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Evaluation of the rat bladder-derived relaxant factor by coaxial bioassay system

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Abstract

The release of bladder-derived relaxant factor in a coaxial bioassay system and the effects of reactive oxygen species were studied. After precontraction with phenylephrine (10^{-6}–3×10^{-6}) or 50 mM K^+, acetylcholine (10^{-8}–10^{-3} M) induced relaxation in rat anococcygeus muscle mounted within rat bladder in a tissue bath. This relaxation was not altered by the removal of the urothelium or incubation with tetrodotoxin (10^{-6} M). However, bupivacaine (10^{-4} M) and lidocaine (3×10^{-4} M) inhibited this response after raising the pH of the nutrient solution to 7.8, and oxybuprocaine (10^{-4} M) exerted inhibitory effect at both physiological pH (7.4) and at pH 7.8. Exposure to electrolysis-generated reactive oxygen species or incubation with hydrogen peroxide and pyrogallol did not alter the acetylcholine response. Present results indicate that the bladder-derived relaxant factor does not behave like endothelium-derived hyperpolarizing factor, but its release may be associated with tetrodotoxin-resistant Na^+ channels, which are probably in the neurons of the bladder rather than in the urothelium or detrusor muscle. Furthermore, reactive oxygen species do not interact with this relaxing factor, the exact nature and the physiological importance of which, however, remains to be established.

Keywords: Urinary bladder; Coaxial bioassay; Relaxation; (Rat)

1. Introduction

Studies have demonstrated that the local release of smooth muscle-relaxing agents in the urinary bladder has a modulator function in the regulation of detrusor muscle tone. Prostanoids and parathyroid hormone-related protein, synthesized in the bladder wall play a role in the bladder compliance during urine storage (Maggi, 1992; Steers et al., 1998). The influence of nitric oxide and vasoactive intestinal peptide on the regulation of contractile responses of the detrusor has also been demonstrated (Sjögren et al., 1985; Persson et al., 1991). Hawthorn et al. (2000) showed that the removal of urothelium augmented the contractions to carbachol in pig isolated bladder strips, and thus, suggested the modulatory role of urothelium by releasing an urothelium-derived inhibitory factor. Evidence for the presence of another smooth muscle relaxant factor not originating from urothelium has been obtained by using in vitro coaxial bioassay system consisting of endothelium-denuded rat aortic strips and the rat bladder (Fovaeus et al., 1999). The coaxial bioassay assembly enables the direct demonstration of the release of a smooth muscle relaxant factor from a tissue (Ilhan and Sahin, 1986). Thus, Fovaeus et al. (1999) have suggested that carbachol induced the release of a smooth muscle relaxant factor from bladder wall. In our previous study, we have confirmed the release of this bladder-derived relaxant factor, which is neither nitric oxide nor a product of cyclooxygenase (Inci et al., 2003). We have further demonstrated that bladder inflammation induced by cyclophosphamide or lipopolysaccharide injection to rats did not alter its synthesis and/or release (Inci et al., 2003). However, the nature of this diffusible smooth muscle relaxant factor and its origin in the bladder wall has not been identified yet.

Reactive oxygen species can cause lipid peroxidation of cellular membranes, the oxidation of macromolecules, and cellular damage when produced in huge amounts in certain pathologies, such as inflammation and ischemia–reperfusion (Halliwell and Gutteridge, 1984; Halliwell, 1996; Toyokuni, 1999). Increased production of reactive oxygen

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species may also be associated with some bladder diseases, and thus, their possible interaction with the bladder-derived relaxant factor may have physiological significance. In previous studies, we have demonstrated that reactive oxygen species interacted with other endogenous smooth muscle relaxing factors, such as nitric oxide in rat isolated aorta (Gumusel et al., 1996) and guinea pig tracheal epithelium-derived relaxant factor (EpDRF; Burcin et al., 1999), and impaired the endothelium- and epithelium-dependent relaxation responses, respectively.

In this study, we have evaluated whether the bladder-derived relaxant factor resembles the endothelium-derived hyperpolarizing factor (EDHF) in terms of its smooth muscle relaxing effect, the involvement of Na^+ channels in its release, and the effects of reactive oxygen species on the activity of this factor. The coaxial bioassay system consisting of rat urinary bladder and rat anococcygeus muscle was used in the present experiments.

2. Materials and methods

The study protocol was approved by the Hacettepe University Animal Ethics Committee.

2.1. Coaxial bioassay system

Male Wistar albino rats (150–250 g) were killed by a sharp blow to the head and bleeding, and the urinary bladder and anococcygeus muscles were isolated. The bladder was the donor organ for the assays in the coaxial bioassay system. The rat anococcygeus muscle was the assay tissue and was prepared as described by Gillespie (1972). In this bioassay system, the anococcygeus muscle was passed through small cuts made at the bladder neck and dome and placed in the lumen of the bladder under a resting tension of 1 g in an organ bath filled with Krebs–Henseleit solution. The composition of the Krebs–Henseleit solution was (in mM): NaCl, 95; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.6 and gassed with 95% O_2–5% CO_2 at 37 °C and pH 7.4. Tissues were equilibrated for 1 h and washed every 15 min before each experimental procedure, and isometric changes in tension were recorded with an isometric force transducer and "MAY 95-transducer data acquisition system" on an IBM-compatible personal computer.

2.2. Experimental protocol

The rat anococcygeus muscle enveloped by the bladder was precontracted by phenylephrine (10^{-6}–3×10^{-6} M, 60–80% of the maximum), and then, concentration-dependent relaxation to cumulatively added acetylcholine in one-log unit steps (10^{-8}–10^{-3} M) was obtained. In some experiments, anococcygeus muscle was precontracted by 50 mM K^+ (60–80% of the maximum) instead of phenylephrine, and an acetylcholine response was elicited afterwards. The initial tension produced by phenylephrine and 50 mM K^+ corresponds to ~ 2 g of contraction. The acetylcholine response in the coaxial bioassay was also obtained after mechanical denudation of the urothelium in rat bladder. Removal of the urothelium was confirmed by histological examination.

In a group of experiments, after constructing the initial acetylcholine concentration-response curve, the tissues were incubated with either tetrodotoxin (10^{-6} M) or the local anaesthetics lidocaine (10^{-4}–3×10^{-4} M), bupivacaine (10^{-4} M), and oxybuprocaine (10^{-4} M) for 30 min. Then, the concentration-dependent acetylcholine response was repeated as described above. Incubation with these agents did not alter the phenylephrine-induced precontraction level of the anococcygeus muscle. The experiments with lidocaine, bupivacaine, and oxybuprocaine were also conducted after the pH of the Krebs–Henseleit solution was raised from normal 7.4 to 7.8 by addition of $NaHCO_3$. In the controls of these experiments, acetylcholine responses were obtained at pH 7.8 in the absence of these agents. In the preliminary experiments, the effect of tetrodotoxin was also elicited at pH 7.8 of the incubation medium, and as tetrodotoxin did not alter the relaxation to acetylcholine at this pH, the experiments were not further repeated.

In another group of experiments, the tissues were exposed to reactive oxygen species generated by electrolysis of the physiological solution in the organ bath. Electrolysis was conducted by two platinum electrodes (20 mA DC current) for 5 min, which were placed in the organ bath 1 cm away from the tissues. The reactive oxygen species produced by electrolysis have been reported as superoxide anion, hydrogen peroxide, and hydroxyl radical (Jackson et al., 1986). After electrolysis, the organ baths were washed out, and thereafter, the concentration-response curves for acetylcholine were repeated as described above. Effects of hydrogen peroxide and the superoxide anion generator pyrogallol on acetylcholine-induced relaxation in the coaxial bioassay system were also investigated. For this purpose, the tissues were incubated with either hydrogen peroxide (10^{-4} M) or pyrogallol (10^{-4} M) for 30 min before acetylcholine response was elicited.

2.3. Drugs and drug solutions

Acetylcholine hydrochloride, phenylephrine hydrochloride, papaverine hydrochloride, tetrodotoxin, hydrogen peroxide, pyrogallol were purchased from Sigma (St. Louis, MO). Lidocaine was obtained from Adeka (Turkey), bupivacaine was from Astra-Zeneca, and oxybuprocaine was from Liba Labarotories (Turkey).

2.4. Statistical analysis

In precontracted anococcygeus muscle mounted within the bladder (coaxial bioassay system), the acetylcholine response is expressed as the percentage of papaverine (10^{-4} M)-induced relaxation, which was added to the organ

bath after obtaining the maximum acetylcholine response. Papaverine at this concentration induced ~100% relaxation relative to the precontraction elicited by phenylephrine.

The concentration required to achieve half-maximum relaxation (EC_{50}) and the maximum response (E_{max}) elicited by acetylcholine were obtained from individual concentration-response curves. EC_{50} values are given as pD_2 values ($pD_2 = -\log EC_{50}$).

Data are expressed as means ± standard error of the mean (S.E.M.).

Statistical analysis were performed by using analysis of variance (ANOVA) with repeated measurements and Bonferroni test; paired samples were compared by Student's *t* test. A *P* value of less than 0.05 was considered significant.

3. Results

After precontraction by phenylephrine (10^{-6}–3×10^{-6} M), acetylcholine induced concentration-dependent relaxation in anococcygeus muscle mounted within the urothelium-intact bladder amounting to 75–85% of maximum related to that elicited by papaverine (10^{-4} M; Fig. 1). Such relaxation response to acetylcholine was not observed if bladder was removed from the organ bath. The concentration-response curve to acetylcholine was not altered significantly when the anococcygeus muscle was precontracted by 50 mM K^+ instead of phenylephrine in the coaxial bioassay system (Fig. 2). Mechanical denudation of the bladder urothelium also had no effect on the relaxation response elicited by acetylcholine (Fig. 2). Although the acetylcholine response was not affected by the presence of urothelium, further experiments were conducted with urothelium-denuded bladders to avoid its possible involvement in the effects of agents studied below.

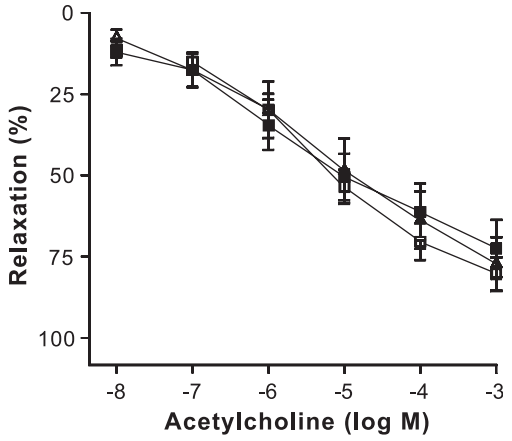

Fig. 2. Acetylcholine-induced relaxation response in rat anococcygeus muscle when mounted within urothelium-intact rat bladder and precontracted with phenylephrine (phe; ■) and 50 mM K^+ (□), and when mounted within urothelium-denuded bladder and precontracted with phenylephrine (Δ). Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M., ($n = 5$). The relaxant effect of acetylcholine is lost if the urinary bladder segment is removed (not shown).

Incubation with Na^+ channel blocker tetrodotoxin (10^{-6} M) did not affect acetylcholine-induced relaxation of phenylephrine-precontracted anococcygeus muscle mounted within the urothelium-denuded bladder (Fig. 3). The local anaesthetics, bupivacaine (10^{-4} M) and lidocaine (10^{-4} –3×10^{-4} M), also did not alter the concentration-response curve for acetylcholine in the coaxial bioassay system (Fig. 4). However, both bupivacaine at 10^{-4} M and lidocaine at 3×10^{-4} M concentration significantly inhibited acetylcholine-induced relaxation when the pH of the Krebs–Henseleit solution in the organ bath was raised from 7.4 to 7.8 by $NaHCO_3$ addition (Fig. 5). On the other hand, oxybuprocaine at 10^{-4} M exerted significant

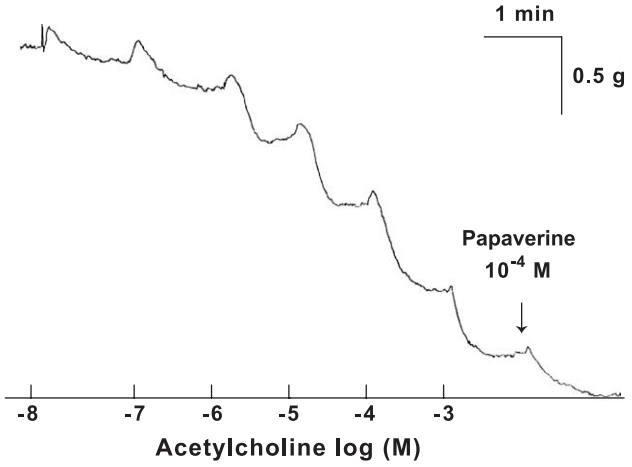

Fig. 1. A typical recording of a relaxation response to cumulatively added acetylcholine (10^{-8}–10^{-3} M) in phenylephrine (10^{-6} M)-precontracted rat anococcygeus muscle mounted with rat urinary bladder in a coaxial bioassay system. Papaverine (10^{-4} M) was applied to the bath to observe the maximum relaxation of the tissue.

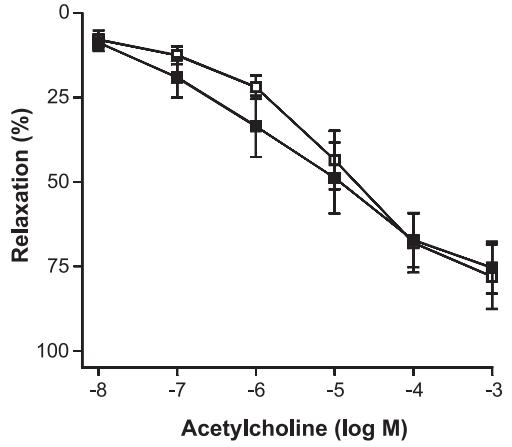

Fig. 3. Acetylcholine-induced relaxation response in phenylephrine-precontracted rat anococcygeus muscle mounted within urothelium-denuded rat bladder before (control; ■) and after incubation with tetrodotoxin (10^{-6} M; □). Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M. ($n = 5$).

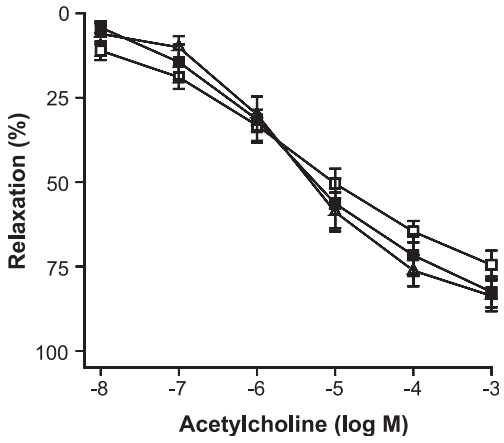

Fig. 4. Acetylcholine-induced relaxation in phenylephrine-precontracted rat anococcygeus muscle mounted within urothelium-denuded rat bladder before (control; ■) and after incubation with bupivacaine (10^{-4} M; □) and lidocaine (3×10^{-4} M; Δ) at usual pH (7.4) of Krebs–Henseleit solution in the organ bath. Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M. ($n = 6$).

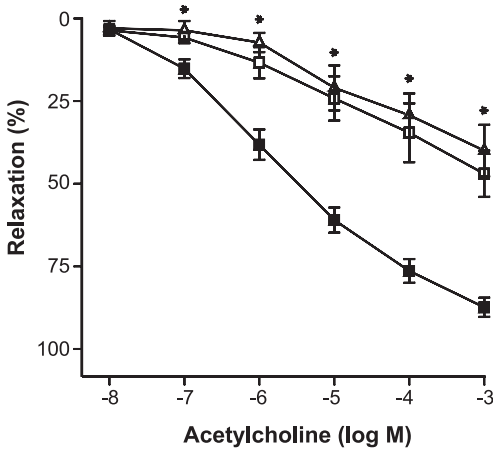

Fig. 6. Acetylcholine-induced relaxation response in phenylephrine-precontracted rat anococcygeus muscle mounted within urothelium-denuded rat bladder before (control; ■) and after incubation with oxybuprocaine (10^{-4} M) at pH 7.4 (□) and at pH 7.8 (Δ) of Krebs–Henseleit solution in the organ bath. Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M. [*significantly different from control response (■), $P < 0.05$, $n = 5$].

inhibitory effect on acetylcholine response in the coaxial bioassay system at both pH 7.4 and 7.8 of the nutrient solution (Fig. 6). In the absence of these local anaesthetics, adjusting the pH of the medium to 7.8 did not alter the acetylcholine response; the pD_2 and E_{max} of acetylcholine were 5.44 ± 0.14% and 79.54 ± 4.07, respectively, and were not significantly different from the values obtained at usual pH (7.4) of the Krebs–Henseleit solution (5.41 ±0.15% and 83.67 ± 4.57, respectively; means ±S.E.M., $n = 6$).

In a further series of experiments, the effects of reactive oxygen species were also evaluated. Electrolysis of the physiological solution in the organ bath did not alter acetylcholine-induced relaxation in the anococcygeus muscle placed within the bladder. Furthermore, incubation with either hydrogen peroxide (10^{-4} M) or superoxide anion generator pyrogallol (10^{-4} M) did not have any effect on the relaxation response to acetylcholine. The pD_2 and E_{max} values from concentration-response curves of acetylcholine in the coaxial bioassay system after exposure to reactive oxygen species were given in Table 1.

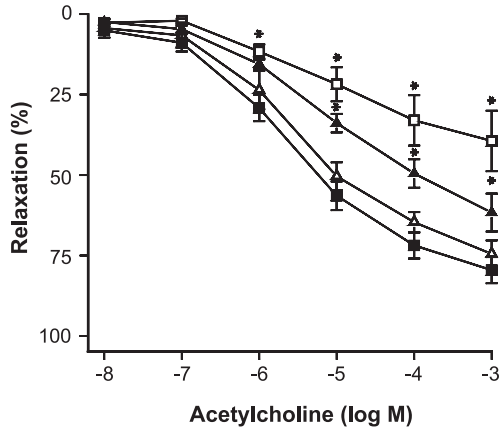

Fig. 5. Acetylcholine-induced relaxation in phenylephrine-precontracted rat anococcygeus muscle mounted within urothelium-denuded rat bladder before (control; ■) and after incubation with bupivacaine (10^{-4} M; □) and lidocaine (10^{-4} M; Δ and 3×10^{-4} M; ▲) at pH 7.8 of Krebs–Henseleit solution in the organ bath. Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M. [*significantly different from control response (■), $P < 0.05$, $n = 6$].

4. Discussion

Using a coaxial bioassay system with rat bladder as the donor organ and rat aorta as the assay tissue, Fovaeus et al. (1999) reported the release of a previously unrecognized smooth muscle relaxing factor from the bladder by muscarinic receptor stimulation. Our previous study and the present experiments, with the same bioassay system but using rat anococcygeus muscle as the assay tissue, validated

Table 1
The pD_2 and E_{max} values for acetylcholine (10^{-8}–10^{-3} M)-induced relaxation responses in rat anococcygeus muscle mounted within the bladder (coaxial bioassay system) before (control) and after exposure to reactive oxygen species

Groups	pD_2	E_{max}
Control	5.35 ± 0.30	84.15 ± 2.17
Electrolysis	4.99 ± 0.52	85.62 ± 2.68
Control	5.69 ± 0.44	74.47 ± 0.78
Pyrogallol (10^{-4} M)	5.62 ± 0.51	67.59 ± 5.01
Control	5.31 ± 0.31	85.46 ± 5.09
H_2O_2 (10^{-4} M)	5.33 ± 0.52	84.95 ± 3.95

Data are expressed as the percentage of papaverine (10^{-4} M)-induced relaxation and shown as mean ± S.E.M. ($n = 4$).
pD_2—negative logarithm of concentration required to achieve half-maximum relaxation.
E_{max}—percent maximum relaxation.

the release of this bladder-derived relaxant factor (Inci et al., 2003). The assay tissue was the rat anococcygeus muscle as it has been reported as a more convenient bioassay organ in coaxial assemblies particularly to detect a relaxing factor derived from airway epithelium (Guc et al., 1988). In our study, removal of the urothelium did not alter the relaxation elicited by acetylcholine in the coaxial bioassay system indicating that the bladder-derived relaxant factor is released by muscarinic receptor stimulation from the other constituents of the bladder wall, i.e., intramural nerves and/or the smooth muscle cells but not from the urothelium. Although its nature could not be identified, this factor is neither nitric oxide nor a cyclooxygenase product and does not appear to be a mediator released upon nonadrenergic noncholinergic nerve stimulation that is degradable by alpha-chymotrypsin in the bladder (Fovaeus et al., 1999; Inci et al., 2003). Its relaxant effect is not mediated through the cyclic nucleotides, cGMP or cAMP, and hyperpolarization by the opening of ATP-sensitive K^+ channels and Ca^{+2}-activated K^+ channels (Fovaeus et al., 1999). Moreover, inflammation of the bladder did not alter the synthesis/release of this bladder-derived relaxant factor (Inci et al., 2003).

We have further investigated whether the smooth muscle relaxant effect of this factor was comparable to that of EDHF. EDHF, like bladder-derived relaxant factor, is an endogenous smooth muscle relaxant but contributes to the reductions in smooth muscle tone in some vascular beds, i.e., rat mesentery, rabbit middle cerebral artery, and basilar artery (Chen et al., 1988; Parsons et al., 1991; Plane and Garland, 1993). Increasing the external K^+ concentration has been reported to inhibit the smooth muscle relaxant effects of EDHF (Parsons et al., 1991; Plane and Garland, 1993). However, in the present experiments, acetylcholine-induced relaxation in the coaxial bioassay system was not altered when anococcygeus muscle was precontracted by high K^+ (50 mM) instead of phenylephrine. Thus, these data might be taken as evidence against any similarity between the mode of relaxant effects of bladder-derived relaxing factor and EDHF.

In this study, tetrodotoxin, a selective blocker of Na^+ channels did not alter the acetylcholine response in the coaxial bioassay system, and thus, it may be suggested that the release of bladder-derived relaxing factor is not associated with the activation of Na^+ channels. On the other hand, Na^+ conductance related to the release of this relaxant factor might be resistant to blockage by tetrodotoxin, because the presence of tetrodotoxin-resistant Na^+ channels besides tetrodotoxin-sensitive subtypes has also been reported (Roy and Narahashi, 1992). Moreover, most of the bladder afferent neurons exhibit high threshold Na^+ currents and action potentials that are resistant to blockage by tetrodotoxin (Yoshimura et al., 1996; Yoshimura, 1999).

We have also investigated the effects of local anaesthetics bupivacaine and lidocaine, another group of drugs that block Na^+ channels. Both bupivacaine and lidocaine attenuated the acetylcholine response in the coaxial bioassay system, although this was achieved only after increasing the pH of the nutrient solution to 7.8. Local anaesthetics coexist as nonionized amine and cationic forms at physiological pH, and alkalinization of their solutions increases the proportion of the nonionized form, which readily penetrates the lipid barriers at its site of action (Chernoff and Strichartz, 1990; Chernoff, 1990). In the present experiments, raising the pH of the incubation medium to 7.8 would increase the nonionized fraction of bupivacaine (pKa = 8.1) and lidocaine (pKa = 7.8) to approximately 33% and 50%, respectively. Therefore, our findings indicated that bupivacaine and lidocaine could inhibit the release of bladder-derived relaxing factor, but at concentrations used in the present experiments, this effect became only evident when their diffusion in the bladder wall was increased by the alkalinization of the medium.

The effect of oxybuprocaine was also tested in this study, and we have demonstrated the inhibitory effect of this local anaesthetic in the coaxial bioassay system not only at pH 7.8 but also at pH 7.4 of the nutrient solution. Oxybuprocaine has a pKa value of 8.8 (Schulman and Underberg, 1979), which is very close to that of tetrodotoxin (8.76). The little changes in pH would not alter its ionisation fraction, much so the action of oxybuprocaine is independent of the medium pH, unlike lidocaine and bupivacaine (Chernoff and Strichartz, 1990). Thus, raising the pH of the physiological solution to 7.8 would increase the nonionised fraction only to 9%, and we found that this is not necessary for oxybuprocaine to inhibit the release of bladder derived-relaxant factor at the concentration used in the present experiments.

As local anaesthetics exerted their effects primarily by blocking Na^+ channels, we may suggest that the inhibitory effect of bupivacaine, lidocaine, and oxybuprocaine on the release of bladder-derived relaxing factor is mediated by Na^+ channel blockage. These could be the tetrodotoxin-resistant subtypes, as it has been previously reported that both bupivacaine and lidocaine are capable of blocking tetrodotoxin-resistant Na^+ channels in other tissues (Scholz et al., 1998; Scholz and Vogel, 2000). The concentrations of bupivacaine and lidocaine used in the present experiments are within the range of previous studies and have been shown to block 60–80% of the tetrodotoxin-resistant Na^+ currents in neurons of dorsal root ganglions of the rat (Scholz et al., 1998; Scholz and Vogel, 2000). Thus, we may speculate that Na^+ channels involved in the release of bladder-derived relaxing factor are located in the neurons or other constituents of the bladder wall rather than detrusor muscle cells. It has been established that the sodium conductance on depolarization does not exist in the detrusor smooth muscle (Kurihara, 1975; Yokoyama et al., 1997).

The inflammation of tissues or ischemia–reperfusion injury is associated with increased production of reactive oxygen species (McCord, 1975, 1985). These species may also be involved in certain bladder pathologies leading to a possible interaction with bladder-derived relaxing factor in

the bladder wall. However, in this study, exposure to reactive oxygen species by electrolysis of the nutrient solution or incubation with hydrogen peroxide and pyrogallol did not alter the bladder-derived relaxing factor-mediated responses in the coaxial bioassay system. On the other hand, we have demonstrated that reactive oxygen species, produced by the similar methods, impaired the relaxation responses mediated by nitric oxide in isolated rat aorta (Gumusel et al., 1996) and EpDRF in coaxial bioassay consisting of guinea pig trachea and rat anococcygeus muscle (Burcin et al., 1999). In this study, the lack of any effect of reactive oxygen species may be due to their poor penetration in the bladder wall. This finding may also indicate that bladder-derived relaxing factor is resistant to the effects of reactive oxygen species. This may be because of its unknown chemical structure or the endogenous antioxidant defence mechanisms, especially if it is originated from the neurons of the bladder. Likewise, previous studies have shown that reactive oxygen species did not alter responses to nitrergic nerve stimulation but inhibited responses to exogenous nitric oxide (Gillespie and Sheng, 1990; Paisley and Martin, 1996). We have also reported that electrolysis of the physiological medium did not alter the relaxation responses mediated by the nitric oxide released from nitrergic nerves in the rat anococcygeus muscle (Durlu et al., 2003).

In this study, using a coaxial bioassay system, we have evaluated the release of a smooth muscle relaxant factor from rat bladder wall by muscarinic receptor stimulation. As shown previously, this factor is neither nitric oxide nor a cyclooxygenase product (Fovaeus et al., 1999). Further to these findings, we have shown that bladder-derived relaxant factor does not behave like EDHF, and its release may be associated with Na^+ channels, which are probably in the neurons of the bladder rather than in the urothelium or detrusor smooth muscle. Reactive oxygen species, despite their involvement in some bladder pathologies, do not interact with this relaxing factor.

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