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# An in vitro study of nonadrenergic-noncholinergic activity on the cavernous tissue of mouse

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**Abstract** The relaxant effects of electrical field stimulation (EFS) and exogenously applied acetylcholine (ACh) or acidified $NaNO_2$ (a-$NaNO_2$) were investigated in the isolated mouse corpus cavernosum precontracted with phenylephrine hydrochloride (PE). Tetrodotoxin (TTX) blocked the relaxant effects of EFS completely, whereas it had no effect on the responses to ACh or a-$NaNO_2$. Guanethidine and indomethacin failed to affect the electrically or ACh-induced relaxations. Atropine completely blocked the effect of ACh; however, it caused a slight reduction in the relaxation evoked by EFS. $N^G$-Nitro-L-arginine (L-NOARG) reduced the effects of EFS and ACh significantly, but it was ineffective on the relaxations induced by a-$NaNO_2$. The inhibitory action of L-NOARG was partly restored by L-arginine, but not by D-arginine. Methylene blue (MB) and hydroxocobalamin (HC) exhibited significant inhibition on the relaxations evoked by EFS, ACh and a-$NaNO_2$. Hydroquinone (HQ) reduced relaxation due to a-$NaNO_2$, but did not affect that of EFS and ACh. Our findings suggest that EFS-induced relaxations of mouse cavernosal tissue are mediated by a transmitter which probably resembles an organic nitrate.

**Key words** Mouse corpus cavernosum · Nitrergic activity · Nitric oxide · Penile erection · Hydroxocobalamin · Hydroquinone

Physiological and pharmacological studies on penile erection carried out in larger animals including monkeys, dogs, rabbits and cats are expensive, leading to difficulties in funding, [17]. For this reason, there is a growing need to use small animals for evaluations of erectile function. Some reports [2, 3, 17] in which a small laboratory animal, the rat, was studied have been published. To our knowledge, however, no attempt to investigate the mechanism of penile erection in the mouse corpus cavernosum (CC) has been made. Therefore, we decided to isolate the mouse cavernosal tissue, with the aim of obtaining an experimental model for in vitro studies. Successful preliminary results led us to examine this preparation for possible use as a nitrergic system in erectile function. Once this was achieved, the effects of electrical field stimulation (EFS), acetylcholine (ACh) and acidified $NaNO_2$ (a-$NaNO_2$) were investigated in the isolated mouse cavernous tissue precontracted with phenylephrine hydrochloride (PE) in either the absence or the presence of several substances which possibly affect nitrergic activity.

However, one must keep in mind that in vitro experiments can only yield part of the information concerning the complex process of penile erection [1]. Therefore such results must be interpreted with caution in terms of physiological and clinical implications.

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## Materials and methods

Male albino mice weighing 35–40 g were killed by cervical dislocation. Penises were surgically removed by cutting from the root of the penis attached to the pubic bone; they were then placed in a Petri dish containing Krebs solution. The glans penis and urethra were excised. The fibrous septum between the two CC was cut and then each CC was carefully dissected from the adherent tissues, keeping the tunica albuginea intact. Two preparations were thus obtained from each mouse. They were separately mounted under 0.2 g tension in 5-ml organ baths containing Krebs solution, which was continuously bubbled with 95% $O_2$ and 5% $CO_2$. Temperature was maintained at 37°C. The preparations were allowed to equilibrate for 1 h. During this period they were frequently washed with fresh Krebs solution (composition, mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, $NaH_2PO_4$ 1.2, glucose 11). The responses were recorded by isotonic transducers (Ugo Basile, 7006) on polygraph paper (Ugo Basile, Gemini 7070). After an equilibrium period the tissues were treated with 5 μM PE. This resulted in an active tone that reached a stable level within 5 min; at the end of this time, EFS, delivered by a Grass S88 stimulator via two parallel platinum electrodes embedded in Perspex as square waves (10 Hz,

10 V, 2 ms), was applied to the tissue for 30 s or vasodilator drugs such as ACh (1 μM) and a-$NaNO_2$ (0.5 mM) were added to the bath medium for 60 s. In preliminary experiments, a separate group of preparations were used to examine the effect of various frequencies (2, 4, 8, 10 and 15 Hz) of EFS. Relaxation induced by EFS at 10 Hz was found to be most reproducible and its magnitude was also more suitable for evaluation of drug actions. Therefore, this frequency was chosen for the remaining experiments. Following each treatment (either electrical or chemical), tissue was washed with fresh Krebs solution and thus relaxed back to baseline. In total six treatments were performed as described. The first and second groups of three treatments were arbitrarily accepted as the first series and the second series. The influences of tetrodotoxin (TTX) (0.1 μM), $N^G$-nitro-L-arginine (L-NOARG) (0.1, 1, 10 and 100 μM), L-arginine (0.5 mM), D-arginine (0.5 mM), L-NOARG (0.1, 1, 10 and 100 μM) plus 0.5 mM L-arginine or 0.5 mM D-arginine, methylene blue (MB) (10 or 30 μM), hydroxocobalamin (HC) (65 or 130 μM) and hydroquinone (HQ) (0.1 or 0.5 mM) were investigated on the electrically or drug-induced effects, each concentration being tried on a separate experimental group. Experiments were also performed to test possible contributions of cholinergic, adrenergic or prostanoid activity. For this purpose, the effects of atropine (0.2 μM), guanethidine (1 μM) and indomethacin (1 μM) were each examined on the EFS- and ACh-induced responses. Drugs were added to the bath medium 30 min prior to the second three applications.

### Drugs and solutions

Stock solutions of PE, ACh, TTX, atropine sulfate, HC and MB were dissolved in distilled water and stored at +4°C. Indomethacin and guanethidine sulfate were dissolved in dimethyl sulfoxide (0.1%). L-NOARG, L-arginine, D-arginine and hydroquinone (HQ) were prepared daily in Krebs solutions. a-$NaNO_2$ was prepared in distilled water (pH2) and stored at −4°C. Except for $NaNO_2$ (Merck Chemical), all of the drugs were purchased from Sigma

### Statistical considerations

The relaxations were calculated as percentage peak reduction of PE contracture. The mean values (±SE) of the first series and those of the second series were calculated separately for each experimental group. The mean values (±SE) of the second series of each group were expressed as the percentage of the mean values of the first series (percentage control). All data were analyzed by using Student's *t*-test for paired and unpaired observations. *P* values of less than 0.05 were considered to be significant.

## Results

### Responses to PE

PE (5 μM) caused a stable contraction that remained stable throughout the experiment. When contracted with PE, some tissues exhibited oscillations superimposed on tonus activity, which is not affected by drugs such as TTX or atropine (0.2 μM).

### Responses to EFS, ACh and a-$NaNO_2$

EFS relaxed the tissue precontracted with 5 μM PE in a frequency-dependent manner. In the experiments in which 10 Hz was used, responses were rapid in onset. On the cessation of electrical stimulation and washing with fresh Krebs solution, the tissue returned to its original level. However, some relaxant responses were biphasic in shape, i.e., relaxation followed by contraction while EFS

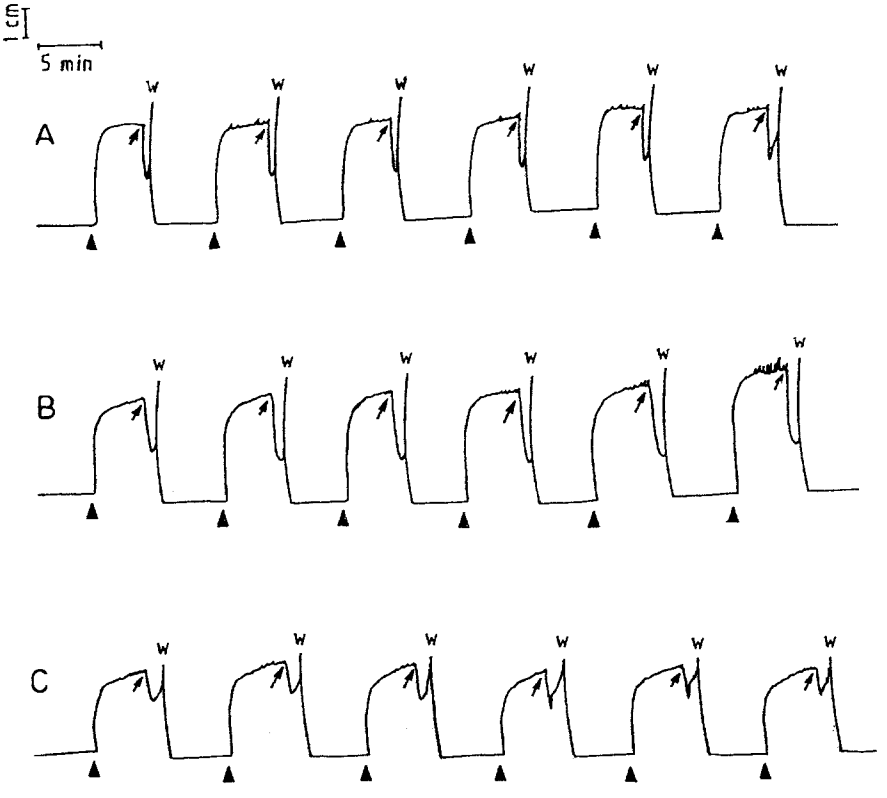


**Fig. 1** Representative tracings showing relaxant effects of *A* EFS (10 Hz), *B* 1 μM ACh and *C* 0.5 mM a-$NaNO_2$ in the mouse CC precontracted with 5 μM PE. *w* washout, ▲ PE application, → EFS or relaxant drug application

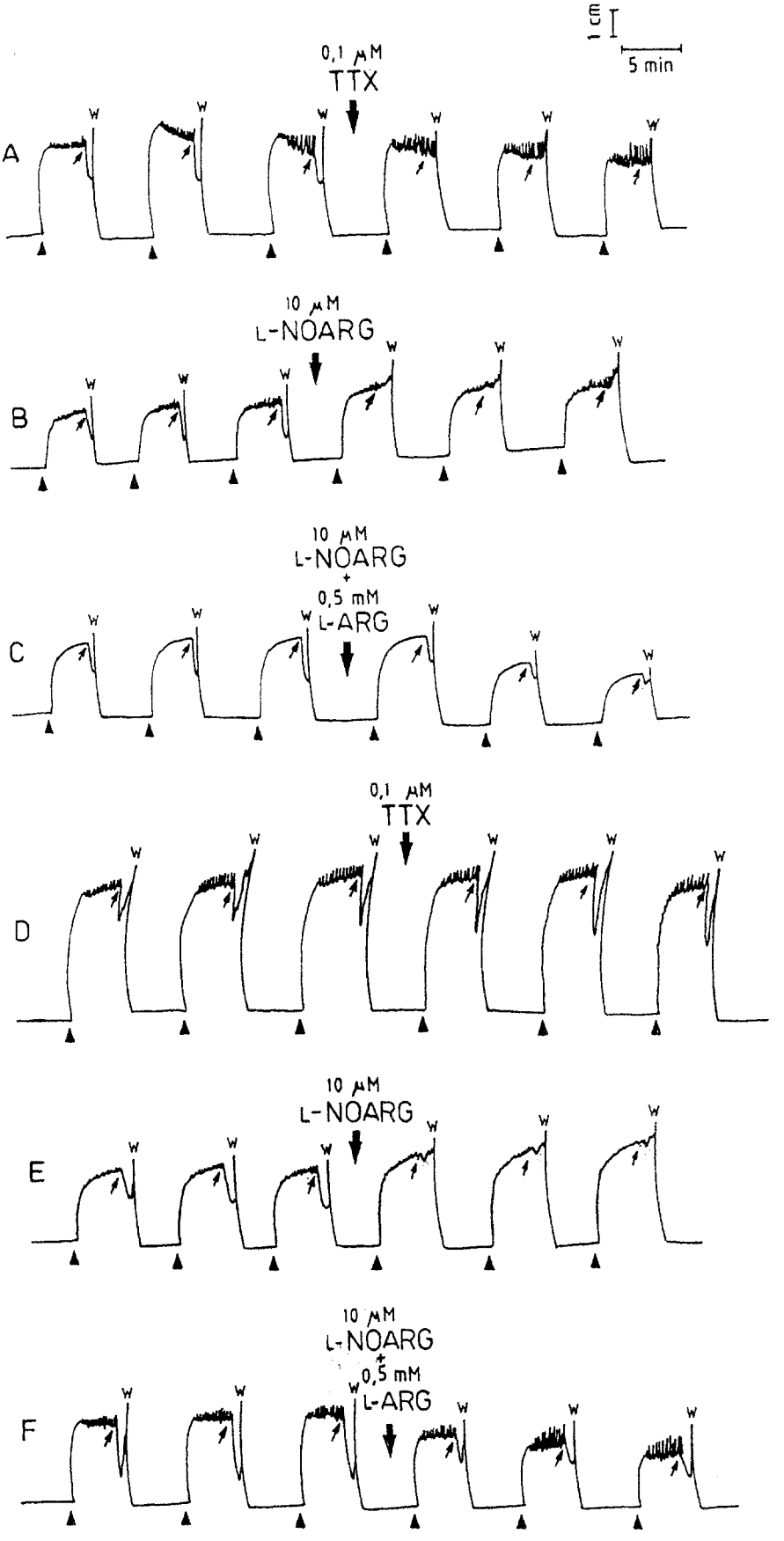


**Fig. 2** Representative tracings showing effects of 0.1 μM TTX, 10 μM L-NOARG or 10 μM L-NOARG + 0.5 mM L-arginine on relaxant actions of *A-C* EFS (10 Hz) or *D-F* 1 μM ACh in the mouse CC precontracted with 5 μM PE. *w* washout, ▲ PE application → EFS or relaxant drug application

**Table 1** Effects of various substances on the relaxations evoked by different stimuli in the mouse CC precontracted by 5 μM PE. Results (means ± SE) are presented as percentages of mean control values

| | EFS (10 Hz) | ACh (1 μM) | a-$NaNO_2$ (0.5 mM) |
|---|---|---|---|
| TTX (0.1 μM) | $0^{***}$ | 102 ± 3.5 | 106 ± 3.6 |
| Guanethidine (1 μM) | 106 ± 6.9 | 107 ± 10 | – |
| Indomethacin (1 μM) | 104 ± 4.7 | 112 ± 15 | – |
| Atropine (0.2 μM) | 90.3 ± 4.6 | $0^{***}$ | – |
| L-NOARG (10 μM) | 1.7 ± $0.6^{***}$ | 20.9 ± $2.0^{***}$ | 106 ± 0.9 |
| L-NOARG (10 μM) + L-ARG (0.5 mM) | 66.6 ± $13^{**}$ | 51.8 ± $7.5^{**}$ | – |
| MB (30 μM) | 66.1 ± $8.0^{**}$ | 73.5 ± $1.4^{*}$ | 60.3 ± $6.8^{**}$ |
| HC 130 μM) | 28.8 ± $2.1^{***}$ | 29.7 ± $3.7^{***}$ | 41.9 ± $1.8^{***}$ |
| HQ (0.5 mM) | 106 ± 12 | 108 ± 11 | 23.3 ± $3.2^{***}$ |

$^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ ($n = 4$–14)

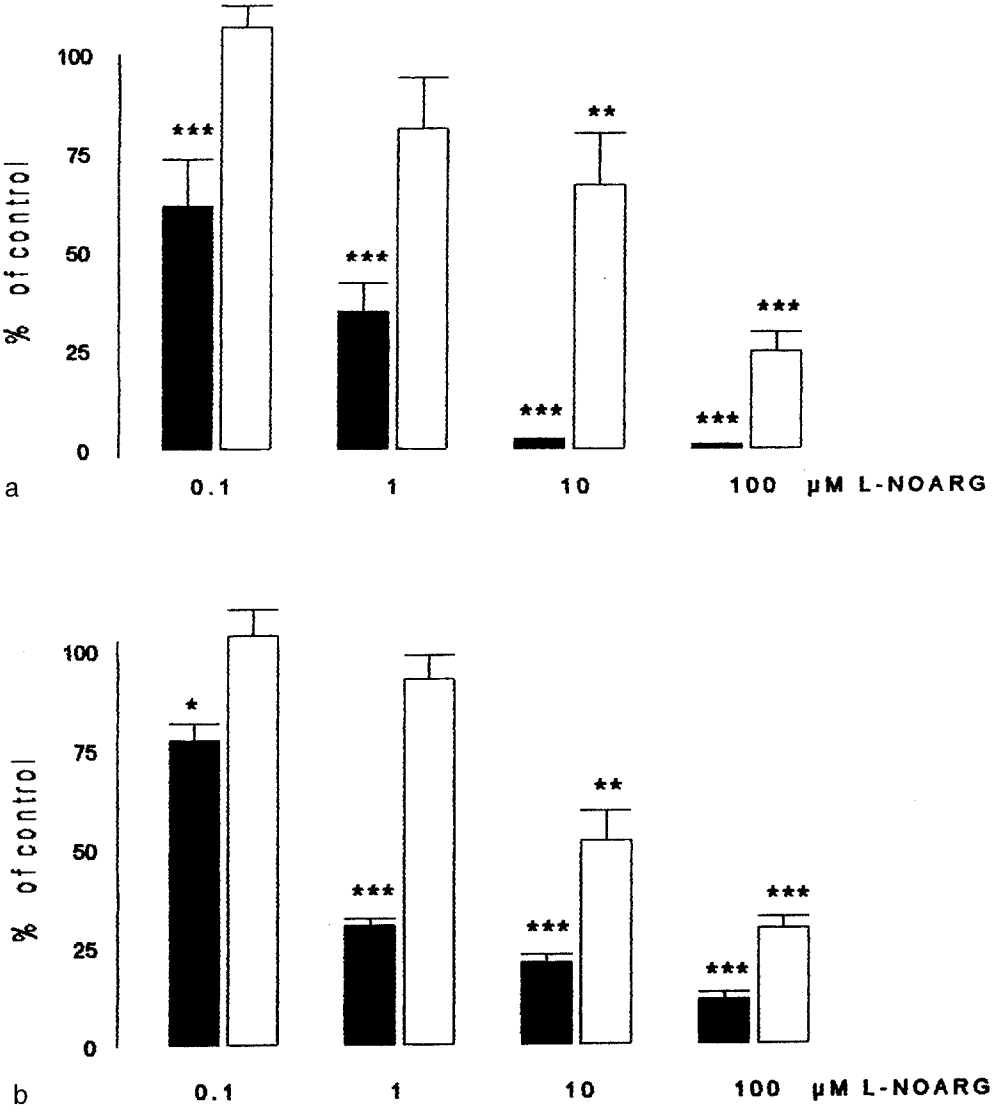


**Fig. 3a, b** Effects of L-NOARG (*solid bars*) or L-NOARG + 0.5 mM L-arginine (*open bars*) on relaxant actions of **a** EFS (10 Hz) or **b** 1 μM ACh in the mouse CC precontracted with 5 μM PE. *Each column* represents the mean relaxant response expressed as a percentage of mean control values. *Vertical bars* indicate SE. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ ($n = 6$–8)

was applied (Fig. 1A, $n = 10$). Likewise, ACh (1 μM) produced similar relaxations (Fig. 1B, $n = 10$). TTX (0.1 μM) prevented relaxations induced by EFS (Fig. 2A); however, the same treatment was found to be ineffective on the relaxant responses to ACh (Fig. 2D). a-$NaNO_2$ (0.5 μM) produced rapid relaxation, which was soon followed by contraction (Fig. 1C, $n = 10$). On testing with 0.1 μM TTX, relaxations showed no significant change (Table 1). All relaxations remained stable throughout the experiments. The mean values of the second series were not significantly different from those of the first series.

### Effects of guanethidine, indomethacin and atropine

Guanethidine (1 μM) or indomethacin (1 μM) did not affect the relaxant responses to either EFS or ACh (Table 1), but guanethidine suppressed the contractions that followed the relaxations (data not shown). With atropine (0.2 μM), the effect of EFS was reduced to 90.3 ± 4.6%, while the same substance completely inhibited the effects of ACh (Table 1). Guanethidine had no effect on the contracture induced by PE.

### Effects of L-NOARG, L-arginine and D-arginine

L-NOARG (0.1, 1, 10 and 100 μM) inhibited both electrically and ACh-induced relaxations in a dose-dependent manner (Fig. 2B,E, Fig. 3a,b), but it did not affect the relaxations produced by a-$NaNO_2$ (Table 1). The suppressive effect of L-NOARG was long lasting and only partially reversed by repeated washes with fresh Krebs solution. L-NOARG enhanced PE-induced contraction concentration – dependently without affecting baseline tension, and its highest concentration (100 μM) increased the amplitude of the contraction by 53.5 ± 6.8%. The inhibition due to L-NOARG on the EFS- or ACh-induced relaxations could be restored by 0.5 mM L-arginine (Fig. 2C,F, Fig. 3a,b), but not by 0.5 mM D-arginine (data not shown). Neither L-arginine nor D-arginine alone affected responses to EFS or ACh (data not shown).

### Effect of MB

Methylene blue at concentrations of 10 and 30 μM reduced the relaxation induced by EFS and a-$NaNO_2$ in a dose-dependent manner; however, the same substance was less effective on ACh-induced relaxations, and the reduction in the response was found to be statistically significant only at the concentration of 30 μM (Table 1).

### Effects of HC and HQ

The relaxation induced by EFS, ACh or a-$NaNO_2$ was significantly reduced by 65 or 130 μM HC (Fig. 4a). In addition, the rate of development of relaxation produced by a-$NaNO_2$ was decreased and the duration was prolonged. HC produced a concentration-dependent enhancement of PE contractions, but did not affect the baseline tension. HQ (0.1 and 0.5 mM) failed to yield an action on the relaxations induced by EFS or ACh, but reduced the relaxant effect of a-$NaNO_2$ (Fig. 4b). Also the same substance changed the characteristics of the relaxation to a-$NaNO_2$, as did HC. HQ did not affect the baseline, but caused some decrease in PE contracture.

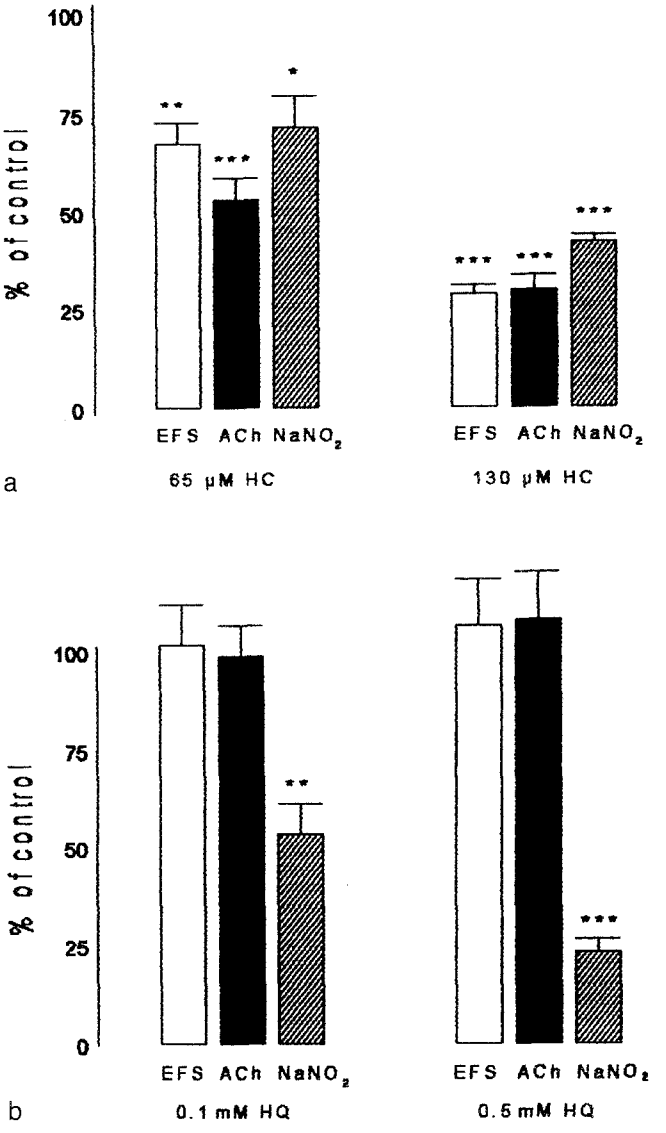


**Fig. 4a, b** Effects of **a** HC or **b** HQ on relaxant actions of EFS (10 Hz), ACh (1 μM) and a-$NaNO_2$ (0.5 mM) in the mouse CC precontracted with 5 μM PE. *Each column* represents the mean relaxant response expressed as a percentage of mean control values. *Vertical bars* indicate SE. $^{*}$ $P < 0.05$, $^{**}$ $P < 0.005$, $^{***}$ $P < 0.001$ ($n = 6$–8).

## Discussion

The results of the present study suggest that both relaxant factors released by nonadrenergic-noncholinergic (NANC) nerves and endothelial cells in mouse cavernosal tissue may not be free NO but may be an NO-generating substance.

A sodium channel blocker, TTX, blocked EFS-induced relaxation completely without affecting responses produced by ACh and a-$NaNO_2$, indicating intrinsic nerve stimulation is responsible for the relaxation due to EFS [13, 16]. Additionally, results of the experiments performed by using guanethidine and atropine revealed that these nerves may be mainly NANC nerves. On the

other hand, an atropine-sensitive relaxant response due to ACh suggests that a relaxant factor may also be released by endothelial cells in mouse CC [6, 11, 13, 21]. The failure of indomethacin to alternate relaxant response induced by EFS or ACh excludes a mechanism involving prostanoids.

To characterize the main mediator causing relaxations of CC, we tried a nitric oxide synthase inhibitor, L-NOARG. The substance abolished or markedly reduced EFS-evoked relaxations. It also caused significant reduction in ACh-induced relaxation, having little or no effect on the relaxant response to a-$NaNO_2$. The antagonism was stereospecific, because the inhibition due to L-NOARG could be restored by an NO precursor, L-arginine, but not by D-arginine. In addition, in the experiments performed with MB, which is known to be a guanylate cyclase inhibitor [15], a significant reduction was observed in relaxations evoked by EFS, ACh and a-$NaNO_2$ in a dose-dependent manner. Results observed in this part of the present study strongly suggest that mediator(s) released by EFS from NANC nerves and by ACh from endothelial cells may be endothelium-derived relaxing factor (EDRF)-like, free NO or NO-generating substance(s). This assumption has been already made in previous experiments carried out on other species such as humans and rabbits [7, 9–13, 16, 19].

On the other hand, HC can bind NO to form nitrosocobalamin. Its inhibitory action was demonstrated on NO-mediated relaxation in endothelium-intact aortic rings and rat anococcygeus muscle [14, 18, 20], but it was found to be ineffective on the relaxant response of the latter tissue to EFS. Based on these findings, it has been suggested that HC may be a discriminating tool between endothelium-dependent and NANC-induced response. However, in the present study, HC did inhibit a relaxant response of the mouse CC to EFS. The reason for this discrepancy is not clear, but it may be the result of a tissue-dependent phenomenon, since it has been demonstrated that HC did not affect the relaxation evoked by exogenously given nitrosothiols in the rat anococcygeus muscle while it inhibited the response induced by the same agents in rat aortic rings [20]. Therefore, HC seems to be useless in distinguishing endothelium from nerve-mediated response in the mouse CC. HC has vitamin activity and can enter the target smooth muscle cells, where it binds free NO coming from nitrergic transmitter or EDRF- like substance penetrating into the same cells.

HQ, which acts as a free radical scavenger or superoxide anion generator [5], significantly inhibited the response to a-$NaNO_2$, but not EFS- or ACh-induced relaxations. Similar results have been obtained with the bovine retractor penis muscle [4], the rat gastric fundus, guinea pig trachea and mouse anococcygeus [5], where HQ had no effect on the EFS-induced relaxation, whereas the response to NO (a-$NaNO_2$) in the presence of this drug was greatly influenced. The above effects of HQ may be explained by a mechanism whereby it reacts with NO in the extracellular milieu, where there is free NO produced by a-$NaNO_2$, while EFS and ACh may cause the release of an NO-containing substance and EDRF-like substance, respectively, which have to enter the cell and to undergo a reaction, thus leading NO generation and resultant relaxation. It is well known that pyrogallol is a charged substance which is unable to come into contact with NO generated intracellularly [8]. If a similar property was the case with HQ, this would explain why it reduced the response to a-$NaNO_2$, but not to those of EFS and ACh.

In conclusion, our results suggest that EFS-induced relaxations may involve an NO-containing substance (possibly an endogenous nitrate) and an EDRF-like substance (not free NO), respectively, originating from NANC nerves and endothelium, both of which have to enter the cell. It is difficult to use the present experimental evidence to support a view that free NO plays a role as the transmitter of NANC nerves in mouse penile erectile tissue

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