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# Differences between proximal and distal portions of the male rabbit posterior urethra in the physiological role of muscarinic cholinergic receptors

[1,3]Katsushi Nagahama, [1]Toshihiko Tsujii, [1]Takashi Morita, [2]Hiroshi Azuma & [1]Hiroyuki Oshima

[1]Department of Urology, School of Medicine and [2]Department of Medicinal Chemistry, Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, 1-5-45, Yusima, Bunkyo-ku, Tokyo 113, Japan

**1** The aim of the present study was to elucidate functional differences between embryologically different portions of the posterior urethra of male rabbits in response to muscarinic acetylcholine receptor (mAChR) stimulation using *in vitro* isometric tension experiments and radioligand binding studies.

**2** In the *in vitro* isometric tension experiments, carbachol, produced a dose-dependent contraction of the proximal portion under the resting state, but did not change the basal tone of the distal portion. Contraction of the proximal portion by $10^{-5}$ M noradrenaline (NA) was dose-dependently enhanced by carbachol either in the presence or absence of $N^G$-nitro-L-arginine (NOARG). In contrast, carbachol induced relaxation of the distal portion contracted by $10^{-5}$ M NA, which was reversed to dose-dependent contraction in the presence of NOARG.

**3** Both portions of the urethra had a similar number of [$^3$H]-quinuclidinyl benzilate ([$^3$H]-QNB) binding sites ($195.3 \pm 74.1$ fmols mg$^{-1}$ protein for the proximal portion and $146.5 \pm 8.5$ fmols mg$^{-1}$ protein for the distal portion) with similar affinities ($115.0 \pm 45.4$ pM for the proximal portion and $79.9 \pm 2.9$ pM for the distal portion).

**4** The concentration-response curves to carbachol in both portions were shifted to the right in a parallel manner in the presence of pirenzepine (an $M_1$ antagonist), 11-[[2-[(diethylamino)methyl]-1-piperidinyl] acetyl]-5, 11-dihydro-6H-pyrido-2,3-b)-(1,4)-benzodiazepin-6-one (AFDX-116, an $M_2$ antgonist) and 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP, an $M_1/M_3$ antagonist). The $pA_2$ values for pirenzepine, AFDX-116 and 4-DAMP were $7.5 \pm 0.1$, $7.2 \pm 0.02$ and $9.3 \pm 0.1$ respectively for the contraction of the proximal portion, and $7.2 \pm 0.1$, $7.1 \pm 0.2$ and $9.1 \pm 0.2$, respectively for the relaxation of the distal portion.

**5** In conclusion mAChR subtypes distribute in a similar fashion throughout the length of the male rabbit posterior urethra with the discrepant responses to carbachol attributable to the differential involvement of the NO pathway in mAChR-generated reactions.

**Keywords:** Muscarinic cholinergic receptors; male rabbit; posterior urethra; proximal portion; distal portion; [$^3$H]-QNB binding

## Introduction

The male urethra plays two roles as the urinary and seminal tracts and in this respect differs from the bladder. The posterior urethra consists of two embryologically different portions. The portion proximal to the seminal colliculus includes tissues originating from both mesoderm and endodem, and the portion distal to the seminal colliculus includes those from both endoderm and ectoderm (Hutch 1972). There have been few reports investigating physiological differences between the two embryologically different portions of the posterior urethra.

It is known that the mammalian bladder and urethra have at least a dual innervation (adrenergic and cholinergic nerves) (Elbadawi, 1982; Slack *et al*., 1982; Elbadawi & Schneck, 1966). In general, bladder contraction is mainly mediated by excitation of the muscarinic acetylcholine receptor (mAChR) and muscular tone of the posterior urethra is maintained primarily by excitation of α-adrenoceptors (Donker *et al*., 1972; Awad & Downie, 1976). The physiological significance of mAChR in the posterior urethra has remained uncler, although some reports have investigated responses to stimulation of mAChRs (Andersson *et al*., 1983; Hassouna *et al*., 1983; Hashimoto *et al*., 1992; Mutoh *et al*., 1997). These previous reports did not refer to segmental differences of embryological origin.

The present organ chamber experiments and radioligand binding studies were designed to investigate whether or not there are differences in the response to stimulation of mAChRs between the two embryologically different portions of the posterior urethra.

## Methods

### *Animals and urethral specimens*

In the current study, rabbits were handled according to the institutional guidelines for the care of laboratory animals under the approval of the Institutional Animal Care Use Committee. Japanese White male rabbits weighing approximately 3 kg were anesthetized by intravenous injection of sodium pentobarbital (25 mg kg$^{-1}$) and were sacrificed by exsanguination. Their urinary bladder and posterior urethra were immediately removed *en bloc* and dipped in an ice-cold buffer (see 'Isometric tension experiments' below) or 50 mM phosphate buffer (pH 7.5) for the radioligand receptor binding assay. The posterior

[3]Author for correspondence at: Department of Urology, Tama Nanbu Regional Hospital, 2-1-2 Nakazawa, Tama City, Tokyo 206, Japan.

urethra was opened by an anterior longitudinal median incision and divided into proximal (from the urethro-vesical junction to the seminal colliculus) and distal (from the colliculus to the end of the posterior urethra) portions.

### In vitro *isometric tension experiments*

The mechanical responses were measured according to methods described previously (Azuma *et al*., 1992; Tsujii *et al*., 1992). In brief, transverse muscle strips prepared from a portion of each urethral specimen were placed in an ice-cold modified Krebs solution (pH 7.4) containing 115.0 mM NaCl, 4.7 mM KCL, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM $KH_2O_4$, 25.0 M $NaHCO_3$ and 10.0 mM glucose. After removal of adipose and connective tissue, muscle strips of approximately 5 mm width, 8 mm length and weighing 90 mg were prepared and mounted vertically in organ baths filled with 5 ml modified Krebs solution which was continuously bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ at 37°C. One end of the strip was anchored to a metal hook at the bottom of the organ bath and the other end was connected to a force-displacement transducer (TB611T, Nihon Kohden Kogyo CO.). Isometric changes in tension were recorded on a pen recorder (R-64, Rika Denki CO.).

The tension of the strip was adjusted several times to a basal tension of 0.5 g. Before starting the experiment, the strip was allowed to equilibrate for at least 40 min in the organ bath and the bath solution was replaced every 20 min. Each agonist or antagonist was applied cumulatively in the organ bath at concentrations indicated in the figures.

### *Radioligand receptor binding assay*

Preparation of crude membrane fractions: Immediately after removal, the posterior urethra was divided into proximal and distal portions in an ice-cold 50 mM phosphate buffer (pH 7.5). Each was minced with scissors and homogenized twice with a Polytron Homogenizer for four 30-s bursts at maximum speed and a 10-s cooling period in 10–20 volumes of the ice-cold phosphate buffer. Each homogenate was then centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was recentrifuged at $105{,}000 \times g$ for 60 min at 4°C to obtain a crude membrane fraction as the precipitate. The final pellet was resuspended in fresh phosphate buffer and stored at −20°C until use.

Protein concentration of each crude membrane fraction was measured according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. Each fraction was adjusted to contain 50 $\mu$g protein $(100\ \mu l)^{-1}$.

Saturation analysis was performed with a radiolabeled ligand of quinuclidinyl benzilate, L-[benzilic-4,4′-$^3$H] ([$^3$H]-QNB, 45.7 Ci $nmol^{-1}$). The medium for the assay contained 100 $\mu$l crude membrane fraction and 150 $\mu$l phosphate buffer containing the rdiolabeled ligand at concentrations indicated in Figure 4 (14.5–1900 pM). Assay tubes were incubated for 30 min at 37°C with constant shaking after which solutions were filtered through Whatman GF/C glass fibre filters. Filters were dipped in 0.05% polyethylenimine solution for 60 min before filtration in order to reduce nonspecific binding to the filter. Each filter was rinsed three times with 3 ml of ice-cold phosphate buffer and placed in 8 ml scintillation fluid (Econofluor; New England Nuclear Research Products).

Specific binding was defined as the difference between the binding in the presence or absence of $10^{-5}$ M atropine. Radioactivity was measured with a Packard 460-CD scintillation spectro-fluorometer. Saturation parameters were calculated using Scatchard analysis (Scatchard, 1949).

The antagonists used for the inhibition study were pirenzepine (an $M_1$ antagonist), 11-[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido-2,3-b)-(1,4)-benzodiazepin-6-one (AFDX-116, an $M_2$ antagonist) and 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP, an $M_1/M_3$ antagonist). Aliquots of the membrane preparation were incubated with 275 pM [$^3$H]-QNB in the presence or absence of each antagonist at concentrations indicated in Figure 5. Conditions of the inhibition study were identical to those used in the saturation study.

### *Chemicals*

[$^3$H]-QNB was purchased from New England Nuclear Research Products (Wilmington, U.S.A.). Atropine, pirenzepine, noradrenaline bitartrate and carbamylcholine chloride (carbachol) from Sigma Chemical Co. Ltd. (St. Louis, U.S.A.). 4-DAMP from Research Biochemicals International (Natick, U.S.A.). AFDX-116 was kindly donated by Dr Karl Thomae (Boehringer Ingelheim).

### *Statistical analysis*

All date are expressed as the mean ± s.e.mean. Statistical analysis was performed by Student's *t*-test ($P<0.05$ as significant) or analysis of variance (ANOVA) and Tukey-Kramer's test ($P<0.01$ as significant). The normal distribution of all the data were confirmed by Shapri-Wilk W test prior to the above-mentioned analyses. Regression lines were calculated by the least squares method.

## Results

### *Response of the proximal and distal portions to carbachol and the effect of $N^G$-nitro-L-arginine*

Under the resting state, addition of carbachol, a non-selective mAChR agonist, produced a dose-dependent contraction in the proximal portion. In contrast, carbachol did not change the basal tone of the distal portion over the range of concentrations examined. The contractile response to carbachol tended to be enhanced in the proximal portion and induced in the distal portion by the presence of $N^G$-nitro-L-arginine (NOARG) as shown in Figure 1.

As shown in Figure 2, carbachol induced dose-dependent relaxation of the distal portion in the contracted state by $10^{-5}$ M noradrenaline (NA) in the absence of NOARG, which was reversed to dose-dependent contraction by presence of NOARG. In contrast, the agent further enhanced dose-dependently the contraction of the proximal portion by $10^{-5}$ M NA regardless of the presence or absence of NOARG.

The phenomena observed in the distal portion were confirmed by separate experiments as shown in Figure 3, which indicated that carbachol was able to induce a tonic response in distal portion at high concentrations.

### *Binding characteristics of [$^3$H]-QNB in the proximal and distal portions*

Binding assay with [$^3$H]-QNB revealed that mAChR binding sites constituted a single population in both proximal and distal portions (Figure 4).

$K_D$ and $B_{max}$ for [$^3$H]-QNB binding were identical in both portions of the urethra (Table 1).

*Subtype of mAChR in the proximal and distal portions*

In the *in vitro* isometric tension experiments, the effects of pirenzepine (an $M_1$ antgonist), AFDX-116 (an $M_2$ antagonist) and 4-DAMP (an $M_1/M_3$ antagonist) on the concentration-response curve to carbachol were examined in each urethral portion. The concentration-contraction curves for carbachol in the proximal portion under the resting state were shifted to the right in a parallel manner in the presence of pirenzepine ($3\times10^{-8}-3\times10^{-7}$), AFDX-116 ($3\times10^{-8}-3\times10^{-7}$) or 4-DAMP ($3\times10^{-9}-3\times10^{-8}$ M). The $pA_2$ value of each antagonist in the proximal portion was determined (Table 2). The rank order of antagonistic affinity was 4-DAMP > pirenzepine > AFDX-116. 4-DAMP was 55- and 126-fold more

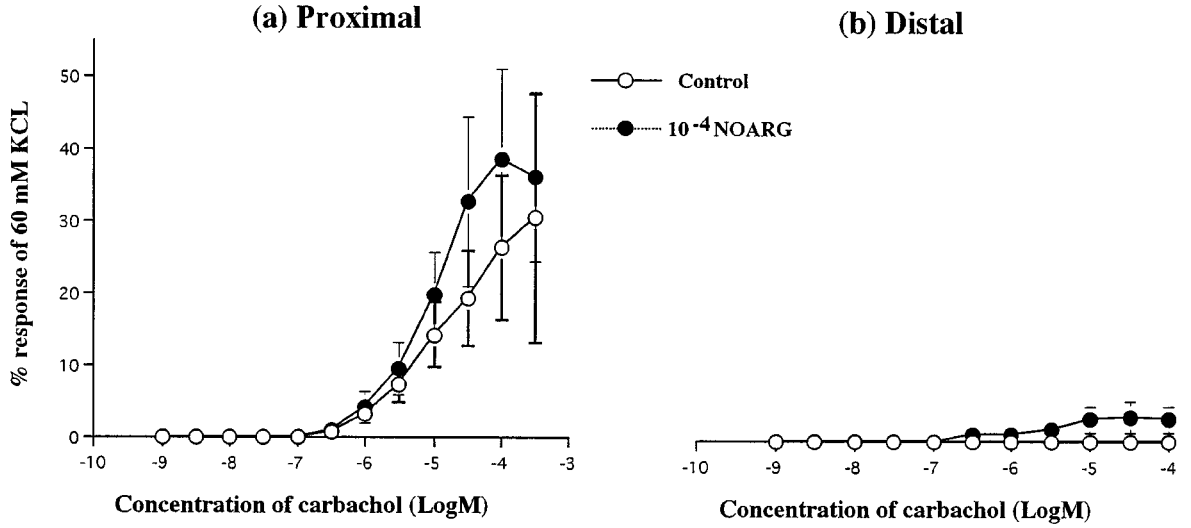


**Figure 1** Concentration-response curves to carbachol in the absence and presence of $N^G$-nitro-L-arginine (NOARG), an inhibitor of nitric oxide synthetase, under the resting state in the proximal (a) and distal (b) portions of the male rabbit posterior urethra. Changes in response were expressed as a percentage of the contraction to 60 mM KCL before addition of carbachol. NOARG tended to enhance the contractile responses in both portions, although not significantly ($n=6$ and 4 in the proximal and distal portion, respectively). Data are mean and s.e.mean.

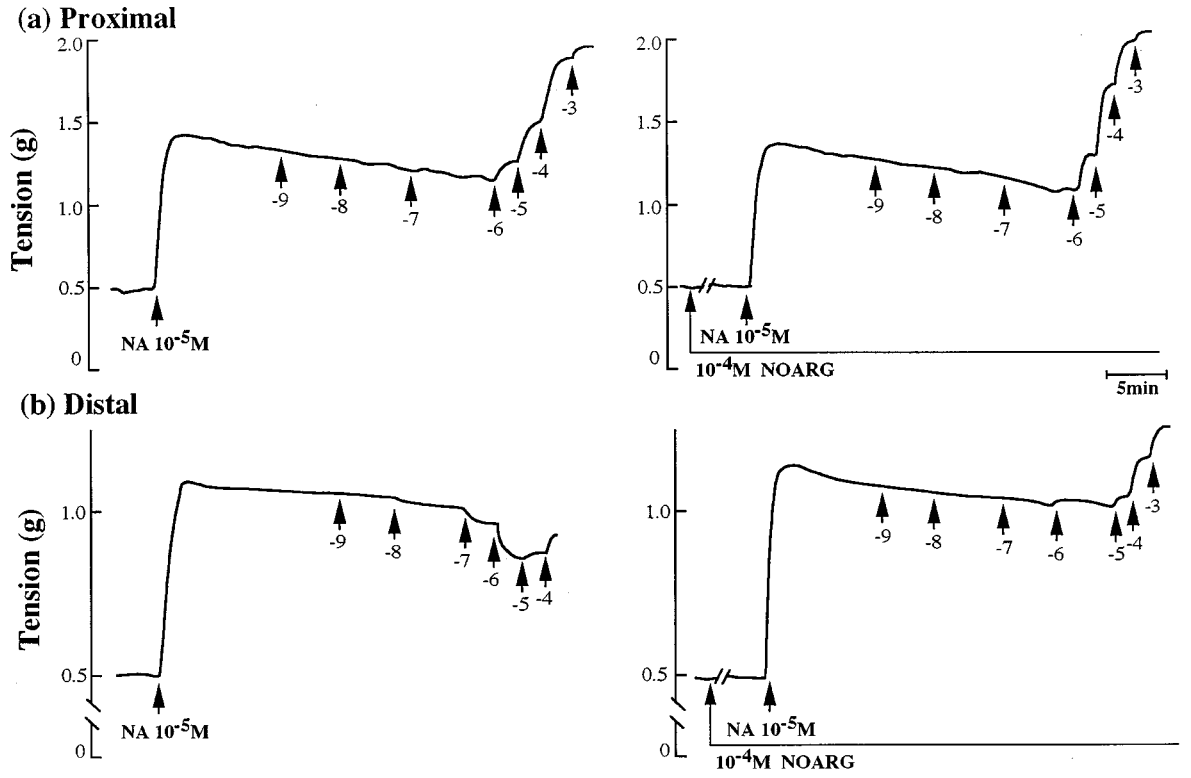


**Figure 2** Responses of the proximal (a) and distal (b) portions of the male rabbit posterior urethra to carbachol in the absence or presence of $N^G$-nitro-L-arginine (NOARG) under contraction by $10^{-5}$ M noradrenaline (NA). Number below arrows indicates the concentration (M) of carbachol in logarithm. The figure shows a representative line of repeated experiments ($n=4$ and 5 in the proximal and distal portion, respectively).

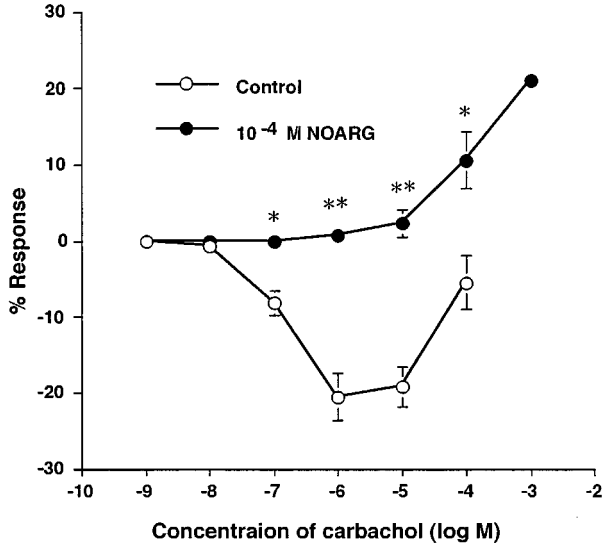


**Figure 3** Effect of $N^G$-nitro-L-arginine (NOARG) on the response of the distal portion of the male rabbit posterior urethra to carbachol. Each strip was contracted by $10^{-5}$ M noradrenaline (NA). NOARG was added to the bath 20 min prior to addition of $10^{-5}$ M NA and then carbachol was added. Results are given as mean±s.e.mean of 5 determinations. Changes in response were expressed as a percentage of the contraction to $10^{-5}$ M NA before addition of carbachol. (*$P<0.01$; **$P<0.001$ *vs* control, by Student's *t*-test).

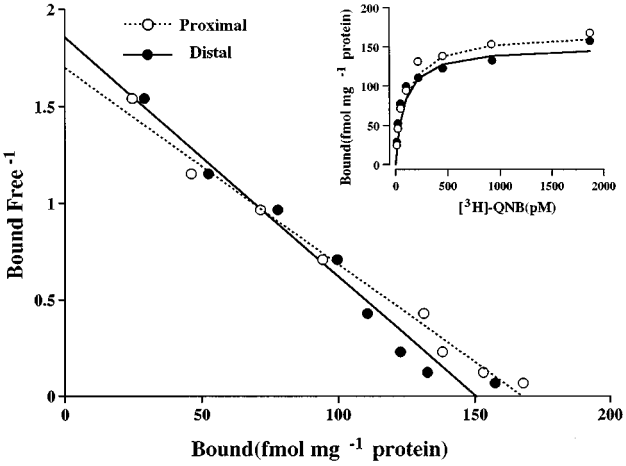


**Figure 4** Scatchard plots of [$^3$H]-Quinuclidinyl benzilate ([$^3$H]-QNB) binding to crude membrane fractions prepared from the proximal and distal portions of male rabbit posterior urethra. Tissue preparations were incubated with [$^3$H]-QNB at eight different concentrations (14.5–1900 pM) in triplicate under conditions described in the text. Non-specific binding was determined in the presence of $10^{-5}$ M atropine. Inset, saturation curves for [$^3$H]-QNB binding in crude membrane fractions of the posterior urethral muscle.

potent than pirenzepine or AFDX-116 respectively. Furthermore, these three agents antagonized dose-dependently the carbachol-induced relaxation of the distal portion under the tonic contraction of $10^{-5}$ M NA. The $pA_2$ value of each antagonist in the distal portion was determined (Table 2). 4-DAMP was 83- and 93-fold more potent than pirenzepine or AFDX-116, respectively, as observed in the proximal portion.

Figure 5 shows the inhibition profiles of pirenzepine, AFDX-116 and 4-DAMP for the specific [$^3$H]-QNB binding in the proximal and distal urethral portions. The rank order of the antagonistic affinity was 4-DAMP > pirenzepine ⩾ AFDX-116 in both proximal and distal portions.

**Table 1** [$^3$H]-Quinuclidinyl benzilate ([$^3$H]-QNB) binding to the proximal and distal portions of the male rabbit posterior urethra

| | *Proximal* | *Distal* |
|---|---|---|
| | N.S. | |
| $K_d$(pM) | 115.0±45.4 | 79.9±2.9 |
| $B_{max}$(fmol mg$^{-1}$ protein) | 195.3±74.1 | 146.5±8.5 |
| | N.S. | |

Results are given as mean±s.e.mean of three separate experiments. N.S.; not significant (Student's *t*-test)

**Table 2** $pA_2$ values for antagonists against subtypes of mAChR in the proximal and distal portions of the male rabbit posterior urethra

| | $pA_2$ | |
|---|---|---|
| *Antagonists* | *Proximal* | *Distal* |
| Pirenzepine | 7.53±0.08* | 7.15±0.09* |
| AFDX-116 | 7.17±0.02* | 7.10±0.20* |
| 4-DAMP | 9.27±0.08 | 9.07±0.16 |

Data are mean±s.e.mean of 3–7 separate experiments. *Significant differences *vs* 4-DAMP (ANOVA, $P<0.01$). AFDX-116: 11-[[2-[(diethylamino)methyl]-1-piperidinyl] acetyl]-5-11-dihydro-6H-pyrido-2,3-b-)-(1,4)-benzodiazepin-6-one 4-DAMP: 4-diphenyl-acetoxy-N-methylpiperidine.

## Discussion

The current study has demonstrated that there are differences in the response of the proximal and distal portions of the male rabbit posterior urethra to carbachol. The proximal portion contracted dose-dependently under both basal and NA-contracted states in response to carbachol, while the distal portion responded to carbachol with a dose-dependent relaxation under the NA-contracted state. The dose-dependent contraction of the proximal portion coincides with previous stimulation studies with non-selective mAChR agonists using either a portion of the posterior urethra of male cats (Hassouna *et al*., 1983), the proximal one-third portion of the female dog urethra (Hashimoto *et al*., 1992), or a proximal portion of the female rabbit urethra (Mutoh *et al*., 1997). On the other hand, the proximal two-thirds of the female rabbit urethra has been reported not to respond to exogenously applied acetylcholine (Andersson *et al*., 1983). The same authors, however, demonstrated that field stimulation induces relaxation of human distal urethral portion and further contraction of proximal urethral portion under the NA-contracted state.

Regardless of the above findings, $K_D$ and $B_{max}$ for [$^3$H]-QNB binding in the proximal and distal portions are virtually identical. While mAChRs have been shown to distribute differently between the bladder and urethra (Johns, 1983), it has been demonstrated by enzymatic staining of acetylcholinesterase that parasympathetic innervation is uniform throughout the length of the female rabbit urethra (Bridgewater *et al*., 1995).

Judging from the $pA_2$ values, mechanical responses to carbachol in both proximal and distal portions of the posterior urethra of male rabbit are similarly inhibited by addition of pirenzepine, AFDX-116 or 4-DAMP, indicating similar distribution of $M_1$, $M_2$ and $M_3$ subtypes of mAChRs in both

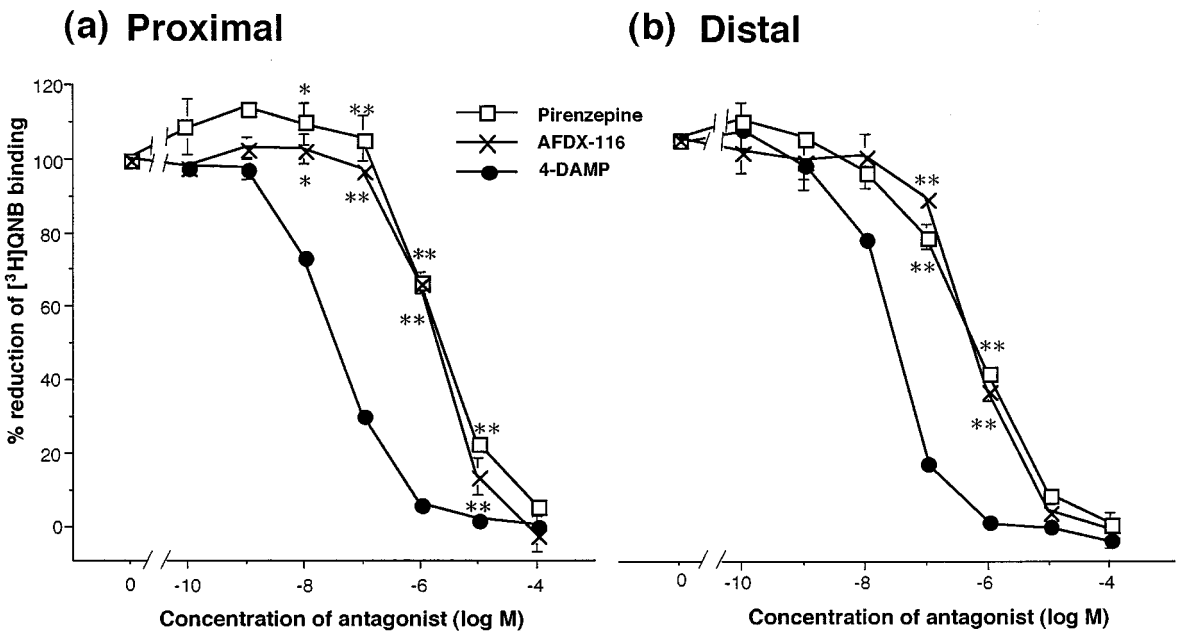


**Figure 5** Displacement of specific [$^3$H]-Quinuclidinyl benzilate ([$^3$H]-QNB) binding to crude membrane fractions of rabbit posterior urethral muscle by pirenzepine, 11-[[2-[(diethylamino)methyl]-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido-2,3-b)-(1,4)-benzodiazepin-6-one (AFDX-116) and 4-diphenyl-acetoxyn-N-methylpiperidine (4-DAMP). Aliquots of the membrane preparation were incubated with 275 pM [$^3$H]-QNB in the presence or absence of each antagonist at concentrations indicated in the figure. Each point represents the mean ± s.e.mean of 3 determinations. The inhibition-concentration curves produced by the antagonists were almost parallel and indicated that 4-DAMP was the most potent antagonist, followed by AFDX-116 and pirenzepine. (*$P<0.01$, **$P<0.001$ *vs* 4-DAMP, by ANOVA and Tukey-Kramer's test).

portions. Inhibition studies using the same antagonists support the above assumption. These three subtypes of mAChRs ($M_1$, $M_2$ and $M_3$) are pharmacologically distinguishable (Hulme *et al*., 1990), while five different sequences of cDNA encoding mAChRs, including the above three types, have been reported (Bonner *et al*., 1987; Dörje *et al*., 1991; Hulme *et al*., 1990). A recent report (Mutoh *et al*., 1997) has indicated that $M_1$, $M_2$ and $M_3$ subtypes are involved in carbachol-induced contraction of the proximal portion of the female rabbit urethra but $M_1$ and $M_3$ subtypes play a major role on the basis of the $pA_2$ for pirenzepine and 4-DAMP. As the selectivity of the antagonists available at present is not specific for each pharmacologic receptor subtype, it is necessary to use several antagonists in order to characterize them. The current results have indicated that mAChR subtypes are present in the posterior urethra of the male rabbit and that those subtypes distribute in the proximal and distal portions of the posterior urethra in a similar fashion.

NOARG has been examined as a modifier of the response of male rabbit posterior urethra to carbachol and reverses the relaxation and has a tendency to enhance the contraction, of the distal and proximal portions, respectively. The present findings suggest that the NO pathway is not only involved in generating the relaxation of the distal portion but also antagonizes the contraction of the proximal portion to carbachol. Urethral relaxation has been recorded 5–15 s before starting micturition in man as an initial decrease in the urethral pressure (Tanago & Miller, 1970). It is believed that the relaxation in the urethra is generated by the presynaptic inhibition of noradrenaline release by the excitation of mAChR localized on the adrenergic nerve terminals (Mattiasson *et al*., 1984). It was recently demonstrated, however, that the L-arginine-NO pathway plays an important role in inducing relaxation of the urethra in response to field stimulation of the proximal portion of the urethra prepared from female sheep (Garcia-Pascual *et al*., 1991), rats (Persson and Andersson, 1992) and dogs (Hashimoto *et al*., 1993). Further, NOARG inhibits the urethral relaxation as a voiding reaction in female rats, suggesting that the NO pathway is also involved in active urethral relaxation during reflex micturition (Bennett *et al*., 1995). In the male rabbit, the magnitude of the field-stimulated relaxation under the phenylephrine-contracted state was greater in the prostatomembranous urethra than the bladder neck, and the relaxation of both regions was abolished by NOARG (Lee *et al*., 1994). Consistent with this finding, the rank order of NO synthase activity in the human lower urinary tract measured by citrulline formation and guanylate cyclase activity has been found to be the prostatic urethra > the bladder neck > the detrusor, with confirmative findings of the magnitude of the field-stimulated relaxations of these regions (Ehren *et al*., 1994). These findings indicate that the magnitude of the relaxation of the lower urinary tract depends on NO synthase activity. Therefore, the discrepant response to carbachol by different portions of the male rabbit posterior urethra in the present study appear to derive from the different involvement of the NO pathway in response to stimulation of mAChR, which may depend on different NO synthase activity by each portion.

In conclusion, different reactions to carbachol by portions of the posterior urethra of the male rabbit are not attributable to differences in distribution of mAChR subtypes present in the posterior urethra but result from differential involvement of the NO pathway in mAChR-generated reactions.

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