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MUSCARINIC RECEPTORS IN DIABETIC RAT PROSTATE

JAMSHID LATIFPOUR,* ANGELO GOUSSE, MASAKI YOSHIDA and ROBERT M. WEISS
Section of Urology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

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Abstract—To investigate the effects of experimentally-induced diabetes on prostatic muscarinic cholinergic receptors, the binding characteristics of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to prostatic membrane particulates were examined in four groups of rats: control, diabetic, diabetic insulin treated, and diabetic *myo*-inositol treated. Diabetes was induced by i.v. injection of streptozotocin (STZ), 65 mg/kg. Diabetic and diabetic *myo*-inositol-treated rats had hyperglycemia, hypoinsulinemia, glucosuria, polydipsia, and polyuria as well as significantly smaller prostates and lower body weights compared to control and diabetic insulin-treated animals. The densities of muscarinic receptors (B_{max}) as determined by saturation studies with [^{3}H]QNB in the prostatic plasma membranes of control, diabetic, diabetic insulin-treated and diabetic *myo*-inositol-treated rats were 80 ± 8, 51 ± 5, 78 ± 3, and 47 ± 7 fmol/mg of protein, respectively. [^{3}H]QNB binding to muscarinic receptors was inhibited by muscarinic antagonists with the following rank order of K_i values: atropine ≤ pirenzepine < AF-DX 116. The pharmacological profile of the muscarinic receptors was similar in all groups examined and was consistent with the predominance of the M_3 muscarinic receptor subtype in prostatic membrane particulates. Our data indicate that STZ-induced diabetes caused a variety of abnormalities including a down-regulation in the density of M_3 muscarinic receptors in the rat prostate and that insulin, but not *myo*-inositol could prevent the development of these abnormalities.

The availability of an experimentally-induced diabetic model in animals has made it possible to investigate various diabetes-induced dysfunctions at different organ levels of the genitourinary tract [1–6]. These dysfunctions have been attributed in part to peripheral autonomic neuropathy [7]. Recent studies showing a significant reduction in the weight of reproductive organs including the prostate following the induction of insulin-dependent diabetes [5, 6] suggest an important regulatory role for insulin in the growth and possibly in the function of this accessory sex organ.

The prostate is innervated by branches of the autonomic nervous system, i.e. sympathetic and parasympathetic [8, 9], and the response to stimulation of these nerves is mediated through adrenergic and cholinergic receptors which have been found in both prostatic adenoma and capsular tissues [10–12]. Studies have shown that whereas α-adrenergic receptor stimulation mediates contractile responses of human prostate [13], muscarinic cholinergic receptor stimulation increases basal prostatic secretion in dogs [14]. The muscarinic agonist-induced secretory process in the prostate was not observed in the castrated dog [15]. Furthermore, the observed down-regulation of muscarinic cholinergic receptors in the castrated rat prostate was shown to be restored by the administration of testosterone [12].

In the present study we investigated the effect of experimentally-induced diabetes on rat prostate muscarinic cholinergic receptors. We compared tissues from 8-week diabetics, and diabetics treated with insulin or *myo*-inositol with those from their age-matched controls.

MATERIALS AND METHODS

Male Sprague–Dawley rats (55–56 days old) weighing 235–280 g were used throughout the study. Experimental diabetes was induced in 48 rats with a single i.v. injection of 65 mg/kg streptozotocin (STZ)† as described previously [2]. Sixteen rats were injected with citrate buffer and used as a control group. Three days after the injection of STZ, and confirming the induction of diabetes by measuring urinary glucose levels, diabetic rats were divided randomly into three groups. One group of diabetic animals received a daily injection of 5–8 units of protamine zinc insulin (Eli Lilly, Indianapolis, IN) and another group received 1 g/kg/day of *myo*-inositol via their drinking water. Insulin administration was adjusted in such a way that STZ-injected rats had comparable blood and urine glucose levels as their age-matched controls. A third group of diabetic animals received no treatment. All animals had free access to food and water and were kept under identical conditions. Eight weeks after the initial injection, nonstarved rats were killed, blood samples were collected, and prostates were dissected, frozen in liquid nitrogen and stored at −80° until assayed.

Serum insulin and glucose measurements. Serum insulin levels were determined utilizing a radioimmunoassay kit (Ventrex Laboratories, Inc., Portland, ME) and serum glucose concentrations were measured using the enzymatic (hexokinase)

* Corresponding author: Jamshid Latifpour, Ph.D., Section of Urology, Yale University School of Medicine, P.O. Box 3333, New Haven, CT 06510. Tel. (203) 785-2818; FAX (203) 785-4043.

† Abbreviations: STZ, streptozotocin; QNB, quinuclidinyl benzilate; LH, luteinizing hormone; and FSH, follicular stimulating hormone.

Table 1. General features of experimental groups

	Control	Diabetic	Diabetic insulin treated	Diabetic *myo*-inositol treated
Initial body wt (g)	278 ± 3	285 ± 6	290 ± 4	291 ± 4
Final body wt (g)	505 ± 16	331 ± 34*	473 ± 19	342 ± 15*
Prostate wt (mg)	760 ± 29	392 ± 51*	640 ± 62	434 ± 39*
Serum glucose (mg/dL)	180 ± 14	687 ± 75*	113 ± 12	601 ± 20*
Serum insulin (μU/mL)	48.6 ± 7.3	9.7 ± 0.6*	152.8 ± 16.3	12.2 ± 1.3*
Water intake (mL/day)	42 ± 2	199 ± 5*	52 ± 3	206 ± 8*
Urine output (mL/day)	24 ± 2	130 ± 3*	32 ± 4	141 ± 4*

Diabetes was induced by an i.v. injection of 65 mg/kg STZ. Diabetic insulin-treated and diabetic *myo*-inositol-treated rats received a subcutaneous injection of 5–8 units of protamine zinc insulin daily and 1 g/kg/day *myo*-inositol via drinking water, respectively. At the end of week 4, rats were placed in metabolic cages and their water intake and urine output were measured during a 24-hr period. Animals were killed after 8 weeks, and blood samples were obtained for determination of serum glucose and insulin levels. Data are means ± SEM of 10–12 determinations in each group.

* Significantly different from control and from diabetic insulin-treated groups ($P < 0.05$).

method (Glucose H.K., Sigma Chemical Co., St. Louis, MO).

Binding experiments. Membrane particulates were prepared as previously described [2]. Frozen prostate was homogenized in ice-cold 50 mM Tris–HCl buffer (pH 8.0 at 25°) with a polytron and centrifuged at 49,000 *g* for 15 min at 4°. The pellet was homogenized again, filtered through a 105 μm nylon mesh, and centrifuged under the same conditions. The final pellets were suspended in 50 mM Tris–HCl buffer (pH 8.0 at 25°) and protein concentration was adjusted as needed. Protein concentration was determined by the method of Lowry *et al.* [16], using bovine serum albumin as standard.

Saturation and inhibition experiments were performed in order to study the density and pharmacological profile of muscarinic receptors in the prostatic membrane particulates. In saturation experiments, fixed concentrations of the tissue particulates were incubated with increasing concentrations of [^{3}H]quinuclidinyl benzilate ([^{3}H]-QNB) (0.01 to 0.70 nM) for 60 min at 23°. At the end of the incubation period, the suspensions were filtered through GF/B glass fiber papers presoaked in 0.05% polyethylenimine using a Brandel Cell Harvester (Gaithersburg, MD). The tubes and filter papers were washed twice with 5 mL of 50 mM Tris–HCl buffer, and radioactivity retained on the filter papers was measured by standard scintillation methods with an efficiency of 50–55%. A parallel set of tubes, in duplicates, containing 1 μM atropine was used to determine the level of nonspecific binding. Specific binding was determined from the difference in the binding in the presence and absence of atropine. The ratio of specific binding to total binding was decreased with increasing concentrations of [^{3}H]QNB and ranged from 97 to 85%.

In inhibition experiments, aliquots of membrane particulates were incubated with a fixed concentration (0.1 to 0.2 nM) of [^{3}H]QNB in the presence of various concentrations of nonlabeled muscarinic antagonists. The remainder of the binding experiment was similar to that of the saturation studies. In inhibition experiments, 1 μM atropine was used to determine the level of nonspecific binding.

Analysis of data. Saturation data were analyzed according to Rosenthal [17] using linear regression of bound/free versus bound in order to calculate the maximum number of binding sites, B_{max}, and equilibrium dissociation constant, K_D. In inhibition experiments, IC_{50} or the concentration of unlabeled drug that inhibits 50% of specific binding was calculated by log-logit analysis. K_i values or inhibition constants were obtained from $K_i = IC_{50}/(1 + F/K_D)$ [18], where F and K_D are the concentration of [^{3}H]QNB and its equilibrium dissociation constant, respectively.

K_D and K_i values were calculated as geometric means, whereas B_{max} values were calculated as arithmetic means. Statistical analyses between groups were performed using analysis of variance and the multiple comparison Fisher's test. $P < 0.05$ was regarded as the level of significance.

Drugs and chemicals. [^{3}H]QNB (43.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA). AF-DX 116 was donated by Karl Thomas GmH of the Federal Republic of Germany, and pirenzepine was a gift from Boehringer Ingelheim (Ridgefield, CT). Streptozotocin, atropine and carbachol were purchased from the Sigma Chemical Co.

RESULTS

The onset of diabetes was evident from day 2 after the injection of STZ by observing glucose levels greater than 300 mg/dL in the blood and greater than 1500 mg/dL in the urine of STZ-treated animals. As expected, at the time of sacrifice, diabetic animals had lower body weights, higher serum glucose levels and lower serum insulin levels than controls (Table 1). Diabetic rats also had significantly smaller prostates and larger water intakes and urine outputs than controls. The general features of the diabetic rats were similar to those of the diabetic *myo*-inositol-treated animals, whereas the features of the control rats were similar to those of the diabetic insulin-treated animals (Table 1). These data indicate that insulin, but not *myo*-inositol normalized these abnormalities in STZ-injected rats.

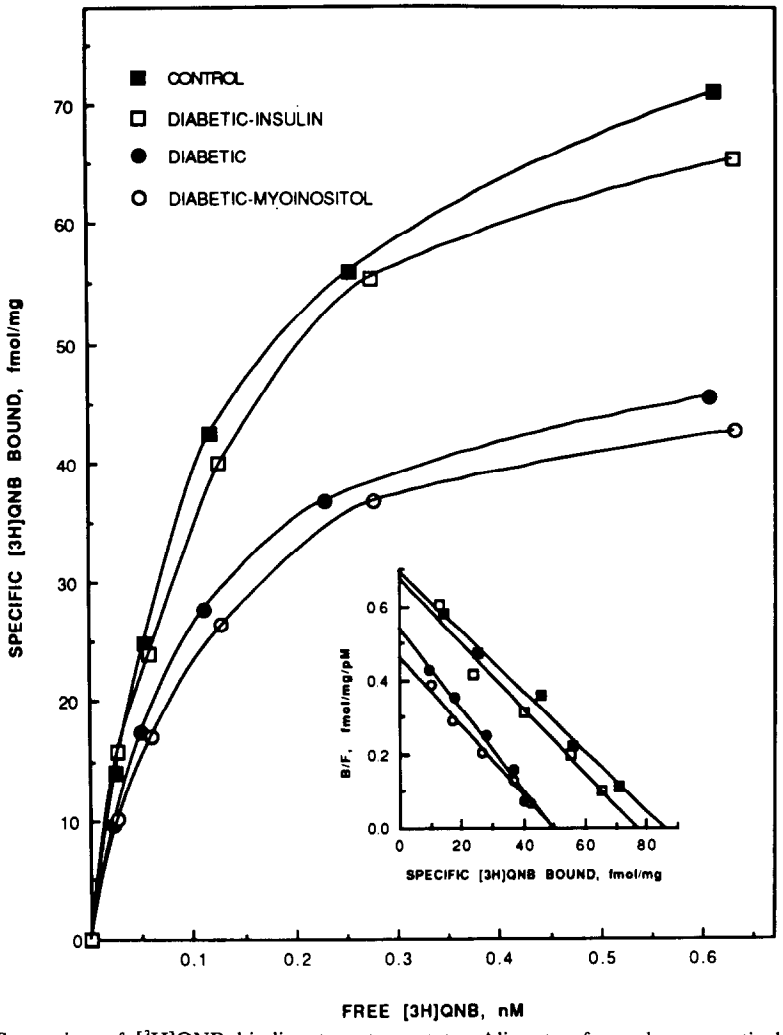

Fig. 1. Saturation of [^{3}H]QNB binding to rat prostate. Aliquots of membrane particulates were incubated in triplicate with increasing concentrations of [^{3}H]QNB for 60 min at 23°. Specific binding was defined in the presence and absence of 1 μM atropine. In the inset, the same data are plotted according to Rosenthal [17]. Each curve represents the mean of a single experiment performed in triplicate.

Saturation experiments with [^{3}H]QNB in rat prostate membrane particulates are shown in Fig. 1 and Table 2. The data indicate a decreased density of muscarinic receptors (smaller B_{max} values) in the diabetic prostates compared to controls, without significant alteration in the affinity constants (K_D values) of [^{3}H]QNB for the receptor sites. It is also to be noted that an estimation of the total number of muscarinic receptors in the prostate was shown to be considerably lower in diabetic and diabetic *myo*-inositol-treated groups than in the control and diabetic insulin-treated groups (data not shown). Similar B_{max} and K_D values for [^{3}H]QNB binding to diabetic insulin-treated and control rats show the effectiveness of insulin treatment in preventing the development of the diabetes-induced down-regulation of muscarinic receptors in the rat prostate (Table 2). *myo*-Inositol, on the other hand, did not prevent the prostatic muscarinic receptor down-regulation in the STZ-injected rats (Table 2).

Figure 2 is a representative study of inhibition of [^{3}H]QNB binding to control and diabetic rat prostatic membrane particulates by atropine, pirenzepine and AF-DX 116, which are nonselective, M_1 selective and M_2 selective muscarinic antagonists, respectively [19]. The rank order of potency of antagonists in displacing [^{3}H]QNB from its binding sites was atropine $\geqslant$ prenzepine > AF-DX 116 in these tissues. The finding in the inhibition studies of a similar rank order of potencies in each of the experimental groups indicates that the induction of diabetes and insulin or *myo*-inositol treatment did not alter the pharmacological profile of muscarinic receptors in the rat prostate (Table 3). The K_i values obtained in the present study are consistent with the predominance of the M_3 subtype of muscarinic receptors in the rat prostate [19–22].

Table 2. Saturation of [^{3}H]QNB binding in rat prostate

	B_{max} (fmol/mg protein)	B_{max} (fmol/g tissue)	K_D (pM)
Control	80 ± 8	2098 ± 291	119 ± 18
Diabetic	51 ± 5*	1284 ± 197*	116 ± 10
Diabetic insulin treated	78 ± 3	2785 ± 186	156 ± 15
Diabetic *myo*-inositol treated	47 ± 7*	1436 ± 183†	114 ± 22

Diabetes was induced by an i.v. injection of 65 mg/kg STZ. Diabetic insulin-treated and diabetic *myo*-inositol-treated rats received a subcutaneous injection of 5–8 units of protamine zinc insulin daily and 1 g/kg/day *myo*-inositol via drinking water, respectively. Rats were killed after 8 weeks, and saturation experiments were performed with [^{3}H]QNB in prostatic plasma membrane particulates. Data were analyzed by the linear regression of bound vs bound/free. B_{max}, maximum number of binding sites; K_D, equilibrium dissociation constant. Values are means ± SEM of 4–7 separate experiments.

* Significantly different from control and from diabetic insulin-treated groups ($P < 0.05$).

† Significantly different from diabetic insulin-treated group ($P < 0.05$).

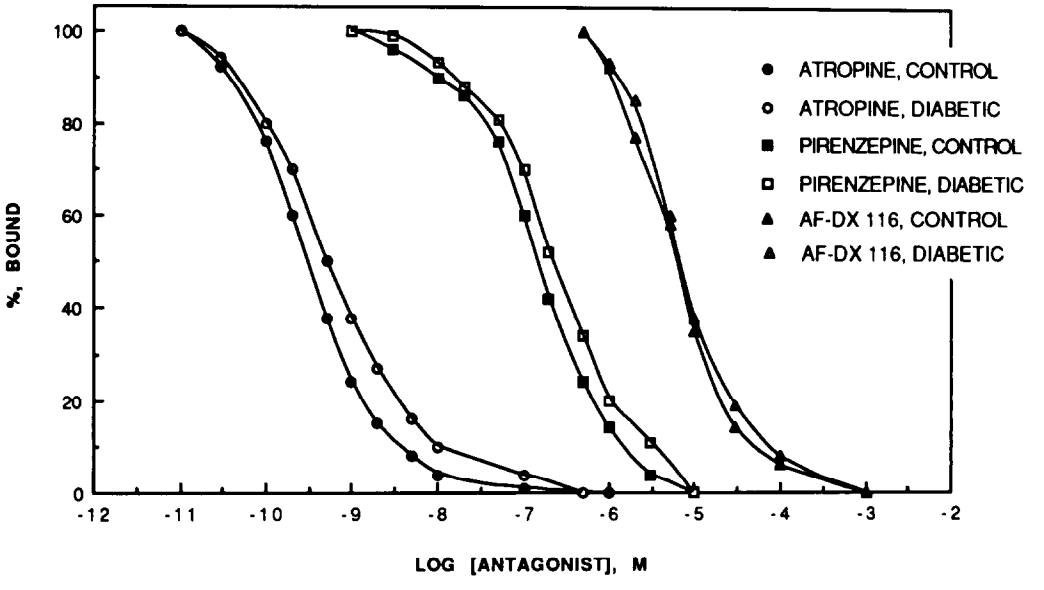

Fig. 2. Inhibition of [^{3}H]QNB binding to rat prostate. Aliquots of membrane particulates were incubated with eight to ten concentrations of an unlabeled antagonist with a fixed concentration of [^{3}H]QNB (0.1 to 0.2 nM) for 60 min at 23°. One hundred percent inhibition is defined as the binding in the presence of 1 μM atropine. Each curve represents the mean of a single experiment performed in duplicate.

DISCUSSION

The findings of this study show that experimentally-induced diabetes caused a down-regulation of muscarinic cholinergic receptor density in membrane particulates of rat prostate. The reduction in the density of the muscarinic receptors was prevented by the daily injection of insulin but not by the oral administration of *myo*-inositol. Furthermore, receptor specificity of the muscarinic receptors, that was found to be similar in all experimental groups, indicates the predominance of the M_3 subtype in this tissue.

The prostate is innervated by branches of the autonomic nervous system, sympathetic and parasympathetic [8, 9]. The distribution of muscarinic receptors and their role in the function of the prostate are poorly defined. For example, although there is anatomic evidence that sympathetic fibers supply the glandular elements and parasympathetic fibers innervate the muscular stromal elements of the prostate [23], there is evidence indicating that muscarinic cholinergic agonists have no effect on the contractile properties of human adenomatous tissue [11, 24]. Caine *et al.* [24], however, did show that acetylcholine causes a significant contractile response

Table 3. Inhibition of [^{3}H]QNB binding to rat prostate by muscarinic antagonists

Antagonist	K_i (nM)			
	Control	Diabetic	Diabetic insulin treated	Diabetic *myo*-inositol treated
Atropine	0.174 ± 0.04	0.185 ± 0.04	0.206 ± 0.05	0.207 ± 0.03
Pirenzepine	79 ± 31	133 ± 34	168 ± 42	134 ± 22
AF-DX 116	1334 ± 394	1339 ± 340	1912 ± 386	1515 ± 162

Diabetes was induced by an i.v. injection of 65 mg/kg STZ. Diabetic insulin-treated and diabetic *myo*-inositol-treated rats received a subcutaneous injection of 5–8 units of protamine zinc insulin daily and 1 g/kg/day *myo*-inositol via drinking water, respectively. Rats were killed after 8 weeks, and inhibition experiments were performed by incubating prostatic membrane particulates with a fixed concentration of [^{3}H]QNB (0.1 to 0.2 nM) in the presence of various concentrations of an unlabeled muscarinic antagonist. IC_{50} Values were determined and K_i values were calculated according to Cheng and Prusoff [18]. Values are means ± SEM of 3–4 separate experiments.

in human prostatic capsule which is inhibited by atropine. The differences seem to be related to tissue selection since the muscarinic-induced contractile response was found only in the "surgical capsule" of the prostates from patients with benign prostatic hypertrophy [11, 24]. Furthermore, recent autoradiographic studies have localized the muscarinic receptors to the glandular epithelium [11, 25–27].

The significant diabetes-induced decrease in prostatic weight noted in this study is consistent with the findings of others [5, 6, 28]. The decrease in prostatic size has been shown to be accompanied by a reduction in the epithelial and smooth muscle layers and by the absence of interstitium in some areas of the diabetic rat prostate [6]. Furthermore, Crowe *et al.* [6] showed that there was no change in the density of acetylcholinesterase-positive nerves in the different areas of the diabetic prostate, i.e. around alveoli, blood vessels and ducts. In addition to observing a reduction in prostatic weight with STZ-induced diabetes, Seethalakshmi *et al.* [5] have reported marked decreases in the sizes of other reproductive organs, i.e. testis, epididymis and seminal vesicles together with reductions in serum luteinizing hormone (LH), follicular stimulating hormone (FSH) and testosterone levels. The latter investigators also reported that insulin treatment prevents all of these abnormalities except for the reduction in prostatic weight. In the present study, however, insulin treatment not only prevented STZ-induced abnormalities such as hyperglycemia, glucosuria, polydipsia and polyuria, but it also normalized body and prostatic weight growth. In agreement with our findings, Paz *et al.* [28] showed that insulin treatment normalized the diabetes-induced loss in prostatic weight. The differences between the results of these studies are probably due to the differences in insulin treatment protocols. For example, while diabetic animals from the present study and from the study conducted by Paz *et al.* [28] were placed on an insulin treatment schedule within a few days following STZ injection, Seethalakshmi *et al.* [5] started insulin administration 2 weeks after the induction of the STZ-induced diabetes. Insulin treatment has also been demonstrated to normalize diabetes-induced alterations in cardiac contraction–relaxation processes, to prevent diabetes-induced down-regulation of cardiac β-adrenergic and muscarinic cholinergic receptors, and to reverse the supersensitivity of the contractile response of the diabetic rat hearts to Ca^{2+} [29–31].

As deficiencies in a variety of other hormones such as thyroid hormones and testosterone have been reported in STZ-induced diabetes [5, 32] and as these hormones have been shown to regulate the levels of autonomic receptors in peripheral tissues [12, 33], the diabetes-induced abnormalities may not be due exclusively to low levels of insulin in STZ-injected rats. Thus, the preventative effect of insulin treatment on the diabetes-induced down-regulation of prostatic muscarinic receptors may be due to both direct and indirect regulatory roles of insulin on these receptors. The indirect regulatory effect of insulin might be mediated via testosterone which has been shown to be lowered in STZ-diabetic rats [5, 28]. In support of this hypothesis, 95% of testosterone in males is produced in the testes [34] and castration has been shown to cause a significant down-regulation of rat prostatic muscarinic receptors [12]. The latter investigators have also demonstrated that testosterone administration restored the density of muscarinic receptors in the prostate of castrated rats to intact levels. It should be noted, however, that insulin replacement in diabetic rats did not normalize spermatogenesis or fertility rates, and that in order to restore these functions the administration of both insulin and gonadotropins was required [28]. It is also possible that as there is a marked reduction in prostatic epithelium in the diabetic rat prostate [6] and as significant amounts of muscarinic receptors appear to be located in the epithelium [25], that the regulatory effects of the above-mentioned hormones might be related to their role in maintaining the epithelium rather than to a direct effect on the number of muscarinic receptors.

Since oral *myo*-inositol administration has been shown to prevent the diabetes-induced decrease in conduction velocity and ouabain-sensitive Na^+,K^+-ATPase activity in rat sciatic nerve, presumably by restoring nerve *myo*-inositol content [35, 36], we attempted to determine if *myo*-inositol treatment could prevent the diabetes-induced alterations

observed in the rat prostate. *myo*-Inositol did not prevent or reverse the effects of diabetes on the general features studied or on prostatic muscarinic receptor densities.

The advent of receptor binding techniques and the discovery of various subtype specific muscarinic cholinergic antagonists such as pirenzepine and AF-DX 116 have lead to the subtype classification of muscarinic cholinergic receptors [19, 37]. Pharmacologically, muscarinic receptors have been divided into three subtypes, i.e. M_1, M_2, and M_3, that are mainly, but not exclusively, found in brain, heart and exocrine glands, respectively [19, 22]. Muscarinic receptor subtypes mediate different biochemical and pharmacological responses depending on the system in which the receptors are integrated [38]. AF-DX 116 is generally found to have a higher affinity for the M_2 subtype than for either the M_1 or M_3 receptor subtypes. Pirenzepine, on the other hand, has been shown to have a higher affinity for the M_1 subtype than for either the M_2 or M_3 receptor subtypes. Furthermore, the pirenzepine/AF-DX 116 ratio is considerably smaller in the M_1 than in the M_3 system [19, 22].

The results of our inhibition studies in rat prostate with [^{3}H]QNB and subtype selective muscarinic antagonists demonstrated a rank order of K_i values of atropine $\ll$ pirenzepine $<$ AF-DX 116, that was similar to that reported for the M_3 muscarinic receptor subtype that is generally found in exocrine glands [19–22]. The subtype specificity of prostatic muscarinic receptors and their localization in the glandular epithelium of the prostate [11, 25] suggest the involvement of these receptors in secretory functions of the prostate. The possible functional relevance of these muscarinic receptors is evident from the studies in which pilocarpine has been shown to cause a significant enhancement in the basal output of prostatic secretions in the dog [14]. The pilocarpine-induced increase in prostatic secretion has not been observed in the castrated dog [15]. The similar down-regulation of muscarinic receptors observed in both castrated and diabetic rat prostates may provide a rationale for a new approach for studying the significance of hormonal derangements in diabetes-induced sexual dysfunction.

In summary, our data show a diabetes-induced down-regulation of M_3 muscarinic receptors in the rat prostate that can be prevented by adequate insulin treatment.

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