Remote arteriolar dilations caused by methacholine: a role for CGRP sensory nerves?

Naris Thengchaisri and Richard J. Rivers

Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland

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Thengchaisri, Naris, and Richard J. Rivers. Remote arteriolar dilations caused by methacholine: a role for CGRP sensory nerves? Am J Physiol Heart Circ Physiol 289: H608–H613, 2005. First published March 11, 2005; doi:10.1152/ajpheart.01290.2004.—Remote vasodilation caused by arteriolar microapplication of acetylcholine cannot be completely attributed to passive cell-cell communication of a hyperpolarizing signal. The present study was undertaken to ascertain whether a neural component may be involved in the remote response. In the cheek pouch of anesthetized hamsters, methacholine (100 μM) was applied to the arteriole by micropipette for 5 s, and the arteriolar responses were measured at the site of application and at remote locations: 500 and 1,000 μm upstream from the site of application. Superfusion with the local anesthetic bupivacaine attenuated a local dilatory response and abolished the conducted dilation response to methacholine. Localized micropipette application of bupivacaine 300 μm from the methacholine application site also attenuated the remote dilatation but did not inhibit the local dilation. Blockade of neuromuscular transmission with botulinum neurotoxin A (1 U, 3 days), micropipette application of calcitonin gene-related peptide (CGRP) receptor inhibitor CGRP-(8–37) (10 μM) 300 μm upstream from the methacholine application site, and derenervation of the CGRP sensory nerve by 2 days of capsaicin treatment reduced the conducted dilation response to methacholine but did not affect the local dilatory response. Together, these data support involvement of a TTX-insensitive nerve, specifically the CGRP containing nerve, in vascular communication. Understanding the effect of regulation of a novel neural network system on the vascular network may lead to a new insight into regulation of blood flow and intraorgan blood distribution.

calcitonin gene-related peptide; microcirculation; conducted vasodilation; muscarinic receptor agonist; intrinsic nerves

BLOOD FLOW DISTRIBUTION within the organ is controlled through complex interactions that regulate arteriolar resistance and define the paths of blood flow. The resistances of the entire arteriolar network aggregate to define what is commonly known as organ resistance. The specific resistance of an individual arteriole is controlled by a number of local variables, including flow (17), pressure (19), metabolites (35), and neural input (33), whereas the coordinated network response requires vascular communication. Vascular communication is the process whereby areas of the vascular network adjust to the vasomotor signals that are generated within the tissue or at other remote locations along the vascular tree. It potentially plays a role in the precise control of intraorgan blood distribution (30, 31). Although various efforts have been made, the underlying mechanism of this vascular communication is not fully understood (9).

Hypermcanceling signals for vascular communication and for remote control of arteriolar diameter reportedly pass from cell to cell through gap junctions in the vessel wall (20). The length constant for decay of this signal is much larger than can be explained by the simple passive decay of an electrotonic signal (6), and simple blockade of gap junctions does not abolish the response (32). Therefore, additional pathways, such as nerves, may be involved, or the signal may be regenerated within the vessel wall as it moves from cell to cell. Because various neural innervations of elusive function (12) have been identified on the arterioles in the cheek pouch and because previous studies indicate that local anesthesia inhibits the vascular relaxation response to acetylcholine (23), this study was performed to determine whether there may be a role for neural mechanisms in the remote vasomotor signals generated by arteriolar muscarinic receptor stimulation.

MATERIALS AND METHODS

Hamster Cheek Pouch

Vascular communication can be initiated by a number of methods (4, 7, 24, 27, 30). In the present study, we initiated vascular communication by applying selective drugs onto an arteriole of the hamster cheek pouch. In accordance with Institutional Animal Care and Use Committee approved protocols, male golden hamsters (103–132 g body wt) were anesthetized with pentobarbital sodium (70 mg/kg ip). The cheek pouch was exteriorized for intravital microscopy (5) after tracheotomy and insertion of an endotracheal tube. Continuous intraperitoneal injection of saline (0.008 ml/min) and anesthesia (0.075 mg/min) maintained the appropriate levels of hydration and depth of anesthesia. The cheek pouch was continuously superfused with 37°C physiological salt solution (PSS; in mM: 132 NaCl, 2.0 CaCl2, 1.2 MgSO4, and 20 NaHCO3) equilibrated with 0%, 5%, or 10% O2, 5% CO2, and balance N2. Deep esophageal temperature of the hamster was maintained at 37°C by a combination of conductive and radiant heating.

The transilluminated pouch vasculature was viewed with a video microscope (Micro Instruments model M2 microscope with Nikon E400 components and an arm-mounted dissecting microscope, Witten, Oxon, UK). The microscope was coupled to a video (charge-coupled device) camera (model 72S, Dage-MTI, Michigan City, IN) and a video monitor. Diameter was measured with a video caliper (Texas A & M University Health Science Center) that was calibrated to cell through the vessel wall (20). The length constant for decay of this signal is much larger than can be explained by the simple passive decay of an electrotonic signal (6), and simple blockade of gap junctions does not abolish the response (32). Therefore, additional pathways, such as nerves, may be involved, or the signal may be regenerated within the vessel wall as it moves from cell to cell. Because various neural innervations of elusive function (12) have been identified on the arterioles in the cheek pouch and because previous studies indicate that local anesthesia inhibits the vascular relaxation response to acetylcholine (23), this study was performed to determine whether there may be a role for neural mechanisms in the remote vasomotor signals generated by arteriolar muscarinic receptor stimulation.

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Conducted Dilations Initiated by Muscarinic Receptor Stimulation

Methacholine (5 s, 100 μM) was applied by pneumatic ejection from a micropipette (26). Micropipette application of a drug is a
standard method we have used for many years. Specific application controls were used to eliminate concerns about diffusion of the drug to remote locations of interest (25). Arteriolar vasomotor responses were sequentially measured at the site of drug application (local) and 500 and 1,000 μm upstream from the site of application (Fig. 1). Sites were randomly viewed, and each response was measured at least twice. The arteriole was always allowed to return to baseline before the next measurement (≥3-min delay between ejections).

Neural Transmission Blockade

**Bupivacaine.** Bupivacaine blocks neural transmission by blocking Na⁺ channels, which are important for the transmission of action potentials along the nerve. Bupivacaine is especially useful here, because it can block Na⁺ channels that are not sensitive to TTX. TTX is commonly used to demonstrate neural involvement, although sensory nerves possess Na⁺ channels that are not affected by TTX. To test for the effects of local anesthetics, bupivacaine (100 μM) was added to the superfusion solution for ≥20 min before the responses were repeated. We found that bupivacaine caused some vasoconstriction and affected the local methacholine response. To eliminate these confounding variables and to determine whether the effects of bupivacaine were specific to transmission of the signal to the remote sites, we applied bupivacaine (10⁻²–10⁻³ M) by micropipette directly to the arteriole 300 μm upstream from the methacholine ejection site.

**Botulinum toxin.** The role of a neural pathway was investigated by inhibition of neural function with botulinum toxin. Botulinum toxin is a zinc-dependent protease that selectively cleaves synaptosomal-associated protein-25, a component of the fusion complex mediating synaptic vesicle exocytosis (1). This effectively blocks release of a transmitter at the synapse. We infiltrated 1 U of botulinum neurotoxin A (BoTox; BoTA) in 250 μl of saline into the exteriorized cheek pouch of a temporarily anesthetized hamster, and the animal was studied 2 days later. Tissue samples were fixed after experimentation to verify sensory nerve denervation. Arteriolar dilation responses to methacholine were compared between vehicle-control and capsaicin-treated animals.

**Calcitonin Gene-Related Peptide Receptor Blockade**

Stimulated sensory nerves release calcitonin gene-related peptide (CGRP), which causes vasodilation. CGRP-(8–37) (10 μM), a CGRP receptor antagonist, was continuously applied by micropipette at the arteriolar site 300 μm upstream from the methacholine application site to test for a role of sensory nerves in the remote vascular responses to methacholine. To verify the specificity of CGRP-(8–37), local arteriolar responses to adenosine, methacholine, and CGRP were tested at the site of CGRP-(8–37) application.

**Sensory Nerve Denervation**

The role of CGRP sensory nerves was further investigated by chemical denervation of the preparation with capsaicin (11). Capsaicin activates the vanilloid receptor on sensory nerves to cause an increase in intracellular Ca²⁺ and a release of neuropeptide. The receptor for capsaicin quickly desensitizes, but prolonged exposure to capsaicin causes a neurotoxic effect and results in sensory nerve denervation (16). Capsaicin (0.013 M in 10% alcohol, 100 μl) or vehicle control was injected into the exteriorized cheek pouch of a temporarily anesthetized hamster, and the animal was studied 2 days later. Tissue samples were fixed after experimentation to verify sensory nerve denervation. Arteriolar dilation responses to methacholine were compared between vehicle-control and capsaicin-treated animals.

**Immunohistochemistry**

To confirm the effect of sensory nerve denervation, cheek pouches from vehicle-control and capsaicin-treated animals were excised, pinned onto dental wax, and then fixed in 2% formaldehyde and 0.2% picric acid. Tissues were permeabilized by incubation in Triton X-100 (0.5%) in PBS (pH 7.4) for 30 min at 37°C and washed several times in PBS. Whole mounts were rinsed three times (5 min each) in PBS and then incubated with secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:200 dilution; Jackson Laboratory, Bar Harbor, ME) at 4°C overnight. The whole mounts were rinsed three times (5 min each) in PBS and then incubated with secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:200 dilution; Jackson Laboratory, Bar Harbor, ME) at room temperature for 2 h. The whole mounts were rinsed three times (5 min each) in PBS and then observed with fluorescent light microscopy using our standard microscope and an intensified charge-coupled device camera (model XR/MEGA10, Stanford Photonics). Images were captured using a standard gain protocol, and 10 images were averaged to minimize noise.

**Chemicals**

Bupivacaine, capsaicin, CGRP, CGRP-(8–37), CsCl, methacholine, and sodium nitroprusside were purchased from Sigma (St. Louis, MO) and BoTA from Allergan (Irvine, CA). The stock solutions (10 mM) of bupivacaine and capsaicin were first made in distilled water and alcohol, respectively. The other chemicals were dissolved in PSS. The subsequent concentrations of all chemicals were achieved by dilution in PSS.

**Data Processing and Statistical Analysis**

For each treatment, at least two measurements were obtained from a single vessel. The averages were used for further comparison. Changes in diameter after the drug applications were compared at each location using ANOVA with repeated measures or a compound response model (JMP3.1.5, SAS Institute). \( P < 0.05 \) was considered statistically significant. Values are means ± SE.

**RESULTS**

A total of 50 arterioles from 44 hamsters were used for data collection. The average “maximal” diameter of all the methacholine application sites, determined by their responses to methacholine (10 mM), was 43.2 ± 1.5 μm, and the average
baseline diameter, measured after equilibration with 5% O₂ but before any treatment, was 25.4 ± 1.2 μm.

**Neural Transmission Blockade**

*Bupivacaine.* The local anesthetic bupivacaine, applied to the cheek pouch via the superfusate, led to a small decrease in resting diameter: from 29.3 ± 2.2 (control) to 23.6 ± 1.4 μm (with bupivacaine). Bupivacaine significantly partially inhibited the local dilatory response and completely abolished the remote dilation response 500 and 1,000 μm upstream from the methacholine application site (Fig. 2). The actions of bupivacaine suggested an important role for voltage-gated Na⁺ channels and involvement of the neural pathway in remote dilations initiated by methacholine.

Bupivacaine affects local and remote responses when applied via the superfusion solution. To dissect the effect of bupivacaine on local vs. remote dilation responses, we ejected bupivacaine onto the arteriole by micropipette 300 μm upstream from the methacholine application site (Fig. 1). Interestingly, localized inhibition with bupivacaine significantly attenuated the conducted response 500 and 1,000 μm from the methacholine ejection site without affecting the local dilatory response to methacholine (Fig. 3).

**Na⁺ depletion.** To investigate the involvement of Na⁺ channels in conducting the signal along the nerve, we replaced NaCl with CsCl in the superfusion solution. Interestingly, CsCl significantly inhibited the local and conducted dilation responses to methacholine similar to superfusion with bupivacaine, further supporting involvement of Na⁺ channels in remote dilation (Fig. 4A).

**BoTA.** To further confirm the involvement of a neural response in the remote dilation response to methacholine, BoTA (1 U, 3 days) was used to block neurotransmitter release. BoTA did not alter the local dilatory response to methacholine but significantly inhibited the remote dilation 500 and 1,000 μm from the methacholine application site (Fig. 4B). The local dilatory response to sodium nitroprusside (14.8 ± 1.4 and 15.8 ± 1.2 μm for vehicle control and BoTA, respectively, n = 5) or local contraction response to phenylephrine (19.8 ± 1.6 and 17.1 ± 1.9 μm for vehicle control and BoTA, respectively, n = 7) was not affected by BoTA.

**Role of CGRP Sensory Nerves in the Arteriolar Dilation Response to Methacholine**

Because TTX does not block these conducted responses (3), whereas bupivacaine does, an alternative explanation was sought. Bupivacaine is reported to also block TTX-resistant Na⁺ channels, which are found in sensory nerves (2). We therefore tested for a role for sensory nerves in these remote responses.

**CGRP receptor blockade.** Application of CGRP-(8–37) 300 μm from the methacholine application site significantly attenuated the conducted dilation response to methacholine but did not affect the local dilatory response (Fig. 5A). To confirm the specificity of CGRP-(8–37), we tested the local dilatory response to adenosine, CGRP, and methacholine. CGRP-(8–37) inhibited the local dilatory response to CGRP but did not affect the local dilatory response to adenosine and methacholine (Fig. 5B). CGRP is a potent vasodilator for hamster cheek pouch arteriole (EC₅₀ ～ 2 × 10⁻⁵ M; Fig. 5B, inset).

**Sensory nerve denervation.** CGRP sensory nerves were chemically denervated by infiltration of capsaicin into the cheek pouch, and the arteriolar dilation response to methacholine was examined 2 days later. The local arteriolar methacholine-induced dilation was not affected by capsaicin, but the remote dilation response was significantly attenuated 500 and 1,000 μm upstream from the methacholine ejection site (Fig. 6A). Immunohistochemical images of hamster cheek pouch confirmed that capsaicin decreased the presence of the CGRP sensory nerve (Fig. 6B).

**DISCUSSION**

Our data provide evidence that vascular communication initiated by methacholine stimulation involves activation of CGRP sensory nerves. In contrast to the reported lack of effect of TTX (8), our data show that remote dilations caused by methacholine are inhibited by bupivacaine, BoTA, cesium, capsaicin, and CGRP-(8–37), suggesting involvement of the CGRP sensory nerve in the remote dilation response. These data strongly suggest that activation of methacholine receptors leads to activation of a neural network and release of CGRP onto the arteriole at locations remote from the methacholine.
application site to enhance the remote dilation. The mechanism whereby arteriolar muscarinic receptor activation generates a neural signal is not known. There may be activation of vascular muscarinic receptors with secondary transfer of a neurotransmitter from the vessel wall to the sensory nerve (22), or there may be direct activation of muscarinic receptors on peri vascular nerves themselves (21).

A role for neural networks in blood distribution has been previously demonstrated in various vascular beds, including intestine (10), brain (36), and heart (18). Release of vasoactive substances during hypoxia (22) may play an important role in initiating vascular communication during the hyperemic response. However, our data shed light on a potential neural mechanism that is affected by muscarinic receptor activation. This neural activation may coordinate blood flow distribution by modulating the conducted dilation along the arteriolar network. Consistent with our study, Figueroa et al. (8) showed that electrical stimulation of the arteriole can cause conducted vasodilation that is not affected by TTX. However, TTX did prevent sympathetic nerve conduction caused by the same electrical stimulation. In this case, it is possible that TTX-insensitive sensory nerves were activated by the electrical stimulation (2). Thus multiple mechanisms, including innervation of the sensory nerve, may play a role in remote vasodilatory responses.

Numerous studies suggest a mechanism for vascular communication that involves cell-cell communication of a hyperpolarizing signal. Nerve conduction involvement in remote vasodilation complements the cell-cell gap junction mechanisms that have been previously described (8). In the present studies, remote dilation in response to methacholine was only partially attenuated by capsaicin denervation and the CGRP receptor antagonist CGRP-(8–37), leaving an ample residual response that can be explained by other mechanisms, such as cell-cell transmission of a hyperpolarizing signal.

It is most interesting to note that microapplication of CGRP-(8–37) and bupivacaine to the arteriole attenuated the dilation responses farther upstream, beyond the direct site of drug application. The drug did not need to be applied directly to the area of the arteriole that was dilating to effect the response. This suggests two possibilities: 1) Perivascular release of CGRP causes conducted dilation beyond the area of neuropeptide release. This seems unlikely, however, because we were unable to reliably demonstrate conducted dilation with direct microapplication of CGRP. 2) CGRP receptor activation on the...
arteriole may modulate cell-cell conduction rather than directly
dilate the vessel itself. So interruption of the neural mechanism
attenuates conduction of vasodilatory signals along the vessel
wall.

Acetylcholine and nitric oxide have been shown to enhance
conducted dilation (3, 6). The neural pathway we describe
offers one possible mechanism for this enhancement. Activa-
tion of the CGRP nerves by either substance would cause
release of perivascular CGRP, which would modulate cell-cell
conduction. This would cause less decay of the conducted
signal and potentially turn a decaying signal into a potentiated
signal. Variability in this CGRP mechanism could explain the
well-described variability in cell-cell conduction. In addition, a
recent study indicates that a similar neural mechanism plays a
role in the myogenic response (29). Thus perivascular nerves
appear to be playing a complex, critical role in the coordina-
tion of blood distribution that is affected by perivascular humoral
agents as well as intrinsic properties of the blood vessel. This
means that direct activation of neural muscarinic receptors by
intrinsic acetylcholine [as suggested to be released near neu-
romuscular junctions (34)] may not be the most interesting
physiological process described by these data.

The effect of various muscarinic receptor antagonists on
methacholine-induced local and conducted dilation was previ-
ously reported from our laboratory (26). The $M_3$ muscarinic
receptor antagonists had a higher equilibration constant ($K_B$)
for the local response, and the $M_2$ receptor antagonist had a
higher $K_B$ for the conducted response. Furthermore, the mus-
carinic $M_2$ receptor has been shown in sensory neurons of rat
dorsal root ganglion, I-B4-positive neurons, keratinocytes, and
endothelial cells (13). Thus it is possible that the muscarinic
$M_2$ receptor is involved in the activation of the CGRP sensory
nerve to effect conducted dilation.

Clinical Implication

The CGRP-1 receptor has been identified in human coronary
artery (15) and bronchial blood vessels (14), indicating a role
for the CGRP nerve in coronary and bronchial vasculature. The
role of vascular conduction in coronary microvessels (28) has
been identified, but the role of CGRP in microvascular coordi-
nation requires further investigation.

In summary, this study has revealed a neural component to
the vascular communication initiated by muscarinic receptor
activation in the microcirculation of the hamster cheek pouch.
These data indicate that local coordination of the blood distri-
bution may be actively modulated by an intrinsic neural net-
work.

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Present address of N. Thengchaisri: Dept. of Companion Animal Clinical
Sciences, Kasetsart University, 50 Paholyothin Rd., Bangkok 10903, Thailand.

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