Carbon monoxide-induced relaxation and distribution of haem oxygenase isoenzymes in the pig urethra and lower oesophagogastric junction

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1 The distribution of the carbon monoxide (CO) producing enzymes haem oxygenase (HO)-1 and -2 was studied by immunohistochemistry in the pig’s lower urinary tract, including bladder extramural arteries, and the oesophagogastric junction (OGJ). In isolated smooth muscle from the urethra and the OGJ, the mechanisms for CO-induced relaxations were characterized by measurement of cyclic nucleotide levels and by responses to the guanylate cyclase inhibitor methylene blue and some K+ channel inhibitors.

2 HO-2 immunoreactivity was observed in coarse nerve trunks within the smooth muscle of the urethra and OGJ, and in nerve cell bodies of the enteric plexuses of the OGJ. Furthermore, the vascular endothelium of the intramural vessels of the urethra, bladder and OGJ, and the extramural vessels of the bladder, displayed HO-2 immunoreactivity. Two different antisera against HO-1 were used, but only one displayed immunoreactivity in neuronal structures. HO-1 immunoreactivity, as displayed by this antisera, was seen in nerve cells, coarse nerve trunks and varicose nerve fibres in the smooth muscle of the urethra and OGJ. Some HO-2 and/or HO-1 (as displayed by both HO-1 antisera) immunoreactive cells with a non-neuronal appearance were observed within the smooth muscle of the OGJ, bladder and urethra.

3 In the urethral preparations, exogenously applied CO (72 μM) evoked a relaxation amounting to 76 ± 6%. The relaxation was associated with an increase in cyclic GMP, but not cyclic AMP, content. CO-evoked relaxations were not significantly reduced by treatment with methylene blue, or by inhibitors of voltage-dependent (4-aminopyridine), high (iberiotoxin, charybdotoxin) and low (apamin) conductance Ca2+-activated, and ATP-sensitive (glibenclamide) K+ channels. Bladder strips, and ring preparations from the extramural arteries of the bladder, did not respond to exogenously administered CO (12 – 72 μM).

4 In the OGJ, exogenously applied CO evoked a relaxation of 86 ± 6%, which was associated with an increase in cyclic GMP, but not cyclic AMP, content. Treatment with 30 μM methylene blue raised the spontaneously developed muscle tone, and reduced the maximum relaxation evoked by CO to 33 ± 9%. Addition of 4-aminopyridine, apamin, glibenclamide, iberiotoxin, charybdotoxin or glibenclamide had no effect on the relaxations. 4-aminopyridine (0.1 – 1 mM), iberiotoxin (0.1 μM) and charybdotoxin (0.1 μM) increased the spontaneously developed tone, and a combination of charybdotoxin and apamin reduced CO-induced (24 μM CO) relaxations.

5 The present findings demonstrate the presence of HO in both neuronal and non-neuronal cells in the pig OGJ and lower urinary tract. CO produces relaxation of the smooth muscle in the OGJ and urethra, associated with a small increase in cyclic GMP concentration in both regions. Relaxations evoked by CO in the urethra do not seem to involve voltage-dependent, low and high conductance, or ATP-dependent K+ channels. However, in the OGJ relaxations evoked by CO can be attenuated by methylene blue and a combination of charybdotoxin and apamin.

Keywords: Carbon monoxide; cyclic nucleotides; haem oxygenase; lower oesophagogastric junction; lower urinary tract; potassium channels; smooth muscle relaxation

Introduction

The circular smooth muscle of the lower oesophagus and female urethra has a sphincter function. Preparations from both regions develop active tension spontaneously, and their inhibitory control is considered to be non-adrenergic non-cholinergic (NANC) in origin and partly mediated by nitric oxide (NO; Daniel, 1992; Andersson & Persson, 1993). However, an NO-independent mechanism for relaxation has been demonstrated in the pig urethra (Bridgewater et al., 1993; Werkström et al., 1995) and cat lower oesophageal sphincter (LOS; Ny et al., 1995a), which has indicated other putative neuromessengers in these regions. One candidate is carbon monoxide (CO), which has been suggested to be involved in neuromuscular control of gastrointestinal sphincters, including the opossum internal anal sphincter (Rattan & Chakder, 1993) and the feline LOS (Ny et al., 1995c; 1996). A main pathway for production of CO is the degradation of haem to biliverdin and CO, a reaction catalysed by the enzyme haem oxygenase (HO). This enzyme is considered to exist in at least two isoforms, designated HO-1 and HO-2 (Maines, 1988). The activity of HO-1, or heat shock protein (HSP) 32, localized to the spleen and liver, is induced by various agents such as haem and metal ions. HO-2 is constitutive, and has been demonstrated predominantly in the testis and brain (Maines, 1988; 1993). It has been suggested that CO is a neuromessenger in the brain (Verma et al., 1993) as well as in the periphery (Ny et al., 1995c; Rattan & Chakder, 1993; Zakhary et al., 1996). CO is believed to cause relaxation of smooth muscle by activation of soluble guanylate cyclase and formation of intracellular cyclic GMP (Ramos et al., 1989; Furchgott & Jothianandan, 1991; Lefer et al., 1993). Yet another proposed mechanism for the action of CO is modulation of K+ currents, as demonstrated in human jejunal smooth muscle (Farrugia et al., 1993).
Experimental protocol

The urethral preparations were mounted and stretched to a tension of 10 mN. The OGJ preparations were stretched from resting length (L₀) until about 180% of L₀ was reached. This induced a tension of 45 ± 5 mN (n = 10). The strips were left to equilibrate for at least 45 min. To establish a 100% relaxation level, i.e. ‘0-level’, the urethral and OGJ preparations were exposed to Ca²⁺-free Krebs solution (for composition see Solutions). After addition of Ca²⁺-containing Krebs solution, a stable tone was re-established. Only preparations that developed a spontaneous tension were used for further experiments. Relaxant responses to exogenously applied CO (6–72 µM) were recorded and served as controls. After washout, the preparations were incubated for 30 min with either methylene blue (30 µM), 4-amino pyridine (0.1–1 mM), glibenclamide (1 µM), iberiotoxin (0.1 µM), charybdotoxin (0.1 µM), apamin (0.1 µM), or a combination of charybdotoxin (0.1 µM) and apamin (0.1 µM) before a second concentration-response curve to CO was recorded. The concentrations of the blockers were chosen on the basis of pilot experiments and previously published data (Zygmunt & Högestätt, 1996; Zygmunt et al., 1996). Each preparation was exposed to only one treatment, and parallel experiments without treatment were run as controls.

Vessel segments were stretched to a passive tension of 2–4 mN, and bladder strips were stretched to 4–6 mN. Each experiment was started by exposing the preparations to a K⁺ (124 mM) Krebs solution (see Solutions), to verify their contractile capacity. For studies of possible relaxant effects of CO, the vessel segments and bladder strips were precontracted by (-)-noradrenaline (NA; 1 µM) and carbacchol (10 µM), respectively.

Measurement of cyclic AMP and cyclic GMP levels

Urethral and OGJ strips were rapidly frozen in liquid nitrogen when maximum relaxations in response to CO (72 µM) were obtained. The tissue was homogenized at 4°C in 2 ml of 10% trichloroacetic acid with a hand glass homogenizer, and centrifuged at 1500 g (4°C) for 10 min. The pellet was reconstituted in 2 M NaOH and assayed for protein content (Bradford, 1976), with bovine serum albumin as a standard. The trichloroacetic acid in the supernatant was removed by five successive extractions with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate and the amount of guanosine 3',5'-cyclic monophosphate (cyclic GMP) or adenosine 3',5'-cyclic monophosphate (cyclic AMP) was quantitated by use of [³²P]-cyclic GMP and [³²P]-cyclic AMP radioimmunoassay kits (DuPont, Wilmington, DEL, U.S.A.) according to kit instructions. All determinations of cyclic nucleotide levels were made in duplicate. Cyclic nucleotides were acetylated with acetic anhydride to increase the sensitivity of the assay. The final values of tissue cyclic AMP were corrected for trace amounts of [¹H]-cyclic AMP, used to determine recovery (70%).

Calculations and statistics

The relaxant effects of CO are expressed as percentage reduction in tension. Results are expressed as mean values ± s.e.m. Statistical analysis of data was performed by Student’s two-tailed t test. In the case of multiple comparisons, a one-way analysis of variance (ANOVA) was used, and subsequently P values were determined by an unpaired Student’s t test corrected for multiple comparisons by Bonferroni’s method. A probability value of P < 0.05 was regarded as significant. (n) is the number of strips tested, and, when not specifically indicated, refers to tissues from different animals. When statistical analyses between means were performed, all values refer to different animals.
Solutions

The Krebs solution had the following composition (mm): NaCl 119, KCl 4.6, CaCl2 1.5, MgCl2 1.2, NaHCO3 15, NaH2PO4 1.2 and glucose 11. Ca2+ free Krebs solution was prepared by omitting CaCl2 and adding EGTA (0.1 mM) and K+ (124 mM). Krebs solution was prepared by replacing NaCl with equimolar amounts of KCl.

Drugs

The following drugs were used: (-)-noradrenaline hydrochloride (NA), carbamylcholine chloride (carbachol), 4-aminopyridine, methylene blue, tetrodotoxin, glibenclamide (Sigma Chemical Company, St Louis, MO); apamin, charbydotoxin (Almone labs, Jerusalem, Israel); iberiotoxin (Peninsula Inc, Belmont, CA). Glibenclamide was dissolved in ethanol, all other drugs were dissolved in saline. A stock solution of CO was prepared by bubbling deoxygenated saline for 10 min with 99.9% CO gas (AGA Gas AB, Stockholm, Sweden). The CO concentration of the stock solution (3.6 mM) was determined by gas chromatography with 99.9% CO as a standard (Ny et al., 1995c). Concentration-response curves to CO were achieved by adding increasing volumes of the saturated CO solution to the bath.

Results

Localization of HO isoenzymes in the lower urinary tract

HO-2 immunoreactivity was seen in a few coarse nerve trunks distributed in the smooth muscle of the urethra and bladder (Table 1). The vascular endothelium of extramural arteries, and intramural vessels within the smooth muscle layers of the bladder and urethra, also displayed HO-2 immunoreactivity (Figure 1a). No HO-2 immunoreactivity was seen in the urothelium. HSP32, but not ‘HO-1’, immunoreactivity, was observed only in a few non-neuronal cells, distributed in the inner circular muscle layer. In addition, the vascular endothelium of intramural vessels was in general HO-2 IR. ‘HO-1’ immunoreactivity was seen in nerve structures or vascular endothelium. However, HSP32 immunoreactivity was seen in the urethra and OGJ, CO evoked concentration-dependent relaxations, which reached a maximum of 10% in the urethra (n = 8; Figure 2a, 3a), and 86 ± 6% of the spontaneously developed tension in the OGJ (n = 10; Figure 3b). No effects of the vehicle control were observed. Bladder preparations, or preparations from the extramural arteries of the bladder, did not respond to exogenously administered CO (12 – 72 μM; n = 3 – 5). A combination of iberiotoxin (0.1 μM) and apamin (0.1 μM) did not significantly reduce the relaxation evoked by CO (n = 6; Figure 3a).

Effects of exogenously applied CO

All urethral preparations developed spontaneous tension, reaching a maximum of 10±1 mN from 0-level. In the OGJ, only preparations that developed spontaneous tension were used (approximately 25%), and in these preparations the tension reached a maximum of 10±3 mN from 0-level. In both the urethra and OGJ, CO evoked concentration-dependent relaxations, which reached a maximum of 76±6% in the urethra (n = 8; Figure 2a, 3a), and 86 ± 6% of the spontaneously developed tension in the OGJ (n = 10; Figure 3b). No effects of the vehicle control were observed. Bladder preparations, or preparations from the extramural arteries of the bladder, did not respond to exogenously administered CO (12 – 72 μM; n = 3 – 5). A combination of iberiotoxin (0.1 μM) and apamin (0.1 μM) did not significantly reduce the relaxation evoked by CO (n = 6; Figure 3a).

Effects of methylene blue and potassium channel inhibitors on the relaxation evoked by CO

Urethra Treatment with methylene blue (30 μM) did not affect the tension in the urethra, and had no significant effects on the relaxant response to CO. The maximum relaxation was 76 ± 6% in the absence, and 62 ± 12% in the presence of methylene blue (n = 6; Figure 3a). Treatment with 4-aminopyridine (0.1 – 1 mM) raised the tension by 3 ± 1 mN, but had no effects on the relaxations evoked by CO (n = 5). K+ channel inhibition by glibenclamide (1 μM), iberiotoxin (0.1 μM), charybdotoxin (0.1 μM), or apamin (0.1 μM), had no effect on tension or on relaxations evoked by CO (n = 3 – 5). A combination of charybdotoxin (0.1 μM) and apamin (0.1 μM) did not significantly reduce the relaxation evoked by CO (n = 6; Figure 3a).

OGJ Treatment with methylene blue (30 μM) raised the spontaneously developed tension in the OGJ by 19±5 mN (n = 5; Figure 4). In the presence of methylene blue, the maximum relaxation evoked by CO was reduced from 86 ± 6% to 33±9% (n = 5; P<0.01; Figure 3b). Treatment with 4-aminopyridine (0.1 – 1 mM), charybdotoxin (0.1 μM) and iberiotoxin (1 μM) raised the tension by 25±1 mN, 9±2 mN and 6±2 mN, respectively (Figure 4), but had no effects on the relaxations (n = 3 – 4). Treatment with apamin (0.1 μM) or glibenclamide (1 μM) had no effects on either the tone or the CO-evoked relaxations (n = 3). A combination of charybdotoxin (0.1 μM) and apamin (0.1 μM) markedly raised the tension by 34±13 mN (Figure 4), and significantly reduced the

Table 1 Localization of haem oxygenase isoenzymes in the pig lower urinary tract and the oesophago-gastric junction (OGJ)

<table>
<thead>
<tr>
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<th>HO-2</th>
<th>‘HO-1’</th>
<th>HSP32</th>
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<tr>
<td>Bladder detrusor and urethra</td>
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<tr>
<td>Coarse nerve trunks</td>
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<tr>
<td>Fine nerve fibres</td>
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<tr>
<td>Urothelium</td>
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<td>Non-neuronal cells</td>
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<td>Endothelium intramural v.</td>
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<td>+</td>
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<tr>
<td>Extramural vessels of the bladder</td>
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<tr>
<td>Endothelium</td>
<td>+</td>
<td>-</td>
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<td>OJG</td>
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<td>Submucosal n.c.b.</td>
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<td>-</td>
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<tr>
<td>Myenteric n.c.b.</td>
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<tr>
<td>Coarse nerve trunks</td>
<td>+</td>
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<td>Endothelium intramural v.</td>
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<td>Epithelium</td>
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<td>HO-2, haem oxygenase-2 immunoreactivity; ‘HO-1’ and HSP2, haem oxygenase-1 immunoreactivity as displayed by two different antisera; v., vessels; n.c.b., nerve cell bodies.</td>
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relaxations evoked by 24 μM CO \((n=6; \ P<0.05; \ \text{Figure 3b})\). However, the maximum relaxation evoked by CO \((72 \mu M)\) was not significantly reduced, and was 86 ± 6% in the absence, and 66 ± 10% in the presence of the combination \((n=6; \ \text{Figure 3b})\).

**Cyclic AMP and cyclic GMP levels**

In smooth muscle preparations exposed to 72 μM CO, the cyclic GMP content increased from 1.8 ± 0.1 to 3.2 ± 0.5 pmol mg⁻¹ protein in the OGJ \((n=6; \ P<0.05)\), and from 0.78 ± 0.1 to 1.2 ± 0.2 pmol mg⁻¹ protein in the urethra \((n=12; \ P<0.05)\). The levels of cyclic AMP remained unchanged following exposure to CO; and were 28.2 ± 2.0 before, and 26.3 ± 3.3 pmol mg⁻¹ protein after CO-exposure in the OGJ \((n=6)\). The corresponding values in the urethra were 8.5 ± 1.4 and 8.8 ± 1.3 pmol mg⁻¹ protein \((n=12)\).

**Discussion**

CO has been suggested to be a neuromessenger in the brain (Verma et al., 1993), and in the periphery (Rattan & Chakder, 1993; Ny et al., 1995c; 1996; Zakhary et al., 1996). HO-2 is the predominant isoenzyme in the brain (Maines, 1993), and has

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**Figure 1** (a) HO-2 immunoreactivity in the endothelium of arteries running within the smooth muscle of the urethra. Bar 50 μm. FITC-immunofluorescence. (b) Fine nerve fibres (arrow), displaying HSP-32 immunoreactivity, running along the smooth muscle bundles in the urethra. Bar 50 μm. FITC-immunofluorescence. (c) HSP32-immunoreactivity in non-neuronal cells distributed in the smooth muscle of the urethra. Bar 50 μm. FITC-immunofluorescence. (d) Fine nerve fibres, displaying HSP32 immunoreactivity, running along the smooth muscle bundles in the bladder. Bar 50 μm. Texas Red-immunofluorescence. (e) Numerous HO-2 immunoreactive (IR) nerve cell bodies in the myenteric plexus of the OGJ. Bar 50 μm. Texas Red-immunofluorescence. (f) Fine varicose nerve fibres in the inner circular smooth layer of the OGJ being HSP32 IR. Bar 50 μm. FITC-immunofluorescence.
also been demonstrated in sympathetic postganglionic neurones in the guinea-pig (Vollerthun et al., 1995). However, in the present study, both HO-1 and HO-2 immunoreactivities could be demonstrated in peripheral neuronal structures, including coarse nerve trunks and nerve fibres running in parallel to the smooth muscle bundles in both regions. Two antisera against HO-1 were used, 'HO-1 and HSP32. Initially only HO-1 antiserum (against the isolated enzyme from rat liver) was tested, but expecting no differences, we wanted to confirm our results by using the HSP32 antiserum (obtained from an E. coli expression system). Differences between the antisera were found. Thus, only HSP32 antiserum, and not 'HO-1' visualized nerve structures in the tissue. However, it should be noted that all structures visualized with the 'HO-1' antiserum were also demonstrated by the HSP32 antiserum, which suggests that HSP32 antiserum is the more sensitive indicator of the presence of HO-1.

Some HO-2, 'HO-1' and/or HSP32 IR cells with a non-neuronal appearance were found to be distributed in the muscularis propria of the OGJ. Possibly, these cells correspond to the interstitial cells of Cajal, a cell type involved in motility regulation, since their size, form and localization, are similar to at least some types of interstitial cells of Cajal (Thuneberg, 1989). Furthermore, similar HO-2 'HO-1'/HSP32 IR cells were observed in the urethra, but in a markedly lower number. This suggests a potential role for HO/CO in non-neuronal control of urethral motility. Moreover 'HO-1'-HSP32 immunoreactivity was demonstrated in the urothelium. The functional implications of this finding remain to be established.

The relaxant responses to exogenously applied CO were associated with a small increase in cyclic GMP content in both the urethra and the LOS, suggesting that CO causes relaxation, at least partly, by activating soluble guanylate cyclase. In the LOS, relaxations were sensitive to treatment with the guanylate cyclase inhibitor methylene blue, further supporting the involvement of the guanylate cyclase/cyclic GMP system in relaxations evoked by CO in this region. However, it should be mentioned that methylene blue has been suggested to act distinct from its proposed inhibition of soluble guanylate cyclase, by extracellular generation of superoxide anions (Wolin et al., 1990).

CO has been demonstrated to modulate whole cell K⁺ currents in human jejunal smooth muscle (Farrugia et al., 1993). In the present study, neither voltage-dependent (4-aminopyridine), nor ATP-sensitive (glibenclamide), K⁺ channels seemed to be involved in relaxations evoked by CO in the pig urethra and OGJ. CO-induced relaxations in the OGJ were partly sensitive to blockade of high and low conductance Ca²⁺-activated K⁺ channels by a combination of charybdotoxin and apamin. However, a variety of different K⁺ channel types may possess a common binding site for charybdotoxin (Edwards & Weston, 1994), making the interpretation of the results more difficult. The combination of charybdotoxin and apamin has been demonstrated to inhibit relaxation induced
suggested that both high and low conductance Ca\(^{2+}\)-activated K\(^{+}\) channels are involved in regulation of smooth muscle tone. Spontaneous tone in the urethra and OGJ may contribute to the sphincter function in these regions, preventing urine leakage and gastric acid reflux. However, as only 25% of the preparations in the present study developed spontaneous tone, the pig OGJ is obviously not a suitable model for studies of this phenomenon. In comparison to the OGJ of other species, such as cat, opossum and man, which develops a pronounced spontaneous tone (Daniel, 1992), regulation of tone in the pig OGJ probably requires extrinsic input to prevent reflux of gastric contents.

The endothelium of extramural arteries of the bladder displayed distinct HO-2 immunoreactivity. In these vessels, an endothelium-dependent relaxation has been demonstrated, which is only partially mediated by NO (Persson et al., 1993). The presence of HO in the endothelium therefore suggests CO as a putative endothelium-derived mediator of relaxation in pig vesical arteries. However, these vessels did not respond to exogenously applied CO, making it less likely that CO is an endothelium-derived relaxing factor in this preparation. This is in agreement with the findings of Zakharov et al. (1996). The presence of the suggested target for CO in vessels, guanylate cyclase, was demonstrated by exposing the preparations to NO, which caused a complete relaxation. Previous findings have suggested NO to be more potent than CO in relaxing blood vessels (Furchgott & Jothianandan, 1991; Lefer et al., 1993; Zygmunt et al., 1994), and CO is considered to be a poor activator of soluble guanylate cyclase, as demonstrated in a purified fraction of the enzyme (Stone & Marletta, 1994). The reason for a total lack of response to CO in the extramural bladder vessels, or in the detrusor muscle preparations, in this study is unclear. However, in the detrusor, the sensitivity to drugs acting via the cyclic GMP system is low (Persson & Andersson, 1994). The reason for a total lack of response to CO in the extramural bladder vessels, or in the detrusor muscle preparations, in this study is unclear. However, in the detrusor, the sensitivity to drugs acting via the cyclic GMP system is low (Persson & Andersson, 1994). On the other hand, the findings suggest roles for HO/CO in this system other than regulation of smooth muscle tone. CO has for example been suggested to modulate cell-cell interactions and cell proliferation in the vessel wall during hypoxia (Morita & Kourembanas, 1995). This study suggests various sources for CO production in both the urethra and OGJ. CO may have a function in the inflammatory response (Willis et al., 1996). We conclude that HO is present in both neuronal and non-neuronal structures in the pig lower urinary tract and OGJ. CO produces relaxation of smooth muscle in both the urethra and OGJ, associated with a small increase in cyclic GMP concentration. In the OGJ, but not the urethra, relaxations evoked by CO were inhibited by methylene blue, and/or inhibition of the enzyme (Stone & Marletta, 1994). The reason for a total lack of response to CO in the extramural bladder vessels, or in the detrusor muscle preparations, in this study is unclear. However, in the detrusor, the sensitivity to drugs acting via the cyclic GMP system is low (Persson & Andersson, 1994). On the other hand, the findings suggest roles for HO/CO in this system other than regulation of smooth muscle tone. CO has for example been suggested to modulate cell-cell interactions and cell proliferation in the vessel wall during hypoxia (Morita & Kourembanas, 1995). This study suggests various sources for CO production in both the urethra and OGJ. CO may have a function in the inflammatory response (Willis et al., 1996).


(Received March 25, 1996 Revised September 9, 1996 Accepted October 4, 1996)