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Effect of neocuproine, a selective Cu(I) chelator, on nitrergic relaxations in the mouse corpus cavernosum

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Abstract

The effects of neocuproine and bathocuproine, Cu(I) and Cu(II) chelators, respectively, were studied on relaxations in response to electrical field stimulation, acetylcholine, *S*-nitrosoglutathione, acidified sodium nitrite and sodium nitroprusside in the mouse corpus cavernosum precontracted with phenylephrine. Neocuproine significantly inhibited relaxations induced by electrical field stimulation, acetylcholine and *S*-nitrosoglutathione, but not by acidified sodium nitrite and sodium nitroprusside. The pre-prepared neocuproine–Cu(I) complex was ineffective on the responses. The discrepancy between the shape of relaxations in response to electrical field stimulation or to acetylcholine and *S*-nitrosoglutathione was abolished by adding $CuCl_2$ into the bathing medium. The copper action was blocked by neocuproine but not by bathocuproine. However, the pre-prepared bathocuproine–Cu(II) complex did not accelerate the relaxations affected by $CuCl_2$. These findings suggest that a Cu(I)-dependent mechanism may play a role in the relaxation induced by the endogenous relaxant factor as well as by *S*-nitrosoglutathione in mouse cavernosal tissue.

Keywords: Neocuproine; Bathocuproine; Corpus cavernosum; *S*-Nitrosoglutathione; Non-adrenergic non-cholinergic (NANC) nerve; Nitrergic relaxation

1. Introduction

The role of nitric oxide in non-adrenergic non-cholinergic neurotransmission is now well established, but the exact nature of the nitrergic transmitter has remained unclear. It is suggested that, in some tissues, such as gastric fundus (Hobbs et al., 1991; Kitamura et al., 1993; Barbier and Lefebvre, 1994; Rand and Li, 1995), mouse anococcygeus (Hobbs et al., 1991; Gibson et al., 1992) and bovine retractor penis (Gillespie and Sheng, 1990; Liu et al., 1994) muscles, the actual nitrergic neurotransmitter might not be free nitric oxide but a superoxide-resistant, nitric oxide-carrying molecule, such as *S*-nitrosothiols. However, results of subsequent studies performed on the same tissues and on rat anococcygeus muscle, and the canine ileocolonic junction revealed that the nitrergic neurotransmitter may be free nitric oxide but not an *S*-nitrosothiol (Liu and Szurszewski, 1994; Martin et al., 1994; Lilley and Gibson, 1995; De Man et al., 1995, 1996, 1998). On the other hand, we had suggested that the relaxant factor released from non-adrenergic non-cholinergic nerves (tetrodotoxin-sensitive) or endothelial cells in mouse cavernosal tissue displays an *S*-nitrosoglutathione-like character (Göçmen et al., 1997, 1998). Recently, results of a study on sheep urethra suggest that the urethral nitrergic transmitter may not be free nitric oxide (Garcia-Pascual et al., 2000). In addition, it has been suggested that a cell-mediated mechanism for the biotransformation of *S*-nitrosoglutathione is present in some cell types (Gordge et al., 1998). Some studies showed a Cu(I)-dependent mechanism to be responsible for the release of nitric oxide from endogenous nitrosothiols (Gordge et al., 1996; Al-Sa'doni et al., 1997). These results prompted us to investigate whether there is an actual contribution of a similar Cu(I)-dependent mechanism for the relaxant factor released from non-adrenergic non-cholinergic nerves or endothelial cells in mouse cavernosal tissue. We thus studied the effect of a selective Cu(I) chelator, neocuproine

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(2,9-dimethyl-1,10-phenanthroline), on relaxations induced by electrical field stimulation, acetylcholine, *S*-nitrosoglutathione, acidified sodium nitrite and sodium nitroprusside compared with the effect of bathocuproine, a Cu(II) chelator (De Man et al., 1996) on the relaxations in response to all these stimuli.

2. Materials and method

2.1. Organ bath experiments

Male albino mice weighing 30–35 g were killed by cervical dislocation. Penises were removed and placed in a Petri dish containing Krebs solution (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, $NaHPO_4$ 1.2, glucose 11). Corpus cavernosum was prepared according to a previously described method (Göçmen et al., 1997). The preparations were mounted under 0.2-g tension in 5-ml organ baths maintained at 37°C and containing Krebs solution aerated with 95% O_2 and 5% CO_2. The tissues were allowed to equilibrate for 1 h. During this period, the preparations were washed with fresh Krebs solution at 15-min intervals. The responses were recorded on a polygraph (Ugo Basile, Gemini 7070) via an isotonic transducer (Ugo Basile, 7006).

After the equilibration period, the tissues were treated with 5 μM phenylephrine. This resulted in an active tone that reached a stable level within 5 min; at the end of this period, electrical field stimulation delivered as square waves (2–8 Hz; 30 V, 0.5 ms) by a Grass S88 stimulator via two parallel platinum electrodes embedded in Perspex was applied to the tissue for 15 s at 2-min intervals. In experiments in which electrical field stimulation was used, atropine (0.2 μM) and guanethidine (1 μM) were always present in the bathing medium to obtain non-adrenergic non-cholinergic conditions. In some experiments, *S*-nitrosoglutathione (2, 10 or 100 μM), acidified sodium nitrite (50, 100 or 500 μM) or sodium nitroprusside (0.05, 0.1 or 1 μM) was added to the bathing medium after the contraction reached a steady state. Exposure time of the tissue was 2 min for *S*-nitrosoglutathione and 1 min for sodium nitroprusside and acidified sodium nitrite since relaxations reached a steady state within these periods. Following each application of these chemicals, recording was stopped and the tissue was washed. After 15 min, phenylephrine was added to the bathing medium and recording was restarted when tissue tone had returned to near its previous level. In one series of experiments, acetylcholine was cumulatively re-added to the organ bath in concentrations of 0.1–1 μM. Thus, the first series of responses were obtained. After the tissue was left to rest for 30 min, the second series of

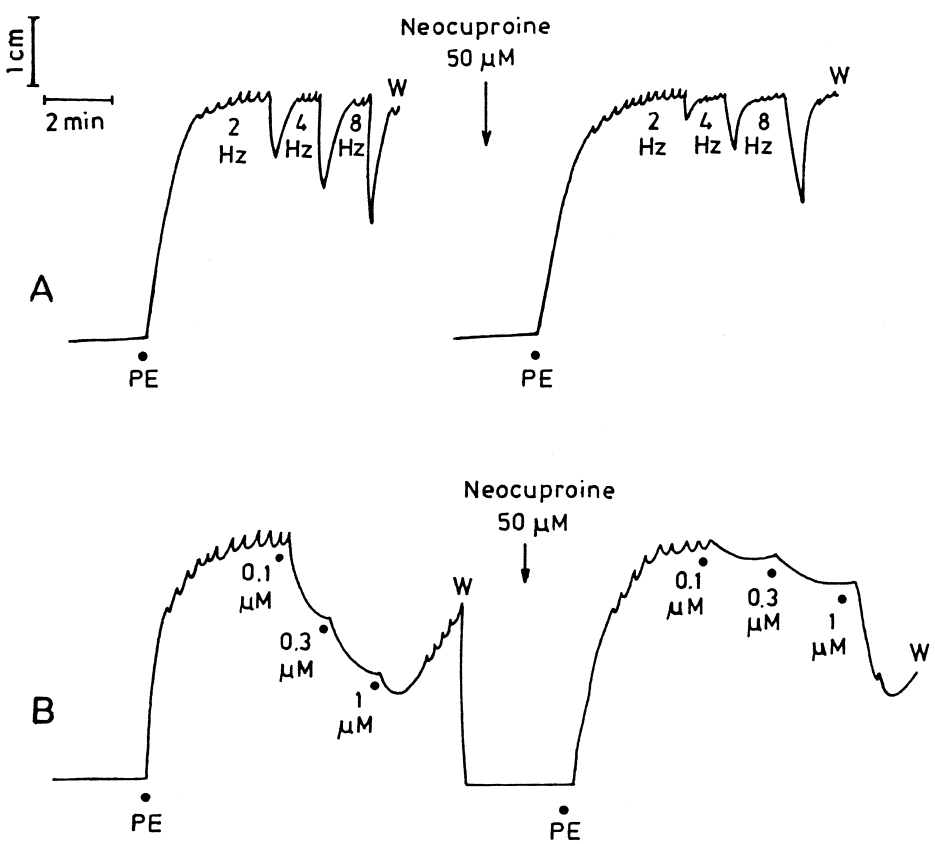

Fig. 1. Representative tracings showing the relaxant effects of electrical field stimulation (A) or acetylcholine (B) in the presence of neocuproine (50 μM on the mouse corpus cavernosum precontracted with 5 μM phenylephrine (PE). (w) Washout.

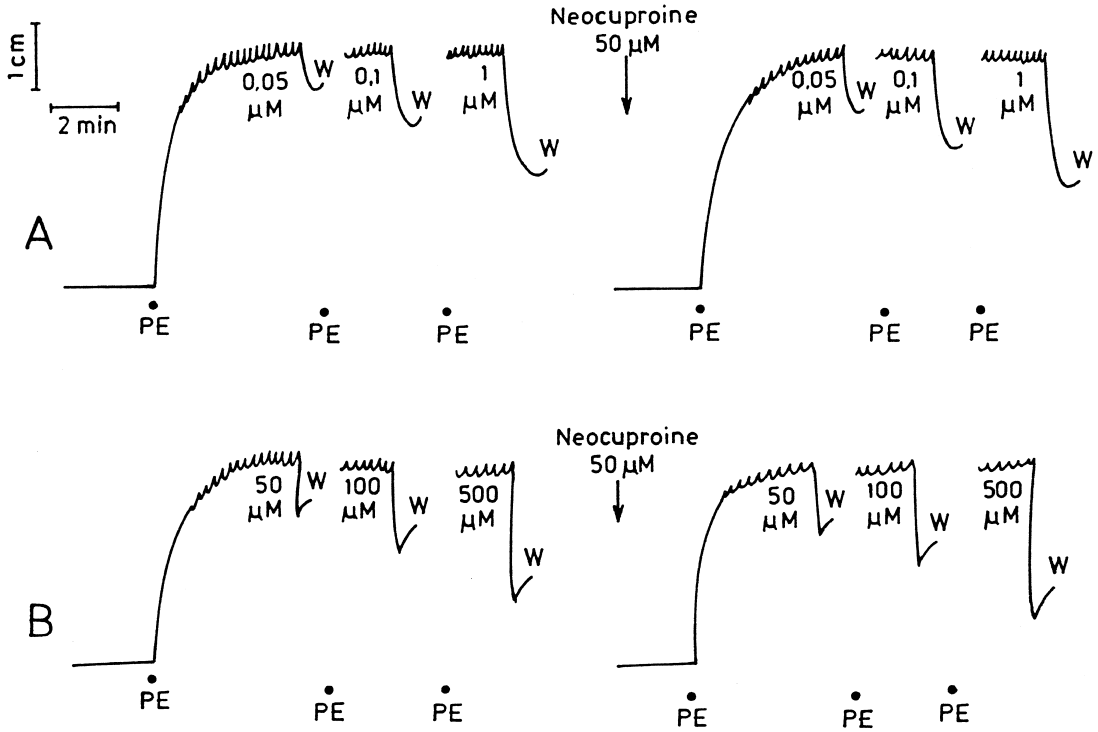

Fig. 2. Representative tracings showing the relaxant effects of sodium nitroprusside (A) or acidified sodium nitrite (B) in the presence of neocuproine (50 μM) on the mouse corpus cavernosum precontracted with 5 μM PE. (w) Washout.

responses were recorded as control in the same manner. In some trials, after the first series of responses were recorded, the preparation was replaced into medium with neocuproine (10 or 50 μM), bathocuproine (50 or 100 μM), $CuCl_2$ (10 or 25 μM), glutathione (10 or 25 μM), $CuCl_2$ (10 μM) + glutathione (10 μM), $CuCl_2$ (25 μM) + glutathione (25 μM), $CuCl_2$ (10 μM) + neocuproine (50 μM), $CuCl_2$ (10 μM) + bathocuproine (100 μM), pre-prepared neocuproine–Cu(I) complex, pre-prepared bathocuproine–Cu(II) complex and the second series of relaxation due to chemicals or electrical field stimulation was examined. The neocuproine–Cu(I) complex (50 μM) was prepared by reacting glutathione, neocuproine and $CuCl_2$ in molar ratios of 1:2:1, respectively (Dicks et al., 1996). The bathocuproine–Cu(II) complex was prepared by reacting 100 μM bathocuproine and 10 μM $CuCl_2$. The incubation time for this substance was 30 min except for bathocuproine. The incubation time with bathocuproine was 1 h.

2.2. Drugs and solutions

Stock solutions of atropine sulphate, phenylephrine, acetylcholine, sodium nitroprusside, $CuCl_2$, glutathione, neocuproine and bathocuproine were diluted in distilled water. *S*-nitrosoglutathione solution was prepared immediately before use and kept at −4°C. Guanethidine was dissolved in dimethyl sulfoxide (0.1% v v^{-1}). Nitric oxide

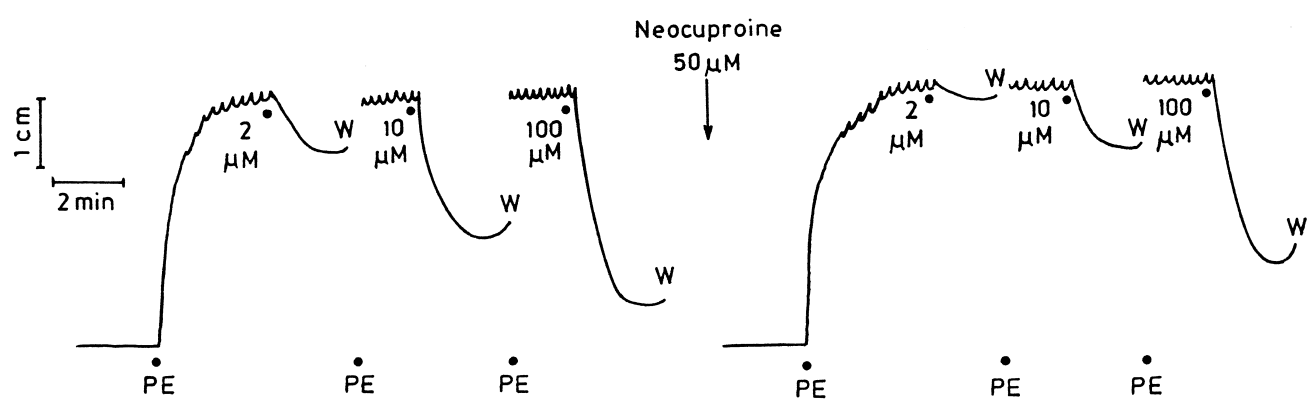

Fig. 3. Representative tracings showing the relaxant effects of *S*-nitrosoglutathione (GSNO) in the presence of neocuproine (50 μM) on the mouse corpus cavernosum precontracted with 5 μM PE. (w) Washout.

was present as acidified solution (pH 2) of $NaNO_2$ and stored at −4°C. Except $NaNO_2$ (Merck), all drugs were obtained from Sigma.

2.3. Statistical considerations

Relaxations were calculated as percentage peak reductions of the contraction elicited by phenylephrine. The values from the second series were expressed as percent of the first series. Mean values (mean ± S.E.) for the second series were calculated separately for each experimental group. All data were evaluated with the Bonferroni-corrected *t*-test that was used in the one way analysis of variance (ANOVA). *P* values of less than 0.05 were considered to be significant.

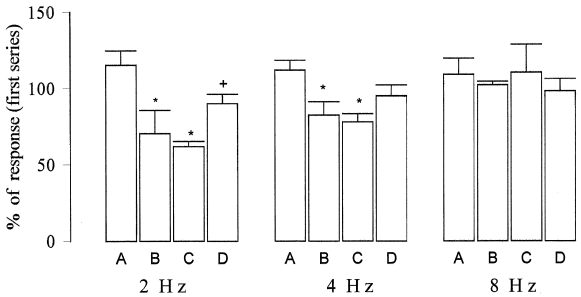

Fig. 5. The relaxant responses to electrical field stimulation in the mouse corpus cavernosum precontracted with 5 μM PE from (A) control, (B) 10 μM neocuproine, (C) 50 μM neocuproine or (D) 50 μM neocuproine–Cu(I) complex. * Indicates significant differences between control and neocuproine-treated groups; + indicates significant differences between 50 μM neocuproine-treated and neocuproine–Cu(I) complex groups. Both * and +, $P < 0.05$ ($n = 12–24$).

3. Results

3.1. Relaxant effects of electrical field stimulation, acetylcholine, acidified sodium nitrite, sodium nitroprusside and S-nitrosoglutathione in the mouse corpus cavernosum

Electrical field stimulation (2–8 Hz), acetylcholine (0.1–1 μM), sodium nitroprusside (0.05–1 μM) and exogenous nitric oxide, applied as acidified sodium nitrite (50–500 μM) all relaxed the tissues in a frequency- or concentration-dependent manner (Figs. 1A,B and 2A,B). The *S*-nitrosoglutathione (2–100 μM)-induced reproducible relaxations were relatively slow to develop when compared to electrically induced relaxation (Fig. 3). However, the start of the relaxation induced by *S*-nitrosoglutathione became much more rapid after addition of 10 μM $CuCl_2$ into the bath (Fig. 4). On the other hand, glutathione did not affect relaxations induced by *S*-nitrosoglutathione in the presence or absence of $CuCl_2$ (not shown). Glutathione was also ineffective on the relaxations evoked by all other stimuli (not shown).

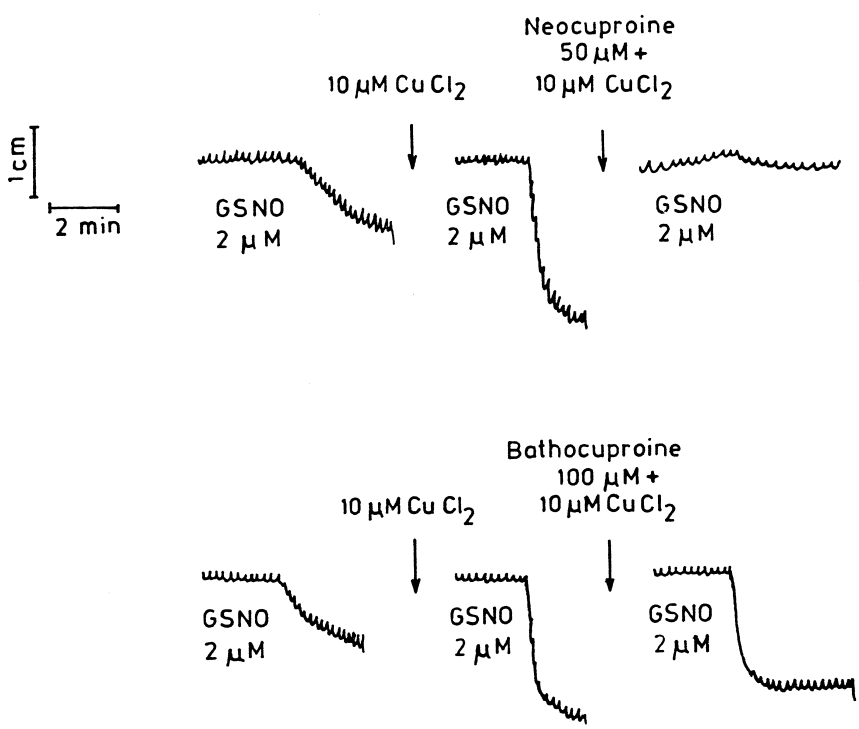

Fig. 4. Representative tracings showing the effects of 10 μM $CuCl_2$, 50 μM neocuproine + 10 μM $CuCl_2$ or 100 μM bathocuproine + 10 μM $CuCl_2$ on the relaxations induced by 2 μM GSNO on the mouse corpus cavernosum precontracted with 5 μM PE.

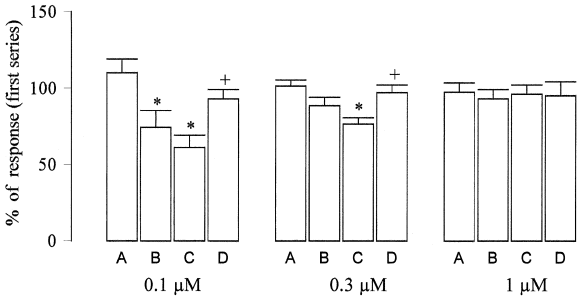

Fig. 6. The relaxant responses to acetylcholine in the mouse corpus cavernosum precontracted with 5 μM PE from (A) control, (B) 10 μM neocuproine, (C) 50 μM neocuproine or (D) 50 μM neocuproine–Cu(I) complex. * Indicates significant differences between control and neocuproine-treated groups; + indicates significant differences between 50 μM neocuproine-treated and neocuproine–Cu(I) complex groups. Both * and +, $P < 0.05$ ($n = 6$–14).

3.2. Effect of neocuproine

Neocuproine at a concentration (10 or 50 μM) ineffective on the phenylephrine-induced contraction, significantly inhibited the electrically induced relaxations at 2 and 4 Hz, but not at a high frequency, 8 Hz, in a concentration-dependent manner (Figs. 1A and 5). The agent also caused a significant inhibition of the relaxation induced by lower concentrations of acetylcholine (0.1 and 0.3 μM), but not of that with the higher concentration of 1 μM (Figs. 1B and 6). Similarly, responses of the tissue to lower concentrations of *S*-nitrosoglutathione (2 and 10 μM) were significantly decreased by neocuproine, while there was a slight and insignificant reduction in the relaxation due to the higher *S*-nitrosoglutathione concentration, 100 μM (Figs. 3 and 7). In addition, the Cu(I) chelator significantly reduced the enhanced responses to *S*-nitrosoglutathione in the presence of 10 μM $CuCl_2$ (Fig. 4). On the other hand, neocuproine did not affect the relaxation produced by acidified sodium nitrite or sodium nitroprusside (Fig. 8). The inhibitory effect of neocuproine was reversible, inhibitions were abolished after the agent was removed from the bathing medium by washing with fresh Krebs solution (not shown).

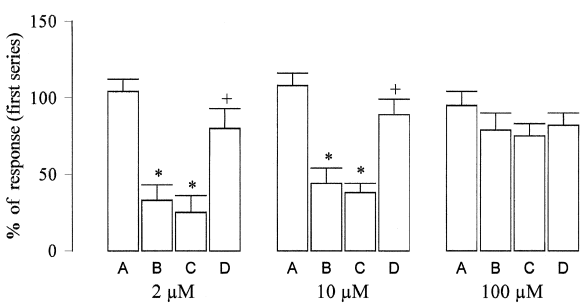

Fig. 7. The relaxant responses to GSNO in the mouse corpus cavernosum precontracted with 5 μM PE from (A) control, (B) 10 μM neocuproine, (C) 50 μM neocuproine or (D) 50 μM neocuproine–Cu(I) complex. * Indicates significant differences between control and neocuproine-treated groups; + indicates significant differences between 50 μM neocuproine-treated and neocuproine–Cu(I) complex groups. Both * and +, $P < 0.05$ ($n = 6$–12).

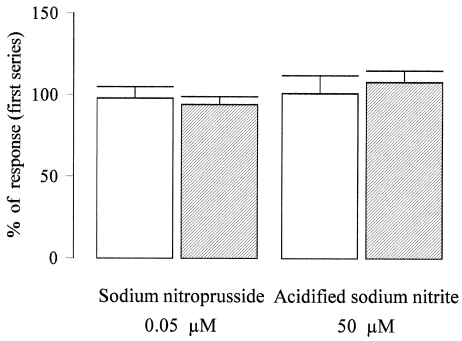

Fig. 8. The relaxant responses to sodium nitroprusside and acidified sodium nitrite in the absence (open bars) and presence of 50 μM neocuproine (hatched bars) in the mouse corpus cavernosum precontracted with 5 μM PE. * $P < 0.05$ ($n = 6$–14).

3.3. Effect of neocuproine–Cu(I) complex

Pre-prepared neocuproine–Cu(I) complex had no effect on the phenylephrine-induced contraction. Neocuproine–Cu(I) complex was ineffective on electrical field stimulation- (Fig. 5), acetylcholine- (Fig. 6) and *S*-nitrosoglutathione- (Fig. 7) induced relaxations as well as on responses to the other chemicals (not shown).

3.4. Effect of bathocuproine

Incubation of the tissue with bathocuproine (50 and 100 μM) for 60 min did not significantly affect relaxations evoked by electrical field stimulation, acetylcholine and *S*-nitrosoglutathione (Table 1). It also did not alter responses induced by acidified sodium nitrite and sodium nitroprusside (not shown). The Cu(II) chelator did not affect the increase in relaxation rate due to *S*-nitrosoglutathione in the presence of 10 μM $CuCl_2$ (Fig. 4) when pre-prepared bathocuproine–Cu(II) complex was used instead of $CuCl_2$, no alteration was observed in the response to *S*-nitrosoglutathione (not shown). At the concentrations used, incubation of the tissue with bathocuproine for 60 min did not affect the contraction induced by phenylephrine.

Table 1
Effects of bathocuproine (100 μM, for 1-h incubation) on the relaxant responses to electrical field stimulation (EFS; 2 Hz), acetylcholine (ACh; 0.1 μM) and *S*-nitrosoglutathione (GSNO; 2 μM) in the mouse corpus cavernosum precontracted with 5 μM phenylephrine

	EFS	ACh	GSNO
Control	104.9 ± 12.0	111.9 ± 14.0	99.2 ± 9.70
+ Bathocuproine	94.0 ± 7.98	86.7 ± 17.3	88.9 ± 11.2

Results are expressed as percentage decreases of the phenylephrine-induced contraction and are shown as percent of the first series (mean ± S.E.) for $n = 4$–8 experiments.

4. Discussion

The present results suggest that a Cu(I)-dependent mechanism may play a role in the relaxation induced by especially small amounts of the nitrergic relaxant factor released from non-adrenergic non-cholinergic nerves or endothelial cells, and that, in the mouse corpus cavernosum, this relaxant factor may have some properties resembling those of *S*-nitrosoglutathione. Neocuproine, a Cu(I) chelator, significantly inhibited electrical field stimulation- and acetylcholine-induced relaxation. The agent caused a similar alteration in the relaxation produced by *S*-nitrosoglutathione, while it had no effect on responses to acidified sodium nitrite and sodium nitroprusside, indicating that the behaviour of the endogenous nitrergic mediator is similar to that of *S*-nitrosoglutathione in the presence of neocuproine. The inhibitory action of neocuproine seems to result from its ability to bind Cu(I) since no effect was observed when it was used as the neocuproine–Cu(I) complex. Additionally, the ineffectiveness of bathocuproine, a Cu(II) chelator, on responses to all relaxant stimuli suggests that a mechanism requiring the reduced form of copper may be important.

The importance of a Cu(I)-dependent mechanism may be related to its role in the generation of nitric oxide from *S*-nitrosoglutathione Although by which mechanisms *S*-nitrosothiols deliver nitric oxide to target cells is still unclear, it has been demonstrated that their biological activity is mediated by a nitric oxide/cyclic GMP-dependent pathway (Lieberman et al., 1991). There is some evidence that decomposition of *S*-nitrosothiols, yielding bioactive nitric oxide, is governed by an enzymatic rather than a spontaneous process (Kowaluk and Fung, 1990; Radomski et al., 1992) and that this decomposition may be controlled by membrane enzymes or receptors in some target cells (Mathews and Kerr, 1994). In subsequent studies, it was shown that the biological activity of *S*-nitrosothiols can be modulated by copper (Askew et al., 1995; Gordge et al., 1995), and that the anti-platelet action of *S*-nitrosoglutathione, a stable *S*-nitrosothiol compound, may be mediated by a copper-dependent enzymatic mechanism (Gordge et al., 1996). In addition, some evidence has been provided from a study on the perfused rat tail artery that a Cu(I)-dependent mechanism is responsible for the liberation of nitric oxide from endogenous nitrosothiols (Al-Sa'doni et al., 1997). Based on these findings and on the results of the present study, it can be said that, in the mouse corpus cavernosum, a Cu(I)-dependent mechanism may be involved in the relaxation evoked by either neurogenic stimulation or by acetylcholine, similar to the relaxation in response to *S*-nitrosoglutathione. On the other hand, the ineffectiveness of neocuproine on the relaxation evoked by acidified sodium nitrite and sodium nitroprusside shows that these agents do not require the activity of a Cu(I)-dependent mechanism to exert their effects, suggesting that the nitrergic mediator does not seem to be free nitric oxide in the mouse corpus cavernosum. Findings supporting our results have been obtained from a study on sheep urethra, suggesting that the urethral transmitter may not be free nitric oxide (Garcia-Pascual et al., 2000), while results of a study on rat gastric fundus do not favor this view (De Man et al., 1999).

An interesting finding in the present study was that neocuproine did not significantly affect relaxations due to the high frequency of electrical field stimulation and the high concentration of acetylcholine. This finding suggests that the contribution of the Cu(I)-dependent mechanism to the response may be decreased or abolished when the amount of endogenous nitrergic mediator released is increased by increasing the frequency of the electrical field stimulation or by elevating the concentration of acetylcholine. Similar results were observed with *S*-nitrosoglutathione. This may be additional proof that the endogenous relaxant factor and *S*-nitrosoglutathione behave the same and was under the influence of neocuproine. In a previous in vitro study, it was demonstrated that the nitric oxide generation rate is decreased as the concentration of *S*-nitrosoglutathione is increased in the presence of copper, confirming our results (Noble et al., 1999).

However, the rate of the exogeneous *S*-nitrosoglutathione-induced relaxation was slower than that with electrical field stimulation- or acetylcholine-evoked relaxation. One of the possible reasons for this difference is the time that *S*-nitrosoglutathione needs to reach the biophase, where the functional Cu(I)-dependent mechanism is present. Thus, limitation of the relaxation rate can be expected because of slow penetration of the agent to the decomposition site. Indeed, the *S*-nitrosoglutathione-evoked relaxation became much more rapid when the tissue was preincubated with $CuCl_2$. The action of copper was abolished by neocuproine added into the incubation medium but not by bathocuproine. On the other hand, acceleration by $CuCl_2$ of the relaxation rate was not observed in the presence of the bathocuproine–Cu (II) complex. This result indicates that Cu^{2+} may be reduced to Cu^{+} in the bathing medium. In a previous in vitro experiment, it was shown that amino acid- and protein-bound Cu^{2+} can be reduced by thiolate ion to Cu^{+} (Dicks and Williams, 1996). A similar reduction may have occurred under our experimental conditions. Thus, *S*-nitrosoglutathione can rapidly yield nitric oxide under the influence of Cu(I), before reaching the decomposition site and this may accelerate the relaxation rate. In the study on the rat gastric fundus, addition of $CuSO_4$ or $CuCl_2$ into the bathing medium caused a rapid and transient relaxation in the presence of *S*-nitrosoglutathione (De Man et al., 1996). This finding confirms our results. The site of decomposition may be located at or near some particular regions where the nitrergic mediator is released. If so, this may explain, not only why the relaxation rate in response to exogenous *S*-nitrosoglutathione is slower than that induced by electrical field stimulation or acetylcholine, but also

why $CuCl_2$ failed to alter the shape of responses to electrical field stimulation and acetylcholine, since the transmitter was already in contact with the Cu(I)-dependent mechanism and needs no copper supplementation. However, further studies are needed to support this hypothesis. On the other hand, glutathione alone did not exhibit any action on the relaxations evoked by any of the stimuli used. One can expect that the agent may capture nitric oxide generated from acidified sodium nitrite and cause a decrease in the relaxation rate. However, there was no observation indicating the occurrence of this type of reaction in the present study. The possibility is that nitric oxide itself may not react with the thiol within a short time under our experimental conditions (Williams, 1999).

In conclusion, the results of the present study provide additional evidence that the behaviour of the nitrergic mediator is similar to that of *S*-nitrosoglutathione in the mouse corpus cavernosum. Also, these findings support the view suggested by De Man et al. (1999) that neocuproine is a useful tool to elucidate the nature of the nitrergic transmitter. Further studies on identification and localisation of the Cu(I)-dependent mechanism may help to understand the reason for the different behaviour of the nitrergic transmitter in various tissues.

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