Evidence for NO· redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut

RAJ K. GOYAL AND XUE D. HE
Center for Swallowing and Motility Disorders, Brockton/West Roxbury Department of Veterans Affairs Medical Center, West Roxbury 02132; and Harvard Medical School, Boston, Massachusetts 02215

Goyal, Raj K., and Xue D. He. Evidence for NO· redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut. *Am. J. Physiol.* 275 (*Gastrointest. Liver Physiol.* 38): G1185–G1192, 1998.—A nitric oxide (NO)-like product of the L-arginine NO synthase pathway has been shown to be a major inhibitory neurotransmitter that is involved in the slow component of the inhibitory junction potential (IJP) elicited by stimulation of nonadrenergic, noncholinergic nerves. However, the exact nature of the nitrergic transmitter, the role of cGMP, and the involvement of a potassium or a chloride conductance in the slow IJP remain unresolved. We examined the effects of soluble guanylate cyclase inhibitors LY-83583 and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), potassium-channel blockers and putative chloride-channel blockers diphenylamine-2-carboxylate (DPC) and niflumic acid (NFA) on the hyperpolarization elicited by an NO· donor, diethylenetriamine/NO adduct (DNO), NO in solution, and an NO^+ donor, sodium nitroprusside (SNP), in the guinea pig ileal circular muscle. Effects of these blockers on purinergic (fast) and nitrergic (slow) IJP were also examined. DNO-induced hyperpolarization and nitrergic slow IJP were suppressed by LY-83583 or ODQ and DPC or NFA but not by the potassium-channel blocker apamin. In contrast, hyperpolarization caused by SNP or solubilized NO gas and purinergic fast IJP were antagonized by apamin but not by inhibitors of guanylate cyclase or chloride channels. These results demonstrate biological differences in the actions of different redox states of NO and suggest that NO· is the nitrergic inhibitory neurotransmitter.

enteric nervous system; chloride channels; potassium channels; sodium nitroprusside; neuromuscular transmission; amine/nitric oxide adduct

NITRERGIC AND PURINERGIC pathways constitute two major components of nonadrenergic, noncholinergic (NANC) inhibitory neurotransmission in the gut (8, 13, 20). Purinergic smooth muscle relaxation and membrane hyperpolarization are caused by an increase in an apamin-sensitive potassium conductance (8). However, several key questions regarding nitrergic neurotransmission remain unresolved (25, 30). Although nitric oxide (NO) is generally thought to be the nitrergic neurotransmitter, the redox form of the NO molecule involved in the neurotransmission is controversial (9, 16). Moreover, the signal transduction cascade stimulated by the nitrergic neurotransmitter within the smooth muscle remains unclear. It is assumed that the nitrergic neurotransmitter acts by stimulating guanylate cyclase to cause intracellular cGMP accumulation and opening of a potassium conductance (25, 30). However, several studies showed that cGMP accumulation does not fully account for the inhibitory neurotransmission (15, 34), and the nature of the potassium conductance activated by the nitrergic motor nerves is uncertain (3, 14, 22, 24, 29). Furthermore, suppression of a chloride conductance was suggested to be involved in the nitrergic NANC neurotransmission (6, 7). Recently, cGMP and NO donors were shown to suppress chloride and nonselective cation currents on dispersed smooth muscle cells (36, 39).

There are several possible reasons for these confounding conclusions. First, the intracellular messengers and conductance changes associated with the inhibitory responses reflect a mixture of distinct sets of changes associated with coexisting nitrergic and purinergic NANC inhibitory neurotransmission (13, 20). Therefore, the signal transduction pathway that is associated with purinergic neurotransmission may be incorrectly assigned to nitrergic neurotransmission. The inhibitory junction potential (IJP) in many tissues consists of two overlapping components called the fast and the slow IJP (8, 20). The fast IJP is insensitive to NO synthase (NOS) inhibitors and is caused by a purinergic neurotransmitter. The slow IJP is sensitive to NOS inhibitors and is therefore caused by a nitrergic inhibitory NANC neurotransmitter (8, 20). Second, the effects of the inhibitory transmitter may vary with the membrane potential of the smooth muscle. For example, the stimulatory effect of NO on the large-conductance calcium-dependent potassium (BK) channels may be seen at depolarized potentials but not in unstimulated smooth muscle cells, because the BK channels are generally active only at depolarized potentials (1). Third, sodium nitroprusside (SNP), *S*-nitrosothiols, sydnonimines, and NO gas in physiological solution have been used to define the cellular actions of the endogenous nitrergic neurotransmitter (9, 16). However, these NO donors yield different redox forms of NO that may not mimic the endogenous neurotransmitter (28).

We investigated the ion conductances underlying the purinergic fast IJP and the nitrergic slow IJP separately and those caused by exogenous administration of the NO^+ donors SNP and *S*-nitroso-*N*-acetylpencillamine (SNAP) (28), NO in solution, and NO· donors 3-morpholinosydnonimine hydrochloride (SIN-1) in the presence of superoxide dismutase (SOD) and a recently developed amine/NO· adduct (DNO) (19). Our results show that the nitrergic slow IJP involves generation of

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cGMP and suppression of a diphenylamine-2-carboxylate (DPC)- and niflumic acid (NFA)-sensitive conductance and that these effects are best mimicked by the NO· donor DNO. On the other hand, the NO^+ donor SNAP and NO solution cause smooth muscle membrane hyperpolarization by a cGMP-independent increase in an apamin-sensitive potassium conductance, a pathway that is normally used by purinergic inhibitory neurotransmission. These studies reveal redox-based differences in the action of NO on the smooth muscle membrane potential and suggest that NO· or an amine/NO adduct is the nitrergic NANC inhibitory neurotransmitter.

METHODS

Animals. Adult guinea pigs weighing between 150 and 400 g were killed by CO_2 narcosis, and a segment of ileum was prepared for intracellular microelectrode recording from the circular layer of smooth muscle as described previously (8, 20). The bath had a volume of 3 ml and was continuously perfused with oxygenated, warmed Krebs solution at a rate of 3 ml/min. The bath was further oxygenated by bubbling a 95% O_2-5% CO_2 mixture directly into it. The bath temperature was maintained at 30 ± 0.5°C.

Drugs. Drugs and chemicals used in this study included apamin, D-arginine, L-arginine, atropine sulfate, α,β-methylene ATP, DMSO, guanethidine, hemoglobin, N^{ω}-nitro-L-arginine (L-NNA), SNP, substance P (SP), tetraethylammonium (TEA), tetrodotoxin (TTX), and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (obtained from Sigma, St. Louis, MO); DPC (obtained from Aldrich, Milwaukee, WI) and NFA; LY-83583 (from Calbiochem, La Jolla, CA); SOD, glibenclamide, DNO, SIN-1, and SNAP (from Research Biochemicals International, Natick, MA); and NO gas (from Matheson Gas Products, Rutherford, NJ).

The Krebs solution contained (in mM) 11.5 glucose, 21.9 bicarbonate, 1.2 phosphate, 138.5 sodium, 2.5 calcium, 1.2 magnesium, 4.6 potassium, and 125 chloride. The pH of the solution after 30 min of bubbling with 95% O_2-5% CO_2 was between 7.34 and 7.39. Drugs were dissolved in water or Krebs solution (unless otherwise specified) and prepared fresh before use. SNP and SIN-1 were protected from exposure to light. LY-83583, DPC, NFA, glibenclamide, and SNAP were dissolved in DMSO. L-NNA was dissolved in 0.01 N HCl and then diluted in Krebs solution. A standard solution of NO in Krebs solution was prepared under anoxic conditions. Oxygen-free Krebs solution was first prepared by vacuum evacuation for 1 h followed by flushing with 100% nitrogen gas for 20 min. This oxygen-free Krebs solution was chilled to 0–4°C in a freezer for 20 min. NO gas was bubbled through the chilled Krebs solution in a cylinder sealed with Parafilm for 1 min under a protective hood. The pH of this solution was adjusted to 7.4 before use. The concentration of NO in such a solution is 1–1.5 μM. DNO was dissolved in phosphate-buffered solution (composition in g/l: 0.21 KH_2PO_4, 9.00 NaCl, 7 Na_2HPO_4, and 0.726 H_2O) at pH 7.4. This agent causes slow release of NO· and degrades slowly in phosphate buffer at pH 7.4 at 37°C with a half-life of 20 h.

The following (final bath) concentrations of drugs were used: 0.3 μM apamin, 1 mM D-arginine, 1 mM L-arginine, 1 μM atropine, 33 μM α,β-methylene ATP, 5 μM guanethidine, and 1 μM SP. Tissues were perfused with L-NNA (200 μM) for at least 15 min before recording. SP desensitization was produced by perfusion with 1 μM SP for 30 min. The adequacy of SP-receptor desensitization was tested by loss of the depolarizing effect on the muscle strip with additional SP (7). α,β-Methylene ATP tachyphylaxis was produced by perfusion with 100 μM of this agent for 30 min as described earlier (7). SOD (3 U/ml) was perfused for 20 min before the study. NFA (200 μM) was perfused for 10 min and ODQ (6 μM) for 20 min before studies. The final bath concentrations of bolus doses of agonists were (in μM) 66 SNP, 100 SIN-1, 66 SNAP, and 100 DNO. The solution of oxyhemoglobin (10 μM) was prepared as described elsewhere (13).

Intracellular recordings. Intracellular membrane potentials were recorded from smooth muscle cells of circular muscle strips dissected from the guinea pig ileum using standard techniques as described earlier (7). The microelectrodes were filled with 3 M KCl and had tip resistance between 30 and 80 MΩ. The fast inhibitory junction potential (fast IJP) was evoked by electrical field stimulation (EFS) with silver-silver chloride electrodes on the muscle strips perfused with oxygenated Krebs solution containing atropine and guanethidine. Guanethidine and atropine suppress adrenergic and cholinergic excitatory responses, respectively, and unmask the NANC nerve-mediated IJP. The slow IJP was expressed by blocking the fast IJP with a saturating concentration of apamin and SP receptor desensitization of the muscle strips in the presence of atropine and guanethidine. Desensitizing SP receptors obliterates the SP-mediated excitatory junction potential. EFS consisting of four pulses (0.5-ms pulse duration, 30 mA at 20 Hz) was used because these stimulus parameters produced maximal IJP amplitudes. These responses were neurogenic, because they were abolished by TTX (0.1 μM). Effects of chemical antagonists were studied in the same cell whenever possible. However, when electrode displacement made it impossible recordings were made from adjacent cells.

Statistical analyses. Statistical comparisons were made using Student's standard paired and unpaired *t*-statistics, and all data are expressed as means ± SE.

RESULTS

Responses to NO donors. Administration of a bolus injection of the NO donors SNP (66 μM), DNO (100 μM), and NO solution (100 μl in a 3-ml chamber) in the perfusion bath produced a transient (~10 s) membrane hyperpolarization (5–10 mV) of the circular smooth muscle of guinea pig ileum (Figs. 1 and 2). Other NO donors, SIN-1 (100 μM) in the presence of SOD (3 U/ml) and SNAP (66 μM), produced similar hyperpolarizations.

The soluble guanylate cyclase (sGC) inhibitor LY-83583 (26) by itself had no effect on the smooth muscle membrane potential and did not affect the hyperpolarization elicited by SNP (66 μM). The SNP-induced hyperpolarization was 9.1 ± 0.5 mV before and 9.0 ± 0.6 mV after LY-83583 treatment (7 cells from 3 animals; $P > 0.05$). LY-83583 also failed to inhibit the hyperpolarization elicited by the NO gas in aqueous solution. The mean value of NO gas-induced hyperpolarization was 10.8 ± 0.5 mV before and 9.9 ± 0.4 mV after LY-83583 treatment (17 cells from 4 animals; $P > 0.05$). In contrast, LY-83583 pretreatment significantly suppressed the hyperpolarization produced by DNO from 9.3 ± 0.5 to 0.8 ± 0.5 mV (4 cells from 3 animals; $P < 0.01$; Figs. 1 and 2). These observations suggest that

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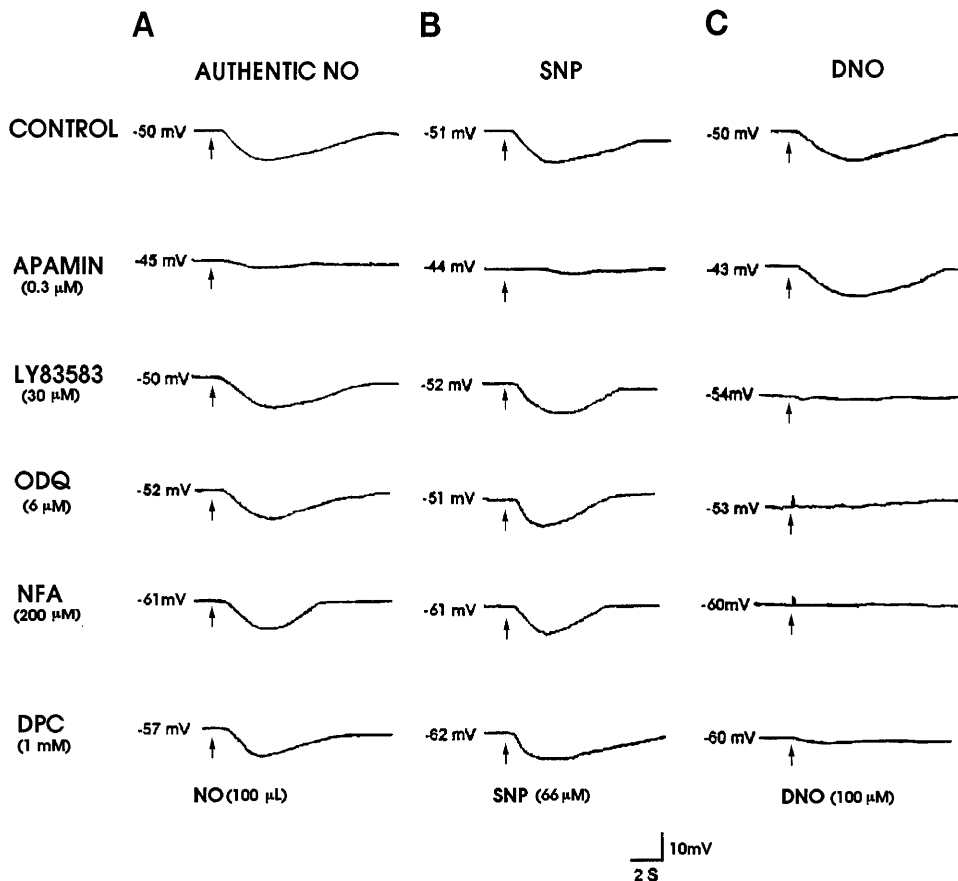

Fig. 1. Examples of effects of antagonists on hyperpolarizing effects of different nitric oxide (NO) donors on guinea pig ileum circular smooth muscle. *A*: bolus injection of NO in solution (100 μl in a 3-ml bath) produced membrane hyperpolarization that was suppressed by apamin but not affected by guanylate cyclase inhibitors LY-83583 or 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ). It was also not suppressed by diphenylamine-2-carboxylate (DPC) or niflumic acid (NFA). *B*: bolus injection of sodium nitroprusside (SNP) causes hyperpolarization similar to that caused by authentic NO that is suppressed by apamin but not by LY-83583, ODQ, NFA, or DPC. *C*: in contrast to SNP-induced hyperpolarization, diethylenetriamine/NO (DNO)-induced hyperpolarization is not affected by apamin but is suppressed by LY-83583, ODQ, NFA, and DPC.

the action of DNO, but not SNP or NO gas, is dependent on activation of sGC.

ODQ is a potent, more selective blocker of sGC (11). ODQ (6 μM) also failed to suppress SNP-induced hyperpolarization but blocked DNO-induced hyperpolarization (Figs. 1 and 2). The mean value of SNP-induced hyperpolarization was 9.0 ± 0.9 mV before and 9.3 ± 0.9 mV after ODQ treatment (4 cells from 3 animals; $P > 0.05$). On the other hand, DNO-induced hyperpolarization was 8.6 ± 0.5 mV before and 0.6 ± 0.8 mV after ODQ treatment (4 cells from 3 animals; $P < 0.01$). These observations further suggest that the hyperpolarizing action of DNO, but not SNP, is mediated via sGC.

Apamin (0.3 μM), a blocker of small-conductance potassium (SK) channels, depolarized the smooth muscle cells from −50.5 ± 1.1 to −40.3 ± 1.0 mV (13 cells from 5 animals; $P < 0.01$). Apamin reduced the SNP-induced hyperpolarization from 8.9 ± 0.5 to 1.1 ± 0.3 mV (8 cells from 4 animals; $P < 0.01$) and the authentic NO-induced hyperpolarization from 10 ± 0.5 to 2.2 ± 0.3 mV (13 cells from 5 animals; $P < 0.01$). In

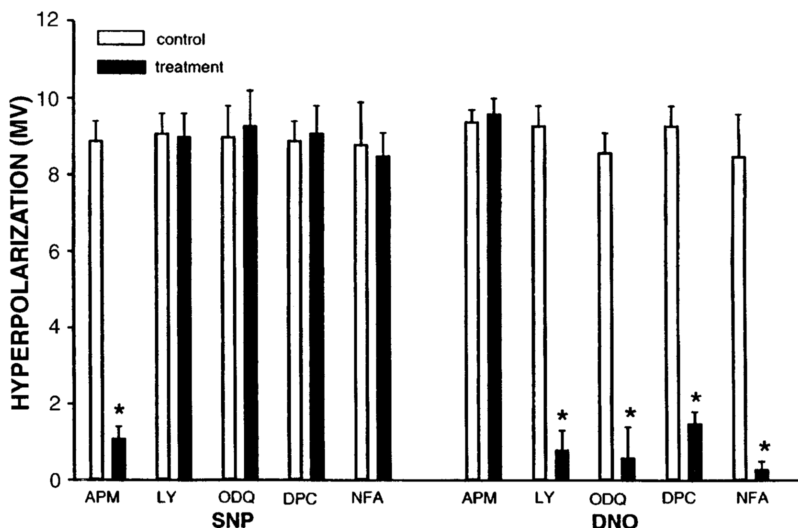

Fig. 2. Effects of apamin (APM), LY-83583 (LY), ODQ, DPC, and NFA on hyperpolarization associated with SNP and DNO. Note that hyperpolarization caused by NO^+ donor SNP was significantly inhibited by apamin but not by LY-83583, ODQ, DPC, or NFA. On the other hand, hyperpolarization caused by NO· donor DNO was not affected by apamin but was significantly suppressed by soluble guanylate cyclase (sGC) inhibitors LY-83583 or ODQ and putative chloride-channel blockers DPC and NFA. These results show that NO^+ donor SNP causes hyperpolarization via apamin-sensitive potassium channels whereas NO· donor causes hyperpolarization via a cGMP-dependent closure of chloride conductance. Each bar represents mean ± SE of 4–17 cells in 3–5 animals. *Statistical difference from respective control value ($P < 0.05$).

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contrast, apamin did not affect the hyperpolarization elicited by DNO, which averaged 9.4 ± 0.3 mV before and 9.6 ± 0.4 mV after apamin (8 cells from 4 animals; $P > 0.05$; Figs. 1 and 2).

TEA (1 mM), a blocker of BK channels, or glibenclamide (30 μM), a blocker of ATP-sensitive potassium (K_{ATP}) channels (26), both increased the hyperpolarization elicited by the NO donors. TEA (1 mM for 30 min) depolarized the membrane potential from −51.8 ± 1.3 to −42.5 ± 0.8 mV (13 cells from 3 animals; $P < 0.05$) and increased the NO gas-induced hyperpolarization from 10.2 ± 0.7 to 13.1 ± 1.0 mV (13 cells from 3 animals; $P < 0.05$). Glibenclamide (30 μM) did not affect the resting membrane potential but increased the NO gas-induced hyperpolarization from 8.3 ± 0.4 to 11.8 ± 0.5 mV (21 cells from 5 animals; $P < 0.05$).

In smooth muscle the resting membrane potential is less negative than the Nernst equilibrium potential for potassium, which is estimated to be around −85 mV in guinea pig tenia coli smooth muscle (32). The relatively depolarized state of the ileal smooth muscle cells could be caused by a resting inward chloride current, an inward cation current, or an inward current-generating ion pump. The smooth muscle cells have a resting chloride efflux because these cells have an intracellular chloride concentration (54 mM) that is fivefold higher than that predicted on the basis of passive diffusion (2). Therefore, a decrease in this chloride conductance would cause membrane hyperpolarization. Gut smooth muscle cells also generate inward current through nonselective cation channels, closure of which would also produce membrane hyperpolarization (5). To examine a role for these conductances in the fast and slow IJP, we tested the effect of DPC, which blocks both chloride and nonselective cation conductances (4, 28). DPC (1 mM) by itself hyperpolarized the smooth muscle cells from a control value of −53 ± 1 to −63 ± 2 (5 cells from 3 animals; $P < 0.01$). DPC had no effect on the membrane hyperpolarization elicited by SNP or NO gas solution. SNP elicited hyperpolarization was 8.9 ± 0.5 mV before and 9.1 ± 0.7 mV after DPC (7 cells from 3 animals; $P > 0.05$). NO gas solution produced hyperpolarization of 10.2 ± 0.4 mV before and 10.2 ± 0.2 mV after DPC (5 cells from 3 animals; $P > 0.05$). DPC, however, effectively suppressed the hyperpolarization elicited by DNO from 9.3 ± 0.5 to 1.5 ± 0.3 mV (4 cells from 3 animals; $P < 0.01$; Figs. 1 and 3). These observations suggest that the NO· donor DNO may hyperpolarize smooth muscle by suppressing a resting chloride or a nonselective cation conductance.

NFA is a more selective blocker of chloride conductance (37). NFA (200 μM) by itself hyperpolarized the membrane by 10.2 ± 0.4 mV (9 cells from 4 animals); however, it failed to modify the hyperpolarizing action of SNP but blocked the action of DNO (Fig. 1). The mean value of SNP-induced hyperpolarization was 8.1 ± 1.1 mV before and 8.5 ± 0.6 mV after NFA (6 cells from 3 animals; $P > 0.05$). The DNO-induced hyperpolarization was 8.5 ± 1.1 mV before and 0.3 ± 0.2 mV after NFA (6 cells from 3 animals; $P < 0.01$; Fig. 2).

We also examined the effects of NFA (200 μM) and apamin (0.3 μM) on hyperpolarizing actions of SNAP and SIN-1 in the presence of SOD. As shown in Fig. 3, SNAP-induced hyperpolarization was blocked by apamin but not by NFA. SNAP (66 μM) elicited hyperpolarization of 12.2 ± 0.3 mV in the control period, 0.3 ± 0.2 mV after apamin ($P < 0.01$), and 12.5 ± 0.4 mV after NFA (6 cells from 3 animals; $P > 0.05$). In contrast,

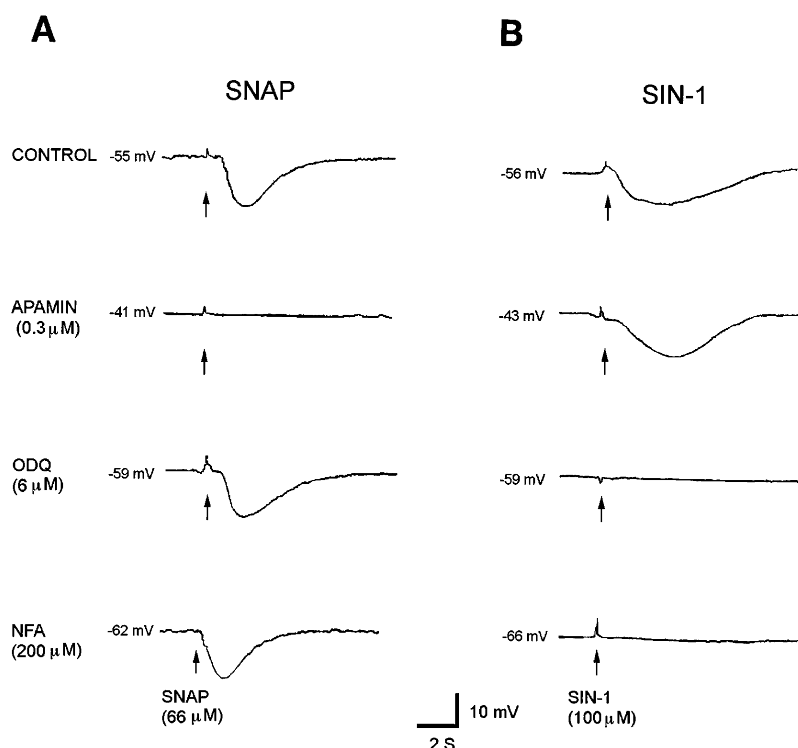

Fig. 3. Examples of effects of apamin and NFA on hyperpolarization elicited by *S*-nitroso-*N*-acetylpencillamine (SNAP) and 3-morpholinosydnonimine hydrochloride (SIN-1) in presence of superoxide dismutase (SOD). *A*: SNAP-induced hyperpolarization was unaffected by NFA but was abolished by apamin. *B*: in contrast to action of SNAP, SIN-1-elicited hyperpolarization was suppressed by NFA but not by apamin. Because SNAP is an NO^+ donor and SIN-1 in presence of SOD yields NO·, these results suggest that NO^+ causes hyperpolarization via apamin-sensitive channels and NO· via NFA chloride channels.

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hyperpolarization elicited by SIN-1 was blocked by ODQ and NFA but not apamin. SIN-1 in the presence of SOD produced hyperpolarization of 10.2 ± 0.3 mV in the control period, 0.5 ± 0.4 mV after apamin ($P >$ 0.05), and 12.5 ± 0.4 mV after NFA (6 cells from 3 animals; $P > 0.05$). SIN-1 in the presence of SOD produced hyperpolarization of 10.2 ± 0.3 mV in the control period, 0.5 ± 0.4 mV after NFA ($P < 0.01$), and 11.2 ± 0.4 mV after apamin (6 cells from 3 animals; $P >$ 0.05). SIN-1 in the absence of SOD produced only a small hyperpolarization that was not quantified. SNAP is an NO^+ donor, whereas SIN-1 in the presence of SOD yields NO·. These studies further suggest that NO^+ acts by opening apamin-sensitive potassium conductance, whereas NO· causes hyperpolarization via closure of a chloride conductance.

Fast and slow IJP. The NANC nerve-mediated fast IJP evoked by EFS in the guinea pig ileum circular muscle was recorded in the presence of atropine (1 μM) and guanethidine (5 μM). Under these conditions the slow IJP was masked by an SP-mediated excitatory junction potential and was revealed more fully after SP receptors were desensitized. The slow IJP was isolated in the additional presence of apamin to block the fast IJP (Fig. 4).

The fast IJP was resistant to the NOS inhibitor L-NNA (200 μM) or the NO scavenger oxyhemoglobin but was suppressed by α,β-methylene ATP tachyphylaxis, suggesting that the fast IJP was caused by purinergic inhibitory neurotransmission (Fig. 4). In contrast, the slow IJP was almost abolished by L-NNA and oxyhemoglobin but was unaffected by α,β-methylene ATP tachyphylaxis (Fig. 4). The L-NNA block of the slow IJP was reversed by L-arginine (1 mM) but not by D-arginine (1 mM) (data not shown), indicating that the slow IJP results from the nitrergic NANC inhibitory neurotransmission.

The fast IJP was unaffected by the sGC inhibitors LY-83583 and ODQ. The amplitude of the fast IJP was 19.1 ± 0.5 mV before and 19.4 ± 0.5 mV after LY-83583

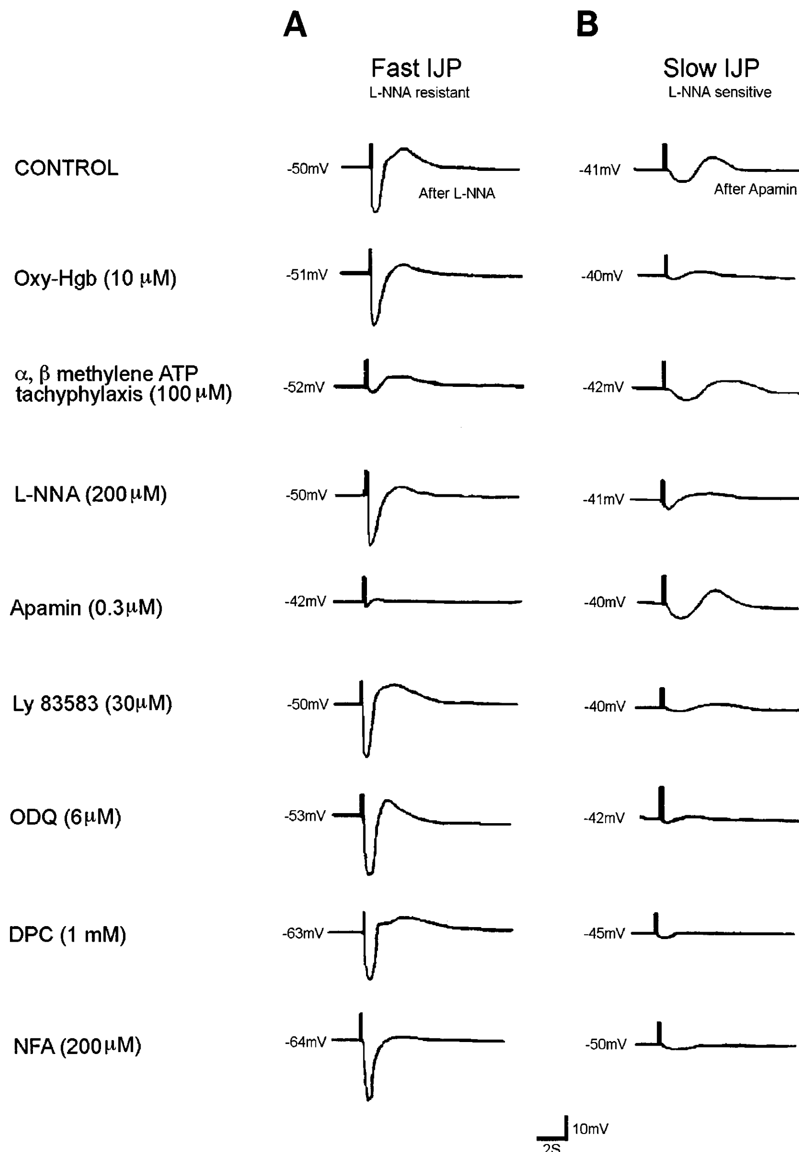

Fig. 4. Examples of effects of antagonists on fast and slow inhibitory junction potentials (IJPs) in guinea pig ileum circular muscle. Fast IJP is produced by electrical field stimulation of muscle strips under nonadrenergic, noncholinergic (NANC) conditions. Fast IJP is <2 s in duration and 17–22 mV in amplitude. It is resistant to NO inhibitor N^{ω}-nitro-L-arginine (L-NNA) and is not affected by NO scavenger oxyhemoglobin (Oxy-Hgb). Fast IJP is markedly suppressed by α,β-methylene ATP tachyphylaxis and is not inhibited by putative chloride-channel blockers DPC or NFA. Fast IJP is also unaffected by sGC inhibitor LY-83583 or ODQ but is nearly abolished by apamin. Slow IJP is produced by electrical field stimulation of muscle strips under NANC conditions and addition of apamin and substance P (SP) receptor desensitization. Apamin is used to suppress fast IJP. Note that use of apamin leads to less negative value of resting membrane potential. SP receptor desensitization is necessary to suppress excitatory junction potential that masks slow IJP. Slow IJP is 4–5 s in duration and 4–6 mV in amplitude. Slow IJP is sensitive to NOS inhibitor L-NNA and is suppressed by NO scavenger oxyhemoglobin, suggesting that it is caused by NO. It is unaffected by α,β-methylene ATP tachyphylaxis. Slow IJP is suppressed by LY-83583 or ODQ. It is expressed in presence of apamin and is antagonized by DPC or NFA. IJPs shown are from different cells.

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(30 μM) treatment (8 cells from 3 animals; $P > 0.05$). In contrast, LY-83583 markedly suppressed the slow IJP amplitude from 5.4 ± 0.3 mV before to 1 ± 0.2 mV after LY-83583 treatment (8 cells from 3 animals; $P < 0.01$; Figs. 4 and 5). ODQ (6 μM) also did not affect the fast IJP but markedly suppressed the slow IJP. The fast IJP was 21.2 ± 0.4 mV without and 21.3 ± 0.3 with ODQ treatment (7 cells from 3 animals; $P > 0.05$), and slow IJP was 6.8 ± 0.3 mV before and 0.3 ± 0.1 mV after ODQ (8 cells from 3 animals; $P < 0.01$).

The fast IJP was almost abolished by apamin, which selectively blocks the SK channels. Apamin reduced the amplitude of the fast IJP from 19.4 ± 0.4 during the control period to 0.4 ± 0.1 after apamin treatment (10 cells from 3 animals; $P < 0.001$; see Fig. 5). However, the fast IJP was unaffected by TEA (1 mM), which blocks BK channels, or by glibenclamide (30 μM), which blocks K_{ATP} channels (29). TEA (1 mM for 30 min) depolarized the smooth muscle membrane by ~10 mV and increased the IJP amplitude from 16.4 ± 0.3 to 21.0 ± 0.8 mV (8 cells from 2 animals; $P < 0.01$). Glibenclamide (30 μM) did not affect the resting membrane potential but increased the IJP amplitude from 12.1 ± 0.6 to 14.8 ± 0.9 mV (16 cells from 5 animals; $P < 0.05$).

DPC (1 mM) or NFA (200 μM) significantly hyperpolarized the smooth muscle cells by ~10 mV but did not affect the fast IJP. The mean amplitude of the fast IJP was 19.3 ± 0.6 mV before and 20 ± 0.3 mV after DPC treatment (7 cells from 3 animals; $P > 0.05$). In contrast, DPC significantly suppressed the amplitude of the slow IJP from a control value of 5.3 ± 0.1 to 1.7 ± 0.3 mV (10 cells from 3 animals; $P < 0.01$; Figs. 4 and 5). NFA also selectively suppressed the slow IJP without affecting the fast IJP (Figs. 4 and 5). The fast IJP was 17.2 ± 0.7 mV before and 17.3 ± 0.6 mV after NFA treatment (10 cells from 4 animals; $P > 0.05$). In contrast, the slow IJP was 7.1 ± 0.5 mV before and 0.3 ± 0.1 mV after NFA (10 cells from 4 animals; $P < 0.01$), thus suggesting that the nitrergic NANC neurotransmitter DPC and NFA act to suppress the same conductance.

DISCUSSION

The main findings of this study are that in producing membrane hyperpolarization in the guinea pig ileum circular smooth muscle *1*) the NO· donors DNO and SIN-1 in the presence of SOD mimic the action of the endogenous nitrergic neurotransmitter and *1*) the NO^+ donors SNP and SNAP mimic the purinergic inhibitory neuromuscular transmission (Fig. 3).

DNO is a nucleophile adduct that was developed to provide a source of pure NO· under physiological conditions (19). DNO elicited smooth muscle hyperpolarization that was suppressed by a blocker of sGC, LY-83583, or ODQ. This is consistent with the view that NO· is a natural activator of sGC. The slow IJP was also suppressed by the sGC inhibitors, supporting the view that this slow IJP is caused by NO·.

The ion conductances associated with the slow IJP were similar to those underlying the DNO-induced hyperpolarization. The DNO-induced hyperpolarization and the slow IJP were both apamin resistant and were not suppressed by either TEA or glibenclamide. These observations are similar to previous reports of the failure of these potassium-channel blockers to inhibit the action of NO donors or to suppress IJP (7, 14). The report of smooth muscle hyperpolarization by 8-bromoguanosine 3′,5′-cyclic monophosphate, which is insensitive to these potassium-channel blockers (3), also suggests that intracellular cGMP accumulation may hyperpolarize smooth muscles by a mechanism other than the opening of potassium channels. However, cGMP-activated G kinase is well known to activate the TEA-sensitive BK channels in patch-clamp studies on single circular smooth muscle cells (14, 22) These channels are operative mainly at depolarized potentials, suggesting that the NO-cGMP pathway may cause hyperpolarization of smooth muscles that are already depolarized, by activation of BK channels (1). It has been also reported that quinine in millimolar concentrations blocks the apamin-resistant IJP, suggesting that an “unusual” potassium conductance was activated in the muscle during nitrergic neurotransmis-

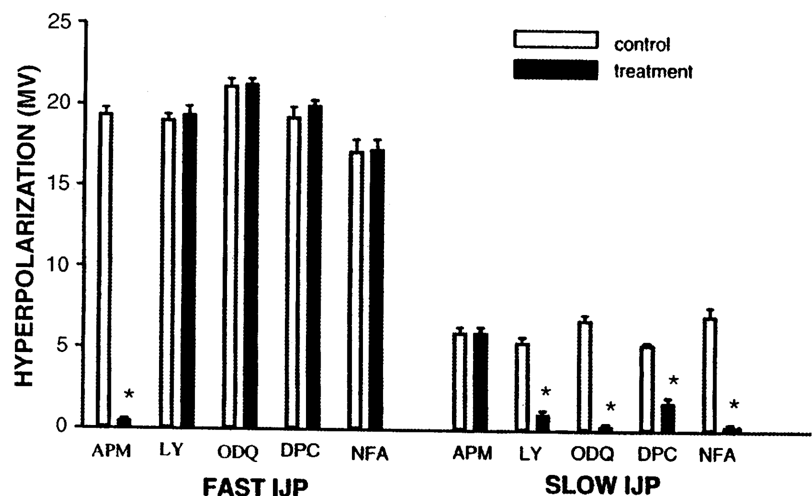

Fig. 5. Effects of apamin (APM), sGC inhibitors LY-83583 (LY) and ODQ, and putative chloride-channel blockers DPC and NFA on fast and slow IJPs. Fast IJP is nearly abolished by apamin but is unaffected by LY-83583 and ODQ or DPC and NFA. In contrast, slow IJP is unaffected by apamin but is suppressed by LY-83583 and ODQ or DPC and NFA. *Statistical difference from respective control value ($P < 0.05$).

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sion (3). However, these results are difficult to interpret because quinine also blocks chloride and nonselective cation channels (12).

Our observation that the hyperpolarization produced by DNO as well as the slow IJP were suppressed by DPC, which is a known blocker of chloride and nonselective cation channels (5, 31), further supports the view that NO· is the mediator of the slow IJP. The action of DNO was also suppressed by the chloride conductance inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (unpublished observations). The slow IJP was previously shown to be suppressed by chloride substitution and by DIDS (7). Moreover, the slow IJP was shown to be associated with an increase in the membrane resistance, suggesting its association with decreased ionic conductance (7). Chloride ion substitution with nonpermeant anions and chloride channel blockers causes membrane hyperpolarization by suppressing the resting chloride conductance. Under these conditions the NO·-cGMP signaling pathway cannot further close the chloride conductance to produce smooth muscle membrane hyperpolarization. This hypothesis is validated by the fact that chloride channels have been recently identified in the gut smooth muscle (34, 39). Finally, NFA, a known chloride-channel blocker, also suppressed the slow IJP and hyperpolarization caused by DNO. Further studies are needed, however, to define the nature of the chloride channels involved in the NO·-cGMP signal transduction pathway.

Although cGMP involvement and ion conductances associated with predictable NO· donors such as amine/NO adducts (19) on the gut smooth muscle membrane potential have not been reported before, several reports are available on smooth muscle hyperpolarization by NO donors such as SNP, *S*-nitrosothiols such as SNAP, and aqueous solution of NO gas (14, 25, 30). SIN-1 is metabolized by tissues to produce peroxynitrite that is largely involved in toxic reactions, but in the presence of SOD, SIN-1 yields NO· (26). SNP spontaneously yields NO^+. *S*-nitrosothiols act as NO^+ donors in addition to spontaneously releasing NO· (18). In our studies a transient hyperpolarization lasting several seconds elicited by a bolus of SNP and SNAP in a continuously perfusing bath was not suppressed by LY-83583 but was markedly suppressed by apamin. This may be caused by the fact that NO^+ yielded by these donors may nitrosate cell surface thiols and directly activate some SK channels (17, 28). Osthaus and Galligan (23) made similar observations, and Kitamura and colleagues (15) reported that apamin blocked an early component of hyperpolarization caused by *S*-nitrosocysteine. Apamin is a selective blocker of calcium-dependent SK channels, which normally mediate purinergic fast IJP (20, 33). It is possible the fast IJP is also nitrergic and caused by NO^+ redox form. However, because the fast IJP is not sensitive to block by L-NNA and is present in neuronal constitutive NOS-negative animals, this possibility is not tenable (13, 20). Under our experimental conditions, administration of a solution of NO gas in the oxygenated bath mimicked the actions of SNP, indicating that the NO was being converted to an intermediate that was an NO^+ donor. In oxygenated physiological solutions, NO· gas has been shown to react rapidly with oxygen to produce oxides of nitrogen such as N_2O_3 and N_2O_4, which are effective NO^+ donors (28). NO· donors such as DNO are distributed to the immediate vicinity of the smooth muscle cells and release NO· slowly, thereby making NO· available near the smooth muscle cells.

Apart from SNP being an NO^+ donor, cellular metabolism of SNP produces a potpourri of biologically active intermediates including *S*-nitrosothiols (19). SNP, therefore, may produce a variety of cGMP-independent and -dependent actions (17, 28). Moreover, intermediates of SNP stimulate both soluble form and particulate form (not suppressible by inhibitors of sGC) leading to a very large but gradual (over minutes) increase in intracellular cGMP (21). Therefore, the actions and antagonism of its action by an inhibitor of soluble cGMP may vary in different tissues and with different experimental conditions. In many smooth muscles, ODQ has been shown to antagonize mechanical relaxation caused by SNP (4, 27, 38). SNP and *S*-nitrosothiols, at depolarized potentials, activate BK channels through cGMP to elicit hyperpolarization (14, 22). In guinea pig proximal colon, SNP causes hyperpolarization by activating apamin-sensitive and -insensitive potassium currents (35). In the canine proximal colon the hyperpolarizing action of SNP is all ODQ sensitive (10). In the mouse anococcygeus muscle, SNP has been shown to act by inhibiting a nonselective cation (36).

In conclusion, our studies suggest that NO· or NO^+ redox forms of NO produce smooth muscle hyperpolarization by different ionic mechanisms. The early hyperpolarization of NO^+ donors such as SNP or SNAP is caused by activation of apamin-sensitive potassium channels. On the other hand, NO· donors such as DNO act by cGMP-mediated suppression of chloride conductance. The action of DNO resembles that of the nitrergic slow IJP, suggesting that NO· is the nitrergic neurotransmitter.

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Address for reprint requests: R. K. Goyal, Research and Development Program (151), Brockton-West Roxbury VA Medical Center, 1400 VFW Parkway, West Roxbury, MA 02132.

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