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CHOLINERGIC AND PURINERGIC RESPONSES IN ISOLATED HUMAN DETRUSOR IN RELATION TO AGE

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ABSTRACT

Purpose: We investigated whether the contractility of isolated human detrusor muscle, responsiveness to commonly used spasmolytic drugs, and expression of selected muscarinic and purinergic (P2X) receptor subtypes (M_2, M_3, $P2X_1$ and $P2X_3$) change with age.

Materials and Methods: Tissues were taken from 63 patients 37 to 84 years old undergoing radical cystectomy. Specimens from 49 patients were used for contractility studies and those from 50 were used for mRNA analysis.

Results: Propiverine, oxybutynin, tolterodine and atropine decreased contractions evoked by electric field stimulation to different degrees. However, neither the efficacy nor potency of the drugs showed age related changes. Since human detrusor muscle shows atropine resistant noncholinergic responses, we also studied the putative age dependence of concentration-response curves to the muscarinic agonist carbachol, and the purinergic agonists adenosine triphosphate (ATP) and α-β-methylene-ATP. Sensitivity to α-β-methylene-ATP increased with age, while the efficacy and potency of spasmolytic drugs did not depend on age. In addition, mRNA detected for M_2, M_3, $P2X_1$ and $P2X_3$ receptors did not change with age.

Conclusions: Our results do not provide evidence for age related contractile deterioration in human detrusor muscle strips, nor do they suggest that responses to anticholinergic spasmolytic drugs change substantially with age.

KEY WORDS: bladder; muscle, smooth; parasympatholytics; muscle contraction; aging

Detrusor hyperactivity as a symptom of overactive bladder is more common in the elderly population.[1] With aging the connective tissue of the bladder increases at the expense of smooth muscle.[2] Functional processes involved in neurohumoral activation of the detrusor could also alter with age. Treatment of detrusor hyperactivity is often unsatisfactory with incomplete symptom suppression, which could be due to age dependent impairment of drug responsiveness.

The main neurotransmitter in human detrusor activation is acetylcholine, which stimulates muscarinic receptors, but a portion of neuronally mediated contraction is atropine resistant, suggesting additional nonadrenergic noncholinergic (NANC) mechanisms.[3] The major neurotransmitter involved in NANC mediated contractions is probably adenosine triphosphate (ATP) acting via purinergic (P2X) receptors.[3,4] The relationship between cholinergic and NANC mediated detrusor contraction can be affected by bladder disease[5] and also by age.[6]

Currently it is not clear which receptor subtypes are involved in neurohumoral function of the human detrusor. The M_2 receptor subtype is more abundantly expressed than the M_3 subtype but M_3 receptors appear to have the key role in human bladder muscle contraction.[7–9] The mechanisms involved include 1,4,5-triphosphate induced calcium release and calcium influx via L-type and T-type calcium channels,[4] while the role of M_2 receptors is still unclear.[10,11] Purinergic transmission in the detrusor appears to depend on $P2X_1$ receptor subtypes.[10,12] $P2X_3$ receptors are localized on nerve fibers and are also found in the urothelium[13] but mRNA for this receptor subtype has not been detected in detrusor smooth muscle.[12] Bladder hyporeflexia in $P2X_3$ deficient transgenic mice is accompanied by decreased voiding frequency and increased bladder capacity.[14]

In this study we searched for age related alterations in human detrusor function. Therefore, muscle strips from patients undergoing radical cystectomy for bladder cancer were investigated in vitro for contractile function, responses to antimuscarinic drugs and the expression of muscarinic (M_2 and M_3) and P2X ($P2X_1$ and $P2X_3$) receptors.

METHODS

Patients and material. The study was approved by the Ethical Committee of the University Hospital and all patients provided informed consent. Tissue from 63 patients undergoing radical cystectomy were used, that is for contractility experiments from 49 who were 37 to 84 years old and for mRNA analysis from 50 who were 43 to 84 years old. Detrusor tissue samples were dissected from an area of the bladder wall macroscopically unaffected by bladder cancer.

Tissue preparation. Immediately after removal from the bladder specimens were transported to the laboratory. Serosa and mucosa were removed and part of the samples was frozen in liquid nitrogen for polymerase chain reaction (PCR) studies. From the remaining tissue up to 6 muscle strips (10 to 15 mm long, 4 to 5 mm wide and 13 to 140 mg wet weight) were dissected and mounted in 25 ml organ baths (oxygenated Tyrode's solution at 37C). Muscle strips were preloaded with 10 mN and isometric tension was measured with a force transducer. Parameters for electric field stimulation (EFS)

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were pulse duration 1 millisecond, frequency 30 Hz, amplitude 90 mA and trains of stimuli for 2 seconds every 2 minutes. Recordings were documented using Chart 4.0™.

Detrusor muscle contraction. After 60 minutes of equilibration muscle strips (17 patients) were electrically stimulated. When contraction amplitude became constant, usually within 30 minutes, cumulatively increasing concentrations of spasmolytic agents were added at 30-minute intervals. Tetrodotoxin (TTX) (1 μM) was finally added to estimate the nonneurogenic component of contraction.

Detrusor sensitivity to spasmolytic drugs (22 patients) was tested with 2 consecutive concentration-response curves (CRCs) for carbachol (CCh), separated by 60-minute washout and an additional 60-minute incubation with test drug or no drug for time matched controls (TMCs). Cumulative CRCs for ATP and α-β-methylene-ATP (α,β-MeATP) were studied in strips from 4 and 6 patients, respectively.

Isolation of RNA and PCR. cDNA was synthesized from 1 μg total RNA using conventional methods.[15] For PCR 3 μl aliquots of total cDNA were amplified in a 25 μl reaction mixture containing buffer, bases, primer and Taq polymerase. The same single strand cDNA product was used to analyze the expression of all genes described. Amplifications were performed in a thermal cycler with a first denaturation step at 94C for 5 minutes, followed by 30 cycles for HMR2, HMR3, P2X1, β-actin (β-ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calponin (CAL) or 39 cycles (P2X3), each cycle consisting of 94C for 30 seconds at 60C for HMR2, HMR3, P2X1 and GAPDH, 66C for P2X3 or 58C for β-ACT and CAL for 30 seconds, and 72C for 30 seconds with final extension for 7 minutes. When possible, intron spanning primers were constructed with the HUSAR program package[15] or modified using published sequences. Table 1 lists primer sequences. PCR products obtained with amplification in the linear range were analyzed by agarose gel electrophoresis and ethidium bromide staining, and were quantified by optic densitometry. All RNA samples tested negative for DNA contamination.

Data analysis. Detrusor contraction data are shown as the mean ± SEM. Contraction amplitudes are expressed as a percent of the maximum during the first CRC or corrected for muscle strip wet weight. Individual CRCs were analyzed by nonlinear regression (Prism®) to determine EC_{50} or IC_{50} values (molar concentration producing 50% of maximum stimulatory or inhibitory effect).

EFS induced contraction amplitudes were averaged from 5 contractions prior to the drug concentration increase. Drug effects are expressed as a percent of the pre-drug control, which was considered equal to 100%. For antimuscarinic effects pA_2 values were analyzed using the Schild plot.[11]

Expression of mRNA of receptor subtypes was normalized to the housekeeping genes GAPDH or CAL. One-way ANOVA with an additional Bonferroni′s multiple comparison test with significance considered at $p < 0.05$ was used for statistical analysis of differences in drug effects or putative age related associations, as tested by linear regression.

Solutions and drugs. The composition of Tyrode's solution was 127 mM NaCl, 5.4 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 22 mM Na_2CO_3 and 5.6 mM glucose, pH 7.4 when gassed with 95% O_2/5% CO_2. The chemicals and drugs used were carbachol, ATP, α,β-MeATP, atropine sulfate, oxybutynin HCl, TTX, (R)-tolterodine-L(+)-tartrate and propiverine HCl. Drugs were dissolved as 0.1 M

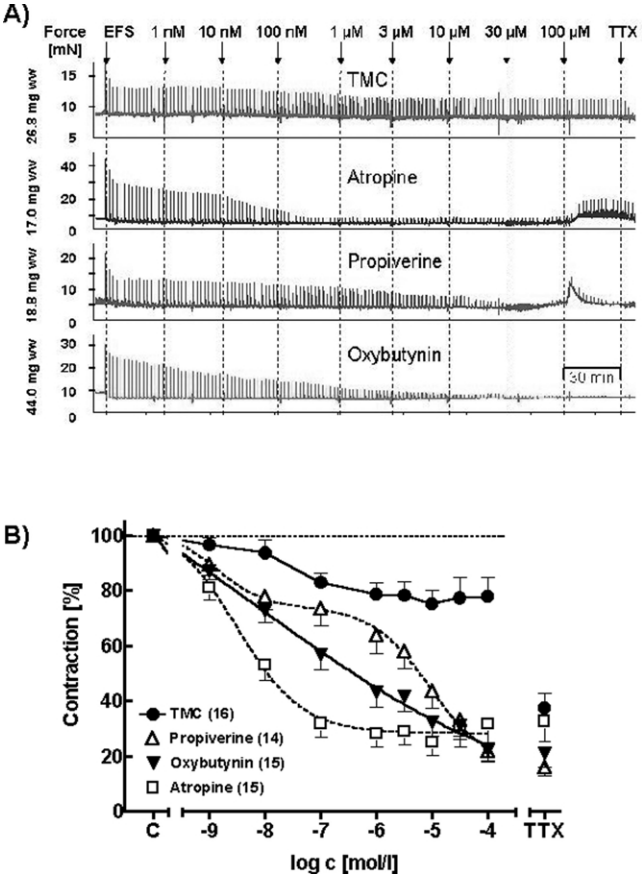

FIG. 1. *A*, EFS induced contraction force in 4 human detrusor strips for TMC without any drug added and responses to increasing concentrations of atropine, propiverine or oxybutynin. At end of each experiment 1 μM TTX was added to test for nonneuronal elicited force development. Values at left indicate strip wet weight. *B*, mean CRC ± SEM for effects of propiverine, oxybutynin and atropine on EFS evoked contractions in human detrusor muscle. Force is shown as percent of pre-drug control value, which was considered to equal 100%. TMC represents no drug added. At end of each experiment 1 μM TTX was applied to test for nonneuronal elicited force development. Values in parentheses indicate number of experiments.

TABLE 1. *Primer pairs used for semiquantitative reverse transcriptase-PCR*

Gene	Accession No.	Nucleotides	Primer	Sequence (5′–3′)
HMR2	X15264	752–771	Forward	AAG AAG GAC AAG AAG GAG CC
HMR2	X15264	1033–1049	Reverse	CTT TGG AAT GGC CCA GG
HMR3	U295891	1210–1229	Forward 1	TGG AAC AAC AAT GAT GCT GC
HMR3	U295891	1621–1641	Reverse	CCT TTT CCG CTT AGT GAT CTG
P2X1	AF020498	348–370	Forward	GCG TAA TAA GAA GGT GGG CGT TA
P2X1	AF020498	439–456	Reverse	GCC GCT CGA GGT CTG GTA
P2X3	AB016608	8–27	Forward 1	GCA TAT CCG ACT TCT TCA CC
P2X3	AB016608	225–245	Reverse	GTC ACG TAA TCA GAC ACA TCC
GAPDH	AF261085	963–981	Forward	AAC AGC GAC ACC CAC TCC TC
GAPDH	AF261085	1199–1219	Reverse	GGA GGG GAG ATT CAG TGT GGT
β-ACT	M10277	275–291	Forward	AGC CTC GCC TTT GCC GA
β-ACT	M10277	1201–1215	Reverse	CGC CCC AGG CAC CAG
CAL	U37019	82–100	Forward 1	TCC TCT GCT CAC TTC AAC C
CAL	U37019	333–351	Reverse	GAA GTT GCC GAT GTT CTC C

TABLE 2. *-log IC_{50} values and remaining contractions in different age groups for effects of spasmolytics on EFS*

Age Group	Remaining Contraction			Mean -log IC_{50} (mol/l)
	Mean % ± SEM	No. Strips	Mean % Control ± SEM	
TMC:	78 ± 7	17		
Younger than 60		6	88 ± 12	
60–69		5	72 ± 18	
Older than 69		6	79 ± 7	
Atropine:	21 ± 3	15		8.09 ± 0.21
Younger than 60		6	19 ± 4	
60–69		3	29 ± 11	
Older than 69		6	19 ± 4	
Propiverine:	17 ± 3	14		8.61 ± 0.29 (5 strips), 4.95 ± 0.35 (13 strips)
Younger than 60		6	14 ± 5	
60–69		4	22 ± 3	
Older than 69		4	16 ± 6	
Oxybutynin:	22 ± 4	16		6.86 ± 0.29
Younger than 60		6	24 ± 16	
60–69		5	28 ± 11	
Older than 69		5	15 ± 4	

stock solution in Milli-Q® water (CCh and all anticholinergics) or acetate buffer (TTX) and were further diluted with Milli-Q® water.

RESULTS

EFS. Atropine, propiverine and oxybutynin decreased EFS induced force of contraction of human detrusor in a concentration dependent manner (fig. 1, *A*). The paradoxical increase in baseline tension and contraction amplitudes at high concentrations of atropine and propiverine was not observed in all preparations and it must be addressed in a separate study.

Figure 1, *B* shows drug effects on EFS elicited tension. Force development decreased by about 20% during 4 hours of continual EFS in control experiments. Compared with oxybutynin and propiverine atropine was more potent, decreasing contraction force. The atropine resistant component was not further decreased after the addition of TTX (1 μM).

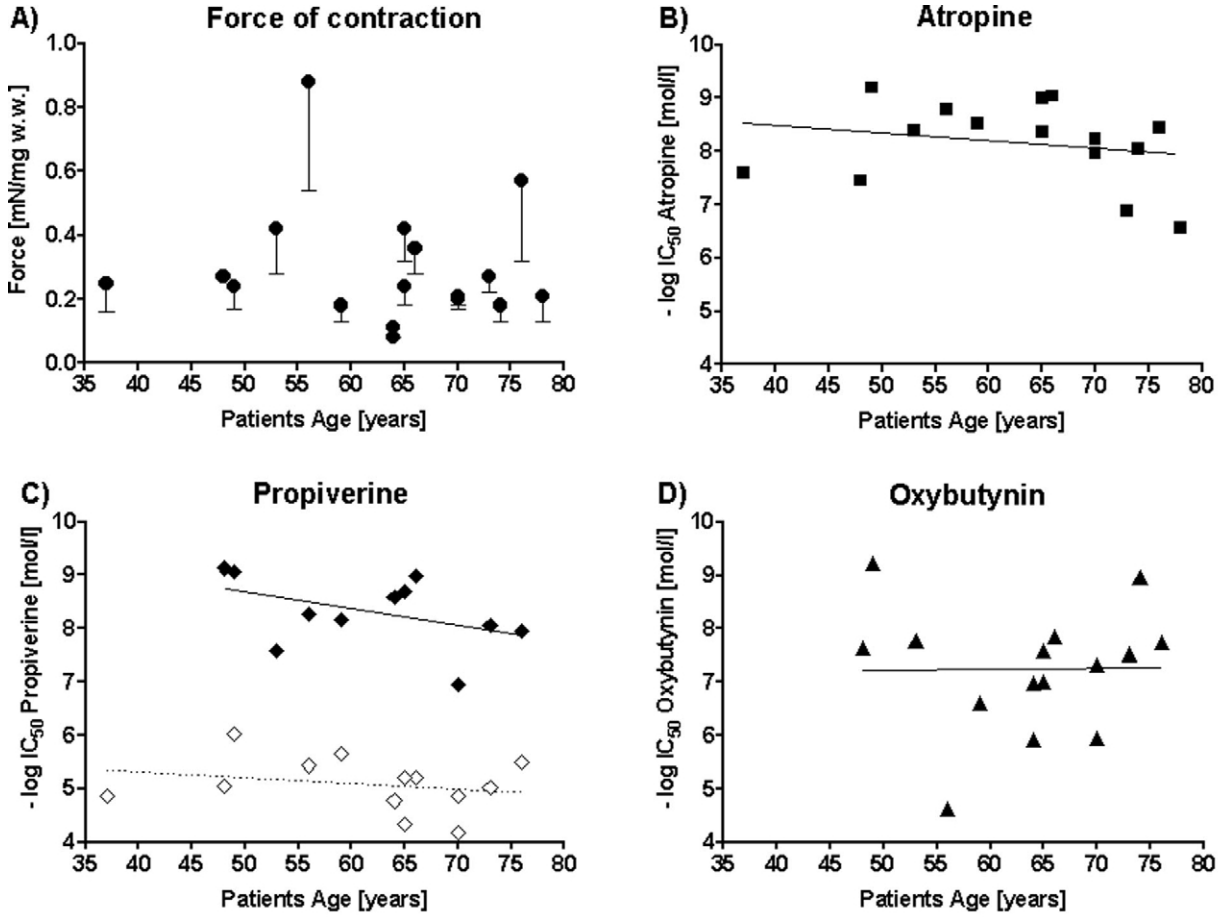

FIG. 2. Age dependent effects on EFS. *A*, mean pre-drug control contraction force in mN/mg wet weight of all detrusor strips of individual plotted as function of age of individual patient. *B*, negative logarithm of atropine concentration in mol/l for $-\log IC_{50}$ in EFS induced contraction force (regression line $y = mx + b$, $r^2 = 0.047$, $p = 0.44$). *C*, $-\log IC_{50}$ in mol/l for 2 phases of response to propiverine. Unbroken line represents $r^2 = 0.19$, $p = 0.17$. Dashed line represents $r^2 = 0.06$, $p = 0.44$. *D*, $-\log IC_{50}$ in mol/l for oxybutynin ($r^2 = 0.0002$, $p = 0.96$).

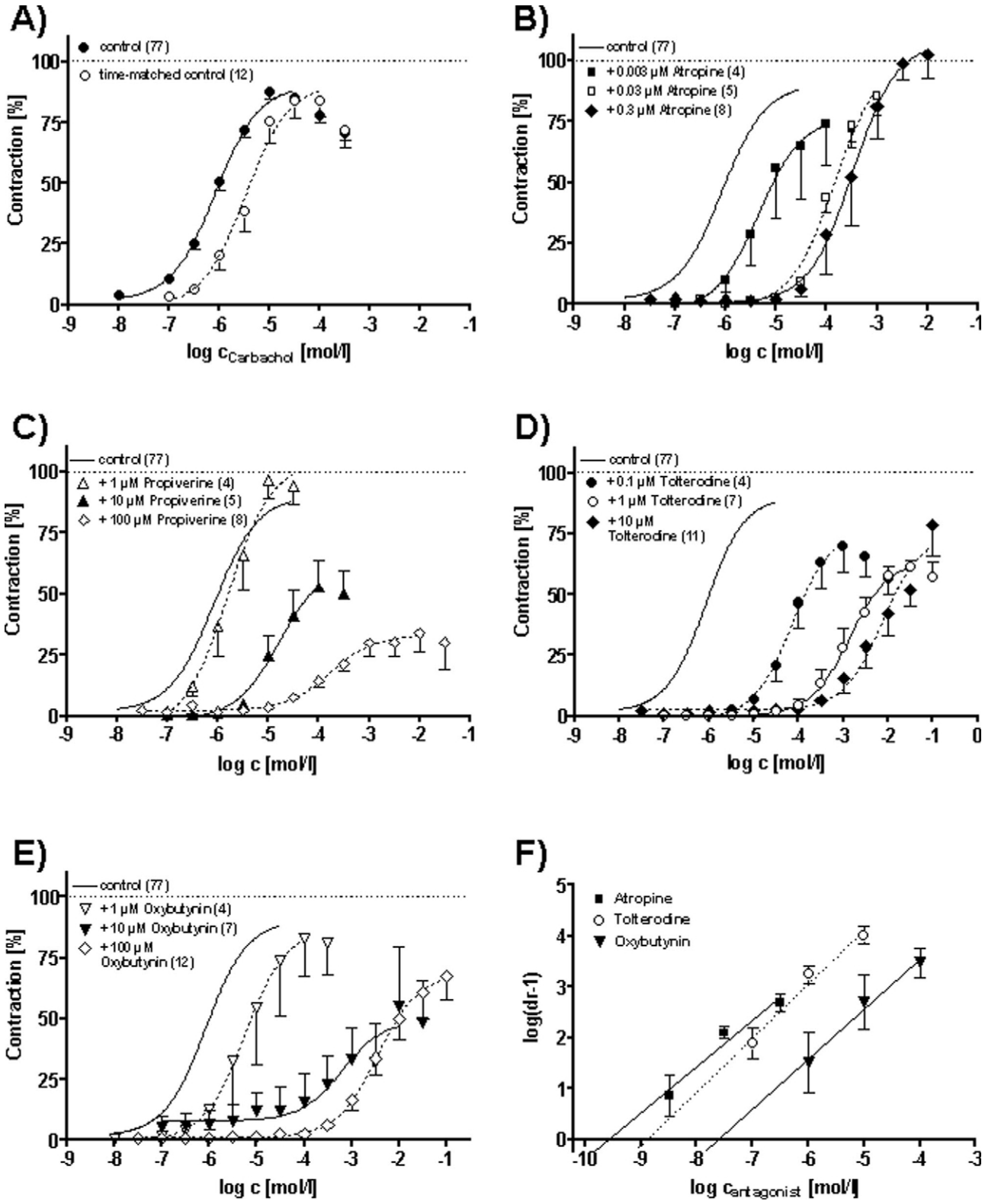

FIG. 3. CRC for CCh in absence and presence of spasmolytic agents. Force is normalized to maximum CCh response in each individual muscle during first exposure and plotted vs CCh concentration logarithm in mol/l. *A*, TMCs without any drugs added. *B*, effects of increasing concentrations of atropine. *C*, effects of increasing concentrations of propiverine. *D*, effects of increasing concentrations of tolterodine. *E*, effects of increasing concentrations of oxybutynin. *F*, Schild plots for antagonism on CCh induced detrusor contraction. Data are shown as mean ± SEM of 4 to 13 experiments per data point.

Individual CRCs for propiverine were best fitted with a 2 component sigmoidal function (small fraction in the low and major effect in the high concentration range). Table 2 lists $-\log IC_{50}$ values and drug resistant contraction amplitudes according to age groups.

To test for the age dependence of detrusor contractility regression lines were calculated for the plots of EFS evoked contractions in mN/mg wet weight or $-\log IC_{50}$ values of the spasmolytics against patient age (fig. 2). Neither absolute force development in response to EFS nor the sensitivity of

contractions to suppression with atropine, propiverine or oxybutynin was associated with patient age.

CRC for CCh. CCh induced, concentration dependent contractions with maximum tension were observed around 10 μM (fig. 3, *A*). Mean $-\log EC_{50}$ was 5.73 ± 0.10 in a total of 77 investigated detrusor strips from 18 patients. A second CRC during TMC led to a small shift to the right by 0.49 ± 0.14 log units in 12 preparations.

Like atropine, oxybutynin and tolterodine significantly shifted the CRC for CCh to higher concentrations. This shift was significantly larger than in TMCs (fig. 3, *B* to *E*). Propiverine also shifted the CRC and, in addition, it strongly decreased the maximum CCh effect. The parallel shift in CRCs by atropine, oxybutynin and tolterodine allowed the analysis of pA_2 values, which were 9.48 ± 0.44 for atropine, 7.56 ± 0.32 for oxybutynin and 8.81 ± 0.46 for tolterodine (fig. 3, *F*).

A putative association of detrusor sensitivity with CCh and age was tested by calculating linear regression between maximum effect and EC_{50} values vs age (fig. 4, *A* and *B*). The shift in TMC CRCs for CCh increased with age ($p = 0.05$, fig. 4, *C*). Sensitivity to the spasmolytic drugs tested (0.3 μM atropine, 10 μM tolterodine, 100 μM propiverine and 100 μM oxybutynin) did not vary with age (fig. 4, *C* and *D*).

CRCs for ATP and α,β-MeATP. In cumulative CRCs α,β-MeATP was more potent than ATP ($-\log EC_{50}$ 4.85 ± 0.11 and 2.71 ± 0.10 mol/l, respectively, fig. 5, *A*). In detrusor strips from 6 patients the maximum force of contraction induced by α,β-MeATP did not depend on age but $-\log EC_{50}$ values significantly increased with age ($r^2 = 0.66$, $p < 0.05$, fig. 5, *B* and *C*). Respective ATP values did not show age dependence but patient number was small and patient age clustered in a narrow range (fig. 5, *C*).

Expression of selected muscarinic and P2X receptors in the human detrusor. β-ACT, CAL and GAPDH were tested as housekeeping genes. mRNA for β-ACT was only detected in 29 of 50 tissue samples. In contrast, CAL and GAPDH were consistently expressed and their expression was independent of age, although the variability of CAL expression was high. Interestingly there were some differences in the expression of mRNA for GAPDH in particular, with less expression in tissue from women than from men.

M_2, M_3 and $P2X_1$ receptors were consistently expressed in all samples (fig. 6), while the $P2X_3$ receptor subtype was detected in only 31 of 50 samples. With GAPDH used as the housekeeping gene the expression of all 4 receptors appeared to be unaffected by patient age whereas, when normalized to CAL, the expression of some receptor subtypes appeared to be decreased with age (fig. 6, *A* and *B*). Changes in M_2 and M_3 expression were significant in patients older than 69 years compared with samples from patients younger than 60 years ($p = 0.03$). The expression of $P2X_1$ receptors tended to be lower at older ages but it was not significant.

DISCUSSION

The principle findings of our experiments with isolated human detrusor muscle strips are that patient age did not influence 1) EFS evoked contractions and the effects of several spasmolytic drugs, 2) CRCs for CCh and their modulation by spasmolytic agents, and 3) expression levels of recep-

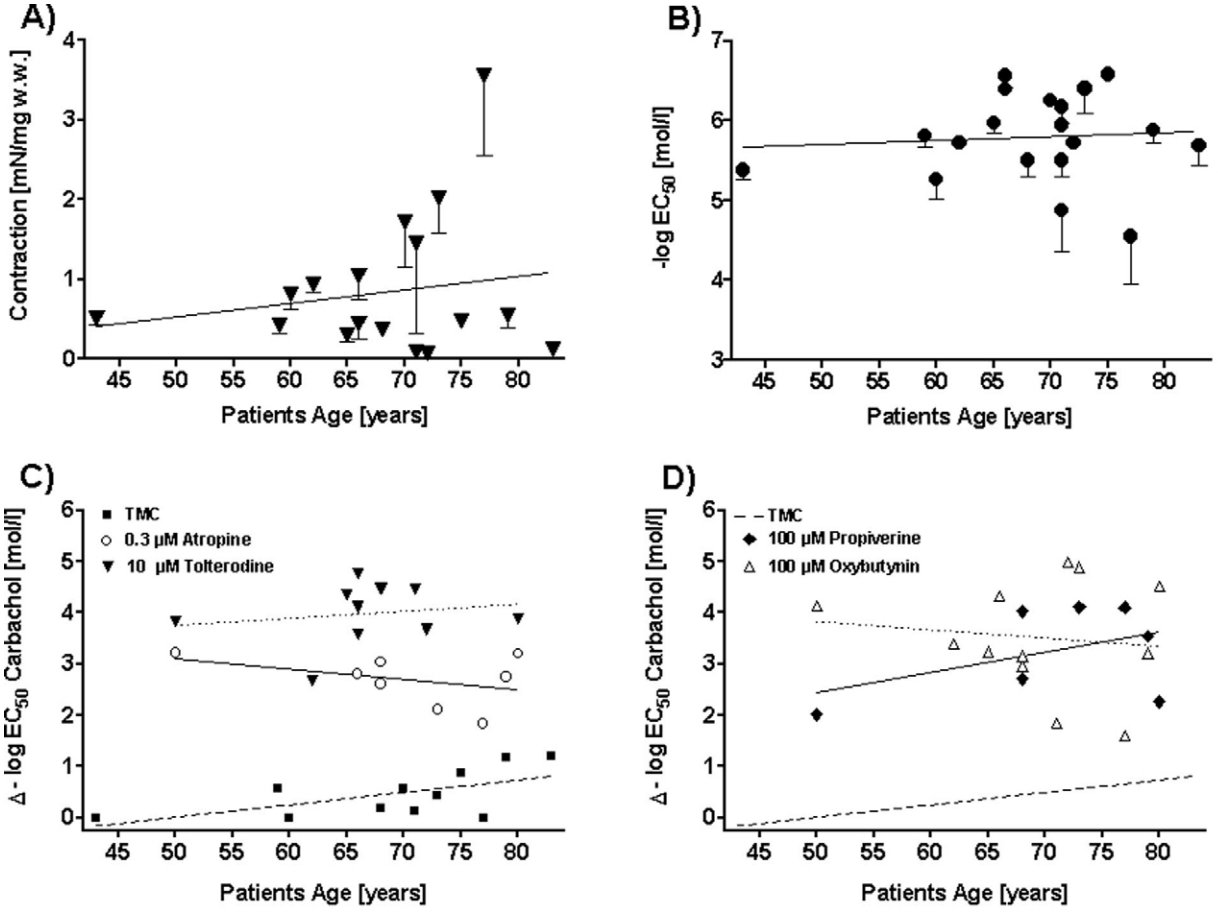

FIG. 4. Age dependent effects on CRCs for CCh. *A*, maximum force increase by CCh. *B*, negative logarithm of CCh concentration for EC_{50}. *C*, shift in $-\log EC_{50}$ by 0.3 μM atropine and 10 μM tolterodine plotted as function of patient age. Regression lines are $r^2 = 0.01$, $p = 0.79$ for atropine and $r^2 = 0.07$, $p = 0.44$ for tolterodine. *D*, shift in $-\log EC_{50}$ by 100 μM propiverine and 100 μM oxybutynin plotted as function of patient age. Regression lines are $r^2 = 0.03$, $p = 0.50$ (top), $r^2 = 0.0007$, $p = 0.74$ (middle) and $r^2 = 0.12$, $p = 0.44$ for propiverine, and $r^2 = 0.03$, $p = 0.61$ for oxybutynin.

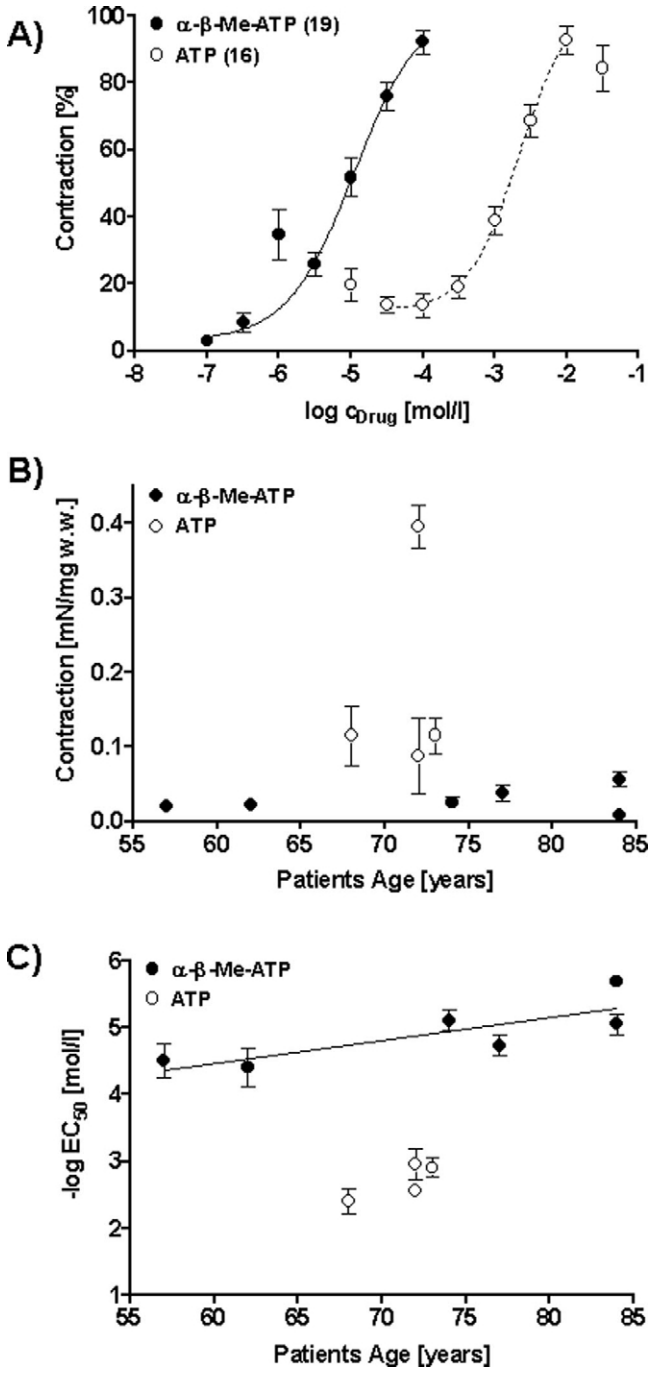

FIG. 5. *A*, effects of α,β-MeATP and ATP on human detrusor muscle contractions, expressed as percent of maximum concentrations. *B*, absolute values of contraction force in mN/mg wet weight in response to ATP or α,β-MeATP. *C*, –log EC_{50} plotted against patient age in years. Regression line parameters are $r^2 = 0.66$, $p = 0.048$.

tor subtype mRNA when normalized to GAPDH. In contrast, the age dependent changes detected were a shift between 2 CRCs for CCh, increased sensitivity to α,β-MeATP with increasing age and decreased mRNA expression for M_2, M_3 and $P2X_1$ receptors normalized to CAL in older patients.

EFS evoked contractions were decreased by spasmolytic drugs in a certain order of potency, that is atropine > oxybutynin > propiverine, which is in accordance with the literature.[16] Interestingly propiverine suppressed EFS evoked contractions in a 2 step concentration dependence with the major decrease at high concentrations. A biphasic response with propiverine was observed previously in guinea pig but not in pig detrusor[17] despite the accepted resemblance of pig to human bladder muscle.

The amplitude of EFS induced contractions of human detrusor did not correlate with patient age at 30 Hz stimulation frequency in this study, nor at a wide range of frequencies.[6] However, Yoshida et al reported a decreased atropine sensitive contraction component, which they thought was related to decreased ACh release with increasing age.[6] Conversely atropine resistance increased with age and was related to enhanced ATP release.[18] In our hands neither total contraction amplitude nor the atropine resistant component significantly correlated with age, probably because of large variability among individual values. Based on –logIC_{50} values sensitivity to atropine, propiverine and oxybutynin did not vary systematically with age.

The mean –logEC_{50} value for CCh in 77 human detrusor strips was 5.73 ± 0.10, which corresponded well with other published estimates.[9] All spasmolytics shifted the CRC for CCh to the right but only propiverine significantly decreased the maximum CCh induced contraction to 32% ± 6% of control values at 100 μM in 8 preparations, again demonstrating its dual mode of action.[16] The same concentration of oxybutynin also decreased the contraction maximum to 59% ± 8% in 13 preparations, in accordance with the reported additional mechanism of action at greater than 10 μM.[16] In previous studies we detected a significant decrease in the maximum CCh induced contraction with oxybutynin (and tolterodine) in pig but not in mouse detrusor, suggesting some species differences concerning this effect.[17]

In the current study the sensitivity of human detrusor to CCh, as measured by maximum contraction amplitude and –logEC_{50} values, did not change significantly with age, nor did responses to spasmolytic drugs show age dependence with respect to potency or efficacy. Nevertheless, we cannot exclude that receptor density and function may vary with age.[19] The significantly increasing shift in CRC for CCh (TMC) with age could have been due to enhanced desensitization, suggesting some functional modification of the muscarinic response. Of all parameters correlated with age the intensified responses to α,β-MeATP with increasing age confirms the hypothesis of an age related increase in purinergic neurotransmission.[6]

In human detrusor all 5 muscarinic receptor subtypes (M_1 to M_5) have been detected at the mRNA level.[20] Of the 7 known P2X receptor subtypes mRNA of $P2X_3$ and $P2X_6$ has not yet been detected in human detrusor,[12] although $P2X_3$ was demonstrated in the urothelium by immunofluorescence.[13] We noted robust expression of mRNA of M_2 and M_3 receptors, and $P2X_1$ receptors, whereas detection mRNA for $P2X_3$ receptors required a high number of PCR cycles, suggesting the possibility of contamination of the muscle probes with suburothelial connective tissue, vessels or nerves, which express this subtype. Gene expression of none of these receptor subtypes depended on age when normalized to the housekeeping gene GAPDH. In contrast, mRNA expression of M_2 and M_3 receptors was lower in the high age groups when normalized to CAL[12] as a housekeeping gene, suggesting that this phenotypic smooth muscle cell marker is itself regulated by age.

CONCLUSIONS

Briefly, we did not detect profound age dependent alterations in physiological or pharmacological functional parameters of human detrusor muscle. Instead, minor variations in the expression of M_2 and M_3 receptors were seen as well as increased responsiveness to α,β-MeATP stimulation. These findings must be confirmed by prospective studies.

A. Weiss, S. Kirsch and C. Fischer provided technical assistance. Carbachol, ATP, α,β-MeATP, atropine sulfate, oxybutynin HCl and TTX were obtained from Sigma-Aldrich, Taufkirchen, Germany. (R)-tolterodine-L(+)-tartrate and

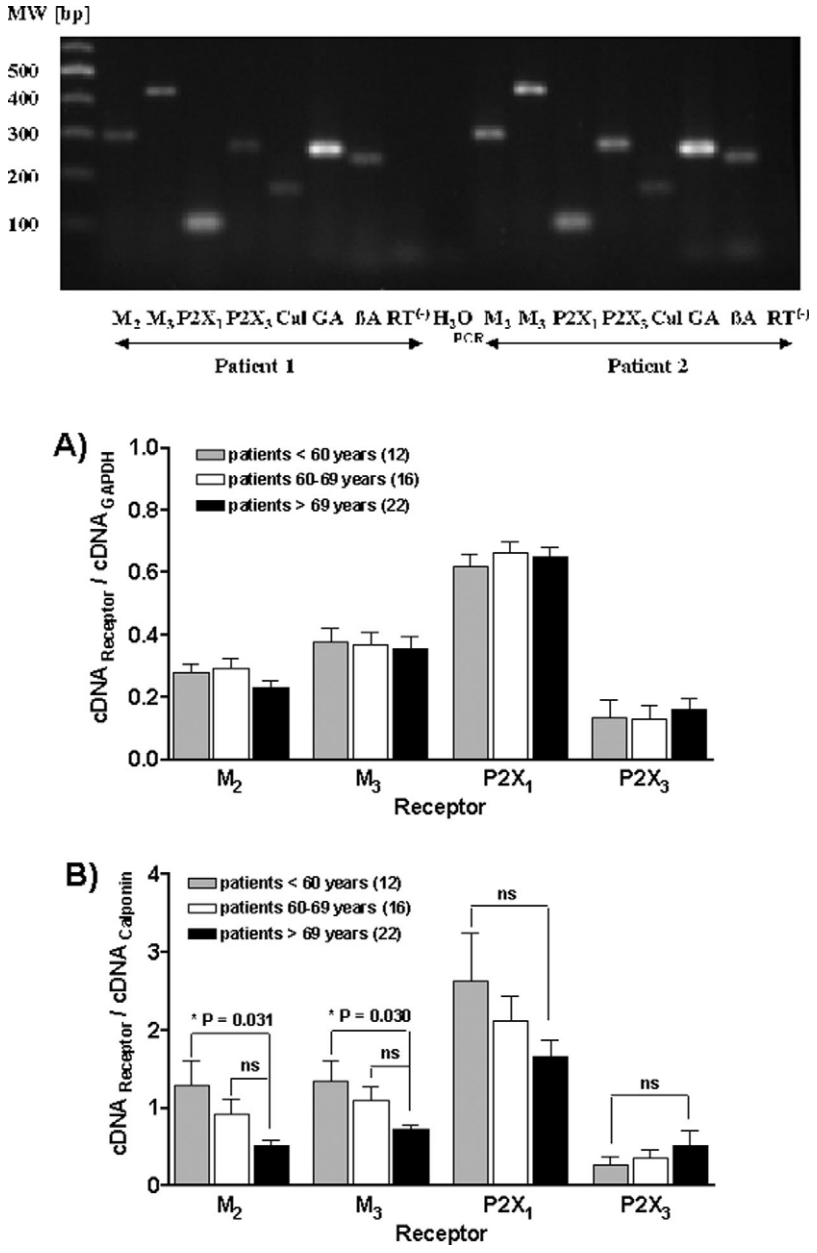

FIG. 6. Top, representative agarose gel shows amplicons of M_2, M_3, $P2X_1$ and $P2X_3$ receptors, CAL (*Cal*), GAPDH (*GA*) and β-ACT (*βA*) in 2 select patients. *MW*, 100 bp molecular weight marker. *RT-*, negative control for reverse transcription. *H_2O*, negative control for PCR. *A* and *B*, age associated changes in expression of muscarinic M_2 and M_3, and purinoceptor $P2X_1$ and $P2X_3$ receptor subtypes in human detrusor tissue in relation to 3 age groups. *A*, data normalized to housekeeping gene GAPDH. *B*, data normalized to housekeeping gene CAL. Note statistical significance.

propiverine HCl were provided by Apogepha Arzneimittel GmbH, Dresden, Germany.

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