Roles of Guanylate Cyclase in Responses to Myogenic and Neural Nitric Oxide in Canine Lower Esophageal Sphincter

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Received October 2, 2001; accepted February 19, 2002

ABSTRACT

Whether cGMP and cytosolic guanylate cyclase (cGC) mediate responses of canine lower esophageal sphincter (LES) to nitric oxide (NO) released from nerves, produced in muscle, or added exogenously was evaluated in vitro. 1H-(1,2,4)oxadiazole(4,3-α)quinazolin-1-1 (ODQ), inhibitor of cGC, reduced relaxations to nerve stimulation and sodium nitroprusside but not to nitric-oxide synthase activity-dependent outward K⁺-currents in isolated muscle cells. ODQ also failed to increase tone after nerve blockade. Nonspecific K⁺ channel blocker, TEA ion at 20 mM was previously shown to increase tone, occlude NO-mediated modulation of tone, and inhibit NO-dependent outward currents but not neural relaxation in LES cells. In this study, TEA abolished neural relaxation and nearly abolished relaxation to sodium nitroprusside when present with ODQ. We conclude that mechanisms coupling NO in canine LES to responses vary with the source of NO. ODQ-dependent mechanisms, presumably involving cGC, mediate actions of NO from nerves, but NO from muscle utilizes TEA-sensitive but not ODQ-dependent mechanisms to modulate tone and outward currents. Exogenous NO utilizes both TEA- and ODQ-dependent mechanisms.

Previously we showed that canine LES contains a membrane-bound, constitutive nitric-oxide synthase (Salapatek et al., 1998b,c) that acts to limit development of tone in canine LES. This nitric-oxide synthase, which appears to be neural (Daniel et al., 2001a), uses Ca²⁺ entering through nearby L-type calcium channels for activation and tone regulation by NO formation that activates K⁺ channels, including BK Ca channels (Daniel et al., 2000). However, the source is not neural as tetrodotoxin alone or with ω-conotoxin (GVIA) had no effect on tone (Salapatek et al., 1998a,c). Moreover, tetrodotoxin alone but not ω-conotoxin (GVIA) alone abolished relaxations to electrical field stimulation (Daniel et al., 2000).

Another study showed that contraction in LES was supported by two extracellular sources of Ca²⁺, one of which supported spontaneous tone whereas the other, which supported contraction to carbamyl choline (carbachol), was resistant to removal by extracellular fluid with 0 Ca²⁺ and 100 μM EGTA and appeared to recycle between extracellular binding sites and nearby Ca²⁺ stores (Salapatek et al., 1998a). Similar results have been obtained in airway smooth muscle (Montano et al., 1993, 1996; Bazan-Perking et al., 1998). In these studies, Ca²⁺ from both of these sources entered through l-type calcium channels. We suggested that

Supported by the Medical Research Council, Canada.

ABBREVIATIONS: LES, lower esophageal sphincter; NO, nitric oxide; cGC, cytosolic guanylate cyclase; EFS, electrical field stimulation; ODQ, 1H-(1,2,4)oxadiazole(4,3-α)quinazolin-1-1; L-NAME, Nω-nitro-L-arginine methyl ester; ICC, interstitial cells of Cajal; pps, pulses per second; BK Ca channels, large conductance Ca²⁺-dependent K⁺ channels; L-NOARG, Nω-nitro-L-arginine; ANOVA, analysis of variance.
events do not use cGMP or protein kinase G (Ahern et al., 1999; Ignarro et al., 1999; Pinilla et al., 1999; Taglialetela et al., 1999; Garry et al., 2000; Janssen et al., 2000; Liu et al., 2000; Mazzuco et al., 2000; Takeda et al., 2000; Tseng et al., 2000). We aimed to evaluate the roles of guanylate cyclase in the downstream events initiated by NO released from nerves and from muscle.

**Materials and Methods**

**Tissue Preparation.** Mongrel dogs of either sex were euthanized with an intravenous overdose of pentobarbital sodium (100 mg/kg), according to a protocol approved by the McMaster University Animal Care Committee and following the guidelines of the Canadian Council on Animal Care. The abdomen was opened along the midline, and segments of lower esophagus, ileum, and colon were excised and immediately put into oxygenated Krebs-Ringer solution at 24°C having the following composition: 115.0 mM NaCl2, 4.6 mM KCl, 1.2 mM MgSO4, 22.0 mM NaHCO3, 2.5 mM CaCl2, and 11.0 mM glucose. The gastroesophageal junction was removed and opened along the greater curvature. After careful removal of the mucosa by fine dissection, the thickened ring of muscle, the LES, was removed. The mucosa was removed by fine dissection, leaving the muscularis externa.

In **In Vitro Studies.** In all cases circular muscle strips were prepared by cutting tissues into multiple 15 × 2 mm strips. These were tied with fine thread at both ends and mounted vertically in 5-ml organ baths, bathed in Krebs-Ringer solution at 37°C, and oxygenated with 95% O2 and 5% CO2. Strips were tied at the bottom to an electrode holder, passed through concentric platinum electrodes, and tied at the top to a force displacement transducer (Grass FT 03C; Grass Instruments, Quincy, MA). Tensions were recorded on Beckman R611 Dynographs (Beckman Coulter, Inc., Fullerton, CA). Electrodes were stimulated from a Grass 88 stimulator set at 40 V/cm, 5 pps, and 0.3-ms pulse duration, which gives near maximal relaxation of LES by activation of enteric nerves.

LES strips had to tension applied and equilibrated for 1 h, during which the muscle strips contracted and spontaneously developed tone. Active tension was the difference between the observed tension and that obtained at the end of the experiment when Ca2+-free Ringer solution with 1 mM EGTA was applied. In all cases, we checked that the initial baseline set on the oscillograph remained unchanged by cutting the string from the tissue to the strain gauge. In some cases the deviation was more than 2 mm; because of this concern and possible Ca2+-independent contractions, we used the level of tension obtained after this string was cut. Relaxation responses to electrical field stimulation (EFS) at 40 V/cm, 5 pps, and 0.3-ms pulse duration, which gives near maximal relaxation of LES by activation of enteric nerves.

LES strips had 2 g of tension applied and equilibrated for 1 h, during which the muscle strips contracted and spontaneously developed tone. Active tension was the difference between the observed tension and that obtained at the end of the experiment when Ca2+-free Ringer’s solution with 1 mM EGTA was applied. In all cases, we checked that the initial baseline set on the oscillograph remained unchanged by cutting the string from the tissue to the strain gauge. In some cases the deviation was more than 2 mm; because of this concern and possible Ca2+-independent contractions, we used the level of tension obtained after this string was cut. Relaxation responses to electrical field stimulation (EFS) at 40 V/cm, 5 pps, and 0.3-ms pulse duration were used to reproduce responses were obtained. Responses to 0.5 ms of EFS usually produced maximal relaxation and were used for statistical comparisons. Then 10-5 M ODQ (determined as maximal in preliminary experiments)
or other agents were added at 20-min intervals, and effects on tone and on nadirs of EFS-induced relaxations were measured. If an agent lowered tone to the level of the nadir of relaxation, we added carbachol (10^-6 M) to restore tone so that relaxation could be tested.

At the end of each experiment, sodium nitroprusside at 10^-4 M was added to evaluate the effects on NO from an exogenous donor. This

Fig. 3. Representative tracings from experiments in which effects of ODQ were examined. All traces show baseline tone before applying 2 g of tension (not shown), tone development, responses to EFS, effects after 20 min on tone and response to EFS of drugs, if any were applied, then relaxation to sodium nitroprusside (10^-4 M) after 20 min or more, and, finally, subsequent relaxation to calcium-free solution with 0.1 mM EGTA. A, control recording. Both the tone and the relaxation response to EFS was stable over time. Note that relaxation to sodium nitroprusside relaxed nearly to baseline (passive) tension. B, effect of ODQ (10^-5 M). In the absence of 10^-6 M tetrodotoxin, it increased tone and changed the response to EFS from mono- or biphasic relaxation to phasic contraction followed by a smaller slower relaxation and recovery. The relaxation to sodium nitroprusside was partially inhibited compared with control. C, effect of ODQ followed by L-NAME (3 × 10^-4 M). The effects of ODQ on tone, EFS, and sodium nitroprusside responses were not further changed after L-NAME. In this experiment, but not all, the relaxation to calcium-free EGTA solution was reduced (compare with B). D, effect of 20 mM TEA followed by ODQ. Note that TEA enhanced tone but did not inhibit relaxation to EFS. Previous studies (Daniel et al., 2000) showed that it reduced relaxation to sodium nitroprusside about 50%. Subsequent ODQ now abolished all responses to EFS and nearly abolished responses to sodium nitroprusside. Subsequent relaxation to calcium-free solution with EGTA solution was complete.

**TABLE 1**
Effects on tone
Initial tone = 100%. Means ± S.E.M.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>n</th>
<th>Control</th>
<th>Experiment</th>
<th>p Valuea</th>
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<tbody>
<tr>
<td>Effect ODQ (10^-5 M)</td>
<td>5</td>
<td>105.9 ± 3.9</td>
<td>121.2 ± 5.2*</td>
<td>0.0060</td>
</tr>
<tr>
<td>Effect ODQ after tetrodotoxin</td>
<td>5</td>
<td>107.3 ± 3.5</td>
<td>99.7 ± 4.8</td>
<td>0.032</td>
</tr>
<tr>
<td>After TEA (20 mM) + ODQ</td>
<td>7</td>
<td>103.3 ± 3.2</td>
<td>152.4 ± 18.3*</td>
<td>0.061</td>
</tr>
<tr>
<td>After TEA + ODQ</td>
<td>7</td>
<td>96.6 ± 5.0</td>
<td>206.6 ± 20.6*</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

* p values were determined by paired comparisons except when three groups were considered; then one-way ANOVA with Dunnett’s test was used.

* Significantly different from 100%.
was followed by Ca\textsuperscript{2+}-free Ringer’s solution with 1 mM EGTA to eliminate active tone. A typical protocol was as follows:

Tension → Tone Development → EFS → Experimental Agent →

EFS → Sodium Nitroprusside → EGTA → Cut String

If tetrodotoxin was used, EFS was retested afterward and from time to time during experiments.

**Patch-Clamp Techniques.** The LES was dissected as described above and strips were cut into 1- to 2-mm\(^2\) square pieces and placed in the dissociation solution.

**Cell Isolation.** Cells were dissociated in 0.25 mM EDTA, 125 mM NaCl, 4.8 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 10 mM glucose for 30 min. An enzyme solution containing papain (130 mg/ml), bovine serum albumin (100 mg·m\(^{-1}\)), and Sigma collagenase blend H (occasionally F) was added to the tissue pieces for 30 to 60 min. After netch’s test was used.

**Data Analysis.** Tone and relaxation nadirs were measured in LES in each strip. Each was compared with the control values as 100% and to the observed initial nadir of relaxation. Changes with concentrations of inhibitor were evaluated using Dunnnett’s multiple comparisons test unless only one concentration was used. Then paired comparisons were made. All statistical tests were carried out using Prism 3 software (Intersil Corp., Irvine, CA). The \(n\) values in tables refer to the number of animals that supplied the tissues.

**Results**

**Drug Effects on Active Tone with/without Functioning Nerves.** In the absence of nerve blockade with \(10^{-6}\) M tetrodotoxin, ODQ (\(10^{-5}\) M) and TEA (20 mM) both increased active tone, TEA more than ODQ (cf. Figs. 1, 2, and 3, B and C). Tetrodotoxin alone or combined with \(\omega\)-conotoxin (GVIA) had no effect on tone (Salapatek et al., 1998b). When tetrodotoxin had blocked responses to electrical field stimulation, TEA, but not ODQ, still increased active tone (Fig. 2 and
Table 1), and ODQ after TEA had no additional effect. Moreover, the increase in tone from TEA was greater after tetrodotoxin than before, presumably a result of removal of TEA-induced release of inhibitory nerve mediator.

Drug Effects on Relaxation to EFS. ODQ (10^{-5} M) markedly reduced relaxation to EFS (40 V/cm, 5 pps, 0.3-ms duration), converting the relatively rapid relaxation to a phasic contraction followed by a small, slow relaxation (Fig. 3, B and C) not further affected by L-NAME (Fig. 3C). The inhibition of relaxation is summarized in Table 2. Figure 4 shows that ODQ, in contrast to L-NOARG (3 \times 10^{-4} M), reduced but did not abolish relaxation to EFS. In contrast to ODQ and confirming previous findings (Daniel et al., 2000), 20 mM TEA had no effect to inhibit the amplitude of relaxation when present alone (Fig. 5), although it did reduce the extent of relaxation in some experimental series (Table 2). However, when TEA was present together with ODQ, relaxation to EFS was abolished (Figs. 3D and 5), as it was with L-NOARG.

Drug Effects on Relaxation to Sodium Nitroprusside and Calcium-Free (EGTA) Solution. L-NOARG had no effect (Fig. 6 and Table 3) on relaxation to sodium nitroprusside (3 \times 10^{-4} M), irrespective of the presence or absence of tetrodotoxin (not shown). ODQ or TEA each reduced relaxation to sodium nitroprusside, and together they markedly reduced it but did not abolish it (Figs. 6 and Table 4). In most cases, the residual tone (assumed to be passive) after EGTA was the same irrespective of any pretreatment (Fig. 6 and Table 4). However, when tetrodotoxin was present, the combination of ODQ and TEA significantly increased it (Fig. 7).

Patch-Clamp Study. ODQ (10^{-5} M) had no effect on outward currents when the patch pipette had 200 nM free Ca^{2+} (Fig. 8) This is in contrast to effects of TEA (20 mM) or L-NOARG (3 \times 10^{-4} M), shown previously (Salapatek et al., 1998c) and confirmed here (not shown) to inhibit them by
about 80% at maximum depolarization. We also reconfirmed that sodium nitroprusside (10^{-4} M) restored the currents inhibited by L-NOARG (Salapatek et al., 1998c).

Discussion

ODQ is a widely used inhibitor of cytosolic guanylate cyclase (Ignarro et al., 1999; Chang et al., 2000; Gorodeski, 2000a,b; Janssen et al., 2000; Kwan et al., 2000; Tseng et al., 2000; Yao et al., 2000). In this study 10^{-5} M ODQ increased tone and raised the nadir of NO-mediated relaxation (Jury et al., 1992) to nerve stimulation (EFS) from 38.5 to 77.5% of initial tone and changed the response qualitatively. Instead of a large fast relaxation, there was a small initial phasic contraction followed by a slow, small relaxation (Fig. 3, B and C). This relaxation was unaffected by subsequent L-NAME, suggesting that it is not NO-mediated.

After tetrodotoxin abolished responses to EFS, the tone increase to ODQ and the altered EFS response following ODQ were also eliminated, suggesting that both were nerve-mediated. Reduced responses to basal release of neural NO, acting either on interstitial cells of Cajal (ICC) or on nerve endings, likely accounts for the tone increase after ODQ (see discussion below). Previous studies (Allescher et al., 1988) showed that stimulation of intrinsic nerves of LES releases acetylcholine as well as NO. Acetylcholine was the probable mediator of contraction, observed when actions of neural NO were reduced by ODQ. It also may have contributed to the increase in tone when ODQ was administered with nerves active. Although not mediated by NO, the residual relaxation after ODQ involves opening of K^+ channels, since it was abolished when TEA was present together with ODQ.

We did not evaluate the efficacy of ODQ to block elevations of cGMP levels, so it may have inhibited some responses nonselectively, or some tissue components may have resisted its effects. However, it selectively inhibited two NO-mediated responses (relaxation to EFS and to sodium nitroprusside). Thus, it is unlikely that the failure to inhibit NO actions when NO was derived from muscle can be explained by resistance to ODQ. It did increase spontaneous tone when nerves were functioning, but we attribute this effect to inhibition of the action of NO released from nerves under basal conditions because it disappeared after tetrodotoxin. Thus,

![Nadir after SNPs](image1)

![Nadir after EGTA](image2)

**Fig. 7.** Nadirs of relaxation to sodium nitroprusside (top panel) or calcium-free EGTA (0.1 mM) (bottom panel) after TEA and ODQ irrespective of the presence (right histogram) or absence (left histogram) of nerve activity. Although the final tone levels achieved after sodium nitroprusside were not significantly different from 100% initial tone in the presence or absence of nerve activity, there was a small relaxation since TEA had increased tone from the initial level (Fig. 2). The bottom panel shows that relaxation to calcium-free solution was also impaired significantly by TEA with ODQ when tetrodotoxin was present. ***, p < 0.01 paired comparison (n = 7).

<table>
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<tr>
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<th>n</th>
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<th>p Value</th>
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<tbody>
<tr>
<td>L-NOARG (10^{-4} M)</td>
<td>6</td>
<td>5</td>
<td>30.1 ± 12.2 ††</td>
<td>69.1 ± 10.4 ††</td>
</tr>
<tr>
<td>TEA/ODQ vs. 20 mM/10^{-5} M TEA/ODQ/tetrodotoxin (10^{-6} M)**</td>
<td>7</td>
<td>107.8 ± 15.6 †††</td>
<td>119.3 ± 11.1 †††</td>
<td>0.632</td>
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* † † † p values were determined by paired comparisons. Values for other groups are not shown; none were significantly different from values in left (control) column.

** Table 3

<table>
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<th>Control</th>
<th>Experiment</th>
<th>p Value</th>
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<tbody>
<tr>
<td>L-NOARG (10^{-4} M) vs. ODQ (10^{-5} M)</td>
<td>6</td>
<td>5</td>
<td>22.0 ± 5.3 †</td>
<td>13.8 ± 3.8 †</td>
</tr>
<tr>
<td>TEA/ODQ vs. (20 mM/10^{-5} M) TEA/ODQ (tetrodotoxin)</td>
<td>5</td>
<td>20.5 ± 3.3 †</td>
<td>34.6 ± 3.9 †</td>
<td>0.0035</td>
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its actions appeared efficacious against some NO-mediated events and selective in that it failed to act on other responses.

We postulate that ODQ eliminates most or all of the effects of NO released from nerves, disinhibiting (Fox-Threlkeld et al., 1999) and/or unmasking the response to the release of acetylcholine previously obscured by NO actions. We speculate that nerves release two inhibitory mediators, one NO and the other released in response to NO release. Dependence on NO release would explain why L-NAME alone usually completely inhibits relaxation to low-frequency (1–5 pulses per second) EFS (Jury et al., 1992). According to this model, neural NO acts on cGC to increase cGMP levels, but the other mediator acts to open K⁺

channels. The combination of ODQ and TEA would, on either model, inhibit both the NO/cGC and the K⁺

channel-mediated components of the response.

NO released from nerves may act on cGC in muscle or on the ICC. ICC are present in canine LES, intercalated between nerve endings and muscle to which they are connected by gap junctions (Allescher et al., 1988). Similar structural relations are found in other species and parts of the gastrointestinal tract (e.g., Daniel and Posey-Daniel, 1984; Ward et al., 1998; reviewed in Sanders et al., 1999). Recent studies in mutant W/WV mice with apparently normal nitric-oxide synthase innervation of the LES, but lacking intramuscular ICC, showed that nerves release two inhibitory mediators, one NO and the other released in response to NO release and that NO donors also became less effective to induce relaxation and hyperpolarization in the absence of ICC. This dependence of NO-mediated neural effects on ICC was also found in the pylorus and gastric fundus (Burns et al., 1998b,c). These currents were inhibited by L-NOARG, iberiotoxin, or TEA. They were restored by sodium nitroprusside after L-NOARG but not after the K⁺

channel blockers. The nitric-oxide synthase activity depended upon Ca²⁺

entering through L-type calcium channels, closely associated with the nitric-oxide synthase in caveolae (Daniel et al., 2001a). The tone modulation from nitric-oxide synthase activity in LES strips was abolished by L-NOARG and 20 mM TEA but only partially by iberiotoxin (Daniel et al., 2000). This suggested that additional K⁺

channels besides BKCa were activated by NO of myogenic origin.

ODQ had no effect on tone in the presence of tetrodotoxin and no effect on outward currents. Thus, there appears to be no role for cGC in the modulation of tone and enhancement of outward currents by NO from myogenic nitric-oxide synthase. We did not test that cGC was still active in isolated LES cells. However, the actions of NO to drive outward currents do not require its actions. L-NOARG reduces the outward currents by 80% and NO donors restore them. NO in other systems also operates independently of cGC (Ahern et al., 1999; Ignarro et al., 1999; Pinilla et al., 1999; Taglialetela et al., 1999; Garry et al., 2000; Janssen et al., 2000; Liu et al., 2000; Mazzuco et al., 2000; Takeda et al., 2000; Tseng et al., 2000).

Ionic mechanisms involved in neural NO effects are incompletely understood. Previous studies showed that NO-mediated hyperpolarization accompanied relaxation of muscle and appeared to be associated with current flow through K⁺

channels (Christinck et al., 1991; Jury et al., 1992; Cayabyab and Daniel, 1995). Although 20 mM TEA alone had no inhibitory effect on amplitudes of nerve-mediated relaxation, it reduced outward currents by 80% in LES cells (Salapatek et al., 1998c). Whether LES relaxation after TEA was still associated with hyperpolarization has not been determined. The sites of K⁺

channels, ICC or muscle, affected by NO from nerves or the secondary inhibitory mediator are unclear. Clearly, the mechanisms by which LES relaxation and hyperpolarization are induced after activation of intrinsic nerves are complex and need further study.

Previous studies showed that activation of BKCa channels driven by release of NO from a membrane-bound nitric-oxide synthase accounted for large outward currents, cellular hyperpolarization, and reduced tone in canine LES (Salapatek et al., 1998b,c). These currents were inhibited by L-NOARG, iberiotoxin, or TEA. They were restored by sodium nitroprusside after L-NOARG but not after the K⁺

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NO from the muscle membrane may act locally on nearby ion channels; examples of such local actions have been reported (Bolotina et al., 1994). The NO-dependent outward currents depend on Ca^{2+} entry through L-type calcium channels (Salapatek et al., 1998b,c). However, in opossum LES Akbarali and Goyal (1994) found that NO inhibits L-type calcium currents. Nifedipine abolishes tone in canine LES. However, inward currents opened by depolarization and carried by L-type calcium channels could not be demonstrated in LES cells from dog or opossum, even after all potassium currents were blocked (Jury and Daniel, 1996; J. Jury and E. E. Daniel, unpublished data), but they were easily demonstrated in the body of opossum esophagus. Yet, as noted above, NO-driven BK_{Ca} currents in LES are inhibited when L-type calcium channels are blocked. The presence of these channels in close proximity to nitric-oxide synthase in caveolae (Daniel et al., 2001a) may result in unexpected properties, including altered voltage dependence and opening properties.

Relaxation to sodium nitroprusside, a source of exogenous NO, was reduced by TEA or by ODQ. Together, they nearly eliminated it. This implies that exogenous NO acts by both activating cGC and opening K_{Ca} channels independently of cGC. Since ODQ had no effects on NO-driven outward currents in LES cells, TEA likely inhibited exogenous NO actions at sites on which myogenic-derived NO acts, and ODQ inhibited them on sites at which NO from nerves acts, possibly in ICC. Exogenous NO had access to both sites. In a previous study (Daniel et al., 2000) we found that TEA induced a tone increase when nerve function was abolished and occluded the tone increase to 1-NOARG, consistent with an action of TEA to block effects of myogenic NO release. However, cGC was not demonstrated to be present or responsive to NO in isolated LES cells. If it is absent or nonresponsive to NO in LES cells in situ but present in ICC, this would provide an explanation for the differential effects of ODQ in vitro noted above.

In summary, these findings show that relaxation of the canine LES by neural NO is more complex than originally proposed; i.e., NO from nerves appears to activate cGC, likely in ICC, whereas NO from muscle apparently does not act through cGC. NO from muscle activates K_{Ca} channels, whereas NO from nerves causes relaxation, which does not require opening of TEA-sensitive potassium channels. NO added exogenously acts through both ODQ- and TEA-sensitive mechanisms.

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