Calcitonin gene-related peptide activates different signaling pathways in mesenteric lymphatics of guinea pigs

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Hosaka, Kayoko, Sharyn E. Rayner, Pierre-Yves von der Weid, Jun Zhao, Mohammad S. Intiaz, and Dirk F. van Helden. Calcitonin gene-related peptide activates different signaling pathways in mesenteric lymphatics of guinea pigs. Am J Physiol Heart Circ Physiol 290: H813–H822, 2006.—The effects of calcitonin gene-related peptide (CGRP) on constriction frequency, smooth muscle membrane potential ($V_m$), and endothelial $V_m$ of guinea pig mesenteric lymphatics were examined in vitro. CGRP (1–100 nM) caused an endothelium-dependent decrease in the constriction frequency of perfused lymphatic vessels. The endothelium-dependent CGRP response was abolished by the CGRP-1 receptor antagonist CGRP-(8–37) (1 μM) and pertussis toxin (100 ng/ml). This action of CGRP was also blocked by the nitric oxide (NO) synthase inhibitor Nω-nitro-l-arginine (l-NNa; 10 μM), an action that was reversed by the addition of l-arginine (100 μM), cGMP, adenylate cyclase, cAMP-dependent protein kinase (PKA), and ATP-sensitive K+ ($K_{ATP}$) channels were all implicated in the endothelium-dependent CGRP response because it was abolished by methylxylene (20 μM), H[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 μM), dideoxyadenosine (10 μM), N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinolinesulfonamide-dichloride (H89; 1 μM) and glibenclamide (10 μM). CGRP (100 nM), unlike acetylcholine, did not alter endothelial intracellular Ca2+ concentration or $V_m$. CGRP (100 nM) hyperpolarized the smooth muscle $V_m$, an effect inhibited by l-NNa, H89, or glibenclamide. CGRP (500 nM) also caused a decrease in constriction frequency. However, this was no longer blocked by CGRP-(8–37). CGRP (500 nM) also caused smooth muscle hyperpolarization, an action that was now not blocked by l-NNa (100 μM). It was most likely mediated by the activation of the cAMP/PKA pathway and the opening of $K_{ATP}$ channels because it was abolished by H89 or glibenclamide. We conclude that CGRP, at low to moderate concentrations (i.e., 1–100 nM), decreases lymphatic constriction frequency primarily by the stimulation of CGRP-1 receptors coupled to pertussis toxin-sensitive G proteins and the release of NO from the endothelium or enhancement of the actions of endogenous NO. At high concentrations (i.e., 500 nM), CGRP also directly activates the smooth muscle independent of NO. Both mechanisms of activation ultimately cause the PKA-mediated opening of $K_{ATP}$ channels and resultant hyperpolarization.

smooth muscle; endothelium; nitric oxide; adenosine 3',5'-cyclic monophosphate-dependent protein kinase; vasomotion

Calcitonin gene-related peptide (CGRP), like substance P, is a dominant neurotransmitter in vascular sensory nerves and is a potent vasodilator, which may play a role in modulation of total peripheral resistance of the systemic circulation (6, 22, 30). The pathways by which CGRP produces vasodilatation vary between vascular beds. In the majority of blood vessels, application of CGRP has been reported to cause dilation through an endothelium-independent process. These vessels include cat cerebral (16, 48), rat mesenteric (1, 20, 25, 35), rat stomach mucosal (27), rabbit jejunal (34), dog basilar (40), dog lingual (33), and human uterine arteries (5). A primary signal transduction pathway involved in this form of CGRP activation is adenylyl cyclase, which increases cAMP and cAMP-dependent protein kinase (PKA)-activating K+ channels including ATP-sensitive K+ ($K_{ATP}$) channels (37, 38, 41).

Alternatively, CGRP-induced vasodilatation can occur through an endothelium-dependent pathway involving nitric oxide (NO) release as shown in rat aortas (17, 23, 60), mouse pial arteries (46), rat cremaster venules (31), rat mesenteric arteries (56), and human mammary arteries (42). Importantly, several studies suggest that CGRP-mediated release of NO is very different from that mediated by ACh. Thus, whereas ACh is known to induce synthesis of NO by increasing endothelial synthesis of inositol 1,4,5-trisphosphate and intracellular Ca2+ concentration ([Ca2+]i) (8, 9), CGRP has been reported to enhance endothelial production of PKA and/or PKB, causing increased synthesis of NO independent of increases in [Ca2+]i (15, 19, 23, 24, 61). Further to this, studies on the downstream actions of CGRP-induced NO indicate enhancement of both cGMP and cAMP in the smooth muscle of some blood vessels (17, 23, 61).

CGRP has been shown to produce hyperpolarization in cat cerebral arteries, rabbit mesenteric and ophthalmic arteries, and in iris arterioles (26, 38, 48, 62). This hyperpolarization is due to the activation of $K_{ATP}$ channels (26, 62) and is considered to be a consequence of receptor-activated increases in cAMP and PKA activation (37, 41). However, the role of this hyperpolarization in the vasodilatory effect of CGRP varies between vascular beds. It has been shown to be causal in the dilation observed in mesenteric arteries of the rat, the lingual artery of the dog, and the basilar artery of the rat (32, 33, 38). In contrast to this, it has been shown to be of little if any consequence in the ophthalmic artery of the rabbit (62), the small mesenteric and renal arteries of the rat (20), and in rat iridial arterioles (26). In some vessels, CGRP released from sensory nerves has been shown to inhibit sympathetic nerve-induced vasoconstriction (5, 26).

Lymphatic vessels are also innervated by sensory nerves containing CGRP and substance P as has been shown in dog...
ileal villi (28), bovine mesentery (47), guinea pig mesentery and intestine (43, 57), and rat skin (58). However, the role of these peptides is likely to be very different in lymphatic vessels compared with blood vessels in that their primary action is to modulate vasomotion and hence the propulsion of lymph. For example, substance P has been shown to enhance lymphatic vasomotion (2, 18, 44). The present study examines the effects of CGRP on lymphatic vasomotion, which are presently unknown.

**METHODS**

**Tissue preparation.** Guinea pigs (4–15 days old) of either sex were killed by decapitation during deep anesthesia consequent to inhalation of halothane (5–10%). This procedure has been approved by the University of Newcastle and the University of Calgary Animal Care and Ethics Committees and conforms to the guidelines established by the Australian and Canadian Councils on Animal Care. The small intestine and attached mesentery were rapidly removed and placed in a physiological saline solution (PSS) of the following composition (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 95% O₂:5% CO₂.

**Measurement of vessel contractile activity.** Experiments were performed on mesenteric lymphatic vessels isolated from the ileal region of the intestine and pinned onto a Sylgard (Dor Cowring) base of a small organ bath (volume 0.5 ml). The tissue was viewed with an inverted microscope and superfused at ~5 ml/min with PSS maintained at a temperature of 34°–36°C. Experiments made measuring constriction frequency (i.e., vasomotion) involved luminally perfusing the vessel through a fine glass cannula with a PSS of lower calcium concentration (1.25 mM CaCl₂) to minimize any precipitation of Ca²⁺ and hence blockage of the cannula. The flow rate used of ~3 µl/min was one that caused vessel chambers to commence regular constrictions because they were not in the main spontaneously active, although a small minority (i.e., <10%) showed spontaneous activity that was often irregular. Perfusion was achieved by loosely inserting the cannula into the upstream end of the vessel, usually with the tip positioned through the first viable valve. This method did not allow precise measurement of perfusion pressure, but experiments made under fixed pressure with the vessel tied off required a pressure of ~4–7 cmH₂O to achieve comparable constriction frequencies. Tissues were normally used within 1–4 h of isolation and were either used immediately or stored at 4°C in PSS until use.

Vasomotion of lymphatic chambers [i.e., lymphangions (36)] was monitored with a video camera attached to an inverted microscope with the output recorded on videotape. Analysis was made either directly or from the videotape with a computer-based edge detection program (14) or visual measurement. Stroke volume (SV) was calculated from SV = π/4·L·(D² − SD²), where L is the chamber length, DD is the diastolic diameter, and SD is the systolic diameter. Lymphatic flow (LF) was calculated from LF = f·SV, where f is the constriction frequency.

**Experimental protocol.** The experimental protocol used in the pharmacological studies involved a 15-min control period and a 5-min test period when CGRP was applied, followed by a 15–30 min washout period. Measurements were made by recording the constriction frequency of lymphatic chambers for 4-min periods at the end of the initial control period, 1 min after application of the agonist, and at the end of the washout period. When pharmacological inhibitors were used, this protocol was, in part, repeated but now in the presence of the inhibitor, which was applied for a total of 20 min (15 min before and during the 5-min application of CGRP). There followed a 30-min washout period before CGRP was again applied. Throughout the experiments, there was an interval of at least 30 min between applications of CGRP. Except where noted, analysis has been based on comparisons of the test constriction frequency of individual lymphatic chambers to that of the relevant control immediately preceding the response, both averaged over a 4-min period.

**Treatment with pertussis toxin.** Tissues were superfused with PSS containing pertussis toxin (PTx; 100 ng/ml) for 4 h at 34°–36°C (7) with experiments made subsequent to this in the absence of PTx.

**Measurement of changes in [Ca²⁺].** Experiments involving measurement of changes in endothelial [Ca²⁺], were made with the calcium-sensing dye Oregon Green-AM (Molecular Probes) and a Bio-Rad confocal laser system attached to a Nikon TE200 inverted microscope with a ×60 water immersion objective (1.2 numerical aperture). The experiments were made with the confocal aperture set to provide a depth resolution of ~2 µm and with excitation, dichroic, and band-pass filters of 488, 510, and 515 nm, respectively. The mesentery was pinned out onto the Sylgard base of a chamber (volume 0.5 ml) and superfused at ~5 ml/min with PSS maintained at a temperature of 34°–36°C. The endothelium was loaded with calcium-sensing dye by luminally perfusing lymphatic vessels with 2 µM Oregon Green-AM and pluronic acid (0.2% wt/vol) for 30 min at 34°–36°C. The mesentery was then pinned down through a metal frame, which was then lowered onto the coverslip base of a chamber, but now without Sylgard, allowing optimal viewing of the dye-loaded lymphatic endothelium. Movement artifacts were avoided by imaging endothelial [Ca²⁺], in vessels that were not lumina perforously and where in some cases nifedipine (1 µM) was included in the superfuse. The Ca²⁺-imaging experiments were made at a frame capture rate of 2–3 Hz. Images were analyzed with in-house software written in MATLAB. The assessment of relative changes in Ca²⁺ fluorescence was made by integrating the intensities of all the pixels for each image and plotting this value on a relative scale as a function of time.

**Electrophysiology.** Lymphatic vessels and attached mesentery were pinned into a small organ bath (volume 100 µl), mounted on the stage of an inverted microscope and superfused with PSS heated to 34°–36°C at a flow rate of 3 ml/min. Vₘ was measured with conventional glass intracellular microelectrodes with resistances of 150–250 MΩ when filled with 0.5 M KCl. Electrodes were connected to a high-impedance amplifier through an Ag-AgCl half cell. Vₑ was monitored on a digital oscilloscope and simultaneously recorded on a computer via a MacLab/8s data acquisition system. Impalements of smooth muscle cells were obtained from the adventitial side of lymphatic vessels, cut into short segments (length 125–350 µm) with fine dissecting scissors. Short segments were used to ensure simplified electrical properties of the smooth muscle such that electrical activity, even if generated at localized foci within the smooth muscle syncytium, produced similar potential changes in all the smooth muscle cells of the segment (49). Impalements of endothelial cells were either obtained from the adventitial side of lymphatic vessels or segments by advancing the microelectrode through the smooth muscle into the endothelial layer or by direct access to the endothelial side in vessels cut open and pinned with the endothelial surface uppermost. As described in a previous study (54), endothelial cells are more polarized than smooth muscle cells (resting Vₑ of approximately −70 mV vs. approximately −55 mV) and have a characteristically different response to ACh. These two reliable criteria were used to discriminate between endothelial and smooth muscle intracellular recordings.

Smooth muscle and endothelial impalements were characterized by a sharp drop in potential that settled after 10–15 s to a value typically more negative than −65 or −65 mV, respectively. Impalements were maintained for more than 5 min in >90% of the cases and up to 2 h optimally. In experiments where the effect of CGRP was studied in the presence of inhibitors, CGRP was applied first as a control and then, at least 20 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. However, in some instances, successive impalements were obtained from neighboring cells in the same segment. No significant difference in control responses to CGRP was observed when it was applied at intervals of 20 min.
Lysis of endothelium. Some experiments were made after lysis of the endothelium. Lysis was based on a method that used factor VIII immunoreactivity to selectively destroy endothelial cells while leaving the underlying smooth muscle intact (29). This method involved perfusion of the lymphatic vessel with low-calcium PSS containing BSA (5% wt/vol), antibodies against human von Willebrand factor (1:1,000 dilution; factor VIII R:Ag) and rabbit complement (2% vol/vol) (Sigma Immunochemicals). The vessel was perfused for 30 min at 34°–36°C with an intraluminal flow rate of 5–8 μl/min. The vessel was then left for 2 h without intraluminal perfusion and maintained at 34°–36°C to allow completion of endothelial lysis. The lumen was then washed out with low-calcium PSS. This procedure is a slight modification of that used previously (53) where the endothelium from this same tissue was successfully destroyed, as tested both morphologically and pharmacologically. The success of the lysing was tested by a procedure presented in a previous study (53). Here it was found that whereas in the majority of lymphatic vessels, constrictions were inhibited by both ACh and sodium nitroprusside (SNP), there was a “nonresponding” group that did not respond in any way to either ACh (10–100 nM) or SNP (100 μM). Therefore, the relative success of the lysing procedure was tested by first applying ACh (100 μM), followed by application of SNP (100 μM). A negative response to ACh and a positive response (i.e., inhibition) to SNP indicated successful lysis of endothelial cells. On the basis of this testing procedure, it was found that ~50% of the attempts to lyse endothelial cells proved successful.

Drugs. Special chemicals obtained from Sigma-Aldrich included ACh, CGRP, the CGRP receptor antagonist CGRP-(8–37), dideoxyadenosine, glibenclamide, N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinolinesulphonamide-dichloride (H89), methylene blue, A^o^-nitro-l-arginine (L-NNA), 1H-[1.2.4]oxadiazolo[4,3-α]quinoxaline-1-one (ODQ), PTx, SNP, and the thromboxane A2 mimetic 9,11-dideoxy-9,11-methanoepoxy prostaglandin F2α. Stock solutions were made up in either distilled water or dimethyl sulfoxide (DMSO) at concentrations of between 0.1 and 100 mM and stored at −20°C. These were thawed and diluted to appropriate concentrations in PSS when required. Test solutions were always applied as superfusate. The concentration of vehicle (DMSO) was always <0.1%, a concentration that had no significant effect on the parameters measured. The choice of inhibitor concentrations arose from preliminary experiments with initial concentrations based on published data obtained in other tissues. All drugs were effective without obvious nonspecific effects at or near the published concentrations.

Data analysis. Data are presented as means ± SE with statistical comparisons made with one-way ANOVA followed by Ryan’s test or a two-tailed paired Student’s t-test, with P < 0.05 and P < 0.01 considered significant and highly significant, respectively.

RESULTS

Effect of CGRP on lymphatic vasomotion. Guinea pig mesenteric lymphatic vessels were cannulated and perfused at a frequency necessary to induce spontaneous contractile activity of individual lymphatic chambers. These chambers, which are variously termed lymphangions (36), are formed by adjacent unidirectional valves that frequently occur along the vessels. The smooth muscle in the chamber wall and frequent discon-
continuity of this smooth muscle syncytium in the region of the valves allow these chambers to constrict independently from adjacent chambers (13). Application of CGRP caused a decrease in the frequency that chambers constricted, significantly decreasing vasomotion of these chambers over the 1–100 nM concentration range studied (Fig. 1A and B), with 100 nM CGRP reducing constriction frequency to 73 ± 4% of control (n = 26 segments; P < 0.01). This decrease in constriction frequency, although accompanied by a small but significant increase in stroke volume, led to a net decrease in calculated lymph flow (Fig. 1C–F). Removal of the endothelium largely prevented the CGRP-induced inhibition of lymphatic contractions over the same concentration range (Fig. 1B, open bars; n = 6–7).

Effects of CGRP-(8–37) and PTx on CGRP response. Properties of the receptor and G protein were examined. The receptor was likely to be the CGRP-1 subtype, because CGRP action at a concentration of 100 nM (but not at 500 nM) was prevented by CGRP-(8–37) (1 μM), an antagonist that has selectivity for the CGRP-1 receptor (Fig. 2A; n = 13). The antagonist itself had no significant effect on lymphatic vasomotion (Fig. 2A; n = 7). A dominant involvement of a PTx-sensitive G protein was indicated, because preincubation with PTx (see METHODS), which itself had no significant effect on vasoemotion (n = 11), largely inhibited the response to 100 nM CGRP (Fig. 2B; note that 500 nM CGRP not tested). However, CGRP continued to produce a small but significant response in the presence of PTx (93 ± 2% of control; P < 0.01), suggesting either a separate, less dominant pathway of action of the 100 nM CGRP or incomplete PTx action.

CGRP action on lymphatic vasomotion occurs through release of NO. It has been shown previously that the frequency of constrictions of perfused lymphatic vessels can be markedly inhibited by endothelial release of NO (21, 45, 53, 59). Therefore, the possibility that 100 nM CGRP acts by causing release of NO from the endothelium or by enhancing the actions of NO synthase (NOS) and cGMP.
endogenous NO was examined with the NO synthase (NOS) inhibitor L-NNA (10 μM) applied 20 min before and during application of 100 nM CGRP. L-NNA itself increased constriction frequency by 30% (Fig. 3), indicating that there was endothelial NO release under the perfusion conditions of the present study. Importantly, in paired studies on 12 chambers, the CGRP-induced inhibition of vasomotion was abolished in the presence of L-NNA (Fig. 3A). Application of a high concentration of L-arginine (100 μM) together with the L-NNA (10 μM) reversed this effect so that now application of 100 nM CGRP again significantly reduced the frequency of contractions (Fig. 3A).

The action of NO was likely to be through production of cGMP, because application of the cGMP inhibitor methylene blue (20 μM) abolished the action of 100 nM CGRP (Fig. 3B; n = 9). Methylene blue (20 μM) itself had a direct effect on perfusion-induced vasomotion before application of 100 nM CGRP, significantly increasing constriction frequency to 129 ± 8% of control (P < 0.01; n = 9). ODQ, another guanylate cyclase inhibitor, also abolished CGRP action (Fig. 3C; n = 12). However, unlike methylene blue, ODQ did not have a significant effect on the constriction frequency of perfused vessels measured before application of CGRP.

Evidence for a role of cAMP. The role of cAMP was also investigated because CGRP action in blood vessels has often been reported to involve cAMP and stimulation of PKA. Evidence that this pathway was activated by CGRP was provided by the finding that application of the membrane permeant adenylate cyclase inhibitor dideoxycadenosine (10 μM) abolished the actions of 100 nM CGRP (Fig. 4A). Importantly, the PKA inhibitor H89 (1 μM) also abolished inhibition of vasomotion by 100 nM CGRP (Fig. 4B). Whereas dideoxycadenosine (10 μM) itself had no significant effect on the perfusion-induced vasomotion before CGRP (n = 11), H89 (1 μM) increased constriction frequency to 130 ± 4% of control (n = 10), an action that may correlate to the depolarizing effect that H89 had on the smooth muscle (see Effect of CGRP on smooth muscle Vm).

Effect of glibenclamide on CGRP-induced response on vasomotion. Glibenclamide, a blocker of KATP channels, was used to investigate the role of these channels in the CGRP-induced inhibition of lymphatic vasomotion because they are known to be activated by cAMP and/or PKA (37, 41). KATP channels had a dominant role because treatment with glibenclamide, which itself had no significant effect on vasomotion (data not shown; n = 10), abolished the reduction in constriction frequency induced by 100 nM CGRP, which was 83 ± 2% in the absence and 99 ± 1% in the presence of 10 μM glibenclamide (n = 10; P < 0.01).

Vasodilator prostanoids. Experiments were made to determine the involvement of vasodilator prostanoids by comparing CGRP-induced responses before and after treatment with the cyclooxygenase inhibitor indomethacin (10 μM). Indometha-

![Fig. 4. Application of cAMP/PKA inhibitors. A and B: effects of the membrane permeant adenylate cyclase inhibitor dideoxycadenosine (DDa, 10 μM) and PKA inhibitor N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinolinesulfonamide-dichloride (H89; 1 μM) on the response induced by 100 nM CGRP (n = 11 for both). Vertical lines denote SE. **P < 0.01 compared with CGRP + inhibitor. †P < 0.05 compared with control (i.e., 100%).](http://ajpheart.physiology.org/)

![Fig. 5. Effects of CGRP on membrane potential (Vm) in guinea pig mesenteric lymphatic smooth muscle. A: intracellular microelectrode recordings exhibiting response to 100 and 500 nM CGRP (horizontal bars). Resting Vm is indicated on each record. B: summary data showing mean membrane hyperpolarization caused by 10, 100, and 500 nM CGRP (n = 4–10). Vertical lines denote SE. *P < 0.05 compared with no change, paired Student’s t-test.](http://ajpheart.physiology.org/)

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cin itself had no significant effect on vasomotion (data not shown; n = 11) and did not significantly affect CGRP action, with 100 nM CGRP reducing lymphatic constrictions to 83 ± 4% and 89 ± 3% of control before and during addition of indomethacin, respectively (n = 11).

Effect of CGRP on smooth muscle \( V_m \). Intracellular microelectrode recordings were obtained in smooth muscle cells from nonperfused short lymphatic vessel segments. The \( V_m \) of lymphatic smooth muscle was \(-52.6 ± 0.8 \text{ mV (n = 24 segments).} This was significantly hyperpolarized on superfusion of the lymphatic segments with 100 and 500 nM CGRP, whereas a concentration of 10 nM did not cause a significant response (Fig. 5, A and B).

The effects of CGRP on spontaneous transient depolarizations (STDs) were investigated in segments where STDs could be recorded. These events arise through \( \text{Ca}^{2+} \) release from intracellular stores (50) and have been shown to subserve a pacemaker role in lymphatic vessels (49, 51). STDs were measured during the maximum response to CGRP and expressed as a percentage of the values obtained before CGRP application during the same impalement. CGRP significantly reduced STD frequency at 100 nM CGRP (to 76 ± 12% of control; \( P < 0.05; n = 9 \)) and reduced both frequency and amplitude of these events at 500 nM CGRP (to 69 ± 7% and 79 ± 3% of control, respectively; \( P < 0.01 \) for both; \( n = 8 \)).

Application of \( L^- \)-NNA (100 \( \mu \text{M} \)) prevented the hyperpolarization induced by 100 nM CGRP (Fig. 6A), this paralleling the actions of \( L^- \)-NNA on the response to ACh (Fig. 6B). Comparison of these two recordings in Fig. 6, A and B, also indicates that \( L^- \)-NNA treatment slightly increased the frequency of action potentials, consistent with a previous observation that a basal production of NO occurs in these lymphatic vessels (53). Paired experiments on five segments with this inhibitor of NOS reduced the hyperpolarization induced by 100 nM CGRP to a nonsignificant level (Fig. 6C). In contrast, the hyperpolarization to 500 nM CGRP was not significantly inhibited by 100 \( \mu \text{M} \) \( L^- \)-NNA (Fig. 6C; \( P = 0.86; n = 4 \)). Further proof that the action of 500 nM CGRP was dominated by a direct action on the smooth muscle was provided by the finding that the hyperpolarization induced by 500 nM CGRP persisted in endothelium-denuded segments (Fig. 6C; \( n = 3 \)).

The relative role of \( \text{K}_{\text{ATP}} \) channels in the CGRP-induced smooth muscle hyperpolarization was tested by using glibenclamide. Brief application of CGRP (500 nM for 1 min) hyperpolarized the smooth muscle from \(-47.8 ± 1.0 \) to \(-52.3 ± 1.3 \text{ mV.} \) Glibenclamide (10 \( \mu \text{M} \)) abolished the CGRP-induced hyperpolarization, which was decreased to 12 ± 7% of the pre-glibenclamide response (Fig. 7A; \( n = 4; P < 0.01 \)). The role of PKA in the \( \text{K}_{\text{ATP}} \)-mediated hyperpolarization to CGRP was investigated by using H89. H89 (10 \( \mu \text{M} \)) abolished the hyperpolarization to 500 nM CGRP (Fig. 7B; \( n = 4; P < 0.01 \)). As previously reported (52), both glibenclamide and H89 had a direct effect on the smooth muscle \( V_m \) and depolarized it from a control value of \(-47.8 ± 1.0 \) to \(-43.0 ± 1.1 \text{ mV (n = 4; P < 0.05)} \) and \(-52.0 ± 1.2 \) to \(-44.3 ± 1.1 \text{ mV (n = 4; P < 0.01)} \), respectively.

![Fig. 6. Effects of \( L^- \)-NNA and endothelium lysis on responses of \( V_m \) to CGRP compared with ACh. A: intracellular microelectrode recordings in response to 100 nM CGRP (horizontal bars) in control conditions and in the presence of \( L^- \)-NNA (100 \( \mu \text{M} \)). B: intracellular microelectrode recordings in response to 10 \( \mu \text{M} \) ACh (horizontal bars) in control conditions and in the presence of \( L^- \)-NNA (100 \( \mu \text{M} \)) obtained during the same impalement as for A. Resting \( V_m \) is indicated on each record. C: averaged smooth muscle hyperpolarizations in response to 100 and 500 nM CGRP with (open bars) \( L^- \)-NNA, and without (solid bars) \( L^- \)-NNA. Averaged data are also presented for 500 nM CGRP applied to the endothelium-denuded segments (shaded bar). Vertical lines denote SE. *\( P < 0.05 \) compared with no change, paired Student’s \( t \)-test.](http://ajpheart.physiology.org/)
CA2+/NOS and production of NO (8). Therefore, relative endothelial and Ca2+ sensitivities, making NOS inhibitor l-NNA, an action that was reversed by the addition of the NO substrate l-arginine. NO is known to strongly regulate spontaneous constrictions in lymphatic vessels, particularly in response to endothelial agonists such as ACh (45, 53, 59) or shear stress (21).

Our measurements to determine the action of 100 nM CGRP to cause endothelial release of NO or enhance the actions of NO indicate that the pathway involves activation of a CGRP-1 receptor because the CGRP-induced endothelial response was inhibited by CGRP-(8–37), an antagonist that shows preference for the CGRP-1 receptor (12). Significantly, the CGRP-1 receptor was shown to be coupled to a pertussis toxin-sensitive G protein, the activation of which caused synthesis and release of NO or enhanced NO actions through a mechanism that did not involve measurable increase in either endothelial [Ca2+]i, or endothelial Vm. This is in contrast to the action of ACh, which is known to cause a large increase in lymphatic endothelial [Ca2+]i, and Vm change (53, 54) (see also Fig. 8B). The mechanism by which this occurs has yet to be resolved, but there is evidence that NO can be released in subthreshold levels of [Ca2+]i (10).

Studies on the endothelium of some blood vessels indicate that CGRP action can involve cAMP/PKA and/or phosphatidylinositol 3-kinase (PI3K)-regulated pathways (10, 61). It may be that the PI3K pathway has a dominant role in the CGRP response, because the PTx sensitivity of the lymphatic CGRP response indicates an involvement of Gi [Gi, is not present in endothelial cells (3)], and Gi is known to inhibit activation of adenylate cyclase (39). In contrast, Gi is known to activate the PI3K-regulated pathway, which through involvement of the serine/threonine protein kinase Akt (PKB) has been demonstrated to generate NO in endothelial cells (15, 19). In these studies, Akt was found to phosphorylate Ser1177, thus enhancing NOS activity and Ca2+ sensitivity, making NOS activity maximal at subthreshold [Ca2+]i. Such Gi/PI3K/Akt/NO signaling is consistent with CGRP action on the...
CGRP-induced inhibition of lymphatic pacemaking

Lymphatic endothelium, which acted through Gi, and was independent of significant changes in \([\text{Ca}^{2+}]_o\) or \(V_m\).

**CGRP action on lymphatic smooth muscle.** The primary action of 100 nM CGRP on the smooth muscle occurred through release of NO from the endothelium or enhancement of the actions of NO. There was no evidence for a role of prostacyclin, because the cyclooxygenase inhibitor indomethacin had no effect on CGRP-induced slowing of lymphatic vasomotion. Our data indicate that NO stimulates both cGMP and cAMP pathways in the smooth muscle. Evidence for a fundamental role for the guanylate cyclase/cGMP pathway was provided by the findings that the guanylate cyclase inhibitors methylene blue and ODQ abolished the CGRP-induced decrease in the frequency of lymphatic contractions. However, data were also provided indicating a fundamental role of the adenylate cyclase/cAMP/PKA pathway, because the adenylate cyclase inhibitor dideoxyadenosine and the PKA inhibitor H89 abolished the CGRP-induced slowing in the frequency of lymphatic contractions. Whereas it could be proposed that the cAMP inhibitors are acting on the endothelium, the finding that the \(K_{\text{ATP}}\) channel blocker glibenclamide also abolished the CGRP-induced response indicates functional activation of the cAMP pathway in the smooth muscle, where the \(K_{\text{ATP}}\) channels are most likely expressed. We base this on the knowledge that \(K_{\text{ATP}}\) channels have been shown to be activated by increases in cAMP content and subsequent PKA activation in lymphatic and vascular smooth muscles (37, 41, 52) and that CGRP caused no change of endothelial \(V_m\) while hyperpolarizing the smooth muscle through glibenclamide-sensitive channels (i.e., \(K_{\text{ATP}}\) channels). Our present interpretation is also well supported by the findings that the signaling pathways in response to NO have already been elucidated in the same lymphatic preparation (52, 55). In these studies, as in our present findings, NO activated cAMP and cGMP pathways and led to glibenclamide-sensitive hyperpolarizations of the lymphatic smooth muscle, suggesting a major role for \(K_{\text{ATP}}\) channels in these responses. The ability for NO to activate the cAMP pathway, as studied in various tissues, has been shown to arise through NO/cGMP-mediated inhibition of phosphodiesterase type III, a major phosphodiesterase isozyme present in vascular smooth muscle (4).

A secondary action of CGRP was observed at a very high CGRP concentration (i.e., 500 nM). This action operated through receptor(s) other than the CGRP-1 with a dominant inhibitory action on the frequency of constrictions, which was no longer inhibited by \(l\)-NNA. CGRP at 500 nM was found to now directly act on the smooth muscle, causing a hyperpolarization that persisted in endothelium-denuded preparations or in the presence of 100 \(\mu\)M \(l\)-NNA. This contrasts to the response to 100 nM CGRP, where the hyperpolarization was markedly inhibited by \(l\)-NNA. The response to 500 nM CGRP was likely to be caused by enhancement of the cAMP pathway because the hyperpolarization was blocked by both the \(K_{\text{ATP}}\) channel blocker glibenclamide and by the PKA inhibitor H89, a finding paralleling that shown with 5-hydroxytryptamine and \(\beta\)-adrenoceptor activation (11, 52).

In conclusion, the key finding of this study is that CGRP inhibits lymphatic vasomotion. It does so primarily by release of NO from the endothelium or enhancement of the actions of NO, which acts to enhance cGMP and, in turn, cAMP activity, causing \(K_{\text{ATP}}\) channel-induced hyperpolarization of the smooth muscle. There was also a reduction in the frequency of spontaneous transient depolarizations, events that are fundamental

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Fig. 8. Endothelial \(V_m\) and intracellular \([\text{Ca}^{2+}]_i\) response to CGRP. A and B: recordings of endothelial \(V_m\) before and during application of 500 nM CGRP and 10 \(\mu\)M ACh. Resting \(V_m\) is indicated on each record. C: relative endothelial \([\text{Ca}^{2+}]_i\), \(\Delta F/F_o\), where \(F\) is actual fluorescence intensity and \(F_o\) is fluorescence intensity averaged over 10 frames during control) before and during application of 100 nM CGRP and 1 \(\mu\)M ACh. Records were obtained by confocal microscopy with each point the average of the Oregon Green-associated fluorescence of the entire field of cells imaged at that specific time. Image 1: averaged image of 50 sequential images taken under control conditions at arrow 1 on the record. Similarly, 50 images were averaged during CGRP and ACh application at locations marked by arrows 2 and 3, respectively. Image 1 was then subtracted from each, and the difference images are shown as images 2 and 3.
to pacemaking in these vessels (49). The inhibitory action on pacemaker frequency by CGRP is opposite to the enhancement produced by substance P (18, 44), another neuropeptide that is colocalized in the sensory nerve varicosities that innervate blood and lymphatic vessels. Whereas the interplay that these two sensory neuropeptides have on lymphatic vasomotion remains to be unraveled, it is clear that CGRP itself, whether neurally released or delivered by paracrine or hormonal pathways, will decrease the frequency of intrinsic lymphatic contractions and lymphatic flow (see Fig. 1). However, whether this favors generation of edema will also depend on other factors such as altered filling of the initial lymphatics.

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