Nitric oxide decreases pacemaker activity in lymphatic vessels of guinea pig mesentery

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von der Weid, Pierre-Yves, Jun Zhao, and Dirk F. Van Helden. Nitric oxide decreases pacemaker activity in lymphatic vessels of guinea pig mesentery. Am J Physiol Heart Circ Physiol 280: H2707–H2716, 2001.—Intracellular microelectrode recordings were used to determine whether nitric oxide (NO), affects the pacemaker events that initiate vasomotion in lymphatic vessels of the guinea pig mesentery. This pacemaker activity is recorded as spontaneous transient depolarizations (STDs) and is likely to arise through synchronized Ca2+ release from intracellular stores. We show here that acetylcholine-induced endothelium-derived NO and exogenous NO released by sodium nitroprusside (SNP; 100 µM) and DEA-NONOate (500 µM) reduced the frequency and amplitude of STDs. This inhibition of STD frequency and amplitude was independent of the NO-induced hyperpolarization of the smooth muscle. The SNP-induced inhibition of STD frequency and amplitude was abolished during superfusion with the soluble guanylyl cyclase inhibitor ODQ (10 µM) and was diminished in the presence of cGMP and cAMP-dependent protein kinase inhibitors. The data are consistent with the hypothesis that NO inhibits vasomotion primarily by production of cGMP and activation of both cGMP- and cAMP-dependent protein kinases, which reduce the size and frequency of STDs, probably by acting on the underlying synchronized Ca2+ release from intracellular stores.

MANY LYMPHATIC COLLECTING vessels transport lymph fluid by rhythmic constrictions of the smooth muscle present in the vessel walls. Studies (30) on such lymphatic vessels found in the mesentery of the guinea pig have demonstrated that the pacemaker mechanism underlying the generation of L-type Ca2+ channel-mediated action potentials and associated constrictions is due to a summation of spontaneous transient depolarizations (STDs). Detailed investigations performed in lymphatic and other smooth muscle preparations indicate that STDs (29, 30) or the underlying spontaneous transient inward currents (15, 30) are generated by the release of Ca2+ from d-myoinositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)P_3]\) receptor-mediated intracellular stores, leading to the activation of a Ca2+-dependent inward current probably carried by chloride ions (\(\text{Cl}^-_{\text{Ca}}\); see Ref. 32).

The endothelium present in lymphatic vessels plays an important role in modulating lymphatic pumping (37) through the release of nitric oxide (NO). It has been shown that NO, either released from the endothelium after stimulation with acetylcholine (ACh) or produced by the exogenous application of sodium nitroprusside (SNP), was able to inhibit the phasic constrictions that occur spontaneously or during perfusion in lymphatic vessels of the guinea pig mesentery (34). This action was associated with a marked hyperpolarization of the lymphatic smooth muscle membrane potential and a decrease in the activity of STD. Although the NO inhibition of STD activity might be a consequence of the hyperpolarization and the associated increase in membrane conductance, it is possible to hypothesize that NO also affects STD activity independently of a change in membrane conductance. A recent finding (33) that NO-induced hyperpolarization can be blocked by the ATP-sensitive K+ (\(K_{\text{ATP}}\)) channel blocker glibenclamide provides a pharmacological tool to test this hypothesis. The present study indicates that NO inhibition of pacemaker Ca2+ release, measured by resultant STD activity, occurs primarily through a NO-induced increase in cGMP levels and cGMP- and cAMP-dependent protein kinases.

METHODS

**Tissue preparation.** Guinea pigs (4–15 days) of either sex were euthanized by overexposure to halothane (5–10%) and then decapitated. The university animal welfare committees approved this procedure. Small collecting lymphatic vessels (<230 µm diameter) supplying the jejunum and ileum were dissected together with their associated artery and vein and left intact within the surrounding mesentery. The mesentery was used to pin out the tissues on the Sylgard-coated base of a small organ bath (volume 100 µl) mounted on the stage of...
an inverted microscope (model TMS; Nikon). The tissue was continuously superfused at a flow rate of 5 ml/min with a physiological salt solution, heated to 36°C, composed of (in mM) 2.5 CaCl₂, 5 KCl, 2 MgCl₂, 120 NaCl, 25 NaHCO₃, 1 NaH₂PO₄, and 11 glucose. The pH was maintained at 7.4 by constant bubbling with a 95% O₂–5% CO₂ mixture.

Electrophysiology. Resting membrane potential was measured with the use of conventional glass intracellular micro-electrodes with resistances of 150–250 MΩ when filled with 0.5 M KCl. Electrodes were connected to an amplifier (Intra 767, World Precision Instruments; Berlin, Germany) through an Ag-AgCl half-cell. Resting membrane potential was monitored with the use of a digital oscilloscope (Gould Instrument Systems; Madison, WI) and simultaneously recorded on a computer (Power Macintosh 7600/120) via an analog-to-digital converter (MacLab/8s, ADI; New South Wales, Australia). Impalements of smooth muscle cells were obtained from the adventitial side of the lymphatic vessels cut into short segments (125–350 μm) with the use of fine dissecting scissors. The short segments were used to ensure simplified electrical properties of the smooth muscle such as electrical activity, even if generated at localized foci within the smooth muscle, produced similar potential changes in all the smooth muscle cells of the segment (30).

Measurement of intracellular Ca²⁺. Intracellular calcium concentration ([Ca²⁺]) in the smooth muscle was measured ratiometrically by using the calcium-sensing dye fura 2-ace-toxymethyl ester (AM) (Molecular Probes; Eugene, OR) by a photometer-based system. The smooth muscle was loaded at 35°C by 30-min perfusion of endothelium-lysed vessels (see *Lysis of endothelium* with 1 μM fura 2-AM added to the luminal perfusate, followed by a 5-min washout. The preparations were mounted onto a small metal ring, placed in a glass-bottomed organ bath (0.5 ml volume), and viewed with an inverted microscope (Nikon Diaphot). The tissues were superfused with physiological salt solution maintained at 35°C at a rate of 5 ml/min. The vessel segments were illuminated for 100 ms, every second being sequentially exposed to 50 ms of 340 nm and 380 nm of light from a Xenon bulb. Fluorescent light was passed through a 490-nm dichroic mirror and a 510-nm band-pass filter and measured by a photomultiplier. The respective emission intensities obtained during exposure to 340 and 380 nm of light were collected and recorded by computer, and the ratio was calculated and displayed during the experiment.

*Lysis of endothelium.* To improve loading of the smooth muscle cells with fura 2-AM, the lymphatic endothelium was damaged in vitro by repeatedly (5–6 times) passing brief (5–10 s) streams of air through the lumen of the vessels at a rate of ~3 μl/min. The success of the endothelial destruction was confirmed by applying ACh (100 μM), followed by sodium nitroprusside (SNP; 100 μM). A negative response to ACh and a positive response to SNP were used as confirmation of the success of the procedure. Endothelial destruction on the basis of this testing procedure proved successful in ~50% of treated vessels. The use of SNP was necessary because it has been shown that 40% of guinea pig mesenteric lymphatic vessels with an intact endothelium do not respond in any way to either ACh or SNP. The main reason for the lack of response was due to a high basal production of NO (34).

Chemicals and drugs. Glibenclamide and SNP were purchased from Sigma, U-46619 was purchased from Cayman Chemicals (Ann Arbor, MI), β-phényl-1,N₂-éthanol-8-bromo-guanosine-3’-5’-cyclic monophosphorothioïde Rpi-somer (Rp-8-Br-PET-cGMP), 8-(4-chlorophénylethyl)-guanosine-3’,5’-cyclic monophosphate (8-pCPT-cGMP), and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3’,5’-cyclic monophosphorothioïde Sp-isomer (Sp-5,6-DCl-cBIMPS) to give 10 μM (3 mM for U-46619) or in distilled water (SNP, Rp-8-Br-PET-cGMP, 8-pCPT-cGMP, and Sp-5,6-DCl-cBIMPS) to 10 μM. The drugs were dissolved in dimethyl sulfoxide (forskolin, glibenclamide, H-89, KT-5823, KT-5720, ODQ, and U-46619) or in distilled water (SNP, Rp-8-Br-PET-cGMP, 8-pCPT-cGMP, and Sp-5,6-DCl-cBIMPS) to give 10 μM stock solutions. DE-NO NONOate was solubilized in 0.1 N NaOH according to manufacturer’s instructions. After dilution of the drugs to their appropriate final concentrations in physiological salt solution, the diluted vehicle achieved concentrations <0.1%, a concentration that had no effect on the responses under investigation.

Data analysis. The effects of agonists and inhibitors were analyzed only when the membrane potential at the beginning of the recording period was greater than ~45 mV. In experiments where inhibitors were studied, agonists to be tested were applied first as a control and second, at least 15 min later, in the presence of the inhibitor that had been superfused for at least 10 min. This protocol was usually performed during the same impalement or in some instances on successive impalements obtained from neighboring cells in the same segment. No significant difference in the response induced by a given agonist applied 15 min apart in the absence of a blocker was observed (33–35). The agonists were used at concentrations giving maximal effects on the smooth muscle hyperpolarization and decrease in STD activity, as established during preliminary experiments (results not shown). Subplasmalemmal Ca²⁺ release activity was assessed by recording STDs with the level of activity determined by measuring the frequency and amplitude of events >1 mV. STD frequency and amplitude measured during an interval of 15–60 s (depending on the stability of the recording) before the application of the substance to be tested were compared with that measured during a period of the same duration while the substance was applied.

Experimental data are expressed as means ± SE. Statistical significance was assessed with the use of paired or unpaired Student’s t-test (as specified in the text), with *P* < 0.05 being considered significant.

RESULTS

Spontaneous and agonist-induced transient depolarizations in lymphatic smooth muscle. Lymphatic smooth muscle membrane potential was recorded in short segments (length <350 μm, diameter <230 μm) of guinea pig mesenteric lymphatic vessels and had a mean resting value of ~51 ± 1 mV (*n* = 105). Many vessel segments exhibited STDs, which at times for larger STDs or summations thereof generated action potentials and associated contractions (30).

The variability in STD activity between vessel segments made it difficult to study NO action. However, in cases where spontaneous activity was low, we overcame this by using the stable thromboxane A₂ mimetic U-46619, an agonist that is known to increase synthesis of Ins(1,4,5)P₃ (7) and lymphatic pumping (16). We show here that U-46619 (0.1 μM) markedly increased STD activity, which now occurred at a higher frequency and amplitude with relative increases of 186 ±...
28 and 185 ± 18% of control, respectively (n = 14 segments; Fig. 1A). The enhanced STD frequency and size was at times associated with generation of action potentials (Fig. 1B). When tested, lower concentrations of U-46619 (0.01–0.05 M) often failed to change STD activity, whereas higher concentrations (0.5–1 M) led to an additional depolarization and summation of action potentials (results not shown). When relative $[\text{Ca}^{2+}]_i$ was measured in fura 2-loaded vessels, U-46619 (0.1 M) caused a basal increase in $[\text{Ca}^{2+}]_i$ and Ca$^{2+}$ transients (n = 4, Fig. 1C); the latter caused by action potentials, resulting in constrictions. However, such photometric measurements did not clearly resolve the smaller Ca$^{2+}$ transients that might correlate with STDs, which presumably arise through Ca$^{2+}$ release occurring in a localized region very near to the cell plasmalemma (24, 32).

**Effect of NO on STD activity.** ACh has been shown to induce an endothelium-dependent release of NO in lymphatic vessels of the guinea pig mesentery (34). NO decreased the frequency of STDs and/or action potentials and hyperpolarized the smooth muscle membrane potential (Fig. 2A; see also Ref. 34). These responses were mimicked by application of the NO donor SNP (Fig. 3A; see also Refs. 33 and 34). STDs measured at the peak of the 12 ± 1 mV (n = 16) hyperpolarizations to 10 M ACh and 100 M SNP showed marked respective decreases in both frequency (65 ± 7 and 56 ± 8% of control) and amplitude (74 ± 6 and 63 ± 5%). In 5 of the 18 segments, the ACh inhibition of STD was preceded by a transient increase in STD amplitude that occurred during the initial phase of the hyperpolarization (see Fig. 2B). Such a response was never observed during the SNP-induced hyperpolarization and may have arisen through a direct action of ACh on the smooth muscle. This possibility is further supported by frequent observations that ACh caused an increase in electrical activity after NO synthase or guanylyl cyclase inhibition (34).

Similarly, SNP (100 M) in the presence of U-46619 (0.1 M) decreased STD frequency and amplitude to 47 ± 16 and 52 ± 5% of control and caused a hyperpolarization of 8 ± 1 mV (n = 4). Because the effective concentration of NO released by SNP is not known, and because SNP releases cyanide moieties that could potentially participate in the SNP response (see Ref. 11), we examined the effect of exogenous NO by using DETA-NONOate. NO release from DETA-NONOate has been well characterized and it is known that this compound applied at 100 and 500 M produces a NO concentration of 200 M and 1 mM, respectively (17). We found that application of 100–500 M DETA-NONOate had an effect similar to that of SNP, inducing a hyperpolarization of 11 ± 3 mV and decreasing STD frequency and amplitude to 48 ± 6 and 63 ± 9% of control, respectively (n = 4; Fig. 3B).

**Effects of NO on STD activity during block of NO-induced hyperpolarization.** Membrane hyperpolarization induced by NO has been previously shown to be inhibited by glibenclamide (33). We used this effect of glibenclamide to evaluate the role of NO on modulation of STD activity in the absence of confounding effects caused by the NO-associated hyperpolarization. Superfusion of the vessels with glibenclamide (10 M) depolarized the smooth muscle membrane potential from −51 ± 2 to −46 ± 2 mV (n = 11) without notably affecting STD activity. Glibenclamide essentially abolished the hyperpolarization caused by ACh (10 M) and SNP (100 M). The values were now not significantly different from control (ACh, 3 ± 1 mV, n = 6; SNP, 1 ± 1 mV, n = 9). Under these conditions, ACh and SNP still caused inhibition of STDs, with frequen-
cies reduced to 70 ± 7 and 67 ± 10% of control and amplitudes decreased to 62 ± 2% (n = 6) and 66 ± 7% (n = 9) for ACh and SNP, respectively (Figs. 2 and 3, A and C,a). In the presence of glibenclamide, application of DETA-NONOate (100–500 μM) decreased STD frequency to 56 ± 12% of control and STD amplitude to 73 ± 8% of control (n = 4; Fig. 3, B, and C,b). Comparison of these values with those obtained at the peak of hyperpolarization in the absence of glibenclamide gave a value of P > 0.05 (unpaired Student’s t-test). This result suggests that the NO-induced hyperpolarization has a minor role, if any, in regulating STD frequency and amplitude.

Effect of NO on [Ca2+]i. Measurements of the relative [Ca2+]i during stimulation with 0.1 μM U-46619 indicated that SNP had an antagonist action, with SNP (100 μM) decreasing [Ca2+]i, to 89 ± 2% (n = 4, P < 0.05, paired Student’s t-test) and totally inhibiting action potential-related Ca2+ transients (n = 4; Fig. 4A). These experiments were repeated in the presence of glibenclamide. Glibenclamide (10 μM) itself caused an increase in [Ca2+]i (107 ± 1%, n = 4, P < 0.01), which was transient and returned to control levels in <10 min. SNP (100 μM), applied in the presence of glibenclamide after [Ca2+]i had returned to control levels, caused a small decrease in the baseline [Ca2+]i (96 ± 1%, n = 3, P > 0.05) and abolished action potential-related Ca2+ transients (Fig. 4B).

Role of cGMP in NO-induced decrease in STD activity. Analyzing the response to SNP during superfusion with the soluble guanylyl cyclase inhibitor ODQ tested the possibility that NO was acting via soluble guanylyl cyclase to cause an increase in cGMP. Application of ODQ (10 μM) to control solution caused a rapid increase in STD frequency (138 ± 11% of control) and STD amplitude (172 ± 18% of control, n = 8; Fig. 5, A, and D,a). This result was associated with a depolarization of 6 ± 1 mV. These effects are consistent with those observed in the presence of the guanylyl cyclase inhibitor methylene blue (34). ODQ inhibited the decrease in STD activity induced by 100 μM SNP, which now remained near control levels (89 ± 5% for frequency and 82 ± 16% for amplitude; n = 5; Fig. 5B and D,b). In three of these five recordings made in the presence of ODQ (10 μM), SNP (100 μM) produced a depolarization and in two of these recordings produced subsequent induction of action potentials and tissue constriction. We further tested the involvement of cGMP in the decrease in STD by using the membrane-permeant cGMP analog 8-pCPT-cGMP. When applied in physiological salt solution containing 10 μM glibenclamide, 8-pCPT-cGMP (100 μM) reduced STD frequency to 65 ± 7% and amplitude to 61 ± 11% of control (n = 5; Fig. 5, C,a and D,c). This reduction persisted during superfusion with ODQ, with values
of $65 \pm 9$ and $49 \pm 13\%$ of control for frequency and amplitude, respectively ($n = 4$, Fig. 5, C,b and D,c).

**Effect of cAMP- and cGMP-dependent protein kinases on SNP-induced decrease in STD activity.** cGMP action to relax smooth muscle is believed to act primarily through cGMP-dependent protein kinase G (PKG) activation (18). However, it has been shown that cAMP-dependent protein kinase A (PKA) might also be involved in some of the NO/cGMP-mediated effects (33). The involvement of PKG and PKA in the intracellular mechanism underlying NO-mediated decrease in STD activity was investigated by examining the effects of different protein kinase inhibitors on the SNP inhibition of STD activity. Membrane potential and response to SNP (100 $\mu$M) were first recorded in the presence of glibenclamide (10 $\mu$M) and then during superfusion with protein kinase inhibitors.

The ability of SNP to decrease STD frequency was significantly reduced by the PKG inhibitor KT-5823. In the presence of KT-5823 (1 $\mu$M), 100 $\mu$M SNP decreased STD frequency to $63 \pm 7\%$ of control compared with $27 \pm 9\%$ before KT-5823 application ($n = 5, P = 0.013$, unpaired Student’s t-test). In contrast, KT-5823 did not significantly affect the decrease in STD amplitude caused by 100 $\mu$M SNP (58 \pm 8\% of control before and 62 \pm 6\% of control in 1 $\mu$M of KT-5823; $n = 5; P = 0.688$; Fig. 6A and C,a). KT-5823 alone increased STD frequency and amplitude to $125 \pm 10$ and $150 \pm 31\%$ of control, respectively ($n = 3$). Similar results were obtained with Rp-8-Br-PET-cGMPS (100 $\mu$M), another PKG inhibitor. Rp-8-Br-PET-cGMPS increased STD frequency and amplitude to $156 \pm 27$ and $130 \pm 34\%$ of control, respectively, and depolarized the membrane potential by $5 \pm 1$ mV ($n = 3$). In the two experiments successfully performed, Rp-8-Br-PET-cGMPS reduced the SNP-induced decrease in STD frequency from 47 and 25\% of control before to 59 and 72\% of control during Rp-8-Br-PET-cGMPS and the SNP-induced decrease in STD amplitude from 69\% and 46\% of control before to 86\% and 104\% of control during Rp-8-Br-PET-cGMPS.

The involvement of PKA was examined by using KT-5720 and H-89, two PKA inhibitors that are structurally unrelated. In the presence of KT-5720 (1 $\mu$M), the SNP-induced decrease in STD frequency was $76 \pm 12\%$ of control compared with $32 \pm 8\%$ observed before the application of KT-5720 ($n = 5, P = 0.016$, unpaired Student’s t-test). Similarly, the SNP-induced decrease in STD amplitude was reduced from $72 \pm 4\%$ of control before (10 $\mu$M glibenclamide present) to $84 \pm 3\%$ of control in the presence of KT-5720 ($n = 5, P = 0.047$; Fig. 6B and C,b).
DISCUSSION

Properties of STDs. Lymphatic vasomotion in the guinea pig mesentery has been shown to be initiated by STDs, events that are proposed to be caused by Ca\(^{2+}\) release from intracellular stores and activation of a Ca\(^{2+}\)-dependent Cl\(^{-}\) current (30, 32). This interpretation is based on the finding that STD activity is abolished by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM and by exposure to low-Cl solution (30). STD activity is also suppressed by the Cl\(_{Ca}\)-channel blockers 9-AC (1 mM) and niflumic acid (100 \(\mu\)M) (Ref. 32 and P.-Y. von der Weid and D. F. Van Helden, unpublished data). Further evidence for a Cl\(_{Ca}\) current in lymphatics has also been presented from studies on freshly dispersed sheep mesenteric lymphatic smooth muscle cells (28). This current was shown to be sensitive to [Cl] and inhibited by 9-AC. STD-like events observed in some of these cells were also blocked by 9-AC. The study presented here has further investigated STDs, examining the action of NO. Data presented indicates that NO decreases STD frequency and amplitude by production of cGMP and activation of both PKG and PKA.

Role of thromboxane A\(_2\) in STD activation. Previous observations have demonstrated the importance of arachidonic acid metabolites on lymphatic vessel contractility. In particular, it has been shown that U-46619, a stable mimetic of thromboxane A\(_2\), increased lymphatic pumping in both sheep and bovine mesenteric lymphatics (16). Thromboxane A\(_2\) has also been shown to be responsible for the endothelial-dependent enhancement of lymphatic vasomotion induced by substance P in the guinea pig mesentery (25). The present study results indicate that U-46619 enhanced the occurrence of the pacemaker Ca\(^{2+}\) release measured as STD, which when superthreshold induced action potentials, and associated Ca\(^{2+}\) transients that underlie constrictions of lymphatic vessels. This increased Ca\(^{2+}\) release is consistent with thromboxane A\(_2\) receptors being linked to a G protein/phospholipase C/Ins(1,4,5)P\(_3\) pathway, as demonstrated in vascular smooth muscle (12). A similar mechanism has been proposed for other known activators of Ca\(^{2+}\) release, which have been shown to increase STD activity in guinea pig mesenteric lymphatics (30, 32).

NO as inhibitor of STD activity. The present study demonstrates that NO, released either by the endothelium after ACh stimulation or by the NO donors SNP and DETA-NONOate, decreases STD activity. Specifically, NO reduced STD frequency and amplitude. While the decrease in amplitude could at least have resulted in part from a direct action on the Ca\(^{2+}\)-activated channels, the decrease in frequency indicates a direct action on pacemaker Ca\(^{2+}\) release. This observation confirms that NO is an important factor in...
modulating lymphatic vessel pacemaking and pumping as shown both in vitro (34, 37) and in vivo (26).

Although several studies (3, 36) demonstrated that NO may act through cGMP-independent mechanisms, the NO-induced inhibition of lymphatic pacemaker activity appears to be due to an increased production of cGMP. This is supported by the findings that STD inhibition was induced by directly increasing the intra-

Fig. 5. Effects of cGMP on STD activity before and during application of SNP. A: membrane potential recording showing the effect of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) on spontaneous STD activity. B: membrane potential recording in the presence of glibenclamide (10 μM), showing the effect of SNP (100 μM) on STD activity in the absence (a) and presence of 10 μM of ODQ (b) superfused for 10 min before the readdition of SNP. C: recording from two different vessel segments exposed to 10 μM glibenclamide, showing a decrease in STD activity induced by the cGMP analog 8-pCPT-cGMP (100 μM) in the absence (a) and presence of 10 μM ODQ (b). D: STD frequency and amplitude measured in the presence of ODQ (a) and during the maximum response to SNP (b) and 8-pCPT-cGMP (c) before and in the presence of ODQ (10 μM). Results are expressed as a percentage of values obtained during the same impalement, before application of the drugs. Values of bars are means ± SE, with the number of experiments indicated in parentheses. *P < 0.05, paired Student’s t-test compared with control.

Fig. 6. Effect of protein kinase G (PKG) and protein kinase A (PKA) inhibitors on the SNP-induced decrease in STD activity. A and B: membrane potential recordings in the presence of U-46619 (0.1 μM) and glibenclamide (10 μM; control conditions, left traces) and during the additional superfusion with SNP (100 μM) before (a, right traces) and in the presence (b) of KT-5823 (1 μM; A) and KT-5720 (1 μM; B). Recordings in A and B were from two different preparations. Scale bars apply to all recordings. C: STD frequency and amplitude before and during application of KT-5823 (1 μM, a) and KT-5720 (1 μM, b), measured during the maximum response to SNP (100 μM), are expressed as a percentage of values measured during the same impalement, before SNP application. Values of bars are means ± SE, with the number of experiments indicated in parentheses. *P < 0.05 and **P < 0.01, paired Student’s t-test compared with control. #P < 0.05 compared with value obtained with KT-5823 or KT-5720, respectively (unpaired Student’s t-test).
cellular cGMP concentration with 8-pCPT-cGMP and that the inhibitor of guanylyl cyclase, ODQ, prevented NO modulation of STD activity. Furthermore, the marked increase in STD activity observed in the presence of ODQ alone suggests that basal levels of cGMP in resting lymphatic smooth muscle are high enough to depress lymphatic pacemaking. The elevated cGMP concentrations are likely to be due to an endogenous release of endothelium-derived NO, as demonstrated by the enhancement of STD after application of the inhibitor of NO synthase N\textsuperscript{\textbullet}-nitro-L-arginine (34).

Possible mechanisms of NO-mediated decrease in STD activity. The NO- and cGMP-induced reduction of spontaneous and U-46619-associated STDs are unlikely to reflect a decrease in the activity of the Cl\textsubscript{Ca} channels proposed to underlie STDs. Thus, whereas direct inhibition of Cl\textsubscript{Ca} channels by NO has been reported in smooth muscle of the opossum esophagus (38), this may have occurred by an action of NO/cGMP altering the release of Ca\textsuperscript{2+} from intracellular stores underlying these potentials. We base this conclusion on the findings of Hirakawa et al. (13), who demonstrated that Cl\textsubscript{Ca} channels in the mouse and rabbit dispersed smooth muscle cells were not affected by NO or cGMP but indirectly by decreasing Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores. This interpretation is also consistent with the known action of cGMP to inhibit Ins(1,4,5)P\textsubscript{3}-induced stimulation of Ca\textsuperscript{2+} release (see Ref. 23) through a decrease in phosphatidyl inositol metabolism (see Ref. 14) or through a mechanism involving a primary cGMP-induced decrease in [Ca\textsuperscript{2+}], which subsequently affects phospholipase C activity (see Ref. 8 for a review). A recent study by Ghisdal et al. (10) demonstrated that inhibition of inositol phosphate production accounted for the hyperpolarization, decrease in [Ca\textsuperscript{2+}], and relaxation caused by NO donors in the rat superior mesenteric artery activated by norepinephrine. In addition to directly altering Ca\textsuperscript{2+} release from stores, NO and cGMP were suggested to alter Ca\textsuperscript{2+} influx. SNP and 8-Br-cGMP have been shown to inhibit voltage-gated L-type Ca\textsuperscript{2+} channels in smooth muscle cells isolated from rabbit pulmonary arteries (5) and guinea pig basilar arteries (27) and in A7r5 smooth muscle cell lines (2). The hypothesis that NO and cGMP act through a reduction of the smooth muscle [Ca\textsuperscript{2+}], is further supported by observations that cGMP and cAMP increased Ca\textsuperscript{2+} sequestration (19), increased Ca\textsuperscript{2+} extrusion (4), and/or inhibited Ca\textsuperscript{2+} influx (22).

Role of cyclic nucleotide-dependent protein kinases in NO-mediated decrease in STD activity. It is usually assumed that cGMP-mediated effects, in particular inhibition of Ca\textsuperscript{2+} release from intracellular stores, occurs predominantly via the activation of PKG (see Ref. 20). However, the present study revealed that inhibition of PKG, as well as PKA, altered the inhibitory action of the NO donor SNP on STD activity. There are two hypotheses that may explain these observations. The first is that cGMP causes a direct inhibition of phosphodiesterase III (PDEIII), the major phosphodiesterase isozyme present in platelets and vascular smooth muscle (see Ref. 1), thus preventing cAMP breakdown. It has been shown that the nitrovasodilators (SNP and 3-morpholino-sydnonimine) cause increases in platelet cAMP levels even in the absence of adenylate cyclase activators, due to inhibition of PDEIII (21). Therefore, it may be that SNP exerts its inhibitory effect on STD activity via a direct elevation of cGMP and a secondary elevation of cAMP, mediated by the inhibition of PDEIII. Alternatively, it may be that the modest selectivity of the cyclic nucleotide binding sites regulating PKA and PKG for their respective nucleotides results in PKA and PKG being activated by high concentrations of either cAMP or cGMP, respectively (18). Both mechanisms are consistent with the present observation that PKA inhibitors altered the NO-induced decrease in STD activity. Similar mechanisms have also been proposed to account, at least in part, for the antiproliferative effects of NO in cultured rat aortic smooth muscle cells (6) and for the bacterial enterotoxin-induced stimulation of Cl\textsuperscript{-} transport in cultured epithelial cells (9). Involvement of cAMP and/or PKA in the NO-induced decrease in STD is further suggested by the observation that forskolin and Sp-5,6-DCI-cBIMPS also reduce STD activity. It has been shown that both NO/cGMP- and forskolin/cAMP-induced hyperpolarizations of lymphatic smooth muscle are blocked by H-89, suggesting a dominant role for PKA in hyperpolarizations induced by NO (33). We conclude that NO decreases the frequency and amplitude of STD, the pacemaker potentials in lymphatic smooth muscle. This action is mediated through production of cGMP, leading to the activation of both
PKG and PKA. This mechanism is distinct and independent from the NO-induced hyperpolarization due to activation of KATP channels. Although lymphatic vessel tone and constrictions depend on smooth muscle membrane potential, the inhibition of STD activity is suggested to be the primary contributor to inhibiting vasoconstriction. This is likely to occur through NO-induced inhibition of Ina_{(1,4,5)P}_3 receptor-mediated Ca^{2+} release, the key element underlying this pacemaker (31, 32). These findings confirm the important role of NO in the modulation of lymph propulsion.

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