British Journal of Pharmacology (1996) 118, 1518–1522

The involvement of the endothelium in the relaxation of the leopard frog (*Rana pipiens*) aorta in response to acetylcholine

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1 The vasodilator response to acetylcholine (ACh) was investigated in the aortic arches of the leopard frog (*Rana pipiens*).

2 With adrenaline pre-constricted preparations, both ACh and sodium nitroprusside (SNP) caused concentration-dependent relaxations. Damage to the endothelial layer abolished relaxations to ACh, or reduced them greatly, but had no effect on vasodilatation to SNP.

3 N^G-Nitro-L-arginine methyl ester (L-NAME; 1–100 μM) concentration-dependently inhibited relaxations in response to ACh, but had no effect on the ability of SNP to induce vasodilatation.

4 L-Arginine (L-Arg; 100–200 times the concentration of L-NAME) failed to reverse the inhibitory effect of L-NAME (1–100 μM) apart from one isolated instance.

5 In summary, this study has shown endothelium-dependent vasodilatation to ACh in an amphibian blood vessel that appears to be mediated via nitric oxide (NO). The response to ACh differs from many mammalian preparations in that the inhibitory effect of L-NAME could not be overcome by L-Arg, in addition to L-NAME itself having no direct effect upon the tone of the vessel.

Keywords: Aorta; nitric oxide; vasodilatation; N^G-nitro-L-arginine methyl ester; sodium nitroprusside; frog

Introduction

Over the past decade, considerable information has become available concerning the role of the endothelium in maintaining vascular tone. The initial observations by Furchgott & Zawadzki (1980) were followed by further investigations into the nature of the 'endothelium-dependent relaxing factor' (EDRF) which initiates vasodilatation after stimulation of muscarinic receptors located on the endothelium; EDRF has subsequently been identified as nitric oxide (NO) (Ignarro *et al.*, 1987a, b; Palmer *et al.*, 1987).

NO has since been found to mediate vasodilatation in many mammalian vascular preparations (for reviews see Ignarro, 1989; Moncada *et al.*, 1991), initiated by several agents in addition to acetylcholine (ACh), including adenosine 5′-triphosphate, 5-hydroxytryptamine, (5-HT), substance P and bradykinin, as well as stimuli such as hypoxia and sheer stress (see Ralevic & Burnstock, 1993).

While there are many examples of NO-mediated vasodilatation in mammalian vascular preparations, there is less information available about the action of ACh in lower vertebrate groups and whether its activity is mediated via NO. ACh and 'dissolved' NO (as described by Palmer *et al.*, 1987) have been seen to cause vasodilatation in the aorta of some lower vertebrate species including the cayman (a reptile) and rooster (a bird) Miller & Vanhoutte, 1986; 1992) that was dependent upon an intact endothelium; ACh has also been seen to cause endothelium-dependent vasodilatation in the aorta of domestic fowl (Yamaguchi & Nishimura, 1988) and garter snake (Knight & Burnstock, 1993). In addition to the perfused feet of ducks and chickens (McGregor, 1979), chick jugular vein (Yonezawa & Watanabe, 1982) and the coronary artery of the mako shark and skate (Farrell & Davie, 1991a, b). However, a vasoconstrictor action of ACh in amphibian blood vessels has been demonstrated; the perfused systemic blood vessels of frogs (*Rana tigrina*), cannulated via one of the aortic arches, were found to constrict to ACh (Gambhir & Das, 1970; 1978), as does the pulmonary vascular bed of the toad (*Bufo marinus*) (Davies & Campbell, 1988). Reite (1969) obtained a dual action of ACh on the systemic vasculature of frogs and toads, a weak dilator response followed by a constriction. Vasoconstriction to ACh has also been noted in other lower vertebrates, including the lizard aorta (Kirby & Burnstock, 1969a; Ogundahunsi & Tayo, 1988; Wright & Hurn, 1993), coronary artery of the steelhead and rainbow trout (Small & Farrell, 1990; Small *et al.*, 1990), branchial artery of the shark (*Negaprion queenslandicus*) (Bennett, 1993) and splenic artery of two species of dogfish (*Scyliorhinus canicula* and *Squalus acanthias*) (Nilsson *et al.*, 1975).

In the present study the effect of ACh on the aorta of the leopard frog (*Rana pipiens*) has been examined. In addition to observing the response of the aorta to ACh with and without endothelium, the effect of the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) was investigated against the vasodilatation to ACh (Rees *et al.*, 1989; 1990) and, also, the effect of L-arginine (L-Arg), which has been shown to restore NO activity in many mammalian vessels after inhibition by L-NAME (Palmer *et al.*, 1988a, b; Sakuma *et al.*, 1988), was studied.

Methods

General procedures

Leopard frogs (*Rana pipiens*) of either sex, supplied by Blades Biological, Cowden, Edenbridge, Kent, were stunned, decapitated and pithed. The brain was destroyed by a metal seeker. Both aortic arches were rapidly removed and placed in a physiological saline solution. Ring segments of approximately 5–6 mm were dissected free of adhering tissue and mounted in 10 ml organ baths containing continuously gassed (95% O_2/5% CO_2) saline of the following composition (mM): NaCl 111.1, KCl 1.88, NaH_2PO_4 0.08, $NaHCO_3$ 2.35, $CaCl_2$ 1.08 and glucose 1.11. Experiments were carried out at room temperature, 23 ± 1.0°C.

Segments were mounted horizontally in the organ bath by inserting 2 tungsten wires through the lumen of the aorta, one

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wire attached to a rigid support and the other to a Grass force displacement transducer FT03C. Mechanical activity was displayed on a Grass ink-writing oscillograph. An initial load of 0.75–1 g was applied and the tissues were allowed to equilibrate for 1 h. In those vessels to be tested without the endothelial layer, the endothelium was removed by rotating the vessel on a tungsten wire.

Concentration-response curves

Cumulative concentration-response curves were constructed for adrenaline in order to obtain an EC_{50} value for each preparation. Adrenaline is known to be the principle transmitter in sympathetic nerves in amphibians (see Burnstock, 1969). Amphibian aorta has also been shown to be devoid of inhibitory β-receptors (Burnstock & Kirby, 1968). The EC_{50} concentration of adrenaline was used to constrict the vessel in order to observe relaxations to ACh and sodium nitroprusside (SNP) in the absence and presence of L-NAME (1–100 μM) incubated for 20 min and, finally, in the presence of L-NAME together with L-Arg (100–200 times the concentration of L-NAME) again incubated for 20 min.

Drugs used

Adrenaline, acetylcholine chloride, sodium nitroprusside, L-arginine, N^G-nitro-L-arginine methyl ester and ascorbic acid were all supplied by Sigma Chemical Co. Adrenaline was dissolved in 100 μM ascorbic acid, all other drugs were dissolved in distilled water.

Statistical analysis

Vasodilator responses are expressed as mean % relaxation of the adrenaline contraction (EC_{50} concentration) ± s.e. of n observations (shown in parentheses).

Statistical significance was tested by paired Student's t test. A probability of $P<0.05$ was taken as significant.

Results

Adrenaline caused concentration-dependent contractions of the frog aorta (Figure 1a). The mean maximum tension developed by adrenaline was 443.84±28.35 mg (43). The EC_{50} concentration was obtained for each preparation and this was used to pe-constrict the vessel. The mean pD_2 value (−log of the EC_{50}) for adrenaline was 6.34±0.06 (23).

ACh concentration-dependently relaxed preparations of the frog aorta that possessed an intact endothelium (Figure 1b), the mean pD_2 value for ACh being 7.17±0.07 (15).

SNP concentration-dependently relaxed the frog aorta in the absence and presence of the endothelium (Figure 1c). There was no significant difference in the mean pD_2 values for SNP being 7.19±0.08 (12) in the presence and 7.31±0.13 (7) in the absence of the endothelium.

Effect of removal of the endothelium

In those preparations where the endothelium had been damaged by a tungsten wire, vasodilatations to ACh were very much reduced or entirely absent. The integrity of the endothelial layer was investigated by scanning electron microscopy (methods and data not shown), which revealed that absence of vasodilatation toACh corresponded to at least 60% damage of the endothelium.

Effect of L-NAME on ACh relaxations

L-NAME (1–100 μM) concentration-dependently inhibited relaxations to ACh in aorta preparations with an intact endothelium, such that mean maximum relaxations to ACh in the absence and presence of the NO-synthase inhibitor respectively were: L-NAME 1 μM, 78.2±5.7 (5) and 74.5±11.0 (5) (Figure 2a); L-NAME 10 μM, 83.8±6.0 (7) and 50.5±9.3 (7) (Figure 2b) and L-NAME 100 μM, 83.7±5.7 (6) and 6.0±2.6 (6) (Figure 2c). pD_2 values were also significantly ($P<0.05$) altered in the presence of L-NAME; 7.18±0.08 (5) in the absence and 7.32±0.03 (5) in the presence of L-NAME 1 μM, and 7.13±0.08 (7) in the absence and 6.86±0.17 (7) in the presence of L-NAME 10 μM. A pD_2 value for ACh in the presence of 100 μM L-NAME could not be accurately calculated as there was total inhibition of the response to ACh in some preparations.

Application of L-NAME itself had no significant ($P<0.05$) effect on the tone of the vessel or on the constriction to adrenaline. Mean constrictions to adrenaline in the absence and presence of L-NAME were: 271.0±15.84 mg (5) and 272.0±10.68 mg, L-NAME 1 μM; 309.26±34.16 mg (7) and 307.14±32.03 mg (7), L-NAME 10 μM; 266.67±20.76 mg (6) and 241.67±39.62 mg (6), L-NAME 100 μM.

Effect of L-arginine

Concentration-response curves to ACh were repeated in the presence of L-NAME (1–100 μM) together with L-Arg (100–200 times the concentration of L-NAME). However, in only 1 preparation was any reversal of the effect of L-NAME seen. In this preparation, the maximum vasodilatation to ACh in the presence of 100 μM L-NAME was 5.6%, this increased to 7.7% in the presence of 100 μM L-NAME plus 10 mM L-Arg. In all other preparations, L-Arg failed to reverse the effect of L-NAME even when L-Arg was used at a concentration of 200 times that of L-NAME.

Effect of L-NAME on SNP relaxations

SNP concentration-dependently relaxed aortic preparations of the frog in the absence and presence of the endothelium.

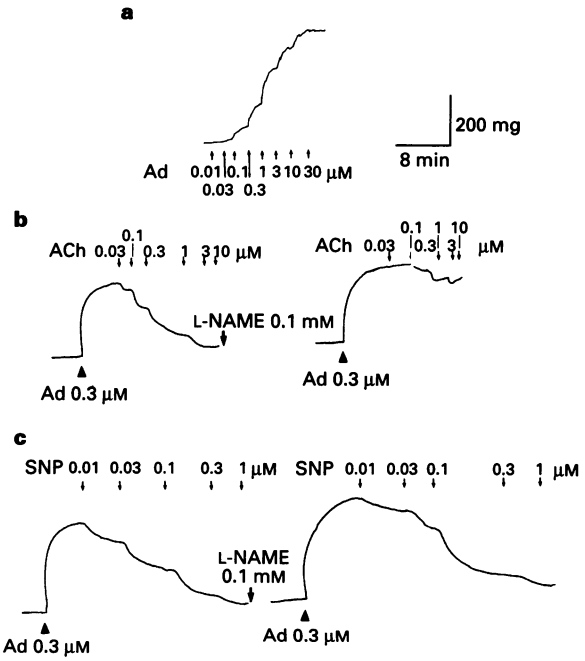

Figure 1 Examples of original tracing of the action of adrenaline (Ad), acetylcholine (ACh) and sodium nitroprusside (SNP) on isolated segments of frog aorta. (a) Cumulative concentration-response relationship to adrenaline. (b) Cumulative concentration-response relationship to ACh on adrenaline pre-constricted (EC_{50} concentration) aorta in the absence and presence of L-NAME (0.1 mM) incubated for 20 min. (c) Cumulative concentration-response relationship to SNP on adrenaline pre-constricted aorta in the absence and presence of L-NAME (0.1 mM) incubated for 20 min.

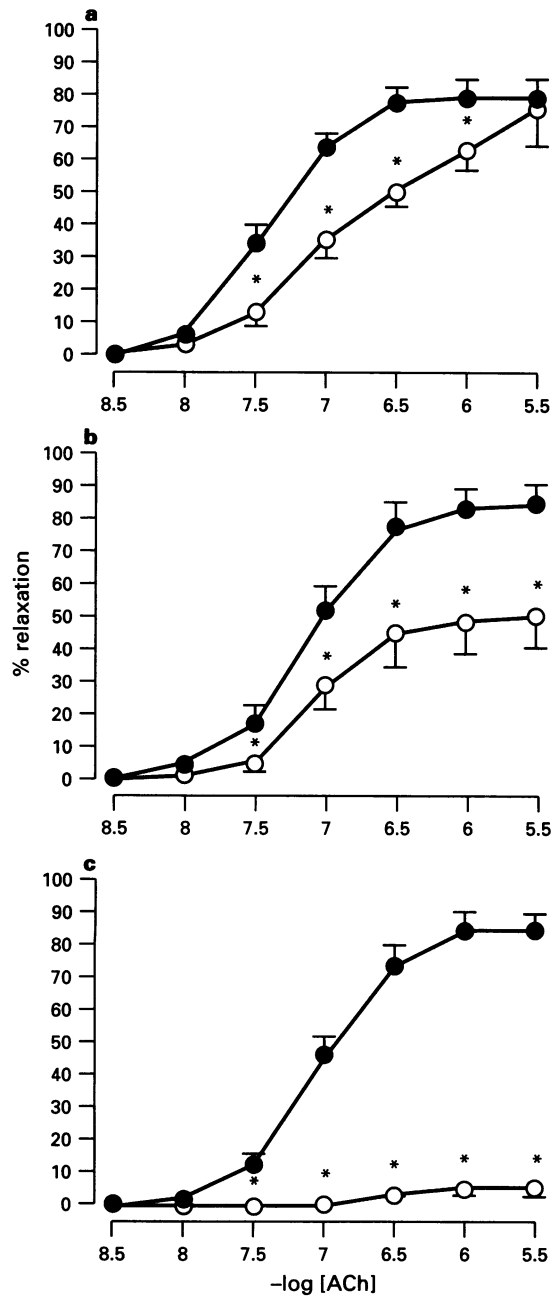

Figure 2 Cumulative concentration-response curve to acetylcholine (ACh) in the frog aorta, on adrenaline (EC_{50} concentration) pre-constricted vessels in the absence and presence of L-NAME incubated for 20 min. (a) Relaxant effects of ACh in the absence (●; $n=5$) and presence (○; $n=5$) of L-NAME (1 μM). (b) Relaxant effect of ACh in the absence (●; $n=7$) and presence (○; $n=7$) of L-NAME (10 μM). (c) Relaxant effect of ACh in the absence (●; $n=6$) and presence (○; $n=6$) of L-NAME (100 μM). All symbols represent mean % relaxation ± s.e. (unless masked by the symbol). *Indicates significance, $P<0.05$.

L-NAME (up to 100 μM) did not affect the ability of SNP to relax the aorta. In the absence and presence of L-NAME (100 μM) pD_2 values were 7.18 ± 0.08 (6) and 7.32 ± 0.03 (6) (Figure 3) and mean maximum relaxations were consistently 100%.

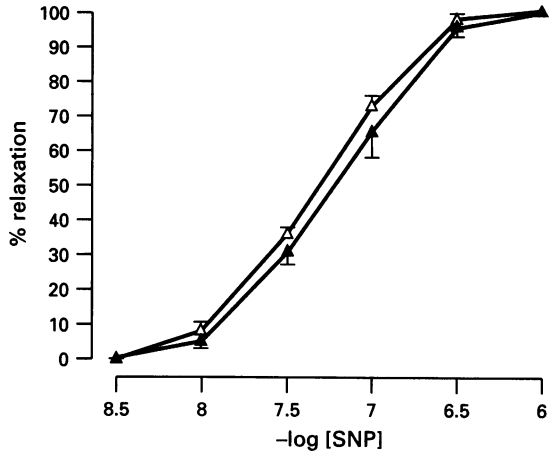

Figure 3 Cumulative concentration-response curve to sodium nitroprusside (SNP) in the frog aorta, on adrenaline pre-constricted (EC_{50} concentration) vessels, in the absence (▲; $n=6$) and presence (△ $n=6$) of L-NAME (100 μM) incubated for 20 min. Symbols represent mean % relaxation ± s.e. (unless masked by the symbol).

Discussion

The aim of the present study was to examine the effect of ACh on the aorta of the amphibian *Rana pipiens*, and if vasodilatation was observed, to determine whether the response was mediated via the endothelium. The results indicate that this is the case; ACh initiated endothelium- and concentration-dependent vasodilatations that were inhibited by the NO-synthase inhibitor L-NAME which would seem to suggest that NO was mediating the dilatation, while vasodilatations to the endothelium-independent dilator SNP were not affected.

There are several derivatives of L-Arg that have been used to inhibit the activity of the enzyme NO-synthase, responsible for the conversion of L-Arg to NO (see Moncada, 1992) and as a result inhibiting endothelial-mediated vasodilatation (Palmer *et al.*, 1988b; Moore *et al.*, 1990; Rees *et al.*, 1990). In many mammalian preparations, these inhibitors have provided evidence for the role of NO in the maintenance of vascular tone. It is generally accepted that L-NAME is more potent than the original analogues of L-Arg found to inhibit endothelial-mediated vasodilatation (Moore *et al.*, 1990; Rees *et al.*, 1990). The potency of L-NAME in mammalian preparations is comparable with data from this study. For example, 100 μM L-NAME completely abolishes vasodilatation to ACh in the rabbit isolated perfused ear, whereas 10 μM causes a significant shift to the right and depression of the maximum response by approximately 60% (Randall & Griffith, 1991). This is in agreement with the potency of L-NAME on the frog aorta and also on the garter snake aorta (Knight & Burnstock, 1993), where a similar concentration of L-NAME almost completely inhibited dilatation to ACh, and a lower concentration caused a shift to the right of the concentration-response curve with a significant reduction in the maximum response. In the rat aorta, L-NAME is more potent than in the frog aorta or the rabbit ear artery, with 10 μM causing an almost complete inhibition of the responses to ACh, although the inhibition pattern of L-NAME resembles that found in the frog and snake aorta, in that there is a concurrent significant reduction of the maximum response as the concentration of L-NAME is increased (Rees *et al.*, 1990). This is true of other inhibitors of NO synthase such as N^G-nitro-L-arginine (L-NOARG) (Moore *et al.*, 1990) and N^G-monomethyl-L-arginine (Palmer *et al.*, 1988b).

While the application of these inhibitors may have a direct action on their own, raising the tone of the vessel by blocking any basal release of NO, this was not found to occur in the aorta of the frog, suggesting that there was no basal release of NO, unlike that of the garter snake (Knight & Burnstock, 1993) and many mammalian vascular preparations (Amezuca *et al.*, 1989; Rees *et al.*, 1989, 1990; Jackson *et al.*, 1991; Lamontagne *et al.*, 1991; Randall & Griffith, 1991).

The necessity of an intact endothelial layer in order to see

vasodilatation in response to NO was first shown by Furchgott & Zawadzki (1980) and later applied to other vasoactive substances such as bradykinin and substance P (Moncada *et al.*, 1991) and 5-HT (Cocks & Angus, 1983; Cohen *et al.*, 1983). The confused state of the early literature as to the effect of ACh on vascular preparations from a number of lower vertebrates may reflect variable damage to the endothelial cells during the preparatory procedure.

There are few studies that have actually shown a role for NO in the control of lower vertebrate vascular systems, these being limited to the rooster, chicken, frog and cayman aorta (Miller & Vanhoutte, 1986, 1992; Hasegawa & Nishimura, 1991) and capillaries of the frog sartorius muscle (Ferguson *et al.*, 1994). An endothelial-dependent effect of ACh has been demonstrated, where removal of the endothelium abolishes the vasodilator activity of ACh in chicken aortae (Yamaguchi & Nishimura, 1988; Hasegawa & Nishimura, 1991). Studies showing a vasoconstrictor response to ACh in lower vertebrate preparations such as fish (Holmgren & Nilsson, 1974; Klaverkamp & Dyer, 1974; Nilsson *et al.*, 1975; Bennett, 1993), some reptiles (Kirby & Burnstock, 1969a, b; Reite, 1970; Burggren, 1977; Ogundahunsi & Tayo, 1988) and amphibians (Gambhir & Das, 1970, 1978; Ishimatsu *et al.*, 1986) may reflect variable damage to the endothelial cells during the preparatory procedure, rather than an absence of endothelial-mediated vasodilatation. Although NO *per se* has not been studied on the frog aorta, the selectivity of the enzyme inhibitor would strongly suggest that ACh mediates its effect via NO.

The lack of ability of L-Arg to reverse the inhibition of ACh-mediated vasodilatation seems to suggest that in this preparation L-NAME is a highly effective blocker of NO-synthase, even increasing the concentration of L-Arg to 200 times the L-NAME concentration did not facilitate the reversal of the effect of L-NAME. This may reflect a difference in the uptake mechanism of L-Arg in this particular preparation or in the mode of action of L-NAME on the NO synthesizing enzymes. There are instances in the literature where the reversing effect of L-Arg varies between inhibitors and also preparations. For example, in the rabbit aorta the partial inhibition of ACh caused by L-NOARG could only be reversed by addition of excess of 150 μM L-Arg (Moore *et al.*, 1990), whereas in the rat aorta the effect of 100 μM L-NOARG was irreversible by L-Arg (Unmans, 1990) as was the hypertensive effect of L-NOARG in the anaesthetized guinea-pig (Steinberg *et al.*, 1990).

In summary, the present study has shown that ACh dilates the aorta of the frog via the endothelium, there being evidence that this is via the production of NO. This process could be blocked by L-NAME. This amphibian vessel differs from many mammalian preparations in that the inhibitory effect of L-NAME could not be significantly overcome by the addition of excess L-Arg.

The authors are very grateful to Mr M. Turmaine for electron microscopy, for the advice of Dr C.H.V. Hoyle during the preparation of this manuscript, and to Dr D. Christie for invaluable editorial assistance.

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(Received January 2, 1996
Revised March 11, 1996,
Accepted April 3, 1996)