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Effects of L-N^G-nitro-arginine on noradrenaline induced contraction in the rat anococcygeus muscle

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1 The influence of L-N^G-nitro-arginine (L-NOARG, 30 μM) on contractile responses to exogenous noradrenaline was studied in the rat anococcygeus muscle.

2 Noradrenaline (0.1–100 μM) contracted the muscle in a concentration-dependent manner. L-NOARG (30 μM) had no effect on noradrenaline responses.

3 Phenoxybenzamine (Pbz 0.1 μM) depressed by 46% ($P<0.001$) the maximum response and shifted to the right ($P<0.001$) the E/[A] curve to noradrenaline (pEC_{50} control: 6.92 ± 0.09; pEC_{50} Pbz: 5.30 ± 0.10; $n=20$).

4 The nested hyperbolic null method of analysing noradrenaline responses after phenoxybenzamine showed that only 0.61% of the receptors need to be occupied to elicit 50% of the maximum response, indicating a very high functional receptor reserve.

5 Contractile responses to noradrenaline after partial α_1-adrenoceptor alkylation with phenoxybenzamine (0.1 μM) were clearly enhanced by L-NOARG.

6 The potentiating effect of L-NOARG on noradrenaline responses after phenoxybenzamine was reversed by (100 μM) L-arginine but not by (100 μM) D-arginine.

7 These results indicate that spontaneous release of NO by nitrergic nerves can influence the α_1-adrenoceptor-mediated response to exogenous noradrenaline.

Keywords: Anococcygeus muscle (rat); α_1-adrenoceptor; L-N^G-nitro-arginine; NANC transmission

Introduction

The anococcygeus muscle has a motor noradrenergic and an inhibitory non-adrenergic, non-cholinergic (NANC) innervation (Gillespie & McGrath, 1973). The nature of the NANC transmitter has been extensively investigated. Pharmacological studies indicate that the most likely candidate as a NANC neurotransmitter in this tissue is nitric oxide (NO, Gillespie *et al*., 1989; Li & Rand, 1989a,b; Gibson *et al*., 1990; Gillespie & Sheng, 1990; Ramagopal & Leighton, 1989). In addition, structural studies (Song *et al*., 1993) have demonstrated that NO-synthesizing neurones innervate the rat anococcygeus muscle, results that are consistent with the evidence that NO mediates the inhibitory transmission.

The neuronal release of noradrenaline contracts the muscle mainly via α_1-adrenoceptors (Docherty & Starke, 1981). The noradrenergically-mediated contraction evoked by electrical field stimulation in the absence of guanethidine was enhanced by nitric oxide synthase (NOS) inhibitors in both the rat (Li & Rand, 1989a; Vila *et al*., 1992; Brave *et al*., 1993) and the mouse (Gibson *et al*., 1990) anococcygeus. These results indicate that both NO and noradrenaline are released by electrical field stimulation in the anococcygeus muscle. Thus, relaxation due to NO and contraction due to noradrenaline released by electrical stimulation oppose each other. However, contractions mediated by exogenous noradrenaline were not affected by NO-synthase inhibitors (Li & Rand, 1989a; Brave *et al*., 1993).

We have previously demonstrated that when a tissue, such as the rat tail artery, shows a high efficiency of coupling for α_1-adrenoceptors, the influence of endothelium (Tabernero & Vila, 1995) and of an NO-synthase inhibitor, N^G-nitro-L-arginine methylester (Tabernero *et al*., 1996) on contractions mediated by these adrenoceptors cannot be observed. Nevertheless, after partial irreversible inactivation of α_1-adrenoceptors, responses to phenylephrine were potentiated by an inhibitor of NO synthase (Tabernero *et al*., 1996). The anococcygeus is a highly innervated muscle that exhibits a high efficiency of coupling for α_1-adrenoceptors (Kenakin, 1993). Thus, the objective of our study was to evaluate the role of functional reserve on the influence of NO on α_1-adrenoceptor-mediated contraction in the rat anococcygeus muscle.

Methods

Male Sprague-Dawley rats (300–350 g) were killed by decapitation. The anococcygeus was dissected as described by Gillespie (1972) and set up in 7 ml organ bath containing physiological salt solution (PSS) of the following composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1 maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. Desipramine (0.1 μM), normetanephrine (1 μM), Na_2 EDTA (23 μM), propranolol (1 μM) and yohimbine (0.1 μM) were present throughout the experiment to block neuronal and extraneuronal uptake, to prevent noradrenaline oxidative degradation and stimulation of β- (Minneman *et al*., 1983; Sallés *et al*., 1994) and α_2-adrenoceptors (Bao *et al*., 1993), respectively. A resting tension of 4.90 mN was placed on the tissue and changes in tension recorded with a PIODEN (UF-1) isometric transducer attached to an Omniscribe pen recorder. The preparations were left to equilibrate for 45 min and tension was readjusted if necessary. The tissues were then contracted 4 times with KCl 75 mM every 5 min until the amplitude of the contractile response was similar in magnitude. After a 30 min equilibration period the different experiments were performed.

Four series of assays were carried out. The first was carried out to evaluate further the lack of effect of NO on responses induced by exogenous noradrenaline. Thus, two cumulative agonist concentration-effect (E/[A]) curves to noradrenaline

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were constructed. After the agonist was washed out, a 30 min period in the absence or presence of L-N^G-nitro-arginine (L-NOARG; 30 μM) was allowed before the second E/[A] curve with the agonist was run. In the second set of experiments, 30 min after the initial E/[A] curve to noradrenaline, tissues were exposed to phenoxybenzamine (Pbz, 0.1 μM), an alkylating agent that is known to bind covalently to α_1-adrenoceptors at a very low concentration (Minneman, 1983), for 20 min. The muscles were then washed successively every 5 min for half an hour, after which the E/[A] curve to the agonist was repeated. In the third series of experiments the anococcygeus muscles were exposed to Pbz as described above but the incubation was before the first E/[A] curve. Before a second E/[A] curve with noradrenaline was constructed, the tissue was incubated in L-NOARG (30 μM) for 30 min. The same protocol as above was used for the last series of assays but L-NOARG (30 μM, 30 min) was preceded by a 15 min incubation with L- or D-arginine (total period of incubation 45 min). Control experiments, with vehicle instead of drugs, were always carried out in parallel under the above mentioned conditions to check the reproducibility of the concentration-response curves to the agonist in the different experimental protocols.

Data analysis

Pragmatic logistic curve fitting Each individual set of E/[A] curve data was fitted to a logistic function of the form:

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

in which E and [A] are the pharmacological effect and the concentration of agonist, respectively; α, EC_{50} and m are the asymptote, location and slope parameters, respectively. Location parameters were actually estimated as pEC_{50} (the negative logarithm of the concentration required to cause 50% of the maximum response).

Experimental points and results from pragmatic logistic curve fitting are expressed as mean $\pm$ s.e.mean. The number of animals used (n) is indicated in the figures. Contractile responses are expressed as a percentage of the maximum (α) of the first curve. The statistical significance for the estimated parameters (pEC_{50}, α, pK_A, q, m) was assessed by two-tailed Student's t test for paired or unpaired observations as appropriate. Two-way analysis of variance for repeated measures

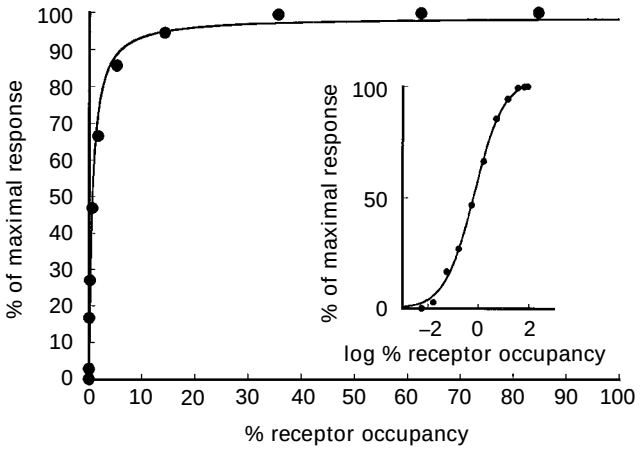

Figure 1 Plot of percentage of receptor occupancy (in natural or logarithmic scale) versus percentage of response for the contractile effect of noradrenaline. The percentage of occupancy of the receptor was calculated by use of the equation $[AR]/[R_T] = [A]/(K_A + [A])$ where $[AR]/[R_T]$ is the fractional receptor occupancy, K_A is the apparent dissociation constant calculated by the nested hyperbolic method and [A] is the concentration of agonist.

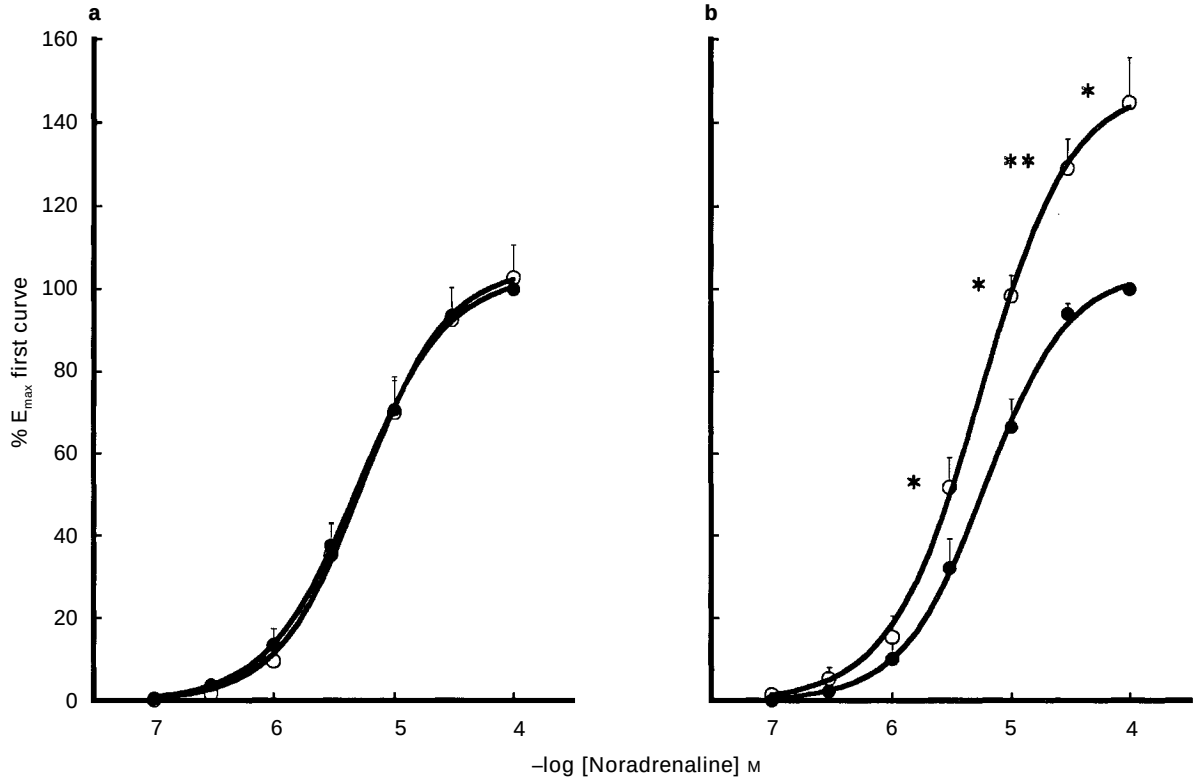

Figure 2 Concentration-response curve for noradrenaline-mediated contraction in rat anococygeus muscle after incubation with 0.1 μM phenoxybenzamine in the absence (●) or presence (○) of (a) vehicle; (b) 30 μM L-NOARG. Results are expressed as percentage of the maximum response obtained in the first curve. The lines drawn through the data are the results of pragmatic logistic curve fitting (see Methods). Results are the mean of 7 experiments; vertical lines show s.e.mean. $^*P<0.05$, $^{**}P<0.01$.

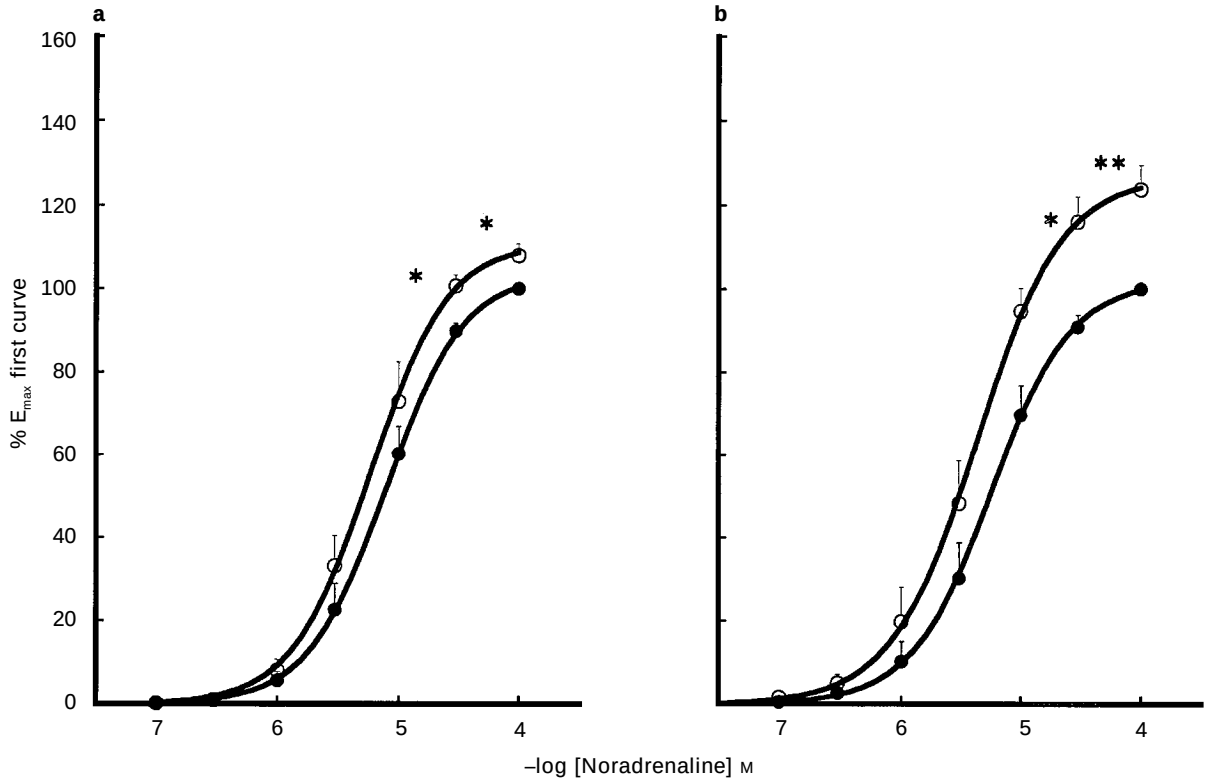

Figure 3 Concentration-response curve for noradrenaline-mediated contraction in rat anococygeus muscle after incubation with 0.1 μM phenoxybenzamine in the absence (●) or presence (○) of (a) L-arginine plus L-NOARG; (b) D-arginine plus L-NOARG. Results are expressed as percentage of the maximum response obtained in the first curve. The lines drawn through the data are the results of pragmatic logistic curve fitting (see Methods). Results are the mean of 8 to 10 experiments; vertical lines show s.e.mean. $^*P<0.05$, $^{**}P<0.01$.

followed by an orthogonal contrast test was applied to analyse the E/[A] curves. The statistical analysis was carried out with the SAS statistical package (Littell *et al*., 1991) by use of a general linear model (PROC GLM) which allows for the occurrence of unbalanced designs. A probability level of 0.05 or less was considered significant.

Nested hyperbolic method Data obtained from receptor inactivation experiments were analysed by the nested hyperbolic method (James *et al*., 1989) as previously described (Tabernero *et al*., 1996) to obtain the apparent dissociation constant (pK_A) and the fractional receptor concentration which remains after inactivation (q).

Drugs

L-Arginine HCl, D-arginine HCl, (−)-noradrenaline bitartrate, desipramine HCl, normetanephrine HCl, (±)-propranolol HCl and yohimbine HCl were purchased from Sigma Chemical Co; L-N^G-nitro-arginine from Carl Biochem; phenoxybenzamine HCl from Research Biochemical Incorporated (RBI). All drugs were prepared in physiological saline solution (PSS) except noradrenaline which was prepared in 23 μM Na_2EDTA, and Pbz in 0.1 M tartaric acid. All other chemicals used were of analytical grade.

Results

Noradrenaline contracted the anococcygeus muscle in a concentration-dependent manner. L-NOARG *per se* did not induce a contractile response of the tissue. Neither the potency (pEC_{50}: 6.78±0.06, $n=6$) nor the maximum contraction (α: 90.20±4.50 mN, $n=6$) exhibited by this agonist were modified by incubation with 30 μM L-NOARG (pEC_{50}: 6.70±0.06; α: 85.2±6.44 mN, $n=6$).

To test the hypothesis that the lack of effect of L-NOARG on noradrenaline-mediated contractions was due to a high efficiency of coupling of α_1-adrenoceptors, responses to this agonist were studied in the absence and presence of an alkylating agent. Pbz (0.1 μM) depressed the maximum contraction to noradrenaline (control: 93.20±3.30 mN; Pbz: 50.60±3.63 mN, $n=20$) by 46% ($P<0.001$) and shifted to the right ($P<0.001$) the E/[A] curve to noradrenaline (pEC_{50} control: 6.92±0.09; pEC_{50} Pbz: 5.30±0.10; $n=20$). The quantitative evaluation of the effects of Pbz by the nested hyperbolic method provided the following parameters: $pK_A=4.746\pm0.077$; $q=0.017\pm0.005$; $m=1.014\pm0.037$. The receptor occupancy was calculated for each concentration of noradrenaline and plotted as fractional occupancy against the fractional response (see legend of Figure 1). To obtain half of the maximal response only 0.61% of receptors need to be occupied, indicating a high efficiency of coupling.

The presence of Pbz before the first E/[A] curve with noradrenaline gave a maximal contraction (α: 45.40±3.63 mN, $n=10$) and a potency (pEC_{50}: 5.26±0.09, $n=10$) that did not differ from the one obtained when Pbz was incubated between the first and the second agonist E/[A] curve. When we studied the effects of L-NOARG (30 μM) after partial alkylation of α_1-adrenoceptors with Pbz, the NO-synthase inhibitor did not contract the muscle but potentiated the E/[A] curve of noradrenaline (Figure 2b). In parallel experiments incubation in PSS instead of L-NOARG did not modify the E/[A] curve to noradrenaline, in presence of Pbz (Figure 2a). L-Arginine (100 μM; Figure 3a) but not D-arginine (100 μM; Figure 3b) reversed the effects of L-NOARG after alkylation of α_1-adrenoceptors with Pbz.

Discussion

The results obtained in this study show that contractile responses to exogenous noradrenaline are potentiated by L-

NOARG, under conditions in which the functional α_1-adrenoceptor reserve is reduced by means of an alkylating agent. Furthermore, the potentiating effect of the NO synthase inhibitor on noradrenaline responses after partial α_1-adrenoceptor alkylation is reversed by L- but not by D-arginine.

L-NOARG enhanced the electrical stimulation mediated contractions in the rat (Li & Rand, 1989a; Vila *et al*., 1992; Brave *et al*., 1993) and mouse (Gibson *et al*., 1990) anococcygeus muscle without modifying responses to exogenous noradrenaline (Li & Rand, 1989a; Brave *et al*., 1993). Since L-NOARG inhibits the formation of NO, the potentiation by L-NOARG of stimulation-mediated contractile responses was probably due to the lack of the relaxation component because of a decrease on NANC transmitter formation and its release by electrical stimulation. We have previously demonstrated (Tabernero *et al*., 1996) that in rat tail artery, due to the high efficiency of coupling exhibited, the influence of the NO-synthase inhibitor on the response mediated by α_1-adrenoceptors could only be observed if the population of these receptors was diminished by use of an alkylating agent. Results obtained with Pbz on noradrenaline-induced contractions show that only 0.61% of the receptors need to be occupied to obtain half the maximum response. These results further confirm the previously demonstrated high efficiency of coupling for α_1-adrenoceptors in the rat anococcygeus muscle (Kenakin, 1993). Thus, the lack of effect of L-NOARG on noradrenaline-mediated contractions observed in the present as well as in previous studies (Li & Rand, 1989a; Brave *et al*., 1993) could be attributed to the high efficiency of coupling of α_1-adrenoceptors in this muscle. Thus, only after partial α_1-adrenoceptor alkylation, L-NOARG potentiates the contraction induced by exogenous noradrenaline. In addition, the fact that L-arginine but not D-arginine, reversed the observed potentiation effect of noradrenaline responses by L-NOARG seems to confirm that the potentiation observed is related to the inhibition of NO-synthase by L-NOARG.

In agreement with other authors (Gillespie *et al*., 1989; Li & Rand, 1989b), we have also observed (results not published), in the rat anococcygeus muscle, that NOS inhibitors have the ability to augment further the guanethidine-induced tone, an effect that is probably due to the inhibition of spontaneous synthesis of NO by the inhibitory nerves. Thus, we could speculate that the enhancement by L-NOARG of noradrenaline-induced contraction observed in the presence of Pbz is probably due to the lack of basal NO release. In addition, we should not exclude the possibility that basal release of NO could also contribute to the final contractile response induced by electrical stimulation observed in previous studies (Li & Rand, 1989a; Vila *et al*., 1992; Brave *et al*., 1993).

In summary, this study provides evidence that spontaneous, as well as electrically stimulated, release of NO by nitrergic nerves can influence the α_1-adrenoceptor-mediated contraction in the rat anococcygeus muscle.

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