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Muscarinic receptor subtypes in porcine detrusor: comparison with humans and regulation by bladder augmentation

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Abstract The properties of muscarinic acetylcholine receptors of porcine and human bladder detrusor were compared in radioligand binding studies using [^{3}H]quinuclidinylbenzylate as the radioligand. The receptor affinity for the radioligand and the density of muscarinic receptors was similar in male and female pigs and in humans (K_d = 35 ± 8 pM, B_{max} = 153 ± 30 fmol/mg protein). Atropine and subtype-selective antagonists had steep and monophasic competition curves in porcine and human detrusor with a rank order of potency of atropine ≫ hexahydro-sila-difenidol ≥ AF-DX 116 ≥ pirenzepine, indicating the presence of a homogeneous population of M_2 muscarinic receptors. In female pigs bladder outflow obstruction generated by partial urethral ligation or its surgical treatment by ileum augmentation or autoaugmentation did not significantly alter expression of muscarinic receptors or of α_{2A}-adrenoceptors, but the power was insufficient to exclude alterations of less than 60%. We conclude that porcine and human detrusor express muscarinic receptors of the M_2 subtype; despite these qualitative similarities the use of the porcine model may be limited by large biological variance with regard to quantitative receptor expression.

Key words Detrusor · M_2 muscarinic receptors · α_{2A}-adrenoceptors · Bladder outflow obstruction · Pigs · Humans

Introduction

Parasympathetic nerves acting via muscarinic acetylcholine receptors play an important role in the regulation of bladder function [1]. While stimulation of postjunctional muscarinic receptors yields detrusor smooth muscle contraction, activation of prejunctional muscarinic receptors can mediate both facilitation and inhibition of acetylcholine release [5]. Thus, muscarinic receptors are an attractive therapeutic target for the medical treatment of bladder dysfunction [14]. Receptor cloning studies have revealed the existence of five subtypes of muscarinic receptors that are designated m_1, m_2, m_3, m_4 and m_5; each has a distinct tissue distribution and a distinct pharmacological profile [4, 16]. The development of muscarinic agonists and antagonists for the treatment of bladder dysfunction, therefore, requires precise knowledge about the muscarinic receptor subtypes present in human bladder, about animal models mimicking this situation and about possible alterations of muscarinic receptor function under pathophysiological conditions.

Previous studies have demonstrated that the expression of muscarinic receptor subtypes and their functional role in bladder may be species-dependent (for reviews see [1, 5]). When multiple subtypes coexist in a species, they may mediate distinct functions. For example in rabbit bladder the prejunctional facilitatory receptors may belong to the M_1 subtype, the prejunctional inhibitory receptors to the M_2 subtype, and the postjunctional receptors to the M_3 subtype [17, 18]. While functional and radioligand binding experiments in human bladder have argued against a major role of M_1 muscarinic receptors, no detailed positive identification of muscarinic receptor subtypes in human bladder has been presented [1, 5]. Moreover, it is not clear which animal species is most similar to man with regard to muscarinic receptors in the detrusor. Finally, studies in other tissues have shown that muscarinic receptor expression can be dynamically regulated by a variety of physiological and pathophysiological factors, including arterial hypertension [19] and oestrogen exposure [2], but it is not known whether this also occurs in the bladder.

Therefore, the present study had two major aims. Firstly, we compared the quantitative and qualitative

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characteristics of the muscarinic receptors in porcine and human detrusor to determine whether the pig may be a suitable animal model in which to study muscarinic receptors in the bladder. Secondly, we have investigated whether muscarinic receptors in the detrusor are regulated in a porcine model of bladder outflow obstruction and during its surgical treatment by ileum augmentation or autoaugmentation. Based on our previous findings that porcine and human detrusor are similar with regard to α_{2A}-adrenoceptor expression [8], we have also investigated the regulation of α_{2A}-adrenoceptors in our porcine model of bladder outflow obstruction.

Materials and methods

Tissue sources

Human bladder detrusor samples were obtained from patients undergoing cystectomy due to bladder cancer; all specimen were from macroscopically tumour-free areas. Porcine bladder detrusor samples were obtained from Goettingen minipigs of either sex (20–50 kg) that had been obtained from Ellegard (Aarhus, Denmark). The study protocol for the animal studies was approved by the state board for animal welfare at the Regierungspräsident Düsseldorf. All human and porcine tissue samples were macroscopically freed from surrounding adipose and connective tissues, blotted dry, rapidly frozen in liquid nitrogen and stored at −80°C.

In some experiments female pigs (3 months old, 10–12 kg) were randomized into four groups. They underwent surgical ligation of the urethra (groups 2–4) or sham surgery (group 1) during halothane anaesthesia. The chosen diameter of the ligation did not result in acute narrowing of the urethral lumen, but progressive narrowing occurred during the physiological growth process of the animals. During the same operation group 3 was additionally treated by ''autoaugmentation''; this consisted of partial detrusor myectomy for creating a widely based bladder diverticulum. Group 4 was concomitantly treated by an ileal augmentation according to Goodwin. After 6 months the animals were killed following repeat urodynamic verification of the effects of surgery. Details of the urodynamic and histological characterization of these animals will be published elsewhere. Tissue specimens were processed as described above.

Tissue preparation

Membrane fractions were prepared as previously described [15]. Briefly, tissue samples were thawed in ice-cold binding buffer A (50 mM TRIS, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5) and minced with scissors. They were homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) for 10 s at full speed and thereafter twice for 20 s each at two-thirds speed. The homogenates were centrifuged for 20 min at 50 000 *g* at 4°C. The pellets were resuspended in buffer, rehomogenized briefly (10 s at full speed) and washed by an additional centrifugation step. The final pellets were resuspended and rehomogenized in the appropriate binding buffer (see below).

Radioligand binding

Unless otherwise indicated, muscarinic receptors were identified by [^{3}H]QNB ([^{3}H]-l-quinuclidinylbenzylate) as the radioligand according to published procedures [12]. Briefly, equilibrium saturation and competition binding experiments were performed in buffer B (10 mM Na_2HPO_4, 10 mM NaH_2PO_4 at pH 7.4) in a total volume of 1000 µl containing 50–100 µg protein per assay. Incubations were performed for 60 min at 37°C and terminated by rapid vacuum filtration over Whatman GF/C filters. Each filter was washed twice with 10 ml ice-cold incubation buffer. Non-specific binding was defined by 3 µM atropine.

α_2-Adrenoceptors were identified by [^{3}H]RX 821002 ([^{3}H]-2-methoxy-idazoxan) as the radioligand as previously described [7]. Briefly, experiments were performed in binding buffer A (see above) in a total assay volume of 250 µl containing 50–100 µg protein per assay. The mixtures were incubated for 60 min at 25°C and terminated by rapid vacuum filtration over Whatman GF/C filters followed by two washes of the filters each with 10 ml ice-cold incubation buffer. Non-specific binding was defined as binding in the presence of 10 µM phentolamine.

For both types of receptors six concentrations of radioligand were tested in the saturation binding experiments. In competition binding experiments a single concentration of radioligand and 21 narrowly spaced concentrations of competitors were used.

Data analysis

Saturation binding experiments were analysed by fitting of rectangular hyperbolic functions to the experimental data. Competition binding experiments were analysed by fitting of mono- and biphasic sigmoidal functions to the experimental data; a biphasic fit was accepted only if it resulted in a significant improvement of the fit as judged by an *F*-test. All curve fitting was performed by the InPlot computer program (GraphPAD Software, San Diego, Calif.). Statistical significance of differences between groups was analysed by one-way analysis of variance when multiple groups were compared. When two groups were compared, paired and unpaired, two-tailed *t*-tests were performed as indicated. Statistical calculations including power calculations were performed with the InStat computer program (GraphPAD Software), and $P < 0.05$ was considered significant.

Chemicals

[^{3}H]QNB (specific activity 48 Ci/mmol) and [^{3}H]RX 821002 (specific activity 59 Ci/mmol) were obtained from Amersham (Braunschweig, Germany), [^{3}H]NMS ([^{3}H]-*N*-methyl-scopolamine; specific activity 80 Ci/mmol) from New England Nuclear (Dreieich, Germany). Phentolamine hydrochloride and atropine hemisulphate were obtained from Sigma (Deisenhofen, Germany), and pirenzepine dihydrochloride and HHSiD (hexahydro-siladifenidol hydrochloride) from RBI (Natick, Mass.). AF-DX 116 [11-((2-(diethylamino)-methyl)-1-piperidinyl)acetyl-5,11-dihydro-6H-pyrido(2,3-b)(1-4)benzodiazepin-6-on] was a gift from Dr. Karl Thomae GmbH (Biberach, Germany). All drugs were dissolved at 10 mM in dilute HCl (10 mM), except for HHSiD which was dissolved in ethanol, and diluted further in the respective binding buffers (see above).

Results

Characterization of muscarinic receptors in porcine detrusor

In porcine detrusor membranes [^{3}H]QNB labelled approximately twice as many sites as [^{3}H]NMS and had a somewhat higher affinity and significantly less non-specific binding in a direct side-by-side comparison (Fig. 1). Therefore, all further experiments were done using [^{3}H]QNB as the radioligand.

We next investigated whether expression of porcine detrusor muscarinic receptors differs between male and female pigs. Our data demonstrate that the two sexes express similar muscarinic receptor densities

(B_{max} = 113 ± 16 vs 125 ± 19 fmol/mg protein; Fig. 2). Affinities for the radioligand (K_d values) were also similar in male (34 ± 11 pM) and female pigs (47 ± 11 pM; n = 6 each).

To characterize the subtype(s) of muscarinic receptors present in porcine detrusor, competition binding experiments were performed with atropine and the subtype-selective antagonists pirenzepine, AF-DX 116 and HHSiD (Fig. 3, Table 1). All drugs competed for [^{3}H]QNB binding with relatively steep curves that were not significantly better explained by a two-site than a one-site model, indicating the presence of a homogeneous population of muscarinic receptor subtypes. The order of potency was atropine ≫ HHSiD ≥ AF-DX 116 ≥ pirenzepine, indicating the presence of the M_2 subtype.

Characterization of muscarinic receptors in human detrusor

The affinity of human detrusor muscarinic receptors for [^{3}H]QNB was similar to that seen in pigs (K_d = 35 ± 8 pM; n = 6). The density of muscarinic receptors in human detrusor (B_{max} = 153 ± 30 fmol/mg protein) was also similar to porcine detrusor (Fig. 2). To characterize the subtype(s) of muscarinic receptors present in human detrusor, competition binding experiments were performed as described above for porcine detrusor (Fig. 4, Table 1). Atropine and the subtype-selective antagonists pirenzepine, AF-DX 116 and HHSiD competed for [^{3}H]QNB binding with relatively steep curves that were not significantly better explained by a two-site than a one-site model, indicating the presence of a homogeneous population of muscarinic receptor subtypes. The

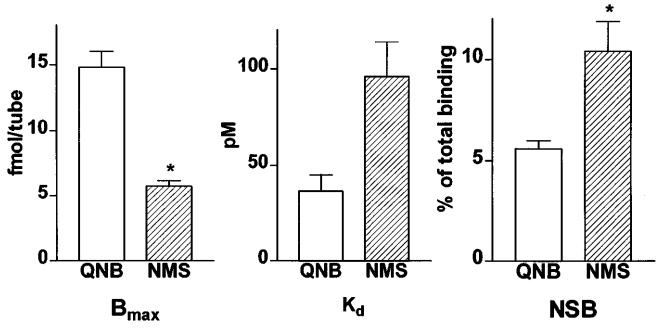

Fig. 1 Comparison of [^{3}H]QNB and [^{3}H]NMS ligand binding properties in porcine detrusor. Saturation binding experiments were performed with six concentrations of each radioligand. From the resulting saturation isotherms receptor density (B_{max} in fmol per tube; *left panel*), receptor affinity for the radioligand (K_d in pM; *middle panel*) and percentage of non-specific binding at the ligand concentration closest to K_d (NSB in % of total binding; *right panel*) were calculated. Data are the mean ± SEM of five experiments. $^*P < 0.05$ in a paired two-tailed t-test

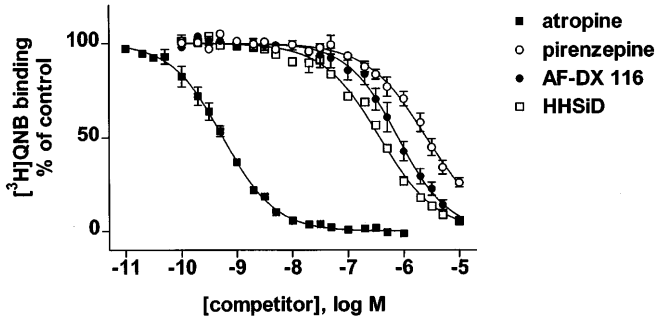

Fig. 3 Characterization of muscarinic receptor subtypes in porcine detrusor. Data were derived from competition binding experiments using [^{3}H]QNB as the radioligand and atropine (*filled squares*), AF-DX 116 (*filled circles*), HHSiD (*open squares*) and pirenzepine (*open circles*) as the inhibitors. They are expressed as percentage of [^{3}H]QNB binding in the absence of competitor (control) and given as mean ± SEM of three to five experiments. A quantitative analysis of these data is given in Table 1

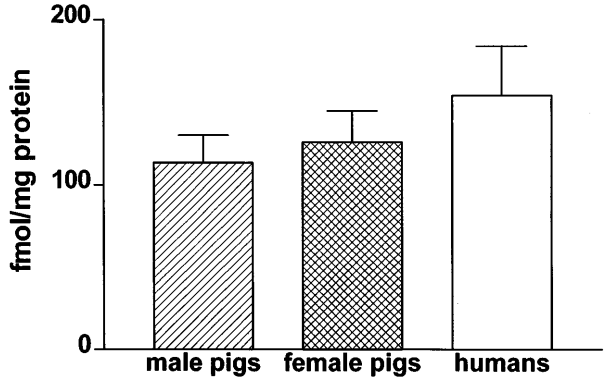

Fig. 2 Muscarinic receptor density in detrusor of male pigs (*hatched bars*), female pigs (*cross-hatched bars*) and humans of either sex (*open bars*). Data were derived [^{3}H]QNB saturation binding experiments, and are mean ± SEM of six experiments. Receptor densities were not significantly different between groups in a one-way analysis of variance

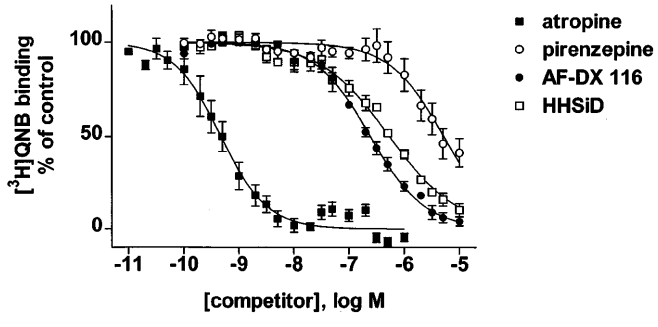

Fig. 4 Characterization of muscarinic receptor subtypes in human detrusor. Data were derived from competition binding experiments using [^{3}H]QNB as the radioligand and atropine (*filled squares*), AF-DX 116 (*filled circles*), HHSiD (*open squares*) and pirenzepine (*open circles*) as the inhibitors. They are expressed as percentage of [^{3}H]QNB binding in the absence of competitor (control) and given as mean ± SEM of three to five experiments. A quantitative analysis of these data is given in Table 1

Table 1 Competition binding parameters in porcine and human detrusor. Data are mean ± SEM of three to five experiments. Competition curves for none of the compounds were consistently better fitted by a two-site model. A graphical representation of the data is given in Figs. 3 and 4 (*HHSiD* hexa-hydro-sila-difenidol)

	Porcine detrusor		Human detrusor	
	Hill slope	$-\log K_i$	Hill slope	$-\log K_i$
Atropine	1.25 ± 0.30	10.17 ± 0.17	0.95 ± 0.08	10.15 ± 0.07
Pirenzepine	1.05 ± 0.22	6.35 ± 0.12	0.84 ± 0.06	6.52 ± 0.09
AF-DX 116	0.88 ± 0.07	6.99 ± 0.29	1.13 ± 0.13	7.06 ± 0.15
HHSiD	0.72 ± 0.12	7.32 ± 0.06	0.86 ± 0.10	7.52 ± 0.01

Table 2 Effects of urethral ligation and its surgical treatment on muscarinic receptors in porcine detrusor. Experiments were performed with sham-operated pigs (control), after urethral ligation, after urethral ligation and ileum augmentation, or after urethral ligation and autoaugmentation. Data were derived from [^{3}H]QNB saturation binding experiments, and are mean ± SEM of five or six animals per group. None of the differences was statistically significant in a one-way analysis of variance

	K_d (pM)	B_{max} (fmol/mg protein)
Control	73 ± 14	184 ± 27
Urethral ligation	35 ± 12	137 ± 25
Autoaugmentation	47 ± 16	180 ± 39
Ileum augmentation	83 ± 42	130 ± 21

Table 3 Effects of urethral ligation and its surgical treatment on α_2-adrenoceptors in porcine detrusor. Experiments were performed with sham-operated pigs (control), after urethral ligation, after urethral ligation and ileum augmentation, or after urethral ligation and autoaugmentation. Data were derived from [^{3}H]RX 821002 saturation binding experiments, and are mean ± SEM of four to six animals per group. None of the differences was statistically significant in a one-way analysis of variance

	K_d (pM)	B_{max} (fmol/mg protein)
Control	409 ± 47	29 ± 4
Urethral ligation	327 ± 32	29 ± 4
Autoaugmentation	325 ± 25	27 ± 4
Ileum augmentation	300 ± 45	23 ± 3

order of potency was atropine $\gg$ HHSiD $\geq$ AF-DX 116 $\geq$ pirenzepine, indicating the presence of the M_2 subtype.

Regulation of muscarinic receptors and α_{2A}-adrenoceptors in porcine detrusor

To study the regulation of muscarinic receptors and α_{2A}-adrenoceptors in detrusor, female pigs underwent partial urethral ligation or sham surgery. Some of these pigs were treated surgically by ileum augmentation of the bladder while others were treated by autoaugmentation. Urethral ligation decreased muscarinic receptor number by approximately 25% but this failed to reach statistical significance with the number of animals used (Table 2). Ileum augmentation did not affect this, while pigs treated with autoaugmentation had a muscarinic receptor density very close to that in control pigs (Table 2).

α_{2A}-Adrenoceptor density was determined in the same pigs using [^{3}H]RX 821002 as the radioligand. In these experiments receptor density was quite similar in all four experimental groups (Table 3). The K_d values for [^{3}H]RX 821002 were also not significantly different between groups in a one-way analysis of variance (Table 3).

Because the 25% reduction in muscarinic receptors upon urethral ligation did not reach statistical significance with the given number of pigs, we have performed formal power calculations. For feasibility reasons the number of pigs was limited to 6. These calculations showed that for a β (the chance of missing an existing difference as being significant) of 0.05 and 0.20, only alterations of at least 75% and 60%, respectively, would be detected as significant at the $P < 0.05$ level.

Discussion

The present study has compared muscarinic receptors in porcine and human detrusor to determine whether pigs may be a suitable model for studying human detrusor with regard to muscarinic receptors. For the labelling of muscarinic receptors in binding studies two radioligands are mainly used: the hydrophobic [^{3}H]QNB and the hydrophilic, quaternary [^{3}H]NMS. The lipophilicity of [^{3}H]QNB can result in the disadvantage of having more non-specific binding than [^{3}H]NMS in some tissues. On the other hand, [^{3}H]QNB can access all receptors in membrane preparations; however, the access of [^{3}H]NMS can be limited to outside-out vesicles, yielding a labelling of only about half of all muscarinic receptors [3]. In our study [^{3}H]QNB indeed labelled approximately twice as many sites as [^{3}H]NMS. Concomitantly the percentage of non-specific binding was significantly lower than with [^{3}H]NMS. Therefore, for studies of bladder muscarinic receptors [^{3}H]QNB appears superior to [^{3}H]NMS, and [^{3}H]QNB was used for all further experiments.

In some settings, for example the rabbit uterus, muscarinic receptor expression can be regulated by oestrogen treatment [2]. Our data demonstrate that the density of muscarinic receptors in porcine detrusor is similar in males and females. Thus, muscarinic receptors in the detrusor do not appear to undergo major sex-

hormone-dependent regulation. Moreover, similar numbers were also detected in human detrusor samples of either sex, indicating that muscarinic receptor expression in the detrusor is quantitatively similar in humans and pigs.

To provide a qualitative analysis of muscarinic receptors in porcine and human detrusor, we have performed competition binding experiments using the non-selective antagonist, atropine, the M_1-selective antagonist, pirenzepine, the M_2-selective antagonist, AF-DX 116, and the M_3-selective antagonist, HHSiD [14]. The very high affinity of atropine clearly identifies the [^{3}H]QNB binding sites as muscarinic receptors. The competition curves for the subtype-selective antagonists were best explained by a single-site model. This indicates the presence of a homogeneous population of muscarinic receptors in detrusor of both species. The affinity of pirenzepine at M_1 receptors is usually reported with $-\log K_i$ values of 7.5–8.7 [4, 14, 16]. Therefore, the relatively low affinity of pirenzepine in the present study argues strongly against the presence of M_1 muscarinic receptors in porcine and human detrusor. The affinity of AF-DX 116 at muscarinic receptors in porcine and human detrusor was much closer to its reported M_2 ($-\log K_i = 6.5$–7.7) than M_3 affinity ($-\log K_i = 5.4$–6.1; [4, 14, 16, 20]). The affinity of HHSiD in the present study was intermediate with regard to reported affinities at M_2 and M_3 muscarinic receptors [4, 16]. Thus, the observed rank order of potency atropine $\gg$ HHSiD $\geq$ AF-DX $\geq$ 116 pirenzepine indicates that the muscarinic receptors in human and porcine detrusor belong predominantly to the M_2 subtype. This conclusion is supported by mRNA data [5] and immunoprecipitation studies with subtype-selective antibodies [20]: While no M_1, M_4 or M_5 receptor protein was detectable in human bladder by immunoprecipitation, a ratio of M_2:M_3 muscarinic receptors of 3:1 was observed; an even greater M_2:M_3 ratio was seen in rabbits, guinea pigs and rats. A dominance of M_2 receptors has also been reported in binding sites in porcine ureter [11]. Therefore, the data from radioligand binding and immunological studies generally suggest that M_2 receptors are the most abundant subtype of muscarinic receptors in bladder tissue of humans, pigs, rats, rabbits and guinea pigs, other subtypes being much less abundant or undetectable.

Functional data, however, have yielded a more complex picture. For example, it has been found that the muscarinic receptors mediating contraction of rabbit [17, 18] and rat bladder [13] belong to the M_3 subtype. In the rabbit bladder prejunctional facilitatory effects occur via M_1 while only prejunctional inhibitory effects occur via M_2 receptors [18]. Why techniques directly quantifying receptor expression, i.e. radioligand binding and immunological data, yield different results from functional data in the bladder is not fully understood. Interestingly a similar situation exists in ileum, where binding studies indicate a predominance of M_2 receptors but functionally M_3 receptors are more important [9]. Therefore, it is not surprising that M_3-selective drugs have yielded promising results in clinical trials on patients with bladder dysfunction and are currently being launched [6]. It is possible that subtypes other than the M_2 receptor indeed exist only in very small numbers and that numbers too small for reliable biochemical detection nevertheless mediate important functions. While some M_2 receptors mediate prejunctional inhibitory effects in the bladder [17, 18], other biological functions in this tissue are likely. Recent studies have demonstrated that M_2 receptors may not cause bladder contraction per se but may reverse β-adrenoceptor-mediated bladder relaxation [5, 10]. This would be a quite plausible concept in light of the opposite effects of M_2 muscarinic and β-adrenoceptors on cyclic AMP generation.

Thus, the present study demonstrates that pigs and humans are quite similar with regard to muscarinic receptor expression in the detrusor. Recently we have demonstrated that pigs and humans are also quite similar with regard to α_{2A}-adrenoceptor expression and the lack of α_1-adrenoceptor expression in their detrusor [8]. Based on this similarity we have investigated the regulation of muscarinic receptors and α_{2A}-adrenoceptors in a porcine model of bladder outflow obstruction and its surgical treatment. Our data demonstrate that α_2-adrenoceptor expression in the detrusor is not significantly altered upon urethral ligation or its treatment by ileum augmentation or autoaugmentation. We have also failed to detect statistically significant differences in muscarinic receptor expression in the same experimental groups. The pathophysiological relevance of these findings is not fully clear, since the functions of α_{2A}-adrenoceptors and M_2 muscarinic receptors in the detrusor have not been fully defined. Moreover, our power calculations demonstrated that only alterations of at least 60–75% would have been detectable as statistically significant under our experimental conditions, i.e. any alteration smaller than that cannot be excluded with certainty. Obviously alterations of less than 60% can be very relevant biologically. Because the number of animals cannot be increased far beyond six per group in large-animals studies for practical reasons, this biological variance in quantitative receptor expression may limit the use of pigs in such studies despite their pharmacological similarities with humans.

In conclusion, we have demonstrated that bladder detrusor of pigs and humans are quite similar with regard to the density and pharmacological characteristics of their muscarinic receptor expression. No major sex differences were found. However, the large biological variance of muscarinic receptor expression in pigs makes quantitative regulation studies very difficult. Therefore, the use of smaller laboratory animals, which can be studied in larger numbers more easily, may be inevitable for biochemical studies although they recommend themselves less well to functional urodynamic investigations.

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