Nitric oxide potentiates cAMP-gated cation current by intracellular
acidification in feeding neurons of Pleurobranchaea

by

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Abstract

A pH-sensitive cAMP-gated cation current ($I_{Na,cAMP}$) is widely distributed in neurons of the feeding motor networks of gastropods. In the sea-slug Pleurobranchaea this current is potentiated by nitric oxide (NO), which itself is produced by many feeding neurons. The action of NO is not dependent on either cGMP or cAMP signaling pathways (Hatcher et al., 2006). However, we found that NO potentiation of $I_{Na,cAMP}$ in the serotonergic metacerebral cells could be blocked by intracellular injection of MOPS buffer, pH 7.2. In neurons injected with the pH indicator BCECF, NO induced rapid intracellular acidification to several tenths of a pH unit. Intracellular pH has not previously been identified as a specific target of NO, but in this system NO modulation of $I_{Na,cAMP}$ via pH$_i$ may be an important regulator of the excitability of the feeding motor network.

Keywords: pH$_i$, proton, neuromodulation, Pleurobranchaea
**Introduction**

Many gastropod neurons express a cAMP-induced sodium current \(I_{\text{Na,cAMP}}\) (Green and Gillette, 1983; Connor and Hockberger, 1984a) activated by direct cAMP gating (Sudlow et al., 1993). The current takes two forms – Types 1 and 2, differentiable on the basis of their voltage dependence (Gillette and Green, 1987; Huang and Gillette, 1993). Observations that cell signaling pathways could target pH\(_i\) in molluscan neurons (Connor and Hockberger; 1984b) was followed by finding that the Type 1 \(I_{\text{Na,cAMP}}\), most often found in neurons of the feeding motor network of *Pleurobranchaea*, was highly sensitive to small changes in pH\(_i\), where current amplitude was increased with acidification (Green and Gillette, 1988; also see Aldenhoff, et al., 1983). The Type 2 \(I_{\text{Na,cAMP}}\) form, found in pedal ganglion neurons, was entirely insensitive to pH\(_i\) (Huang and Gillette, 1991, 1993).

The functional benefit of pH sensitivity of \(I_{\text{Na,cAMP}}\) has been a question without an obvious answer since its discovery. However, recently the pH sensitive \(I_{\text{Na,cAMP}}\) was shown to be highly sensitive to potentiation by NO activity, while the pH-insensitive form was not (Hatcher, 2003; Hatcher et al., 2006). Thus, NO excited many neurons of the feeding motor network expressing both \(I_{\text{Na,cAMP}}\) and apparent resting levels of cAMP, but lacked detectable actions on the pedal locomotor neurons.

NO potentiation of \(I_{\text{Na,cAMP}}\) was not dependent on cGMP, as it was not mimicked or blocked by cGMP analog, or by inhibitors of cGMP synthesis orPKG (Hatcher et al., 2006). Effects were specific to NO, as they were induced by
several different donors and abolished by depletion of donor through exposure to
air at room temperature. NO synthase activity is considerable in the feeding
network of *Pleurobranchaea* (Moroz et al., 1996; Cruz et al., 1997; Floyd et al.,
1998).

We asked whether NO might act through altering intracellular pH. To
study potential effects of NO on pH$_i$, we used the same well-studied, identified
neuron previously used to characterize NO-induced potentiation of I$_{Na,cAMP}$
(Hatcher et al., 2006), the serotonergic metacerebral giant neuron (MCG; Gillette
and Davis 1977; McCrohan and Gillette 1988). This neuron is conserved in
cerebral ganglion lobes of many gastropods and provides neuromodulatory
excitation to the feeding motor network (Gillette and Davis, 1977).

Clamping pH$_i$ blocked the potentiating effects of NO on I$_{Na,cAMP}$. Using
indicator dyes, we observed that NO induced rapid intracellular acidification.
These results clarify a mechanism of the excitatory effects of NO on the feeding
motor network of *Pleurobranchaea*, as well as documenting a novel mechanism
for NO action in biological systems.

**Methods**
Specimens of *Pleurobranchaea californica* were obtained from Sea Life
Supply (Sand City, CA) and maintained in Instant Ocean at 12-13$^\circ$ C. The cell
bodies of the identified metacerebral giant neurons (MCGs) were visually
identified by their large size and anterior-most position in cerebropleural ganglia,
isolated by hand dissection, and stabilized in a perfusion dish against carbon
fiber pins. For most experiments, the cell was perfused with MOPS-buffered saline (composition in mM: NaCl 420, KCl 10, MgSO$_4$ 25, MgCl$_2$ 25, CaCl$_2$ 10, 3-(N-morpholino)propanesulfonic acid (MOPS) 5, at pH 7.5) at a rate of 1 ml min$^{-1}$ at 13 ± 1$^\circ$ C. Bicarbonate saline was made 30 mM in NaHCO$_3$, substituting equimolar for NaCl, and adjusted to pH 7.5.

$I_{\text{Na,cAMP}}$ responses to iontophoretic injection of cAMP were measured under two electrode voltage clamp as described previously (Hatcher et. al, 2006, Sudlow and Gillette, 1997). Briefly, sharp electrodes were pulled from borosilicate glass (WPI). A single-barreled capillary filled with 3M KCl served as a voltage-sensing electrode. A second, double-barreled, electrode acted as the current passing electrode (filled with 3 M KCl) in one half and a cAMP iontophoresis capillary in the other (filled with 200 mM cAMP and 20 mM Tris, adjusted to pH 7.5 with KOH). Iontophoretic injection of cAMP (5 s duration; 5-200 nA) was accomplished with negative current from a constant current source (Model 260, WPI) that allowed precise, repeatable quantities of cAMP to be injected intracellularly.

Injection amplitudes were kept relatively low to avoid current saturation while yielding a robust $I_{\text{Na,cAMP}}$ response. Under our conditions, cAMP injection occurs with a constant transport number of 0.1 over the wide range of injection currents used here (Sudlow and Gillette 1997). The kinetics of $I_{\text{Na,cAMP}}$ are largely determined by intracellular cAMP diffusion and phosphodiesterase activity (Huang and Gillette 1991). $I_{\text{Na,cAMP}}$ measures in the presence of DEA/NO were begun
around 10 min. after addition of NO donor, at which time responses were near maximal and relatively constant in absence of blocking treatment.

Earlier work in neurons of the mollusk *Archidoris* showed that pressure-injected cAMP to values of 0.1 - 1 mM could acidify intracellular pH (pHi) in the range of 0.1 pH unit, an effect likely mediated by cAMP dependent kinase (Connor and Hockberger; 1984b). Such an effect could add interesting dimension to $I_{Na,cAMP}$ functioning, but was not visible in these present studies. A preceding study by Hatcher et al. (2006) did not find effects of NO on pH-sensitive $I_{Na,cAMP}$ to be dependent on cAMP beyond a simple gating role for the current, perhaps because iontophoretic injections were smaller (Sudlow and Gillette, 1997) and were not previously found to cause significant changes in pHi themselves (Green and Gillette, 1988).

Intracellular recording and voltage clamping were performed with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA), and data were recorded digitally with Biopac hardware and the accompanying Acknowledge software package (Biopac Systems, Inc., Goleta, CA). All recordings were performed at a holding potential of -50 mV unless otherwise indicated.

For fluorescence measurements, glass microelectrodes were pulled to resistances in the range 5-15 MΩ when filled with 200 mM KCl, 10 mM MOPS and the fluorescent pH indicator BCECF (2’,7’-bis(2-carboxyethyl)-5(6)-carboxy fluorescein; Sigma) pressure injected from an electrode containing 1 mM dye. Following injection, the electrode was withdrawn and the cell was penetrated with
an electrode filled with 3 M KCl for electrical recording via an Axoclamp 2A amplifier and Clampex 8.1 software (Axon Instruments).

Fluorescence measurements were made using an InCyt dual-wavelength fluorescence imaging and photometry system (sampling rate 10 per min; Intracellular Imaging Inc.) with a Sutter Lambda 10C optical filter changer (Sutter Instrument Co.) and InCyt Im2 acquisition software. For BCECF measurements, a calibration curve for the 488:436 nm fluorescence ratio was generated using a series of MOPS-saline standards, pH 6.5 to 8. These standards proved reliably stable over time. A slow negative (i.e., acidic) drift occurred in ten pH recordings, averaging $0.007 \pm 0.002$ pH units min$^{-1}$ (mean $\pm$ SD), suggesting that slow dye bleaching or leakage was leading to a greater signal contribution by autofluorescence. This artifact did not interfere with observation of the more rapid dynamic changes caused by NO. Resting membrane potentials in these experiments were -45 to -55 mV.

MOPS was pressure injected as a 1 M solution, adjusted to pH 7.2 with KOH. Injection volumes were estimated at 10% or more of cell volume; thus, injections are likely to have raised cell buffering power to 4 or more times resting values (cf. Table II, Ahmed and Connor, 1980). Injection sequences were composed of multiple small injections where pressures were maintained until a small polarizing membrane response indicated injection success. Subsequent injections were made approximately a minute after membrane potential recovered. Dye injections were performed similarly. The NO donor diethylamine/nitric oxide (DEA-NO, 1-2 mM; Axxora LLC) was applied in the
perfused saline. When measured using a calibrated NO electrode (WPI), freshly prepared 1 mM DEA-NO in *Pleurobranchaea* saline at this temperature yielded a value of 430 nM of NO, with first-order exponential decay of $t_{1/2} = 418$ seconds (Hatcher et al., 2006). Usually 1-2 half-lives were passed by the time perfusion occurred, so actual NO concentrations may have been in the range of 100-200 nM.

**Results**

NO donors potentiate the $I_{Na,cAMP}$ response to iontophoretically injected cAMP in neurons of the feeding motor network (Hatcher et al., 2006), including the MCG neurons (Fig. 1A). In a total of 16 MCG neurons the NO donor DEA-NO enhanced the $I_{Na,cAMP}$ response on average by 85% (24% SEM) over control, pre-donor measures ($p < 0.001$; 2-tailed t-test). Intracellular acidification of a few tenths of pH unit was previously shown to have a similar effect (Green and Gillette, 1988), which is recapitulated in the records of Figure 1B. In the present study, bicarbonate-containing salines increased $I_{Na,cAMP}$ amplitude in four neurons by 71.7% (6.3 % SEM). Thus, intracellular acidification mimics the enhancing effect of NO donors on $I_{Na,cAMP}$.

A likely causal linkage of NO and pH$_i$ was supported by finding in each of four cells tested that intracellular injection of 1 M MOPS buffer at pH 7.2, in volumes estimated near 0.1X soma volume, eliminated the potentiating effect of NO on $I_{Na,cAMP}$ amplitude (Fig. 2; average change 10% (5% SEM); $p = 0.30$, 2-tailed t-test. Similar intracellular injections of BCECF (see below) did not block
NO induced depolarization or NO effects on pH$_i$. Thus, a non-specific effect of simple pressure injection was unlikely to be a significant factor in the pH$_i$ response. The blockade of NO effects by intracellular pH buffering was significant relative to the positive effects of NO donor on the 16 uninjected MCGs (p = 0.0003; Fishers Exact Test, 2-sided), and led to the following assessment of the effects of NO on pH$_i$.

In 6 of 8 MCG somata injected with the pH-indicator dye BCECF, initial pH$_i$ lay close to an averaged 7.0 (0.08 SEM; values for the remaining 2 cells were not attained). Bath application of DEA-NO caused strong and rapid intracellular acidification in all 8 cells (Fig. 3). Peak response amplitudes were attained within 13-16 minutes of donor application, and ranged from 0.16 to 0.46 pH units (mean 0.22; SEM 0.04; N=8 cells). Decay of the pH response was monitored for 7 cells following washout of DEA-NO, and lasted up to 26 minutes with an average half-time to estimated full recovery of 6.5 minutes (SEM 0.72). The minutes-long time course of acidification and recovery of pH$_i$ was comparable to observations of effects of NO and its slow washout on $I_{Na,cAMP}$ amplitudes (cf. Fig. 4, Hatcher et al., 2006). The recovery of pH$_i$ was consistent with the slow recovery times previously observed following acidification in mollucan neurons (Ahmed and Connor, 1980; Thomas and Meech, 1982).

**Discussion**

The results show that NO promotes intracellular acidification in neurons whose excitatory state is markedly sensitive to pH$_i$. This demonstration is
significant in providing a functional answer to a long-standing question concerning the likely import of the marked pH sensitivity of $I_{Na,cAMP}$ in gastropod neurons (Aldenhoff et al. 1983; Green and Gillette 1988).

$NO/pHi$ regulation of Type 1 $I_{Na,cAMP}$

Until now, no clearly relevant functional context for the pH sensitivity of $I_{Na,cAMP}$ has been available. We previously documented two forms of $I_{Na,cAMP}$. One is the pH-sensitive Type 1 form found in many neurons of the feeding motor network in the opisthobranch gastropods *Pleurobranchaea* (Green and Gillette 1983, 1988), *Navanax* and *Aplysia* (unpublished results), and in the pulmonate snails *Helix* (Aldenhoff et al. 1983) and *Lymnaea* (McCrohan and Gillette 1988).

This current is a highly sensitive indicator of $pHi$, responding with measurable amplitude change to $\Delta pHi$ of 0.03 unit and closely following induced changes in $pHi$ (Green and Gillette 1988). Two unpublished observations in excised patches indicate a population of pH-sensitive $I_{Na,cAMP}$ channels (Sudlow and Gillette). In contrast, the Type 2 form of $I_{Na,cAMP}$, so far found mainly in neurons of the locomotor network (Huang and Gillette, 1993), is both relatively insensitive to changing $pHi$ and to the potentiating effects of NO (Hatcher et al., 2006).

Demonstration of NO-induced acidification in the MCG provides a mechanism for potentiation of Type 1 $I_{Na,cAMP}$ by NO in the same neuron (Hatcher et al., 2006). It also provides a likely pathway for the ability of exogenous NO to activate the feeding motor network (Moroz et al., 1995). The MCG itself is a neuromodulatory excitor of the feeding network (Gillette and Davis, 1977). It lacks
chemical markers (unpublished) characteristic of nitrergic neurons (Cruz et al. 1997; Floyd et al. 1998), and is thus more likely NO target than source. NO has roles in activation of the feeding motor network in diverse gastropod species (Moroz et al. 1993; Elphick et al., 1995; Park et al. 1998) but its potentiation of $I_{Na,cAMP}$ has as yet only been documented in *Pleurobranchaea*.

Significance

NOS activity in *Pleurobranchaea*’s CNS is high, comparable to that of mammalian brain when assayed *in vitro* (Moroz et al. 1996), and localized to numerous neurons in the feeding and locomotion motor networks (Cruz et al. 1997; Floyd et al. 1998b). The presence of NOS in the feeding motor network and the ability of NO to activate the feeding motor rhythm suggest a normal role in regulating the excitability of the feeding neurons, acting on $I_{Na,cAMP}$ through pH$_i$. Mechanisms of NO action in altering pH$_i$ remain to be explored.

These results indicate a novel action for NO action in modulating neuronal excitability through intracellular acidification. A similar, although slower, acidification response to NO donors was observed in cultured hippocampal neurons, leading to apoptosis (Vincent et al.; 1999). However, effects of NO in the cultured neurons were protein kinase G-dependent (Maiese et al., 1993), unlike for the MCG neurons of *Pleurobranchaea* (Hatcher et al., 2006). Extensive literature search has otherwise failed to turn up similar results. Thus, NO effects on pH$_i$ in this system are likely to reflect a new signaling pathway.
Intracellular H⁺ may interact in complex ways with other signaling pathways and metabolic pathways in the cell to add further dimension to cell signaling and neuronal function. Injections of cAMP in neurons of the mollusk *Archidoris* were found to acidify pH up to 0.1 pH unit, an effect likely mediated by cAMP dependent kinase (Connor and Hockberger; 1984b). Ca²⁺ influx during spike activity can acidify neurons through activation of Ca²⁺/H⁺ exchange mechanisms (Ahmed and Connor, 1980; Thomas, 2009), which in fine neuritic processes can cause swings in pH up to several tenths of a unit (Schwiening and Willoughby, 2002). Such pH shifts can modify many aspects of pH-sensitive ion channels and metabolic processes.

In this system, at levels of cell and circuit NO-induced acidification is liable to amplify effects of neuromodulator induced cAMP. Thus, our emerging picture of the functional role of NO in the feeding motor network of *Pleurobranchaea* is one where it acts as a gain-enhancing mechanism, boosting the cellular responses to neuromodulator-induced fluctuations of cAMP. Such gain control will affect the activity state of the feeding network in large part through broad modulation of pH-sensitive $I_{Na,cAMP}$ in its neuronal elements.

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**References**


**Figure legends:**

**Figure 1.** Potentiation of the I\(_{\text{Na,cAMP}}\) response amplitude to injected cAMP by NO donor and intracellular acidification, and NO-induced depolarization. **A.** DEA/NO (1 mM) augmented I\(_{\text{Na,cAMP}}\) response amplitude by 78% relative to control measures in saline alone taken just prior to addition of the NO donor. **B.** Saline containing 30 mM bicarbonate mimicked effect of NO donor, increasing I\(_{\text{Na,cAMP}}\) amplitude by 41%. Such bicarbonate salines acidify *Pleurobranchaea* neurons by several tenths of a pH unit (Green and Gillette, 1988). Holding potentials were -50 mV. Horizontal bars in records indicate 5 sec. 100 nA iontophoretic injections of cAMP.

**Figure 2.** Intracellular injection of the pH buffer MOPS suppresses NO-potentiation of I\(_{\text{Na,cAMP}}\). **A.** I\(_{\text{Na,cAMP}}\) responses to injected cAMP in normal saline (left), after MOPS injection and in presence of NO donor. Holding potentials were -50 mV. **B.** A typical dose-response relation for cAMP injection and I\(_{\text{Na,cAMP}}\)
response, showing blockade of NO effects over a broad range of injection amplitude.

**Figure 3.** Nitric oxide stimulated rapid intracellular acidification in isolated MCG somata, as measured with BCECF fluorescence. Bath application of the NO donor, DEA-NO (1 mM, 10 min, solid bar) to a soma in saline at pH 7.5 caused an acidification of pH<sub>i</sub> greater than 0.3 pH unit, peaking around 15 minutes after donor application and decaying slowly over minutes following washout.