British Journal of Pharmacology (1998) 125, 1397–1404
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Distribution of the perivascular nerve Ca^{2+} receptor in rat arteries

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1 We recently showed that perivascular sensory nerves of mesenteric branch arteries express a receptor for extracellular Ca^{2+} (CaR), and reported data indicating that this CaR mediates relaxation induced by physiologic levels of Ca^{2+}. We have now tested whether the perivascular sensory nerve CaR-linked dilator system is a local phenomenon restricted to the mesentery, or is present in other circulations.

2 Vessels from the mesenteric, renal, coronary, and cerebral circulations were studied. Immunocytochemical analysis was performed using anti-CaR and anti-neural cell adhesion molecule (NCAM) antibodies. Wire myography was used to assess contracation and relaxation.

3 Although perivascular nerves of all arteries stained for CaR protein, there were regional differences. A morphometric method used to estimate CaR positive nerve density revealed the following rank order: mesenteric branch artery > basilar artery = renal interlobar artery > main renal trunk artery > left anterior descending coronary artery.

4 Vessels from the mesentery, renal, coronary, and cerebral circulations showed nerve-dependent relaxation in response to electrical field stimulation (EFS) when precontracted with serotonin in the presence of guanethidine. The degree of Ca^{2+}-induced relaxation of mesenteric, renal, and cerebral arteries positively correlated with the magnitude of EFS-induced relaxation. In contrast, coronary arteries contracted at Ca^{2+} levels between 1.5 and 3 mmol L^{-1}, and relaxed to a small degree to 5 mmol L^{-1} Ca^{2+}.

5 Thus, a functional perivascular sensory nerve CaR-linked dilator system is present to varying degrees in the mesenteric, renal, and cerebral circulations, but only to a very limited extent in the coronary circulation.

Keywords: Calcium; calcium receptor; sensory nerve; immunocytochemistry; rat arteries

Introduction

Although it has been recognized for several decades that elevating the concentration of extracellular Ca^{2+} in fluid bathing isolated arteries causes them to relax (Bohr, 1963; Holloway & Bohr, 1973; Webb & Bohr, 1978), until recently the molecular mechanism was not clarified (Bukoski *et al*., 1995). Over the past several years it has become increasingly clear that extracellular Ca^{2+} can modulate the function of specific cell types by activating an extracellular receptor for Ca^{2+} (Ca^{2+} receptor, CaR). The CaR, which was first cloned from bovine parathyroid gland, exhibits the seven membrane spanning domain characteristic of G protein coupled receptors and couples changes in the concentration of extracellular Ca^{2+} with alterations in parathyroid hormone secretion (Brown *et al*., 1993). Subsequent reports have shown that message encoding the CaR is expressed in human and rat parathyroid and thyroid (Garrett *et al*., 1995a,b) and rat kidney (Riccardi *et al*., 1995) and brain (Ruat *et al*., 1995).

In confirmation that CaR protein is widely expressed throughout the organism, our laboratory recently demonstrated that CaR message is expressed in dorsal root ganglia, which house cell bodies of primary sensory afferent nerves, and showed that protein that is immunoreactive with the human parathyroid CaR is present in perivascular nerves of rat mesenteric resistance arteries (Bukoski *et al*., 1997). We have also performed experiments that demonstrate that Ca^{2+}-induced relaxation in the resistance artery is nerve dependent, being ablated by acute phenolic denervation and reduced by chronic capsaicin-induced sensory denervation, and is mediated by a vasodilator released from perivascular nerves (Bukoski *et al*., 1997; Mupanomunda *et al*., 1998).

These findings have led to our proposal that extracellular Ca^{2+} binds to the perivascular nerve CaR and induces the release of a vasodilator which then diffuses to underlying vascular smooth muscle and induces relaxation. In view of the potential importance of this vasodilator system as a regulator of local vascular reactivity, and for understanding its possible functional implications, it is important to establish its regional distribution. We now report on the results of experiments that used immunocytochemical analysis and wire myography to assess the regional distribution of the functionally coupled perivascular CaR. A preliminary report of these results has been published in abstract form (Wang & Bukoski, 1997).

Methods

Animals and tissue isolation

All procedures were approved by the Institutional Animal Care and Use Committee. Male Wistar rats (8–10 weeks of age) were purchased from Harlan-Sprague Dawley and upon arrival in our animal care facility were maintained in a colony room with constant temperature, humidity, and fixed dark:-light cycles. The animals were given free access to tap water and Purina rodent chow (5001). The animals were killed by exsanguination while anaesthetized with a mixture of ketamine and xylazine (100 mg kg^{-1} : 5 mg kg^{-1}) and the following arteries were isolated: branch II and III mesenteric, main renal trunk, renal interlobar, left anterior descending coronary, basilar. After isolation, the tissues were placed in ice cold

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physiologic salt solution (PSS) of the following composition (mmol L^{-1}): NaCl, 150; KCl, 4.7; $MgSO_4 7H_2O$, 1.17; $NaHCO_3$, 5; KH_2PO_4, 1.10, $CaCl_2$, 1.0; HEPES, 20; and glucose, 5, pH 7.4 and cleaned of fat and connective tissue. The larger arteries, mesenteric and renal, were cannulated and flushed with ice cold PSS to remove residual blood.

Immunocytochemistry

Immunocytochemistry was performed as recently described (Bukoski *et al*., 1997). All vessel segments were fixed for 10 min in ice cold methanol, washed with Tris-buffered saline, pH 7.4 (TBS), incubated with a peroxidase blocker to quench intrinsic peroxidase activity, then soaked in a serum-free protein blocker at room temperature for 30 min to block non-specific binding. Several rinses in TBS were included between each step. Two different antibodies were used to identify CaR protein. A polyclonal rabbit anti-CaR antibody (KK-3 1 : 2,000, 6 μg ml^{-1} raised against a fusion protein containing amino acids 340–620 and generously provided by Dr Kim Rogers of NPS Pharmaceuticals) was used to quantify the density of CaR protein as described below. In a limited number of other experiments, a monoclonal anti-CaR (raised against the CaR peptide sequence ADDDYGRPGIEK-FREEAEERDI and generously provided by NPS Pharmaceuticals in conjunction with Drs Allen Spiegel and Paul Goldsmith, NIDDK) was used at a concentration of 20 μg ml^{-1}. A polyclonal rabbit anti-neural cell adhesion molecule (NCAM) antibody provided by Dr Ken Johnson was used in some experiments at a dilution of 1 : 800. In all cases, the fixed tissue was incubated with the primary antibody overnight at 4°C. After this incubation, the segments were washed with TBS, intrinsic biotin was blocked using a biotin blocking kit, and the segments were incubated with biotin conjugated secondary antibody (horse anti-mouse at 1 : 250, or goat anti-rabbit at 1 : 1000) in the presence of 5% horse or goat serum respectively.

To localize the bound antibodies, a commercially available kit (ABC Elite, Vector Labs, Burlingame, CA, U.S.A.) was used for the avidin-biotin peroxidase reaction, in which 3′ 3-diaminobenzidine or SG served as the chromagen. The segments were dehydrated through sequential ethanol and xylene, then mounted on slides using Permount. The specificity of the immunoreaction was assessed by use of preimmune serum in the case of the polyclonal anti-CaR and by omission of the primary antibody in the case of the monoclonal anti-CaR.

The density of immunoreactive nerve fibres in the vessel wall was estimated using a stereologic counting method (Schmidt-Schoenbein *et al*., 1977) as recently described (Mupanomunda *et al*., 1998). Briefly, a photomontage was made by taking photomicrographs at different planes of focus through the slide, then tracing the visible nerve fibres onto a clear plastic sheet. The density of fibres (as length per unit area) on the clear plastic sheet was then estimated by counting the number of intersections between the nerves and a reticule grid that was placed over the photomontage according to the equation: density $= \pi N\ 2L^{-1}$, where density is expressed as $\mu m\ mm^{-2}$, N is the number of intersections in the grid, and L is the total length of the grid.

Isometric force generation

Isometric force generation was measured using previously described methods (Bian *et al*., 1995). After isolation, the arteries were mounted on a wire myograph (Kent Scientific, Pistickaway, NJ, U.S.A.) for measurement of isometric force generation. The vessels were maintained in PSS warmed to 37°C and gassed with 95% air/5% CO_2, and stretched to a length equivalent to a diameter ranging between 125 and 225 μm, depending on the vessel was being studied, then allowed to equilibrate for 30 min. After equilibration, the vessels were induced to contract 3–4 times by the addition of an ED_{70} concentration of serotonin. Serotonin was used as the contractile agonist since neither the coronary nor the basilar artery responded to noradrenaline. Ca^{2+}-induced relaxation was assessed by cumulatively adding $CaCl_2$ to serotonin pre-contracted vessels to achieve total extracellular Ca^{2+} concentrations of 1.5, 2, 3, and 5 mmol L^{-1}. The magnitude of relaxation was expressed as the percentage of the total that the vessel could relax. ED_{50} values for Ca^{2+} were estimated from plots of relaxation expressed as a percentage of the response to 5 mmol L^{-1} Ca^{2+} versus extracellular Ca^{2+}.

The dilator response to electrical field stimulation was assessed as an index of the functional integrity of the perivascular dilator nerve network. Vessels were preincubated with 10 μmol L^{-1} guanethidine to block sympathetic nerve release of catecholamines, precontracted with serotonin, then stimulated according to the parameters outlined by Li & Duckles, (1993): rate, 3 pulses per s; duration, 3 msec; and voltage, 70 mV.

Statistical analysis

All data are presented as mean ± s.e.mean and statistical analysis was performed using the SYSTAT software package. Comparisons among groups were performed using ANOVA with a repeated measures design when appropriate. A P value of less than 0.05 was taken to indicate a statistically significant difference between groups.

Drugs

Serotonin, 3′ 3-diaminobenzidine (DAB), and the organic solvents were obtained from Sigma Chemical Co (St. Louis MO, U.S.A.) Monoclonal anti-CaR was developed by Drs Allan Spiegel and Steve Goldman of the National Institutes of Health and obtained through an arrangement with Dr Kim Rogers of NPS Pharmaceuticals (Salt Lake City, UT, U.S.A.) Polyclonal anti-NCAM was a generous gift of Dr Ken Johnson, University of Texas Medical Branch. Horseradish peroxidase conjugated antibodies, horse and goat serum and the SG chromagen were obtained from Vector Laboratories (Burlingame, CA, U.S.A.) serum free blocker, peroxidase blocking solution, and the biotin blocking kit were obtained from the DAKO Corporation (Carpinteria, CA, U.S.A.) Unless otherwise stated, all chemicals were dissolved in distilled water.

Results

Mesenteric branch arteries

Mesenteric branch arteries were used as the reference tissue in this study since they have previously been shown to express the perivascular nerve CaR and to exhibit nerve dependent Ca^{2+}-induced relaxation (Bukoski *et al*., 1997). Incubation of mesenteric resistance arteries with monoclonal anti-CaR revealed a nerve-like pattern of staining in the adventitial layer of the vessel (Figure 1A), that was completely absent when the vessel was incubated with secondary antibody alone (Figure 1B). This pattern of staining was nearly identical to

that which was observed with antiserum that is specific for neuronal cell adhesion molecule (NCAM), which is a nerve specific antigen (Jorgensen, 1995) (Figure 1C). Moreover, when a single mesenteric arterial segment was incubated sequentially with the anti-CaR and anti-NCAM antibodies, co-localization of the CaR and NCAM proteins was apparent (Figure 1D). These data verify our prior observation that CaR protein is expressed in the periadventitial nerve network (Bukoski *et al*., 1997). As shown in Figure 1E, a similar pattern of staining was observed when the vessels were incubated with polyclonal anti-CaR antiserum and there was a complete lack of staining when the vessel was treated with pre-immune serum alone (Figure 1F). The density of CaR positive nerve fibres estimated using the morphometric technique described in Methods was 86.2 ± 7.2 $\mu m/mm^2$ (Table 1).

Relaxation induced by electrical field stimulation (EFS) was used to assess the capacity for nerve-dependent relaxation. To

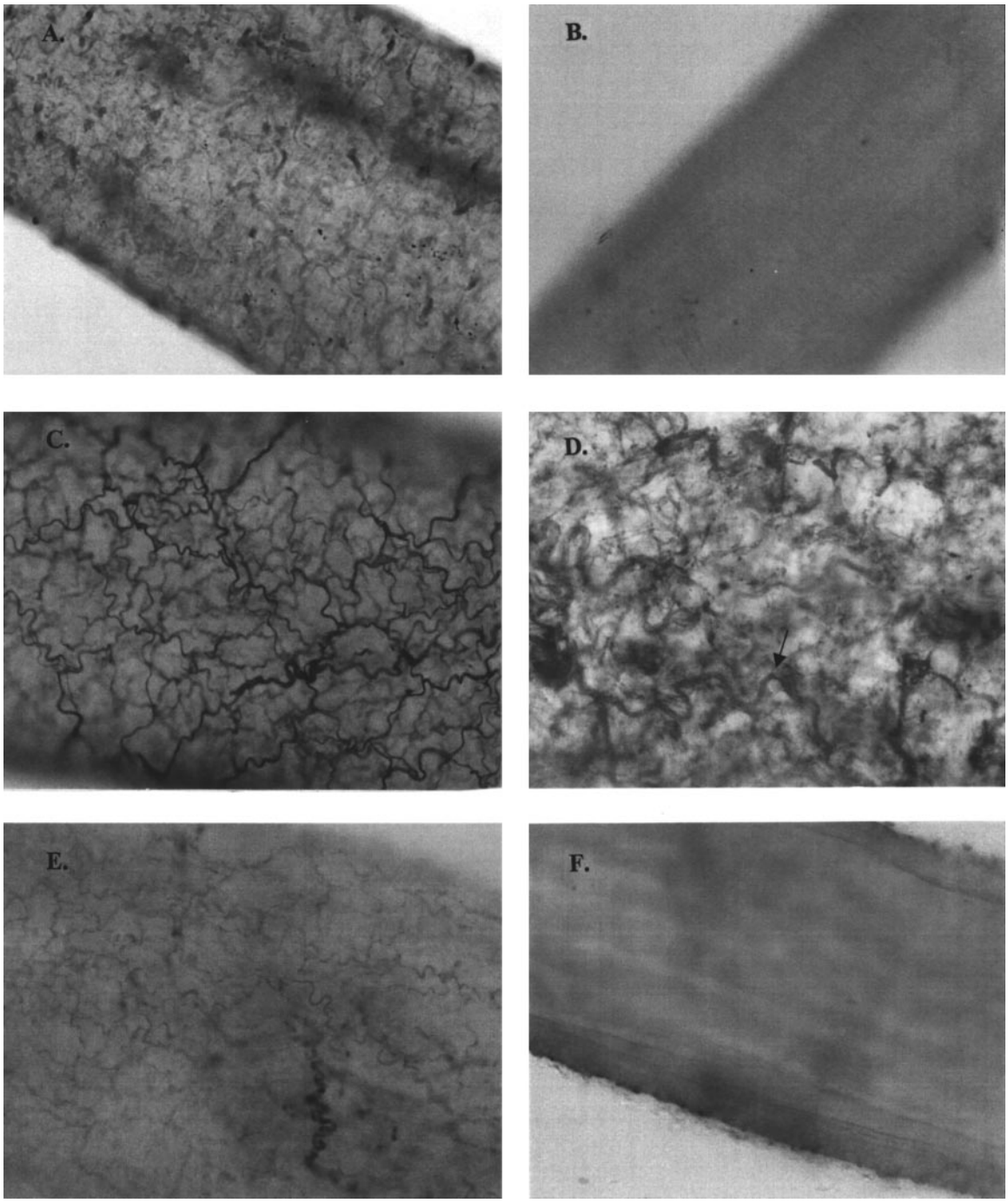

Figure 1 Demonstration of CaR-positive nerve fibres in whole mount preparations of rat arteries using monoclonal mouse and rabbit polyclonal anti-CaR antibodies; immunostaining was performed as described in the text. The panels show (A) staining after incubation with monoclonal anti-CaR (magnification = 200 ×). (B) Lack of staining after incubation with biotin-conjugated anti-mouse IGG alone (200 ×). (C) Staining obtained after incubation with polyclonal anti-NCAM (200 ×); (D) arrow indicates colocalization of CaR and NCAM staining after sequential incubation with anti-CaR and anti-NCAM (400 ×); (E) staining obtained after incubation with polyclonal anti-CaR(200 ×); and (F) absence of nerve-like staining after incubation with pre-immune serum (200 ×). The density of CaR-positive nerve staining in mesenteric branch arteries from five different animals was estimated using a morphometric method and is summarized in Table 1.

Table 1 Density of CaR-innervation

Arterial segment	*Density of CaR-positive nerves* (μm mm^{-2})
Mesenteric branch	86.2±7.2
Main renal	16.9±2.1**
Interlobar	58±2.6*
Coronary	7.4±3.2
Basilar	62.6±4.1*

CaR positive nerve density was determined from immunostained whole mount arterial preparations as described in the text. Values are mean±s.e.mean, $n=5$ per group. *Indicates a significant difference in CaR-positive nerve density from mesenteric branch, main renal, and coronary arteries. **Indicates a significant difference from coronary arteries; all less than a P value of at least 0.05.

validate this method, several control experiments were performed. When fully relaxed mesenteric resistance arteries were stimulated using the electrical parameters outlined in Methods, they responded with a rapid increase in tension. This contraction was blocked by pretreatment of the vessel with the α-adrenergic antagonist phentolamine (Figure 2A), and with guanethidine which is an inhibitor of noradrenaline release from sympathetic nerves (Figure 2B). These data indicate that the contraction elicited by EFS results from the release of catecholamines from perivascular nerves and not depolarization of vascular smooth muscle. When mesenteric segments were preincubated with guanethidine, then precontracted with serotonin, EFS caused a relaxation which was interpreted to be the result of activation of perivascular vasodilator nerves (Li & Duckles, 1993) (Figure 2C).

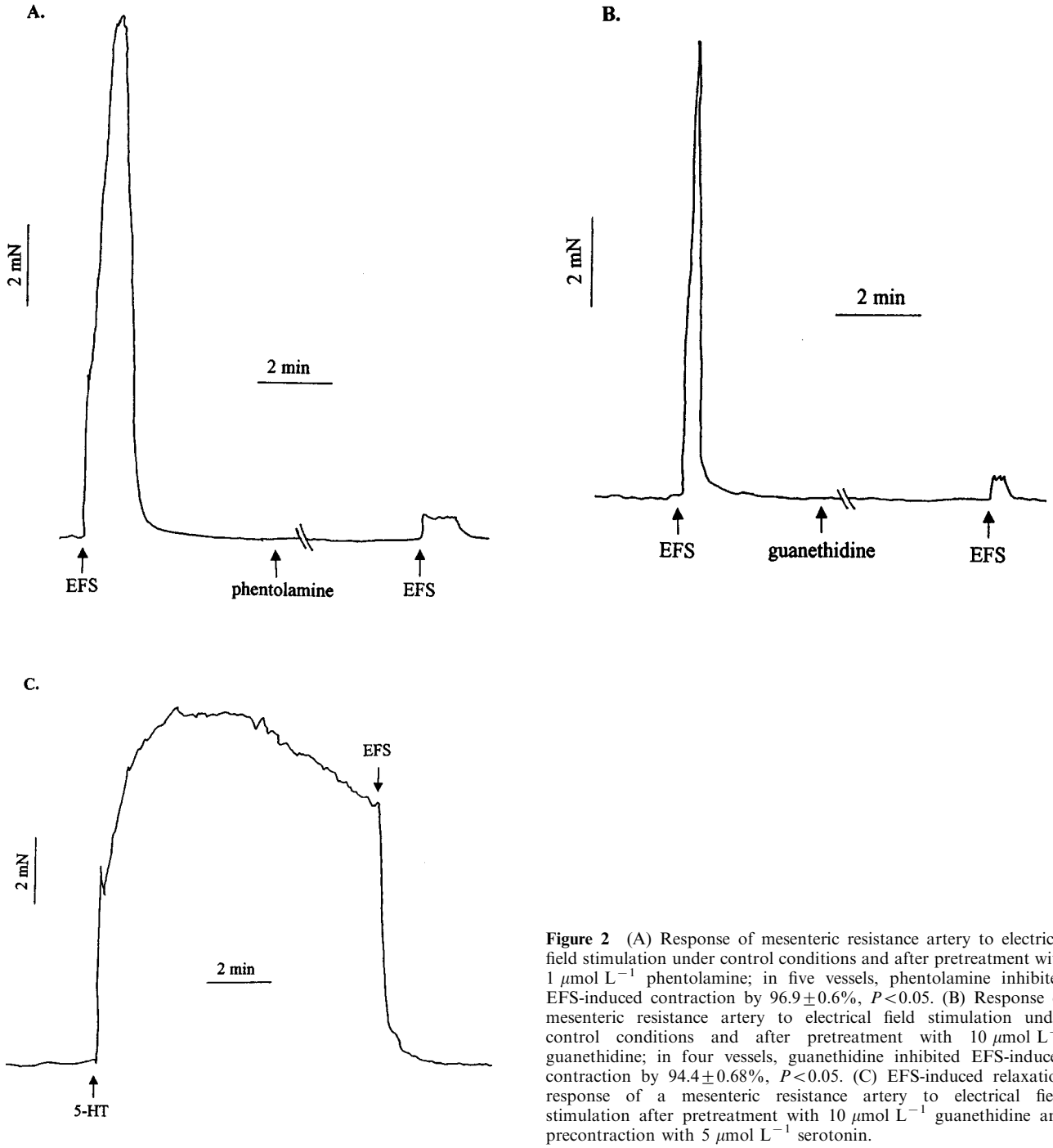

Figure 2 (A) Response of mesenteric resistance artery to electrical field stimulation under control conditions and after pretreatment with 1 μmol L^{-1} phentolamine; in five vessels, phentolamine inhibited EFS-induced contraction by 96.9±0.6%, $P<0.05$. (B) Response of mesenteric resistance artery to electrical field stimulation under control conditions and after pretreatment with 10 μmol L^{-1} guanethidine; in four vessels, guanethidine inhibited EFS-induced contraction by 94.4±0.68%, $P<0.05$. (C) EFS-induced relaxation response of a mesenteric resistance artery to electrical field stimulation after pretreatment with 10 μmol L^{-1} guanethidine and precontraction with 5 μmol L^{-1} serotonin.

Cumulative addition of extracellular Ca^{2+} to mesenteric resistance arteries that were precontracted with serotonin caused a dose-dependent relaxation. When the relaxation response to Ca^{2+} was expressed as a percentage of the serotonin-induced precontraction (Figure 3A), the maximal relaxation was $89.6 \pm 1.5\%$ (Table 2). This value was 23% greater than the maximal relaxation observed in response to EFS which was $72.6 \pm 3.9\%$, $P < 0.05$ (Table 2). Since these data indicate that electrical field stimulation of nerves cannot produce complete relaxation of the precontracted vessel, the ED_{50} values for Ca^{2+} were determined from plots of the relaxation expressed as a percentage of the maximal response to 5 mmol L^{-1} Ca^{2+} versus extracellular Ca^{2+} (Figure 3B). The ED_{50} for Ca^{2+} in the mesenteric branch arteries was 1.91 ± 0.18 mmol L^{-1} (Table 2).

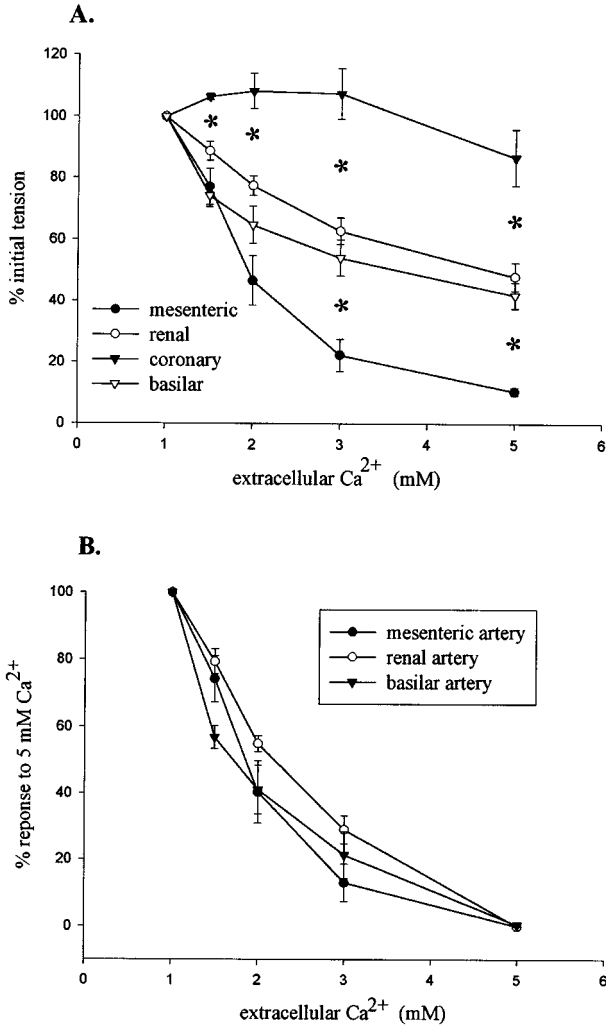

Figure 3 (A) Relaxation response to cumulative addition of extracellular Ca^{2+} of mesenteric branch, main renal, left anterior descending coronary, and basilar arteries expressed as a percentage of the initial tone induced by 5 μmol L^{-1} serotonin. (B) Ca^{2+}-induced relaxation response expressed as a percentage of the maximal relaxation response to 5 mmol L^{-1} Ca^{2+}.

Renal arteries

Perivascular nerves in both the main renal arteries and interlobar renal arteries stained positively for CaR protein (Figure 4A and B). In the main renal trunk, CaR positive nerve density was 16.9 ± 2.1 μm mm^{-2}, while in renal interlobar arteries CaR-positive nerve density was 58 ± 2.6 μm mm^{-2} (Table 1). Both values were significantly less than the mesenteric branch artery at $P < 0.05$.

Because of the small size of the interlobar arteries exceeded the limitations of our myograph system, mechanical properties were only studied in the main renal artery. When the relaxation response to electrical field stimulation was assessed, the renal artery relaxed an average of $40.8 \pm 3.5\%$ (Table 2). Cumulative addition of Ca^{2+} to this artery also induced relaxation (Figure 3A). When the response was expressed as a percentage of initial tension, the maximal relaxation was $52.0 \pm 4.6\%$ which is 30% greater than the magnitude of relaxation induced by electrical field stimulation, $P < 0.05$ (Table 2). As with the mesenteric artery, ED_{50} values for Ca^{2+} were derived from plots of relaxation expressed as a percentage of the maximal response to 5 mM Ca^{2+} versus extracellular Ca^{2+} (Figure 3B) and averaged 2.21 ± 0.11 m mol L^{-1} (Table 2).

Coronary arteries

When left anterior descending coronary arteries were stained with polyclonal anti-CaR, very little staining was observed (Figure 5A), and the density was estimated to be 7.4 ± 3.2 μm mm^{-2} (Table 1) which was less than the mesenteric branch and renal interlobar arteries at $P < 0.05$, but did not achieve statistical significance compared with the main renal trunk ($P = 0.066$). To determine whether the lack of staining was because our dissection procedure destroyed the perivascular nerve network, the coronary arteries were also exposed to anti-NCAM. This antibody revealed a network of perivascular nerves which had an average density of 64 ± 4 μm mm^{-2}, indicating that the nerve network was intact (Figure 5B).

Table 2 Maximal degree of EFS- and Ca^{2+}-induced relaxation and ED_{50} values for Ca^{2+}

Arterial segment	*Tension-pre-EFS* (mN)	*Maximal relaxation to EFS* (% initial tension)	*Tension-Pre-Ca^{2+}* (mN)	*Maximal relaxation to Ca^{2+}* (% initial tension)	*ED_{50} for Ca^{2+}* (mmol L^{-1})
Mesenteric branch	7.86 ± 0.78	72.6 ± 3.9	7.83 ± 0.75	89.6 ± 1.5	1.91 ± 0.18
Main renal	7.30 ± 1.2	40.8 ± 3.5	7.12 ± 1.0	52.0 ± 4.6#	2.21 ± 0.11
Coronary	3.42 ± 0.63	15.9 ± 4.4**	3.37 ± 0.43	13.3 ± 9.2	nd
Basilar	3.37 ± 0.55	59.6 ± 4.0*	3.22 ± 0.41	58.1 ± 4.3#	1.93 ± 0.18

The maximal response to EFS indicates the % relaxation that was observed when the vessel was electrically stimulated using parameters described in the text while precontracted with serotonin in the presence of 10 μmol L^{-1} guanethidine, the maximal relaxation response to Ca^{2+} indicates the relaxation that was observed in the presence of 5 mmol L^{-1} Ca^{2+} after precontraction in the presence of serotonin. n.d. = not determined. Values are mean $\pm$ s.e.mean, $n = 5-6$. *Indicates a significant difference in the maximal level of EFS-induced relaxation compared with mesenteric and main renal arteries. **From main renal arteries. #Indicates a significant difference in the maximal level of Ca^{2+}-induced relaxation compared with mesenteric branch and coronary arteries at $P < 0.05$.

When the relaxation responses of left anterior descending coronary arteries to electrical field stimulation were assessed, they relaxed only 15.9±4.4% (Table 2), which was significantly less than the other vessels at $P<0.05$. Cumulative addition of Ca^{2+} between 1.5 and 3 mmol L^{-1} caused a dose-dependent contraction of the isolated coronary arteries, while raising Ca^{2+} further to 5 mmol L^{-1} caused a slight relaxation which averaged 13.3±9.2% (Figure 3A). This value was not significantly different from the response to EFS (Table 2). In view of the absence of Ca^{2+}-induced relaxation through the range observed for the other vessels, ED_{50} values for Ca^{2+} were not estimated (Table 2).

Basilar artery

The perivascular nerve network of the basilar artery stained positively for CaR protein (Figure 6) and had a nerve density of 62.6±4.1 μm mm^{-2} (Table 1). This density was not significant different from that obtained for the renal interlobar artery.

When the relaxation response of the basilar artery to electrical field stimulation was assessed, it relaxed an average of 59.6±4.0% (Table 2). Cumulative addition of Ca^{2+} to this

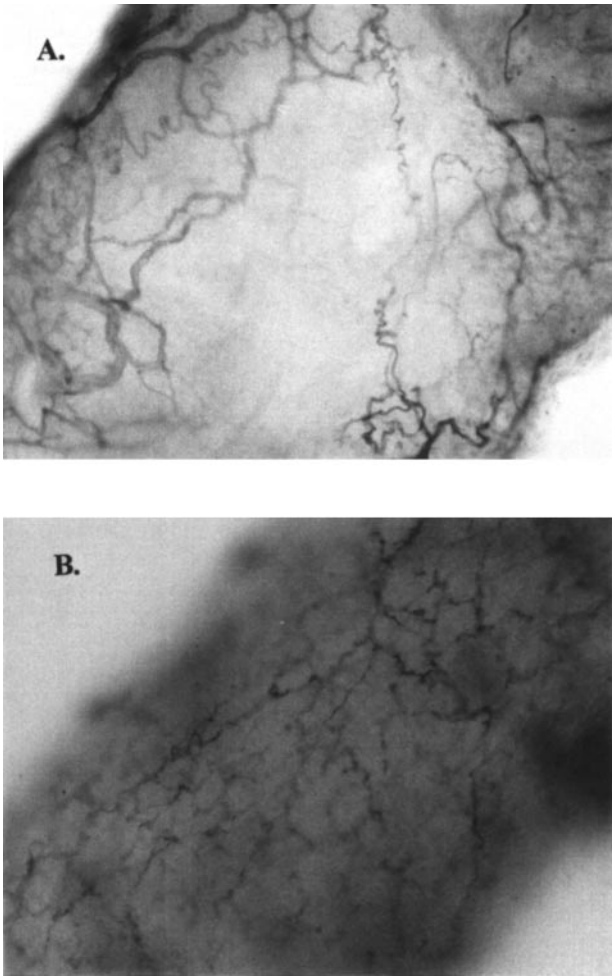

Figure 4 Demonstration of CaR-positive nerve fibres in a whole mount preparation of the main renal artery (A) and an interlobar artery (B) visualized using a rabbit polyclonal anti-CaR antibody; immunostaining was performed as described in the text; magnification of main renal artery = 100×, interlobar artery = 200×. The density of CaR-positive nerve staining in renal arteries from five different animals was estimated using a morphometric method and is summarized in Table 1.

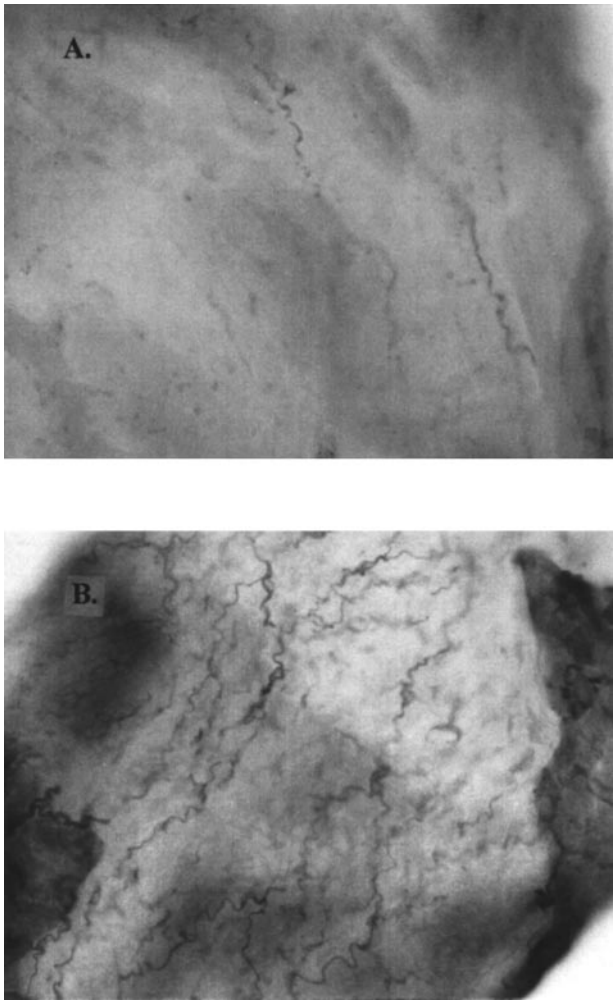

Figure 5 Results of staining the left anterior descending coronary artery with polyclonal anti-CaR and polyclonal anti-NCAM. (A) Demonstration of the sparse distribution of CaR-positive nerve fibres in a whole mount preparation stained with anti-CaR (magnification = 200×). (B) Demonstration using anti-NCAM, that perivascular nerves were intact after the dissection procedure (200×). The density of CaR-positive nerve staining of coronary arteries from five different animals was estimated using a morphometric method and is summarized in Table 1.

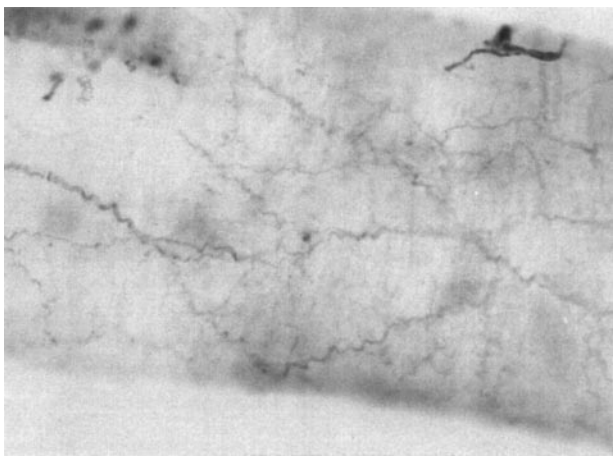

Figure 6 Demonstration of CaR-positive nerve fibres in a whole mount preparation of the basilar artery; immunostaining was performed as described in the text, magnification = 200×. The density of CaR-positive nerve staining of basilar arteries from five different animals was estimated using a morphometric method and is summarized in Table 1.

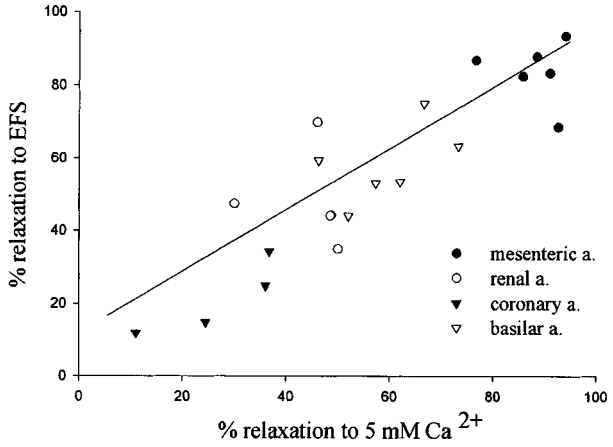

Figure 7 Correlation analysis of the relationship between the maximal level of relaxation induced by 5 mmol L^{-1} Ca^{2+} (ordinate) and the maximal relaxation response to electrical field stimulation (abscissa). The two variables were high correlated with $r^2=0.78$ and $P<0.001$.

artery also induced relaxation (Figure 3A). The maximal relaxation response was $58.1\pm4.\%$ when expressed as a percentage of the serotonin-induced precontraction. This values was not different from the maximal response to electrical field stimulation (Table 2). As with the mesenteric and renal arteries, ED_{50} values for Ca^{2+} were calculated from plots of relaxation expressed as a percentage of the maximal response to 5 mmol L^{-1} Ca^{2+} vs extracellular Ca^{2+} and averaged 1.93 ± 0.18 mmol L^{-1} (Table 2).

Discussion

We recently reported that the perivascular sensory nerve network of small mesenteric branch arteries expresses a receptor for extracellular Ca^{2+} that apparently couples changes in the concentration of extracellular Ca^{2+} with the release of vasodilator from sensory nerves (Bukoski *et al*., 1997; Mupanomunda *et al*, 1998). We also proposed that this novel dilator system, if present in tissues that are involved in transcellular Ca^{2+} movement, i.e. the intestine and kidney, could serve to couple changes in the level of interstitial Ca^{2+} with changes in blood vessel tone, regional resistance, and local blood flow (Bukoski, 1998). The present experiments were performed as an initial test of this hypothesis by determining whether expression of this dilator system is limited to the mesenteric circulation or whether it is also present in other regional circulations.

The results of our study show that CaR protein is expressed in relatively high levels in the periadventitial network of mesenteric branch arteries, interlobar arteries, and the basilar artery, but in to a lesser extent in the main renal trunk and even less in the left anterior descending coronary artery. Identification of the CaR protein in the perivascular nerve network relied on the use of anti-CaR antibodies. The antibody used for quantification was a polyclonal rabbit antihuman CaR that has previously been used by ourselves (Mupanomunda *et al*., 1998) and others (Garrett *et al*., 1995b) to localize CaR protein. The pattern of staining that was observed clearly matched that obtained with the polyclonal antibody that recognized neuronal cell adhesion molecule; a protein that is highly expressed nervous tissue (Jorgensen, 1995). Thus, it seems safe to conclude that the periadventitial nerve network of mesenteric, renal, and cerebral arteries expresses CaR protein.

A morphometric method that we recently described (Mupanomunda *et al*., 1998) was used to quantify the density of CaR positive nerve fibres and the results indicated the following rank order: mesenteric branch arteries > basilar arteries = renal interlobar arteries > renal arteries > coronary arteries. While this relative ranking of CaR positive nerve density, including the low level of expression in the coronary artery, appears to be accurate, there are factors that could impact on the analysis particularly in the coronary artery where very little staining was observed. Among these are the possibility that there could have been gross damage to the perivascular nerve network during removal of the vessel from surrounding parenchymal tissue, or a chance that differences in the level of fixation occurred among the vessels, such that there was degradation of the antigen. We do not believe that these factors contributed to the low density of CaR positive fibres in the coronary artery, however, since significant NCAM-positive nerve staining was detected in this artery.

As has been shown by others (Li & Duckles, 1993), electrical field stimulation of isolated arteries precontracted in the presence of guanethidine induces relaxation of isolated arteries. In the present study, EFS-induced relaxation of arterial segments from each circulation that was examined indicating the presence of vasodilator nerve fibres. It was apparent, however, that the magnitude of EFS-induced relaxation was dissimilar among the arteries and had the following rank order: mesenteric branch artery > basilar artery > main renal artery > left anterior descending coronary artery. Moreover, electrical field stimulation was not sufficient to induce a complete relaxation in any vessel that was studied.

Ca^{2+}-induced relaxation was examined in these arteries as an index of functional coupling of the perivascular sensory nerve CaR. Significant relaxation over the range of 1–3 mmol L^{-1} was observed in all vessels except the coronary, which instead responded with a contraction. Moreover, the magnitude of Ca^{2+}-induced relaxation was highly correlated with that of EFS-induced relaxation (Figure 7), which is consistent with data obtained using mesenteric branch arteries indicating that Ca^{2+}-induced relaxation has a neuronal basis. Further studies using chemical denervation techniques will be required to verify that Ca^{2+}-induced relaxation in arteries from the kidney and brain has a neuronal origin.

Of interest, the finding of a very sparse network of CaR positive nerves in the coronary artery, coupled with the absence of Ca^{2+}-induced relaxation, strongly indicates that this bed lacks the perivascular sensory nerve CaR mediated dilator system. Whether this is a specific property of the LAD, which is a conduit vessel, or is also shared by the smaller resistance arteries of the heart is unknown. If the coronary circulation does lack this dilator system, one possible explanation could be linked to the fact that the coronary circulation has a unique ontological origin compared with other vessels (Viragh *et al*., 1993).

A general question that needs to be addressed concerns the possible functional role of this Ca^{2+}-activated dilator system. As noted above, we have proposed that the Ca^{2+}-activated dilator system monitors the concentration of Ca^{2+} that is present in interstitial fluid and stimulates the release of dilator transmitters as Ca^{2+} rises. Thus, in the duodenal submucosa, where we have recently shown that interstitial Ca^{2+} varies from 1 to 1.9 mmol/L under physiologic conditions (Mupanomunda & Bukoski, unpublished observation), a rise in Ca^{2+} would be associated with local vasodilation and washout of the elevated Ca^{2+}. Although the cumulative data are consistent

with a functional role of the CaR dilator system in the mesenteric vasculature, there is one caveat. The second branch mesenteric resistance arteries that we examined are proximal to the arterioles of the submucosa that would be exposed to elevations of extracellular Ca^{2+} and, as such, are likely to be innervated by non-adrenergic, non-cholinergic nerves arising from the myenteric plexus and not the dorsal root ganglia. The questions of whether or not neuronal cell bodies in the myenteric plexus express CaR protein and whether activation of this receptor causes vasodilation have not been experimentally addressed. It has been demonstrated, however, that mRNA encoding the CaR is expressed in the small intestine, and this message could be of neuronal (myenteric plexus) origin.

The question of what role this Ca^{2+}-activated dilator system might play in the cerebral circulation is less clear. Although it is well established that the brain contains areas that participate in the regulation of salt and water homeostasis and that one of these regions, the subfornical organ, expresses very high levels of CaR message (Rogers *et al*., 1997), there is no evidence that interstitial Ca^{2+} in the area of this tissue changes in response to the Ca^{2+} status of the organism. It thus seems unlikely that the CaR activated dilator system would be active in this region. It is possible, however, that local changes in interstitial Ca^{2+} may accompany nerve depolarization and transmitter release as has been proposed by Ruat *et al*. (1995). Such changes could possibly serve to modulate the activity of a Ca^{2+} activated dilator system. In contrast with these putative mechanisms, an equally possible function for wide-spread expression of the perivascular CaR in tissues that do not participate in transcellular Ca^{2+} movement would be that it serves to protect arteries in the general circulation from spastic events that could be associated with pathological increases in serum Ca^{2+} such as might occur during hyperparathyroidism and hypervitaminosis D.

This work was supported by grants from the National Institutes of Health (HL54901) and from NPS Pharmaceuticals (Salt Lake City, UT, U.S.A.). We acknowledge the thoughtful input of Drs Edward Nemeth and Kim Rogers of NPS Pharmaceuticals.

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(Received June 15, 1998
Revised August 18, 1998
Accepted September 1, 1998)