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SPECIAL REPORT

Urothelium-derived inhibitory factor(s) influences on detrusor muscle contractility *in vitro*

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The function of the bladder urothelium in modulating contractile responses of the underlying detrusor smooth muscle to muscarinic stimulation has been examined in the pig bladder. Saturation curves for [^{3}H]-QNB binding demonstrated a greater muscarinic receptor density in the urothelium than in the detrusor smooth muscle. The presence of an intact urothelium on isolated bladder strips inhibited contractions induced by carbachol but not KCl. Contractions of a urothelium-denuded muscle strip were inhibited in the presence of a second bladder strip with an intact urothelium, but not if the second strip was denuded. The urothelium-induced inhibition of contractions was not prevented in the presence of L-NOARG, methylene blue, indomethacin, propranolol, suramin, TEA or apamin. The data suggest the presence of a diffusable, urothelium-derived inhibitory factor, which could not be identified but appears to be neither nitric oxide, a cyclo-oxygenase product, a catecholamine, adenosine, GABA nor an EDHF sensitive to apamin.
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Abbreviations: [^{3}H]-QNB, quinuclidinyl benzilate, L-[benzilic-4,4′-^{3}H]

Introduction The bladder urothelium has for a long time been thought to act solely as a barrier protecting the underlying detrusor smooth muscle. There is however, a growing body of evidence to indicate that this tissue plays a far more active role in bladder function. The bladder urothelium is the first bladder component to react to stress with an increase in early gene responses (Chen *et al*., 1994; Zhao *et al*., 1994); the metabolic rate of the urothelium is also significantly greater than that of the detrusor muscle (Hypolite *et al*., 1993), and its removal significantly increases the responses to a range of contractile agents (Dveksler *et al*., 1987; Maggi *et al*., 1987; Pinna *et al*., 1992; Levin *et al*., 1995).

The urothelium has an afferent innervation (Wakabayashi *et al*., 1993) which is likely to play an important role in the reflex responses to bladder filling and distension (Lecci *et al*., 1993). Fifty per cent of the afferent neurones in the urothelium contain acetylcholinesterase (ACHE), however, not all are destroyed by dorsal root ganglionectomy leading to the suggestion that some of the ACHE innervation represents a parasympathetic innervation (Wakabayashi *et al*., 1992; 1995). Whilst the function of such an innervation may be unclear; its existence would suggest the possible existence of a population of urothelial muscarinic receptors. Such a population may represent another site of action for the muscarinic antagonists used in the treatment of bladder disorders which are normally thought to act specifically at the smooth muscle.

This work was carried out in pig bladder to determine if the urothelium contains such a population of muscarinic receptors and whether the stimulation of such a population of receptors may exert an effect on the underlying smooth muscle.

Methods Samples of fresh pig bladders were obtained from a local abattoir and placed immediately in cold Krebs-bicarbonate solution (composition in mM: NaCl 118.4, $NaHCO_3$ 24.9, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7). The serosa was removed and the detrusor muscle cut into strips. Adjacent strips of equal size were prepared in pairs, the urothelium (approximately 35% of the tissue mass) being removed from one of the strips. Both tissues were then suspended under 1 g tension in Krebs-bicarbonate solution gassed with 95% in oxygen at 37°C. Isometric tension was recorded using isometric force transducer (Lectromed UF1, 57 g sensitivity) connected to a Tandon PCA-SL computer *via* an analogue to digital converter (Cambridge Electronic Design). Developed tension was recorded and analysed using 'CHART' software. Tissues were equilibrated for 1 h and washed every 15 min before construction of cumulative concentration-response curves to carbachol or KCl (in the presence of 1 μM atropine). In experiments with antagonists and inhibitors, after the construction of an initial concentration-response curve the tissues were washed every 10 min for 1 h and then incubated with antagonists/inhibitors for 30 min before construction of a second cumulative concentration-response curve to carbachol.

In some experiments cumulative carbachol concentration-response curves were initially constructed using only tissues with the urothelium removed. After washing a second curve to carbachol was obtained on the denuded tissue, but with a second tissue being present in the bath. The second tissue (with either an intact urothelium or no urothelium) was attached to the same tissue holder as the first, denuded tissue. The second tissue was thus in contact with the first tissue, but was not attached to the recording transducer.

In another series of experiments, after the initial responses to carbachol, tissues were removed from the organ baths, the urothelium removed (sham removal for denuded strips) and then the tissues set up again under 1 g tension. After 20 min equilibration, responses were again recorded to carbachol.

At the end of each experiment the urothelium was removed from all tissues and the detrusor muscle weighed. Tension responses were normalized by expression as mg tension g^{-1} muscle tissue.

[^{3}H]-QNB binding Pig bladder urothelium or detrusor muscle were homogenized in ice cold 50 mM Tris-HCl (pH 7.6) using an Ultra-turrax homogenizer for 30 s followed by 4×4 strokes of a glass-Teflon homogenizer. The homogenate was

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filtered through muslin and centrifuged at 45,000 × *g* for 15 min. The pellet was washed in the Tris buffer and recentrifuged at 45,000 × *g* for a further 15 min. This final pellet was resuspended in Tris buffer for radioligand binding experiments at a concentration of 1–3 mg ml^{-1}. Protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. Membranes (100–300 μg 250 μl^{-1}) were incubated in 50 μM Tris-HCl (pH 7.6) with varying concentrations of [^{3}H]-QNB (0.06–3.0 nM) for 30 min at 37°C. Non-specific binding was determined using 1 μM atropine and accounted for 10.3 ± 3.3 and 7.9 ± 1.1% of total binding in the urothelium and detrusor respectively at a [^{3}H]-QNB concentration of 0.5 nM. After incubation samples were filtered over Whatman GF/B filters and washed three times with 2 ml ice cold buffer using a cell harvester (Model 30R, Brandel Instruments). Radioactivity on the filters was determined by liquid scintillation counting spectrometry.

Data analysis For each curve, responses were plotted as a per cent of the individual maximal response, the concentration of carbachol producing a response 50% of the maximum response (EC_{50} value) was calculated using Prism (GRAPHPAD software, San Diego, CA, U.S.A.) and geometric mean EC_{50} values with 95% confidence limits were calculated. To compare responsiveness between pairs of tissues (± urothelium), contractions to carbachol were expressed as a percentage of the maximum contraction obtained in the absence of an intact urothelium. Mean responses (± s.e.mean) were calculated and used to plot concentration-response curves.

[^{3}H]-QNB saturation curves were analysed using Prism (GraphPAD software, San Diego, CA, U.S.A.) to determine K_d and B_{max} values. For statistical comparison, Students paired *t*-test was used to compare maximum responses and also to compare logarithmic EC_{50} values between intact and urothelium-denuded tissues. Students unpaired *t*-test was used to compare radioligand binding data (K_d and B_{max} values) between urothelium and detrusor tissues.

Drugs and solutions [^{3}H]-QNB (specific activity 49 Ci $mmol^{-1}$) was obtained from New England Nuclear. Apamin was obtained from Calbiochem. All other compounds were obtained from Sigma, Poole, U.K. All drugs were prepared fresh in Krebs-bicarbonate solution (tissue experiments) or Tris buffer (binding experiments) except indomethacin which was prepared as a stock solution in ethanol and then diluted in Krebs-bicarbonate solution.

Results *Radioligand binding studies* Specific binding of [^{3}H]-QNB to membranes prepared from either pig urothelium or detrusor muscle was concentration-dependent and saturable (Figure 1). Scatchard analysis of the saturation curves demonstrated that in the urothelial tissue the density of muscarinic receptors (B_{max}) was 127.8 ± 7.7 fmoles mg^{-1} protein and the affinity (K_d) of the ligand was 0.21 ± 0.05 nM ($n = 6$). The K_d in the detrusor muscle of 0.18 ± 0.02 nM was not significantly ($P < 0.05$) different to that in the urothelium, but the receptor density was significantly lower ($P < 0.05$) with the B_{max} being 81.3 ± 15.3 fmoles mg^{-1} protein ($n = 6$).

Effect of the urothelium on contractile responses The maximum response to carbachol in isolated detrusor strips with an intact urothelium was only 43.9 ± 3.5% ($n = 38$) of that in the denuded strips without a urothelium (Figure 2). The presence of the urothelium also caused a small but statistically significant ($P < 0.05$) decrease in the sensitivity of the tissue to carbachol with the mean EC_{50} value increasing from 2.36(2.00–2.79) μM ($n = 6$) to 4.51(3.67–5.57) μM ($n = 6$). Responses to KCl (in the presence of 1 μM atropine) were not significantly affected by the presence of the urothelium, maximum responses being 102.3 ± 6.1% of those in the corresponding denuded tissues.

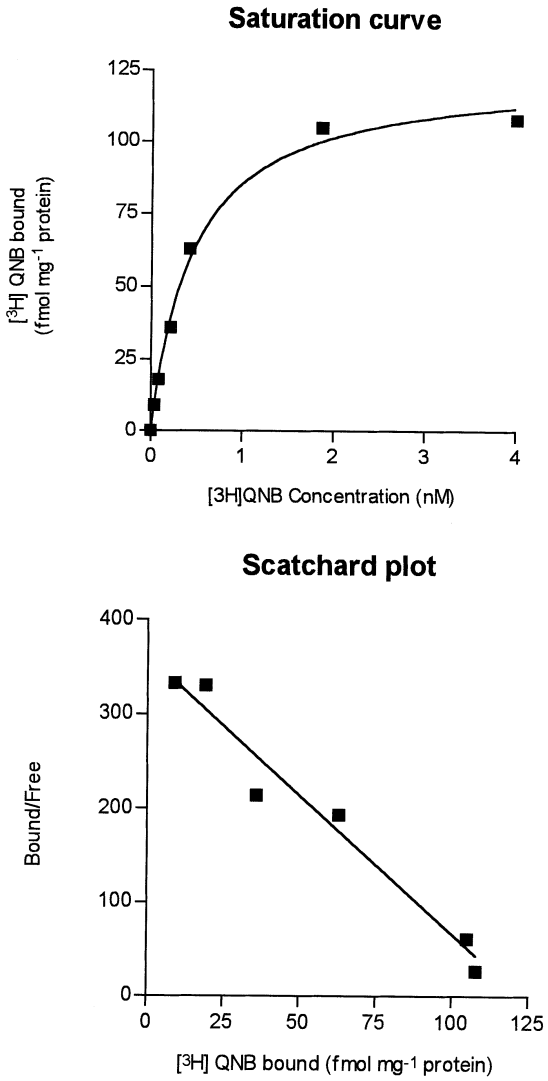

Figure 1 Saturation curve and Scatchard plot for the specific binding of [^{3}H]-QNB to membranes prepared from pig urothelium.

In another series of experiments, the urothelium was removed from the tissues (sham removal for denuded tissues) and responses again recorded to carbachol. After sham-removal of the urothelium from already denuded tissues, maximum tension development to carbachol increased by 7.9 ± 12.6% ($n = 5$), but urothelium-removal from previously intact tissues resulted in a significantly greater increase (69.0 ± 15.6%, $n = 5$) in maximum responses to carbachol ($P = 0.01$).

Co-incubation of muscle strips with and without an intact urothelium The maximal response of denuded strips to carbachol was significantly reduced ($P < 0.05$) to 46.7 ± 5.6% ($n = 6$) by the presence of a second tissue strip with an intact urothelium (Figure 3). The presence of a second strip denuded of urothelium however, had only a minor effect on the first muscle strip, with the maximal response to carbachol being 85.6 ± 10.3% ($n = 6$) (Figure 3).

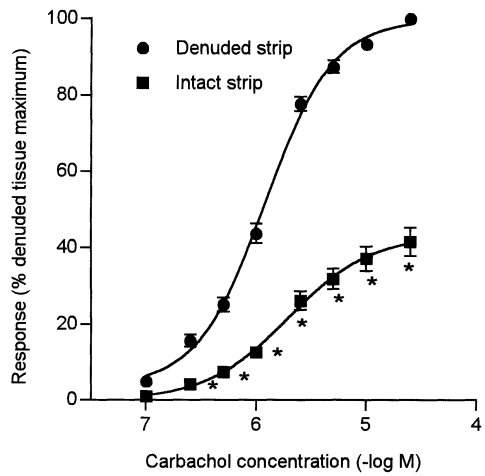

Figure 2 Responses to carbachol of detrusor strips with an intact urothelium or with the urothelium removed. Responses are plotted as a percentage of the maximum response of the denuded tissue. *$P<0.001$ vs responses of urothelium-denuded tissues.

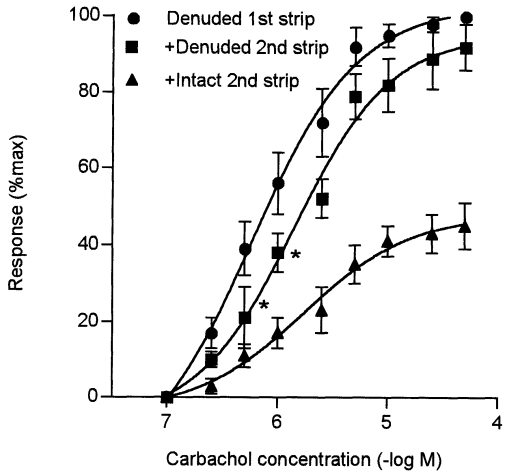

Figure 3 Responses to carbachol of urothelium-denuded detrusor muscle in the presence of a second bladder strip with or without a urothelium. Responses are plotted as a percentage of the maximum response of the denuded tissue. All responses were depressed in the presence of the urothelium ($P<0.001$ for all points). Responses in the presence of the denuded muscle strips were significantly greater than in the presence of an intact urothelium ($P<0.05$ for all points) and were not significantly different to controls (except where indicated, *$P<0.05$).

Effect of antagonists and inhibitors Inhibition of the nitric oxide pathway with either L-NOARG (50 μM) or methylene blue (10 μM) failed to significantly affect the urothelium-induced inhibition of responses (Table 1). Similarly, cyclo-oxygenase inhibition with indomethacin (5 μM) was without significant effect on the relaxation induced by the urothelium (Table 1). The presence in the bath of antagonists of purinergic receptors (suramin, 100 μM) and β-adrenoceptors (propranolol, 1 μM) also failed to alter the inhibitory effect of the urothelium on contractile responses to carbachol (Table 1). Finally the effect of potassium channel inhibition with TEA (5 mM) or apamin (400 nM) was examined (Table 1), but again had no significant effect on the urothelium-induced inhibition of responses to carbachol.

Table 1 Effect of antagonist/inhibitors on urothelium-induced inhibition of carbachol-mediated detrusor contractions

Drug	*Absence of drug* (%)	*Presence of drug* (%)
L-NOARG (50 μM)	31.9±2.1	32.5±5.1
Methylene blue (10 μM)	47.5±3.2	45.8±4.6
Indomethacin (5 μM)	29.4±6.2	29.6±5.3
Suramin (100 μM)	43.8±14.9	30.2±7.8
Proprandolol (1 μM)	47.1±8.2	52.4±8.5
TEA (5 mM)	57.9±8.8	58.6±9.2
Apamin (400 nM)	48.9±8.4	47.9±5.4

Responses are plotted as a percentage of the maximum contraction obtained in the urothelium-denuded tissues ($n=7$).

Discussion [^{3}H]-QNB binding clearly demonstrates that pig bladder urothelium contains a relatively large muscarinic receptor population. This is unlikely to be due to contamination from the underlying smooth muscle as the receptor density is 40% greater in the urothelium than in the smooth muscle. Such a receptor population is not surprising since the urothelium appears to receive a parasympathetic innervation (Wakabayashi *et al*., 1992, 1995).

In vascular tissue stimulation of endothelial muscarinic receptors causes the release of relaxation factors, in particular nitric oxide. A similar process may be postulated to occur in the bladder as supported by the observation that contractions to carbachol, but not potassium (in the presence of atropine) are reduced by the presence of the urothelium. Similar observations with muscarinic agonists have been made in the cat bladder (Levin *et al*., 1995), although the authors were unable to determine if the inhibition was due to the release of a diffusable relaxant substance released from the urothelium or purely due to the physical presence of the urothelium restricting contractions. Also, in the rat bladder, the detrusor smooth muscle has been shown to release a non-nitrergic factor which caused relaxation of blood vessels, but the inhibition was independent of the urothelium (Fovaeus *et al*., 1999).

In the pig, inhibition of smooth muscle contraction was induced in denuded strips by the presence of a second strip with a functional urothelium, suggesting the release of a diffusable inhibitory agent. In contrast, the presence of a second bladder strip denuded of its urothelium, failed to inhibit contractions in the first strip, suggesting the inhibitory factor is released by the urothelium and not the detrusor smooth muscle. This conclusion was supported further by the finding that responses to carbachol were potentiated following removal of the urothelium from previously intact bladder strips.

The mechanism by which the urothelium modifies detrusor contractile responses may be important for normal bladder compliance, where during filling there is no increase in intravesical pressure until the initiation of voiding. In this case, dysfunction of the urothelial mechanism may well be involved in bladder disorders, and identification of the inhibitory agent may provide a new potential target for pharmacologically mediated therapeutic intervention.

A number of possible pathways for the inhibition have been investigated. Prime amongst these is nitric oxide, which induces relaxation by activation of soluble guanylate cyclase. Nitric oxide does not appear to be released from pig bladder urothelium, since the nitric oxide synthase inhibitor L-NOARG, had no effect on the inhibition induced by the urothelium. Methylene blue which inhibits soluble guanylate cyclase was also without effect, further supporting the

conclusion that nitric oxide is not involved in mediating this inhibition. Prostaglandins and prostacyclin are known to be released from the urothelium and modulate contraction of detrusor smooth muscle. However indomethacin was without effect suggesting that products of cyclo-oxygenase activity are not involved in the inhibition of detrusor muscle contractions. In the marmoset bladder, adenosine nucleotides have been shown to cause relaxation by a P2Y receptor mechanism which could be blocked by suramin (McMurray *et al*., 1998). In the present study however suramin failed to modify the inhibition caused by the urothelium indicating that the released agent is not an adenosine nucleotide.

Catecholamines, which relax the bladder by stimulating β-adrenoceptors, would also appear not to be mediating the urothelium-induced inhibition, since the β-adrenoceptor antagonist propanolol was without effect. GABA also inhibits detrusor muscle contraction, an action that can be prevented by the potassium channel blocker TEA (Ferguson & Marchant, 1995). In this study however, TEA was without effect indicating the inhibitory effect was not mediated by GABA. It also shows the inhibitory agent is not activating either voltage activated or ATP-dependent potassium channels which would cause hyperpolarization and relaxation of the detrusor. Not all potassium channels however are blocked by TEA and one of the major TEA-insensitive channels is the small calcium activated potassium channel which can be blocked by apamin. The inhibition induced by the urothelium was not affected by apamin, which has also been shown to inhibit the effects of endothelium-derived hyperpolarizing factor (EDHF) in some vascular tissues (Garcia-Pascual *et al*., 1995). In rat hepatic arteries, potassium has been identified as the EDRF responsible for mediating relaxation responses to acetylcholine, local elevation of myoendothelial potassium levels activating K^+ channels and Na^+K^+ATP'ase (Edwards *et al*., 1998). However, the diffusible nature of the urothelium-derived factor between tissues in a relatively large organ bath (30 ml) would make it unlikely that a similar mechanism operates in the bladder.

In conclusion, the detrusor smooth muscle is sensitive to a diffusible inhibitory factor released from the urothelium, the nature of which at the present time is unclear. However, it would appear to be neither nitric oxide, a product of the cyclo-oxygenase system, an adenosine nucleotide, catecholamine, GABA, nor an apamin-sensitive response to EDHF.

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