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Pharmacodynamics of propiverine and three of its main metabolites on detrusor contraction

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1 Besides its antimuscarinic effects, propiverine may possess an additional mode of action. We compared the effects of propiverine, three of its metabolites (M-5, M-6, M-14) and atropine in human, pig and mouse urinary bladder preparations in order to elucidate the nature of a possible additional mode of action.

2 Like the parent compound, M-5, M-6 and M-14 reduced to variable degrees the contractions elicited by electric field stimulation (EFS) of isolated, urothelium-denuded detrusor strips. In mouse the atropine-resistant and therefore the nonadrenergic, noncholinergic component of contractile response to EFS was reduced by M-5, M-14 and propiverine, but was hardly affected by M-6.

3 Atropine, propiverine and M-6 significantly shifted the cumulative concentration–response curves for carbachol (CCh) to higher concentrations. Atropine and M-6 did not affect the maximum tension induced by CCh. Propiverine, M-5 and M-14 reduced the maximum CCh effect, suggesting at least one additional mode of action. This pattern of response was observed in all the three species, albeit with some differences in sensitivity to the various agents.

4 In freshly isolated human detrusor smooth muscle cells, propiverine and M-14 inhibited the nifedipine-sensitive L-type calcium current (I_{Ca}) in a concentration-dependent manner. In contrast, the effects of M-5 and M-6 on I_{Ca} were insignificant in the concentration range examined.

5 The investigated responses to propiverine and its metabolites suggest that impairment of maximum CCh-induced contractions is due to strong effect on I_{Ca} and that this may be associated with the presence of the aliphatic side chain.

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Abbreviations: ACh, acetylcholine; CCh, carbamoylcholine chloride (carbachol); CRC, concentration–response curve; DSMC, detrusor smooth muscle cells; EFS, electric field stimulation; I_{Ca}, L-type calcium current; M receptors, muscarinic receptors; NANC, nonadrenergic–noncholinergic; OAB, overactive bladder syndrome; TMC, time-matched control; TTX, tetrodotoxin

Introduction

Activation of muscarinic (M) receptors plays a major role in the control of urinary bladder contractility (Andersson, 1993). Although M_2 receptors are the predominant subtype found in detrusor muscle (Yamanishi *et al*., 2001), M_3 receptors appear to mediate urinary bladder contractile responses in many species (Yamanishi *et al*., 2000; Chess-Williams *et al*., 2001; Choppin & Eglen, 2001; Fetscher *et al*., 2002). Detrusor contraction in response to muscarinic receptor (M receptor) stimulation requires Ca^{2+} entry *via* nifedipine-sensitive L-type calcium channels (Schneider *et al*., 2004a; Wegener *et al*., 2004).

Antimuscarinic drugs are used for the treatment of the overactive bladder syndrome (OAB; Sellers *et al*., 2001; Andersson *et al*., 2002; Andersson & Yoshida 2003; Ouslander, 2004). In addition to its antimuscarinic effect, propiverine appears to have additional spasmolytic effects (Andersson *et al*., 1999; Wuest *et al*., 2002). Thus, propiverine inhibits contractile responses elicited by electric field stimulation (EFS) as well as acetylcholine (ACh)-induced contractions in human detrusor strips (Wada *et al*., 1995). The drug potently reduces KCl-induced contractions in guinea-pig (Haruno, 1991; Tokuno *et al*., 1993), as well as KCl- and $CaCl_2$-induced contractions in human bladder strips (Wada *et al*., 1995). In rabbit, propiverine also impairs intracellular Ca^{2+} homeostasis in addition to its antagonistic muscarinic effects (Madersbacher & Mürtz, 2001).

Propiverine is rapidly absorbed after oral administration and is subject to extensive first-pass metabolism, giving rise to several active metabolites, for example, M-5, M-6 and M-14 (for chemical structures, see Figure 1). At 24 h after oral application, these compounds can be recovered among other derivatives from the human urine (in percent of the original dose): propiverine, 2–3%; M-5, 20%; M-6, 5%; M-14, 1% (Haustein & Hüller, 1988). Siepmann *et al*. (1998) reported that after 5 days of treatment with multiple dosing of

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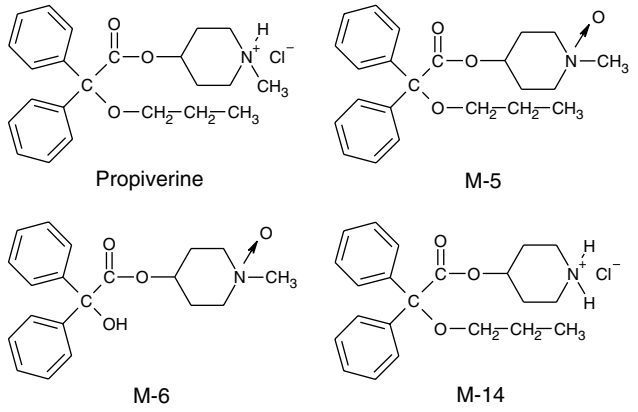

Figure 1 Chemical structures of propiverine and its metabolites M-5, M-6 and M-14.

propiverine the following maximum serum concentrations were detected (median; range): propiverine (155 (96–240) ng ml^{-1}) corresponding to 0.38 (0.24–0.59) μM, and its N-oxide M-5 (645 (385–955) ng ml^{-1}) corresponding to 1.68 (1.00–2.49) μM. The metabolites M-6 and M-14 were not detected in serum but in urine only.

The pharmacological activities of these metabolites have not yet been studied in detail (Andersson *et al*., 1999). In order to estimate their contribution to the therapeutic action, we have chosen the three main metabolites in man (M-5, M-6 and M-14) for investigation of their effects on detrusor muscle and used atropine and the congener propiverine for comparative purposes. Pig detrusor was studied because of its similarity to human detrusor. Mouse detrusor was studied because of its high nonadrenergic–noncholinergic (NANC) component of contraction (Wuest *et al*., 2002). Furthermore, we investigated the effects of these compounds on contractions in human, pig and mouse urinary bladder preparations and on the L-type calcium current (I_{Ca}) in human and porcine detrusor smooth muscle cells (DMSC) to estimate additional mechanisms of propiverine and possibly also of its metabolites.

Methods

Materials

Human urinary bladder tissue was obtained from 10 male and 11 female patients (age range: 58–80 years) undergoing radical cystectomy for bladder cancer. All patients had given informed written consent in accordance with the regulations of the local ethical committee. Samples from tumour-free parts of the bladder wall were taken. After removal of the serosa and mucosa, four to eight muscle strips (10–15 mm long, 4–5 mm wide) were dissected from each sample. The remaining tissue was stored overnight at 4°C and used for isolation of detrusor smooth muscle cells (DSMC).

Muscle strips from pig and mouse bladder were prepared as described previously (see Wuest *et al*., 2002). Urinary bladders of female pigs were obtained from a local abattoir transported to the laboratory in transport buffer at 4°C. Serosa and mucosa were removed from a 2×2 cm tissue piece of the anterior wall, out of which four to eight longitudinal muscle strips (7–10 mm long, 2–4 mm wide) were dissected.

Male C57Bl6 (Charles River) mice weighing 20–30 g were killed by cervical dislocation. The urinary bladder was removed at the bladder neck. After cutting off the dome of the bladder, the remaining muscle ring was opened longitudinally and cut into strips after removal of the mucosa (two strips per bladder for electric field and four strips for carbachol (CCh) stimulation).

Detrusor muscle contraction experiments

Muscle strips were mounted in 25-ml (human and pig) or 5-ml organ baths (mouse) containing carbogen-gassed Tyrode's solution maintained at 37°C. Tension generated was measured with an isometric force transducer (GM 2, Föhr Medical Instruments, Seeheim/Ober Beerbach, Germany), amplified and recorded with a data and recording system (Chart 4.0™, ADInstruments, Sydney, Australia). Resting load was set to 10 mN for human and pig and 5 mN for mouse preparations, and was readjusted after 30 min. During the equilibration period of 60 min, the bath solution was changed once.

In EFS-experiments, pig and mouse strips were challenged twice with CCh (1 μM in pig and 1 or 10 μM in mouse for 15 min each) with a 15 min washout period between exposures. After 20 min of stabilization, muscle strips were subjected to EFS. Human detrusor strips were not initially exposed to CCh because of the marked desensitization. In preliminary experiments, the mean amplitude of the first EFS-induced contractions was only about one-quarter of the amplitude obtained without prior CCh challenge, and contraction amplitudes almost doubled in the course in time-matched control (TMC) experiments (unpublished results). The parameters for EFS (stimulator, Föhr Medical Instruments, Seeheim/Ober Beerbach, Germany) were: pulse duration 1 ms at 30 Hz with 90 mA. Stimuli trains lasted 2 s (mouse) or 5 s (pig, man). The compounds under investigation were added in cumulatively increasing concentrations with 30 min between increments. To estimate the non-neuronally mediated portion of muscle contraction under our stimulation conditions, nerve conduction was completely blocked by adding the neurotoxin tetrodotoxin (TTX, 1 μM) at the end of each experiment. Average values for the EFS-induced muscle contraction amplitudes were obtained from the last five contractions before the next concentration increase. The magnitude of drug effect is given in percent inhibition of the electrically evoked contraction amplitude before any substance addition (= 100%).

The antimuscarinic actions of the compounds were examined in separate experiments by assessing their effects on cumulative concentration–response curves (CRC) for CCh. The first CRC was obtained at the end of the equilibration period of 60 min. After a washout period of 1 h, the test drug was added; a second CRC for CCh in the presence of the test compound was started after one further hour. TMC experiments were run without any drug added. Peak increase in force of contraction induced by the individual CCh concentrations was expressed as percent of the maximum effect observed during the first CRC.

Isolation of DSMC

Mucosa and serosa-free tissue pieces of human or pig urinary bladder were cut into small pieces, washed three times in chilled, nominally Ca^{2+}-free Tyrode's solution. Tissue pieces

were then transferred into Ca^{2+}-free Tyrode's solution containing 279 U ml^{-1} collagenase type I (Worthington) for pig or 266 U ml^{-1} for man plus 4.6 mg ml^{-1} protease type XXIV (Sigma-Aldrich) and gently stirred for 45 min. After 10 min the Ca^{2+} concentration was raised to 0.2 mM. Stirring was continued in Tyrode's solution (0.2 mM Ca^{2+}) with collagenase only until long, narrow and spindle-shaped DSMC were seen (after 10–20 min). Cells were released from the tissue mass by gentle trituration. The cell suspension was centrifuged, the pellet resuspended and stored until use in Ca^{2+} (0.5 mM) containing Tyrode's solution at room temperature. The cells remained viable for up to 8 h.

Whole-cell recording of I_{Ca}

I_{Ca} was measured at room temperature with standard voltage-clamp technique (Axopatch 200, Axon Instruments, Foster City, CA, U.S.A.), ISO 2 software was used for data acquisition and analysis (MFK, Niedernhausen, Germany). Heat-polished pipettes were pulled from borosilicate filamented glass (Hilgenberg, Malsfeld, Germany). Tip resistances were 2–6 MΩ, seal resistances were about 1 GΩ. Cell capacitance (C_M) was calculated from steady-state current during depolarizing ramp pulses (1 V s^{-1}) from −40 to −35 mV. I_{Ca} were measured from a holding potential of −60 at +10 mV test potential. For further isolation of I_{Ca} from contaminating currents, Na^+ was replaced with tetraethylammonium ions and K^+ was replaced with Cs^+ to block K^+ currents. Current–voltage relations were obtained by a series of test pulses between −50 and +50 mV. The experiments were performed with the following Na^+-free superfusion solution (in mM): tetraethylammonium chloride 120, CsCl 10, HEPES 10, $BaCl_2$ 3.8, $MgCl_2$ 1 and glucose 20, pH 7.4 (adjusted with CsOH). The pipette solution (pH 7.2) included (in mM): cesium methanesulphonate 90, CsCl 20, HEPES 10, Mg-ATP 4, Tris-GTP 0.4, EGTA 10 and $CaCl_2$ 3, with calculated free Ca^{2+} concentration of ~60 nmol l^{-1} (computer program EQCAL, Bio soft, Cambridge, U.K.). Current amplitudes were determined as the difference between peak inward current and current at the end of the depolarising step or as nifedipine-sensitive peak inward current.

A system for rapid solution changes allowed application of substances in the close vicinity of the cells (Cell Micro Controls, Virginia Beach, VA, U.S.A.; ALA Scientific Instruments, Long Island, NY, U.S.A.).

Drugs

The composition of the solutions was (in mM): transport buffer: NaCl 149, KCl 2.7, $CaCl_2$ 1.8, NaH_2PO_4 0.1, Na_2HPO_4 0.7, glucose 5.6, pH 7.4 adjusted with NaOH; Tyrode solution: NaCl 127, KCl 5.4, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.4, Na_2CO_3 22, glucose 5.6, pH 7.4 when gassed with 95% O_2 and 5% CO_2. All chemicals were of analytical grade and purchased from SIGMA-ALDRICH (Taufkirchen, Germany). CCh, atropine sulphate and TTX acetate were also purchased from SIGMA-ALDRICH. Propiverine-HCl (2,2-diphenyl-2-propoxy-acetic acid [1-methyl-piperid-4-yl]-ester-hydrochloride), M-5 (2,2-diphenyl-2-propoxy-acetic acid [1-methyl-piperid-4-yl]-ester-*N*-oxide-trans), M-6 (2,2-diphenyl-2-hydroxy-acetic acid [1-methyl-piperid-4-yl]-ester-*N*-oxide-trans) and M-14 (2,2-diphenyl-2-propoxy-acetic acid [piperid-4-yl]-ester) were provided by APOGEPHA Arzneimittel GmbH, Dresden, Germany. Drugs were made up as a 0.1 M stock solution in Milli-Q-water (CCh) or dimethyl sulphoxide (DMSO; all anticholinergics), and further diluted with Milli-Q-water. The highest concentration of DMSO 1% (v v^{-1}) did not affect the amplitude or time course of detrusor contractions.

Data analysis

All data are expressed as the mean ± s.e.m. Individual IC_{50}'s (molar drug concentration producing 50% inhibition of maximum contractile response to EFS) were determined by nonlinear regression analysis for each individual experiment using GraphPad Prism® 3.02 (GraphPad Software Inc., San Diego, U.S.A.). Mean IC_{50} values ± s.e.m. from n experiments are expressed as $-\log IC_{50}$ [M] (in Table 1). Differences between drugs were tested by Student's t-test, or one-way ANOVA with an additional Bonferroni's multiple comparison test, and were considered significant for $P<0.05$.

Cumulative CRC were analysed by nonlinear regression of each individual experiment using GraphPad Prism® 3.02 (GraphPad Software Inc., San Diego, U.S.A.), mean EC_{50} values for CCh (molar concentration producing 50% of the maximum contraction response) were calculated for CRC before and after test drug addition. pA_2 values for the antagonistic effect of M-6 on CRC for CCh in human detrusor were estimated from Schild plot analysis (Arunlakshana & Schild, 1959).

Results

Effects on electrically induced contractions

The drug effects on EFS-elicited tension and the TMC in human, pig and mouse detrusor are summarized in Figure 2.

Table 1 Inhibitory effects of drugs on EFS-induced contractions in detrusor strips

Compound	n	*Human*	n	*Pig*	n	*Mouse*
Propiverine ^{NS}	5	5.44 ± 0.56	8	4.73 ± 0.12	8	5.09 ± 0.09
M-5^{NS}	6	4.14 ± 0.11	6	3.90 ± 0.15	8	3.89 ± 0.11
M-6	7	7.05 ± 0.15**	13	5.38 ± 0.34	8	4.69 ± 0.31
M-14^{NS}	6	4.40 ± 0.29	16	4.24 ± 0.23	8	4.69 ± 0.11
Atropine^{NS}	5	7.60 ± 0.38	18	7.82 ± 0.08	15	7.85 ± 0.10

Data expressed as $-\log IC_{50}$ values (means ± s.e.m.; IC_{50} in M). n, number of experiments.
NS – no statistical significant differences between the three species.
**$P<0.01$ for $-\log IC_{50}$ value in man vs pig and mouse.

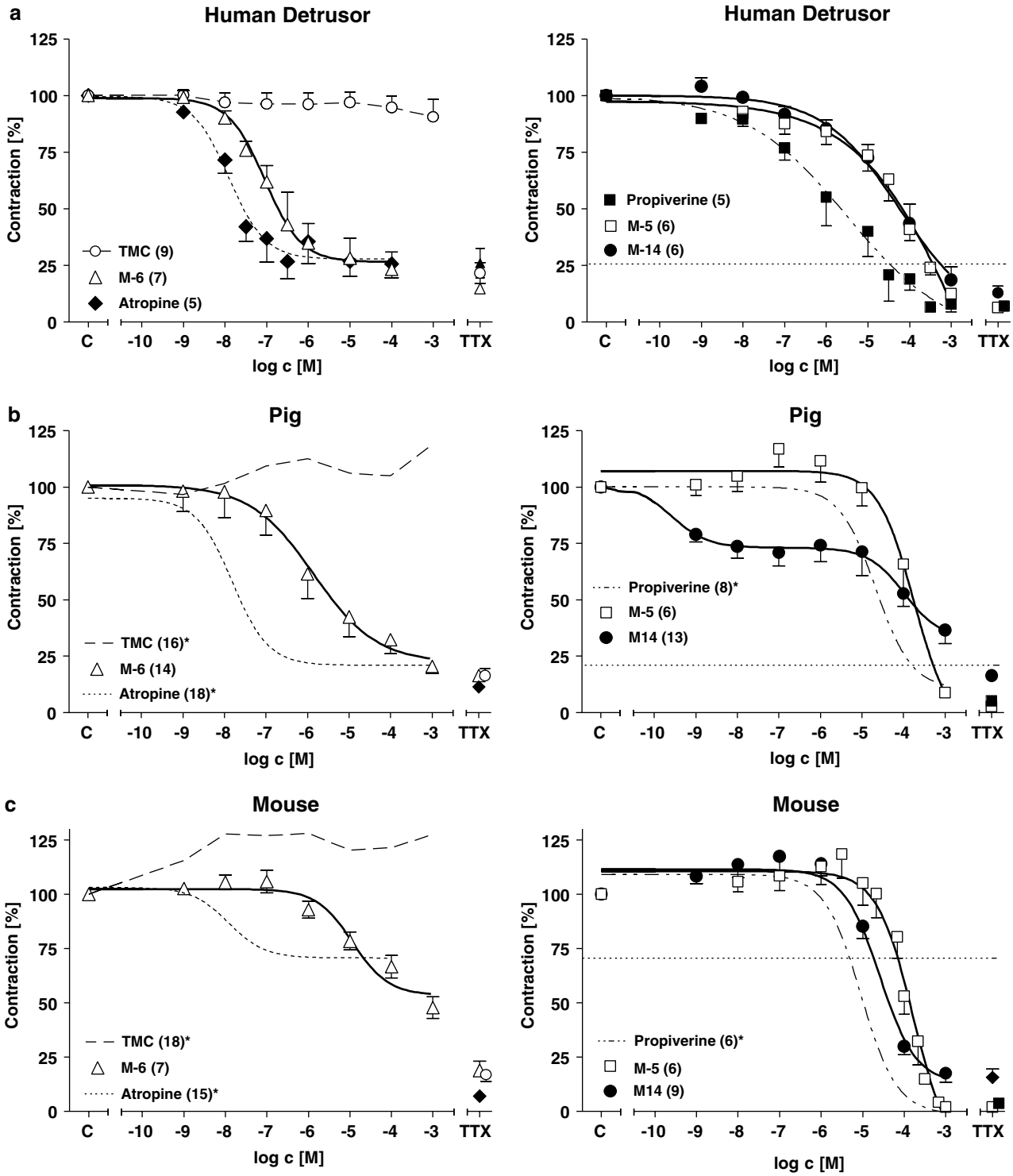

Figure 2 Average CRCs for the effects of M-5, M-6 and M-14 in comparison to those of propiverine and atropine on EFS-evoked contractions in detrusor muscle from man (a), pig (b) and mouse (c). Force is expressed in percent of the pre-drug control value (=100%). TMC, time-matched control without any drug added. The horizontal dashed lines in the right side of the figure represent the contraction amplitude in the presence of the highest atropine concentration. The percent of direct smooth muscle contractions in the presence of 1 μM TTX is plotted for each experimental group on the far right of each diagram. Mean values $\pm$ s.e.m. from *n* experiments. *Labelled data in pig and mouse were taken from previous work and are included for comparative purposes (Wuest *et al*., 2002).

In TMC with human detrusor, force development remained constant within a period of 4 h (Figure 2a). In mouse force increased by about 25% during the first hour and remained stable thereafter (Figure 2c), and a similar, though less pronounced, time course was observed for TMC experiments in pig detrusor (Figure 2b).

In *human detrusor*, atropine reduced EFS-induced force of contraction with high potency (for $-\log IC_{50}$ value, see Table 1). In the presence of the highest atropine concentration, contraction amplitude still amounted to $26\pm6\%$ of control ($n=5$; $P<0.001$ vs TMC). TTX ($1\,\mu$M) could not further reduce electrically evoked contractions ($26\pm7\%$, $n=5$, Figure 2a). This TTX-resistant amplitude was similar as in TMC ($22\pm5\%$, $n=6$, NS). The CRC for propiverine was less steep, required higher concentrations and reached $8\pm3\%$ of control ($n=5$; $P<0.001$ vs TMC). There were distinct differences between the effects of the metabolites. M-5 and M-14 were less potent than propiverine (see Table 1), but depressed force to a similar degree (i.e. M-5 to $12\pm3\%$ of control ($n=6$; $P<0.001$ vs TMC) and M-14 to $18\pm6\%$ of control ($n=6$; $P<0.001$ vs TMC). The CRC for M-6 was distinct from that of M-5 or M-14. The slope was as steep as with atropine though M-6 was slightly less potent (Table 1). This difference was not statistically significant. M-6 decreased force to $23\pm8\%$ of control ($n=7$; $P<0.001$ vs TMC).

In *pig detrusor*, the CRC for atropine was similar to that in human detrusor (Figure 2b; Table 1). Contraction in the presence of maximum atropine concentration was $20\pm2\%$ of control ($n=18$, NS vs human detrusor). Propiverine was slightly less potent (difference did not reach statistical significance) and the CRC was steeper than in human detrusor. However, like in human detrusor, propiverine reduced force similiarly to $10\pm1\%$ of control ($n=8$). The CRC for M-5 lay in a similar concentration range as for human detrusor. M-14 reduced contraction amplitude by about 25% already at very low concentrations (1 nM), the remainder of the response occurred within the same concentration range as in human strips. At the highest M-14 concentration (1 mM), force was reduced to $29\pm5\%$ of control ($n=16$). Compared with atropine, the $-\log IC_{50}$ value of M-6 was more than 2 log units lower than for atropine and force was depressed to the same extent (to $19\pm3\%$ of control; $n=14$).

In *mouse detrusor*, the effects of the compounds on EFS-induced contractions produced yet a different pattern of responses (Figure 2c). In this species, TTX ($1\,\mu$M) reduced TMC contractions to $17\pm3\%$ of control. The percent contraction in the presence of the highest atropine concentra-

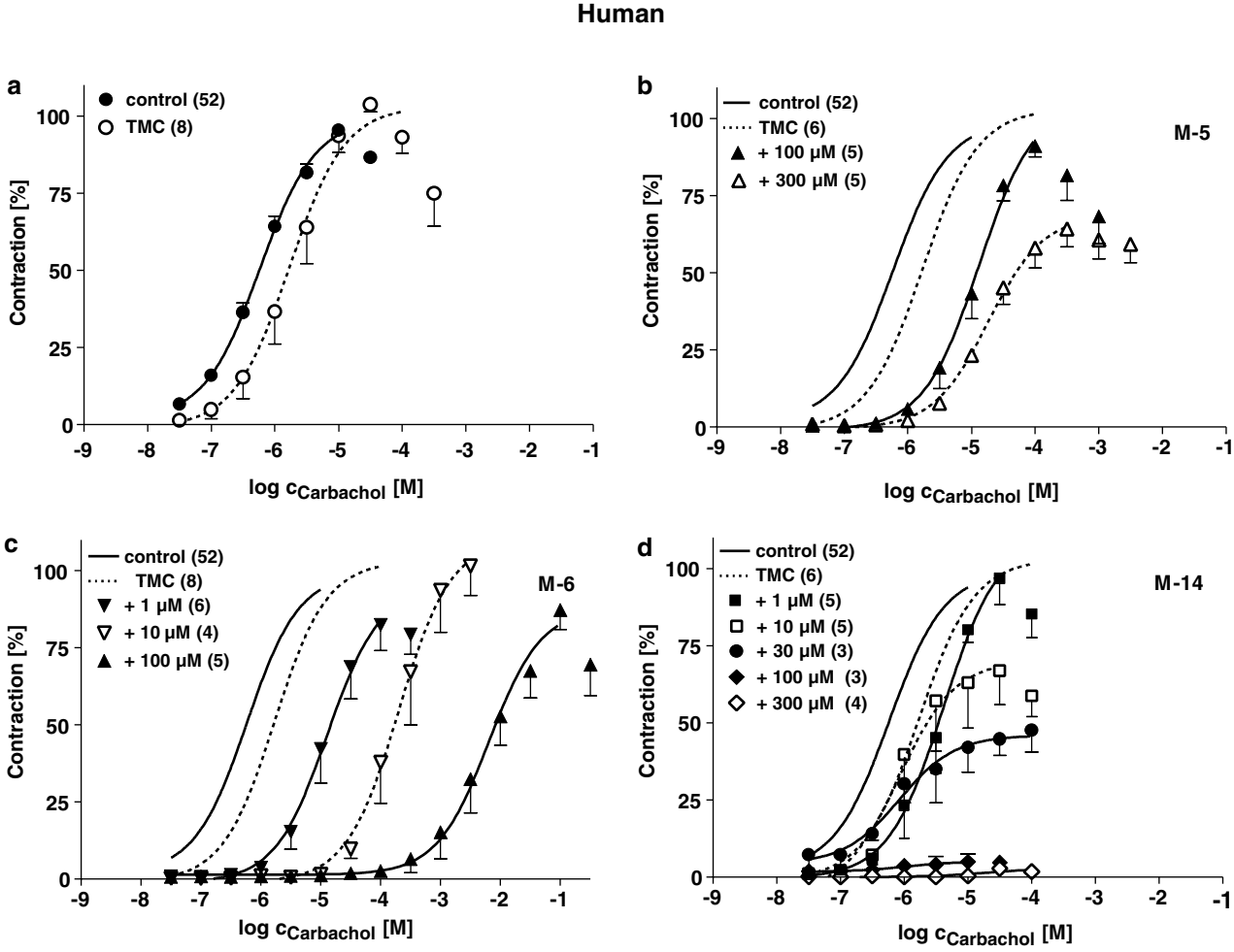

Figure 3 Effects of M-5 (b), M-6 (c) and M-14 (d) on cumulative CRC for CCh in human detrusor muscle strips in comparison to TMC experiments without any drugs added (a). Data are shown as means$\pm$s.e.m. from n experiments. Responses to CCh are expressed in percent of the maximum effect in the first CRC. Note that the continuous and dashed lines have been fitted to the mean values without considering the decline of force at very high concentrations. Control data in (a) are averaged values from the first CRC's in all experiments ($n=46$, continuous line) and are also together with the TMC data (dashed line) depicted in (b), (c) and (d) without data points.

tion was 70±3% of control ($n=15$). TTX (1 μM) further reduced contractions to 7±1% of control, indicating a large NANC component of contractions of about 60% of predrug control (see Wuest *et al*., 2002). Propiverine completely suppressed force of contraction with a steep CRC in a similar concentration range as for pig detrusor. M-5 also suppressed force completely with about 1 log unit lower potency than propiverine. M-14 was slightly less potent and also somewhat less efficacious (force reduction to 18±4% of control; $n=8$). With M-6, higher concentrations than with propiverine were required for half maximum force reduction, 1 mM of M-6 reduced force of contraction to 48±5% of control ($n=8$).

Impairment of the NANC component (i.e. the difference contraction in the presence of atropine and TTX) in mouse detrusor by the three investigated metabolites suggests that besides their antimuscarinic effects one or more additional mechanisms of action may occur. Therefore, the influence of the compounds on CRCs for CCh was also studied.

Cumulative CRC for CCh

Increasing concentrations of CCh (30 nM–100 μM, 5 min exposure time each) elicited strong force of contraction in all the three investigated species. The mean $-\log EC_{50}$ (M) from all first CRCs were: man 6.15±0.07 ($n=52$); pig 5.76±0.08 ($n=51$) and mouse 5.69±0.09 ($n=34$). The effects of M-5, M-6 and M-14 on the cumulative CRCs for CCh in human, pig and mouse detrusor are summarized in Figures 3–5, the $-\log EC_{50}$ values and maximum effects of CCh are listed in Tables 2–4.

In human detrusor, the second CRC for CCh in TMC experiments was slightly shifted to the right by about one half log unit, in pig detrusor the rightward shift was somewhat larger and in mouse detrusor both CRCs were almost superimposed. Only in pig detrusor the maximum effect (Eff_{max}) was slightly reduced to about 90% of the Eff_{max} in the first CRC.

The metabolites profoundly affected CCh-induced contractions in a concentration-dependent manner. While M-6 only shifted the CRCs to the right and did not affect maximum CCh responses, M-5 additionally reduced Eff_{max}. In contrast M-14 did not shift the CRC, but suppressed Eff_{max} almost completely. The response patterns to the individual metabolites were comparable in all three investigated species. Differences were only detected for M-5, which was more effective in reducing Eff_{max} in pig and mouse compared to human detrusor, but shifted the CRC for CCh to a larger extent human strips.

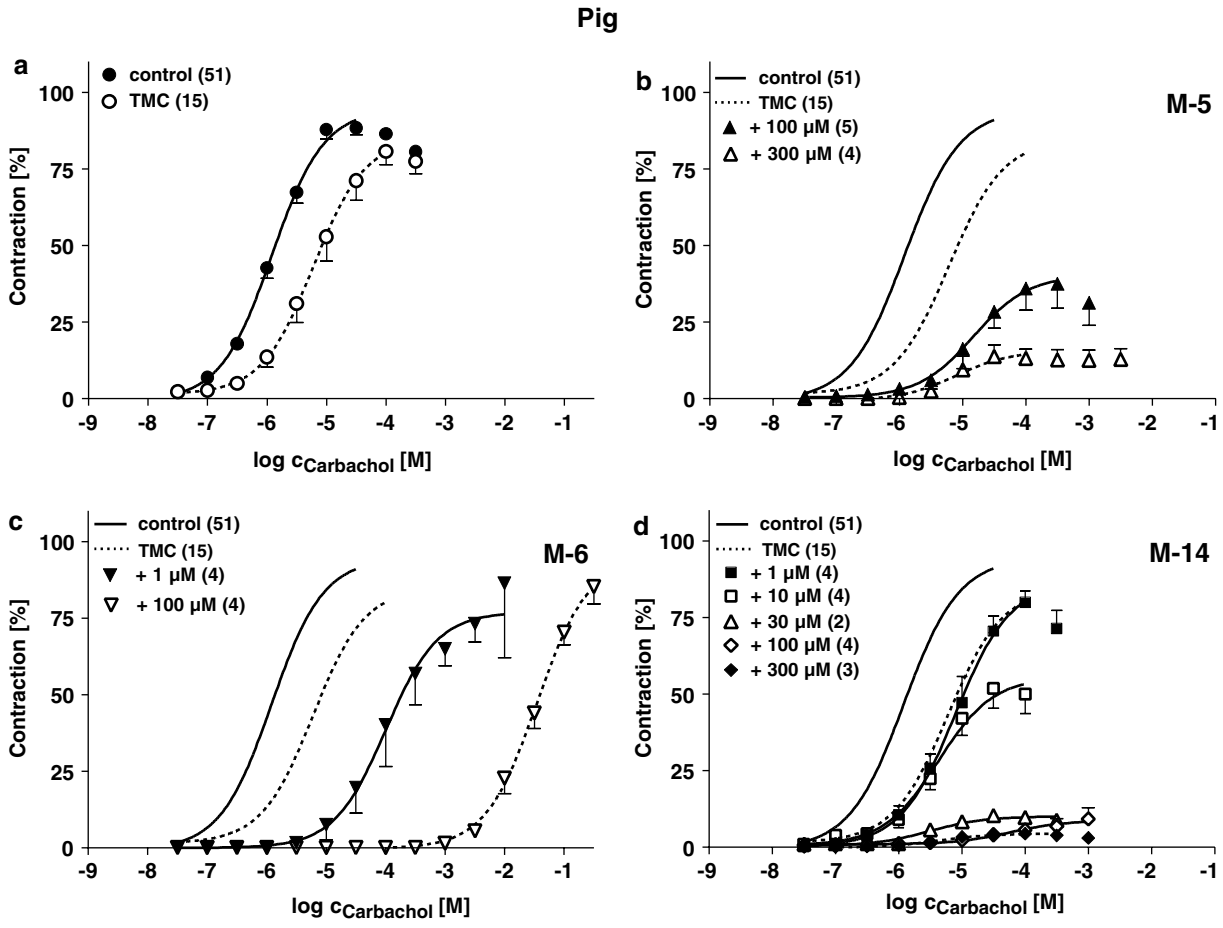

Figure 4 Effects of M-5 (b), M-6 (c) and M-14 (d) on cumulative CRC for CCh in pig detrusor strips in comparison to TMC experiments without any drugs added (a). Data are shown as means±s.e.m. from *n* experiments. Responses to CCh are expressed in percent of the maximum effect in the first CRC. Lay-out like in the Figure 4.

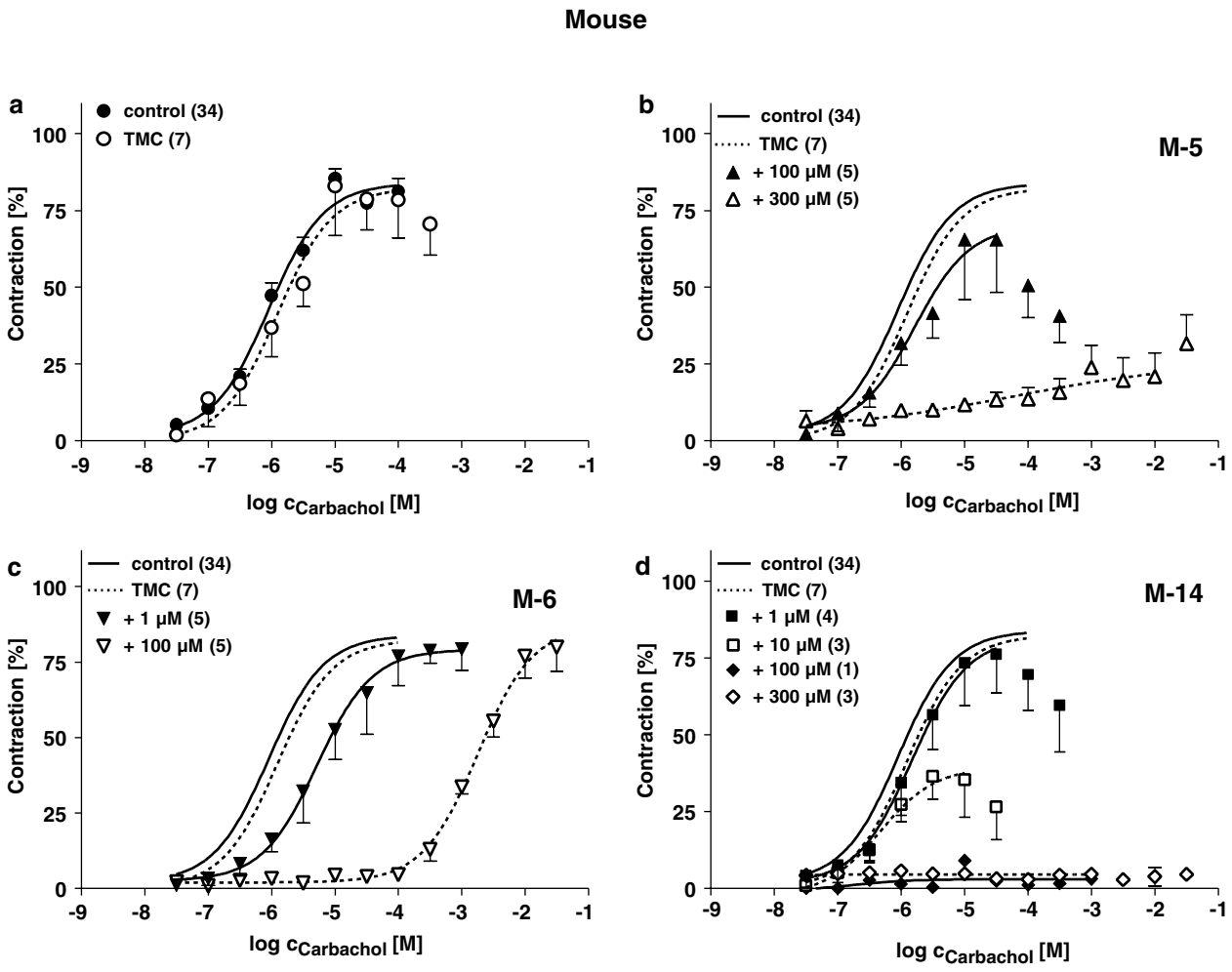

Figure 5 Effects of M-5 (b), M-6 (c) and M-14 (d) on cumulative CRC for CCh in mouse detrusor strips in comparison to TMC experiments without any drugs added (a). Data are shown as means ± s.e.m. from *n* experiments. Responses to CCh are expressed in percent of the maximum effect in the first CRC. Lay-out like in Figure 4.

Table 2 Effects of propiverine metabolites on the CRC for CCh in *human* detrusor

	n	*First CRC-* $-log\,EC_{50}$	*Second CRC* $-log\,EC_{50}$	$\Delta - log\,EC_{50}$[a]	Eff_{max}
TMC	8	6.13 ± 0.25	5.64 ± 0.20	0.50 ± 0.11	108 ± 3
M-5					
100 µM	5	5.99 ± 0.19	4.95 ± 0.16	0.54 ± 0.18**	92 ± 4**
300 µM	5	6.37 ± 0.15	4.68 ± 0.09	1.19 ± 0.13***	66 ± 6***
M-6					
1 µM	5	6.08 ± 0.17	4.78 ± 0.19	0.74 ± 0.19**	89 ± 5*
10 µM	4	6.30 ± 0.20	3.60 ± 0.20	2.19 ± 0.26***	110 ± 9
100 µM	5	6.07 ± 0.18	2.19 ± 0.27	3.37 ± 0.31***	81 ± 7*
M-14					
1 µM	5	5.52 ± 0.22	5.34 ± 0.19	−0.11 ± 0.06	99 ± 8*
10 µM	5	6.43 ± 0.23	5.71 ± 0.14	0.22 ± 0.14	78 ± 10**
30 µM	3	6.69 ± 0.19	5.94 ± 0.18	0.25 ± 0.09	48 ± 4***
100 µM	3	6.84 ± 0.14	Not calculated	Not calculated	5 ± 3***
300 µM	4	6.01 ± 0.26	Not calculated	Not calculated	5 ± 3***

$-\log EC_{50}$ (M) and Eff_{max} (% of the maximum response in the first CRC) was determined from curve fitting to the experimental data obtained in each individual muscle strip. Data as means ± s.e.m., *n* = number of detrusor strips. $\Delta - \log EC_{50}$ are the differences between first and second CRC (i.e. the shift of CRC to higher CCh concentrations) and were corrected for the mean shift for TMC (with exception for the TMC itself).

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

[a]Tested for difference from zero.

Table 3 Effects of propiverine metabolites on the CRC for CCh in pig detrusor

	n	*First CRC-* $-log\ EC_{50}$	*Second CRC* $-log\ EC_{50}$	$\Delta -log\ EC_{50}$[a]	Eff_{max}
TMC	15	5.64±0.19	5.00±0.13	0.63±0.11	90±3
M-5					
100 μM	5	5.91±0.17	4.94±0.26	0.34±0.21	40±8***
300 μM	4	5.99±0.14	5.02±0.16	0.34±0.08	14±4***
M-6					
1 μM	5	5.69±0.22	3.82±0.30	1.24±0.13***	85±13
100 μM	4	5.97±0.10	1.44±0.06	3.89±0.15***	83±5
M-14					
1 μM	4	5.86±0.12	5.03±0.20	0.20±0.25	81±4
10 μM	4	6.11±0.06	5.34±0.11	0.18±0.08	54±7***
30 μM	2	5.99	5.44	−0.09	10***
100 μM	4	5.15±0.39	4.64±0.43	−0.24±0.26	8±4***
300 μM	3	6.06±0.14	5.08±0.30	0.35±0.21	5±1***

$-\log EC_{50}$ (M) and Eff_{max} (% of the maximum response in the first CRC) was determined from curve fitting to the experimental data obtained in each individual muscle strip. Data as means±s.e.m., n = number of detrusor strips. $\Delta -\log EC_{50}$ are the differences between first and second CRC (i.e. the shift of CRC to higher CCh concentrations) and were corrected for the mean shift for TMC (with exception for the TMC itself).
*$P<0.05$; **$P<0.01$; ***$P<0.001$.
[a]Tested for difference from zero.

Table 4 Effects of propiverine metabolites on the CRC for CCh in *mouse* detrusor

	n	*First CRC-* $-log\ EC_{50}$	*Second CRC* $-log\ EC_{50}$	$\Delta -log\ EC_{50}$[a]	Eff_{max}
TMC	7	5.42±0.23	5.71±0.35	−0.29±0.20	97±7
M-5					
100 μM	5	5.75±0.18	5.52±0.20	0.52±0.18	73±16
300 μM	5	6.07±0.21	5.54±0.61	0.82±0.75	25±7***
M-6					
1 μM	5	5.41±0.22	4.81±0.19	0.89±0.12***	90±6
100 μM	5	6.07±0.18	2.19±0.27	3.91±0.24***	106±14
M-14					
1 μM	4	5.20±0.22	5.55±0.20	−0.06±0.24	88±8
10 μM	3	5.84±0.03	6.06±0.24	0.07±0.25	40±9**
100 μM	1	5.82	Not calculated	Not calculated	9
300 μM	3	6.10±0.31	Not calculated	Not calculated	7±2***

$-\log EC_{50}$ (M) and Eff_{max} (% of the maximum response in the first CRC) were determined from curve fitting to the experimental data obtained in each individual muscle strip. Data as means±s.e.m., n = number of detrusor strips. $\Delta -\log EC_{50}$'s are the differences between first and second CRCs (i.e. the shift of CRC to higher CCh concentrations) and were corrected for the mean shift for TMC (with exception for the TMC itself).
*$P<0.05$; **$P<0.01$; ***$P<0.001$.
[a]Tested for difference from zero.

The pA_2 value calculated from Schild regression analysis for M-6 was 6.93±0.14 (slope 1.34±0.09; $R^2=0.99$) in human detrusor. The respective pA_2 value for atropine was 8.91±0.38 ($R^2=0.94$; data were taken from Wuest *et al*., 2005), suggesting that M-6 has a lower affinity for M receptors of human detrusor than atropine.

Since detrusor contractions depend on the extracellular Ca^{2+} concentration, the reduction of the maximum CCh responses with propiverine, M-5 and M-14 could be due to a block of Ca^{2+} entry. In the following experiments, we therefore examined whether inhibition of I_{Ca}'s could be responsible for the observed effects.

Effects on I_{Ca}

Inward currents in freshly isolated DSMC were studied with the conventional whole-cell patch-clamp technique. Human and pig DSMC were of similar size, resulting from their membrane capacitances which were 43±2 ($n=31$) and 47±2 pF ($n=20$), respectively. Current traces in human DSMC for the test pulse of +10 mV from a holding potential of −60 mV showed a rapidly activating, slowly inactivating inward current, that was completely suppressed by the dihydropyridine nifedipine (Figure 6a). This current was also blocked by 100 μM propiverine (Figure 6b). The current–

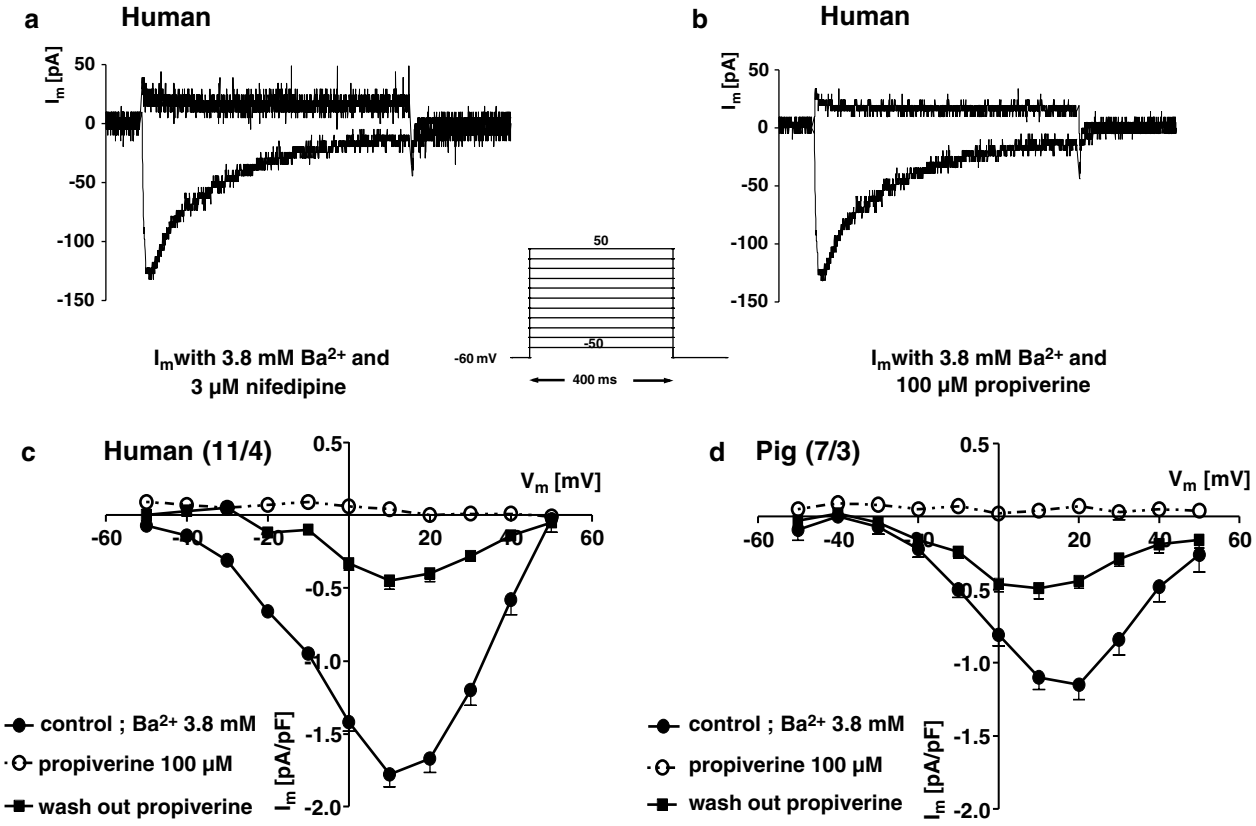

Figure 6 Current traces for the I_{Ca} in human DSMC in the presence of 3 μM nifedipine (a) and 100 μM propiverine (b). Current–voltage relations (*I–V*) for human (c) and porcine cells (d). Data of the *I–V* curves are shown as means$\pm$s.e.m. in pA pF^{-1} from *n* investigated cells. Effect of 100 μM propiverine on the *I–V* curves and washout are shown.

voltage relation was characteristic for L-type calcium channels with maximum at $+10$ mV (-1.39 ± 0.13 pA pF^{-1}, $n=37$ for human DSMC) and an apparent reversal potential arround $+50$ mV (Figure 6c). I_{Ca} at $+10$ mV was smaller in porcine compared to human DSMC: -0.75 ± 0.08 pA pF^{-1} ($n=20$). With 100 μM propiverine the block was complete throughout the potential range of the *I–V* curve, and was only partially reversible during washout. Similar results were obtained in pig DSMC (Figure 6d). Block of I_{Ca} by propiverine was concentration-dependent (Figure 7a) in a similar manner as the reduction of the CCh-induced maximum contractile force (Figure 7c).

The effects of the metabolites M-5, M-6 and M-14 on I_{Ca} in human DSMC are also shown in Figure 7. While M-14 like propiverine almost completely inhibited I_{Ca}, M-6 had only small effects and M-5 showed no block at all in the investigated concentration range (Figure 7a and c). The respective $-\log IC_{50}$ values were: for propiverine 5.49 ± 0.08 ($n=7$–11 cells from four patients), for M-6 4.97 ± 0.11 ($n=6/3$) and for M-14 5.19 ± 0.07 ($n=9/4$).

The observed pattern of responses of I_{Ca} to block by propiverine and its metabolites are similar to their effects on the maximum CCh-induced contraction (Figure 7b and d). While propiverine and M-14 strongly reduced Eff_{max}, M-6 did not affect maxiumum CCh response. A remarkable but smaller effect of M-5 was only seen at higher concentrations.

Discussion

The main findings of our study were: (i) Propiverine and the metabolites M-5, M-6 and M-14 reduced electrically evoked contractions in human, pig and mouse detrusor, albeit to different extents. (ii) M-6 shifted the CRC for CCh to the right, M-5 additionally reduced the maximum CCh-induced contractions like propiverine, and M-14 only impaired the maximum response without a rightward shift of the CRC. (iii) Propiverine and M-14 strongly inhibited I_{Ca} in human DSMC, whereas M-5 and M-6 had only marginal effects. (iv) Our findings suggest that suppression of the CCh maximum effect may be related to a direct block of I_{Ca}.

Effects on EFS-induced contractions

EFS excites intramural nerve endings, leading to release of ACh and other neurotransmitters involved in stimulation of detrusor smooth muscle contraction (for a review, see Andersson, 1993). With the selected EFS parameters, detrusor contractions were not only neurogenic but also myogenic (TTX-resistant). Block of the cholinergic contraction by atropine (Bayliss *et al*., 1999) was reported to leave a small NANC component in man and pig, but a sizeable one in mouse detrusor (Sibley, 1984; Bayliss *et al*., 1999). Under the same experimental conditions as used for this study we have recently detected an NANC component of contraction of

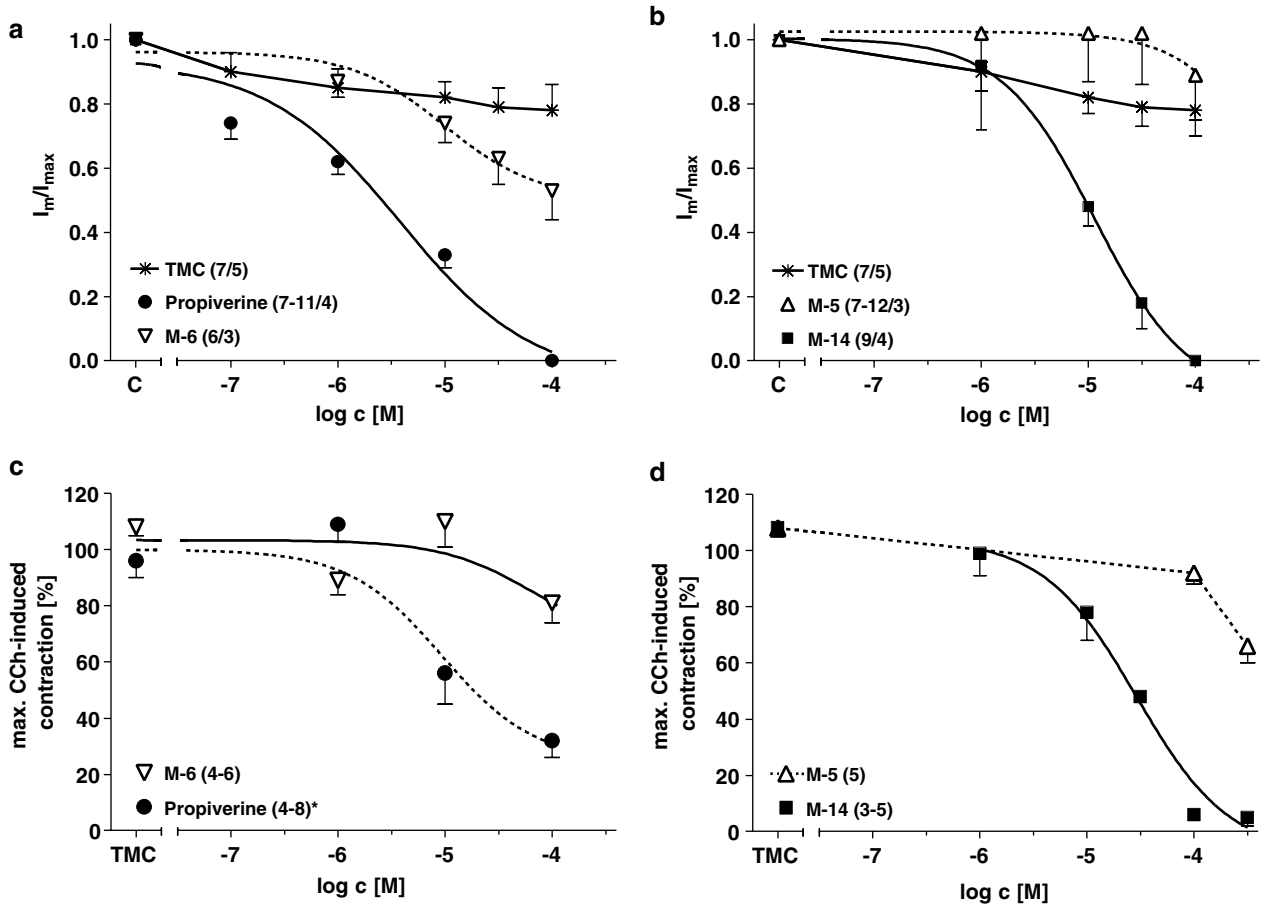

Figure 7 Cumulative CRCs for the effects of propiverine, M-6 (a) and M-5, M-14 (b) on the maximum I_{Ca} at $+10$ mV in comparison to TMC experiments without any drug added. Effects of propiverine (*labelled data were taken from previous work and are included for comparative purposes, Wuest *et al*., 2005) and M-6 (c) and M-5 and M-14 (d) on the maximum CCh-induced contraction during the first CRC. All data are shown as means$\pm$s.e.m. from n measured cells from x different patients or n investigated detrusor muscle strips.

about 60% in mouse, but only 7% in pig detrusor (Wuest *et al*., 2002). In the present study, human preparations did not exhibit NANC contractions. This could be due to the selected stimulation parameters. Pessina *et al*. (2001) showed that with rising stimulation frequencies from 1 to 15 Hz contraction amplitudes were enhanced in all species. Despite the long stimulus duration (i.e. 1 ms), we found a similar frequency dependence of EFS-induced contraction amplitudes in mouse and pig as recently reported for rat, guinea-pig, monkey and human detrusor (pulse duration 0.05 ms, Pessina *et al*., 2001). These authors described a negligible NANC component in man and monkey (like our findings for man and pig) and a large NANC component in rat and guinea-pig (like our findings in mouse). Therefore, mouse detrusor muscle appears to be a suitable model for studying additional spasmolytic effects, which are considered not to be mediated *via* the M receptor pathway. M-5 and M-14 did in fact suppress EFS-evoked contractions in the mouse, suggesting that these two metabolites possess additional effects like the parent compound propiverine. However, such an effect was not observed with M-6, which marginally affected the atropine-resistant (NANC) contractions. These observations are in line with the proposal, that an unchanged aliphatic side chain in the propiverine derivatives is associated with one or more additional actions (Siegmund *et al*., 1990), whereas after change to the hydroxylic group only antimuscarinic action is retained (Haruno *et al*., 1989; Haruno, 1991, see below).

In man, pig and mouse, the order of potency was: M-6 > propiverine > M-5. Propiverine and M-5 differ in structure only at the nitrogen. We speculate that the oxidation of the tertiary amine may have a negative influence on the drug's potency to reduce EFS-evoked contractions. It can be noted that the change from the tertiary amine to the secondary amine structure has very little effect on the potency and the overall impairment of EFS-evoked contractions.

Effects on CRC for CCh

In TMC experiments the second CRC for CCh was shifted to higher concentrations, which is probably due to desensitization of the M receptors as previously reported for pig and mouse detrusor (Wuest *et al*., 2002) and confirmed herein for human samples. Only in pig the maximum CCh-stimulated contraction during the second CRC was somewhat smaller, suggesting a larger desensitization of the CCh response in this species.

Like atropine, M-6 merely shifted the CCh CRCs to the right without any effect on the maximum response, suggesting that this compound behaves as a competitive antagonist in this

system. Similar results have been obtained with M-6 in guinea-pig ileum and detrusor (Haruno *et al*., 1989; Haruno, 1991). The respective pA_2 values for M-6 and atropine reported for ileum (6.53 and 9.15; Siegmund *et al*., 1990) are in good agreement with the one reported here, that is, 6.93 for the effect of M-6 and 8.91 for atropine.

M-5, on the other hand, exhibited a mixed action like propiverine (Yono *et al*., 1999; Wuest *et al*., 2002) by shifting the CRC, and in addition reducing Eff_{max}, which is in line with the effects of a unsurmountable antagonist. M-14 only reduced Eff_{max} without any shift of the CRC, and hence is expected to have the most prominent 'additional' actions. Evidence in guinea-pig detrusor suggests that the 'additional' effects could be related to depolarization dependent on Ca^{2+} influx, since propiverine ($\geqslant 10\,\mu$M) and the N-oxide M-5 ($\geqslant 100\,\mu$M) reduced KCl-induced contractions, whereas M-6 had no effects (Haruno *et al*., 1989).

Effects on I_{Ca}

Propiverine is considered to act with at least one additional mode of action, reducing detrusor contractility (Wada *et al*., 1995; Madersbacher & Mürtz, 2001). It has been reported to block I_{Ca} in rat and guinea-pig DSMC (Tokuno *et al*., 1993). Our data confirm the blocking effect on I_{Ca} in man and pig, which was as large as the effect of nifedipine. In accordance with the literature, we found larger current density in human than in porcine DSMC (Kajioka *et al*., 2002).

The differences in effects of the metabolites M-5, M-6, and M-14 on I_{Ca} are in agreement with the extent of impairment of the maximum CCh response during detrusor contraction. Like propiverine, M-14 almost completely blocked I_{Ca} and also strongly reduced Eff_{max}. However, M-6, which merely shifted the CRCs like a competitive antagonist, had only minor effects on I_{Ca}, which may even be due to the large variability. Furthermore, M-6 only marginally reduced the atropine-resistant component of contraction during EFS in mouse detrusor. This may be another evidence for competitive muscarinic antagonism of M-6. Lack of I_{Ca} block by M-5 could be due to the reduced potency of this metabolite (see above).

The amount of block of I_{Ca} by M-14 was comparable with that of propiverine and nifedipine. M-14 did not affect the potency of CCh. This response pattern is similar to the effects of nifedipine on CRC for CCh (Schneider *et al*., 2004b). We conclude that the spasmolytic effect of M-14 is mainly due to block of calcium influx *via* L-type calcium channels and not due to antagonism at M receptors. Siegmund *et al*. (1990) suggested that the aliphatic side chain is responsible for the nonspecific properties in addition to antagonism at M receptors. We would extend this conclusion by suggesting that the aliphatic side chain is necessary for the blocking effect on I_{Ca}.

In summary, we have shown different effects of propiverine's main metabolites in the human system, that is, M-5, M-6 and M-14, on contractility of detrusor muscle in man, pig and mouse and on I_{Ca} in human and porcine DSMC. Changes in the molecular structure of propiverine lead to a change in the antagonistic action on M receptors and to a change in additional block of I_{Ca}. The unsurmountable effect on CCh-induced contractions seems to involve a direct block of I_{Ca}. We conclude that the main metabolites of propiverine (M-5, M-6 and M-14) may well contribute to its clinical effect.

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