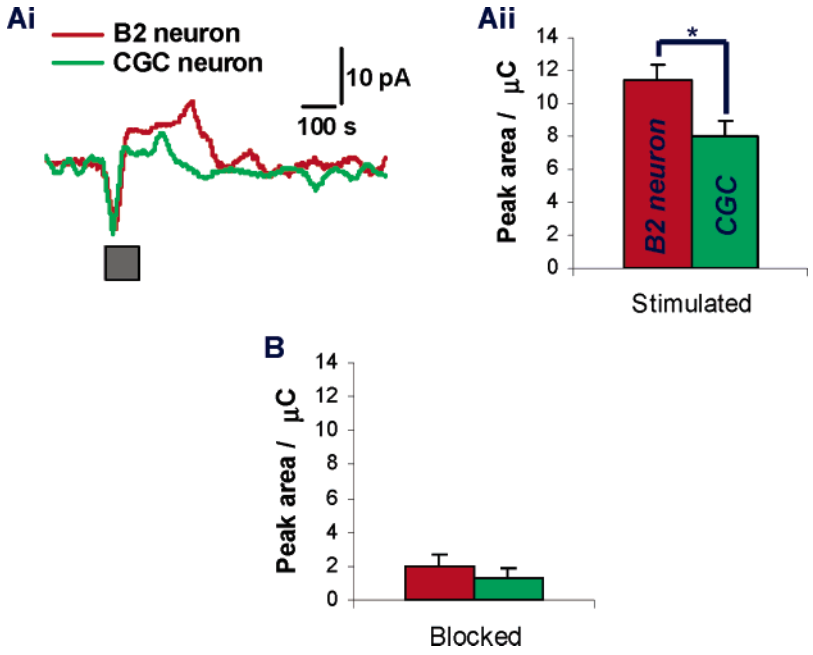

Figure 5. High-Ca^{2+}/K^+ saline evoking different amounts of nitric oxide release from the B2 and CGC cells. (Ai) shows a typical response stimulated using high-Ca^{2+}/high-K^+ ringer, from both B2 and CGC neurons. Mean peak area responses from both cells are shown in (Aii). In (B), the responses from the B2 neuron and CGC are shown after application of the competitive NOS inhibitor L-NAME. All results shown in red are from the B2 neuron, while all CGC results are shown in green. Results represented as mean ± SEM (*$p < 0.01$) and $n = 8$.

nitric oxide observed (Figure 5). Both cells showed a similar level of inhibition from the NOS antagonist L-NAME, thus indicating that the signal detected is that of nitric oxide (Figure 5B).

The differential amount of release from the two cells could be due to the function of the cell within the neuronal network. Nitric oxide in the B2 cell is know to be a major neurotransmitter,[25,26] while in the CGC there is no evidence that it is used routinely as a transmitter and therefore may be involved in modulating the activity of the CGCs. Its precise role remains to be determined. The CGC neuron is known to contain various other transmitters (serotonin and myomodulin[27,28]), which may be evoked during nitric oxide release.

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CONCLUSIONS

Neuronal nitric oxide release has been detected using a sensitive and selective film-coated nitric oxide electrode. Nitric oxide release has been observed from two different neurons in an intact neuronal preparation using a high-Ca^{2+}/high-K^+ stimulus. The B2 neuron released more nitric oxide than the CGC neuron; however, both cells were equally suppressed by equivalent concentrations of the NOS inhibitor L-NAME.

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