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Nitric oxide regulation of the central aortae of the toad *Bufo marinus* occurs independently of the endothelium

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Summary

Nitric oxide (NO) signalling pathways were examined in the lateral aortae and dorsal aorta of the cane toad *Bufo marinus*. NADPH diaphorase histochemistry and nitric oxide synthase (NOS) immunohistochemistry found no evidence for endothelial NOS in the endothelium of toad aortae, but it could be readily demonstrated in rat aorta that was used as a control. Immunohistochemistry using a specific neural NOS antibody showed the presence of neural NOS immunoreactivity in the perivascular nerves of the aortae. The anatomical data was supported by *in vitro* organ bath physiology, which demonstrated that the vasodilation mediated by applied acetylcholine (10^{-5} mol l^{-1}) was not dependent on the presence of the vascular endothelium; however, it was significantly reduced in the presence of a neural NOS inhibitor, vinyl-L-NIO (10^{-4} mol l^{-1}). In addition, atropine (10^{-6} mol l^{-1}) (a muscarinic receptor inhibitor), L-NNA (10^{-4} mol l^{-1}) (a NOS inhibitor) and ODQ (10^{-5} mol l^{-1}) (an inhibitor of soluble guanylyl cyclase) abolished the vasodilatory effect of applied acetylcholine. In conclusion, we propose that an endothelial NO system is absent in toad aortae and that NO generated by neural NOS in perivascular nerves mediates vasodilation.

Key words: nitric oxide, endothelial nitric oxide synthase, neural nitric oxide synthase, soluble guanylyl cyclase, vasodilation, aorta, cane toad, *Bufo marinus.*

Introduction

The vascular endothelium is responsible for the production of a large number of mediators that regulate vascular tone, including nitric oxide (NO). First identified as endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki (1980), it was not until 1987 that Palmer et al. (1987) demonstrated that NO and EDRF were the same molecule. Nitric oxide is a potent vasodilator that is synthesised by nitric oxide synthase (NOS), an enzyme that occurs in three major isoforms, endothelial NOS (eNOS), neural NOS (nNOS) and inducible NOS (iNOS) (Förstermann et al., 1994). Both eNOS and nNOS are calcium-dependent and NO is released *via* the activation of NOS by a variety of signalling molecules, including acetylcholine (Furchgott, 1996). Once synthesised, NO diffuses out of the endothelial cell or nerve fibre and into the adjacent smooth muscle cells (Ignarro, 1989; Moncada et al., 1991; Lowenstein et al., 1994). In the mammalian vasculature, NO derived from the vascular endothelium is primarily responsible for vasorelaxation; however, some blood vessels of the cerebral, pelvic and gut regions have perivascular nerves that contain neural NOS, and neurally released NO can induce relaxation (Young et al., 2000).

In mammals, the vasodilatory effect of NO is mediated *via* an intracellular soluble guanylyl cyclase (soluble GC) located in the cytoplasm of the vascular smooth muscle cells (Chinkers and Garbers, 1991; Schmidt et al., 1993; Lucas et al., 2000). Once activated, the soluble GC catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The subsequent increase in cGMP concentration activates cGMP-dependent protein kinase, which lowers the intracellular calcium in vascular smooth muscle cells to mediate a vasodilatory response (Lucas et al., 2000).

Compared with mammals, there have only been a few studies in non-mammalian vertebrates that have suggested that an endothelial NO system exists in the vasculature of birds (Hasegawa and Nishimura, 1991), reptiles (Knight and Burnstock, 1993), and amphibians (Rumbaut et al., 1995; Knight and Burnstock, 1996). The presence of an endothelial NO system in blood vessels of fish remains controversial because of studies proposing both its existence (Mustafa and Agnisola, 1998; Fritsche et al., 2000) and non-existence (Olson and Villa, 1991; Kågström and Holmgren, 1997; Evans and Gunderson, 1998). Miller and Vanhoutte (1986) were the first to demonstrate, in amphibians, that the vasodilatory effect of applied acetylcholine in the vasculature of the American bullfrog *Rana catesbeiana* is endothelium-dependent. In addition, the effect of acetylcholine was reversed by methylene blue, which suggested that the vasodilation was occurring *via* activation of a soluble GC. However, it was the study by Knight and Burnstock (1996) that indicated that an endothelial

NO system is present in amphibians. These authors showed that in pre-constricted aortic arches of the leopard frog *Rana pipiens*, applied acetylcholine caused a vasodilatory response that was abolished or greatly reduced by L-NAME (a NOS inhibitor), or when the endothelium was removed. Further evidence for an endothelial NO system was found in the mesenteric capillaries of *R. pipiens*, in which a NOS inhibitor (L-NMMA) decreased the hydraulic conductivity compared to control levels (Rumbaut et al., 1995).

Interestingly, studies investigating the presence of a NO system in the vasculature of amphibians have primarily used *in vitro* organ bath experiments. There has, however, been no histochemical or immmunohistochemical evidence in the literature to suggest the presence of NOS in the endothelium or perivascular nerves of amphibians. Therefore, the present study examined the mechanisms involved in NO-mediated vasodilation in the aortae of the toad *Bufo marinus* using physiological and anatomical approaches. The use of NADPH diaphorase histochemistry and immunohistochemistry using specific antibodies to eNOS and nNOS found no evidence to support the presence of an endothelial NO system, but it did show the presence of neural NOS immunoreactivity in the perivascular nerves. Concurrent physiological studies have provided further evidence that toad aortae contain a neural NO system rather than an endothelial NO system.

Materials and methods

Cane toads *Bufo marinus* L. with a body weight of 90–150 g, and of either sex, were purchased from a commercial supplier in Queensland. Toads were maintained at the Deakin University Animal House at 20–25°C. They were not fed during captivity (up to 1 month) but had *ad libitum* access to water. Sprague Dawley rats, *Rattus norvegicus*, of either sex were obtained from a breeding colony established at the Deakin University Animal House. They were housed in rat boxes and maintained at 23°C on a 12:12 h light:dark cycle with *ad libitum* access to water and rat chow. Prior to experimentation, toads were killed by decapitation and the rats were stunned by a blow to head followed by cervical dislocation. All experiments were approved by the Animal Ethics Committee of Deakin University (Approval A9/2000).

In vitro *organ bath physiology*

The lateral aortae and dorsal aorta were excised from toads and placed in Mackenzie's balanced salt solution (115 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 20 mmol l^{-1} $NaHCO_3$, 3.1 mmol l^{-1} NaH_2PO_4, 1.4 mmol l^{-1} $MgSO_4$, 16.7 mmol l^{-1} D-[+]glucose and 1.3 mmol l^{-1} $CaCl_2$, pH 7.2). Individual rings of approximately 4–5 mm in length were mounted horizontally between two hooks for the measurement of isometric force, and placed in an organ bath. The rings were bathed in 15 ml of Mackenzie's solution, which was maintained at 22°C and aerated with 95% O_2 and 5% CO_2. The force transducer (Grass-FT03) was linked to a MacLab™ data collection system and a Macintosh computer, which recorded data for further analysis. An initial tension of 0.5 g was applied to the blood vessels and they were allowed to equilibrate for 30 min. In some experiments, the endothelium was removed by rubbing with a toothpick, and the extent of removal was determined using NADPH histochemistry. Prior to administering various vasodilatory substances, each vessel was pre-constricted with endothelin-1 (10^{-8} mol l^{-1}), and vasoconstriction was allowed to reach its maximum. A previous study has shown that 10^{-8} mol l^{-1} endothelin^{-1} elicits an appropriate vasoconstriction for studies of vasodilatory mechanisms (Minerds and Donald, 2001). The extent of vasodilation was determined by scoring the degree of relaxation as a ratio, where dilation to pre-constriction levels were set at 100%. For experiments, matched controls were used from the same animal for comparison of drug effects. Data are expressed as mean ± S.E.M. of five or more experiments, and statistical analysis was performed with independent *t*-tests using the SPSS (9.0) statistical package; $P \leq 0.05$ was considered significant.

NADPH diaphorase histochemistry

The lateral aortae and dorsal aorta from toads and the descending aorta from rats were dissected free and immersed in phosphate-buffered saline (PBS, 0.01 mol l^{-1} phosphate buffer and 0.15 mol l^{-1} NaCl, pH 7.4) at 4°C. Each vessel was opened and pinned out endothelium side up on dental wax, prior to fixing for 1 h in 4% formaldehyde (pH 7.4) at 4°C. The blood vessels were washed in 0.01 mol l^{-1} PBS (3× 10 min) and removed from the dental wax. Blood vessels were stained in a NADPH diaphorase mixture containing 1 mg ml^{-1} β-NADPH, 0.25 mg ml^{-1} nitroblue tetrazolium (NBT), 1% Triton X-100 in 0.1 mol l^{-1} Tris buffer, pH 8, for times ranging from 15–60 min at 25°C. This mixture was kept in the dark, as it is light sensitive. The vessels were then washed in 0.01 mol l^{-1} PBS and mounted on slides in buffered glycerol (0.5 mol l^{-1} Na_2CO_3 added dropwise to 0.5 mol l^{-1} $NaHCO_3$ to pH 8.6, combined 1:1 with glycerol). Blood vessels were observed under a light microscope (Olympus) and were photographed with a digital colour system (Spot 35 Camera System). Control experiments were performed on the myenteric plexus of both rats and *B. marinus* because previous studies have demonstrated that neurons in the mammalian and amphibian myenteric plexuses showed positive NADPH diaphorase staining (Wilhelm et al., 1998; Li et al., 1993, respectively). The descending aorta of rats was used as a control to demonstrate the presence of NOS in the vascular endothelium.

Immunohistochemistry

Blood vessels from toad and rat were fixed as described above. The blood vessels were unpinned, washed in 0.01 mol l^{-1} PBS (3× 10 min), incubated in DMSO (3× 10 min) and washed in 0.01 mol l^{-1} PBS (5× 2 min). The blood vessels were then incubated in a polyclonal antibody raised against mouse endothelial NOS (1:1000; O'Brien et al., 1995) or a polyclonal antibody raised against sheep neural NOS (1:4000; Anderson et al., 1995) for 24 h at room temperature in a humid

box. The following day, tissues were washed in 0.01 mol l^{-1} PBS (3× 10 min) to remove any excess antibody and incubated in a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-sheep IgG (1:200) (Zymed Labratories, San Francisco, USA) for 3–4 h at room temperature in a humid box. The blood vessels were then washed in 0.01 mol l^{-1} PBS (3× 10 min) and mounted in buffered glycerol. Blood vessels were observed under a fluorescence microscope (Zeiss) using a FITC filter and photographed as above.

Materials

Sodium nitroprusside (SNP), frog atrial natriuretic peptide (fANP), acetylcholine, N^{ω}-nitro-L-arginine (L-NNA), atropine, hexamethonium, β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), NBT and Triton X-100 were obtained from Sigma (St Louis, USA). Endothelin-1 (ET-1) was purchased from Auspep (Melbourne, Australia), oxadiazole quinoxalin-1 (ODQ) and L-N^5-(1-imino-3-butenyl)-ornithine (vinyl-L-NIO) were obtained from Alexis (San Diego, USA), and the NOS antibodies were obtained from Chemicon (Melbourne, Australia).

Results

In vitro *organ bath physiology*

In the lateral and dorsal aortae, ET-1 (10^{-8} mol l^{-1}) induced a potent and long-lasting vasoconstriction that was allowed to reach its maximum, after which various chemicals associated with the vasodilatory mechanisms of NO were added.

In the lateral and dorsal aortae, the NO donor, SNP (10^{-4} mol l^{-1}), induced an average vasodilation of 66±4.48% and 85.6±3.25%, respectively (N=5, Fig. 1). In addition, applied acetylcholine (10^{-5} mol l^{-1}) produced an average vasodilatory response of 33±3.8% and 35.5±4%, respectively (N=5, Fig. 2). The addition of the soluble GC inhibitor, ODQ (10^{-5} mol l^{-1}), completely abolished the vasodilatory effect of SNP (10^{-4} mol l^{-1}) and applied acetylcholine (10^{-5} mol l^{-1}) in both aortae (Fig. 3; N=5); acetylcholine now caused a vasoconstriction. Subsequently, frog ANP (10^{-8} mol l^{-1}), which mediates vasodilation *via* a particulate guanylyl cyclase (Minerds and Donald, 2001), caused a vasodilation in the presence of ODQ (not shown). This indicates that the vasodilatory effect of SNP and acetylcholine is mediated solely *via* a soluble GC.

In the presence of the NOS inhibitor L-NNA (10^{-4} mol l^{-1}), the vasodilatory effect of applied acetylcholine was abolished in both aortae. However, as expected, SNP, which mediates vasodilation by releasing NO, produced a potent vasodilatory response (Fig. 4; N=5).

In endothelium-denuded lateral aortae, applied acetylcholine (10^{-5} mol l^{-1}) caused a vasodilation of 23.9±6.7% compared to 29.4±9.2% in control vessels with an intact endothelium (P=0.26, N=5; Fig. 5); a similar effect was found in the dorsal aorta (denuded; 28.7±1.9%; control 31.8±2.6%, P=0.35, N=5; Fig. 5). These data show that the removal of the endothelium had no significant effect on acetylcholine-mediated

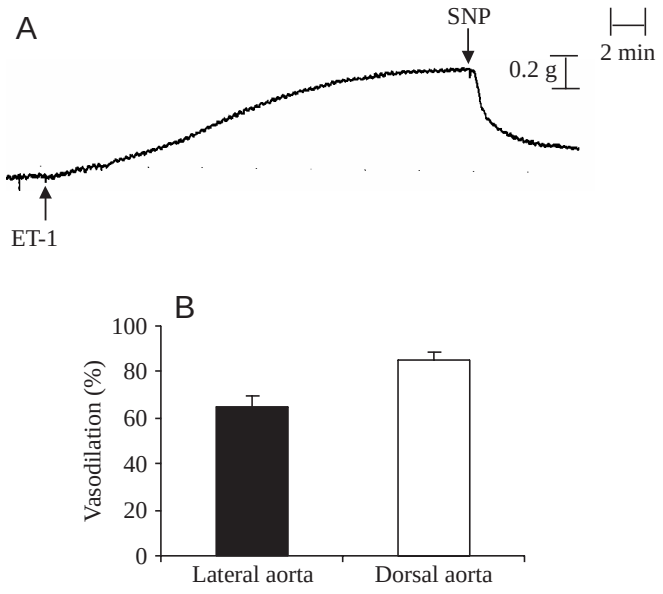

Fig. 1. (A) Tension recording showing the effect of sodium nitroprusside (SNP) on the lateral aorta. The aorta was exposed to endothelin-1 (ET-1; 10^{-8} mol l^{-1}) until a maximal constriction was achieved and then SNP (10^{-4} mol l^{-1}) was added. SNP caused a marked vasodilation. (B) Mean responses (% vasodilation) of pre-constricted lateral and dorsal aortae to SNP (10^{-4} mol l^{-1}; N=5).

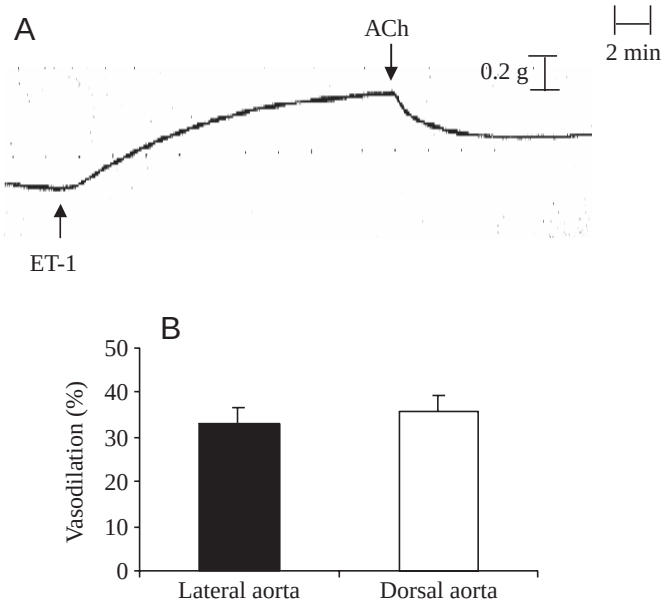

Fig. 2. (A) Tension recording showing the effect of acetylcholine (ACh) on the dorsal aorta. The aorta was exposed to endothelin-1 (ET-1; 10^{-8} mol l^{-1}) until a maximal constriction was achieved and then ACh (10^{-5} mol l^{-1}) was added. ACh caused a marked vasodilation. (B) Mean responses (% vasodilation) of pre-constricted lateral and dorsal aortae to acetylcholine (10^{-5} mol l^{-1}; N=5).

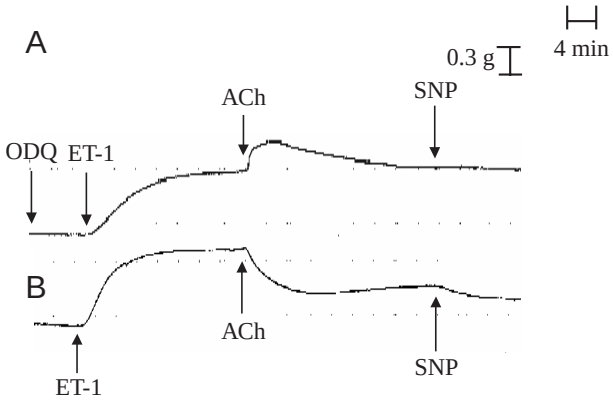

Fig. 3. Tension recordings showing the effect of acetylcholine (ACh) on the dorsal aorta with (A) or without (B) the presence of the soluble guanylyl cyclase inhibitor oxadiazole quinoxalin-1 (ODQ). The vessels were pre-treated with ODQ (10^{-5} mol l^{-1}) for approximately 10 min before being constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}). At the peak of vasoconstriction, ACh (10^{-5} mol l^{-1}) was administered. ACh caused a constriction in the preparation incubated with ODQ (A) and a vasodilation in the preparation without ODQ (B) (*N*=5).

vasodilation in the lateral and dorsal aortae. In the lateral aortae incubated with vinyl-L-NIO (10^{-4} mol l^{-1}), a specific nNOS inhibitor (Babu and Griffith, 1998), applied acetylcholine (10^{-5} mol l^{-1}) caused a vasodilation of 21.1±5.9%, compared to 35.1±6.2% in control vessels without vinyl-L-NIO ($P<0.05$, $N=5$; Fig. 6); a similar effect was found in the dorsal aorta (vinyl-L-NIO; 23.8±1.9%; control 38.9±4.9%, $P<0.05$, $N=6$; Fig. 6). These data indicate that vinyl-L-NIO significantly reduced the vasodilatory effect of applied acetylcholine in both aortae.

In both aortae, acetylcholine-mediated vasodilation was abolished in the presence of the muscarinic receptor antagonist, atropine (10^{-6} mol l^{-1}), but the nicotinic receptor antagonist, hexamethonium (3×10^{-5} mol l^{-1}), had no effect ($N=5$, results not shown).

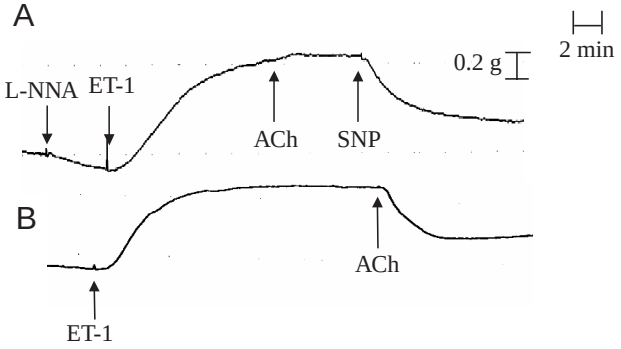

Fig. 4. Tension recordings showing the effect of acetylcholine (ACh) on the lateral aorta with (A) or without (B) the presence of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA). The preparations were pre-treated with L-NNA (10^{-4} mol l^{-1}) for approximately 10 min before being constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}). At the peak of vasoconstriction, acetylcholine (Ach; 10^{-5} mol l^{-1}) was administered, which only caused a marked vasodilation in the preparation without L-NNA (B) (*N*=5).

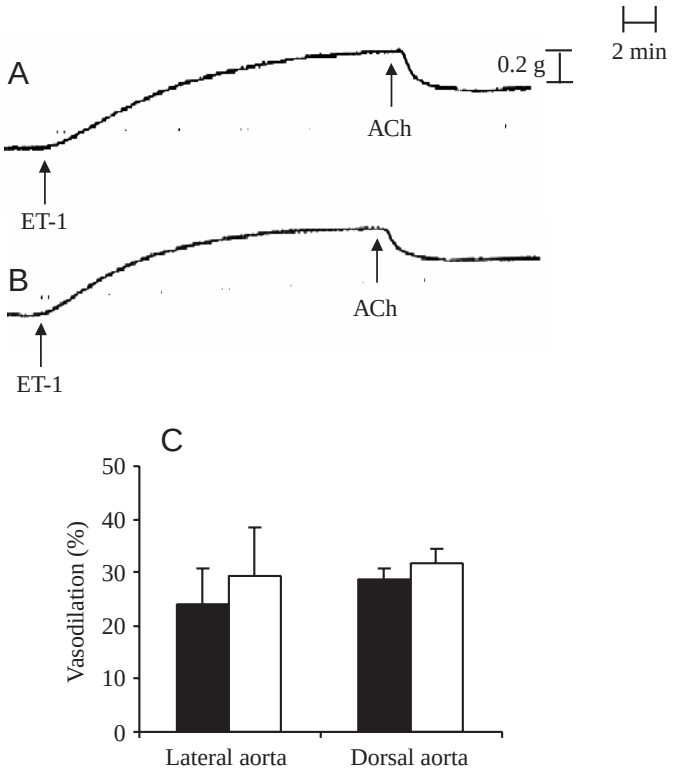

Fig. 5. Tension recordings showing the vasodilatory effect of acetylcholine (ACh) on the lateral aorta with the endothelium removed (A) and with the endothelium intact (A). The preparations were exposed to endothelin-1 (ET-1; 10^{-8} mol l^{-1}) until a maximum constriction was achieved and then ACh (10^{-5} mol l^{-1}) was added. (C) Mean response (% vasodilation) of ACh on pre-constricted lateral and dorsal aortae with the endothelium removed (filled bars) or the endothelium intact (open bars). Note that there is no significant difference in the ACh-mediated dilation (lateral aortae, $P=0.26$; dorsal aorta, $P=0.35$; $N=5$).

NADPH diaphorase histochemistry

In the lateral aortae and dorsal aorta, similar patterns of staining were observed following NADPH diaphorase histochemistry (*N*=5). No specific staining was observed in the endothelium of the toad aortae (Fig. 7A). In contrast, the endothelial cells of the rat aorta showed intense, perinuclear staining, indicating the presence of NOS (Fig. 7C); the NADPH-diaphorase staining pattern in the rat aorta was similar to that previously reported (O'Brien et al., 1995). The absence of specific NADPH-diaphorase staining in the toad endothelium was found in all preparations incubated for 15, 20, 30 or 60 min. However, NADPH-diaphorase staining was observed in the perivascular nerve fibres of the outer layers of the wall of both aortae (Fig. 8A,C). Specific staining was observed in nerve bundles and single, varicose nerve fibres. There was no distinct pattern in the distribution of nerve

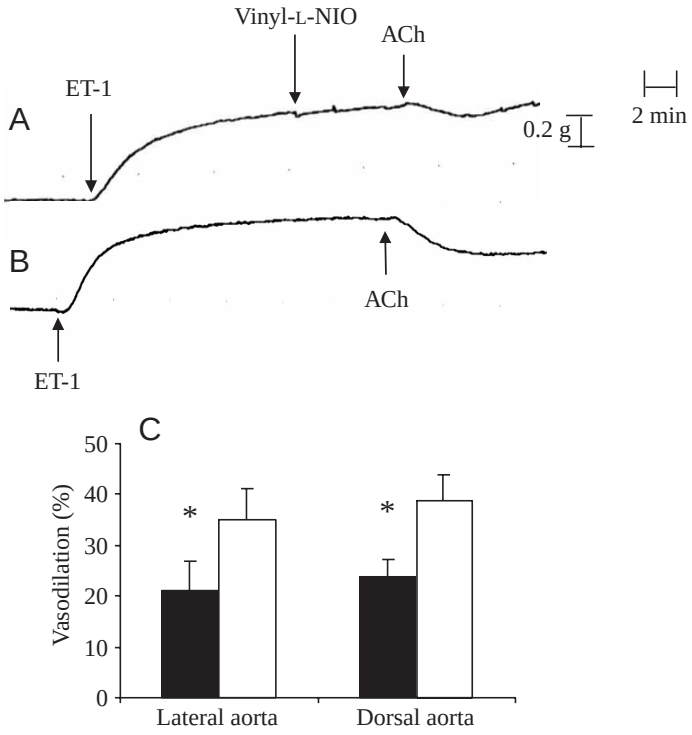

Fig. 6. Tension recordings showing the effect of acetylcholine (ACh) on the lateral aorta with (A) or without (B) the nNOS inhibitor vinyl-L-NIO. The preparations were exposed to endothelin-1 (ET-1; 10^{-8} mol l^{-1}) until a maximum constriction was achieved and then vinyl-L-NIO (10^{-4} mol l^{-1}) was added for approximately 10 min. Subsequently, ACh (10^{-5} mol l^{-1}) was added. The vasodilatory response was reduced in the presence of vinyl-L-NIO. (C) Mean response (% vasodilation) of ACh on ET-1-constricted lateral and dorsal aortae with (filled bars) or without (open bars) the presence of vinyl-L-NIO. Note that vinyl-L-NIO significantly reduced the ACh-mediated vasodilation (lateral and dorsal aortae, $P<0.05$, *denotes significant difference; $N=5$).

terminals in either blood vessel, although it appeared that the lateral aorta contained more NADPH-diaphorase positive nerves than the dorsal aorta.

Endothelial NOS and neural NOS immunohistochemistry

To specifically identify the type of NOS present in the endothelium and nerve fibres, eNOS and nNOS antibodies were used ($N=3$). Both antibodies revealed identical patterns of staining in comparison to the NADPH diaphorase histochemistry (Fig. 7B). In the toad aortae, eNOS immunoreactivity was absent in the endothelial cells, but it was clearly present in the rat aorta (Fig. 7D). Neural NOS immunoreactivity was observed in the perivascular nerve fibres of the lateral and dorsal aortae of toad (Fig. 8B,D). The nNOS immunoreactive fibres were also NADPH-diapharose positive.

Discussion

The current study showed by physiological and anatomical approaches that NO regulation of the aortae of the toad, *B. marinus*, occurs independently of the endothelium. The physiological experiments using aortic rings demonstrated that applied acetylcholine caused vasodilation that was not endothelium-dependent because the response was not abolished by endothelial removal. The acetylcholine-mediated vasodilation was caused by the generation of NO because the NOS inhibitor L-NNA abolished or significantly inhibited the response; the response was also abolished by the soluble GC inhibitor ODQ. These data strongly indicated that the acetylcholine-mediated vasodilation was occurring *via* a non-endothelial NOS. In fact, application of vinyl-L-NIO, a nNOS inhibitor, significantly inhibited the acetylcholine-mediated vasodilation. The physiological findings were supported by anatomical studies in which NADPH diaphorase histochemistry and immumohistochemistry showed the absence of NOS in the endothelium, but demonstrated that nNOS was present in the perivascular nerves of the aortae. In combination, the physiological and anatomical data provide evidence that NO regulation of the aortae of *B. marinus* occurs through a nNOS system. This is the first study to propose such a mechanism in amphibians and to demonstrate that an endothelial NO system is absent.

In mammals, it is well documented that acetylcholine mediates vasodilation by indirectly activating NOS to produce NO (see Moncada et al., 1991). Many studies have shown that mammalian blood vessels are regulated by two types of NO systems: the endothelial NO system, which is the predominant NO system, and the neural NO system, which has only been identified in blood vessels that supply the cerebral, pelvic and enteric regions (Young et al., 2000). Accordingly, it can be difficult to determine the specific source of NO and, prior to the recent development of gene knockout and drugs that selectively block eNOS and nNOS, the only way that neurally derived NO could be distinguished from endothelially derived NO was by examining vasodilation with and without the endothelium (Young et al., 2000). In mammals, nerve fibres containing nNOS that innervate blood vessels of the head and pelvic region have been identified as parasympathetic neurons, but in mesenteric arteries nNOS is found in sensory neurones (Young et al., 2000).

In comparison with mammals, NO regulation in the amphibian vasculature is less well understood, although it has been proposed that an endothelial NO system does exist (Knight and Burnstock, 1996). In the leopard frog *R. pipiens*, Knight and Burnstock (1996) demonstrated that acetylcholine induced vasodilation. In addition, these authors showed that the presence of L-NAME abolished the vasodilation caused by acetylcholine, suggesting that acetylcholine mediates vasodilation *via* NOS. This was also the case in the present study in which the acetylcholine-mediated vasodilation of the aortae of the toad was abolished in the presence of L-NNA, thus establishing the existence of NOS. To determine if NOS was located in the endothelium, experiments were performed on endothelium-denuded blood vessels. To date, this technique has been the predominant method for determining whether or not the endothelium plays a role in NO-mediated vasodilation

in lower vertebrates (Miller and Vanhoutte, 1986; Olson and Villa, 1991; Hasegawa and Nishimura, 1991; Knight and Burnstock, 1993; Knight and Burnstock, 1996; Evans and Gunderson, 1998). Knight and Burnstock (1996) demonstrated that damage to the endothelial layer abolished the acetylcholine-mediated vasodilation in *R. pipiens* and, therefore, proposed that acetylcholine was likely to be mediating vasodilation *via* an endothelial NO system. This is in contrast to our findings in the toad, which showed no significant difference between acetylcholine-mediated vasodilation in the lateral and dorsal aortae with or without the endothelium.

Although the physiological evidence suggested an endothelial NO system was absent in the blood vessels of the toad, it was important to anatomically support this finding. To date, there appears to be a lack of studies aimed at detecting the presence of NOS in the vasculature of lower vertebrates using anatomical methods. NADPH diaphorase histochemistry and immunohistochemistry are two anatomical methods that have been widely used to identify the presence of NOS in the mammalian vasculature (Beesley, 1995); however, this is the first study to use such techniques on the amphibian vasculature. The results showed an absence of NADPH diaphorase staining in the endothelial cells of the toad lateral aortae and dorsal aorta. In contrast, the endothelium of the rat aorta (used as a control) showed perinuclear staining, as previously demonstrated by O'Brien et al. (1995). This finding suggests that toad aortae lack an eNOS and, therefore, do not contain an endothelial NO system. Importantly, the results of the NADPH diaphorase histochemistry were supported immunohistochemically by the use of a mammalian eNOS antibody. In contrast to the perinuclear staining in the aorta of rat, the vascular endothelium of the toad was devoid of any immunofluorescence. It was assumed that the mammalian eNOS antibody would cross-react with eNOS if it was present in the toad because Fritsche et al. (2000) demonstrated cross-reactivity in the zebrafish *Danio rerio* using a mammalian eNOS antibody.

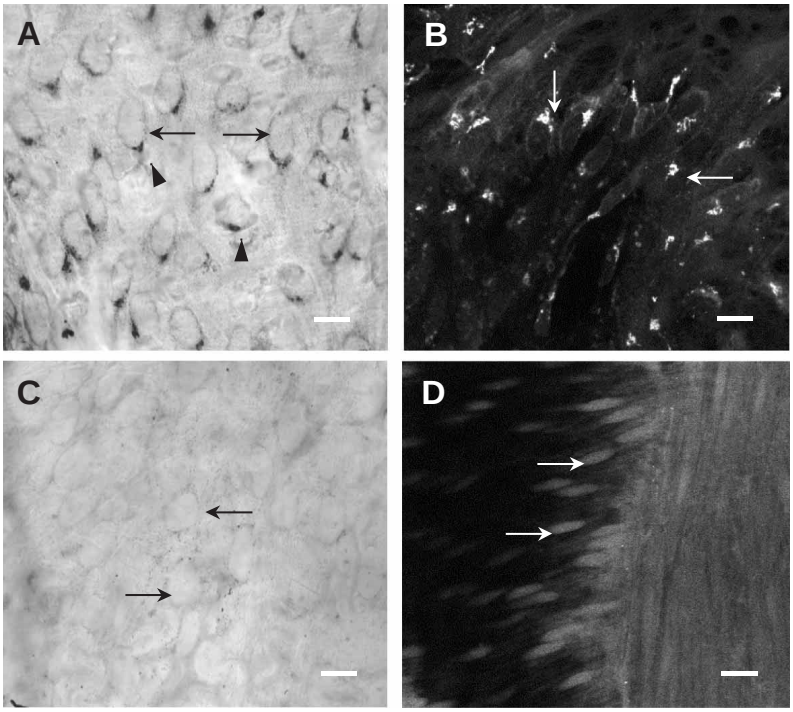

Fig. 7. Photomicrographs showing whole-mount preparations of the rat aorta (A,B) and the toad lateral aorta (C,D) following processing for NADPH diaphorase histochemistry (A,C) and endothelial nitric oxide synthase (NOS) immunohistochemistry (B,D). In the rat aorta, punctate NOS-positive staining (arrowheads) occurred around the nuclei (arrows) of the endothelial cells, which was demonstrable with both techniques (A,B). In contrast, no NOS-positive staining was observed around the nuclei of the endothelial cells in the toad lateral aorta (C,D). Scale bars, 10 μm.

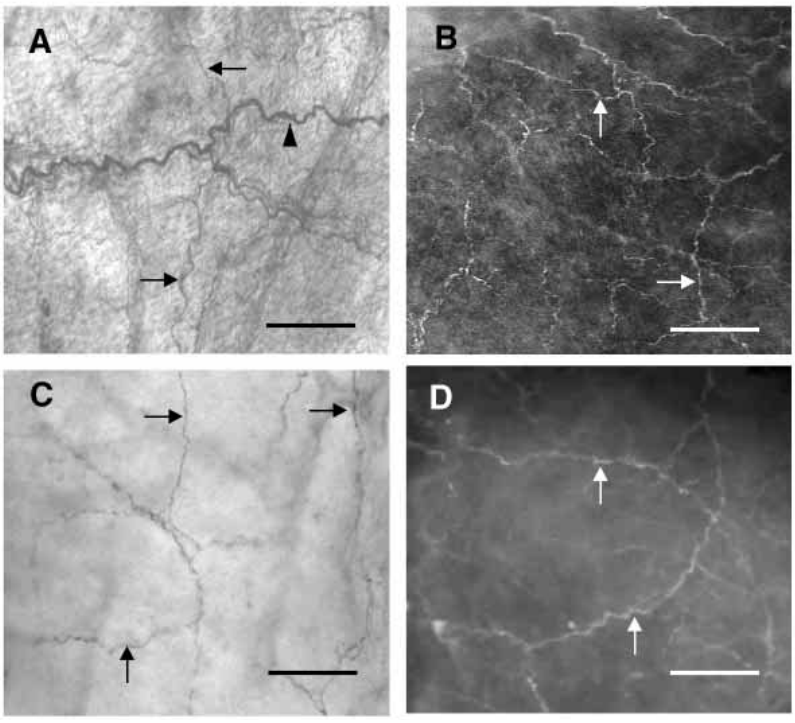

Fig. 8. Photomicrographs showing whole-mount preparations of the toad lateral (A,B) and dorsal (C,D) aortae following processing for NADPH diaphorase histochemistry (A,C) and neural nitric oxide synthase (NOS) immunohistochemistry (B,D). Using both techniques, a plexus of neural (n)NOS-positive perivascular nerve fibres (arrows) was observed in the outer layers of the wall of both vessels. In addition, some larger nNOS-positive perivascular nerve bundles (A, arrowhead) were observed. Scale bars, 100 μm.

Interestingly, NADPH diaphorase staining and nNOS immunoreactivity were observed in the perivascular nerve terminals innervating the lateral aortae and dorsal aorta of the toad. As mentioned above, blood vessels in the cerebral, pelvic and enteric regions of mammals are innervated with nerve fibres containing nNOS, but they have yet to be located within the systemic vasculature. The only other non-mammalian study to demonstrate the presence of nNOS positive nerves fibres in the vasculature was that in the estuarine crocodile *Crocodylus porosus* (Axelsson et al., 2001).

The presence of a neural NO system in the toad was verified by a significant decrease in the acetylcholine-mediated vasodilation in the presence of the nNOS inhibitor vinyl-L-NIO. This finding provides evidence that nNOS is responsible for inducing vasodilation in the central vasculature of *B. marinus*. In the mammalian cerebral vasculature, NO generated by nNOS is an important regulator of vascular tone (Meng et al., 1998). Experiments on endothelium-denuded cerebral arteries show that NOS inhibitors abolish or dramatically reduce vasodilations mediated by transmural electrical stimulation, which suggests that nerves innervating cerebral arteries produce NO (Toda and Okamura, 1990). More recently, eNOS knockout mice have been used to examine the role of nNOS in the cerebral vasculature. For example, Meng et al. (1998) demonstrated that the nNOS inhibitor 7-NI attenuated acetylcholine-mediated vasodilation in cerebral blood vessels that do not express eNOS. Whether a neural NO system is the primary regulator of cerebral arteries or it is just compensating for the absence of an endothelial NO system remains to be elucidated. However, the absence of an endothelial NO system in the toad suggests that a nNOS system is the only source of NO that could elicit vasodilation.

The presence or absence of an endothelial NO system in fish and amphibians is controversial. A study by Fritsche et al. (2000) provided evidence for the presence of an endothelial NO system in developing zebrafish. They showed that the diameter of the dorsal artery and vein is decreased by L-NAME, which indicates that there is a basal release of NO that is contributing to vascular tone. In addition, they showed the presence of eNOS immunoreactivity using an antibody to mammalian eNOS. Further evidence for an endothelial NO system in fish was proposed by Mustafa and Agnisola (1998), who showed that NO production and L-arginine-mediated vasodilation in the trout coronary vasculature were endothelium dependent. However, several studies in fish could not demonstrate the presence of an endothelial nitric oxide system (Olson and Villa, 1991; Evans and Gunderson, 1998; Kågström and Holmgren, 1997). In fact, these studies provide evidence for a prostaglandin as the mediator of endothelium-dependent vasodilation in fish. In amphibians, the present study has shown that eNOS is absent in the aortae of the toad and that acetylcholine-dependent vasodilation is mediated by nNOS. The fact that the findings of the current study differ from that of Knight and Burnstock (1996) in *R. pipiens* cannot be explained and is unlikely to be as a result of phylogeny. The present study provides a more comprehensive investigation into vascular NO regulation because it provides both physiological and anatomical evidence for an absence of an eNOS system. Further studies are required to characterise the mechanisms by which NO contributes to the vascular regulation of non-mammalian vertebrates.

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