ELSEVIER

European Journal of Pharmacology 367 (1999) 85–89

ejp

Short communication

Presence of constitutive endothelial nitric oxide synthase immunoreactivity in urothelial cells of hamster proximal urethra

Christian Pinna [a], Ivano Eberini [a], Lina Puglisi [a], Geoffrey Burnstock [b,*]

[a] *Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy*
[b] *Autonomic Neuroscience Institute, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK*

Received 22 October 1998; revised 23 November 1998; accepted 29 December 1998

Abstract

Electrical field stimulation caused frequency-dependent relaxations in precontracted strips of hamster proximal urethra, which were attenuated by L-N^G-nitroarginine methyl ester (10^{-4} M) and completely blocked by tetrodotoxin (10^{-6} M). Strips of hamster urethra devoid of urothelium showed reduced relaxant responses to electrical field stimulation which were abolished by L-N^G-nitroarginine methyl ester (10^{-4} M). Western blot analysis showed the presence of a constitutive endothelial nitric oxide synthase in the urothelial layer, suggesting that urothelium may release nitric oxide in response to electrical field stimulation and that this release is blocked by tetrodotoxin. It is suggested that the urothelium may contribute to relaxations of the smooth muscle of hamster urethra produced by nerve stimulation.

Keywords: Urethra; Urothelium; Nitric oxide (NO); Endothelial nitric oxide (NO) synthase; L-N^G-Nitroarginine methyl ester (L-NAME); (Hamster)

1. Introduction

It has been proposed that nitric oxide (NO) is involved in the non-adrenergic, non-cholinergic inhibitory nerve responses of the urethra, bladder neck and trigone from various species (Dokita et al., 1994; Andersson and Persson, 1995; Takeda and Lepor, 1995; Andersson, 1996; Smet et al., 1996). We have previously demonstrated that NO is the main inhibitory neurotransmitter in the hamster proximal urethra, and that urothelium plays a modulatory role in the smooth muscle response (Pinna et al., 1996). In fact, removal of urothelium from urethral preparations resulted in reduced frequency-related relaxations in response to electrical field stimulation. Preparations devoid of urothelium also showed increased contractile responses to noradrenaline, suggesting that an inhibitory factor(s) was synthesized and released from urothelial cells. Despite this pharmacological evidence, we were unable to provide direct proof for the presence of constitutive endothelial NO synthase in the urothelial layer. However, our previous immunohistochemical study of hamster urethra showed the presence of endothelial NO synthase immunoreactivity in the smooth muscle layer and sparse endothelial NO synthase immunoreactivity in the lamina propria (Pinna et al., 1996), similar to that shown in the rabbit urethra (Zygmunt et al., 1993). Conversely, the NADPH–diaphorase reaction was found to be particularly strong in the urothelium. A similar observation has been reported for the urothelium of mouse and pig urethra (Grozdanovic et al., 1992; Persson et al., 1993).

The aim of the present work, using isolated organ bath experiments and Western blot analysis, was to further investigate whether endothelial NO synthase is localized in the urothelium of the hamster urethra, as has already demonstrated for other epithelia (Matera et al., 1995; Birder et al., 1998).

2. Materials and methods

2.1. Tissue preparation and recording of mechanical activity

The bladder and the urethra from male golden hamsters (120–140 g) were quickly removed and placed in cold, modified Krebs solution of the following composition

* Corresponding author. Tel.: +44-171-830-2948; Fax: +44-171-830-2949; E-mail: g.burnstock@ucl.ac.uk

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00981-9

(mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.4, $NaHCO_3$ 16.4, $MgSO_4$ 0.6 and glucose 7.7. The urethra was then opened along its longitudinal axis and a strip (2 mm in length, 1 mm in width) was prepared from each urethra. The urothelial layer was separated from the underlying lamina propria under a dissecting microscope by the use of scissors and forceps. Each strip was threaded through a pair of platinum-ring electrodes (3 mm in diameter, 1 cm apart) connected to a Grass SD 9 stimulator, with one end attached to a holder and the other to a Basile isometric force transducer coupled to a two-channel Basile 7070 ink-writing oscillograph. The strips were equilibrated for 1 h in 5 ml organ baths containing modified Krebs solution gassed with 95% O_2 and 5% CO_2 at 37 ± 0.5°C, and loaded to a tension of 300 mg.

2.2. Experimental procedure

Previously published procedures were used to monitor relaxant responses to electrical field stimulation (Pinna et

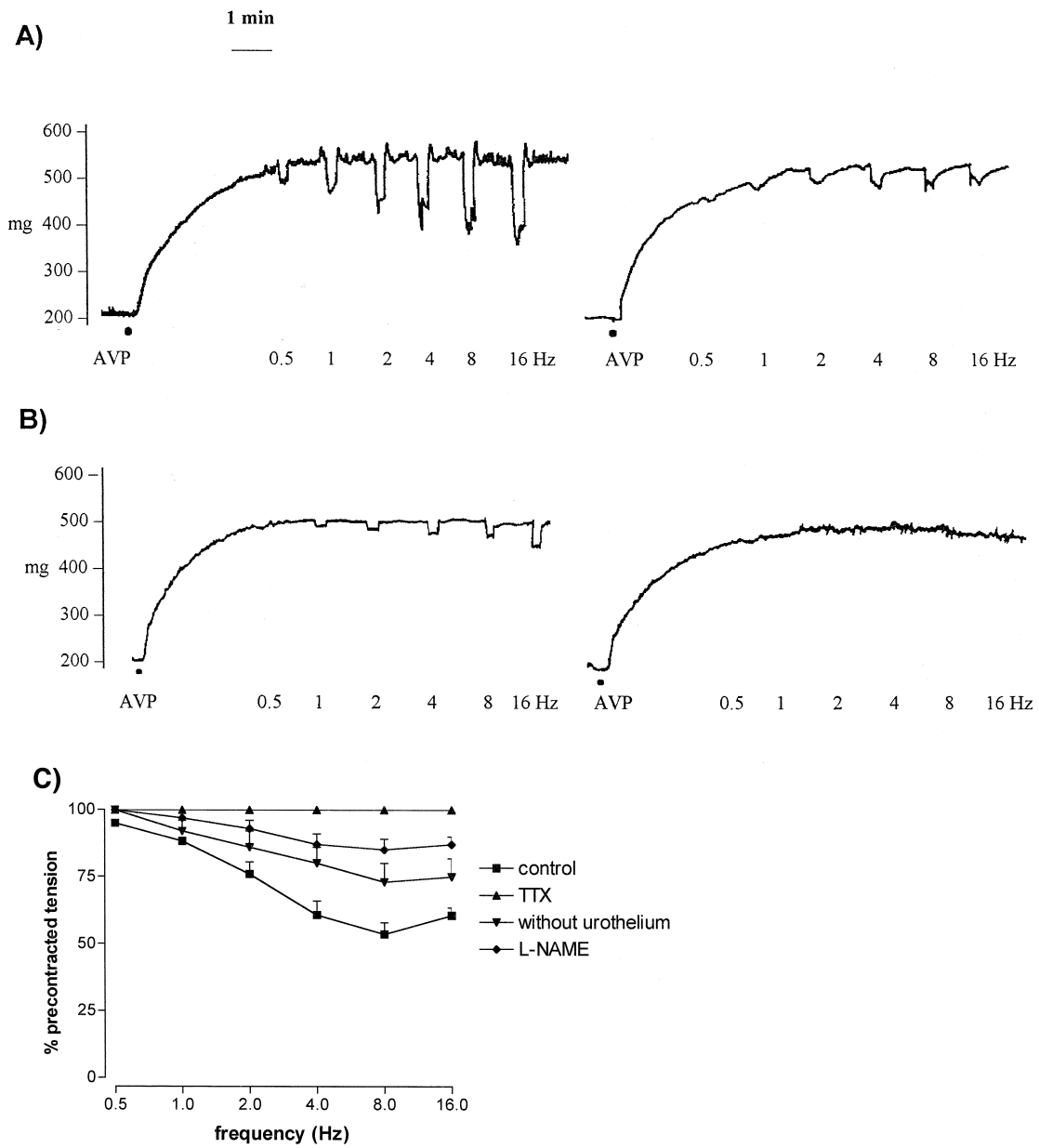

Fig. 1. Original tracings showing responses of circular smooth muscle strips of hamster proximal urethra precontracted with arginine vasopressin (10^{-8} M) in response to electrical field stimulation (80 V, 0.3 ms, 0.5–16 Hz) in intact (A) and in urothelial-free preparation (B), before (left panel) and after (right panel) incubation with L-NAME (10^{-4} M). Neurogenic non-adrenergic, non-cholinergic relaxation of circular strips of hamster proximal urethra precontracted with arginine vasopressin (10^{-8} M) and incubated with propranolol (10^{-6} M), phentolamine (10^{-6} M) and atropine (10^{-6} M), with (■) and without urothelium (▼) (ANOVA, $P < 0.05$, $n = 10$), in the presence of L-N^G-nitroarginine methyl ester (L-NAME: 10^{-4} M) in intact (◆) and in urothelial-free preparation (●) and tetrodotoxin (TTX: 10^{-6} M, ▲) (C). Points show mean ± S.E.M. of 6 experiments.

al., 1996). Briefly, strips were precontracted with arginine vasopressin (10^{-8} M), which produced a long-lasting and stable contraction. In order to display the non-adrenergic, non-cholinergic relaxant responses, phentolamine (10^{-6} M), propranolol (10^{-6} M) and atropine (10^{-6} M) were present throughout the experiment. Frequency-response curves were then constructed by giving square wave pulses (80 V, 0.3 ms) delivered for 30 s, at increasing frequencies (0.5–16 Hz). L-N^G-nitroarginine methyl ester (L-NAME, 10^{-4} M) was added 30 min before the strips were once again precontracted with arginine vasopressin and subjected to electrical field stimulation. At the end of the experiment, tetrodotoxin (10^{-6} M) was added to the organ bath and electrical field stimulation repeated. In some experiments *N*-methyl-L-arginine acetate (L-NMA; 10^{-4} M) was added to the organ bath 30 min before the strips were precontracted with arginine vasopressin and subjected to electrical field stimulation.

2.3. Electrophoresis and Western blot analysis

Total proteins from the urothelium (10 mg) were extracted with 300 ml of sample buffer containing 0.125 M Tris–HCl pH 6.8, 4.6% sodium dodecyl sulphate (SDS), 2% β-mercaptoethanol, 0.15% (w/v) bromophenol blue and 20% glycerol. The lysates were boiled for 5 min, and the supernatant was collected. As positive controls, endothelial cells from human umbilical cord, which are known to produce endothelial NO synthase (Sladek et al., 1997), or neuronal cells from rat hippocampus that produce neuronal NO synthase (Endoh et al., 1994), were used. Protein samples were then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with a running gel ($T\% = 5$) and a stacking gel ($T\% = 3$), using a Bio-Rad Mini-Protein II cell (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membrane Hybond-C extra (Amersham UK, Amersham), in a transblotter (Mini-Protein II). The membrane was immunostained with rabbit polyclonal antibody against neuronal NO synthase or endothelial NO synthase (Transduction Laboratories) at a dilution of 1:1000, followed by horseradish peroxidase-conjugated anti-rabbit Immunoglobulin G (IgG) antibody (1:2000; Bio-Rad). Zymogram for horseradish peroxidase was developed with ECL Kit (Amersham) and revealed by Hyperfilm-ECL (Amersham).

2.4. Drugs and solutions

Arginine vasopressin, atropine sulphate, L-N^G-nitroarginine methyl ester, *N*-methyl-L-arginine acetate and tetrodotoxin were all purchased from Sigma (Poole, UK). Bromophenol blue, β-mercaptoethanol and sodium dodecylsulphate were purchased from Merck. Phentolamine mesylate (Rogitine) was obtained from Novartis (formerly Ciba, Cambridge, UK) and propanolol hydrochloride (Inderal) from Zeneca (Cheshire, UK). For pharmacological experiments, stock solutions were usually prepared in distilled water. Subsequent dilutions of the drug were prepared in 0.9% NaCl.

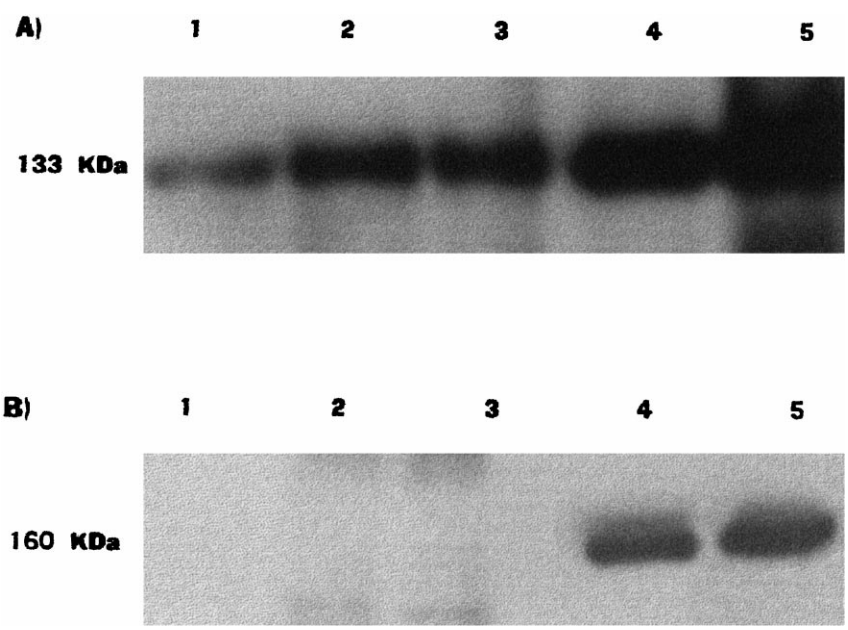

Fig. 2. Immunochemical analysis of cellular proteins with antiserum against endothelial NO synthase and neuronal NO synthase. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and immunostained with antiserum to endothelial NO synthase (A) or neuronal NO synthase (B). Lanes 1, 30 μg; 2, 60 μg; and 3, 90 μg of total proteins from hamster urothelium. Lanes 4 and 5, total proteins from endothelial cells of umbilical cord (A) or neuronal cells from rat hippocampus (B).

2.5. Analysis of data

Relaxant responses are expressed as percent inhibition of the arginine vasopressin-induced contraction. All data in the text are expressed as mean ± S.E.M. Student's *t*-test was used to compare two means, and concentration-response curves were compared by two-way analysis of variance (ANOVA) using the computer program Minitab. $P < 0.05$ was considered significant.

3. Results

Electrical field stimulation evoked frequency-dependent inhibitory responses in urethral preparations in vitro, which were reduced by the NO synthase inhibitor, L-NAME (10^{-4} M) (Fig. 1A and C). Removal of the urothelium also reduced the inhibitory responses to electrical field stimulation (Fig. 1B and C), which were abolished by L-NAME (10^{-4} M). In some experiments L-NMA (10^{-4} M; $n = 5$) was added to the organ bath; electrical field stimulation was partially inhibited by L-NMA to the same amplitude observed with L-NAME (10^{-4} M). The frequency response curves obtained in the presence of either L-NAME or L-NMA did not differ significantly. Relaxant responses were also completely blocked by tetrodotoxin (10^{-6} M).

The presence of endothelial NO synthase immunoreactivity in the urothelial cells from hamster urethra was assayed by Western blot analysis and is shown in Fig. 2A. Lanes 1, 2 and 3 represent total proteins (30, 60, 90 μg) from urothelial cells, lanes 4 and 5 (60, 90 μg) are the total proteins obtained from human venous endothelial cells of umbilical cord, used as positive control. Fig. 2B shows the result of Western blot analysis for neuronal NO synthase expression. Lanes 1, 2 and 3 represent total proteins (30, 60, 90 μg) from urothelial cells, lanes 4 and 5 (60, 90 μg) are the total proteins obtained from rat hippocampal neurons, used as positive control.

4. Discussion

The electrical field stimulation-evoked frequency-dependent inhibitory responses in the hamster urethral preparations, and their reduction by L-NAME and L-NMA are in agreement with previous reports (Hoyle et al., 1994; Andersson and Persson, 1995) that identified nitric oxide as an inhibitory neurotransmitter in the lower urinary tract of several mammals. Inhibitory responses to electrical field stimulation were also reduced in preparations devoid of urothelium by about 75%, suggesting that the urothelial layer might have an important modulatory role on the smooth muscle responsiveness of the proximal urethra. This confirms our previous results (Pinna et al., 1996) and suggests that activation of tetrodotoxin-sensitive neurons can also release nitric oxide from uroepithelial cells.

L-NAME also abolished the electrical field stimulation-induced relaxant responses in tissues devoid of urothelium, confirming that NO is also released from nerve fibres present in the smooth muscle layer as indicated by the presence of neuronal NO synthase immunoreactivity (Pinna et al., 1996). Our findings are in keeping with those of Birder et al. (1998) who found that NO released from rat urinary bladder is largely of non-neuronal origin and arises by about 85% from the urothelial layer. The hypothesis that the urothelial layer has an active role in modulating bladder and urethra responsiveness is also supported by findings of Ferguson et al. (1997), who demonstrated a release of ATP from urothelial cells acting as a sensory mediator of urinary bladder distension in rabbit.

It is suggested that stimulation of inhibitory nerves located in the circular smooth muscle layer just beneath the urothelium (Pinna et al., 1996) provokes the relaxation of proximal hamster urethra, which is largely but not entirely nitrergic, since L-NAME does not completely block this response. In fact, in intact preparations, the residual inhibitory response still present after L-NAME is due to at least another neurotransmitter such as ATP (Pinna et al., 1998), since suramin affects the response.

At least two mechanisms could contribute to neuronally-mediated nitric oxide release in the urothelium. Neurotransmitters released from nerves adjacent to the urothelium could trigger the release of nitric oxide from the urothelial cells. Alternatively, the neurogenic relaxation could cause, in turn, the release of nitric oxide from urothelial cells by mechanical disturbance, which then secondarily enhances relaxation of urethra. Either of these mechanisms would be consistent with the findings, namely: (i) partial block of relaxation after removing the urothelium; (ii) partial block in the presence of L-NAME; (iii) complete block of relaxation in the presence of tetrodotoxin; and (iv) presence of endothelial NO synthase in urothelial cells.

The reason why the immunohistochemical analysis performed in the previous work (Pinna et al., 1996) failed to detect the presence of an endothelial NO synthase in the urothelial layer of sections of hamster urethra, can be attributed to a different isoform of NO synthase, not detected by our antibody. Since the Western blot analysis was performed with the same antibody against endothelial NO synthase, we can assume that different responses given by the two analyses result from differences in sensitivity.

In conclusion, the present results confirm that the synthesis and the release of nitric oxide are involved in the relaxant responses of the hamster urethra and provide evidence for the presence of endothelial NO synthase in the urothelium. Our results clearly show that the urothelial layer from hamster urethra contain endothelial NO synthase, but not neuronal NO synthase. We suggest that the production of nitric oxide from urothelium may account for the decrease in response to electrical field stimulation observed in preparations of hamster urethra devoid of

urothelium and imply that neurotransmitter or mechanical deformation of urothelium during the filling phase of the bladder might lead to the release of nitric oxide that contributes to the relaxation of urethra produced by nerve stimulation.

Acknowledgements

The authors wish to thank Mr. Roy Jordan and Dr. Manuela Magnani for their assistance in the preparation of the manuscript and Dr. Jill Lincoln for valuable discussion and criticism on the paper.

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