Nitric Oxide Potentiates cAMP-Gated Cation Current in Feeding Neurons of
Pleurobranchaea californica Independent of cAMP and cGMP
Signaling Pathways

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Hatcher, Nathan G., Leland C. Sudlow, Leonid L. Moroz, and Rhanor Gillette. Nitric oxide potentiates CAMP-gated cation current in feeding neurons of Pleurobranchaea californica independent of cAMP and cGMP signaling pathways. J Neurophysiol 95: 3219–3227, 2006. First published February 15, 2006; doi:10.1152/jn.0815.2005. Critical roles for nitric oxide (NO) in regulating cell and tissue physiology are broadly appreciated, but aspects remain to be explored. In the mollusk Pleurobranchaea, NO synthase activity is high in CNS ganglia containing motor networks for feeding and locomotion, where a cAMP-gated cation current (I_{Na,cAMP}) is also prominent in many neurons. We examined effects of NO on I_{Na,cAMP} using voltage-clamp methods developed to analyze cAMP signaling in the live neuron, focusing on the identified metacerebral giant neuron of the feeding network. NO donors enhanced the I_{Na,cAMP} response to injected cAMP by an averaged 85%. In dose-response measures, NO increased the current stimulated by cAMP injection without altering either apparent cAMP binding affinity or cooperativity of current activation. NO did not detectably alter levels of native cAMP or synthesis or degradation rates as observable in both current saturation and decay rate of I_{Na,cAMP} responses to cAMP injection. NO actions were not exerted by cGMP signaling, as they were not mimicked by cGMP analogue nor blocked by inhibitors of guanylate cyclase and protein kinase G. NO potentiation of I_{Na,cAMP} was broadly distributed among many other neurons of the feeding motor network in the buccal ganglion. However, NO did not affect a second type of I_{Na,cAMP} found in locomotor neurons of the pedal ganglia. These results suggest that NO acts through a novel mechanism to regulate the gain of cAMP-dependent neuromodulatory pathways that activate I_{Na,cAMP} and may thereby affect the set points of feeding network excitability and reactivity to exogenous input.

INTRODUCTION

Actions of the ubiquitous cellular messenger nitric oxide (NO) are reflected in many facets of nervous system function, and new roles and mechanisms continue to emerge. Gastropod mollusks present markedly accessible systems for the integrated study of NO, where its actions are amenable to broad analysis at levels spanning single neurons to behavior (Elphick et al. 1995; Gelperin 1994; Jacklet and Tiemann 2004; Katzoff et al. 2002; Kornenev et al. 2002). In these animals, NO and its synthetic enzyme NO synthase (NOS) are notably associated with sensory pathways, neural networks, and anatomical structures that serve feeding behavior (cf. Moroz 2000).

Previous work in invertebrate systems has largely concerned the regulation of membrane conductances and synaptic release through the signaling pathway involving cGMP (Kemenes et al. 2002; Koh and Jacklet 1999; Mothet et al. 1996) as have most studies in mammalian brain. However, NO is “promising” with respect to its functional roles, signaling pathways, and mechanisms of action in different biological systems, and new aspects of its physiology continue to be uncovered. In the predatory sea-slug Pleurobranchaea, NOS activity in the CNS is quite high and tentatively localized to numerous neurons in the feeding and locomotor motor networks (Cruz et al. 1997; Floyd et al. 1998; Moroz and Gillette 1996; Moroz et al. 1996). It is notable that many of those neurons also express a CAMP-gated sodium current (I_{Na,cAMP}) that contributes significantly to the excitable properties of the neurons (Green and Gillette 1983; Sudlow et al. 1995). The co-occurrence of I_{Na,cAMP} and NO led us to investigate NO effects on this ion current as a potential mechanism for regulating neuron excitability, particularly in the feeding motor network. We focused analyses on the identified serotonergic metacerebral giant interneurons (MCGs) of the feeding motor network but surveyed NO sensitivity of I_{Na,cAMP} across many neurons.

Prior work has characterized the kinetics of the I_{Na,cAMP} response to injected cAMP in terms of activation, inactivation, and saturation, thereby establishing the use of I_{Na,cAMP} as a reporter for cAMP in the whole cell. The mathematical models derived and tested permit precise quantitation in molar terms for cAMP metabolism for synthesis, degradation, and concentration (Huang and Gillette 1991, 1993; Sudlow and Gillette 1995, 1997; Sudlow et al. 1993). Thus we took advantage of the exceptional opportunity in this system to investigate the possible interactions of the signaling pathways of NO and cAMP in the living neuron.

Two forms of I_{Na,cAMP} have been identified in the CNS of Pleurobranchaea. These two forms are differentially located in neurons of the feeding motor network of the buccal and cerebropleural ganglia (Type 1) and in locomotor neurons of the pedal ganglia (Type 2). The two currents are similar in ion selectivity and cAMP requirement but differ markedly in their voltage dependencies and sensitivities to Ca^{2+} and H^{+} (Green and Gillette 1983, 1987, 1988; Huang and Gillette 1993). Here we report that NO potentiated the Type 1 but not the Type 2 form. The enhancement of the Type 1 I_{Na,cAMP} was novel in its independence from mediation by cGMP and was not caused by altered cAMP synthesis, degradation, or sensitivity of current activation. NO-induced potentiation of I_{Na,cAMP} presents a novel mechanism of neuromodulation.
Methods

Pleurobranchaea californica (80–500 g) were obtained from Sea Life Supply (Sand City, CA) and maintained at 12–13°C in a closed, circulating sea-water system. For CNS dissections, animals were anesthetized by chilling to 4°C. The cerebropleural, pedal, and buccal ganglia were removed and axotomized soma (80- to 400-µm diam) were prepared as previously for voltage clamping (Sudlow and Gillette 1995, 1997). Axotomized somata were placed in a recording chamber (1 ml) continuously superfused at 12–13°C with Pleurobranchaea saline (in mM: 420 NaCl, 10 KCl, 25 MgCl2, 25 MgSO4, 10 CaCl2, and 10 MOPS (3-N-morpholino-propanesulfonic acid), adjusted to pH 7.5 with NaOH.

Solutions of the NO donors diethylamine/nitric oxide complex (DEA/NO), spermine-NO complex (NO-spermine), and S-nitroso-N-acetylpenicillamine (SNAP; Research Biochemicals International, Natick, MA) were prepared in Pleurobranchaea saline immediately before use. Actual NO concentrations in physiological solutions are usually uncertain given that NO donors are sensitive to variables such as temperature and considering that NO gas oxidation rate is dependent on the partial pressure of O2. We checked NO concentrations in our experimental chamber with NO-sensitive electrodes (ISO-NOP detector with 100-nm-tip electrode sensors; WPI) World Precision Instruments, Sarasota, FL). Electrodes were calibrated in N2-bubbed saline solutions of DEA/NO yielding known concentrations of NO in anoxic conditions. In aerated saline (1 ml) at 12°C in the voltage-clamp preparation chamber, the relation between NO and DEA/NO concentrations was linear between 0 and 2 mM. DEA/NO (1 mM) in the recording chamber measured in this manner initially yielded 430 nM NO with first-order exponential decay (t½ = 418 s). Unless otherwise stated, experimental treatments were performed with this concentration of DEA/NO. Experimental exposures to DEA/NO rarely lasted much longer than a single half-life for DEA/NO (although see Fig. 4). However, it seems unlikely that changing NO levels affected the qualitative outcomes of these experiments given both the near saturation effects of DEA/NO at the concentration used (RESULTS), the relatively long recovery period after washout (RESULTS), and the smoothness of the relations of Figs. 3, 6, and 8, which were built from multiple data points. When used, NO-sensitive electrodes used to directly monitor saline NO concentrations were calibrated both before and after experiments. Disposed NO controls were produced by exposing donor solutions to air at room temperature for ≥24 h before use.

The membrane permeable analog of cyclic-3',5'-guanosine monophosphate, 8-Br-cGMP (20 µM; RBI), was mixed in Pleurobranchaea saline. Soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4, 3-a]quinoxaline-1-one (ODQ) and protein kinase G inhibitor KT5823 (Sigma Chemicals, St. Louis, MO) were prepared as millimolar stock solutions in DMSO; final dilutions in saline to 20 µM ODQ and 1 µM KT5823 concentrations resulted in DMSO present in amounts <0.1% in experimental solutions. 8-Br-cGMP, ODQ and KT5823 were chosen for their reported efficacies and previous use in molluscan neurons (Koh and Jacklet 1999; Mothet et al. 1996).

I\textsubscript{Na,cAMP} responses to iontophoretic injection of cAMP were measured under two-electrode voltage clamp with a third headstage maintaining a bath clamp as a virtual ground via an agar bridge. Sharp electrodes were pulled from borosilicate glass (WPI). A single-barreled capillary filled with 3 M 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; 5–2,000 nA unless otherwise stated) was accomplished with negative current from a constant current source (Model 260, WPI) that allowed precise, repeatable quantities of cAMP to be injected intracellularly. Under our experimental 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). 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, Goleta, CA). All recordings were performed at a holding potential of −50 mV unless otherwise indicated. The kinetics of I\textsubscript{Na,cAMP} are largely determined by intracellular cAMP diffusion and phosphodiesterase activity (Huang and Gillette 1997) as well as by cell phenotype (unpublished observations). Thus the amplitudes of I\textsubscript{Na,cAMP} responses to a given cAMP injection varied widely with cell size and identity.

Dose-response curves of I\textsubscript{Na,cAMP} amplitudes to injected cAMP were fitted to Eq. 1 by least squares (Huang and Gillette 1991)

\[
I = I_{\text{max}} \times \frac{[\text{cAMP}]^n}{[\text{cAMP}]^n + [K_c]}
\]

where \(I = I_{\text{Na,cAMP}}\) amplitude, \(I_{\text{max}}\) = the maximum current at cAMP saturation, \(K_c\) = the dissociation constant of cAMP for the channel binding site, and \(n = \) the Hill coefficient, an indicator of the cooperativity among cAMP binding sites of the I\textsubscript{Na,cAMP} channel. Because I\textsubscript{Na,cAMP} currents were generally not saturable in these giant cell bodies, asymptotic values of hyperbolic cAMP dose-response functions were more accurately obtained from the linear extrapolations obtained with double reciprocal plots of ordinate and abscissa where \(I_{\text{max}}\) and \(K_c\) were determined from the inverse values of the respective \(y\) and \(x\) intercepts. Dose-response measures in DEA/NO began when I\textsubscript{Na,cAMP} stabilized, usually ~10 min after addition of donor saline.

Comparisons of phosphodiesterase degradation rates were performed as per Huang and Gillette (1991). Briefly, I\textsubscript{Na,cAMP} decay slopes, sensitive reflections of intracellular cAMP levels, were fit with Eq. 2 by the method of least squares with the first-order exponential decay

\[
I = I_0 \times e^{-t/k_d}
\]

where \(I\) equals I\textsubscript{Na,cAMP} amplitude, \(I_0\) is the initial current amplitude at the start of the decay slope measure, \(t\) is time in seconds, and \(k_d\) is the inverse of the time decay constant expressed in units of seconds\(^{-1}\). Here, \(k_d\) is used as an index of intracellular phosphodiesterase activity in Pleurobranchaea neurons (see Sudlow and Gillette 1997).

Statistical analyses were performed with Instat software, version 2.03 (Graphpad Software, San Diego, CA). Paired t-tests were used for statistical comparisons unless otherwise stated. Error bars in histograms indicate SE.

Results

NO potentiation of Type 1 I\textsubscript{Na,cAMP} in MCGs

NO donors enhanced the Type 1 I\textsubscript{Na,cAMP} response to injected cAMP in the MCGs (Fig. 1) and numerous other identified members of the feeding motor network. To elucidate NO effects in MCG neurons, we measured cAMP dose-response before and during NO treatment in six experiments.

![Fig. 1. Nitric oxide (NO) donor (0.1 mM diethylamine (DEA)/NO) enhancement of the Type 1 I\textsubscript{Na,cAMP} response to an injected pulse of cAMP (○: −100 nA) in a metacerebral giant (MCG) neuron (n = 15, see Table 1).](https://jn.physiology.org/doi/10.1212/01.WN.0000733352.00099.68)
Figure 2A shows the dose-response curve fitted with Eq. 1 for one experiment. NO enhancement of $I_{Na,cAMP}$ was accompanied by an increase in the projected maximum current available for activation by cAMP. However, there were no apparent changes in the dissociation constant ($K_c$) or the Hill coefficient ($n$). In such dose-response curves, when $n$ is close to 1.0, double-reciprocal plots of the hyperbolic curve yield linear relations where the $y$ intercepts are equal to $1/I_{Max}$ and the $x$ intercepts are equal to $-1/K_c$. The double-reciprocal plots were linear both before and after NO superfusion (Fig. 2B), with $n$ values close to 1.0, implying that there was no induced change in cooperativity of cAMP activation of $I_{Na,cAMP}$. Additionally, extrapolated $K_c$ values from before and during DEA/NO donor applications were relatively unchanged, whereas $I_{Max}$ values were increased after NO treatments. Figure 2C summarizes these results for all six cAMP dose-response experiments after NO enhancement of $I_{Na,cAMP}$.

In three separate experiments, we tested the effects of NO on steady-state $I_{Na,cAMP}$, achieved by tonic cAMP injection and found that it was potentiated similarly to pulsed cAMP responses (Fig. 3). Ionotrophetic injection values used were well below current saturation to avoid confounding, use-dependent effects on phosphodiesterase activity and subsequent $I_{Na,cAMP}$ amplitudes commonly observed with continuous injections of large amounts of cAMP (Sudlow and Gillette 1997). Unpublished results (K. Potgieter, R. Gillette, and C. McCrohan) indicate that 0-Ca²⁺/H⁴⁺/EGTA salines do not block NO effects in the MCGs, minimizing the possible importance of actions mediated through presynaptic terminals remaining on isolated somas. Depleted NO donors had no detectable effects on $I_{Na,cAMP}$, whereas fresh DEA/NO applied thereafter markedly increased $I_{Na,cAMP}$ amplitudes in one MCG (Fig. 4, A and B) and in three buccal cells. The donors NO-spermine (0.2 mM, $n = 4$) and SNAP (0.2 mM, $n = 3$) were also tested on buccal neurons with positive results. In a fifth experiment, neither depleted 1 mM SNAP nor depleted 1 mM spermine-NO negative controls enhanced $I_{Na,cAMP}$ in a buccal cell, whereas fresh spermine-NO increased the current 52%. The specific effectiveness of the fresh NO donors indicated that the effects did not result from decay products of the donors and were most likely specific effects of NO. The latency of NO potentiation was too short for exact measure by our protocols and may have been initiated quite shortly after actual contact with NO.
NO potentiation of $I_{Na,cAMP}$ was slowly reversible on donor washout. Figure 4 shows a time course for addition and washout of NO donor from one experiment. The $I_{Na,cAMP}$ response was rapidly potentiated on addition of DEA/NO, then declined over 10 min to a lower value that remained constant in the presence of DEA/NO. On washout, the response declined slowly over tens of minutes approaching predonor baseline. The experiment of Fig. 4 was specifically aimed at assessing time course and was unusual in its long duration (nearly 2 h); such frequent and numerous tests of injected cAMP typically lead to an artifact evident in the later stages of the experiment: use-dependent prolongation of the decay of the $I_{Na,cAMP}$ response and steadily increasing amplitude (Sudlow and Gillette 1997)—this was not a specific effect of NO, but an event often occurring over long recording periods probably due to steady loss of phosphodiesterase activity (see further).

**NO and the voltage dependence of $I_{Na,cAMP}$**

The Type 1 $I_{Na,cAMP}$ normally shows increasing amplitude with depolarization from rest up to around $-30 \text{ mV}$. Slow command voltage ramps revealed simple potentiation of $I_{Na,cAMP}$ after treatment with 1 mM DEA/NO throughout the range of $-80$ to $-30 \text{ mV}$ with no marked change in the current/voltage relation (Fig. 5A). Similar results were obtained by measuring $I_{Na,cAMP}$ amplitudes in response to pulsed cAMP injections at $-60$, $-40$, and $-30 \text{ mV}$ (not shown). Subtraction of leak currents before and after DEA/NO treatment showed no measurable current induction in the absence of cAMP injection within this voltage range (Fig. 5B).

**NO and cAMP saturation effects on $I_{Na,cAMP}$**

NO alters cAMP synthesis and degradation in a variety of systems (Kurtz et al. 1998; McVey et al. 1999). We tested for possible actions on cAMP pathways that might mediate NO effects on $I_{Na,cAMP}$ or other aspects of cell function. The effect of current saturation on $I_{Na,cAMP}$ amplitude is readily observed when a pulsed $I_{Na,cAMP}$ response is superimposed on a steady-state $I_{Na,cAMP}$ background induced by tonic cAMP injection. Figure 6A illustrates the paradigm for measurement of $I_{Na,cAMP}$ current saturation. The occlusion ratio, $(I - I_0)/I$, is a direct measure of current saturation, where $I_0$ is the test pulse re-

![Figure 4](image1.png)

**FIG. 4.** Noneffects of NO-depleted donor, and a time course for NO potentiation of $I_{Na,cAMP}$ and washout. *A, top:* depleted DEA/NO saline had no significant effect on $I_{Na,cAMP}$ amplitude, whereas fresh DEA/NO caused a marked potentiation. *Bottom:* recovery of the $I_{Na,cAMP}$ response after DEA/NO potentiation and donor washout. A slow linear increase in response durations and amplitudes was an artifact of the frequent injections and prolonged nature of the experiment; extrapolation of the baseline. *B:* time course for $I_{Na,cAMP}$ response amplitudes during control period (i), NO-depleted 0.1 mM DEA/NO saline (ii), fresh 0.1 mM DEA/NO saline (iii), and washout in normal saline (iv).

![Figure 5](image2.png)

**FIG. 5.** NO did not alter resting voltage-dependent current or the voltage dependence of $I_{Na,cAMP}$: *A:* $I/V$ plot of steady-state $I_{Na,cAMP}$, induced by cAMP injection during an ascending command voltage ramp (1.9 mV/s), before and during DEA/NO treatment in an MCG neuron. NO induced a constant increase of $I_{Na,cAMP}$ amplitude with little change in voltage dependence over a range of $-80$ to $-30 \text{ mV}$. Steady-state $I_{Na,cAMP}$ was induced by continuous cAMP injection ($-50 \text{ nA}$). *B:* subtraction of leakage current before and during DEA/NO treatment showed no significant induction of membrane current in the absence of cAMP injection between $-80$ and $-30 \text{ mV}$. 

![Figure 6](image3.png)
spontaneous during imposed background steady-state $I_{\text{Na,cAMP}}$ and $I$ is the control response (Huang and Gillette 1993; Sudlow and Gillette 1995). When $(I - I_0)/I$ was plotted against steady-state $I_{\text{Na,cAMP}}$ for three MCG and two buccal motor neurons, the relationship was linear with increasing background $I_{\text{Na,cAMP}}$ and was not altered by NO donor (Fig. 6B; $n = 5$).

**NO and intracellular cAMP**

In the intact cell, $I_{\text{Na,cAMP}}$ responses are shaped by the activities of endogenous phosphodiesterase and adenylyl cyclase. These enzyme activities determine the saturation state of $I_{\text{Na,cAMP}}$ by setting resting levels of cAMP and determine the rate of $I_{\text{Na,cAMP}}$ decay through cAMP degradation. Measures of both phosphodiesterase kinetics and cAMP levels in the intact cell are possible using $I_{\text{Na,cAMP}}$ as a reporter of intracellular cAMP dynamics via measures of latency-to-peak $I_{\text{Na,cAMP}}$ amplitudes as well as through first-order exponential fits of $I_{\text{Na,cAMP}}$ decay slopes (Huang and Gillette 1991; Sudlow and Gillette 1997). To test the effect of NO on cAMP metabolism, $I_{\text{Na,cAMP}}$ decay rates and latencies to peak after pulsed cAMP iontophoretic injection were compared in 11 MCGs. Figure 7A overlays two $I_{\text{Na,cAMP}}$ responses to identical cAMP injections in the same MCG neuron before and after DEA/NO-induced current potentiation. It can be readily observed that the latency-to-peak amplitude is unchanged after NO treatment, whereas amplitude is enhanced. To further test for possible effects on cAMP metabolism, $I_{\text{Na,cAMP}}$ exponential decay slopes were fitted with Eq. 2 to obtain decay rate constants ($k_d$) indicative of phosphodiesterase degradation rates of cAMP (Fig. 7B). NO treatment failed to alter either latency to peak or $k_d$. These data are summarized in Fig. 7, C and D. Inhibition of phosphodiesterase (PDE) has previously been reported to elevate $I_{\text{Na,cAMP}}$ amplitudes but is accompanied with a characteristic increase of the latency to peak amplitude as well as a decreased decay slope (Fig. 7E, reprinted from Huang and Gillette 1991), two readily observable effects on $I_{\text{Na,cAMP}}$ that NO potentiation does not exhibit.

**NO and cGMP effects on $I_{\text{Na,cAMP}}$**

Intracellular signaling pathways for NO are often found to be mediated by cGMP through direct activation of soluble guanylyl cyclase (Garthwaite and Boulton 1995). Accordingly, in separate experiments on MCGs, we tested the membrane-permeant cGMP analogue 8-bromo-cGMP (20 μM), an inhibitor of soluble guanylyl cyclase, ODQ (20 μM), and the specific protein kinase G inhibitor KT5823 (1 μM) on $I_{\text{Na,cAMP}}$; NO enhancement of $I_{\text{Na,cAMP}}$ was not mimicked by 8-Br-cGMP, whereas NO donor was active on the same cells (Fig. 8A). Additionally, perfusion of 20 μM 8-Br-cGMP did not detectably alter ion current in MCG preparations nor did it cause detectable occlusion of the $I_{\text{Na,cAMP}}$ response. The guanylyl cyclase inhibitor ODQ (20 μM) and the inhibitor of PKG, KT5823 (1 μM), had no effects on the $I_{\text{Na,cAMP}}$ response to injected cAMP and did not suppress NO potentiation of $I_{\text{Na,cAMP}}$ amplitudes (Fig. 8, B and C). Moreover, no changes in basal currents were observed after perfusion of either ODQ or KT5823. Each experiment was performed four separate times (a total of 12 cells, summarized in Fig. 7D). Additionally, in other experiments even higher concentrations of 8-Br-cGMP were tested on the MCG (100 μM; $n = 1$) and on buccal motorneurons (1 mM; $n = 4$) with no potentiating effects on $I_{\text{Na,cAMP}}$. These results are consistent with previous, unpublished results of iontophoretic injections of cGMP.

**Distribution of NO effects on $I_{\text{Na,cAMP}}$**

In exploratory experiments, NO donors markedly enhanced the Type 1 $I_{\text{Na,cAMP}}$ found in feeding neurons of diverse identities but not the Type 2 $I_{\text{Na,cAMP}}$ of the G neurons (serotonergic locomotor neurons) (Sudlow et al. 1998) of the pedal ganglia (Fig. 9). Results for 27 different neurons, axotomized from the buccal, cerebropedal, visceral and pedal ganglia, are summarized in Table 1. Of these neurons, those specifically identified were the ventral white cell feeding command neuron of the buccal ganglion (Gillette and Gillette 1983), the VGI neuron of the visceral ganglion (Moroz and Gillette 1996), and the MCGs of the cerebropedal ganglia (Gillette and Davis 1977). Increasing DEA/NO concentration from 0.1 to 0.2 mM after 10–15 min at the lower concentration in five neurons caused further increase of $I_{\text{Na,cAMP}}$ amplitude by 5–55%; however, the increase was not significant ($P > 0.05$; 2-tailed t-test), suggesting near-saturation of the NO dose-$I_{\text{Na,cAMP}}$ response relation at the lower concentrations. In contrast, NO donor treatment of the Type 2 $I_{\text{Na,cAMP}}$ tested in ten pedal G locomotory neurons from four animals failed to cause detectable increases in the Type 2 current.
The present findings support a role for NO in modulating the activity and reactivity of neurons of the feeding motor network through selective amplification of Type 1 $I_{\text{Na,cAMP}}$. In addition, the experimental observations appear to exclude common intracellular signaling pathways that mediate effects of NO in other systems and suggest alternate avenues for future investigations.

Distinctiveness of NO Signaling in this system

NO modulation of $I_{\text{Na,cAMP}}$ is not consistent with several well-understood pathways of action in other systems. It is distinctly different from those effects described for other cyclic nucleotide-gated cation currents, those of vertebrate olfactory receptor and retinal photoreceptor cells. In the retina, NO enhances cGMP-gated current through stimulation of cGMP synthesis (Savchenko et al. 1997). However, NO potentiation of $I_{\text{Na,cAMP}}$ appears to be independent of the cGMP signaling pathway. The effects of NO were not blocked or attenuated by inhibitors of guanylate cyclase and PKG. The membrane-permeant cGMP analogue 8-Br-cGMP failed to either mimic or occlude NO effects. Thus NO is unlikely to act here through stimulation of the synthesis of cGMP nor through cGMP-dependent phosphorylation. These results recall previous observations (Gillette et al. 1982) that cGMP analogues are relatively ineffective at stimulating activity in the intact feeding motor network in Pleurobranchaea where $I_{\text{Na,cAMP}}$ is widely present.

Olfactory cAMP-gated channels can be activated by NO through direct nitrosylation of C-terminal cysteine residues in the absence of the gating nucleotide (Broillet 2000). Pleurobranchaea $I_{\text{Na,cAMP}}$ differs in that the action of NO on $I_{\text{Na,cAMP}}$ is dependent on the presence of cAMP. Here, the augmenting effect of NO is through potentiation and not co-activation.

The present experimental system stands out in providing a sound basis for investigation of possible roles for cAMP in mediating effects of NO in living neurons, using relatively simple voltage-clamp methodology. Our previous comprehensive studies of the kinetics of $I_{\text{Na,cAMP}}$ in molluscan neurons enabled quantitative breakdown of cAMP paths of action with respect to diffusion and degradation (Huang and Gillette 1991), cAMP channel binding affinity (Huang and Gillette 1993), direct gating by cAMP (Sudlow et al. 1993), and synthetic rate and intracellular concentrations (Sudlow and Gillette 1997). When these methods were applied here, effects of NO on known cAMP pathways could be tested and, in some cases, excluded. This contrasts with a number of other systems in which NO can affect the levels, synthesis, and degradation of cAMP (e.g., Kurtz et al. 1998; McVey et al. 1999).

**DISCUSSION**

**FIG. 7.** Evidence that phosphodiesterase (PDE) activity remains unaltered after NO enhancement of $I_{\text{Na,cAMP}}$. A: overlays of $I_{\text{Na,cAMP}}$ responses in an MCG neuron to discrete cAMP injections (~100 nA, 5-s duration) before (o) and after (■) 1 mM DEA/NO perfusion. Although $I_{\text{Na,cAMP}}$-amplitude increases after NO treatment, the latency to peak (7.5 s) remains unchanged. B: plots of the absolute values of the $I_{\text{Na,cAMP}}$ in A past 9 s follow 1st-order exponential decay relations. Fits of decay slopes before and after NO treatment with Eq. 2 give exponential constants indicative of phosphodiesterase activity ($k_d$). Here, $k_d$ values before and after NO treatment remain relatively unchanged at 0.429 and 0.463 s$^{-1}$, respectively (exponential decay data fit by the method of least squares: control $r^2 = 0.995, \chi^2 = 0.00008$; DEA/NO $r^2 = 0.994, \chi^2 = 0.00036$). C: summary histogram for 11 cells comparing latency to peak amplitude shows no significant difference in the time to peak after NO treatment ($P = 0.46$; paired $t$-test; error bars indicate SE, $n = 11$). D: comparison of $k_d$ values before and after NO enhancement of $I_{\text{Na,cAMP}}$ suggest that no change in phosphodiesterase activity occurs ($P = 0.979$ paired $t$-test; error bars indicate SE; $n = 11$; $r^2$ for all fits are $<0.07$ and $\chi^2 <0.05$). E: Records reproduced from Huang and Gillette (1991) emphasize that increasing concentrations of the PDE inhibitor IBMX increases latencies to peak amplitude and decreases decay rate of $I_{\text{Na,cAMP}}$ responses. Peak amplitudes initially increase, and then decline due to $I_{\text{Na,cAMP}}$ saturation by background cAMP. Numbers indicate $I_{\text{Na,cAMP}}$ response recordings in 0 (1), 10 (2), 20 (3), 50 (4), 100 (5), and 600 $\mu$M IBMX (6).
NO-induced changes in the binding affinity of cAMP with the $I_{Na,CAMP}$ channel were absent. Hill fits of the dose-response curves before and after NO treatment showed marked enhancement of $I_{Na,CAMP}$ amplitudes at all cAMP injection values below saturation. There was no evident change in the apparent affinity of cAMP for the channel, as observed as an unchanging $K_v$ value, or in the number of cAMP molecules required to bind the channel and activate the current. There was no change in any potential cooperativity between channel subunits with cAMP binding, as reflected in the constancy of the Hill coefficient ($n$) of 1.

NO did not induce evident changes in either native cAMP concentrations or the rate of cAMP hydrolysis because the decay rates and latencies to peak amplitude of $I_{Na,CAMP}$ responses to test pulses of cAMP, sensitive indicators of both in vivo cAMP concentration and phosphodiesterase activity (Huang and Gillette 1991; Sudlow and Gillette 1995), were unaltered by NO donors. This interpretation is further supported by the unchanging cAMP saturation plots after NO treatment. If NO had elevated cAMP through adenylyl cyclase activation and/or phosphodiesterase inhibition, plots of the occlusion ratio would have been shifted markedly to the left, reflecting increased saturation of $I_{Na,CAMP}$. This methodology was previously employed to establish that serotonin stimulated adenylyl cyclase and cAMP levels in Pleurobranchaea G neurons (Sudlow and Gillette 1995). Moreover, possible NO-induced increase in maximal enhancement of $I_{Na,CAMP}$, through an increase in the number of channels available, was not supported by our finding that cAMP saturation fits were unchanged after NO treatment.

**Table 1. Effects of NO by cell identity**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$I_{Na,CAMP}$ Enhancement by DEA/NO, %</th>
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<tbody>
<tr>
<td>Buccal ganglion (Type 1 $I_{Na,CAMP}$)</td>
<td>66 ± 15***</td>
</tr>
<tr>
<td>Motorneurons (0.2 mM DEA-NO; n = 16)</td>
<td>50 ± 36–64</td>
</tr>
<tr>
<td>Ventral White Cells (0.1 mM DEA-NO; n = 2)</td>
<td>65 ± 23**</td>
</tr>
<tr>
<td>Cerebropleural ganglion (Type 1 $I_{Na,CAMP}$)</td>
<td>85 ± 25***</td>
</tr>
<tr>
<td>Giant pleural lobe neurons (0.1 mM DEA-NO; n = 3)</td>
<td>66 ± 61–72</td>
</tr>
<tr>
<td>Metacephalic giant cells (0.1 mM DEA-NO; n = 15)</td>
<td>16 ± 16</td>
</tr>
<tr>
<td>Visceral ganglion (Type 1 $I_{Na,CAMP}$)</td>
<td>61–72</td>
</tr>
<tr>
<td>VGI neurons (0.1 mM DEA-NO; n = 2)</td>
<td>66 ± 61–72</td>
</tr>
<tr>
<td>Pedal ganglion (Type 2 $I_{Na,CAMP}$)</td>
<td>16 ± 16</td>
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</tbody>
</table>

Values are means ± SE. *$P < 0.01$; **$P < 0.02$; ***$P < 0.001$, 2-tailed t-test.
NO did not act by altering the voltage dependence of the Type 1 $I_{\text{Na,cAMP}}$, which is mediated by a likely depolarization-sensitive $\text{Ca}^{2+}$ block of the channel (Green and Gillette 1987). Current-voltage plots of steady-state $I_{\text{Na,cAMP}}$ showed an NO-induced inward shift but no change in slope between $-100$ and $-30$ mV. No other effects on leak currents were detected.

The mechanism for selective NO enhancement of Type 1, but not Type 2, $I_{\text{Na,cAMP}}$ remains for future documentation. Its independence from both cGMP and cAMP pathways of action suggest a different mode, possibly involving the ability of NO to exert physiological effects through direct nitrosylation of cysteine residues in proteins (Xu et al. 1998). When found, the target of NO in this system may represent a novel mechanism of action.

The mechanism for selective NO enhancement of Type 1 $I_{\text{Na,cAMP}}$, but not Type 2, has yet to be defined. Its independence from both the cGMP and cAMP pathways suggests regulatory actions that are not as well known. For example, NO might act through direct nitrosylation of cysteine residues in proteins (Xu et al. 1998). Regulation in these neurons might also involve NO-induced change in intracellular pH (McCrohan et al. 2005); type 1 $I_{\text{Na,cAMP}}$ amplitudes have previously been reported to be sensitive to alterations of intracellular pH (Green and Gillette 1988). When found, the target of NO in this system may reveal a novel regulatory mechanism of cell excitability.

**Physiological relevance**

Expression of NO and NOS in feeding-related neural circuitry are traits shared across species of gastropod mollusks. Chemosensory activation of the feeding motor network in *Lymnaea* appears to depend on NO (Elphick et al. 1995), and NO itself stimulates feeding motor output in both isolated CNS and semi-intact preparations of *Lymnaea* (Moroz et al. 1993) as also in *Pleurobranchaea* (unpublished results). However, there is marked variability in the apparent expression, localization, and $\text{Ca}^{2+}$ dependence of NOS activity across species, which may reflect the long evolutionary history and broad diversification in the feeding ecology of the members of the class (see reviews by Moroz and Gillette 1995; Moroz et al. 1996). The present observations add another novel aspect in NO potentiation of Type 1 $I_{\text{Na,cAMP}}$, which represents a new neuromodulatory pathway.

In neurons of the pond snail *Lymnaea* and the sea-hare *Aplysia*, NO stimulates excitability through cGMP-dependent reduction of a $K^+$ conductance (Jacklet and Tieman 2004; Park et al. 1998). Although it cannot be entirely excluded, such a NO-cGMP signaling pathway was not prominent enough to be detected in our observations on *Pleurobranchaea*. Conversely, those previous studies did not assay for possible NO effects on $I_{\text{Na,cAMP}}$. Comparative studies have indicated a broad distribution of Type 1 currents in feeding neurons of other gastropods, including *Lymnaea stagnalis* (McCrohan and Gillette 1988), *Navanax inermis* and *Aplysia californica* (unpublished observations). Thus NO stimulation of $I_{\text{Na,cAMP}}$ is potentially more widely spread among gastropods, and perhaps other taxa.

What is the likely role of NO in this system? At levels of cell and circuit, NO 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 would in turn affect the activity state of the feeding network in part through broad modulation of $I_{\text{Na,cAMP}}$ in its neural elements.

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**References**


