Mechanisms underlying mechanosensitivity of mesenteric afferent fibers to vascular flow

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The extrinsic innervation of the gastrointestinal tract largely follows the vascular supply in the mesenteric arcades. Nerve and blood vessel development are sensitive to similar guidance cues (8), and thus the vascular tree is often viewed as a scaffold via which neurones reach their targets. However, the mesenteric vascular tree is richly innervated by both postganglionic sympathetic fibers and by afferent fibers with cell bodies in the dorsal root ganglia. These fibers form a dense perivascular network around mesenteric blood vessels and play a functional role in regulating vascular tone (9, 12, 28). Rather than penetrating the vascular smooth muscle, these fibers form varicose or smooth endings, either in the adventitia or at the medial-adventitial border of the vessel (19, 20). The role of sympathetic efferent (motor) nerves in regulating blood vessel tone, and hence blood flow, is well established; however, afferent fibers are also thought to regulate blood flow via axon reflexes (14). Afferent terminals often colocalize the neuropeptides substance P and CGRP, especially in small laboratory animals. Activation of these so-called “sensory motor” nerves is thought to cause antidromic activation of axon collaterals, which, in turn, release CGRP and substance P to cause vasodilation and increased permeability (16). This motor action is thought to mediate the vasodilatory “flare,” part of the “triple response” to cutaneous injuries. Similar reflexes may underlie hyperemia in the gastrointestinal tract and may play a role in cytoprotection (13).

In our previous study (6), we suggested that mesenteric afferents may also sense changes in gastrointestinal blood flow. This sensitivity persisted after removal of the gut itself and, therefore, was a property of the mesenteric membrane and its vascular supply, prior to where they penetrate the gut wall. We speculate that this sensitivity is part of a novel mechanism for detecting and responding to hemodynamic disturbances. However, the mechanism by which mesenteric sensory nerves detect changes in flow was not identified. Therefore, in the present study, we used a novel in vitro mesenteric vascular preparation in combination with anterograde nerve labeling experiments to investigate 1) the location and 2) the mechanism of afferent sensitivity to vascular perfusion. We offer evidence that flow sensitivity arises as a consequence of direct distortion of the afferent endings on blood vessels rather than being secondary to mediator release from the vascular endothelium or mechanical responses of vascular smooth muscle.

METHODS

Tissue Preparation

Male Wistar rats (350–400 g) were anesthetized with pentobarbital sodium (60 mg/kg), and a midline laparotomy was performed. The terminal ileum (~3 cm rostral to the ileocecal junction) and the arterial supply were identified, and the vessels were exposed by carefully removing connective tissue from around the superior mesenteric artery and vein. A branching section of an artery with a clear projection to a segment of the ileum was chosen, and unwanted side branches were ligated, as previously described (6).

The ileal segment, complete with the mesenteric arcade and an accompanying piece of the mesenteric artery, was then removed and quickly immersed in ice-cold saline. After the gut lumen was flushed, the tissue was placed in a purpose-built chamber consisting of a recording chamber and a common bathing and perfusion chamber (Fig. 1). The gut “tube” was removed by cutting along the mesenteric border, and the mesenteric fan was pinned flat in the bathing chamber and perfused with Krebs-bicarbonate-saline solution [composed of (in...
Fig. 1. Diagrammatical representation of the apparatus used to simultaneously record afferent discharge (AD) and vascular perfusion pressure (VPP) in the isolated mesentery (see METHODS for details).

Nerve Recordings

Electrodes were connected to a Neurolog headstage (NL 100), and the signal was amplified (NL 104, ×20,000), filtered (NL 125, bandwidth: 100–1,000 Hz), and then relayed to a spike processor (Digitimer D130) to allow discrimination of action potentials from noise using a manually set amplitude and polarity window. The whole nerve recording was displayed on a storage oscilloscope (Tektronix 5111A) and digitized (PCM-2 A/D VCR adapter, Medical Systems) to allow recording on a VHS videotape for future off-line analysis. Whole nerve activity was continually monitored as spike discharge (in impulses/s) and stored on a PC using Spike 2 software (CED).

Anterograde Labeling in Whole Mounts

After functional recordings had been completed, some preparations were used to visualize the distribution of axons originating from the paravascular nerve bundle under study. The nerve bundle was left attached to the recording electrode and anterogradely filled with biotinamide (5%) applied at a recording site (sites A or B; Fig. 2) as previously described (30). The nerve bundle was washed using drops of artificial “intracellular medium” [composed of (in mM) 150 monopotassium 1-glutamic acid, 7 MgCl₂, 5 glucose, 1 EGTA, 25 HCO₃⁻, 5.9 K⁺, 1.2 Mg²⁺, 1.2 H₂PO₄, 2.5 Ca²⁺, and 126 Cl⁻; pH was maintained at 7.4 with 95% O₂-5% CO₂; flow rate was 5 ml/min]. The mesenteric artery was cannulated to permit intravascular perfusion, and the system was connected to a pressure transducer to allow monitoring of vascular perfusion pressure (VPP). VPP was used as an index of vascular tone/blood flow. The pressure transducer to allow monitoring of vascular perfusion pressure to permit intravascular perfusion, and the system was connected to a compartment was filled with heavy liquid paraffin. The preparation was allowed to warm slowly and reached a working temperature of 32–34°C before a paravascular nerve recording was obtained.

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were mounted in Vectamount (Vector Labs) and viewed using a Zeiss
Axioskope 2 fluorescent microscope.

Experimental Protocols for Functional Experiments

Location of afferent sensitivity to stop of vascular perfusion (stopped flow). To assess the site of sensitivity, basal afferent firing and afferent responses when vascular perfusion was stopped for 5 min (“flow off”) were examined. The first response was recorded in preparations in which the blood vessels had been cut close to the point where they entered the gut body. The second response was recorded after removal of the terminal vessels (cut 1), as shown in Fig. 2D. After each experiment, afferent responses and effects on vascular perfusion pressure induced by capsaicin (100 nM intra-arterially) were assessed in preconstricted preparations (L-phenylephrine, 10 μM intra-arterially).

Mechanism of afferent activation by stopped flow. Preparations were largely immobilized by being pinning flat, which prevented excessive distortions as the entire fan collapses once arterial perfusion is stopped. Although not preventing all movement in the fan, the pinning allowed us to examine the effects of more subtle perivascular changes such as vasocostriction and localized distortion, which may act as stimuli for perivascular afferents. We investigated roles of the following: 1) extracellular Ca$^{2+}$, 2) changes in vascular smooth muscle contractility, 3) neuronal stimulation by ATP, 4) neuronal stimulation by ruthenium red-sensitive channels, and 5) involvement of N-type Ca$^{2+}$ channels.

After a 30-min equilibration period, two successive responses to 5-min flow off were recorded 20 min apart. Only preparations with two reproducible responses were used. After the second response, perfusion solutions were modified or pharmacological agents were added (see below). After a further 20 min, a third response to flow off was established. In experiments investigating extracellular Ca$^{2+}$, all perfusion solutions were replaced with Ca$^{2+}$-free equivalent solutions (CaCl$_2$ replaced with MgCl$_2$) after the second response to flow off. A similar protocol was used to test the effects of water-soluble antagonists (PPADS, ruthenium red, and α-conotoxin). Effects of wortmannin were examined by comparing two sets of experiments: one group pretreated with wortmannin (1 μM intra-arterially) and another set of vehicle controls (0.1% DMSO). In these experiments, the inhibitory action of this light chain kinase was confirmed by comparing vasoconstriction (maximum increase in VPP) caused by L-phenylephrine (10 μM intra-arterially) after each experiment.

Chemicals

The following compounds were purchased from Sigma: dextran (mol wt 40,000), L-phenylephrine HCl, capsaicin, and ruthenium red. Capsaicin was dissolved in DMSO and diluted with saline; 0.01% DMSO was the final concentration for intra-arterial administration. PPADS and wortmannin were purchased from Tocris Bioscience (Bristol, UK).

Data Analysis

The afferent response to flow off was calculated as the total number of impulses (area under response profile) using Spike 2 software. Baseline afferent discharge was calculated as the average firing rate over 1 min (unless stated otherwise) during steady-state activity after an equilibration for 30 min and expressed as impulses per second. Afferent responses to capsaicin were determined as the total number of impulses in the first minute of the response [area under the curve (AUC)]. Data are expressed as means ± SE. Two means were compared using a paired or unpaired t-test on the raw data. Values of $P < 0.05$ were considered significant.

RESULTS

Location of Afferent Sensitivity to Stop of Vascular Perfusion

Mesenteric afferents respond to the stop of vascular perfusion even when all connections to the body of the gastrointestinal tract have been severed (6). We explored the location of this sensitivity within the mesenteric fan by 1) visualizing the morphological arrangement of nerves within it and 2) examining the functional effects of removing the terminal blood vessels (Fig. 2D).

Morphological experiments. Biotinamide applied to one of the paravascular nerve bundles was used to visualize the pattern of mesenteric innervation. The nerve bundle loosely followed the dividing vascular tree (Fig. 2A). No intimate associations between nerves and blood vessels were observed when paravascular nerve bundles were filled from recording site A. Since the intensity of fluorescence reduces as the distance from the recording site increases, the arrangement of nerves further down the vascular tree was examined by applying biotinamide closer to the mesenteric border with the gut body (site B). Under these conditions, a perivascular nerve plexus was observed, largely confined to the terminal arteries, close to the point where they penetrated the serosa (Fig. 2B). Some of these neurobiotin-filled axons contained CGRP immunofluorescence (Fig. 2C) suggesting that some of the perivascular nerves visualized were afferent in nature. No other specialized axonal endings were seen in either the mesenteries or serosa; the only other filled structures were bundles of smooth axons of passage.

Functional experiments. In the absence of the gut body, the basal afferent discharge was 6.4 ± 2.3 impulses/s, and there was a robust response to stop of vascular perfusion (513 ± 98 impulses). Cutting off all terminal vessels had a nonsignificant effect on perfusion pressure (from 64.3 ± 5.9 mmHg before to 56.6 ± 2.7 mmHg after) but significantly reduced both baseline discharge and the response to flow off (Fig. 3). However, after these terminal arteries were removed, preparations showed maintained vasoconstrictor responses to L-phenylephrine and vasodilator responses to capsaicin (data not shown).

Mechanism of Afferent Activation Following Stop of Vascular Perfusion

To determine the mechanisms underlying the increase in afferent firing in response to flow off, the response was examined following pharmacological treatments designed to modulate mediator release, including that from the vascular endothelium and vascular contractility.

Effects of Ca$^{2+}$-free media. Under Ca$^{2+}$-free conditions, there was an augmentation of baseline afferent firing (Fig. 4B) and a dramatic increase in the response to stopped flow (Fig. 4C). A representative example is shown in Fig. 4A. The effect of removal of Ca$^{2+}$ was reversible (data not shown). The vasoconstrictor effect of L-phenylephrine persisted under Ca$^{2+}$-free conditions (data not shown). The increased excitability of mesenteric afferents following removal of Ca$^{2+}$ and the maintained vasoconstrictor effect of L-phenylephrine made it difficult to distinguish contributions from mediator release and vascular contractility. Further experiments sought to distinguish between the two.
Effects of wortmannin. Blood vessels collapse when vascular perfusion is stopped. This may occur passively but could also involve the vascular smooth muscle, which could, in turn, act as a stimulus for perivascular afferent terminals located in the adventitia. To test this hypothesis, vascular smooth muscle was paralyzed by the light chain kinase inhibitor wortmannin. Wortmannin had no effect on baseline firing compared with DMSO controls (data not shown) or the afferent responses to flow off (Fig. 5B). However, wortmannin abolished vasoconstriction induced by L-phenylephrine (data not shown), confirming that it had effectively inhibited smooth muscle contraction.

Effects of PPADS. Release of ATP has been implicated in mechanotransduction in hollow viscera acting on P2X receptors on sensory neurones (7). We investigated the possible role of ATP by examining the effects of the P2X receptor/ion channel antagonist PPADS. At 50 μM, PPADS had no observable effect on baseline firing (data not shown) or afferent discharge induced by stopped flow (Fig. 5A). 

Effects of ω-conotoxin MVIIIC. The potential for mediators to increase afferent firing via an action dependent on N-, P-, and Q-type Ca2+ channels was investigated using the Ca2+ channel blocker ω-conotoxin MVIIIC (100 nM). ω-Conotoxin had no effect on baseline discharge (data not shown) or responses to stopped flow (Fig. 5B). 

Effects of ruthenium red. Ruthenium red is an inorganic dye that blocks nonselective cation channels (NSCCs), including capsaicin-activated voltage-regulated (VR1) receptors [transient receptor potential (TRP)V1 channels]. Ruthenium red (50 μM) reduced baseline firing (from 10.4 ± 3.5 to 5.5 ± 2.4 impulses/s, n = 4), although not statistically significantly, and dramatically attenuated the response to flow off (Fig. 5A). Ruthenium red also significantly reduced the afferent response to 100 nM capsaicin (from 425 ± 46 to 33.5 ± 14 impulses, AUC first minute, P < 0.05 by unpaired t-test) and blocked the capsaicin-induced vasodilation (data not shown).

DISCUSSION

The present study combined two novel techniques: nerve tracing experiments with functional recordings of afferent discharge in the vascularly perfused mesentery. The aims were to elucidate 1) the location of afferent sensitivity and 2) the potential mechanism for afferent activation induced by stop of vascular perfusion. We visualized the pattern of innervation in the mesentery and demonstrated a rich and intimate afferent innervation around terminal arteries within the mesentery. No other specialized nerve endings were visible in the preparations. Functional experiments confirmed that these are the main sites (within the mesentery) for afferent sensitivity to stop of vascular perfusion. The mechanism of activation during flow off was independent of extracellular Ca2+ and therefore un...
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Site of Afferent Sensitivity Within the Mesentery

Sensory nerves, originating from cell bodies in dorsal root
ganglia, are known to project along the mesenteric vasculature
and send collateral projections to small arteries and arterioles
(2, 14). Vagal afferents also supply the small bowel, but these
terminate in endings within the muscularis and mucosa (2) and
so are unlikely to contribute in this mesenteric preparation.
Little is known about functional afferent sensitivity within the
mesenteric fan itself, apart from the fact that some afferents
are activated by nonphysiological levels of focal compression
with blunt probes or von Frey hairs (3, 5, 10, 21–22). We
have proposed that some of these mesenteric afferents are
sensitive to vascular perfusion of the mesentery, responding, in
particular, to stop of arterial perfusion. We (6) have previously
shown that this is not simply a response to hypoxia but is
caused by the change in perfusion. Afferents are excited by
flow off, and activation still occurs when the gut body is
removed. However, afferent sensitivity in the mesentery itself
appears to reside mainly in the finer branches of the vascular
tree originating from the second arterial bifurcation, although
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propose that flow off activates afferents directly, not via
changes in vascular smooth muscle tone. While neuronal P2X
receptors and N-type Ca\(^{2+}\) channels are not involved, ruthen-
ium red-sensitive NSCCs do appear to be implicated in the
mechanism of afferent activation.

Visualizing Paravascular Nerves in the Mesentery

Paravascular nerves (including CGRP-containing extrinsic
afferents) were shown to follow the mesenteric vascular tree
and form a perivascular plexus around terminal arteries prior to
penetrating the gut wall. Previous studies (12, 28) have dem-
onstrated that CGRP-containing fibers extend to the whole
mesenteric arterial tree. However, immunohistochemical stud-
ies have not identified that axons take to their terminals. From
our study, it is clear that afferent fibers travel some distance in
the paravascular bundles before coming into close proximity
with the vessel wall. This is important because it shows where
the axons, which contributed to our recordings, make their
specialized endings. Fibers in the main mesenteric paravascu-
lar bundles, in which robust flow-off responses were recorded,
rarely make close contact with the underlying artery but appear
to preferentially supply the smaller branches just prior to their
entry into the gut wall. These terminal arteries thus are impor-
tant structures for sensory innervation and may play an impor-
tant role in the detection of, or its responses to, disturbed blood
flow. This is fully compatible with the location of mechano-
sensitive sites identified by strong focal compression, both
in vivo and in vitro, which are typically densest on the terminal
vascular branches and nearby sites on the serosa (3, 5, 10,
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Site of Afferent Sensitivity Within the Mesentery

Sensory nerves, originating from cell bodies in dorsal root

Afferent sensitivity to stopped flow was associated with the same areas of the mesentery where blood vessels had an intimate association with neurobiotin-labeled fibers. Previous studies (3, 5, 10, 21–22) that have used probing to identify regions of mechanosensitivity have identified “hot spots” at the branch points of arteries close to the serosal surface. It is likely therefore that the endings responding to stopped flow also respond to other stimuli that cause distortion of the mesenteric fan, such as blunt probing.

Mechanism of the Afferent Response to Stopped Flow

Gastrointestinal afferents can also be activated by ischemia in vivo (11, 15, 18). However, these responses are probably more complex than the afferent responses observed when vascular perfusion is stopped in vitro. In particular, hypoxia cannot account forafferent activation in our model, since previous work has shown that removal of oxygen from the perfusing solution does not activate afferents (6). Moreover, since the responses are preserved after removal of the gut body, it is likely that the “machinery” is present within the mesenteric fan itself.

Indirect Afferent Activation Following the Release of Endothelial Mediators?

Preliminary experiments aimed at selectively damaging the endothelium proved inconclusive, since treatments also altered afferent viability (data not included). In addition, attempts to disable transduction sites in the glycocalyx with neuraminidase were also difficult to interpret (6). To investigate a role for endothelial signals, Ca\(^{2+}\)-free solutions were used to prevent mediator release through influx of extracellular Ca\(^{2+}\) triggering fast exocytosis (31). The response to flow off persisted in Ca\(^{2+}\)-free medium. Indeed, the response was considerably exaggerated after removal of Ca\(^{2+}\), as described in other populations of sensory neurons (29), probably reflecting a generalized increase in the excitability of afferent axons. Because extracellular Ca\(^{2+}\) is not necessary for the flow-off response, it is unlikely that endothelial factors such as EDRF and EDHF are necessary for this response, although mediator release caused by Ca\(^{2+}\) release from intracellular stores cannot be excluded (27).

Ca\(^{2+}\) is also necessary for the contractile machinery in vascular smooth muscle that may be necessary for the action of EDRF and EDHF. However, it is unlikely that changes in tone are necessary to mediate the effect of flow off, as indicated in Fig. 6B, since afferent responses persisted when vascular smooth muscle was paralyzed by wortmannin to block myosin light chain kinase (25), a treatment that abolished the vasconstrictor response to l-phenylephrine.

Direct Afferent Activation?

The abrupt and prolonged nature of the afferent responses to flow off suggests a rapid and constant stimulus such as a mechanical stress. Direct activation of neuronal ion channels could play a role, and while neuronal Ca\(^{2+}\) channels are unlikely to be involved, mechanosensitive channels could be suitable candidates. Since the NSCC blocker ruthenium red effectively and consistently reduced afferent responses to flow off, one or more types of NSCCs may play a role. While the pharmacological characterization of afferent responses to flow off is by no means complete, two fundamental observations support the involvement of NSCCs. Afferent responses are 1) augmented by the removal of extracellular Ca\(^{2+}\) ([Mg\(^{2+}\)] and 2) are inhibited by ruthenium red. Removal of extracellular Ca\(^{2+}\) adds further support to the involvement of a NSCC, since Ca\(^{2+}\) can both carry current through these channels and also modulating the passage of monovalent cations (29).

Ruthenium red is an inorganic dye that blocks NSCCs, including capsaicin-activated TRPV1 channels and other TRP channels (1, 17, 23). Some of these channels are mechanically gated [such as TRPA1 (23)], raising the possibility that there is a direct link between the mechanical forces generated by flow off and activation of afferent firing. However, ruthenium red is not highly specific and may act on other channels not directly linked to mechanosensitivity. For example, the TRPV1 channel is sensitive to treatment with ruthenium red but is not considered to be mechanically gated, even though the intestinal afferent response to distension is attenuated in the TRPV1 knockout mouse (24). Clearly, the clarification of channels mediating direct mechanosensitivity of mesenteric afferents awaits better pharmacological tools.

Physiological/Pathological Relevance?

Sensitivity to capsaicin is often taken as synonymous with nociception, and it is conceivable that signals generated from the vasculature as a consequence of stopped flow may be relevant for pain signaling. Certainly, high-threshold nociceptive afferents can be sensitized by mediators released by ischemia and injury (4). The extent to which ischemia may modulate responses to stopped flow has not been investigated, and therefore the impact of the current findings for nociception is unknown. However, it is clear that the sensitivity to vascular perfusion may impact on the mechanisms that control mesenteric blood flow. Our study clearly points to an afferent role for nerve fibers in the perivascular plexus. Previous emphasis has been on the release of vasoactive mediators from these endings and their contribution to axon reflexes. The sensitivity of these endings to vascular perfusion might suggest that this system offers a rapid “protective” mechanism against gut ischemia. Afferent terminals alone could provide the machinery necessary to “detect and protect,” where any potentially damaging fall in blood flow would signal and induce rapid changes in afferent discharge and the consequent release of vasoactive mediators such as CGRP. Vessel diameter, and hence blood flow, could thus be controlled locally, without reflex mechanisms.

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GRANTS

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REFERENCES


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