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Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence

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1 Isolated rings of rabbit external jugular vein (RbJV) and rat thoracic aorta (RA) were used to study the effect of the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) on muscarinic and 5-hydroxytryptamine (5-HT) receptor-stimulated, endothelium-dependent vascular relaxations.

2 In RbJV relaxations produced by the endothelial 5-HT receptor agonist α-methyl-5-HT were potently and non-surmountably inhibited by L-NAME (10 μM), whereas acetylcholine relaxations in this tissue were unaffected by this concentration of inhibitor. By contrast, acetylcholine relaxations in RA were virtually abolished by 10 μM L-NAME. In each case an equivalent concentration of D-NAME was without effect on agonist-induced relaxations.

3 The different effect of L-NAME on acetylcholine relaxations in RbJV and RA was not due to muscarinic receptor differences. Affinity estimates for acetylcholine ($pK_A = 6.12 \pm 0.09$; 6.09 ± 0.08 respectively) and for 4-diphenyl-acetoxy-N-methylpiperidine methobromide (4-DAMP, $pK_B = 9.01 \pm 0.12$; 9.24 ± 0.16 respectively) indicated that the receptors in both tissues belong to the same M_3 class. Tissue differences resulting from the release of a cyclo-oxygenase product or a glibenclamide-sensitive K^+-channel-linked hyperpolarizing factor were also ruled out by selective inhibition of these pathways.

4 When phenoxybenzamine was used to reduce the efficacy of acetylcholine in RbJV so that it behaved as a partial agonist in this tissue, L-NAME (10 μM) now produced non-surmountable inhibition of relaxation responses. In untreated tissues the same concentration of L-NAME also profoundly inhibited responses produced by butyrylcholine and pilocarpine, both of which behave as partial agonists at the M_3 receptor in RbJV.

5 A simple model was developed which describes the theoretical behaviour of receptor-stimulated synthesis and release of NO. The model predicts that competitive inhibition of NO formation results in parallel displacements of the agonist response curve in the case of high efficacy agonist, but right-shift with concomitant depression of the curve maximum in the case of low efficacy agonists. Simulations based on the model showed reasonable agreement with the experimental data.

6 It is concluded that analogues of L-arginine demonstrate tissue- and agonist-dependence in terms of their ability to inhibit receptor-mediated events involving the liberation of NO. This behaviour can reflect differences in agonist efficacy in the receptor systems being studied, a possibility that should be ruled out before apparent resistance to inhibition is taken as evidence for the involvement of heterogeneous endothelium-derived relaxing factors (EDRFs).

Keywords: Endothelium-derived relaxing factor (EDRF); L-N^G-nitroarginine methyl ester (L-NAME); 5-HT receptors; muscarinic receptors; vasorelaxation

Introduction

A number of pharmacological agents indirectly mediate vascular relaxation by stimulating the release from endothelial cells of a short-lived humoral substance which Furchgott & Zawadski (1980) termed endothelium-derived relaxing factor (EDRF). Numerous studies have subsequently shown that the biological effects of EDRF are consistent with it being nitric oxide (NO: Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987), possibly incorporated into one, or several different nitrosothiols (Myers *et al.*, 1990). The source of endothelial cell NO appears to be the terminal guanidino nitrogen atom(s) of the amino-acid L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) and a growing body of evidence suggests that the biochemical pathway involved in its liberation is similar in a variety of other cell types (see Moncada *et al.*, 1989). Support for this comes from the ability of a number of arginine analogues to inhibit stereoselectively the formation of NO and/or its biological effects. In this regard analogues such as L-N^G-monomethyl arginine (L-NMMA), L-N^G-nitroarginine methylester (L-NAME) and more recently L-iminoethyl-L-ornithine (L-NIO) are being used increasingly as probes with which to investigate the possible physiological and pathological roles of NO (see Rees *et al.*, 1990).

In our laboratory we have been using L-NAME to study the characteristics of receptor-stimulated, endothelium-dependent relaxations in rings of rabbit jugular vein (RbJV) and rat aorta (RA). The endothelium of RbJV possesses 5-hydroxytryptamine (5-HT) receptors effecting vascular relaxation indirectly (Leff *et al.*, 1987; Martin *et al.*, 1987) and, as shown here, endothelial muscarinic receptors are also present in this tissue. The endothelium of RA likewise possesses muscarinic receptors (Choo *et al.*, 1986) but is devoid of receptors for 5-HT (this study). To our surprise we found that in RbJV, endothelium-dependent relaxations elicited by the 5-HT receptor agonist α-methyl-5-HT (α-Me-5-HT) were effectively inhibited by L-NAME whereas the effects of acetylcholine (ACh) were not. On the other hand, ACh relaxations obtained in RA were virtually abolished in the presence of L-NAME.

In view of recent reports implying that different receptor systems stimulate the release of different EDRFs (Hoeffner *et al.*, 1989; Boulanger *et al.*, 1989) we sought to ascertain whether or not this might also be the case for endothelial

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5-HT and muscarinic receptors. This prompted a series of experiments which led us to consider and then reject the data as evidence for heterogeneous EDRFs. Instead an alternative possibility was explored, namely that the agonist- and tissue-dependence of inhibition by arginine analogues might result from differences in the efficiency of occupancy-effect coupling for each receptor system studied. The good agreement between experimental data and predictions based upon a simple theoretical model support this premise. Here we describe the results of these studies and consider their implications for the use of inhibitors in the study of biological events mediated by the receptor-stimulated release of NO.

A preliminary account of these findings was presented to the Spring meeting of the British Pharmacological Society (Giles *et al.*, 1990).

Methods

Tissue preparation

Right and left external jugular veins were removed from male New Zealand White rabbits (2.4–3.0 kg) treated with heparin (200 units, i.v.) and then killed by injecting pentobarbitone sodium (Sagatal; 120 mg kg^{-1}) into a marginal ear vein. After removal of adhering connective tissue, three ring segments 5 mm wide were prepared from each vessel. Rings of thoracic aorta (3 mm) were obtained from male Wistar rats (350–425 g) killed by a blow to the head. As above, each animal provided six ring preparations. In both cases, care was taken to preserve the endothelium.

Rings of RbJV or RA were suspended in 20 ml organ baths between tungsten wire hooks (0.5 mm), one attached to a Grass FT03C force displacement transducer and the other to a stationary support. The bathing medium was Krebs solution of the following composition (mM); NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10 and $CaCl_2$ 2.50. This was maintained at 37 ± 0.5°C, pH 7.4 ± 0.1 and continually aerated with 95% O_2:5% CO_2. Responses were measured as changes in tissue isometric force and recorded on Gould BS272 pen recorders.

Protocols

At the beginning of each experiment vascular rings were subject to a force of either 0.75 g (RbJV) or 1.0 g (RA) and subsequently allowed to stabilize for 60 min. During this period the organ bath buffer was replaced three times and the force reinstated. Tissues were then contracted either with phenylephrine (0.1–1 μM: RA) or the thromboxane A_2-mimetic, U-46619 (10 nM: RbJV). In both cases the concentration of spasmogen used produced 60–70% of the maximum response, the required concentration of phenylephrine being determined in each tissue by first constructing a cumulative concentration-effect (E/[A]) curve. At steady-state contracture ACh (1 μM: RbJV; 10 μM: RA) or α-Me-5-HT (1 μM: RbJV only) was added to check viability of the endothelium. Preparations which failed to relax by more than 50% of the induced tone were discarded. In tissues denuded of endothelium, ACh and α-Me-5-HT relaxations were abolished (see also Leff *et al.*, 1987).

Endothelium-dependent responses to agonists

Experiments using RbJV followed a multiple curve design (pairs or triplets) whereas in RA only a single curve per preparation was obtained. Separate experiments in RbJV demonstrated that first, second and third relaxation curves were superimposable, but data from a particular tissue were rejected if the successive contractures produced by U-46619 (10 nM) differed by more than 10%. In both types of experiment, E/[A] curves were cumulative, agonist concentrations increasing in 0.5 $\log_{10}$ increments.

Affinity estimates using irreversible receptor alkylation

Muscarinic receptor agonist affinities were determined by Furchgott's (1966) method of partial irreversible receptor occlusion. In these experiments a fraction of the total functional muscarinic receptor pool was inactivated with phenoxybenzamine (0.1–10 μM, 30 min). U-46619 was the spasmogen used in both RA (20 nM) and RbJV (10 nM). In multiple curve studies the first curve was always to ACh; second and third curves to either ACh, pilocarpine or butyrylcholine followed after complete washout of the previous agonist additions and a recovery period of 60 min. Phenoxybenzamine was added between first and second curves (ACh) or between second and third curves (pilocarpine, butyrylcholine).

Studies with L-N^G-nitroarginine methyl ester

Studies with α-Me-5-HT followed a paired curve design. The effects of 10 μM and 100 μM L-NAME (30 min) on muscarinic agonist curves were studied between first and second curves and second and third curves respectively, except when ACh curves were obtained after phenoxybenzamine treatment. In these studies phenoxybenzamine was added between first and second curves and the effects of only a single L-NAME concentration (10 μM or 100 μM) studied between second and third curves. Control experiments with ACh indicated that there was a recovery from receptor alkylation between second and third curves, consistent with a 1.98 fold increase in agonist efficacy (average increase from 8 experiments). This factor was used to correct third curves to ACh obtained in the presence of L-NAME.

Experiments with glibenclamide (10 μM, 60 min) and indomethacin (2.8 μM, 60 min) required only paired curves.

Studies with 4-diphenyl-acetoxy-N-methylpiperidine methobromide (4-DAMP)

Following washout of the first ACh challenge to establish viability of the endothelium, tissues were exposed to 4-DAMP (0.001–1.0 μM) for 60 min before recontracture and construction of an agonist cumulative E/[A] curve.

Data analysis

E/[A] curve data were routinely fitted to a logistic function of the form;

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

which provided estimates of the asymptote (α), mid-point ($p[A_{50}]$) and slope parameter (m). In paired curve experiments parameter estimates were compared using a paired t test. When, in antagonist studies, α and m were not different, $\Delta p[A_{50}]$ values at each antagonist concentration were analysed using the Schild regression (Arunlakshana & Schild, 1959). If the Schild plot slope was not different from unity it was constrained to this value in order to estimate pK_B. When antagonists were studied using a single curve per preparation design a one-way analysis of variance compared computed estimates of α and m within and between treatment groups. If the antagonist produced parallel curve displacements with common asymptotes, suggesting competitive antagonism, computed $p[A_{50}]$ values were fitted directly to the following form of the Schild equation (Black *et al.*, 1985a; Trist & Leff, 1985);

$$\log_{10}[A_{50}] = \log_{10}[A^C_{50}] + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

where $[A^C_{50}]$ is a control curve $[A_{50}]$ value, [B] is the concentration of antagonist, K_B its dissociation constant and n a value equivalent to the slope of the Schild regression. If n was not different from unity it was constrained to this value in order to estimate K_B.

Estimates of agonist affinity and efficacy were derived using direct operational model-fitting methods (Black & Leff, 1983; Leff *et al.*, 1990). Agonist E/[A] curve data obtained before and after fractional receptor inactivation using phenoxybenzamine were fitted directly to the expression;

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (3)$$

in which K_A is the agonist dissociation constant, τ is the efficacy of the agonist in a particular tissue, E_m is the maximum possible effect in the receptor system and n defines the slope parameter of the occupancy-effect relation. The different statistical treatments which apply to single as opposed to multiple curve/preparation studies have been discussed in detail elsewhere (Leff *et al.*, 1990).

Drugs

Acetylcholine hydrochloride (Research Biochemicals Inc.); pilocarpine hydrochloride, butyrylcholine hydrochloride, phenylephrine hydrochloride, histamine acid phosphate, glibenclamide and N^G-nitro-L-arginine methyl ester (Sigma Chemical Co. Ltd.); 9,11-dideoxy-9α,11α-methanoepoxy-$PGF_1\alpha$ (U-46619: Cayman Chemical); glyceryl trinitrate (Wellcome Research Laboratories); 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP: a generous gift from Dr R.B. Barlow, University of Bristol); phenoxybenzamine hydrochloride (Smith, Kline & French). N^G-nitro-D-arginine methyl ester and (±)-α-methyl-5-hydroxytryptamine creatinine sulphate were synthesized respectively by Drs H.F. Hodson and A.D. Robertson, Medicinal Chemistry Department, Wellcome Research Laboratories.

U-46619 and phenoxybenzamine were dissolved at 2 mM in absolute ethanol and subsequently diluted in distilled water to give a final concentration in the organ bath of ≤0.05% v/v. Tissue responsiveness was unchanged by the vehicle. All other drugs were dissolved in distilled water.

Results

Effects of D- and L-N^G-nitroarginine methyl ester in rat aorta and rabbit jugular vein

Endothelium-dependent relaxations to ACh or α-Me-5-HT were obtained in vascular ring preparations contracted with a submaximally effective concentration (~$p[A_{60-70}]$) of either U-46619 (10 nM: RbJV) or phenylephrine (0.1–1 μM: RA). ACh was 20 times more potent in RbJV ($p[A_{50}] = 8.58 \pm 0.11$) than in RA ($p[A_{50}] = 7.27 \pm 0.28$) (Figure 1a,b). α-Me-5-HT also caused potent endothelium-dependent relaxations in RbJV ($p[A_{50}] = 8.18 \pm 0.18$) (Figure 1c) but was devoid of endothelium-dependent activity in RA.

Prior exposure of rat aortic rings to L-NAME resulted in a graded inhibition of endothelium-dependent responses to ACh, 10 μM of the inhibitor causing almost complete abolition of relaxations (Figure 1b). D-NAME (10 μM) was without effect on ACh responses in this tissue ($\Delta p[A_{50}] = 0.07 \pm 0.21$, $\Delta max = -4 \pm 4\%$; $n = 4$). Essentially the same results were obtained when U-46619 (20 nM) was the spasmogen (data not shown). By comparison, L-NAME was much less effective against ACh responses in RbJV. Figure 1a shows that 10 μM inhibitor was without significant effect on ACh E/[A] curves, although 100 μM L-NAME caused an eight fold displacement of the curve and depressed the maximum by 18%. In contrast, α-Me-5-HT relaxations in RbJV were effectively inhibited by L-NAME (10–100 μM) (Figure 1c) showing that the inhibitor demonstrates agonist- as well as tissue-differences. As in RA responses to both agonists were unaffected by D-NAME (100 μM) (vs ACh $\Delta p[A_{50}] = 0.03 \pm 0.04$, $\Delta max = 3 \pm 4\%$; $n = 4$: vs α-Me-5-HT $\Delta p[A_{50}] - 0.03 \pm 0.05$, $\Delta max = 3 \pm 4\%$; $n = 3$).

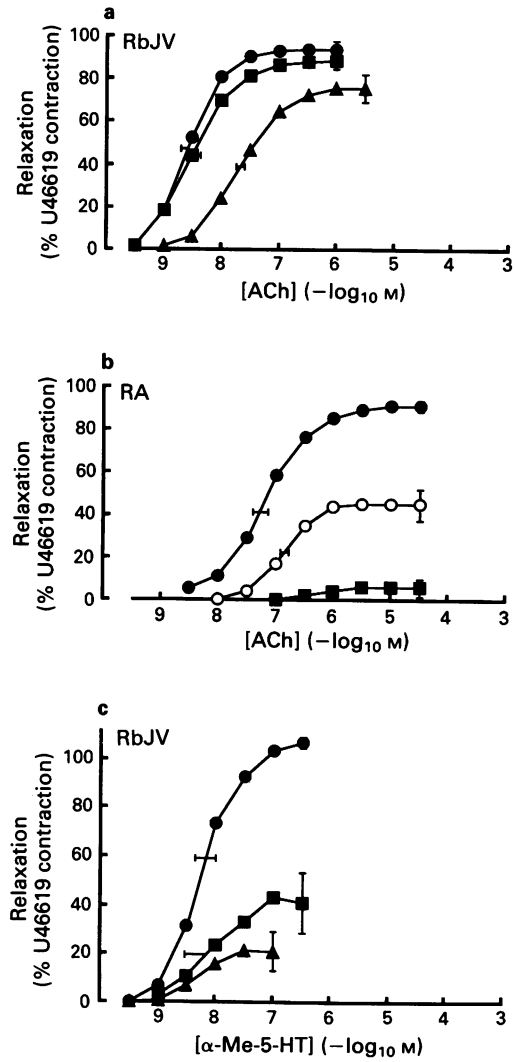

Figure 1 Differential effects of L-N^G-nitroarginine methyl ester (L-NAME, ● 0, ○ 1, ■ 10, ▲ 100 μM) on endothelium-dependent relaxations to acetylcholine (ACh) in rings of RbJV (a) and RA (b) and to α-methyl-5-hydroxytryptamine (α-Me-5-HT) in RbJV (c). Data are the averages of 3–6 E/[A] curves in each case. For clarity s.e.mean, denoted by vertical bars, are shown on maximum responses only. Horizontal bars denote s.e.mean on the average computed $p[A_{50}]$ values.

In separate paired curve experiments E/[A] curves to U-46619 in RbJV were essentially unchanged in the presence of 100 μM L-NAME ($\Delta p[A_{50}] = -0.03 \pm 0.04$, $\Delta max = 13 \pm 4\%$, $n = 6$), consistent with a previous report showing that U-46619 curves are unaffected by endothelial denudation in this tissue (Leff *et al.*, 1987). In contrast, phenylephrine curves obtained in RA ($n = 5$) were potentiated ($\Delta p[A_{50}] = -0.51 \pm 0.09$) and augmented $\Delta max = 25 \pm 6\%$) by L-NAME (10 μM), whilst responses to U-46619 were simply potentiated ($\Delta p[A_{50}] = -0.08 \pm 0.03$, $n = 9$: $P = 0.03$). These results imply that the inhibition of basal NO release by L-NAME increases the tonic state of the phenylephrine- and U-46619-contracted RA but has little impact on the contraction induced by U-46619 in RbJV.

Muscarinic receptor characterization in rat aorta and rabbit jugular vein

Affinity estimates for the muscarinic receptor antagonist 4-DAMP and for ACh itself were obtained in order to establish the identity of the endothelial muscarinic receptors in each tissue. Figure 2 (a and b) shows that in both RA and

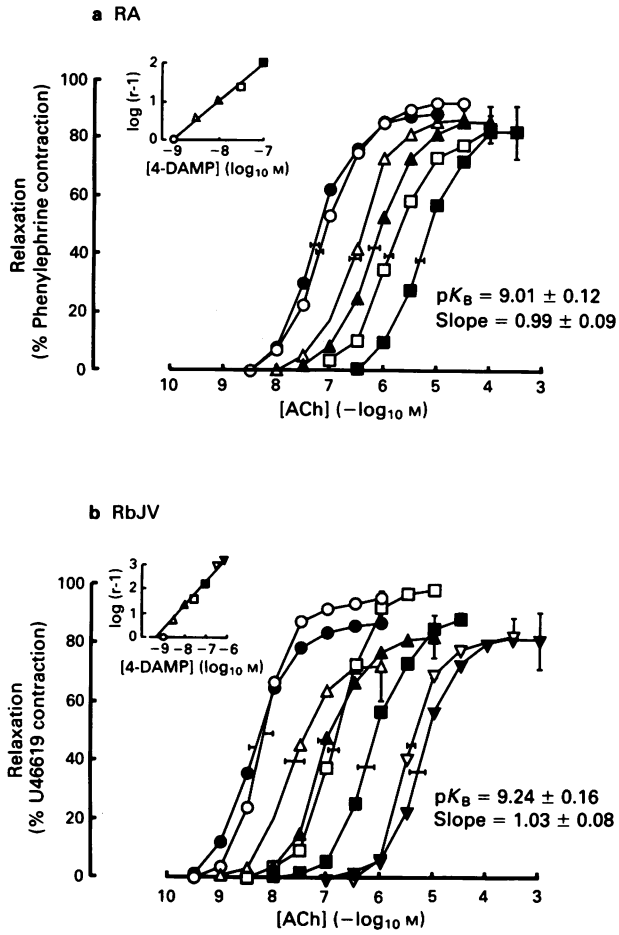

Figure 2 Antagonism of acetylcholine (ACh) relaxations by 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP, ● 0, ○ 1, △ 3, ▲ 10, □ 30, ■ 100, ▽ 300 and ▼ 1000 nM) in RA (a) and RbJV (b) respectively. Data are the averages of 3–5 E/[A] curves in each case and horizontal bars identify s.e.mean about their mean mid-point location. For clarity vertical bars denoting s.e.mean are shown on maximum values only. Inserts show Schild plots displaying the average log (r − 1) values obtained at each concentration of 4-DAMP in each tissue.

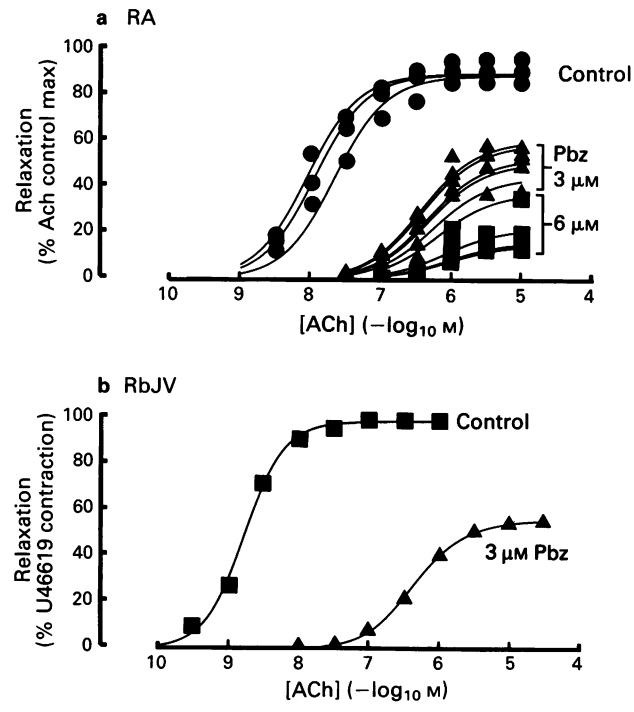

Figure 3 Estimation of acetylcholine (ACh) affinity (pK_A) at the muscarinic receptor in RA (a) and RbJV (b) by the method of fractional receptor inactivation. In (a) is shown a family of 12 ACh E/[A] curves, each obtained in a ring of RA in the absence or presence of phenoxybenzamine (Pbz); (b) shows a representative ACh curve pair (before and after Pbz treatment) from 11 such pairs obtained in separate rings of RbJV. Fitting these data to equation (3) provided the following parameter estimates in each tissue;
RA: $pK_A = 6.08 \pm 0.22$ (10 d.f.), $n = 1.19 \pm 0.25$, $E_m = 90.4 \pm 4.0$
RbJV: $pK_A = 6.12 \pm 0.09$ (10 d.f.), $n = 1.54 \pm 0.18$, $E_m = 93.0 \pm 3.0$
These parameter estimates, together with the estimates of τ (not shown), were used to produce the lines through the data.

RbJV 4-DAMP (0.003–1.0 μM) behaved as a simple competitive antagonist of ACh. Analysis of the data using equation (2) showed that the Schild plot slope was not different from unity (RA = 0.99 ± 0.09, 17 d.f.; RbJV = 1.03 ± 0.08, 29 d.f.) and that estimates of pK_B for 4-DAMP were not significantly different in RA (9.01 ± 0.12) and RbJV (9.24 ± 0.26). The concentrations of antagonist used had no effect on either U-46619 (10 nM) contractions in RbJV or on phenylephrine contractions in RA (ANOVA, $P > 0.05$).

Estimates of affinity (pK_A) for ACh were obtained following partial, irreversible receptor occlusion using phenoxybenzamine (Figure 3a,b). Concomitant incubation with 4-DAMP (0.1 μM) protected completely against the effects of phenoxybenzamine indicating that the latter simply inactivated muscarinic receptors and had no post-receptor actions. In RA a single E/[A] curve to ACh was obtained in each preparation after 30 min exposure to 0, 3 or 6 μM phenoxybenzamine and the resulting 'family' of twelve curves fitted directly to equation (3) yielding a pK_A of 6.08 ± 0.22 (10 d.f.). In RbJV, paired curves were obtained in each of eleven preparations before and after exposure to phenoxybenzamine (3 μM) so that model fitting provided six estimates of pK_A, the average of which was 6.12 ± 0.09, not different from that in RA.

These data indicate that the endothelial muscarinic receptors in RA and RbJV belong to the same class, the affinity estimates obtained being compatible with an M_3 classification. Moreover it is clear from the ratio $[A_{50}]/K_A$ that there exists a considerable 'reserve' in the system subserved by muscarinic receptors in RbJV, but not in RA.

Effects of glyceryl trinitrate, indomethacin and glibenclamide

Glyceryl trinitrate (GTN), like NO, activates the guanylyl cyclase transduction pathway in smooth muscle cells. Cumulative additions of GTN caused concentration-dependent relaxations of both RA and RbJV which completely reversed the U-46619 (10 nM) contracture in each case. Effects were expressed more potently in RbJV ($p[A_{50}] = 8.37 \pm 0.16$, $n = 6$) than in RA ($p[A_{50}] = 7.48 \pm 0.29$, $n = 6$) and in both cases were unaffected by L-NAME (100 μM).

In RbJV, the possibility that ACh responses were mediated in part either by cyclo-oxygenase products or by a K^+-channel linked hyperpolarizing factor was examined using indomethacin and glibenclamide respectively. ACh relaxations were unaltered in the presence of indomethacin (2.8 μM). Similarly treatment of tissues with 1 μM glibenclamide failed to modify either the location or maximum ($\Delta p[A_{50}] = 0.07 \pm 0.07$; Δ max = 2 ± 2%, $n = 5$) of the ACh response curve. In these experiments histamine (3 μM) was used to induce tissue contraction because glibenclamide is a competitive antagonist at thromboxane A_2 receptors (Cocks *et al.*, 1990).

Influence of agonist efficacy on inhibition by L-NAME in rabbit jugular vein

The influence of receptor-effector coupling efficiency was examined in RbJV by determining the effects of L-NAME (10 and 100 μM) on ACh relaxations after partial inactivation of the muscarinic receptor pool using phenoxybenzamine (3 μM

for 30 min). Following this treatment ACh behaved as a partial agonist attaining only about 75% of E_m. Figure 4a shows that under these conditions the inhibitory effect of L-NAME was more profound than in untreated tissues, inhibition of ACh effects being similar to that obtained when α-methyl-5-HT was the agonist (see Figure 1c).

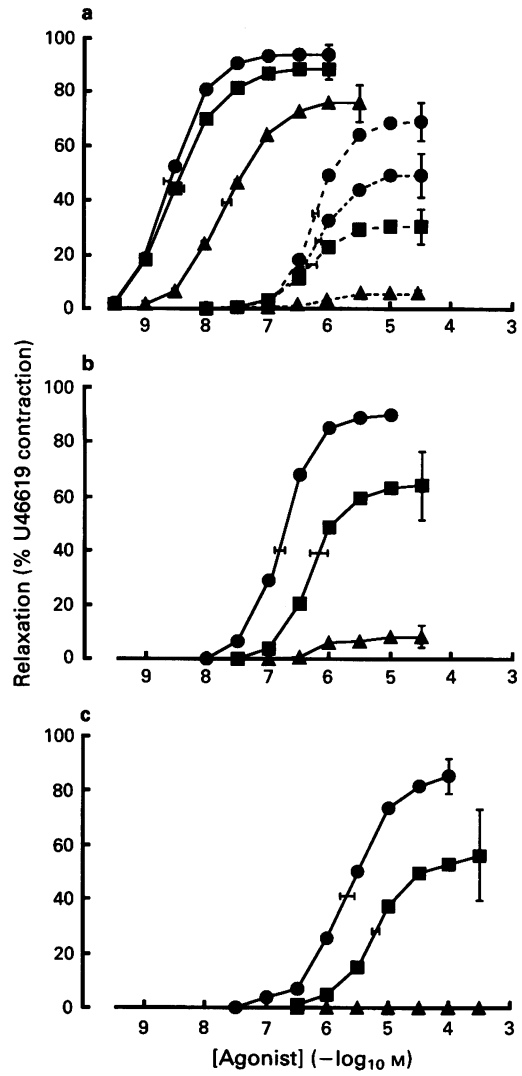

Figure 4 The effect of L-N^G-nitroarginine methyl ester (L-NAME, ● 0, ■ 10 μM, and ▲ 100 μM) on endothelium-dependent relaxations obtained with acetylcholine (ACh) (a), pilocarpine (b) and butyrylcholine (c) in rings of RbJV. Each experiment followed a triple curve design. Panel (a) is a composite of three separate experiments, one showing L-NAME effects in untreated tissues (solid lines), the other two showing inhibitor effects after treatment with phenoxybenzamine (dashed and dotted lines). In the latter case only second and third curves are shown representing respectively, a control curve after receptor inactivation (--●-- or ···●···) and the corresponding curve obtained in the presence of either 10 μM (--■--) or 100 μM (···▲···) L-NAME. Data are the averages of 4-6 E/[A] curves in each case. Vertical bars denote s.e.mean, shown for clarity on maximum values only. Horizontal bars identify the mean (±s.e.mean) computed $p[A_{50}]$ values in each case.

This phenomenon was explored further by studying the inhibition of pilocarpine and butyrylcholine relaxations. These agonists act at the same endothelial M_3 receptor type as ACh in RbJV since antagonism by 4-DAMP (10 nM) provided estimates of pA_2 (vs butyrylcholine = 9.35 ± 0.11, $n = 6$; vs pilocarpine = 9.41 ± 0.11, $n = 4$) that were not different from the estimate of pK_B obtained when ACh was the agonist (9.24 ± 0.26; see above). Furthermore, receptor inactivation experiments (phenoxybenzamine 0.3 μM for 30 min) showed that, unlike ACh, both pilocarpine and butyrylcholine behaved as partial agonists achieving on average 95% and 93% of E_m respectively. Estimates of agonist affinity and efficacy obtained by fitting these data to equation (3) are given in Table 1. L-NAME (10 and 100 μM) inhibited relaxations induced by both partial agonists producing a concentration-related right-shift and depression of E/[A] curves similar to that obtained against ACh after receptor inactivation (Figure 3b,c, cf. a).

A model describing inhibition of receptor-stimulated NO release Theory

Figure 5 illustrates a simplistic scheme for the receptor-stimulated release of NO in which agonist occupancy of receptors on endothelial cells activates an intracellular enzyme cascade responsible for generating NO from L-arginine. The subsequent liberation of NO stimulates a transduction pathway in smooth muscle cells which leads ultimately to the measured effect, vasorelaxation. Inhibitors are regarded as 'false substrates' for the enzyme NO synthase competing directly with the endogenous substrate L-arginine to block the formation of NO. Each of these steps is visualised in Figure 5 and was used as a basis to derive a simple theoretical model. At the outset the following assumptions were made; (i) equilibrium conditions pertain throughout, (ii) the intracellular concentration of L-arginine vastly exceeds that of the enzyme, NO synthase, (iii) the pool of L-arginine is not depleted by NO formation and (iv) inhibitors exhibit reversible, competitive inhibition kinetics.

Firstly, it is assumed that the equilibrium occupancy of endothelial receptors is governed by Mass Law;

$$[AR] = \frac{[R_0][A]}{K_A + [A]} \quad (4)$$

where $[R_0]$ denotes the total functional receptor pool, [A] is the concentration of agonist and K_A its equilibrium dissociation constant. Receptor occupancy then leads to activation of an intracellular enzyme (NO synthase). For simplicity the concentration of enzyme in the active form ([Ea]) is assumed to be proportional to [AR];

$$[Ea] = \gamma[AR] = \frac{\gamma[R_0][A]}{[A] + K_A} \quad (5)$$

Next, substrate (ARG) and inhibitor (B) compete for Ea such that;

$$[Ea] + [Arg] \underset{}{\overset{K_m}{\rightleftharpoons}} [EaArg] \xrightarrow{K_{cat}} [E] + [NO]$$

$$+$$

$$[B] \quad \Big\| \; K_B$$

$$[EaB]_{inactive}$$

Table 1 Parameter estimates describing the agonist effects of acetylcholine, pilocarpine and butyrylcholine at the endothelial muscarinic receptor in rabbit external jugular vein

Agonist	*No. experiments*	pK_A	τ_{test}/τ_{ACh} (×100)	n	E_{max}
Acetylcholine	11	6.12 ± 0.09	100	1.54 ± 0.18	93.0 ± 3.0
Pilocarpine	6	4.86 ± 0.09	3.6 ± 1.2	1.83 ± 0.40	92.7 ± 3.8
Butyrylcholine	5	6.02 ± 0.14	4.1 ± 1.1	1.71 ± 0.09	93.0 ± 1.5

Values are estimates provided by fitting the E/[A] curve data for each agonist to the operational model of agonism (equation 3).

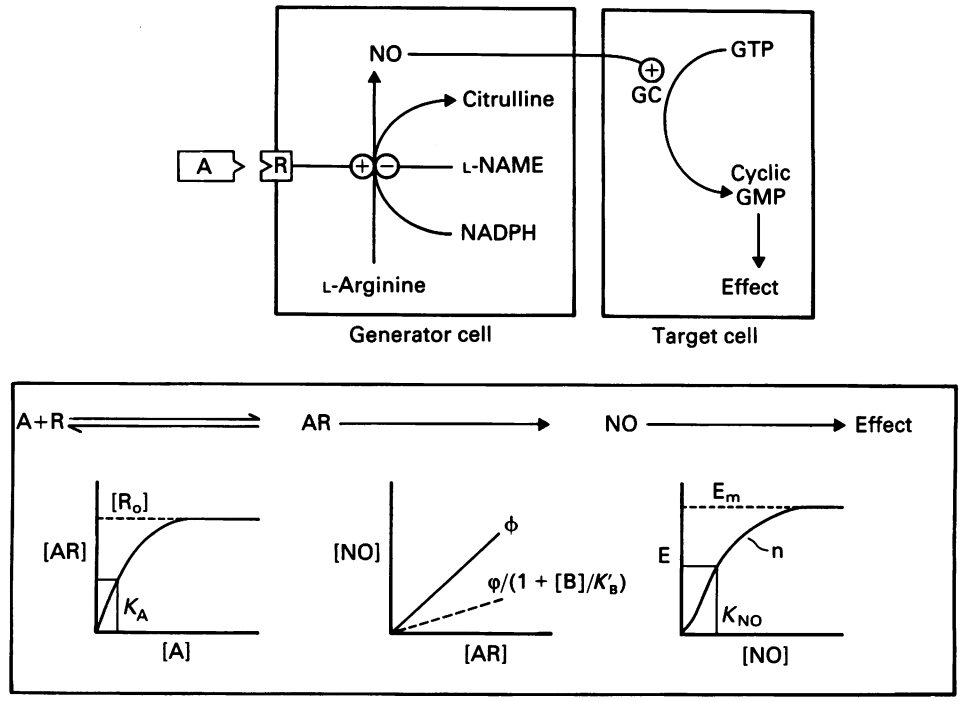

Figure 5 Representation of the biochemical cascade which results in vascular smooth muscle (target cell) relaxation following receptor-stimulated synthesis and release of NO from endothelial (generator) cells (adapted from Knowles *et al.*, 1990). In model terms the cascade can be conveniently regarded as three serial events; (i) the binding of agonist to endothelial cell receptors, (ii) the subsequent formation and liberation of NO and (iii) the interaction of NO with a transducer molecule (GC: guanylate cyclase) in the target cell resulting ultimately in the biological effect.
Key: [A] = concentration of agonist, K_A = its dissociation constant, $[R_0]$ = concentration of functional receptors, ϕ = proportionality constant for [NO]/[AR] relation, [B] = concentration of inhibitor, K'_B a parameter related to the inhibitor dissociation constant (see eq. 10), E_m = maximum effect possible, K_{NO} = value of [NO] for $0.5E_{max}$ and n = slope parameter of E/[NO] relation.

Hence the rate (V) of NO formation is;

$$V = \frac{[Arg][Ea]K_{cat}}{[Arg] + K_m\{1 + [B]/K_B\}} \quad (6)$$

It seems reasonable to suppose that following its liberation NO disappears from the site of action according to first order kinetics, hence the concentration available to initiate a response in the target cell will be proportional to its rate of formation;

$$[NO] = \beta V \quad (7)$$

Substitution of equation (5) into equation (6) now gives;

$$[NO] = \frac{\beta\gamma K_{cat}[Arg][A][R_0]}{([Arg] + K_m\{1 + [B]/K_B\})([A] + K_A)} \quad (8)$$

By rearrangement;

$$[NO] = \frac{\beta\gamma K_{cat}([Arg]/[Arg] + K_m)[A][R_0]}{(1 + [B]K_m/([Arg] + K_m)K_B)([A] + K_A)} \quad (9)$$

The assumption that [Arg] is not depleted during the formation of NO means that $[Arg]/[Arg] + K_m$ can be regarded as a tissue-dependent constant like β, γ, K_{cat} and $[R_0]$. Together these can be described by a single constant, ϕ. For convenience however, $[R_0]$ will be kept separate from ϕ. Now equation (9) can be simplified to;

$$[NO] = \frac{[A][R_0]\phi}{([A] + K_A)\{1 + [B]/K'_B\}} \quad (10)$$

where $K'_B = K_B([Arg] + K_m)/K_m$.

The final step in the pathway relates [NO] to effect, E. In the absence of evidence to the contrary, it seems sensible to assume that NO binds in a saturable way to an effector protein which initiates a cascade to produce the final observed effect. Here, in line with the reasoning of Black & Leff (1983), we have assumed that this relation corresponds to a logistic function analogous to the E/[AR] relation in their operational model of agonism;

$$E = \frac{E_m[NO]^n}{K_{NO}{}^n + [NO]^n} \quad (11)$$

where E_m denotes the maximum possible effect in the system, K_{NO} the value of [NO] for half E_m and n is the slope parameter of the E/[NO] relation. Substitution of equation (10) into equation (11) gives the overall E/[A] relation;

$$E = \frac{E_m\phi^n[R_0]^n[A]^n}{K_{NO}{}^n(K_A + [A])^n \times \{1 + [B]/K'_B\}^n + \phi^n[R_0]^n[A]^n} \quad (12)$$

This equation is equivalent to the operational model (equation 3) except, in this case, $\tau = (R_0\phi/K_{NO})$ (13) modified by the factor $1/(1 + [B]/K'_B)$.

Model predictions In model terms the constant ϕ is simply a proportionality constant determining the availability of NO at the target (smooth muscle) cell transducer following its receptor-stimulated synthesis and release from the generator (endothelial) cell (see Figure 5). Assuming that access to the target cell transducer is not limiting, a large value of ϕ implies that NO synthesis and release is efficiently coupled to the generator cell receptor system, whereas a small value implies the opposite.

However the quantity ϕ is not accessible experimentally because it is inextricably bound up with the other tissue-dependent parameters that define agonist efficacy, in this case $[R_0]$ and K_{NO}. This is clear from equation (13) which shows that ϕ is a proportionality constant on the ratio $[R_0]/K_{NO}$

where this ratio has the same operational meaning as τ (the transducer ratio $[R_0]/K_E$) described originally by Black & Leff (1983). In principle then, the operational efficacy of an agonist in an endothelial receptor system will depend upon the availability of NO to the target cell (determined by ϕ and $[R_0]$) and the efficiency of the target cell transducer machinery (determined by K_{NO}). Since in the presence of a competitive inhibitor of NO synthesis the value of ϕ is reduced by the factor $(1 + [B]/K'_B)$ (equation 10), the inhibitor effectively decreases the operational efficacy (τ) of the receptor system and in this regard has the same consequences as irreversible receptor occlusion ($[R_o]$) or decreasing the efficiency with which NO-guanylate cyclase coupling produces smooth muscle relaxation.

The simulations in Figure 6 illustrate the important features of inhibition predicted for endothelial agonist E/[A] curves that are either rectangular hyperbolic (Figure 6a) or inherently steep (Figure 6b). Model parameters were chosen to represent either full or partial agonism. Under these two conditions increasing the value of $[B]/K'_B$ produces a different pattern of curve displacement. With a full agonist the effect of the inhibitor is the same for both hyperbolic and steep curves i.e. parallel rightward displacements. However in the case of a partial agonist the inhibitor causes depression of the curves with little change in their location. Furthermore E/[A] curve shape now has an important impact upon the inhibition observed. Comparison of the hyperbolic (a) and steep (b) cases in Figure 6 shows that the same increments in inhibitor concentration produce a more profound depression of steep curves than of hyperbolic curves. This behaviour is analogous to that predicted following receptor inactivation (Black *et al.*, 1985a).

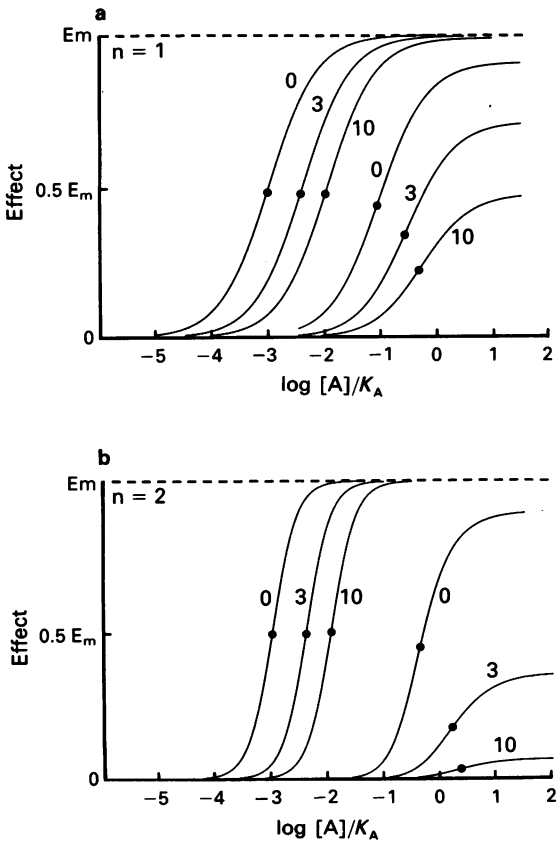

Figure 6 Simulations based on equation (12) depicting the effect of an inhibitor L-arginine analogue on full and partial agonist E/[A] curves which are rectangular hyperbolic (n = 1: a) or steep (n = 2: b) respectively. The number against each curve indicates the value of $[B]/K'_B$. Solid circles identify the mid-point of each curve. Parameter values were; $\phi = 100$, $K_{NO} = 1$, $E_m = 1$ and $[R_0] = 10$ (full agonist) and 0.03 (a) or 0.01 (b) (partial agonist).

In summary the effect of NO synthase inhibition on receptor-stimulated endothelium-dependent responses is predicted to be dependent not only on the efficacy of the agonist in the system under study, but also on the gradient of the resulting E/[A] curve.

Model simulations of experimental data Figure 7 shows model simulations predicting the effects of NO synthase inhibition superimposed on the E/[A] curve data points from Figure 4. The line depicting the control E/[A] curve in each case was obtained using the parameter estimates derived from previous receptor inactivation studies (Table 1) with τ providing a value for the term $(R_o/K_{NO})\phi$ (see eq. 13) and $[B]/K'_B$ set to zero. The lines depicting the effects of L-NAME were produced simply by increasing the value of $[B]/K'_B$. Clearly good agreement can be obtained between model simulations and the experimental data reinforcing the notion that L-NAME behaves as if it acts simply to reduce efficacy in the receptor system under study. However, quantitative differences between the predicted and observed behaviour of the inhibitor are evident. Firstly, in untreated tissues where ACh behaves as a full agonist (Figure 7a), depression of the curve maximum was observed experimentally which was not predicted by the model. Secondly, the values of $[B]/K'_B$ used to simulate the

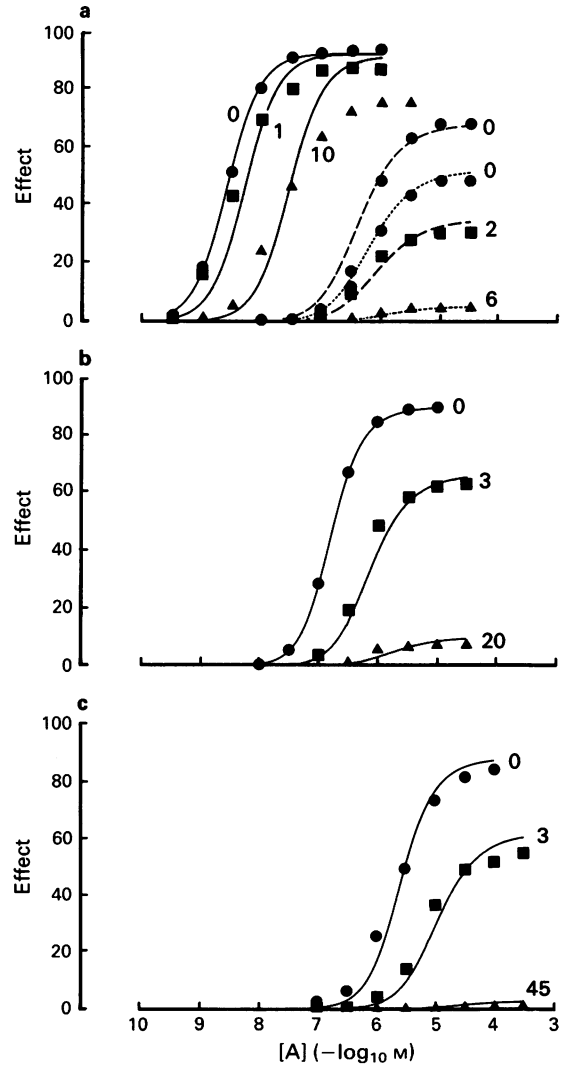

Figure 7 Simulations of L-N^G nitroarginine methyl ester (L-NAME) effects on E/[A] curves to acetylcholine (ACh) (a), pilocarpine (b) and butyrylcholine (c). The lines were obtained as described in the text and values of $[B]/K'_B$ used to simulate the effects of L-NAME are shown next to the lines. The data points shown are taken from Figure 4.

effects of 10 and 100 μM L-NAME in each case would obviously be expected to differ by ten fold, whereas agreement between the simulations and experimental data was achieved by increments of $[B]/K'_B$ that ranged between 5 and 15 fold (see legend to Figure 7). Possible reasons for these discrepancies are considered in detail in the discussion.

Discussion

L-NAME is a potent, enantiomerically specific inhibitor of NO synthase in a variety of cell types (Palacios *et al.*, 1989; Knowles *et al.*, 1990; Radomski *et al.*, 1990; McCall *et al.*, 1991) including vascular endothelial cells (Rees *et al.*, 1990). It also inhibits endothelium-dependent responses induced by ACh and bradykinin *in vivo* as well as *in vitro*, implying that NO synthesis and release accounts for the biological effects of these agents (Moore *et al.*, 1990; Rees *et al.*, 1990). The present experiments confirmed that micromolar concentrations of L-NAME virtually abolish ACh relaxations in endothelium-intact rat aorta (Rees *et al.*, 1990) but they also showed that the inhibitor demonstrates agonist- and tissue-differences. Hence in another tissue, the rabbit jugular vein, ACh responses were much less susceptible to inhibition by L-NAME whereas relaxations elicited by α-methyl-5-HT via endothelial 5-HT receptors remained sensitive to blockade. The purpose of this study was to ascertain whether or not these data could be interpreted as evidence for the existence of heterogeneous EDRFs.

The possibility that different muscarinic receptor subtypes accounted for the tissue-dependence of L-NAME effects was eliminated at the outset. In RbJV affinity estimates for the muscarinic receptor agonist 4-DAMP were independent of the agonist used and not different from the estimate obtained in RA when ACh was the agonist ($pK_B \sim 9.4$). Neither were affinity estimates for ACh itself different in the two tissues ($pK_A = 6.1$). These results indicated the involvement of only a single population of endothelial muscarinic receptors in each tissue and identified the receptor type as M_3 (see e.g. Furchgott & Cherry, 1984; Eglen & Whiting, 1986). Of particular interest was the finding from receptor inactivation experiments that in RbJV, but not RA, there was a considerable M_3 receptor reserve. Traditionally this would allow the higher potency of ACh in RbJV ($p[A_{50}] = 8.58$) compared to RA ($p[A_{50}] = 7.27$) to be explained in terms of a greater M_3 receptor density and/or more efficient occupancy-effect coupling. However, in this case the coupling process is not entirely intracellular; it involves the transfer of an intermediate mediator, NO, from one cell type to another, raising the possibility that anatomical i.e. arterial vs. venous differences may be important. For example the actions of endothelium-derived NO may be favoured in the venous tissue where there is a higher endothelial/smooth muscle cell ratio and the media provides less of a diffusion barrier (Bassenge *et al.*, 1987). Alternatively, and perhaps more likely, the greater potency of ACh in RbJV may reflect a better coupling of the NO-guanylate cyclase cascade in this tissue. This would be consistent with the $\sim$30 fold greater potency of the nitrovasodilator glyceryltrinitrate in RbJV compared to RA and compatible with the generally recognised tendency for nitrates to demonstrate a preferential dilator effect on veins (Ignarro & Kadowitz, 1985).

Early experiments also excluded the possibility that resistance of ACh relaxations to inhibition by L-NAME was due to the stimulated release of other known endothelium-derived vasodilator substances. The lack of effect of indomethacin indicated that cyclo-oxygenase products such as prostacyclin (PGI_2) were not involved. Neither was there evidence for the involvement of a glibenclamide-sensitive K^+-channel-linked hyperpolarizing factor. The release of such a factor from vascular endothelial cells has been implied by electrophysiological as well as pharmacological studies (Taylor & Weston, 1988; Feletou & Vanhoutte, 1988), but in the present experiments the K^+-channel blocker, glibenclamide, failed to affect ACh responses in RbJV indicating that involvement of vascular smooth muscle K^+ channels in endothelium-dependent relaxations was unlikely. Since neither receptor differences nor the release of mediators other than NO appeared to explain the discrepant behaviour of L-NAME in the two tissues the possibility was considered that the agonist- and tissue-dependence of inhibition was in some way related to the efficiency of receptor occupancy-effect coupling. Evidence to support this notion was first obtained using phenoxybenzamine to inactivate a fraction of the muscarinic receptors in RbJV. This intervention converted ACh from a full to a partial agonist so that it attained only about 75% of E_m and in this regard resembled closely the partial agonist behaviour of α-Me-5-HT at the endothelial 5-HT receptor in this tissue (Leff *et al.*, 1987). Under these conditions L-NAME produced an impressive non-surmountable inhibition of ACh relaxations (Figure 3a) similar to that obtained when α-Me-5-HT was the agonist (Figure 1a). Subsequent studies using untreated tissues showed that L-NAME was equally effective against relaxations produced by the muscarinic receptor partial agonists, pilocarpine and butyrylcholine (Figure 4b and c) providing further evidence that the nature of inhibition obtained is dependent upon agonist efficacy. These results are reminiscent of the differential effects produced by a basal release of EDRF on contractions of isolated vascular rings elicited by high and low efficacy α-adrenoceptor agonists (Martin *et al.*, 1986). In that study, endothelial denudation increased tissue sensitivity to the full agonist phenylephrine without affecting the curve maximum, but increased profoundly the maximum response to the partial agonist clonidine. Clearly L-NAME would be expected to produce similar effects in rings with an intact endothelium.

Although agonist efficacy clearly accounts for a part of the tissue-differences in inhibitor behaviour observed here, L-NAME remained substantially more potent in RA than in RbJV (Figure 1b cf. Figure 3a) even when fractional receptor occlusion reduced the efficacy of ACh to a similar level in both tissues. This is most probably explained by the different effects of basal EDRF release on the spasmogens used. U-46619 (10 nM) was used to contract rings of RbJV and in this tissue the E/[A] curve is unaffected either by endothelial denudation (Leff *et al.*, 1987) or by L-NAME (this study). Hence the tonic state of the preparation is the same in the presence and absence of inhibitor. However, phenylephrine was the spasmogen in RA and in this tissue E/[A] curves are potentiated and augmented by L-NAME. This effect of the inhibitor was not taken into account in these experiments so that in RA, but not RbJV, L-NAME not only reduced the release of EDRF, it also increased functional opposition to ACh-induced relaxations. Its greater potency in RA compared to RbJV may therefore be more apparent than real.

Consideration of a simple theoretical model suggests a rational basis for the effects of L-NAME on endothelium-dependent relaxations. In terms of the model described here competitive inhibition of NO synthase is expected to produce a right-shift and, ultimately, depression of the agonist E/[A] curve because the inhibitor effectively decreases the efficacy of the indirectly acting agonist, ACh, by the factor $1 + [B]/K'_B$, (see Results). This is similar to agonist curve behaviour predicted for the simple competitive antagonism of a mediator after its liberation by an indirectly acting agonist, as exemplified by the antagonism of tyramine by α- and β-adrenoceptor antagonists (Fedan *et al.*, 1977; Black *et al.*, 1980) and the antagonism of pentagastrin by histamine H_2-receptor antagonists (Black, 1973; Black *et al.*, 1978; 1985b). In these latter cases the efficacy of the indirectly acting agonist is reduced by the factor $1 + [B]/K_B$ so that ratios of efficacy estimates obtained in the absence and presence of antagonist can be treated just like agonist dose-ratios to provide an estimate of K_B (see Black *et al.*, 1985b). However when the inhibitor competes with substrate to block the synthesis and/or release of the mediator, as is the case for L-NAME in the present study,

the efficacy ratios of the indirectly acting agonist are governed by K'_B which subsumes the true value of K_B (equation 10). This means that in the absence of more precise knowledge concerning the events leading to the liberation of (in this case) NO, only empirical information concerning inhibitor activity can be obtained from these experiments.

A more important prediction from the present model is evident when the dependency of E/[A] curve mid-points on agonist efficacy is considered. The midpoint location ($[A_{50}]$) for the ACh curve can be derived from equation (12) as:

$$[A_{50}] = K_A/\{(2 + (\phi[R_0]/K_{NO})^n)^{1/n} - 1\}$$

where the parameters have the same meaning as defined earlier. From this it is clear that decreasing the value of ϕ (which occurs when [L-NAME] is increased; see equation 10) produces the same result as decreasing $[R_o]$ and therefore, by analogy, the effect of L-NAME on ACh E/[A] curves allows estimation of K_A, the equilibrium dissociation constant for the indirectly acting agonist.

This predicted parity between the effects of a competitive NO synthase inhibitor and an irreversible receptor antagonist implies that the former can be used just like the latter to estimate agonist affinity constants (Furchgott, 1966). In principle this offers a means of assessing the applicability of the model. This test was applied to the data for pilocarpine and butyrylcholine, since in both cases there was a reasonable agreement between model simulations and experimental results (Figure 7). Interestingly, agonist affinities were overestimated when the E/[A] curve data obtained before and after L-NAME treatment were fitted directly to equation (3): the pK_A estimate for pilocarpine was 6.42 ± 0.08 (mean $\pm$ s.e.mean, $n = 6$) and for butyrylcholine was 5.55 ± 0.11 (mean $\pm$ s.e.mean, $n = 5$). These values are significantly ($P < 0.05$) higher than the respective estimates of 6.02 ± 0.14 and 4.86 ± 0.09 obtained following partial receptor occlusion with phenoxybenzamine (Table 1) suggesting that L-NAME produces a more rapid collapse of the agonist curves than would be expected from a simple decrease in efficacy resulting from competitive inhibition of NO synthesis and release. Although less obvious in the case of the partial agonists, such an effect is evident in the experiments with ACh (Figure 7a) where, in untreated tissues, L-NAME produced a right-shift accompanied by depression of the agonist curve maximum when progressive parallel shifts in the concentration-effect curve were predicted.

A second difference between the predicted and observed behaviour of L-NAME was that a generally smaller than ten fold increment in the value of $[B]/K'_B$ was required to simulate the experimental effects of a ten fold increase in L-NAME concentration. Indeed, given the observation above that L-NAME appears to induce a more rapid collapse of the E/[A] curve than predicted by the model, an increment in $[B]/K'_B$ of greater than ten fold might have been anticipated. Obviously these discrepancies might be explained by deficiencies in the model which is inevitably an oversimplification. Alternatively it might indicate that L-NAME does not inhibit NO production in the endothelium of RbJV in a simple competitive manner. Perhaps relevant to this, Moncada and colleagues have demonstrated significant differences in the properties of NO synthase in different tissues (Moncada *et al.*, 1989). Moreover the enzyme present in cultured vascular endothelial cells exists in two forms, one Ca^{2+}-dependent and constitutive, the other Ca^{2+}-independent and inducible (Radomski *et al.*, 1990). Whether or not these two forms of the enzyme exhibit differential sensitivity to L-NAME is unknown at present, but East & Garthwaite (1990) have shown that in rat cerebellar slices the inhibition by N^G-nitroarginine of NMDA-induced cyclic GMP formation involves two components implying an action at two receptor-activated NO synthase enzymes (see also Garthwaite *et al.*, 1989). If such multiple forms of the synthase enzyme contribute to the muscarinic receptor-stimulated production of NO in RbJV then an overall non-competitive pattern of inhibition by L-NAME would be expected and the choice of a simple linear relation between [AR] and [NO] in the model would be inadequate to describe the experimental data. Unfortunately the choice between these possibilities remains arbitrary given the present inability to determine directly the nature of the inhibitor-enzyme interaction.

In spite of these quantitative limitations the good agreement between the observed and predicted behaviour of L-NAME in these experiments implies that the model nevertheless provides a useful basis for studying the effects of inhibitors on receptor-stimulated release of NO. Importantly it indicates that tissue- and agonist-differences observed with such inhibitors can be accounted for by the factors which determine agonist efficacy in the system. This possibility should be ruled out before apparent resistance to blockade by the inhibitor is taken as evidence for the involvement of heterogeneous EDRFs.

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