NO mediates postjunctional inhibitory effect of neurogenic ACh in guinea pig small intestinal microcirculation

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Kotecha, N., and F. P. Coffa. NO mediates postjunctional inhibitory effect of neurogenic ACh in guinea pig small intestinal microcirculation. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1441–H1446, 1999.—The present study was designed to evaluate the role of the endothelium as an effector organ of neurally mediated inhibition of vascular tone. Acetylcholine (ACh), either released by stimulation of the submucosal ganglia or applied exogenously, inhibited phenylephrine (PE)-induced constrictions in arterioles of the guinea pig intestinal submucosa. N\(^\text{G}\) -monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide (NO) synthesis, attenuated the response to superfused ACh by 74% compared with 94% attenuation obtained with N\(^\text{G}\) -nitro-L-arginine (L-NNA). L-NNA attenuated the response to neurally released ACh by 98% and that to ionophoretically applied ACh by 92%. L-Arginine reversed the effects of both L-NMMA and L-NNA. Functional integrity of the endothelium was essential for the neurally mediated inhibition of PE-induced constrictions. However, neurogenic inhibition of neurally evoked constrictions was preserved despite endothelial disruption. It was concluded that at the postjunctional level, the mechanism of action of neurally released ACh was almost exclusively via a NO-dependent pathway, with the source of NO being the vascular endothelium.

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The L-NMMA-resistant component of the vasodilation found by Andriantsitohaina and Surprenant (1) may have been caused by 1) ACh acting directly on the arteriole smooth muscle (3, 13), 2) ACh stimulating the release of another relaxing factor from the endothelium, such as endothelium-derived hyperpolarizing factor (EDHF) (21), or 3) the inability of L-NMMA to completely inhibit NO synthesis in this preparation. The NO inhibitor N\(^\text{G}\) -nitro-L-arginine (L-NNA) has been shown to be more effective than L-NMMA in a number of in vitro preparations, including rabbit aorta, rabbit femoral artery, and rat mesenteric artery (12, 19, 20). Hence, in the present study, L-NNA was used to provide insight into the L-NMMA-resistant vasodilation observed by Andriantsitohaina and Surprenant (1).

The question posed in this study was twofold, i.e., is there another endothelium-dependent or -independent mechanism mediating cholinergic neurogenic inhibition and is the vascular endothelium the effector organ of neurally mediated postjunctional inhibition of vascular tone?

METHODS

Tissue preparation. Guinea pigs (Monash bred, either sex, 200–300 g) were killed by stunning with a blow to the back of the head followed by exsanguination. Use of these guinea pigs
and all procedures undertaken were approved by the Monash standing committee on ethics in animal experimentation. A small piece of ileum was removed from the animal, cut open, and pinned out tightly, mucosal side up. The mucosa was carefully peeled away, revealing several arteriole trees and submucosal plexus embedded within translucent connective tissue. A piece of connective tissue containing one or two arteriole trees was cut and peeled away from the underlying circular muscular layer. This piece of tissue was then pinned onto a small organ bath with a transparent base, with the mucosal side facing down. The preparation was continuously superfused with warmed physiological saline composed of (mM) 146 Na⁺, 5 K⁺, 2.5 Ca²⁺, 2 Mg²⁺, 134 Cl⁻, 25 HCO₃⁻, 1 HPO₄²⁻, and 11 D-glucose and equilibrated with carbogen gas (95% O₂:5% CO₂).

The organ bath containing the submucosal arteriole preparation was mounted onto an inverted microscope equipped with a television camera. A magnified image (×100) of the preparation was projected onto a television screen, and arteriole diameter was monitored by image analysis using the Diamtrak software program (22).

The submucosal arterioles were transiently constricted by iontophoretic application of phenylephrine (PE) from a micropipette. A bevelled glass pipette with a tip diameter of 80–100 μm, filled with physiological saline, was used to stimulate the ganglion or the perivascular nerves. It was connected to a standard pulse stimulator with the earth electrode in the recording chamber. Arterioles were exposed to ACh by superfusion, iontophoresis, or ganglion stimulation.

Vascular endothelium was disrupted by cannulating the parent mesenteric artery, which gives rise to an arteriole network, at its point of entry to the intestine. Disruption of the endothelium was obtained by perfusing arterioles with physiological saline containing 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (13).

Protocol. Submucosal arterioles were constricted every 2 min by iontophoretic application of 10 mM PE from a glass micropipette (10 Hz, 1–2 s). The arterioles constricted transiently within 1 s of PE application and remained constricted for 3–5 s. By altering stimulus parameters for the iontophoretic release of PE, we adjusted the amplitude of constrictions so that they were 30–50% of the resting diameter of the arteriole; this ensured that the constrictions were equivalent to the maximally obtainable constrictions as determined by exposing the arterioles to 100 mM isotonic KCl. Once adjusted, the amplitude of constrictions remained constant over at least 0.5 h. We investigated changes in the amplitude of these constrictions to ACh applied by superfusion, ganglion stimulation, or iontophoresis (see Fig. 1A). When the amplitude of constrictions had returned to control levels, the tissue was rested for ~20 min before further testing.

Analysis and statistics. Inhibition of a transient arteriole constriction was expressed as a proportion of the control constriction and was calculated as follows

\[ \text{Inhibition (c)} = 1 - (b/a) \]

where a is control PE-induced arteriole constriction, b is constriction to PE after application of ACh, and c is inhibition of constriction produced by ACh (see Fig. 1A). Values are expressed as means ± SE and were compared using a paired or unpaired Student’s t-test or ANOVA. A probability value of <0.05 was considered significant. Drugs used were ACh chloride, CHAPS, L-arginine, PE hydrochloride, and pirenzepine dihydrochloride (Sigma), L-NMMA (Calbiochem), L-NNa (Novabiochem), and tetrodotoxin (Research Biochemicals International).

RESULTS

Arteriole response to superfused ACh. ACh superfused into the organ bath attenuated the amplitude of arteriole constrictions induced by PE. Increase in concentration of superfused ACh produced an increase in inhibition of arteriole constrictions to PE (Fig. 1B). An EC₅₀ of 17.7 ± 8.0 nM (n = 4) was obtained from individual values of EC₅₀ calculated from sigmoid curves fitted to each set of data.

Effects of L-NNA and L-NMMA on arteriole response to superfused ACh. These experiments were performed to enable us to compare the results of our study with those obtained by Andriantsitohaina and Surprenant (1), because there are methodological differences between the two. A known concentration of ACh (5–20 μM) was superfused into the organ bath attenuated the amplitude of arteriole constrictions induced by PE. Increase in concentration of superfused ACh produced an increase in inhibition of arteriole constrictions to PE (Fig. 1B). An EC₅₀ of 17.7 ± 8.0 nM (n = 4) was obtained from individual values of EC₅₀ calculated from sigmoid curves fitted to each set of data.

Fig. 1. A: protocol used to assess inhibitory effect of ACh on phenylephrine (PE)-induced constrictions. Transient diameter changes of guinea pig submucosal arteriole to iontophoretic application of PE (10 Hz, 1 s) were obtained every 2 min. Effects of ACh (applied exogenously, either by superfusion or iontophoresis, or released from nerves) on PE-induced constrictions were measured. In example shown, ACh was applied iontophoretically (at 10 Hz for 1 s) 1 s before application of PE. Values obtained for analysis were control PE-induced constriction of submucosal arteriole (a), constriction of submucosal arteriole to PE after application of ACh (b), and inhibition of constriction produced by ACh (c). B: summary of inhibition produced by superfused ACh on constrictions to iontophoretically applied PE in guinea pig submucosal arteriole. Relationship between change in size of PE-induced constrictions (plotted as fractional inhibition) and concentration of superfused ACh (EC₅₀ 17.7 ± 8.0 nM) is shown; each point is mean ± SE of 4 observations. An apparent EC₅₀ taken from this curve with pooled data will not match value above because true value quoted was obtained from sigmoid curves fitted to each set of data.
NITRIC OXIDE MEDIATES NEURALLY RELEASED ACHE RESPONSE

Effects of L-NNA and L-arginine on arteriole response to iontophoretically applied Ach. Ach (0.1 µM) applied to the surface of an arteriole by iontophoresis (10 Hz for 1–2 s), 1 s before the application of PE, inhibited the subsequent arteriole constriction to PE. Stimulus parameters for the iontophoretic release of the Ach were adjusted to obtain an inhibition of constriction to PE in the range of 0.60 to 0.80 (Fig. 2C), to match the maximum inhibition produced by ganglion stimulation (see Effects of L-NNA and L-arginine on pirenzepine-sensitive arteriole response to ganglion stimulation). These experiments were performed to mimic the effects of neurally released Ach, both in time and amplitude of response obtained.

Incubation for 10 min in 100 µM L-NNA resulted in a significant attenuation of the inhibition produced by Ach from 0.75 ± 0.07 to 0.05 ± 0.03 (n = 6, Fig. 2C). In the presence of 10 mM L-arginine, the inhibition to Ach increased from 0.05 ± 0.03 (in L-NNA alone) to 0.63 ± 0.01 (n = 5, Fig. 2C).

Effects of L-NNA and L-arginine on pirenzepine-sensitive arteriole response to ganglion stimulation. The submucosal ganglia selected for stimulation were within a 500-µm radius of the arteriole being investigated. Ganglion stimulation (10 Hz for 10 s), 1 s before application of PE, inhibited the subsequent arteriole constriction to PE. These stimulus parameters were chosen to obtain maximal neural response (23). The inhibition produced by ganglion stimulation was in the range of 0.60 to 0.80 (Fig. 3) of control PE-induced contractions. Neurally mediated inhibitory responses were completely abolished in the presence of 1 µM tetrodotoxin, thus confirming the sole involvement of nerve conduction.

To ensure that the response to ganglion stimulation was mediated by Ach, its sensitivity to pirenzepine (a muscarinic antagonist) was tested. In the presence of 1 µM pirenzepine (a concentration that blocks all muscarinic receptors), the inhibition of arteriole constriction produced by ganglion stimulation was significantly reduced from 0.73 ± 0.07 to 0.07 ± 0.03 (n = 5, Fig. 3), indicating that the response being investigated was mediated by ACh. After pirenzepine was washed out, the inhibition of arteriole constriction to ganglion stimulation increased from 0.07 ± 0.03 (in pirenzepine) to 0.62 ± 0.07 (n = 5), which was not significantly different from control inhibition (0.73 ± 0.07; n = 5, Fig. 3).

Incubation in 100 µM L-NNA for 10 min significantly attenuated the pirenzepine-sensitive inhibition of arte-
arteriole constriction from 0.62 ± 0.07 to 0.02 ± 0.02 (n = 5, Fig. 3). This attenuation was reversed in the presence of 10 mM L-arginine, with an increase in inhibitory response to ganglion stimulation from 0.02 ± 0.02 (in L-NNA alone) to 0.47 ± 0.05 (n = 5, Fig. 3).

In the presence of L-NNA, attenuation of responses to neurally released ACh (98% ± 2%; n = 5) and iontophoretically applied ACh (92% ± 5%; n = 6) were not significantly different. Hence, a NO-dependent pathway was almost exclusively involved in the mechanisms of neurally released and matched exogenous ACh action.

Effects of endothelial disruption on arteriole response to ganglion stimulation. In four experiments, the inhibitory effect of ganglion stimulation on PE-induced arteriole constrictions was evaluated before and after the endothelium was disrupted using CHAPS (13). The protocol used was the same as that in the experiments described in Effects of L-NNA and L-arginine on pirenzepine-sensitive arteriole response to ganglion stimulation. Disruption of the endothelium resulted in a significant and complete abolition of neurally mediated inhibition (from 0.70 ± 0.14 to 0.16 ± 0.09, n = 4; Fig. 4).

Effect of ganglion stimulation on arteriole constrictions evoked by perivascular nerve stimulation. Interaction between ganglion stimulation and perivascular nerves was tested in three arterioles, after endothelial disruption, from the experiments described in Effects of endothelial disruption on arteriole response to ganglion stimulation. Arteriole constrictions were evoked by applying a train of stimuli (10 Hz, 1 s), once every 2 min, to the perivascular nerves. Evoked constrictions were abolished in the presence of 1 µM tetrodotoxin. Ganglion stimulation (10 Hz, 10 s, 1 s before an evoked constriction) significantly inhibited the subsequent constriction (0.69 ± 0.16, n = 3; Fig. 4).

DISCUSSION

In agreement with previous investigations, the results obtained in the present study suggest that nerve-released ACh mediates its response by stimulating the release of NO in guinea pig submucosal arterioles (1).

A discrepancy between the effects of L-NNA and L-NMMA has been observed in a number of vessels similar to that observed in this preparation (12, 19, 20). For instance, in the rabbit aorta L-NMMA produced a 65% inhibition of ACh-induced dilations, whereas L-NNA produced a 90% inhibition (12, 25). Although both L-NNA and L-NMMA are competitive inhibitors of NO synthase (the enzyme that catalyzes the synthesis of NO from L-arginine), they differ in that L-NNA is effectively irreversible and may bind covalently to the enzyme, whereas L-NMMA, but not L-NNA, is metabolized within endothelial cells, thus lowering the effective intraendothelial concentration of L-NMMA (5).

Hence, using the more effective NO synthase inhibitor, L-NNA, as opposed to L-NMMA (see RESULTS), we found a near-maximal block of the response to neurally released ACh (98%) and iontophoretically applied ACh (92%), which were not significantly different. Inhibition of superfused ACh-induced response by L-NMMA obtained in this study (~74%) correlates closely with that obtained by Andriantsitohaina and Surprenant (1) (~70%), indicating that the difference in methodology between the two studies cannot account for a more effective block obtained using L-NNA. Additionally, the EC50 value for the effect of ACh on PE-induced constriction (~18 nM) in this study is almost identical to that obtained for ACh-induced dilation of arterioles preconstricted with U-46619 (done as part of a study on diabetic guinea pigs; K. Eede and N. Kotecha, unpublished observation); the latter being the method used by Andriantsitohaina and Surprenant (1). Overall, it would appear that there are no fundamental problems associated with comparing the effects of ACh on constrictions...
obtained using the two different methods. Hence, we conclude that the inhibitory response of neurally released ACh is mediated almost exclusively by a NO-dependent pathway; this is in accord with recent work that demonstrates that NO accounts for all endothelium-dependent inhibitory effects of superfused ACh (13).

The movement of neurally released ACh through the media is feasible because, unlike the endothelium, vascular smooth muscle cells are not connected by tight junctions, which may hinder the movement of lipophilic molecules (16), but are electrically coupled by gap junctions (11) allowing ready access of adventitiously applied ACh to the abluminal side of the endothelial cells (16). The only diffusion barrier is physical, posed by the interleaving layers of smooth muscle cells. However, in the terminal submucosal arterioles, there is only a single layer of smooth muscle cells, which is 4 µm at its widest point (11). Such, the media of the arteriole wall does not pose a barrier to the movement of ACh from the adventitia to the endothelium. Experiments using detergent to remove the endothelium confirm that the functional integrity of the endothelium is essential for the inhibitory effects of intrinsic vasodilator nerves, implying that the source of NO in the present study must be the endothelium. We have shown in a previous study (15) that the cholinergic vasodilator nerves interact with the perivascular constrictor nerves to modulate the release of sympathetically transmitted nitric oxide by a prejunctional mechanism. The inhibitory effect of ganglion stimulation on arteriolar constrictions obtained by stimulation of perivascular nerve was preserved after the endothelium was damaged; this confirmed that the lack of response of neurally released ACh on PE constrictions was not a result of neural dysfunction related to the protocol used to disrupt the endothelium.

The inhibitory effects of exogenous ACh are known to be mediated by other NO-independent, endothelium-dependent and independent pathways. A factor released from the endothelium has been identified in many vascular beds that hyperpolarizes the smooth muscle of blood vessels (21). This EDHF has also been shown to contribute to ACh-induced vasodilations along with NO and prostacyclin (21). Additionally, ACh can act directly on the smooth muscle to produce vasodilation, as is the case in many vessels of the cephalic circulation, such as the lingual artery of the rabbit, the posterior auricular artery of the cat, and the submucosal arterioles of the guinea pig ileum (2, 3, 13). Although these various mechanisms can mediate the inhibitory effects of ACh, the focus of the current study was to elucidate the mechanisms mediating the effects of neurally released ACh from the intrinsic vasodilator nerves within the submucosa of the guinea pig. The near-maximal block (98%) produced by L-NNA in our study would suggest that NO is the main and perhaps the sole mediator of the endothelium-dependent inhibitory effects of neurally released ACh and is in agreement with recent findings in that cholinergic endothelium-dependent inhibition is fully accounted for by NO in the guinea pig submucosal arterioles (13). Kotecha (13) also demonstrated that ACh can cause endothelium-independent direct inhibition of the vascular smooth muscle. However, the concentrations required for this direct action are relatively higher (EC50 > 100 nM) compared with the endothelium-dependent inhibitory effects (EC50 ~ 20 nM). Because the maximum inhibitory effect produced by neurally released ACh corresponds to a concentration of 5–20 nM (see RESULTS), only the endothelium-dependent inhibitory effects are evident and any direct effect on the vascular smooth muscle does not make a significant contribution.

In conclusion, the cholinergic vasodilator nerves innervating the guinea pig submucosal arterioles mediate inhibition at both the pre- and postjunctional levels. The postjunctional effects are almost exclusively a result of stimulating the release of NO. Although previous studies have implicated the vascular endothelium as the source of NO, this is the first study to directly demonstrate this in the microvasculature of the guinea pig small intestine. As far as we know, this is also the only study in which the role of the vascular endothelium as an effector organ mediating neurogenic inhibition has been definitively determined.

This work was supported by the National Health and Medical Research Council of Australia.

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Received 24 March 1999; accepted in final form 26 May 1999.

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